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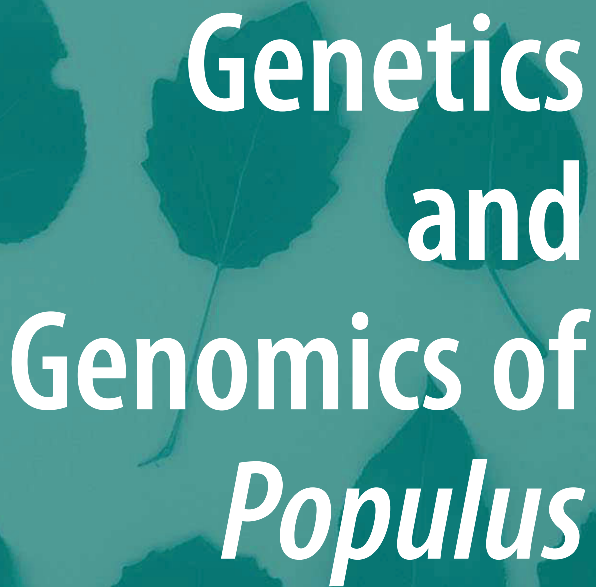
Editors

Plant Genetics / Genomics Volume 8



Genetics and Genomics of *Populus*

Plant Genetics and Genomics: Crops and Models

Volume 8

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Genetics and Genomics of *Populus*

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Preface

Trees are truly amazing! Woody species underpin vital terrestrial ecosystems ranging from the Amazon rainforests to the Krummolz forests that grow on harsh mountaintop environments, to the vast boreal forests that ring the northern hemisphere. Forest trees also present a complex array of evolutionary novelties, including the ability to measure and anticipate the changing seasons (perennial growth), and the ability to produce massive, woody bodies. Indeed, forest trees include the oldest (*Pinus longaeva*) and largest (*Sequoiadendron giganteum*) sexually producing, non-clonal species. Among species with clonal reproduction, it has been suggested that the massive "Pando" grove of *Populus tremuloides* in Utah may be the largest organism in the world.

Forest trees also provide important benefits and commodities. Trees provide lumber and wood products, pulp and paper, and are a major energy source for many developing countries. Trees also play a key role in the major biogeochemical cycles, including water, oxygen, and nitrogen. Notably, forests are second only to oceans in the biological sequestration of carbon, and forests are recognized for their vital role in regulating the concentration of the greenhouse gas, CO_2. At the same time, forests are threatened by land clearing for development and agriculture, introduced pathogens and insects, and by climate change.

A fundamental need encompassing all of these scientific, economic, and management issues is a better understanding of the basic biology of forest trees. One proven strategy for biological research is the development of so-called model organisms, in which an organism is identified that both presents biological traits of interests for study, and for which key experimental tools can be developed. Increasingly, the basis for modern model species development often revolves around genomics. In this regard, the ultimate resource is the determination of the full genome sequence of the organism, which both reveals the entire complement of genes and allows development of advanced genomic tools indexed to the genome.

As the power of genetic model systems for biological research became increasingly obvious, the need for a good tree model system grew. Gradually, *Populus* – a genus consisting of over 30 species with a wide geographic distribution – developed into the prime model system for tree research. *Populus* have many fundamental differences to Arabidopsis and other current plant model species. With regards to life habit, *Populus* species are ecologically dominant species, have long life spans,

and form large woody bodies. They have extended juvenile phases, are dioceous and therefore obligate outbreeders, and some species are often found in extensive clones. Through their relatively long life spans *Populus* are exposed to extreme abiotic conditions and have numerous antagonistic and symbiotic interactions with other organisms. However, from a phylogenetic point of view *Populus* and Arabidopsis are relatively closely related organisms – for example they are more closely related than Arabidopsis and tobacco. This phylogenetic relatedness makes comparisons of gene content and function in Arabidopsis and *Populus* relatively straightforward.

Trees are found among many phylogenetic groups. It can not be excluded that the last common ancestor of today's angiosperms and gymnosperms was woody, and transition from herbaceous to tree life habit (or tree to herbaceous) is apparently relatively simple in evolutionary terms. This suggests that the genetic differences underlying the defining characteristics of forest trees could ultimately be homologous, but are highly plastic and easily modified to produce the wide array of organisms called "trees." Undoubtedly, the tree life habit is rather different from the herb life habit and the unique selection pressures acting on trees versus herbaceous plants are at least in part responsible for variation in different characters. With the advent of *Populus* as a full-fleshed model system for plant genetic and genomics, tools are now available for plant researchers to explore these fascinating aspects of tree biology. Tools and approaches are currently being developed for *Populus* that address genetic variation of traits at the levels of species, populations, and gene function. Importantly, most forest trees, including *Populus spp*, are characterized by high levels of genetic variation, making them highly amenable to population genomics approaches. Indeed, approaches such as association mapping that exploit natural genetic variation in outcrossing species that are now being applied to *Populus* have more in common with human genetics than crop genetics.

Several key innovations in the evolution of land plants that are largely lacking in Arabidopsis and other herbaceous annual models can be studied in *Populus*. These include processes underlying perennial growth and seasonality (for example in cambial activity, leaf senescence and dormancy), extensive wood formation, as well as many processes relating to biotic interactions. There are also research areas, not yet well developed, where *Populus* holds great promise as a model system. For example, studies in ecosystem genomics are supported by the recent sequencing of *Populus* symbionts (*Laccaria bicolor* and *Glomus intraradices*) and a pathogen (*Melampsora larici-populina*). With regards to developmental traits, juvenile to mature transitions may be better studied in a tree versus herbaceous plant, and it is also possible that epigenetic processes may be of increased importance in an organism with a long time span. Therefore, we expect that in the decade to come, many discoveries will be made in *Populus* that will impact plant science in general.

Although the *Populus* genetics and genomics community started to grow in the nineties, the decision to sequence the *Populus* genome in the winter of 2001/2002 prompted additional researchers to choose *Populus* for their studies. The authors of this book is a blend of researchers that have spent most of their research career on *Populus* and those that have moved to *Populus* from other model systems. The chapters describe (for both experienced *Populus* researchers and newcomers to the

field) both genetic and genomic approaches for *Populus* and some of the interesting biology that has been elucidated using genomics. Notably, research on *Populus* forms a useful complement to research on Arabidopsis. In fact, many plant species found in nature are – in terms of the life history and genetics – more similar to *Populus* than to Arabidopsis. Thus the genetic and genomic strategies and tools developed by the *Populus* community may serve as inspiration for researchers working in other, less well developed, systems.

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Contents

Part I Overview and an Introduction to the Biology of *Populus*

Why and How *Populus* Became a "Model Tree" 3
Brian Ellis, Stefan Jansson, Steven H. Strauss, and Gerald A. Tuskan

Salient Biological Features, Systematics, and Genetic Variation of *Populus* 15
Gancho T. Slavov and Peter Zhelev

Growth and Physiology 39
Sophie Y. Dillen, Stewart B. Rood, and Reinhart Ceulemans

Part II *Populus* Genetics and Genomics

The *Populus* Genome and Comparative Genomics 67
Carl J. Douglas and Stephen P. DiFazio

Nucleotide Polymorphism, Linkage Disequilibrium and Complex Trait Dissection in *Populus* 91
Pär K. Ingvarson

Transformation as a Tool for Genetic Analysis in *Populus* 113
Victor B. Busov, Steven H. Strauss, and Gilles Pilate

***Populus* Resources and Bioinformatics** 135
Nathaniel R. Street and Chung-Jui Tsai

Part III Genetics and Genomics of Key *Populus* Traits

Reproductive Development in *Populus* 155
Amy M. Brunner

Perennial Life Style of *Populus*: Dormancy Cycling and Overwintering 171
Päivi L.H. Rinne, Annikki Welling, and Chris van der Schoot

Wood Formation in *Populus* 201
Andrew T. Groover, Kaisa Nieminen, Ykä Helariutta,
and Shawn D. Mansfield

***Populus* Responses to Abiotic Stress** 225
Urs Fischer and Andrea Polle

Defense and Nutrient Mutualisms in *Populus* 247
George Newcombe, Francis Martin, and Annegret Kohler

The Impact of Genomics on Advances in Herbivore Defense and Secondary Metabolism in *Populus* 279
C. Peter Constabel and Richard L. Lindroth

Part IV *Populus* for the Future

***Populus* Breeding: From the Classical to the Genomic Approach** 309
Brian J. Stanton, David B. Neale, and Shanwen Li

Conservation Genomics 349
Elena Hidalgo, Santiago C. González-Martínez, Christian Lexer,
and Berthold Heinze

Index 369

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Part I
Overview and an Introduction to the Biology of *Populus*

Why and How *Populus* Became a "Model Tree"

Brian Ellis, Stefan Jansson, Steven H. Strauss, and Gerald A. Tuskan

Abstract Although *Populus* was not a favored experimental system for very many plant biologists in 2000, *P. trichocarpa* ultimately became only the third plant species to have its genome fully sequenced. Here we examine the many different factors that came into play when this species was abruptly elevated to the status of a new "model organism".

1 Model Systems Within Biological Research

The diversity and complexity of life-forms presents an enormous challenge to biologists. However, the common evolutionary origin of all organisms implies that what is learned about one organism can provide useful insight into its relatives. This concept has led to the selection of a wide array of "model or reference organisms" over the past 50 years, ranging from the early adoption of *Escherichia coli* as the model prokaryotic microbe to a recent focus on the mouse as a model for mammalian biology. There are few fixed criteria for selection of the ideal model organism, but the choice is typically strongly driven by the nature of the biological question(s) to be addressed and the availability of suitable tools or approaches to address the questions (Abzhanov et al., 2008). Thus, many aspects of prokaryotic biology can be profitably explored in *E. coli,* but if the question of interest involves bacterial spore formation, *Bacillus subtilis* is a better model. Similarly, the adoption of *Arabidopsis thaliana* as a model plant has allowed extraordinary progress to be made in understanding the fundamental features of plant biology over the last 20 years. The intense concentration of research on this single species fostered the development of powerful research tools and resources, including the first complete sequence of a plant genome. These resources, paired with genetic, genomic and other approaches, have revealed insights into fundamental plant biology, including induction and organogenesis of flowering, regulation of primary meristems and leaf

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development, and genes responsible for disease resistance. The list of accomplishments is long and impressive but *Arabidopsis* differs in many important respects from plant species of economic value such as legumes or cereals. *Medicago truncatula* and *Oryza sativa* have therefore also been chosen for intensive study, in order to explore symbiotic nitrogen fixation and monocot biology, respectively. In addition, to understand the evolutionary origins and mechanisms underlying developmental processes in seed plants necessitates the examination of similar developmental processes in diverse taxa.

Perennial woody plants are often closely related to annual herbaceous plants, yet woody species possess structural and lifestyle characteristics that differ dramatically from herbaceous annuals. *Arabidopsis* is thus not necessarily a good model for the study of arboreal traits, despite close taxonomic relationships with woody relatives. In light of the ecological and commercial importance of trees across the terrestrial landscape it has been clear for some time that, in order to address traits characteristic of woody plants and forest trees, a suitable "model tree" should be identified, around which key genetic and genomics resources could be developed (Fig. 1).

The primary contenders for this designation fell into two obvious classes – gymnosperms and angiosperms. From a commercial perspective, there was no question that conifers (*Pinus*, *Picea*, *Abies*, and *Larix*) dominated both the marketplace and much of the temperate landscape. Significant conifer genetic resources have therefore – for commercial purposes – been built that would be useful also for genomic research However, countering gymnosperms obvious utility were some serious experimental disadvantages, such as massive genome sizes, long generation times, inefficient transformation procedures and a relatively underdeveloped biological knowledge base. Among the angiosperm tree species, those in contention

Fig. 1 Which tree is the best model species? Photo from October 21 2007, by "Ragesoss" from Wikimedia commons (http://commons.wikimedia.org/wiki/Image:Autumn_leaves,_Talcott_Mountain_State_Park.jpg)

included poplar/aspen (*Populus*), willow (*Salix*), birch (*Betula*) and *Eucalyptus*, but at the time when active debate over selection of a model tree was underway, *Populus* already possessed two major advantages. One was a combination of several desirable biological traits, such as a modest genome size, facile genetic transformation, ease of vegetative propagation, rapid growth response after experimental treatments and a short generation time compared to most other forest trees. The other was the considerable body of baseline research and development already being conducted with *Populus* hybrids in Europe and North America (see below), driven largely by their exceptional vigor and commercial potential for short-rotation forestry.

2 Key Events That Led to Adoption of *Populus* as the Prime Tree Model System

The experience of the plant biology community with the power of gene manipulation in *Arabidopsis* for revealing gene function made the ability to efficiently transform any model tree a particularly high priority. Transformation capability in *Populus* had been examined soon after general methods for plant transformation and regeneration were first established in 1984–85, and the first publication on regenerated transgenic poplar occurred in 1987 (Filatti et al. 1987). Leading researchers in poplar transformation included M. Gordon and E. Nester at the University of Washington (USA), where many of the pioneering advances in *Agrobacterium* biology were made, and B. McCown in Wisconsin (USA), who had long worked on in vitro systems for poplar regeneration. M. DeBlock and W. Boerjan (Belgium), L. Jouanin and G. Pilate (France), A. Seguin (Quebec), and R. Meilan and S. Strauss (Oregon) had all produced and field tested transgenic poplars in the late 1980s and early 1990s. These studies demonstrated convincingly that phenotypically stable traits could be readily produced in *Populus* spp. using transgenic methods. The rate of somaclonal variation has been reported to be very low, and the stability in transgene expression very high, in transgenic *Populus*.

Clonal propagation of select genotypes is another important trait amongst non-domesticated forest tree species. Many forest trees are difficult to vegetatively propagate, or show substantial "maturation effects" after propagation that confound genetic differences. For example, many *Eucalyptus* species root poorly and in many other species, rooted cuttings become increasingly difficult with age of the parent tree. The derived plants also often show variable degrees of maturation effects, such as slow growth and modified wood properties. The success and uniformity in response to micropropagation and other tissue culture methods also declines with age. The maturation effects tend to be much smaller for *Populus* than for most other taxa of forest trees, which means that trees of a variety of ages, and varying tissue sources, can be used to establish clonal populations whose primary differences are genetic rather than physiological in origin.

As genomics technologies became more broadly accessible to the life science community and sequencing of more complex eukaryotic genomes gained momentum in the late 1990s it became clear that producing a genome sequence for a model

plant could launch a new era in plant biology research. Publication of the 125 Mb *A. thaliana* genome sequence in 2000 was indeed a landmark event in plant science, but interestingly, part of the justification for this first plant genomics effort was its potential impact on improvement of agricultural crops and forest productivity. Given the many unique characteristics of trees, the power of having an *Arabidopsis* genome sequence available only whetted the appetite of the tree biology community for similar "global biology" resources devoted to a "model tree", and the lobbying began in earnest.

In the early 1990s, three different efforts began to converge on the choice of *Populus* as the "model tree". In Sweden, researchers at the Swedish University of Agricultural Sciences in Umeå were successful in genetically transforming *Populus* and in 1997, the Swedish *Populus* genome program was launched as a collaboration between researchers in Umeå (both at Umeå University and the Swedish University of Agricultural Sciences) and Stockholm (the Royal Institute of Technology, KTH). ESTs were sequenced from wood-forming tissues and other sources. Spotted DNA microarrays were produced and, again, first used to study wood-formation but later many other processes, and the third generation array contained 25 k features. The primary motivation for choosing *Populus* as the model tree for the Swedish effort was purely scientific – transformability allowing for functional analysis. The genotype that was used for transformation (T89) is a hybrid between *P. tremula* and *P. tremuloides*, but EST sequencing was also performed on tissue from *P. tremula* growing naturally in the area, and also from *P. trichocarpa*. In total, over 100,000 ESTs were sequenced. *Populus* proteomics and metabolomics were also being developed at the time, and a gene knockout project and the first public *Populus* EST database were launched. Although full genome sequencing was discussed in Sweden, such an enterprise appeared beyond reach, considering the estimated cost of the project.

In Canada, intense lobbying by the biomedical research community, spearheaded by M. Smith, had finally convinced the federal government to commit some major research funding specifically to large-scale genomics projects that would be relevant to Canada. The vehicle for this activity was a new foundation (Genome Canada), where funds would be awarded on a competitive basis. In the first Genome Canada competition, two multi-million dollar forest tree genomics projects were funded – *Treenomix* (based in the University of British Columbia, Vancouver) and *Arborea* (based in Laval University, Quebec), both of which incorporated some component of *Populus* genomics research, as well as work on conifers. The focus on conifers in these projects reflected the reality of Canadian forestry, which relies almost exclusively on harvesting coniferous species. Interestingly, however, the inclusion of *Populus* was justified on the grounds that it had already become the de facto "model tree" from a genomics perspective, as attested by the recent development of the *Populus* EST resource in Sweden.

In the USA in 1990 *Populus* was selected as a U.S. Department of Energy's model woody crop. Funding through this program was managed by G. Tuskan and included research at many universities and government laboratories. At a Poplar Genome Steering Committee meeting in Portland, Oregon, on November 14, 2001, a sequencing strategy was presented, and bioinformatics and genetic resources

and other issues were discussed, not only by US but also Canadian and Swedish researchers. During this period, intensive work to lay down the strategy took place, and in 2002, as the Human Genome efforts at DOE were winding down, there was a petition within DOE to utilize the high-throughput sequencing capacity at the Joint Genome Institute (JGI) to address DOE-relevant missions. *Populus* was nominated, reviewed by an external committee and accepted in 2002. At that time, *Populus* represented the largest, most complicated genome to be sequenced, assembled and annotated by a single facility.

3 The *Populus* Genome Sequencing

The *Populus* clone chosen for sequencing was the female clone *Nisqually-1*, originally collected by R. Stettler along the Nisqually River south of Seattle. This clone had been used in control crosses, and a 10X BAC library had been created as part of a QTL cloning effort. After being dubbed the extra-ordinary *Populus* genotype, scions of this genotype are now growing replicated in several places around the world (Fig. 2).

Sequencing began in earnest in 2003 and was met with a number of serious challenges. *Populus*, like other perennial plants, is comprised of multiple genomes, i.e., the nuclear genome, the mitochondrial genome, the chloroplast genome and the genomes of multiple endophytes. Shotgun sequencing such a “mega-genome” had never been attempted before. First, although DNA is found in all living tissues within a plant, the quality and quantity of high-molecular weight DNA that can be

Fig. 2 Nisqually-1 growing in a greenhouse in Umeå Sweden (flanked by two of the editors of this volume)

extracted varies with tissue type. Young, partially expanded leaves provide the highest quantity of DNA per unit of volume of tissue. However, these tissues also contain very large amounts of mitochondrial and chloroplast DNA. The first libraries prepared for the shotgun sequencing were prepared using DNA from young leaves, and although efforts were made to reduce the amount of organellar DNA in the preparations, these libraries contained too much organelle DNA (≈10% chloroplast DNA) to allow for cost-efficient sequencing. To reduce the amount of organellar DNA, root tips were selected and DNA template was isolated using a sucrose gradient to separate the nuclei from organelles, followed by cesium chloride gradient centrifugation and pulsed-field gel electrophoresis. This approach successfully eliminated the majority of organellar DNA, although over 40 partial putative endophyte genomes remained in the template pool. Of these endophytes, six genomes have subsequently been fully sequenced, assembled and annotated – *M. populi* BJ001, *S. maltophilia*, *S. proteamaculans*, *Enterobacter* sp. 638, *Burkholderia cepacia* Bu72 and *P. putida* W619 [http://www.jgi.doe.gov/]

Once high-molecular weight DNA template was obtained it was used to create three cloning libraries with 3 , 8 and 40 kb inserts that were characterized using 700 bp end-reads from a bank of capillary sequencing machines. The first two billion high-quality bases were subjected to several rounds of assembly, each giving more complete coverage of the genome. The first draft assembly was completed in November 2003 and represented 384 Mb of captured sequence, with the 100 largest scaffolds containing ca 50% of the sequence. During the first half of 2003 2.2 billion additional bases were sequenced and added to the assembly. In early 2004, the final draft assembly was completed and represented 429 Mb contained in 2447 scaffolds, with N50 scaffold size of 1.9 Mb and N50 scaffold number of 58. With the aid of newly created physical and genetic maps, these sequence scaffolds were used to create a linear combination of 19 chromosomal units.

Preparatory work for gene modeling and annotation in *Populus* was occurring simultaneously with the assembly. Modeling gene structure (i.e. intron and exons, and transcribed but untranslated regions) in a new organism requires both robust algorithms and high-quality training sets, typically ESTs and/or full-length cDNA sequences. In this stage of the project, the entire *Populus* community rallied to provide relevant EST sequences. About ten groups throughout the world that had been creating small or large scale EST data sets provided their sequence data to create the training set for gene-calling algorithms. With three bioinformatics groups working on gene modeling, the strategy was to initially let each group independently perform individual gene calls on the assembled sequence. Three ab initio gene prediction algorithms, EUGENE, GRAIL, and FgenesH, were trained based on over 5,000 true and in silico full-length cDNAs and a pool of around 500,000 EST sequences. Even though the EST dataset was rather extensive and the average *Populus* gene shares high similarity with orthologous *Arabidopsis* genes, the different algorithms – or even the same algorithm but with different settings – produced results that were quite variable. Not surprisingly, genes with good EST support were often identically predicted while in the absence of ESTs, results could be confusing (Fig. 3).

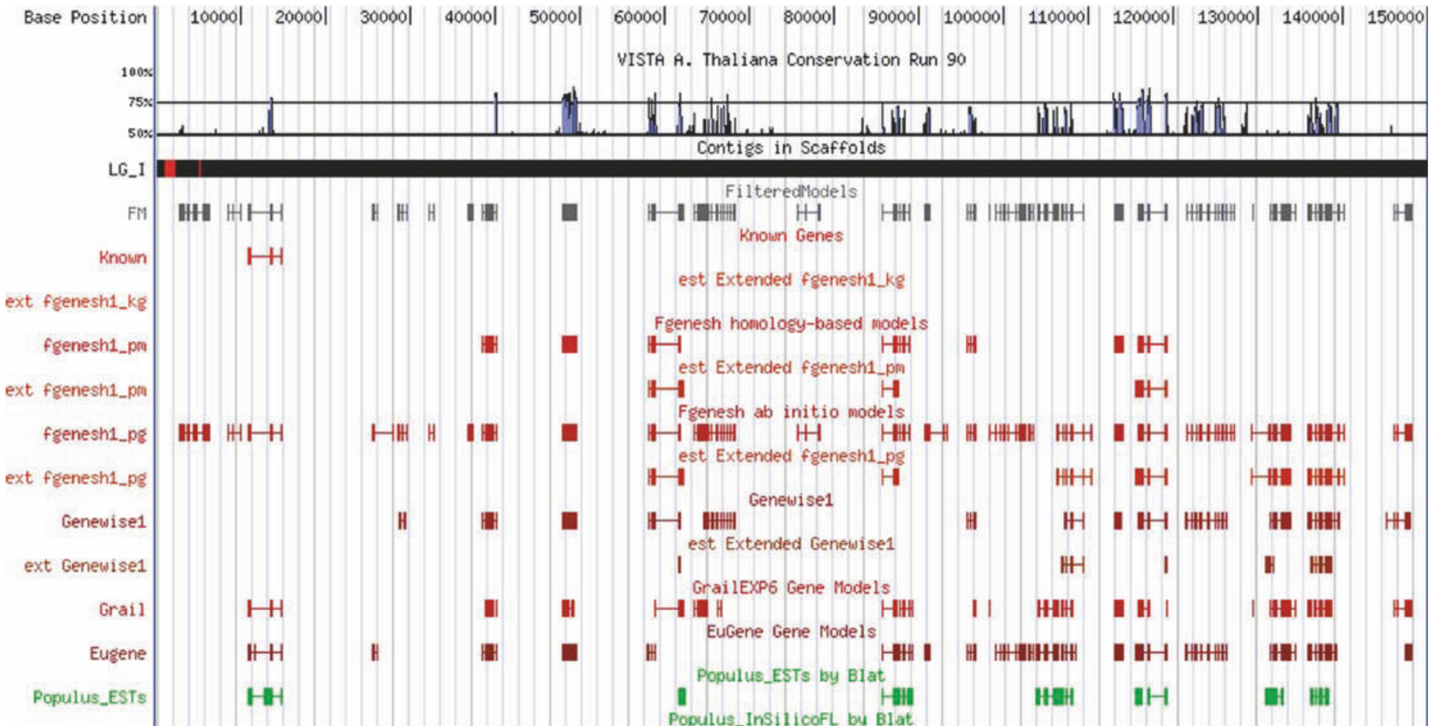

Fig. 3 Which gene model to choose? Genes predicted by FgenesH, Genewise, Grail and Eugene and EST coverage at the first 150 kb of LG I – a difficult region – in the first version of the Populus Genome Browser. The track at the top (FM, filtered models) represents the models that went on into the "Jamboree set"

To improve the annotation, repeat libraries were identified and used to mask repeat regions (e.g., transposable elements) prior to gene calling. These steps were finalized in the summer of 2004. In an effort to restrain the ab initio gene calls, a protocol was developed for collapsing the gene models from the different algorithms into a "Jamboree set" of genes. Of 55,054 predicted loci, 45,500 gene models were promoted and used to annotate the assembled genome. In September 2004, a database containing the genome sequence was made public and a worldwide press release was issued. Many research groups throughout the world contributed to this step, both "at home" but in particular during the *Populus* Genome Annotation Jamboree in Walnut Creek, California in December 2004. Since the release of 45,500 gene models, roughly 5,000 have been manually curated. From these efforts it was apparent that the *Populus* genome contained large paralogous segments that contained syntenic duplicated gene sets. Further analysis of the genome (e.g., the duplication event, non-coding RNAs, expression studies and whole-genome arrays) continued through 2005 and in April 2006 a manuscript describing the results was

Fig. 4 The cover of Science on September 15, 2006

Fig. 5 Ceremonial planting of Nisqually-1 at JGI by Jerry Tuskan and Dan Rokhsar

submitted to Science. The manuscript was accepted on August 9 and published on September 15, 2006 (Tuskan et al. 2006, Figs. 4 and 5). The increased scientific interest in *Populus* (Fig. 6) has of course been much influenced by the genome sequencing effort.

Although the publication marked the formal end of the *Populus* genome sequencing project, the work did not stop. For example, two additional *Populus* genomes have recently been resequenced by JGI using the Solexa short-read platform, over 2 million EST/cDNAs have been sequenced using the 454 platform, and a BAC minimum tiling path and QTL tracks have been added to the JGI *Populus* browser [http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html]. A second assembly based on subcloning BACs and primer walking, as well as a second annotation that draws upon the newly available EST set, are both scheduled for completion in 2009.

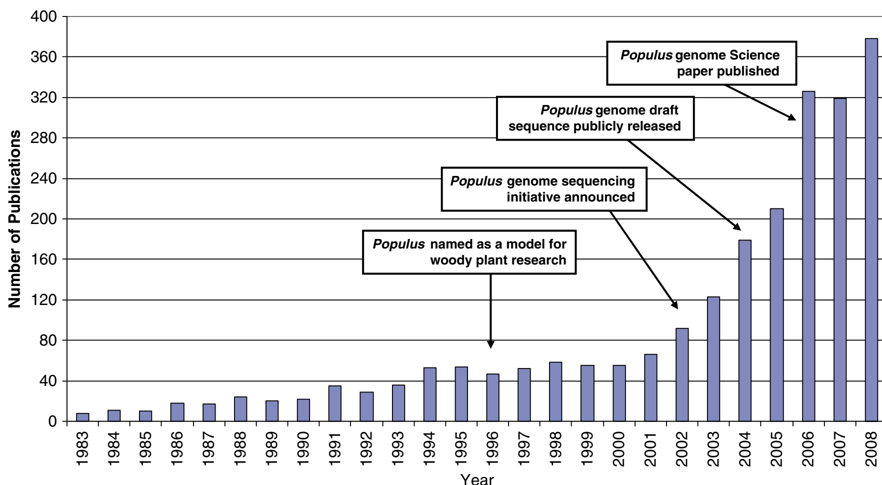

Fig. 6 Populus publomics

4 *Populus* Biotechnology and Breeding – Past and Future Visions

The ability of genomics methods to generate new DNA sequence data, and thus new possibilities for research and breeding, is growing rapidly. This trend is likely to continue for many years. However, the extent to which this knowledge is translated into benefits for society depends on social and economic factors that are difficult to predict. For example, the impact of transformation is extremely powerful for trees, in contrast to annual crops, since transformation allows for the introduction of new traits directly into elite germplasm without rounds of sexual propagation. This is especially important in trees, where genetic gains of clones (specific combining ability) are lost during outcrosssing. However, the ability to use transgenic traits is at present highly restricted, even for field research. Commercial applications are largely restricted to China, as a result of regulation and marketplace factors, and thus investments in applied research by government and industry sources outside of China are limited. This restriction may even grow greater if the pressures for living transgenic trees being incorporated into negotiations under the Cartagena Protocol on Biodiversity continue to grow (Strauss et al. 2009). In addition, in contrast to food crops, simply inherited and quality traits are rarely of major importance in forest tree breeding. The complex traits that are important, such as yield, adaptability, and wood quality are far more difficult to link to major genes. It is therefore unclear to what extent the limited numbers of molecular markers that are robustly identified in QTL and association genetics studies will be useful for marker-aided breeding. High levels of linkage equilibrium require that very large numbers of markers are employed to enable whole-genome marker selection, thus challenging current genotyping platforms and economics. This is especially true for

hybrid poplar programs, which are likely to have a complex QTL structure, and for which there is extensive genetic variation already present that can be captured by short-term trials and cloning without the use of markers. Thus, the economic driver for translational poplar genomic research is uncertain and reflects in part costs associated with sequencing and informatics. Whether translational research will take place with the expected growth of lignocellulosic bioenergy crops remains to be seen; it is likely that this will be highly influenced by costs and efficiencies of new sequencing and informatics technologies.

It is difficult to predict what scientists working with tree genetics and genomics will have achieved by 2020, and where future research emphasis may grow. It is, however, safe to assume that the present stage of *Populus* research will look rather primitive in 2020. The enormous advances in sequencing and profiling techniques will allow for full genome sequencing not only for additional species but also of a vast number of individuals of each species. When combined with thorough characterizations of the transcriptome, proteome, metabolome, lipidome, phosphorylome, etc. new systems-based approaches will be enabled that will more accurately model complex, multigenic traits such as maturation and wood formation. In the past, forest tree research has been limited by technical challenges. In the future, our understanding will increasingly be limited by our ability to pose relevant biological questions, accurately measure the phenotype of each genotype, dissect the relevant biological processes down to the subcellular level, and understand the complexity of genetic networks and signaling pathways in a scientific context that is related to the natural environment, where trees and other organisms constantly interact.

As discussed above, the selection of *Populus* as a model forest trees was highly influenced by practical issues. However, these considerations do not typically coincide with evolutionary or taxonomic realities for trees. For example, *Populus* is much more closely related to Arabidopsis and annual crop species than to coniferous tree species. Woodiness is possibly the ancestral state for angiosperms, and secondary growth and wood formation may even have homologous origins in angiosperms and gymnosperms. Increasingly, it will be vital to consider evolution of woody growth and taxonomic relationships in the study of trees. Perhaps the greatest contribution of *Populus* genomics research will be to identify in detail the basal mechanisms underlying secondary growth, wood development, maturation and perennial habit, ultimately providing a view of the evolution and development of perennial seed plants.

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Salient Biological Features, Systematics, and Genetic Variation of *Populus*

Gancho T. Slavov and Peter Zhelev

Abstract The genus *Populus* includes morphologically diverse species of deciduous, relatively short-lived, and fast-growing trees. Most species have wide ranges of distribution but tend to occur primarily in riparian or mountainous habitats. Trees from this genus are typically dioecious, flower before leaf emergence, and produce large amounts of wind-dispersed pollen or seeds. Seedlings are drought- and shade-intolerant, and their establishment depends on disturbance and high soil moisture. Asexual reproduction is common and occurs via root sprouting and/or rooting of shoots. Fossil records suggest that the genus appeared in the late Paleocene or early Eocene (i.e., 50–60 million years BP). According to one commonly used classification, the genus is comprised of 29 species divided into six sections, but a number of phylogenetic inconsistencies remain. Natural hybridization both within and among sections is extensive and is believed to have played a major role in the evolution of extant species of *Populus*. Both neutral molecular markers and adaptive traits reveal high levels of genetic variation within populations. Deviations from Hardy–Weinberg equilibrium are commonly detected in molecular marker studies. These deviations typically have small to moderate magnitudes and tend to be caused by heterozygote deficiency, indicating the possible existence of population substructure. Genetic differentiation among populations is much stronger for adaptive traits than for neutral markers, which suggests that divergent selection has played a dominant role in shaping patterns of adaptive genetic variation. Molecular and bioinformatic resources are actively being developed for multiple species of *Populus*, which makes this genus an excellent system for studying tree genetics and genomics.

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1 Dendrological Overview

1.1 Morphology

Species of the genus *Populus* (commonly known as aspens, cottonwoods, and poplars) are deciduous or, rarely, semi-evergreen trees that occur primarily in the boreal, temperate, and subtropical zones of the northern hemisphere (Eckenwalder, 1996; Dickmann, 2001; Cronk, 2005). Trees from this genus typically have tall and straight single trunks, with bark that tends to remain thin and smooth until more advanced ages than in other tree species (Eckenwalder, 1996; Dickmann, 2001). They rarely live longer than 100–200 years, but are among the fastest growing temperate trees and can reach large sizes. A notable example is black cottonwood (*Populus trichocarpa*), which can exceed 60 m in height and reach up to 3 m in diameter (DeBell, 1990).

Leaves are alternate and simple, with pinnato-palmate venation, and petioles are often transversally flattened distally (Eckenwalder, 1996). Leaf size, shape, and toothing are extremely variable among species, but also within a single tree and among trees within a species (e.g., Fig. 3 in Eckenwalder, 1996; Fig. 4 in Dickmann, 2001). Within-tree and within-species variation in leaf characteristics can largely be attributed to two sources of developmental heteromorphism (Eckenwalder, 1980). First, heteroblasty (i.e., differences in leaf characteristics between juvenile and adult trees) is common in *Populus*. Second, there is substantial seasonal heterophylly because shoots on *Populus* trees have both preformed leaves (i.e., expanded from well-formed primordia that overwinter in vegetative buds) and neoformed leaves (i.e., initiated during the current growing season). Preformed and neoformed leaves can differ substantially (e.g., Critchfield, 1960), with preformed leaf characteristics typically having higher taxonomic value (Eckenwalder, 1996).

Except for *P. lasiocarpa*, *Populus* species are mostly dioecious, although the occurrence of hermaphroditic trees has been reported in multiple species (Rottenberg, 2000; Rowland et al., 2002; Cronk, 2005; Slavov et al., 2009). Both male and female flowers are grouped in pendent catkins. Perianths are strongly reduced, with 5–60 stamens or 2–4 carpels borne on wide floral disks (Boes and Strauss, 1994; Eckenwalder, 1996). After pollination, female flowers develop into capsules that, upon dehiscence, release 2–50 light seeds (>300,000 seeds/kg; Schreiner, 1974) with cottony hairs (Boes and Strauss, 1994; Eckenwalder, 1996).

Winter twigs range from slender to moderately stout (more rarely stout), from glabrous to lightly pubescent, and can be yellow- or greenish-brown, reddish, or gray, typically with conspicuous lighter-colored lenticels (Seiler and Peterson, http://www.cnr.vt.edu/dendro/). Vegetative buds are long (0.5–2.5 cm), conical, and sharp-pointed (Seiler and Peterson, http://www.cnr.vt.edu/dendro/). They are covered by several bud scales, the most basal of which is oriented away from the stem, and are often impregnated with resinous hydrophobic exudates (Eckenwalder, 1996). Reproductive buds are often found in clusters and, in some species, are

distinguishable between the sexes and from vegetative buds based on size and shape (Stanton and Villar, 1996).

1.2 Habitat

Most species of *Populus* have wide native ranges, often spanning more than 20 degrees of latitude and a great diversity of climates and soils (Eckenwalder, 1996; Dickmann, 2001). *Populus* trees grow in a striking variety of habitats, ranging from hot and arid, desert-like sites in central Asia and northern and central Africa to alpine or boreal forests in Europe and North America (e.g., Fig. 1). They are shade- and drought-intolerant, and seed establishment typically depends on major disturbances, such as fire, floods, or ice scours (Romme et al., 1995, 1997; Rood et al., 2007).

Populus trees tend to occur in two general categories of habitats. First, many species typically grow in riparian areas and wetlands characterized by seasonal flooding and high water tables, with optimal establishment conditions occurring on fresh silt and sand, immediately following the recession of water from point bars and gravel bars (Braatne et al., 1996; Dickmann, 2001). One example is black cottonwood (*P. trichocarpa*), which is common in riparian areas of the Pacific Northwest of North America (Fig. 1a). Some riparian species, however, are extremely phreatophytic (i.e., deep-rooting). Euphrates poplar (*P. euphratica*), for example, can grow under remarkably hot, dry, and high-salinity conditions (e.g., Fig. 1b), with water tables as deep as 10–13 m (Ma et al., 1997; Hukin et al., 2005; Ferreira et al., 2006; Thevs et al., 2008). Second, aspens and some white poplars (section *Populus*; Table 1) grow primarily in mountainous or upland habitats. The North-American quaking aspen (*P. tremuloides*), for example, occurs at elevations up to 3,500 m (e.g., Fig. 1c) and grows best on well-drained, loamy soils, with water tables between 0.6 and 2.5 m, although it can also establish and grow on ash-covered soils, shallow soils on rock outcrops, landslides, mine waste dumps, and borrow pits (Perala, 1990; Dickmann, 2001).

1.3 Life History

1.3.1 Sexual Reproduction

Under favorable conditions, *Populus* trees reach reproductive maturity within 4–8 years in intensively managed plantations and within 10–15 years in natural populations (Stanton and Villar, 1996). Reported sex ratios for natural populations of various *Populus* species range from female-biased to balanced (approximately 1:1) and male-biased (reviewed by Farmer, 1996; Braatne et al., 1996; Stanton and Villar, 1996; see also Gom and Rood, 1999; Rowland and Johnson, 2001; Hultine et al., 2007). Although no consistent pattern has emerged, several of these studies suggest

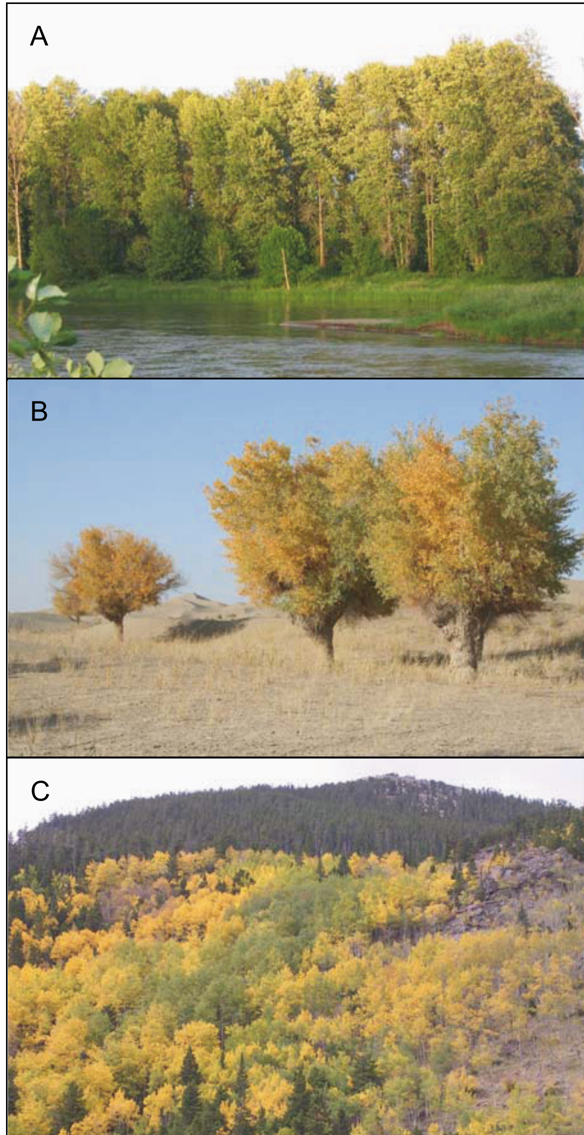

Fig. 1 An example of the diverse habitats occupied by species of *Populus*. (**a**): *P. trichocarpa* along the Willamette River in Oregon, USA (photograph courtesy of Steve DiFazio); (**b**) *P. euphratica* south of the Taklamakan Desert, Xinjiang, China (photograph courtesy of Pavel Sekerka); (**c**) *P. tremuloides* in the Rocky Mountain National Park, Colorado, USA (photograph courtesy of Amy Brunner)

that site-specific biases in sex ratio may be present, with female trees predominating (1) at sites with abundant moisture and nutrients and (2) at lower elevations, whereas males may be more common at high elevations, as well as in warmer, drier,

Table 1 Classification of *Populus* following Eckenwalder (1996)

Section (synonym)	Species	Distribution
Abaso Eckenwalder	*Populus mexicana* Wesmael	Mexico
Turanga Bunge	*P. euhratica* Olivier	NE Africa, Asia
	P. ilicifolia (Engler) Rouleau	E Africa
	P. pruinosa Schrenk	Asia
Leucoides Spach	*P. glauca* Haines *sl* [a]	China
	P. heterophylla L.	USA
	P. lasiocarpa Olivier	China
Aigeiros Duby	*P. deltoides* Marshall *sl* [a]	N America
	P. fremontii S. Watson	USA
	P. nigra L.	Eurasia, N Africa
Tacamahaca Spach	*P. angustifolia* James	N America
	P. balsamifera L.	N America
	P. ciliata Royle	Himalayas
	P. laurifolia Ledebour	Eurasia
	P. simonii Carrière	E Asia
	P. suaveolens Fischer *sl* [a]	NE China, Japan
	P. szechuanica Schneider	E Eurasia
	P. trichocarpa Torrey & Gray	N America
	P. yunnanensis Dode	Eurasia
Populus (*Leuce* Duby)	*P. adenopoda* Maximowicz	China
	P. alba L.	Europe, N Africa, Central Asia
	P. gamblei Haines	E Eurasia
	P. grandidentata Michaux	N America
	P. guzmanantlensis Vazques & Cuevas	Mexico
	P. monticola Brandegee	Mexico
	P. sieboldii Miquel	Japan
	P. simaroa Rzedowski	Mexico
	P. tremula L.	Europe, N Africa, NE Asia
	P. tremuloides Michaux	N America

[a] *Sensu lato*

and more extreme environments. This biologically interesting hypothesis, however, needs to be tested through replicated, large-scale studies that adequately account for (1) developmental differences between the two genders (i.e., male trees in some species may reach reproductive maturity before female trees, thus possibly skewing sex ratios; Stanton and Villar, 1996) and (2) the extensive clonality which is characteristic of many species of *Populus* (discussed below).

With the exception of some subtropical species, flowering occurs before leaf emergence in early spring (Braatne et al., 1996; Eckenwalder, 1996). Individual trees flower for 1–2 weeks (Stanton and Villar, 1996; G. T. Slavov and S. P. DiFazio, unpublished data), but the pollination period in a population can exceed one or even two months (Braatne et al., 1996). The relative timing of flowering follows a temperature-dependent progression, with populations at higher-elevations, more

northern latitudes, and more continental climates flowering later (e.g., DeBell, 1990; Perala, 1990; Zasada and Phipps, 1990; Braatne et al., 1996). Pollen is dispersed by wind, and effective long-distance pollination can be extensive (Tabbener and Cottrell, 2003; Lexer et al., 2005; Pospíšková and Šálková, 2006; Vanden Broeck et al., 2006; Slavov et al., 2009). Fertilization occurs within 24 h after a viable pollen grain has landed on a receptive stigma (Braatne et al., 1996). Capsules typically dehisce 4–6 weeks (but in some species and populations from 2–3 weeks to 3–5 months) after fertilization, which tends to coincide with snowmelt runoff, when microsites favoring seed establishment are most abundant (Braatne et al., 1996; Stella et al., 2006). Seeds are produced in great numbers (>25 million per tree per year, Braatne et al., 1996), and can potentially be dispersed over very long distances by wind and water (Braatne et al., 1996; Karrenberg et al., 2002), but direct empirical data on seed dispersal distances are limited (DiFazio, 2002). Under natural conditions, seeds retain viability for only 1–2 weeks and germination occurs within 24 h (Braatne et al., 1996; Karrenberg et al., 2002). On appropriate microsites, seedlings establish in great numbers (e.g., up to 4,000 m^{-2}), but mortality in the first year is typically high (i.e., up to 77–100%), primarily as a result of desiccation, prolonged flooding, and scouring (Braatne et al., 1996; Karrenberg et al., 2002; Dixon, 2003; Dixon and Turner, 2006).

1.3.2 Asexual Reproduction

Vegetative propagation is one of the distinctive characteristics of the genus (Dickmann, 2001). The means of asexual reproduction and the extent of clonality, however, differ dramatically among species (Braatne et al., 1996; Schweitzer et al., 2002; Rood et al., 2003, 2007). The North-American quaking aspen (*P. tremuloides*), for example, propagates vegetatively through root sprouting (i.e., formation of adventitious shoots on shallow lateral roots, a process also referred to as root suckering; Perala, 1990). Because of the rare opportunities for seedling establishment, root sprouting is believed to have been the primary means of reproduction of *P. tremuloides* in the Rocky Mountains over the last century (Romme et al., 1995, 1997, 2005). The existence of extremely large, and possibly ancient, quaking aspen clones has been reported (Mitton and Grant, 1996), although the direct verification of extreme clone ages and sizes remains a technical challenge (Ally et al., 2008; Mock et al., 2008; DeWoody et al., 2008). In other species, such as the North-American cottonwoods *P. balsamifera*, *P. trichocarpa*, and *P. angustifolia*, and the European black poplar (*P. nigra*), asexual reproduction occurs commonly both through root sprouting and through rooting of shoots from broken branches or entire tree trunks that have been toppled during storms and floods and then buried in sediment (Braatne et al., 1996; Rood et al., 2003, 2007; Barsoum et al., 2004; Smulders et al., 2008). In a third group, including the North-American plains cottonwood (*P. deltoides*) and Fremont cottonwood (*P. fremontii*), asexual reproduction is relatively rare and occurs primarily via rooting of shoots (Braatne et al., 1996; Gom and Rood, 1999; Schweitzer et al., 2002; Rood et al., 2003, 2007).

2 Systematics and Evolution

2.1 Fossil Record

Fossil materials, some of which date back to the Cretaceous, have often been missclassified as belonging to *Populus* (Cronk, 2005), but the most ancient undisputed fossil records (e.g., Manchester et al., 1986, 2006) suggest that the genus appeared no later than the Eocene, and probably as early as the late Paleocene (about 60 million years BP). Fossil records dating from the Eocene and the Oligocene are relatively abundant and are available in many parts of the northern hemisphere (Manchester et al., 1986, 2006; Ramírez and Cevallos-Ferriz, 2000; Iljinskaja, 2003). Presumably, precursors of all extant sections of *Populus* (Table 1) were present by the Miocene (Eckenwalder, 1996; Cronk, 2005).

2.2 Relationships to Salix *and Other Families*

Traditionally, *Populus* and its "sister" lineage, the genus *Salix* (willows), have been considered the only two genera in the Salicaceae family, although some taxonomists have included other genera, mostly from Eastern Asia (Eckenwalder, 1996). More recently, however, the Flacourtiaceae family, the closest relative of Salicaceae, was re-classified, and a number of genera formerly included in Flacourtiaceae are now assigned to Salicaceae *sensu lato*, within the Malpighiales order of the "Eurosid I" clade (Chase et al., 2002; Angiosperm Phylogeny Group, 2003).

The availability of molecular data and sophisticated methods of phylogenetic analysis has revolutionized plant classification (Soltis and Soltis, 2001; Angiosperm Phylogeny Group, 2003; Soltis et al., 2005). Recent molecular phylogenetic studies in Salicaceae (Leskinen and Alström-Rapaport, 1999; Hamzeh and Dayanandan, 2004; Cervera et al., 2005; Hamzeh et al., 2006) showed that *Populus* and *Salix* clearly form two separate groups. Interestingly, in one of these studies the presumably most ancient species of *Populus* (*P. mexicana*; Eckenwalder, 1996) showed higher similarity to *Salix* than to any other species of *Populus* (Cervera et al., 2005). While studies designed specifically to clarify the status of *P. mexicana* will probably resolve this issue in the near future, identifying the common ancestor of *Populus* and *Salix*, and establishing whether both genera are monophyletic natural groups remain wide-open questions.

2.3 Classification

The number of species included in the genus *Populus* varies among classifications from as few as 22 to as many as 85 (Eckenwalder, 1996). Two reasons for these drastic differences are the misclassification of natural hybrids (discussed below) as "true" species and the philosophical differences between "splitter" and "lumper" taxonomists (Eckenwalder, 1996). One classification that is commonly

used in recent years is that of Eckenwalder (1996), who recognized 29 species subdivided into six sections based on relative morphological similarity and crossability (Table 1). A consensus cladogram from the 840 most parsimonious trees built based on 76 morphological characters (Fig. 6 in Eckenwalder, 1996) provided evidence that all sections except for *Tacamahaca* are monophyletic. Section *Tacamahaca* was split into two monophyletic groups, one comprised of "typical balsam poplars" (e.g., *P. balsamifera* and *P. trichocarpa*) and the other one comprised of "narrow-leaved, thin-twigged" species (e.g., *P. angustifolia*, *P. simonii*). Combining fossil records with information from this consensus tree, Eckenwalder (1996) speculated that (1) after the original spread of the genus from either North America or Asia in the Paleocene, the two "primitive" subtropical sections, *Abaso* and *Turanga* were split by a vicariance event, (2) temperate habitats were first invaded by species from section *Leucoides*, and (3) the remaining "advanced" sections evolved rapidly in the Miocene.

More recent molecular studies provide only partial support for this evolutionary scenario and clearly conflict with some of its aspects (Hamzeh and Dayanandan, 2004; Cervera et al., 2005; Hamzeh et al., 2006). The most parsimonious tree based on 151 Amplified Fragment Length Polymorphisms (Cervera et al., 2005), for example, suggests that section *Populus* (referred to as *Leuce* in this study) is the most "primitive" section in the genus, which is diametrically opposed to Eckenwalder's interpretation based on morphological traits. A number of inconsistencies in the classification of *Populus* remain. Their successful resolution will likely require integration of abundant molecular genetic and genomic data with informative morphological traits and the fossil record (Soltis and Soltis, 2001; Delsuc et al., 2005).

2.4 Natural Hybridization

Hybridization is believed to have played a major role in the evolution of extant species of *Populus* (Eckenwalder, 1996; Hamzeh and Dayanandan, 2004; Cervera et al., 2005; Hamzeh et al., 2006). The existence of relict hybrids (i.e., hybrids occurring far away from the current distribution of one or both of the presumed species), and extensive contemporary hybridization both within and among sections has been documented based on morphological traits and molecular markers (e.g., Table 2; Eckenwalder, 1984a, b, c; Rood et al., 1986; Campbell et al., 1993; Martinsen et al., 2001; Floate, 2004; Lexer et al., 2005; Hamzeh et al., 2007).

Hybridization plays a key role in *Populus* domestication (Stettler et al., 1996). Naturally occurring hybrid zones, for example, provide a tremendous potential for admixture mapping, which can be a powerful complement to intraspecific genetic association studies (Lexer et al., 2004, 2007; Lexer and van Loo, 2006; Buerkle and Lexer, 2008). Finally, zones of hybridization between species of *Populus* have been among the primary study systems for the emerging field of community genetics (Whitham et al., 1999, 2003, 2006, 2008).

Table 2 Examples of naturally occurring hybrids of *Populus*

Hybrid	Scientific name
P. alba × *P. adenopoda*	*P.* × *tomentosa* Carrière
P. alba × *P. tremula*	*P.* × *canescens* (Aiton) Smith
P. angustifolia × *P. balsamifera*	*P.* × *brayshawii* B. Boivin
P. angustifolia × *P. deltoides*	*P.* × *acuminata* Rydb.
P. angustifolia × *P. fremontii*	*P.* × *hinckleyana* Correll
P. balsamifera × *P. deltoides*	*P.* × *jackii* Sargent
P. deltoides × *P. nigra*	*P.* × *canadensis* Moench
P. grandidentata × *P. tremuloides*	*P.* × *smithii* B. Boivin
P. trichocarpa × *P. deltoides*	*P.* × *generosa* Henry
P. trichocarpa × *P. fremontii*	*P.* × *parryi* Sargent

3 Genetic Variation

3.1 Molecular Markers

3.1.1 Allozymes and RFLP

As a result of their (1) obligately outcrossing mating systems, (2) relatively large population sizes, and (3) extensive long-distance pollen and seed dispersal, species of the genus *Populus* have high levels of genetic variation for neutral molecular markers. Early studies based primarily on allozyme markers and Restriction Fragment Length Polymorphisms (RFLP) depicted several basic aspects of the population genetics of *Populus* species (Table 3). First, levels of polymorphism (as measured by the average number of alleles per locus, A) and heterozygosity expected under Hardy-Weinberg equilibrium (H_e, also referred to as gene diversity; Nei, 1973) in *Populus* are close to the mean values for long-lived woody species ($A = 1.8$, $H_e = 0.15$) and are higher than those for plants in general ($A = 1.5$, $H_e = 0.11$; Hamrick et al., 1992). Second, while deviations from Hardy-Weinberg equilibrium are not uncommon and can be caused by both deficiency and excess of heterozygotes, the magnitudes of these deviations are typically small to moderate. Deviations caused by heterozygote deficiency (i.e., positive values of F_{IS}) are more common, indicating the possible existence of unaccounted population substructure (i.e., Wahlund Effect; Hedrick, 2005b). Finally, differentiation among populations as measured by F_{ST} (Wright, 1965) is typically weak, with differences among populations accounting for only 1–7% of the genetic variation. The median value of F_{ST} for the genus (0.047) is almost two times lower than the mean for long-lived woody species ($F_{ST} = 0.084$) and nearly five times lower than that for plants in general ($F_{ST} = 0.228$; Hamrick et al., 1992). The weak differentiation among populations is in good agreement with direct studies of gene flow, the findings of which suggest that long-distance pollination can be extensive in *Populus* (Tabbener and Cottrell, 2003; Pospíšková and Šálková, 2006; Vanden Broeck et al., 2006; Slavov et al., 2009).

Table 3 Allozyme and RFLP diversity and differentiation in *Populus*[a]

Section	Species	N_{loci}	N_{pop}	N	A	H_o	H_e	F_{IS}	F_{ST}	References
Turanga	*P. euphratica*	20[b]	3	85	1.8	0.10	0.24	0.592	–	Rottenberg et al. (2000)
Aigeiros	*P. deltoides*	33[b]	9	84	1.2	0.06	–	–	–	Rajora et al. (1991)
		22[b]	21	–	1.5	–	0.08	–	0.064	Marty (1984)
	P. fremontii	36[c]	4	47	1.5	0.18	0.15	−0.175	0.074	Martinsen et al. (2001)
	P. nigra	8[b]	3	146	–	–	0.16	0.113	0.063	Legionnet and Lefèvre (1996)
Tacamahaca	*P. angustifolia*	36[c]	10	281	1.4	0.10	0.08	−0.236	0.022	Martinsen et al. (2001)
	P. balsamifera	17[b]	5	248	–	–	0.04	0.061	0.014	Farmer et al. (1988)
	P. trichocarpa	18[b]	10	456	1.2	–	0.09	–	0.063	Weber and Stettler (1981)
Populus	*P. grandidentata*	14[b]	–	96	1.4	0.07	0.08	0.125	–	Liu and Furnier (1993)
		37[c]	–	75	1.8	0.12	0.13	0.077	–	Liu and Furnier (1993)
	P. tremula	11[b]	6	233	1.7	0.15	0.17	0.153	0.014	Easton (1997)
		10[b]	5	41	1.7	0.33	0.23	−0.427	–	Lopez-de-Heredia et al. (2004)

Table 3 (continued)

Section	Species	N_{loci}	N_{pop}	N	A	H_o	H_e	F_{IS}	F_{ST}	References
	P. tremuloides	26[b]	7	222	2.3	0.52	0.42	–0.238	–	Cheliak and Dancik (1982)
		15[b]	8	200	2.7	0.13	0.24	0.462	0.068	Hyun et al. (1987)
		10[b]	9	347	2.6	0.22	0.22	0.017	0.003	Lund et al. (1992)
		17[b]	6	156	2.4	0.32	0.29	–0.102	0.030	Jelinski and Cheliak (1992)
		13[b]	–	118	2.8	0.19	0.25	0.240	–	Liu and Furnier (1993)
		41[c]	–	91	2.7	0.21	0.25	0.160	–	Liu and Furnier (1993)
	Median[d]	18	7	146	1.8	0.17	0.17	0.077	0.047	

[a] N_{loci} is the number of loci used; N_{pop} is the number of populations sampled; N is the number of genets (or trees) analyzed; A is the average number of alleles per locus detected in each population; H_o is the observed heterozygosity; H_e is the expected heterozygosity (Nei, 1973); F_{IS} is the fixation index as reported in the study or calculated as $F_{IS} = (H_e–H_o)/H_e$; F_{ST} is the among-population differentiation (Wright, 1965).
[b] Allozyme markers.
[c] Restriction Fragment Length Polymorphisms.
[d] Because relatively few studies were included, medians were calculated in order to minimize the influence of extreme values.

Polymorphism and heterozygosity vary substantially among species of *Populus*, and even among studies in the same species (Table 3). Interestingly, however, both the number of alleles per locus and gene diversity appear to be consistently higher in *P. tremuloides* than in any other species of *Populus* (Table 3). Two life history peculiarities of *P. tremuloides* may provide an explanation for its elevated genetic variation.

First, this species is believed to reproduce almost exclusively asexually over much of its range (Mitton and Grant, 1996; Romme et al., 1995; 1997, 2005). Population genetics theory predicts that predominantly and strictly clonal organisms will have much lower genotypic diversity (i.e., fewer unique genotypes for a given number of individuals sampled) and (2) higher allelic diversity and heterozygosity (i.e., as a result of accumulation of mutations known as the "Meselson effect") compared to organisms with similar life histories but with predominantly sexual reproduction (Balloux et al., 2003; Halkett et al., 2005; de Meeûs et al., 2007). The first prediction appears to hold only partially in *P. tremuloides*. Aspen clones spanning large areas (i.e., up to 44 ha) have been discovered in the Rocky Mountains (Mitton and Grant, 1996; Mock et al., 2008; DeWoody et al., 2008; S.P. DiFazio et al., unpublished data). However, relatively high genotypic diversities have been observed in most studies (Hyun et al., 1987; Jelinski and Cheliak, 1992; Lund et al., 1992; Liu and Furnier, 1993; Yeh et al., 1995; Namroud et al., 2005; Mock et al., 2008), suggesting that sexual reproduction may be more frequent and/or its impact on the genetic structure of aspen populations may be more persistent than previously assumed. The second prediction, which under certain conditions holds even for low rates of asexual reproduction (Yonezawa, 1997; Yonezawa et al., 2004), appears to be consistent with empirical data. The median gene diversity from six studies of *P. tremuloides* ($H_e = 0.25$) is comparable to that from two studies of its "sister" species *P. tremula* in Europe ($H_e = 0.20$), and is more than two times higher than that for other species of *Populus* ($H_e = 0.09$). This agrees with the general trend in woody plants (mean $H_e = 0.25$ for species with both asexual and sexual reproduction vs. $H_e = 0.14$ for species that only reproduce sexually; Hamrick et al., 1992).

Second, the frequency of triploid aspen trees, at least in the Rocky Mountains, may be substantially higher than previously thought (Mock et al., 2008; S.P. DiFazio et al., unpublished data). The occurrence of triploids at high frequencies is expected to result in (1) increased gene diversity and (2) heterozygote excess relative to Hardy-Weinberg predictions for a population of diploids (i.e., because phenotypes with two different alleles would occur more frequently than in a population comprised of strictly diploid individuals; Krieger and Keller, 1998; Ridout, 2000). As discussed above, gene diversity estimates for *P. tremuloides* tend to be higher than those for other species of *Populus*, but heterozygote excess (i.e., negative F_{IS}) was observed in only two studies. Thus, although both extensive clonality and triploidy appear as likely explanations for the high levels of genetic variation in *P. tremuloides*, more definitive answers about their relative or combined effects, as well as about the contribution of other factors (e.g., the possible role of past hybridization, Barnes, 1967), will come from studies designed to specifically address these questions.

Table 4 Microsatellite diversity and differentiation in *Populus*[a]

Section	Species	N_{loci}	N_{pop}	N	A	H_o	H_e	F_{IS}	F_{ST}/R_{ST}	References
Aigeiros	*P. deltoides*	10	–	20	5.2	0.23	–	–	–	Rahman and Rajora (2002)
	P. fremontii	4	–	20	5.3	0.60	0.52	–0.146	–	G.T. Slavov (unpublished data)
	P. nigra	6	22	574	–	0.78	0.73	–0.077	0.047	Imbert and Lefèvre (2003)
		12	–	60	11	0.80	0.82	0.030	–	Pospíšková and Šálková (2006)
		7	17	921	–	0.74	0.76	0.027	0.081	Smulders et al. (2008)
Tacamahaca	*P. angustifolia*	4	–	28	4.3	0.45	0.44	–0.033	–	G.T. Slavov (unpublished data)
	P. balsamifera	10	–	29	6	0.35	–	–	–	Rahman and Rajora (2002)
	P. trichocarpa	9	47	372	6.1	0.60	0.80	0.293	0.078/0.112	Ismail et al. (2009)
		10	2	282	17.5	0.71	0.77	0.058	–	Slavov et al. (2009)
Populus	*P. alba*	20	2	40	–	0.38	0.39	0.021	–	Lexer et al. (2005)
		19	1	169	6.4	0.37	0.38	0.027	–	van Loo et al. (2008)

Table 4 (continued)

Section	Species	N_{loci}	N_{pop}	N	A	H_o	H_e	F_{IS}	F_{ST}/R_{ST}	References
	P. tremula	20	2	40	–	0.47	0.50	0.055	–	Lexer et al. (2005)
		9	3	113	–	0.35	0.41	0.120	0.117	Suvanto and Latva-Karjanmaa (2005)
		25	12	116	–	0.50	0.62	0.197	0.015	Hall et al. (2007)
	P. tremuloides	4	4	159	7.4	0.56	0.72	0.201	0.032/0.041	Wyman et al. (2003)
		16	11	189	4.9	0.41	0.45	0.093	0.045	Cole (2005)
		4	–	266	8.8	0.47	0.67	0.300	–	Namroud et al. (2005)
	Median[b]	10	4	116	6.1	0.47	0.62	0.055	0.047[c]	

[a] Parameter designation is the same as in Table 3. R_{ST} is an analog of F_{ST}, which is based on the Stepwise Mutation Model (Slatkin, 1995).
[b] Because relatively few studies were included, medians were calculated in order to minimize the influence of extreme values.
[c] Based on F_{ST} values.

3.1.2 Microsatellites

The availability of highly variable microsatellites spurred a recent wave of population genetic studies in *Populus* (Table 4). Because of the substantially higher mutation rates of microsatellite loci, results from these studies are not directly comparable to those based on allozyme and RFLP markers. The general trends discussed above, however, appear to hold in microsatellite-based studies. Observed and expected heterozygosities are generally high and fall within the broad range of values reported for other angiosperm (e.g., Dow et al., 1995; Brondani et al., 1998; Streiff et al., 1998) and gymnosperm (e.g., Elsik et al., 2000, Table 3 in Slavov et al., 2004) trees. Heterozygote deficiency is the more common cause for departures from Hardy-Weinberg equilibrium and is slightly more prevalent than for allozyme and RFLP markers, presumably because of the much higher rates of null alleles and allele “drop-out” at microsatellite loci (Ewen et al., 2000). Differentiation is typically weak and comparable to levels observed for allozyme and RFLP markers, despite the constraint on F_{ST} imposed by the higher heterozygosities of microsatellite markers (Hedrick, 1999; Hedrick, 2005a). Unlike for allozyme and RFLP markers (Table 3), *P. tremuloides* does not appear to have higher microsatellite polymorphism and gene diversity than other species of *Populus* (Table 4). It is very likely, however, that this difference exists but remains undetected. Two of the three microsatellite studies in which polymorphism and diversity were reported for *P. tremuloides* were based on the same four loci, two of which are tri-nucleotide repeats. Tri-nucleotide microsatellites tend to be less variable than di-nucleotide microsatellites (Chakraborty et al., 1997; Schug et al., 1998), which were used in most other studies. The third study used 16 loci, all of which were developed for other species of *Populus*. Transferring microsatellites across species of *Populus* can be very successful (Tuskan et al., 2004) but markers tend to be much less variable in the recipient species than in the species in which they were developed (e.g., González-Martínez et al., 2004), presumably because of ascertainment bias (Ellegren et al., 1995). Because of the inherently high heterogeneity of microsatellite markers, empirical data need to be expanded considerably before meaningful comparisons among studies and species can be made.

3.1.3 Nucleotide Diversity

The whole-genome sequence of *Populus trichocarpa* (Tuskan et al., 2006), which has been integrated with a detailed genetic map (Kelleher et al., 2007), provides an excellent resource for understanding the population genetics of the genus. Information on nucleotide diversity and linkage disequilibrium in *Populus* is still less abundant than in other model organisms, but the growing interest in genetic association studies (Howe et al., 2003; DiFazio, 2005; González-Martínez et al., 2006; Neale and Ingvarsson, 2008; Ingvarsson et al., 2008; see also Chapter “Nucleotide polymorphism, linkage disequilibrium and complex trait dissection in *Populus*” by Ingvarsson in this volume) will probably create an avalanche of Single

Nucleotide Polymorphism (SNP) data. Levels of nucleotide diversity appear to vary substantially among species and genes (Ingvarsson, 2005a, b, 2008; Ingvarsson et al., 2006; Gilchrist et al., 2006; Hall et al., 2007; Chapter "Nucleotide polymorphism, linkage disequilibrium and complex trait dissection in *Populus*" by Ingvarsson in this volume), but are generally comparable to those in other tree species (González-Martínez et al., 2006; Savolainen and Pyhäjärvi, 2007; Chapter "Nucleotide polymorphism, linkage disequilibrium and complex trait dissection in *Populus*" by Ingvarsson in this volume). Interestingly, nucleotide diversity in trees does not seem to be substantially higher than in other plants, including *Arabidopsis thaliana*, an annual characterized by high levels of self-fertilization. Presumably, this is because (1) longer generation cycles in trees translate into lower neutral substitution rates per year than in plants with shorter life cycles and (2) the genomes of many tree species, including those in the genus *Populus*, may still be affected by past demographic oscillations (Savolainen and Pyhäjärvi, 2007; Ingvarsson, 2008).

3.2 Adaptive Traits

Extensive genecological studies have revealed that forest trees typically have high levels of adaptive genetic variation both within and among populations, and *Populus* is no exception (Farmer, 1996; Morgenstern, 1996; Howe et al., 2003; Savolainen et al., 2007; Aitken et al., 2008). These studies also provided compelling indirect evidence for the existence of local adaptation (i.e., genotypes originating from a given habitat tend to have higher fitness in that habitat than genotypes originating from other habitats; Kawecki and Ebert, 2004). First, genotype-by-environment (GxE) interactions, a necessary condition for local adaptation, are commonly detected (Morgenstern, 1996; White et al., 2007). Second, differentiation among populations is generally much higher for adaptive traits than for neutral genetic markers (Fig. 2; Merilä and Crnokrak, 2001; McKay and Latta, 2002; Howe et al., 2003; Savolainen et al., 2007), which suggests that divergent selection has played a dominant role in shaping adaptive genetic variation. Finally, and most importantly, genecological studies have revealed strong and repeatable correspondence between clinal genetic variation for adaptive traits and climatic and geographic factors believed to be important agents of natural selection (Morgenstern, 1996; St.Clair et al., 2005; Aitken et al., 2008).

Because gene flow is believed to be extensive in most forest trees, the prevalence of local adaptation is a paradox. This apparent contradiction can be explained by (1) reproductive isolation by distance and phenological asynchrony between populations growing under different climatic conditions, (2) very strong divergent selection, or most likely (3) a complex interaction between these two factors. Unraveling the relative roles of gene flow and natural selection, as well as the molecular underpinnings of adaptive genetic variation will be critical for our basic understanding of the evolution of *Populus* and other forest trees, and thus for designing adequate conservation and domestication strategies.

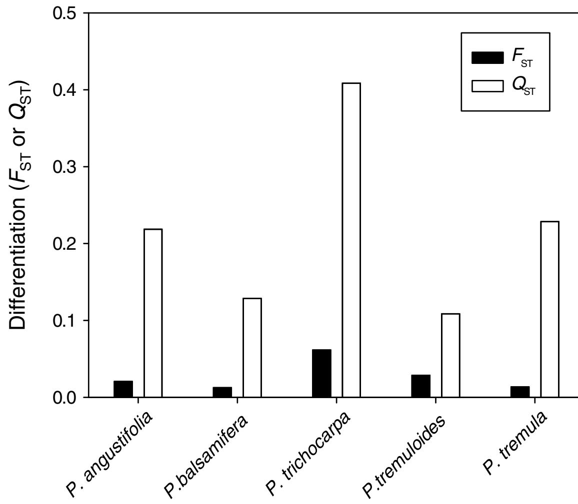

Fig. 2 Differentiation among populations for neutral genetic markers (F_{ST}; Wright, 1965) and its equivalent for quantitative traits (Q_{ST}; Whitlock, 2008) calculated for the timing of vegetative bud burst in five species of *Populus*. F_{ST} values are based on studies listed in Tables 3 and 4 (median values were used when multiple entries were available for a species). Q_{ST} values for *P. balsamifera* and *P. tremuloides* are from Table 1 in Howe et al. (2003), those for *P. tremula* were reported by Hall et al. (2007), and those for *P. angustifolia* and *P. trichocarpa* were calculated based on unpublished data (G.T. Slavov and S.P. DiFazio) and data from Dunlap and Stettler (1996), respectively, using Equation (1) in Howe et al. (2003)

4 Conclusions

1. *Populus* is comprised of morphologically and ecologically diverse species whose peculiar life history characteristics (e.g., dioecy, disturbance-dependent establishment, natural hybridization, clonality) and extensive neutral and adaptive genetic variation make it a unique model organism for basic and applied genetic research.
2. The outstanding genetic and genomic resources created over the last two decades have set the stage for a breakthrough in our understanding of the phylogenetics, population genetics, and molecular underpinnings of adaptation within and among species of *Populus.*

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Growth and Physiology

Sophie Y. Dillen, Stewart B. Rood, and Reinhart Ceulemans

Abstract *Populus* spp. is particularly characterized by fast growth rates and by the potential to adapt to a very wide range of environmental gradients. The vigorous growth performance of *Populus* can be partly explained by high photosynthetic carbon uptake, efficient leaf area development, production of sylleptic branches, appropriate seasonal coordination of growth through phenological adaptations and regulation by phytohormones. However, the high productivity is inextricably related to high water use which may have serious implications for the economic viability of irrigated *Populus* plantations. Substantial genetic variation has been demonstrated in growth, water use efficiency and several growth determinants suggesting promising perspectives toward *Populus* improvement programs.

1 Growth and Biomass Production

1.1 Biomass Yields

On a global scale, water and temperature are major environmental determinants of the natural distributions of plants, including trees (Kramer and Kozlowski, 1979). Poplars (*Populus* spp.) are naturally widespread throughout the Northern Hemisphere and environmental conditions vary substantially across this broad range of environments (Farmer, 1996). Following from this extensive environmental variation, there is considerable natural genetic diversity across *Populus* clones, species and hybrids. The genus *Populus* has about 29 native species (Eckenwalder, 1996) and this diversity probably reflects differences in ecophysiological adaptation, and especially differing requirements for temperature, water, and nutrients (Blake et al., 1996; Braatne et al., 1996; Rood et al., 2003; see Fig. 1). This diversity has not been

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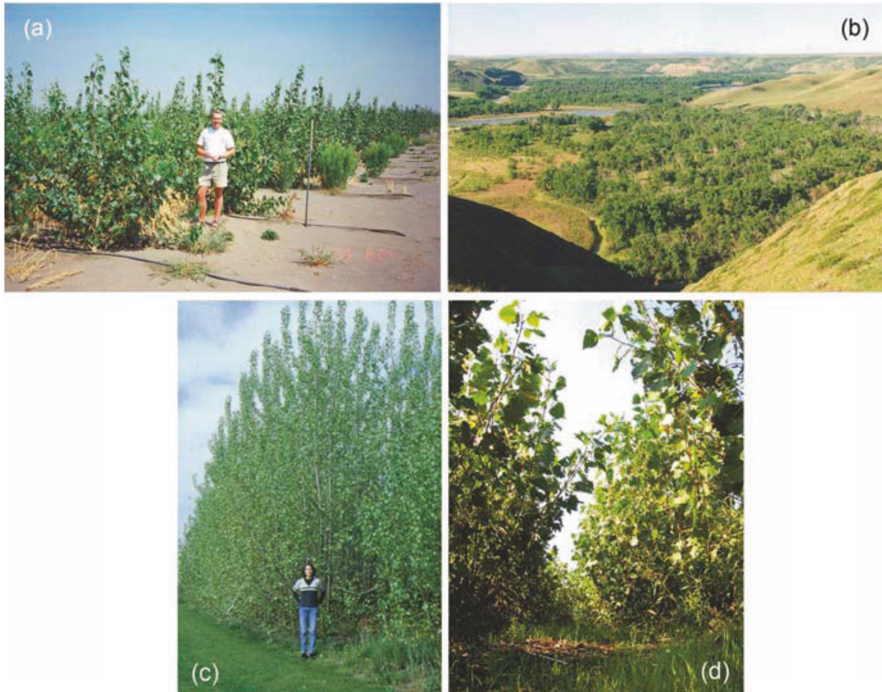

Fig. 1 Illustration of a variety of species at different ages or stages of development. (**a**) Hybrid *P. deltoides* × *P. trichocarpa* in their third year of growth at a high-density plantation in Boardman (Oregon, USA) also showing the irrigation lines (photo S. Rood). (**b**) The parental genotypes for most hybrid *Populus* originate as native riparian, or streamside, poplars or cottonwoods. This grove is along the Oldman River near Lethbridge, Alberta (Canada) and is biologically diverse since three cottonwood species overlap and naturally hybridize: *P. deltoides, P. angustifolia* (narrowleaf cottonwood), and *P. trichocarpa* (photo S. Rood). (**c**) High-density (10,000 plants per ha) *P. nigra* in its fourth year after coppice in a non-irrigated plantation in Boom (Antwerp, Belgium). The experimental plantation included 17 different genotypes or clones, and *P. nigra* performed very well over continuous rotation cycles. (**d**) Two-year-old *P. deltoides* × *P. nigra* in an irrigated short-rotation coppice culture near Viterbo (central Italy). Canopy closes a couple of months after coppice (photo M. Liberloo)

fully characterized and continues to represent a promising area for future research and application (Cooke and Rood, 2007), as was previously emphasized by Stettler and Bradshaw (1996) and Zsuffa et al. (1996). Beside their adaptation to a very wide range of environmental gradients, the genus *Populus* is also characterized by vigorous growth performance.

Due to their impressive growth rates, *Populus* species have become some of the most extensively cultivated trees in temperate latitudes around the world (Fig. 2). Ease of vegetative propagation, rapid juvenile growth, high biomass yields, good coppice ability, and high plasticity in response to environmental changes, are the main characteristics which have promoted *Populus* as superior trees for silviculture. Tree heights of 8–10 m have been reached after only two growing seasons in

Fig. 2 Illustrations of high-density *Populus* plantations in different parts of the world. (**a**) Rotation sequence of irrigated *P. deltoides* × *P. trichocarpa* hybrids in Boardman (Oregon, USA). In the semi-arid region of eastern Oregon and eastern Washington the warm temperatures support very rapid growth if irrigation is applied (photo S. Rood). (**b**) Rotation sequence of *P. deltoides* × *P. trichocarpa* hybrids (7th growth year) in a wetter site in the Columbia River valley near Portland (Oregon, USA). In western Oregon and western Washington increased precipitation eliminates (or reduces) the need for irrigation and growth rates are quite favorable with a long growth season (photo S. Rood). (**c**) Irrigated high-density (10,000 plants per ha) plantation near Viterbo (central Italy) during its first year of the second three-year rotation. In the central part of the plantation plants were treated with elevated atmospheric CO_2 to simulate global changes. Constructions enabled treatment and access to the crown during the entire rotation (photo M. Liberloo). (**d**) Three-year-old coppiced stool with young resprouts in a high-density plantation in Boom (Antwerp, Belgium)

carefully tended plantations (Scarascia-Mugnozza et al., 1997; Dillen et al., 2009), and heights of 40 m have been recorded after less than 20 years (Bradshaw et al., 2000). Following from their vigorous growth, several *Populus* species and interspecific hybrids have been incorporated into managed systems including traditional, wide-spaced plantations, and short-rotation coppice systems (SRC; Fig. 2). The light-weighted *Populus* wood has been used for various commercial purposes or as a source of carbon neutral renewable energy. The success of these commercial wood or energy plantations generally depends on the amount of woody biomass that is produced within a short period of time.

Under optimal conditions of climate, water and nutrient availability, dry biomass yields were observed in the order of 20–25 Mg ha^{-1} year^{-1} (Table 1; Heilman et al., 1994; Scarascia-Mugnozza et al., 1997; Liberloo et al., 2006). These values may

Table 1 Average above-ground biomass yields (Mg ha^{-1} year^{-1}) of different *Populus* species and hybrids in short-rotation coppice systems

Species or hybrid	Average above-ground dry biomass production (Mg ha^{-1} year^{-1})	Age	Plant density (ha^{-1})	Soil conditions	Treatments	Location	References
P. trichocarpa × *P. deltoides*	17.0–23.4	4-year period – 1st rotation	10,000	Rich, alluvial silt loam soil	I/WC	USA, Pacific Northwest	Heilman et al. (1994)
P. trichocarpa × *P. deltoides*	14–35	4-year period – 1st rotation	10,000	Rich, alluvial silt loam soil	I/WC	USA, Pacific Northwest	Scarascia-Mugnozza et al. (1997)
P. trichocarpa × *P. deltoides* *P. deltoides* × *P. trichocarpa* *P. trichocarpa* × *P. balsamifera* *P. deltoides* × *P. nigra* *P. trichocarpa* *P. nigra*	1.48–10.8	4-year period – 1st rotation	10,000	Former waste disposal site	–	Belgium	Laureysens et al. (2004)
P. trichocarpa × *P. deltoides* *P. deltoides* × *P. trichocarpa* *P. trichocarpa* × *P. balsamifera* *P. deltoides* × *P. nigra* *P. trichocarpa* *P. nigra*	1.6–9.7	3-year period – 2nd rotation	10,000	Former waste disposal site	–	Belgium	Laureysens et al. (2005)

Table 1 (continued)

Species or hybrid	Average above-ground dry biomass production (Mg ha^{-1} $year^{-1}$)	Age	Plant density (ha^{-1})	Soil conditions	Treatments	Location	References
P. trichocarpa × *P. deltoides* (F_2)	0.04–23.7	1 year	10,000	Farmland	I/WC	Southern UK	Rae et al. (2004)
P. maximowiczii × *P. nigra*	16.6–18.1	4-year period – 1st rotation	18,000	Farmland	–	Canada, southern Quebec	Labrecque and Teodorescu (2005)
Populus deltoides × *P. nigra*	25.4	3-year period – 2nd rotation	10,000	Farmland	I/WC	Central Italy	Liberloo et al. (2006)
Populus alba	24.0						
Populus nigra	24.7						
P. trichocarpa × *P. deltoides* (F_2)	0.18–18.06	2-year period – 1st rotation	6,670	Farmland	I/WC/F	Southern UK, central France and northern Italy	Rae et al. (2008)
P. deltoides × *P. nigra*	0.70–12.82	2-year period – 1st rotation	6,670	Farmland	I/WC/F	Central France and northern Italy	Marron et al. (2009)
P. deltoides × *P. trichocarpa*	0.67–15.59						
P. trichocarpa × *P. deltoides*	3.5	4-year period – 1st rotation	20,000	Farmland	–	Belgium	Vande Walle et al. (2007)

Plantation managements techniques are indicated as follows: I = irrigation; W = weed control; F = fungicides.

be less achievable on a wide scale since it is unlikely that farmers and land owners will abandon their best agricultural lands for *Populus* plantations (Vande Walle et al., 2007). More realistically, yield ranges of 10–15 Mg ha^{-1} $year^{-1}$ have been reported in suboptimal conditions (Table 1; Laureysens et al., 2004; Labrecque and Teodorescu, 2005). When *Populus* plantations are poorly managed, they may suffer from weed competition during establishment or after coppice, from shortages of nutrients or water, or from diseases or herbivore damage, and biomass yields may drop (Table 1). Weed control is very important during establishment years and under SRC conditions also every year after harvest. Weed competition may be an especially important stress factor for the light-demanding *Populus* species and can be responsible for substantial yield losses. *Populus* species, especially when planted in monoclonal plantations, are prone to diseases such as leaf rust caused by *Melampsora* spp., leaf spot by *Marssonina* spp. and stem canker by *Septoria* spp. (see Chapter 12). Monoclonal plantations are also vulnerable to attacks from various insects. The use of a wide diversity of clones and species in plantations increases the genetic diversity and minimizes the risks from diseases and pests (Labrecque and Teodorescu, 2005; Christersson, 2006).

Populus species could provide an even more important source of biomass in the future. With increasing atmospheric CO_2 concentrations, a stimulation of woody biomass is expected for various plant functional groups and particularly for trees (Ainsworth and Long, 2005). In a recent European FACE experiment with *Populus* (FACE: Free Air Carbon dioxide Enrichment), an increase in CO_2 concentration to 550 ppm produced an average increase of 23% of total woody biomass (Liberloo et al., 2006). Thus, *Populus* showed the ability to benefit from elevated CO_2 conditions, provided that they are grown under favorable conditions of light, water and fertilizer.

Populus are ecological pioneer species and are known for vigorous vegetative propagation through root suckers (i.e. adventitious shoots that emerge from shallow, horizontal roots) and by coppice regrowth (i.e. shoot regrowth after shoot harvesting). However, the suitability of different *Populus* species for coppicing has been poorly documented (Herve and Ceulemans, 1996). SRCs are usually characterized by 3- to 5-year rotations and remain viable for 15–30 years. Sims et al. (2001) showed that biomass yields were higher in coppiced *Populus* than in non-coppiced single-stem *Populus* since the established root systems enhance regrowth after coppice and support higher yields in the second and subsequent rotations. Coppicing reduces establishment costs since replanting is unnecessary but it is uncertain whether yields can be maintained over longer periods of repetitive coppicing (Tuskan, 1998; Pontailler et al., 1999).

1.2 Root Growth and Physiology

While shoot productivity is well-documented in *Populus*, the root system remains the least understood plant organ. The major reason for this lack of knowledge is

the limited accessibility of the below-ground plant parts. It is likely that the root system is a key determinant in the establishment and growth of *Populus* trees as it enables access to soil resources, including water and nutrients (Heilman et al., 1994; Wu et al., 1998). Fine roots form a substantial part of the root system and are usually distinguished from larger, coarse roots based on size (diameter, Friend et al., 1991). Although the diameter classes are somewhat arbitrarily chosen, most studies define 'fine roots' as roots less than 2 mm in diameter (Pregitzer and Friend, 1996). Fine roots are characterized by high turnover rates, representing up to 40% of the total carbon turnover of a tree, and this represents a major carbon sink (Pregitzer et al., 1995). The rapid root turnover increases flexibility to varying soil environmental conditions, and particularly to varying availability of soil nitrogen and water (Pregitzer and Friend, 1996; Al Afas et al., 2008a). *Populus* species are known as rapid nutrient accumulators and this is partly induced by the demand of rapid growth. Moreover, nutrient uptake by fine roots may be enhanced when associated with ectomycorrhizal or vesicular-arbuscular mycorrhizal fungi (see Chapter 12).

Root systems cannot be viewed as autonomous organs. Vigorous growth during the establishment year is generally associated with a strong rooting capacity, although well-rooting genotypes do not necessarily display intrinsically higher growth rates than poorly rooting genotypes. As trees age, they can rely more on stored reserves in permanent organs such as coarse roots and trunks (Pregitzer and Friend, 1996). Early root growth in *Populus* is under fairly strong genetic control (Wilcox and Farmer, 1968; Al Afas et al., 2008a). Large differences in root growth were observed among *Populus* sections, species and genotypes. Species from sections *Aigeiros* and *Tacamahaca*, and their hybrids show the capacity to produce adventitious roots from dormant hardwood cuttings, in contrast to species of the aspen section (Dickmann, 2001; Zalesny et al., 2005). Among species, the rather poor rooting ability in *P. deltoides* can be significantly improved in crosses with well-rooting species such as *P. balsamifera*, *P. trichocarpa* and *P. nigra* (Dickmann and Stuart, 1983). These differences in rooting ability between sections and species are important for the deployment of SRC since plantations are often established with unrooted cuttings. Moderate to high values of broad-sense heritability (i.e. the ratio of genetic variance to phenotypic variance) have been reported for root growth traits such root number, length and dry mass in a limited number of studies (Wilcox and Farmer, 1968; Zalesny et al., 2005). Information on the roots can be useful in selecting trees for specific soil environmental conditions and in other aspects of plantation management (Dickmann and Pregitzer, 1992). The emerging molecular tools of genomics and proteomics will also be useful for characterizing root systems of *Populus* genotypes (Kohler et al., 2003).

1.3 Hybridization and Heterosis

Plant growth performance results from a complex interaction between the genetic background and the biotic and abiotic environments (Ceulemans and Deraedt,

1999). To achieve maximal productivity, it is essential: (i) to know which physiological and morphological characteristics primarily define growth, both directly or indirectly (i.e. growth determinants); (ii) to exploit genetic variation in growth and its determinants within or between species and hybrids; and (iii) to improve the understanding of the genotype by environment interactions relative to these characteristics. Interspecific hybridization and clonal selection are common techniques in *Populus* improvement programs. The main objectives of hybridization are: (i) the combination of favorable parental characteristics in the progeny; (ii) larger homeostasis (i.e. stability among environments); and (iii) heterosis (Stettler et al., 1996). Hybrid vigor or heterosis is a well-known phenomenon in *Populus* and many other crop and tree genera, and can be defined as the superiority of offspring over the parents (Hayes, 1952; Stettler et al., 1996). Hybrid vigor probably partly results from cumulative or additive effects, in which there is dominance for specific, superior traits. For example, if there was dominance for large leaves and independently, for many leaves, the crossing of one *Populus* parent with a few, large leaves and another with many, small leaves could produce a hybrid *Populus* with many, large leaves and thus greater total leaf area and potential productivity. This synergism is a benefit for *Populus* cultivation and has been intensively exploited by tree breeders. In *Populus*, heterosis values appear to vary for volume growth, from moderate values up to values of 300% for F_1 *P. trichocarpa* × *P. deltoides* hybrids (Stettler et al., 1988) and even 600% for F_1 *P. tremula* × *P. tremuloides* (Li et al., 1998). In both cases, volume was used as an index for biomass productivity and was calculated as a product of diameter and height. This approach, however, may result in overestimation of heterosis and lower values are generally found for growth traits such as stem height and diameter (Li and Wu, 1997; Li et al., 1998; Marron et al., 2006; Dillen et al., 2009).

1.4 Allocation Patterns

The allocation of carbon into different components such as stems, branches, foliage and roots, partially defines the commercial value of a clone or species (Hinckley et al., 1992). Large variability in allocation patterns has been detected within and among *Populus* species (Pallardy and Kozlowski, 1979; Scarascia-Mugnozza et al., 1997; Karacic et al., 2003). Considerable genetic gain in biomass yields could be achieved with simultaneous selection for good growth and high harvest index (i.e. the percentage of harvestable biomass of the total biomass). For instance, a low root-to-shoot ratio may indicate a proportional rise in stem size and thus a higher harvest index. Conversely, a high root-to-shoot ratio could be selected for when rapid establishment of the root system and vigorous juvenile growth are desired (Pallardy and Kozlowski, 1979).

Compared to agricultural crops, domestication of *Populus* is still in its infancy, but is advanced as compared to other tree genera. To date, *Populus* improvement

programs are predominantly limited to F_1 hybrids (Aylott et al., 2008). In agriculture, a substantial part of the yield increase of corn and soybeans came from genetic improvement that has involved many breeding generations. Further, a major part of the genetic gain in agricultural crops resulted from reallocation of carbon to the economically valuable parts, such as grain or tuber (Hansen, 1991; Peng et al., 1999). In trees, this type of reallocation will not contribute to a higher above-ground biomass yield. Yet, for some commercial purposes such as for the production of veneer or saw-wood, a smaller proportion of branches is preferred.

Beside the genetic background, the environment has a marked impact on allocation patterns. When canopies close, strong competitive interactions occur among *Populus* trees in the stand. In order to maintain vigorous growth, allocation patterns change. Larger individuals invest more stem biomass in radial growth than in height growth, while smaller individuals must allocate more carbon to height growth in order to increase access to light (Wu et al., 2003). Competition between trees also influences the canopy establishment. For example, at age seven, two *Populus* clones accumulated a larger proportion of biomass in branches at the widest spacing than did those at the lowest planting density (on average 19 and 9% at 2.0 and 0.5 m densities, respectively; DeBell et al., 1996). Both clones could not fully develop their canopies and did not attain their maximal growth capacity in denser plantations.

2 Allometric Relationships

The quantitative and qualitative estimation of tree growth is indispensable for short-rotation and traditional forestry. To this end, different methodologies are used depending on the specific purposes of the predictions. In many situations, generalized equations are adequate for biomass predictions in a wide range of environments and for different species. Occasionally, very accurate biomass estimations are necessary, such as in genetic and quantitative trait loci (QTLs) studies (Dillen et al., 2007; Rae et al., 2008). Direct estimation of volume and biomass entails the complete harvest of the plantation and is very time consuming. In experiments that involve estimates of biomass and biomass increment over a series of years, indirect estimation is required. Indirect methods may involve harvesting a small sample of trees and multiplication of tree weights based on plant density or statistical regression (Verwijst, 1991). The latter approach is based on establishing relationships between volume or yield versus tree dimensions or canopy or leaf traits.

A trade-off exists between increasing the number of sampled trees in order to improve the regression analysis, and the impact of destructive sampling of trees on the quality of the long-term experiment. Decisions should be made on the model form (linear vs. non-linear), data transformations, and the parameters included such as stem height, diameter, and branch or leaf traits. These choices may all affect the final results (Ruark et al., 1987; Verwijst, 1991; Pontailler et al., 1997). Nevertheless, biomass predictions have been successful in numerous experiments (Tuskan and Rensema, 1992; Scarascia-Mugnozza et al., 1997; Rae et al., 2008).

Stem diameter or circumference is probably the most reliable single index of productivity (Monclus et al., 2006; Dillen et al., 2007). This trait is characterized by high heritability and can be easily and accurately scored. Beside stem diameter, stem volume or volume index (based on stem diameter and height) is frequently used as an indicator of biomass (Pontailler et al., 1997).

Once the equations are established a difficulty is to know whether, and to what degree, the derived equations can be used outside the tested environment, such as for different species or hybrids, and over successive years. Since regression coefficients of a given model reflect the population (plantation and site characteristics) upon which the equations were developed, application of equations under different conditions can lead to considerable errors in the predictions (Zabek and Prescott, 2006). In a study comparing the biomass equations for five hybrid poplar families at three contrasting sites, the family effect was larger than the site effect (Dillen et al., 2007). Moreover, the site effect was thought to be due to a severe rust attack by *Melampsora larici-populina* at one of the three studied sites, and the effects of diseases as leaf rust on allometric relationships have previously been recognized (Tuskan and Rensema, 1992). Pontailler et al. (1997) revealed the same trend when studying biomass equations for several *Populus* clones at two different locations. Significant differences in regression coefficients between clones were observed, but between-site differences were rather small. Pontailler et al. (1997) concluded that attention should be directed to the between-clone (or between-family) differences when extrapolating general allometric relations.

In a SRC over three rotations (11 years), Al Afas et al. (2008b) investigated whether a single allometric biomass equation could be applied yearly for all 17 *Populus* clones. One equation fitted the different years, except for 1 year in which leaves were heavily infected by *M. larici-populina*. In terms of clones, one equation was also sufficient, except for the clone Hazendans. In contrast to the other clones in the experiment, Hazendans exhibited a leaf size distribution with smaller leaves in the upper canopy and larger leaves in the lower canopy (Niinemets et al., 2004). Zabek and Prescott (2006) considered different published biomass predictions on their data set. Large discrepancies between predicted and observed biomass highlighted the problems arising when the regression equations were extrapolated to different *Populus* populations. The use of regression coefficients from their own data set improved the predictive power of the equation, illustrating the portability of the model form and the population-specific nature of the regression coefficients.

3 Productivity Determinants

Many studies have already been devoted to *Populus* productivity and its determinants. The overall growth performance of a tree depends on the interaction between light interception by the canopy and the intensity of CO_2 assimilation by the leaves of this canopy. Various canopy traits are intimately related to productivity including functional or process-related components associated with phenology,

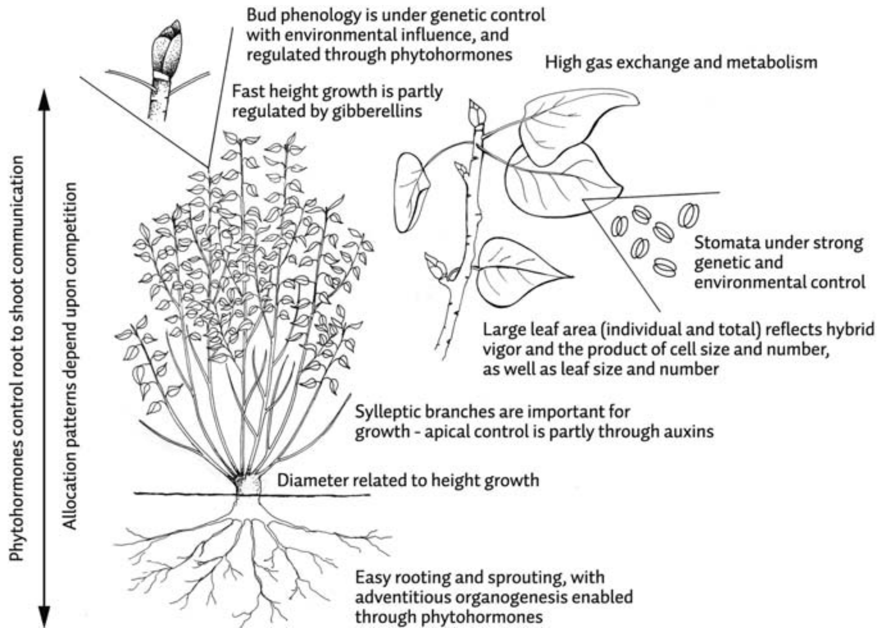

Fig. 3 Schematic illustration of the main physiological growth factors in *Populus* that are being presented in the present contribution. As an example a *Populus* tree from a short-rotation intensive culture is being shown

leaf area growth and morphology, and leaf and whole-tree photosynthetic capacity (Fig. 3). Relevant structural components include orientation, distribution and size of branches and leaves.

3.1 Gas Exchange

Photosynthesis is the ultimate driving force for carbon gain and consequently, for plant growth. The biochemical process uses light to convert atmospheric CO_2 and water into carbohydrates, the structural and maintenance components of plants. Generally, tree photosynthesis is believed to be low; photosynthetic capacity on a leaf area basis averages around 3–6 μmol CO_2 m^{-2} s^{-1}. In contrast, *Populus* species, and particularly interspecific hybrids, can reach rather high photosynthetic capacities, of up to 25 μmol CO_2 m^{-2} s^{-1} (Ceulemans and Saugier, 1991). A review of numerous photosynthetic and gas exchange studies published on *Populus* yielded values from 1.3 to 25 μmol CO_2 m^{-2} s^{-1} illustrating the remarkably large variation in photosynthetic rates among *Populus* clones, species and hybrids (Ceulemans and Isebrands, 1996). However, the relationship between photosynthesis and biomass yield in trees, including *Populus*, is not obvious. Some studies have demonstrated positive correlations, while others have not detected significant correlation. High

photosynthetic carbon uptake is essential for fast growth but the inverse is not necessarily true. The lack of a clear relationship might be due to: (i) the complexity of photosynthesis which is under polygenic control; (ii) the consideration of instantaneous measurements which do not represent photosynthesis over the entire day or season; (iii) measurements on individual leaves while growth is dependent upon the entire canopy; and (iv) the complex allocation patterns of carbon within the tree (Isebrands et al., 1988; Barigah et al., 1994).

In addition, the strong influence of abiotic factors including light (Liberloo et al., 2007), temperature (Drew and Chapman, 1992), CO_2 concentration (Hovenden, 2003), and nutrient availability (Ripullone et al., 2004) on net photosynthesis has been demonstrated in *Populus*. Generally, insufficient radiation, shortage of nutrients, low temperature and drought, reduce the photosynthetic carbon uptake in plants (Schulze, 1982). The high sensitivity of photosynthetic carbon uptake to environmental variation affects the potential of photosynthetic capacity as a predictor of growth and productivity. Biotic factors such as leaf age and position in the canopy also have a significant effect on net photosynthesis, and maximum photosynthetic rates are usually attained just before leaf extension growth ceases (Dickmann, 1971).

3.2 Leaf Traits

Leaves are the major organs of photosynthesis in plants. The development of leaves, and the effective display of leaf area, increase canopy light interception and subsequently, carbon gain. While photosynthetic carbon uptake is sometimes poorly correlated to growth performance, the development of leaf area may be a better integrative predictor of growth. Leaf area, either considering individual leaf areas or leaf area index (LAI), provides a promising productivity determinant (Ceulemans et al., 1992; Marron et al., 2007). The superior productivity of hybrids between *P. trichocarpa* and *P. deltoides* has been attributed to their large individual leaf area which has been explained by the combination of larger cell numbers from *P. deltoides* and the larger cell sizes from *P. trichocarpa* (Ridge et al., 1986). Moreover, stem volume and stem biomass production were more closely related to individual leaf size than to the number of leaves produced per tree in this study.

When different species or genotypes are compared, leaf area traits are rather robust across environments and are easily monitored. Consequently, these traits are very well suited for association with molecular markers that may be selected for in *Populus* improvement programs (Bunn et al., 2004). In *Populus*, leaf size largely affects stem growth, but its impact is determined by the position at which the leaves develop (i.e. main stem, sylleptic branches or proleptic branches). Leaves on sylleptics appeared to contribute more to radial growth than those on proleptic branches and the main stem in young *Populus* hybrids (Scarascia-Mugnozza et al., 1999). Furthermore, stem height growth of 2-year-old F_2 *P. trichocarpa* × *P. deltoides* hybrids benefitted more from upper canopy leaves than from lower canopy leaves (Wu et al., 1997).

Individual leaf area development of *Populus* is highly responsive to abiotic factors. Within a tree canopy, profiles of leaf characteristics follow the light profile in the canopy (Al Afas et al., 2005; Liberloo et al., 2007). The canopy exhibits a vertical profile with a decline in irradiance from top to bottom. Specific leaf area (SLA) is sensitive to changes in irradiance whereby an increase of irradiance leads to a lower SLA or denser leaves (Niinemets and Kull, 1999; Gielen et al., 2003). In the upper canopy, leaves are larger and are supported by longer and thicker petioles as compared to lower canopy leaves (Al Afas et al., 2005). This suggests that larger leaves require more structural support. Together with branching architecture, petioles play an important role in leaf arrangement and through modifications of either branches or petioles, gaps between neighboring leaves could be filled (Niinemets et al., 2006). Leaf orientation also defines the canopy architecture and may therefore enhance growth vigor. The angle between leaf surfaces and incoming solar radiation has a marked effect on whole-tree photosynthesis and angling may diminish the heat load on upper canopy leaves by reducing the absorption of solar radiation during the warmest periods of the day (Hinckley et al., 1992).

Populus leaf size typically decreases while leaf number increases from mesic to xeric environments (i.e. from temperate moist to dry climates, respectively). Greater leafiness combined with smaller leaves could be an adaptive as well as an acclimation response to xeric environments (Dunlap et al., 1995; Pearce et al., 2005). In contrast, larger and more widely spaced leaves are probably favored in the mesic, moderate environments, where overcast days with diffuse light are frequent (Dunlap et al., 1995).

3.3 Branching Pattern: Syllepsis versus Prolepsis

Populus species often display an indeterminate growth habit and produce sylleptic branches (i.e. branches developed from axillary buds without undergoing a dormant period; Remphrey and Powell, 1985), throughout the growing season. They produce sylleptic branches during the first growing season and 2-year-old plants produce two types of branches, sylleptic and proleptic branches (i.e. branches developed from axillary buds after a quiescent period; Hallé et al., 1978; see Fig. 4). Trees producing many sylleptic branches are considered to have weak apical dominance, whereas trees showing no syllepsis are characterized by strong apical dominance. Sylleptic branches play a critical role for tree growth as they allocate a greater proportion of carbon to the stem than proleptics, and contribute more, on a per unit mass basis, to tree growth (Scarascia-Mugnozza et al., 1999). Further, leaf area produced by sylleptic branches, can be 50–100% higher than the leaf area from leaves on the main stem (Scarascia-Mugnozza, 1991; Wu and Stettler, 1998). Sylleptic branches show a shorter lifespan than proleptic branches, but in this short time they play an important part in the carbon balance, providing a quick return for a relatively small physiological investment. Several studies have demonstrated that clones which had the highest number of sylleptics were often the highest yielding clones

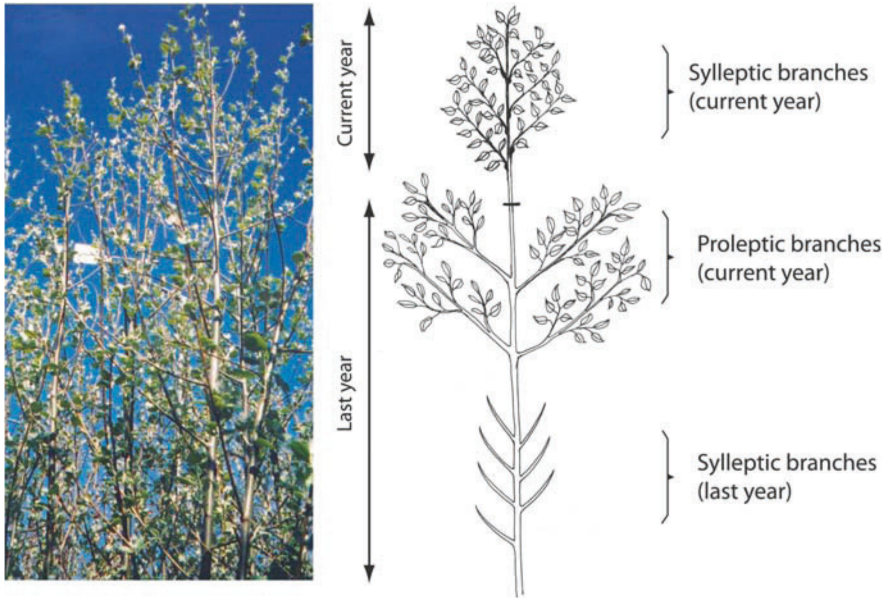

Fig. 4 Difference between sylleptic and proleptic branches: schematic illustration (*right panel*) and close-up of a *P. alba* in its third year of growth in a high-density coppice plantation near Viterbo (central Italy) (*left panel*; photo M. Liberloo). Sylleptic branches result from the development of a newly initiated lateral axis without the apical meristem of that axis having had an intervening rest period. In contrast proleptic branches are the result of the discontinuous development of a new lateral axis, with its apical meristem having experienced an intervening period of rest

(Scarascia-Mugnozza et al., 1999; Marron et al., 2006; Rae et al., 2008; Dillen et al., 2009). Conversely, some clones which showed superior growth performance did not produce sylleptic branches.

Since sylleptic branches can be produced during the course of the growing season, sylleptic branch traits are much more plastic than proleptic branch or stem growth traits (Wu and Hinckley, 2001; Marron et al., 2006). In Europe and the USA, clones from southern, warmer origins display more sylleptic branches than clones from northern, colder regions (Rogers et al., 1989; Ceulemans et al., 1992). Consistent with this response, *P. deltoides*, *P. trichocarpa* and their hybrids produced more sylleptic branches when grown in warmer, more favorable sites (Dickmann et al., 1990; Dunlap et al., 1995; Wu and Stettler, 1998). Wu and Stettler (1998) found that leaf area on sylleptic branches was up to 35-fold higher in warmer, sunnier and well-irrigated conditions than in a cooler and non-irrigated site. In contrast, the leaf area produced by proleptic branches did not significantly differ between the two environments. The plasticity of sylleptic branches of some *Populus* genotypes highlights the capacity to opportunistically alter the canopy architecture in response to changes in resource availability.

Considerable genetic variation in sylleptic and proleptic branching pattern has been demonstrated for the *Populus* genus. Clonal differences in branching pattern, and also in branch orientation and branch curvature, lead to striking differences in canopy structure (Fig. 3). Branch orientation is mostly expressed in terms of angles of origin and termination; branch curvature is calculated as the correlation between angle of origin and angle of termination. *P. trichocarpa* clones from northern origin showed wider branch angles than southern clones (Weber et al., 1985; Rogers et al., 1989). However, the definition of a biologically meaningful variable for branch angle is rather complex, since the angles of origin and angles of termination are not necessarily associated (Nelson et al., 1981).

3.4 Phenology

Phenology refers to repetitive natural processes. In *Populus* species and other deciduous trees, phenology especially relates to the seasonality of catkin and leaf bud flushing in the spring, to the cessation of shoot growth and subsequent bud maturation, and to leaf senescence and abscission in autumn (Boes and Strauss, 1994). The interval between the spring flushing and autumnal senescence provides the growth season, the interval responsible for photosynthesis and biomass production, and is of considerable interest relative to *Populus* productivity (Pellis et al., 2004). A longer growth season can increase productivity, but with a subsequent hazard due to the increasing risk of frost damage that can occur with flushing prior to the end of spring frosts, or insufficient fall hardening prior to fall and winter freezing (Howe et al., 2000). The appropriate seasonal coordination of *Populus* growth and development is also important relative to seasonal patterns of precipitation vs. water demand, and photoperiod or daylength, and also to coping with some pest and disease outbreaks (Brunner et al., 2004).

Aspects of phenology can be especially important for *Populus* hybrids since the blending of traits from different parental species can disrupt the coordination with the seasonal environment. The consideration of phenology is also very important relative to the production of hybrid *Populus* in a variety of regions and especially in different latitudes that vary in photoperiod regime. Following from these considerations there is growing interest in the nature of physiological adaptation to the growth season and in the genetic regulation of the particular characteristics that contribute to phenology and seasonal adaptation. There have been attempts to identify relevant QTLs (Bradshaw and Stettler, 1995) and following the *Populus* genome project, efforts to identify specific genes that contribute to *Populus* phenology (Brunner et al., 2004). It is likely that some of the genes involved in the regulation of phenology are shared with other dicotyledonous plants, including *Arabidopsis* (Böhlenius et al., 2006; Ingvarsson et al., 2008). Thus, comparative genomics represents a promising strategy for the understanding and modification of *Populus* phenology (Brunner et al., 2004), see also section below.

4 Water Relations and Productivity

4.1 Water Requirements

While currently grown hybrid *Populus* are among the highest yielding trees in temperate regions, this high productivity is associated with correspondingly high water demand (Tschaplinski et al., 1994; Blake et al., 1996). In many areas of Europe and some areas of North America, hybrid *Populus* plantations are situated in river valley floodplains where groundwater is shallow and abundant (Monclus et al., 2006). With the abundant water supply, commercial clones were developed primarily based on high productivity while water use efficiency (WUE), the ratio between biomass production and water use, has seldom been a priority (Monclus et al., 2006).

However, hybrid *Populus* plantations are also situated in upland sites, where soil moisture is more limited, and drought provides a major limitation to survival and productivity (Blake et al., 1996). The solution in some regions such as the semi-arid areas of eastern Washington and Oregon, USA, has been the implementation of irrigation, and with the added water, hybrid *Populus* yields in these warm and sunny regions can be very high (Zsuffa et al., 1996). However, with increasing water demands for population, agricultural, and industrial growth, and with climate change diminishing water supplies in some regions, water for *Populus* irrigation will become less available and more costly (Postel and Richter, 2003). In addition, rising energy costs for water pumping and increased labor costs further diminish the economic viability of irrigated *Populus* plantations.

Without irrigation, drought stress is periodic in many prospective upland sites and consequently increased drought-adaptation and higher WUE are likely to increase in priority for hybrid *Populus* breeding programs (Blake et al., 1996). Although *Populus* species are among the most drought-susceptible trees there is substantial genotypic variation in the structural and biochemical characteristics that contribute to drought adaptation (Blake et al., 1996; Monclus et al., 2006; Street et al., 2006). Braatne et al. (1992) recognized the opportunity to develop hybrid *Populus* with moderate productivity and favorable WUE, and achieving this balance would substantially expand the range of environments in which hybrid *Populus* could be commercially grown without the need for costly irrigation.

Previous studies have investigated the ranges of variation in water use and drought-response characteristics of current hybrid *Populus* clones (Ceulemans et al., 1978; Harvey and van den Driessche, 1999; Monclus et al., 2006). Additionally, studies have investigated water use in hybrid *Populus* families to consider aspects of inheritance including segregation and dominance (Braatne et al., 1992; Tschaplinski et al., 1994; Ferris et al., 2002). More recently, there have been efforts to identify QTLs, and ultimately specific genes, which underlie water use and drought-response characteristics in *Populus* (Street et al., 2006). These studies are ongoing and there is another prospect to extend the range of WUE and drought adaptation of hybrid *Populus*. This involves the expansion of the range of prospective parental genotypes to include ecotypes and species that are naturally adapted to drier regions (Zsuffa et al., 1996; Zhang et al., 2004; Bogeat-Triboulot et al., 2007).

4.2 Stomata

Stomata play an important role in both CO_2 uptake and water relations of trees. Within the soil-plant-atmosphere continuum the final passage of water from the tree to the atmosphere is through the foliar stomata and consequently, stomata provide a primary opportunity for the *Populus* tree to regulate its water loss (Ceulemans et al., 1978; Kramer and Kozlowski, 1979; Blake et al., 1984; 1996). In the short-term, this involves stomatal closure due to the loss of turgor pressure of the guard cells that flank the stomatal pore, and in the longer-term, adaptation to particular environments can involve changes in the size, number, and possibly distribution of stomata (Pallardy and Kozlowski, 1981; Pearce et al., 2005). Stomatal traits vary widely among *Populus* species and clones and show heritable variation (Dunlap and Stettler, 2001; Al Afas et al., 2005; Dillen et al., 2008). While various *Populus* species are characterized by amphistomatous leaves (i.e. stomata on both leaf surfaces), *P. trichocarpa* and *P. balsamifera* are known to have very few or no stomata on the adaxial leaf surface (Ceulemans et al., 1988; Ferris et al., 2002; Pearce et al., 2005). These different anatomical and morphological leaf characteristics can provide an adaptive mechanism to irradiance and water stress (Parkhurst, 1978). As the inheritance of different stomatal traits is revealed, it should be possible to develop *Populus* hybrids with particular combinations that would benefit productivity and WUE in particular environments (Blake et al., 1996; Ferris et al., 2002).

While variation in stomatal characteristics has long been recognized as a primary physiological character that influences plant water use, new research indicates that there are also other forms of physiological regulation. Most studies of water relations of trees and other plants have investigated shoots while the structure and function of roots has often been neglected. Newer instruments such as the high-pressure flow meter can measure aspects of hydraulic conductivity in different organs and particularly roots (Tyree et al., 1995). There are apparently diurnal and phenological patterns in hydraulic conductances and this indicates a more dynamic role of stomatal regulation than had been previously thought.

One probable group of contributors to the varying root hydraulic conductances within *Populus* and other trees are aquaporins, trans-membrane water transport proteins (Marjanović et al., 2005). These are very abundant in *Populus* and other plants and facilitate water passage across cell membranes and ultimately, across cells. Within the root, the endodermis is a cylinder of cells that are sealed with suberins and other substances and represents a point at which water must pass through, rather than between, living cells. Aquaporins would facilitate this water transport and changes in the level, type and/or distribution of aquaporins may represent an endogenous mechanism of regulating root water flux. Subsequently, the transgenic modification of aquaporins is a current research strategy to study aquaporin function and role and might also represent a strategy to modify water use in *Populus* and other plants. The inheritance of aquaporins is largely unknown and there may be promise to investigate variation, heritability and combinations across the multiple aquaporin genes and related characteristics (Kohler et al., 2003; Schrader et al., 2004).

5 Phytohormones and Growth Regulation

Stomatal regulation and water relations are partly regulated by the phytohormone abscisic acid (ABA) (Chen et al., 1997). Similarly, ABA and the other phytohormones regulate virtually all aspects of growth and development of trees, as in other plants (Taiz and Zeiger, 2006). There are five classic phytohormones, with ABA and ethylene often serving as inhibitory substances that slow growth and development, while the auxins, cytokinins and gibberellins (GA) are commonly promotory. Unlike the mammalian hormones the plant phytohormones are synthesized in multiple plant tissues and generally have diverse roles. Two or more of the phytohormones generally interact to regulate particular aspects of growth and development (Taiz and Zeiger, 2006). For example, *Populus* bud flushing probably particularly involves inhibition by ABA versus promotion by GA; winter stratification and lengthening of the spring photoperiod change the biosynthetic capacities and responsivities for these two antagonistic phytohormones (Olsen et al., 1997). See also sections below.

Following from the fundamental roles of phytohormones to regulate plant growth, the exogenous or external application of phytohormones has been attempted as a means of deliberately modifying crops and trees. Plant growth regulators are chemicals that are commercially applied to alter plant growth or development and there are numerous commercial applications for field and fruit crops but these have seldom provided benefit in tree plantations. Application of phytohormones to promote root development of young plants or cuttings is of use in *Populus* cultivation.

In *Populus* and other trees, the deliberate modification of growth form and stature is a primary goal and thus, there is considerable interest in the alteration of phytohormones, especially of GA. Transgenic stimulation of GA 20-oxidase did elevate the levels of endogenous GAs, and this was associated with increased biomass production and increased fiber length (Eriksson et al., 2000). Other transgenic strategies have also demonstrated that the modification of GA biosynthesis or action can dramatically alter *Populus* growth form (Busov et al., 2003, 2006; Israelsson et al., 2004). It has also been found that transgenic alteration of phytochrome can influence phytohormone metabolism and balance, and that this is also related to photoperiodic response (Olsen et al., 1997). These results demonstrate promise for breeding through either classical crosses or through transgenic manipulation with respect to the modification of phytohormones and growth form and rate.

The possible transgenic modification of trees has prompted considerable resistance by some environmental groups as well as by some scientists. The alteration of phytohormone biosynthesis or response would represent a change in the balance of a naturally-occurring regulatory substance and would not involve the insertion of a new biochemical capacity. It is also likely that the coarse modification of phytohormones would not improve the tree's adaptation to native environments and thus expansion out of a plantation setting may be unlikely. However, there are real and perceived concerns about transgenic trees and this should invite care prior to release into uncontained sites (Pilate et al., 2002; van Frankenhuyzen and Beardmore, 2004).

6 Conclusion

Being pioneer trees *Populus* species are characterized by fast growth rates and high levels of productivity. Intensive gas exchange rates, both photosynthesis and water loss, partly explain this high growth performance. Other ecophysiological characteristics that significantly contribute to the fast growth include the efficient leaf area development (both individual leaf area as well as total tree leaf area), the potential to produce sylleptic branches and thus to expose more leaf area to capture solar radiation, opportunistic use of the length of the growing season through phenological adaptations and efficient ways of communication through phytohormonal mechanisms. The high water use of *Populus* is of particular relevance toward environmental implications, both in longer, wide-spaced plantations as well as under short-rotation coppice cultures. All of these ecophysiological traits have shown very large genetic variation and moderate to high heritability. These can be tapped in selection and breeding programs, either through traditional breeding or through genetically engineered improvement programs.

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Part II
Populus Genetics and Genomics

The *Populus* Genome and Comparative Genomics

Carl J. Douglas and Stephen P. DiFazio

Abstract *Populus* was the first tree genome, and one of the first plant genomes, to be sequenced. The sequencing project and subsequent annotation was a collaborative, international effort, with the bulk of the sequencing carried out by the US Department of Energy Joint Genome Institute. Due to the high degree of sequence coverage, the hybrid BAC library-whole genome shotgun approach employed, excellent EST and full-length cDNA sequence collections, and an engaged *Populus* research community, the sequence is of high quality and set a standard for subsequent genome sequencing projects. In this chapter, we provide background information on the *Populus* sequencing project, the genome sequence was obtained and annotated, data on *Populus* genome structure, uses of the genome sequence, and examples of comparative genomics studies enabled by the *Populus* genome sequence.

1 Overview

1.1 Significance of the Populus *Genome Sequence*

The sequencing and analysis of the *Populus trichocarpa* genome (Tuskan et al., 2006) was a milestone not only for tree biology but also for plant biology in general. As the first tree and third plant with a complete genome sequence (after *Arabidopsis thaliana*, Arabidopsis and *Oryza sativa*, rice), the impact of the *P. trichocarpa* genome sequence has been felt in different ways. In this chapter, we provide background information on the *Populus* sequencing project, how the genome sequence

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was obtained and annotated, data on *Populus* genome structure, uses of the genome sequence, and examples of comparative genomics studies enabled by the *Populus* genome sequence.

The availability of the *Populus trichocarpa* Nisqually-1 sequence as a reference genome sequence has catalyzed new research on genetic approaches for example, powerful forward genetics approaches such as association genetics (e.g., Ingvarsson, 2008) and QTL mapping (e.g. Rae et al., 2007). Related to these approaches, the Nisqually-1 sequence provides a reference for large-scale, genome-wide discovery of nucleotide diversity (for example single nucleotide polymorphisms, SNPs and insertion/deletions, indels). Indeed, the sequencing and physical mapping (Kelleher et al., 2007) of *P. trichocarpa* has already revealed a wealth of genetic polymorphisms in this single individual. While approaches such as Eco-TILLING (Gilchrist et al., 2006) have made use of Nisqually-1 genome data for SNP discovery, next generation sequencing platforms and whole genome level resequencing approaches can be combined with the reference genome sequence to allow unprecedented exploration of nucleotide and genetic diversity. These issues are addressed in more detail in other chapters of this volume.

The availability of the *P. trichocarpa* genome sequence also opened the door to comparative genomics studies based on whole genome data from multiple lineages. As the second eudicot genome sequence available, three way comparisons between the genomes of *Populus* and Arabidopsis as representative eudicots, and rice, as a monocot, were possible. Subsequently, the *Vitis vinifea* (grapevine) whole genome sequence (Jaillon et al., 2007; Velasco et al., 2007) the sequence of the moss *Physcomitrella patens* genome (Rensing et al., 2008) and partial genome sequences from other plants, has further enriched the data available for plant comparative genomics. Questions regarding the evolution of gene families that are potentially important for tree and woody plant biology, and the degree of synteny between *Populus* and other angiosperm plants can now be addressed, and a wealth of reports in these areas have been published since the public release the genome sequence. While sequencing of whole tree genomes is in its infancy, efforts towards completing a *Eucalyptus* genome sequence are well underway (Novaes et al., 2008), and information from this and other tree genome sequences will further enable comparative genomics between diverse tree taxa in the future.

1.2 Background to the Genome Sequencing Effort

As discussed at length by others (Cronk, 2005, Jansson and Douglas, 2007, Chapters 1, 2, and 3 in this volume), the biology of *Populus* species as a woody perennial, obligate outcrossing, and largely undomesticated plant, relative to herbaceous, weedy, inbreeding life history of Arabidopsis made it attractive as an alternative model system for genome sequencing. Furthermore, as mentioned in Chapter 1 *Populus* began to emerge as the primary angiosperm tree model system in the 1990s for several reasons that were also important in the choice of *Populus* for the first tree

genome sequence. *Populus* genetics became well established during that time, leading to the generation of relatively high-density linkage maps (Yin et al., 2002) for several species and hybrids. Simultaneously, numerous groups began to explore the molecular biology of *Populus*, and the structures of several important gene families began to be illuminated. These included, for example, those encoding enzymes of phenylpropanoid metabolism, important for lignin deposition during wood formation, genes encoding transcription factors such as those of the MYB class and genes involved in hormone signaling. As well, transformation systems for functional analysis of *Populus* genes came into widespread use (Chapter 6). The relatively small genome size (around 500 Mb), and interests in *Populus* as a source of biomass as well as its ecological importance (Brunner et al., 2004) both contributed to the decision to in 2001 to sequence the *Populus* genome. However, one of the most compelling reasons for sequencing the genome was the rapid development of tools for *Populus* genomic and functional genomics, as described in detail by Jansson and Douglas (2007).

2 Summary of Whole Genome Shotgun Results

2.1 Sequencing Strategy

The sequencing of the *Populus* genome was accomplished using a hybrid strategy that combined whole genome shotgun sequencing with a BAC physical map and a genetic map. The physical map was produced by fingerprinting and end-sequencing a *Populus* BAC library. In parallel, whole genome shotgun sequencing was performed for the same genotype that was used for the physical map.

2.2 Selection of a Populus Genotype for Sequencing

The genotype selected for sequencing was a black cottonwood tree (*Populus trichocarpa* Torr. & Gray). Toby Bradshaw, one of the pioneers of *Populus* genomics, originally collected the selected genotype (also known as clone number 383-2499) along the Nisqually River in Washington State. This clone, commonly called “Nisqually-1,” was the maternal parent for the largest pedigree produced for *Populus*, a cross with *P. deltoides* clone ILL-101 to produce a family of 2,028 F_1 progeny. The purpose of this generating this pedigree was to isolate a major gene conferring resistance to a hybrid leaf pathogen, *Melamspora x columbiana* 3, which segregates at a 1:1 ratio in this pedigree (Stirling et al., 2001). For this purpose, a 9.5X BAC library was also prepared for this pedigree by partially digesting high molecular weight genomic DNA with *Hind*III (Stirling et al., 2001). The existence of the large pedigree and the BAC library, coupled with the availability of abundant material in clone banks at the University of Washington and elsewhere, was enough to tip the balance in favor of this genotype.

The selection of this genotype was somewhat controversial, because most of the model transformation clones were derived from aspens (*Populus tremula*, *P. tremuloides*, or *P. alba*), and the majority of ESTs were also from that section of the genus. However, the cottonwoods are much more important commercially in the United States, and cottonwood hybrids were the leading candidates for high-yield plantations for bioenergy and carbon sequestration (Perlack et al., 2005, Tuskan, 1998). Furthermore, most genetic maps and pedigrees were for cottonwoods (Bradshaw et al., 1994; Cervera et al., 2001; Yin et al., 2008, 2004b), and the existing BAC libraries were also from cottonwoods (Lescot et al., 2004; Stirling et al., 2001), so the most relevant resources for genome sequencing and assembly were already in hand.

There are several ironies about the Nisqually-1 genotype: (1) the disease resistance gene that originally piqued interest in this genotype still has not been isolated, due in part to high complexity of this genomic region, which may be linked to suppression of recombination in the large pedigree, thereby making map-based cloning nearly impossible (Stirling et al., 2001; Yin et al., 2004a) (2) the original ortet has since been destroyed by flooding in its native habitat along the Nisqually River; and (3) Nisqually-1 has proven to be somewhat difficult to handle in tissue culture. Even though transformation protocols have been successfully developed (Ma et al., 2004; Song et al., 2006), the relative difficulty and inefficiency of transformation suggest that this clone is unlikely to supplant aspen hybrids as the model of choice for functional genomics in *Populus* (Busov et al., 2005).

2.3 Preparation of Sequencing Template and Shotgun Sequencing

Nisqually-1 genomic DNA was initially extracted from surface-sterilized leaves using a CTAB-based protocol. This template was used to construct 3-kb and 8-kb libraries that yielded most of the sequence data. When it became clear that this template was heavily contaminated with chloroplast and mitochondrial DNA (see below), a second set of templates was prepared from root tissue grown in hydroponics and tissue culture. This DNA extraction protocol involved a nuclei isolation step using a sucrose gradient followed by a cesium chloride gradient centrifugation step. This DNA, which was virtually free of plastid contamination, was used to construct the fosmid libraries.

A total of 7.65 million sequence reads were generated from these libraries, with 4.4 million reads coming from 3 kb libraries and 2.5 million reads from 8 kb libraries, and 650,000 reads from fosmid libraries. In addition, 81,904 end sequences were obtained from BAC clones that averaged 100 kb in size (Kelleher et al., 2007). This resulted in a theoretical sequence coverage of the genome of nearly 10X (i.e., an average of 10 sequences representing each nucleotide position), and an expected clone coverage of nearly 70X (i.e., the average number of clone inserts covering each position in the genome, though only the ends of the clones are represented by actual sequence).

2.4 Sequence Assembly

The shotgun sequences were initially assembled based on homology and paired end read information using the JAZZ assembler (Aparicio et al., 2002). The assembly process began with identification and masking of reads derived from repetitive regions of the genome. This was accomplished by counting the number of occurrences of all 16mer "words" in the entire set of 7.65 million sequences. This "word" length was chosen because words of this length are not expected to occur in a genome of the expected size of *Populus*. The distribution of 16mer words was examined, and it was determined that a discontinuity occurred around 34 occurrences. Therefore, 16mers that occurred more than this were masked from the sequence reads prior to assembly. This resulted in removal of entire reads from the assembly process and mitigated the confounding effects of repetitive DNA on shotgun assembly (Green, 2001). Pairwise alignments of all sequences were then performed, and contigs were constructed by converting pairwise relationships to a graph topology and finding the most direct route through the graph. Sequence contigs were joined using similar methodology, taking advantage of linkage information contained in paired end-read information.

The initial assembly utilized 4.8 million of the sequence reads to form approximately 45,970 sequence contigs of at least 1 kb in length, resulting in approximately 427 Mb of assembled genome sequence contigs, excluding gaps. These contigs were grouped together using paired clone end information into 22,136 sequence scaffolds that covered 464 Mb of assembled sequence and "captured" sequence gaps (the size of which was estimated based on average clone insert size). The approximately 3.6 Gb of sequence assembled to an average depth of 7.4X, resulting in an overall genome size estimate of approximately 485 Mb, which is in rough agreement with estimates based on flow cytometry (Bradshaw and Stettler, 1993). It is remarkable that the assembled sequence size matches the predicted size so closely, suggesting that misassembly is a relatively small problem, and that gaps and incorrectly split haplotypes approximately balance one another.

2.5 Contamination of the Sequencing Template

Nearly 2.85 million of the original sequence reads were not included in the final whole genome shotgun assembly. Approximately 750,000 of these were simply low-quality or chimeric sequences that were excluded by the JAZZ assembler. However, 2.1 million were high quality sequences that otherwise should have assembled. The leaf-derived sequences failed to assemble at a higher rate (34%) than the root-derived sequences (24%), suggesting that the DNA extraction method was related to this problem. Two sets of sequences with unusual sequence depth (954X and 60X, respectively) were removed and assembled into putative chloroplast and mitochondrial genomes, respectively. These accounted for approximately 300,000 of the unassembled sequences. Another 613,000 sequences corresponded to *Populus*

repeat elements, as determined by high 16mer composition and comparison to *Populus* repeat libraries using WU-BLASTN (see below). The remaining 1.1 million unassembled sequences were compared to the NCBI nonredundant nucleotide database using WU-BLASTN searches. Approximately 600,000 of these sequences showed no homology to known sequences, and are therefore of unknown origin. An additional 482,199 had significant hits to known, non-*Populus* sequences. Of these, the vast majority had hits ($E < 1e^{-10}$) to other plants, and likely represent inexplicably unassembled portions of the *Populus* genome. However, nearly 25,000 of the remaining sequences had hits to fungi, bacteria, and viruses that were likely endophytic or pathogenic contaminants of the sequencing template, despite the fact that the leaves and roots were surface-sterilized prior to extraction. Similar trends were seen for small scaffolds from the sequencing dataset, where nearly 300 of the scaffolds <10 kb in size were apparently of microbial origin.

2.6 BAC-Based Physical Map

The generation of a 48,000 – member bacterial artificial chromosome (BAC) library, constructed from the *P. trichocarpa* Nisqually-1 genotype (Kelleher et al., 2007) was critical in generating the whole genome sequence, and in assembly of sequence scaffolds. Map assembly was based on the restriction enzyme digestion of over 46,000 clones, which were assembled into fingerprinted contigs (FPC) (Kelleher et al., 2007). An important part of the effort was to obtain BAC end sequences (BES) for a large fraction of the BAC clones. In total, almost 82,000 BES were obtained, with an average read length of 500 bp, corresponding to 41 Mb of *P. trichocarpa* Nisqually-1 genome sequence. These sequence reads, the majority of them paired end reads, were integrated into the whole genome shotgun sequence, and aided in effective assembly of sequence contigs on a large scale. Furthermore, the BES facilitated anchoring of the physical map to the genome sequence assembly, resulting in the generation of a combined genetic map-physical map-genome sequence for a large fraction of the genome (Kelleher et al., 2007).

An interesting observation from the physical map study was the rather frequent occurrence of BAC FPCs that appeared distinct due to distinct restriction fragment patterns, suggesting that they were from different genomic regions, yet which mapped to identical regions LG locations based on final analysis of the genome assembly. Figure 1 shows and example of this unexpected finding from a portion of LG_ II. The explanation for the phenomenon is that, like the genome sequence, the fingerprinted BAC library derived from a highly heterozygous individual, *P. trichocarpa* Nisqually-l, and thus largely represents a combination of two distinct haploid genomes. Genetic mapping of representative overlapping contigs such as those shown in Fig. 1, using SNP markers, showed that they co-localized to the same genetic locations as predicted from this interpretation (Kelleher et al., 2007). Thus, the sequences of overlapping BAC contigs representing distinct Nisqually-1 haploid genotypes, and this allowed insights into the nature of haploid diversity to

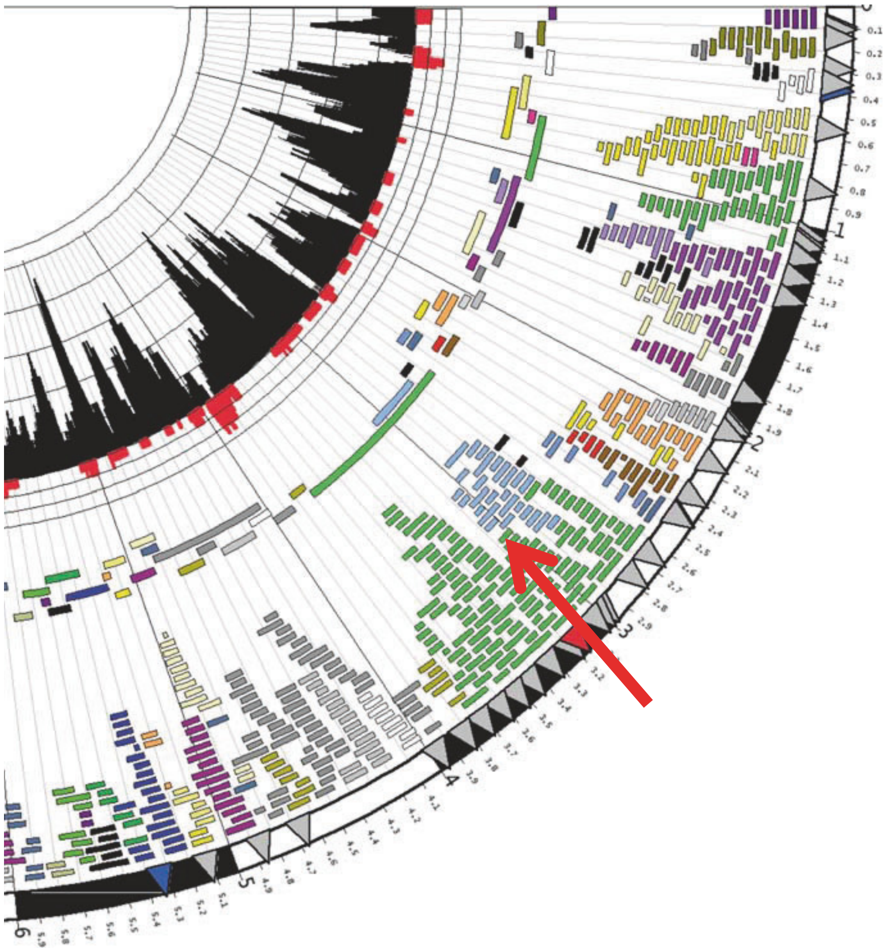

Fig. 1 A section of the LG_II genome assembly showing overlapping BAC finger print contigs (FPC) that map to the same site. *Arrow* indicates and example of such contigs (*blue* and *green*), that represent distinct haploid genomes in the Nisqually-1 genotype. BAC FPCs anchored to the genome assembly and genetic map (*outer ring*) are indicated in alternating color schemes; each color represents a different FPC. See Kelleher et al. (2007) for more details

be made by targeted sequencing of BAC clones representing the two haploid genotypes. Based on this analysis, numerous SNP polymorphisms (roughly 1 per 100 bp) were evident, as well as indels that ranged from a few bases up to 11 kb in size (Kelleher et al., 2007).

Comparison of the sequences of BAC clones representing alternative Nisqually-1 haploid genotypes to the genome assembly revealed, as expected, that the assembled genome sequence is a mosaic of the two Nisqually-1 haploid genotypes (Fig. 1 and Kelleher et al., 2007). Interestingly, in this example, an 11-kb indel

present in BAC T0068B19 is absent in the genome assembly. While there was no evidence for expressed genes in this region, this does raise the possibility of alternative gene content in different *P. trichocarpa* haploid genotypes. It should be noted that, due to the extremely rapid decay of linkage disequilibrium (LD) in *Populus* (LD in both *P. trichocarpa* and *P. tremula* decays over a few hundred bp; Chapter 5), large haplotype blocks are unlikely to exist in over the bulk of the *Populus* genome. That is, loci along the LGs are disrupted by extensive recombination precluding the inheritance of even closely linked loci as haplotype blocks in *P. trichocarpa* populations. One important exception to this is localized to LG_XIX, where a region of suppressed recombination appears to contain haplotypes that could contribute to specification of gender (Yin et al., 2008), as discussed below.

An important part of the sequencing and annotation effort was the use of EST and full-length (FL) cDNA information. While extensive EST information provided good tools for annotating expressed genes, a collection of 4,664 FL cDNAs, largely from the *P. trichocarpa* Nisqually-1 genotype (Ralph et al., 2008) was instrumental in training the gene prediction software for the annotation phase of the genome project (Tuskan et al., 2006).

2.7 Map-Based Assembly

The large number of scaffolds in both the sequence assembly and the physical map posed substantial challenges for the analysis and application of the genome sequence. We therefore anchored sequence contigs onto a genetic map representing the 19 *Populus* chromosomes (Yin et al., 2008, 2004b). This was accomplished using sequence tags provided by PCR primers for 356 simple sequence repeat (SSR) markers that could be placed unambiguously in the sequence, as well as on the genetic map. The location of the primer sequences in the sequence scaffolds was determined based on BLASTN hits that occurred for forward and reverse primers, separated by approximately the expected SSR product size. This resulted in linking 155 major sequence scaffolds and 335 Mb of sequence into chromosomal linkage groups (LGs) (Fig. 2). The chromosome with the smallest number of assembled scaffolds, LG_IX, was covered by two scaffolds containing 12.5 Mb of sequence. This large scaffold seems to contain a large rearrangement of the sequence assembly compared to the genetic map, which was derived from a different genotype (Yin et al., 2004b). In contrast, the largest chromosome, LG_I, contained 21 scaffolds representing 35.5 Mb of sequence, with no major rearrangements between the genetic map and sequence (Fig. 2).

Some caveats are in order regarding the map-based assembly. First, the vast majority of the markers used for genome assembly were mapped with only 44 progeny, so the resolution of the map is quite low, and small sequence scaffolds

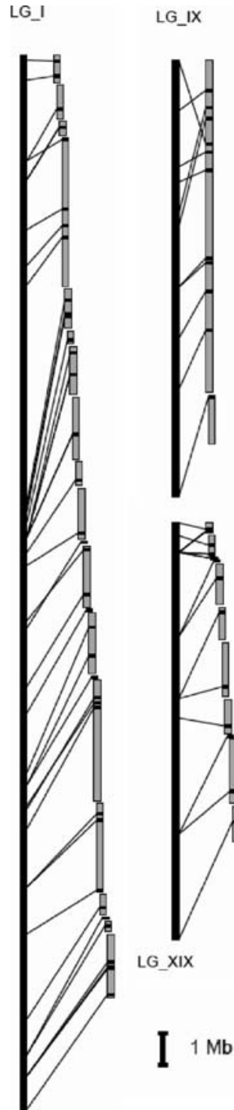

Fig. 2 Representation a portion of a recent map-based assembly of the *Populus* genome. *Black bars* represent chromosomal linkage groups derived from genetic mapping, and *grey bars* represent sequence scaffolds from the sequence-based assembly. Positions of microsatellite markers are indicated by *lines* connecting the linkage groups to the sequence scaffolds. LG_I is the largest assembled linkage group, being more than twice as large as each of the other linkage groups. LG_IX has the fewest scaffolds assembled, and is dominated by one scaffold which seems to have a rearrangement between the genetic and physical maps. LG_XIX shows evidence of strong haplotypic divergence at its distal end (*top*), which contributed to a complex map-based assembly of small sequence scaffolds

are not always positioned or oriented accurately. Orientation is also unknown for the 75 scaffolds that are mapped with a single marker. Second, the low resolution of the map can also lead to tandem assembly of scaffolds representing different haploid copies of the Nisqually-1 genome that should actually be assembled to the same position. This type of misassembly can easily be misinterpreted as large-scale tandem or segmental duplications. It is extremely difficult to distinguish between misassemblies of this type and true duplication events.

One example of a problematic region of the assembly is the peritelomeric (top) portion of LG_XIX (Fig. 2). This chromosome has been investigated in some detail, and demonstrated by intensive mapping to have very strong haplotypic divergence in the misassembled region, as discussed above (Yin et al., 2008). This region also shows strong suppression of recombination in multiple mapping pedigrees (Cervera et al., 2001; Woolbright et al., 2008, Yin et al., 2004b). Interestingly, sex determination also maps to this region in multiple pedigrees (Gaudet et al., 2008; Markussen et al., 2007; Yin et al., 2008). Haplotypic divergence and reduced recombination are both expectations for chromosomes that are in the early stages of evolving into a sex chromosome (Ming and Moore, 2007), so the *Populus* genome sequence has potentially led to the discovery of a previously unrecognized incipient sex chromosome (Yin et al., 2008).

3 Populus Genome Structure

3.1 Overview

Assembly of the *Populus* genome and corresponding gene content to linkage groups made it possible to investigate the gross structure of the genome at a chromosomal scale. This revealed the striking existence of two whole genome duplication events. This was accomplished by making pairwise comparisons among all *Populus* genes using double-affine Smith-Waterman alignments. This revealed the presence of large syntenic blocks of genes on different linkage groups that had approximately concordant genetic distances (Fig. 3). Blocks of these syntenic genes were defined based on the existence of two or more genes aligning on different chromosomes, with fewer than 10 intervening, nonaligning genes. The genetic distance between these aligning genes was calculated based on the number of transversion substitutions at four-fold degenerate nucleotide sites (4DTV), which is a conservative estimate of genetic divergence that should be less susceptible to multiple substitutions than more commonly-occurring synonymous substitutions (Comeron, 1995). Comparison of the size of the syntenic blocks versus the mean 4DTV value for those blocks revealed two clear groups of blocks that were of approximately uniform age (Fig. 4). The group of larger blocks centered at 4DTV = 0.068 represents the most recent whole genome duplication in *Populus*, while the group of

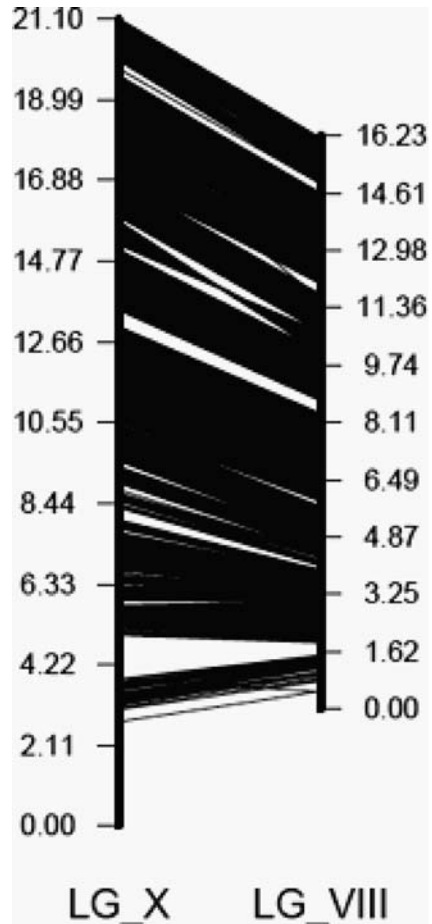

Fig. 3 Comparison of genes with significant alignments between two linkage groups, chromosome X (*left*) and Chromosome VIII (*right*). Genes are represented by *black lines* connecting the *heavy lines* representing the chromosomes. Position on the linkage group is given in Megabases to the side of each group. Genetic distances between genes in these large syntenic blocks are highly concordant, indicating that these syntenic chromosome blocks originated from whole genome duplication events

smaller blocks at 4DTV = 0.31 represents a more ancient duplication event (Sterck et al., 2005). Dating of these events is difficult, because the *Populus* genome is evolving considerably slower than genomes that have previously been used to calibrate the angiosperm molecular clock. Using the molecular clock as calibrated by fossil records for the Brassicaceae, for example, the most recent duplication dates to 8 million years ago (Sterck et al., 2005). However, the *Populus* genus has been in existence for at least 50 million years (Eckenwalder et al., 1996), and the genome duplication is shared by many species in the genus (Sterck et al., 2005), so the *Populus* genome is clearly evolving at a much slower rate than herbaceous angiosperms, which is to be expected based on generation time (Bell et al., 2005).

The Arabidopsis genome also shows evidence of at least two whole-genome duplication events (Blanc et al., 2003), but following these events the genome has

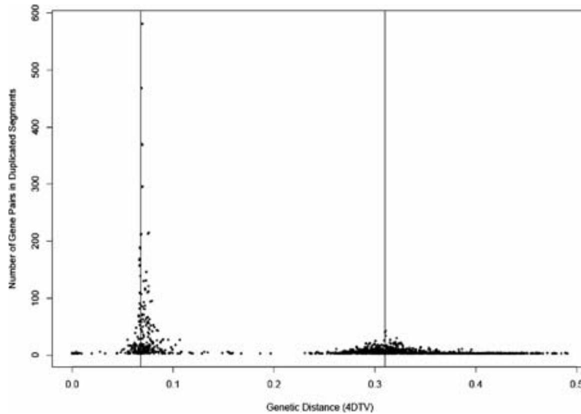

Fig. 4 Observed number of genes aligned in approximately syntenic blocks between chromosomes vs. the genetic distance between genes, as measured by the rate of transversions at four-fold degenerate nucleotide sites (4DTV). Peaks corresponding to the putative Salicoid and Eurosid whole-genome duplication events are indicated as peaks at the centers of clusters of similar 4DTV values, indicating the existence of large numbers of duplicated blocks of approximately the same age

become substantially rearranged, making it difficult to reconstruct the older events (Blanc et al., 2003). A similar rearrangement has occurred in *Populus*, but much less severe (see data in Tuskan et al., 2006). Extensive rearrangements following genome doubling is a common component of the diploidization process (Adams and Wendel, 2004; Semon and Wolfe, 2007). The structural complexity of these two genomes, coupled with the high rates of gene evolution in Arabidopsis, make it particularly difficult to establish orthology and determine whether the ancient duplication event in *Populus* is shared with Arabidopsis. The timing of the event is similar to the timing of the split of the Arabidopsis and *Populus* lineages, as determined by pairwise comparisons of genetic distances between *Populus* duplicated genes, Arabidopsis duplicated genes, and between putative Arabidopsis and *Populus* orthologs. Given the close timing of these events, it is tempting to speculate that the genome duplication was a primary driver of the diversification of the rosids (Lynch and Conery, 2003).

3.2 Comparison of the Populus and Grape Genomes

After the publication of the *Populus* genome the genome of wine grape (*Vitus*) became available (Jaillon et al., 2007; Velasco et al., 2007) allowing the structures of three fully sequenced eudicot genomes to be compared. In contrast to *Populus* and Arabidopsis, the grape genome has been comparatively quiescent, with minimal rearrangements, and equivocal evidence of a single whole genome duplication that could be shared with *Populus* and Arabidopsis (Jaillon et al., 2007; Velasco et al., 2007). This structural simplicity has allowed reconstruction of the truly ancient whole genome duplication event that is shared by all angiosperms. It

appears that this event resulted in hexaploidy in the ancient angiosperm progenitor, as suggested by the presence of three syntenic blocks in rice, *Populus*, and Arabidopsis for every one block in grape (Jaillon et al., 2007). However, evidence for this event is still weak, because the genetic distance is too great to allow relative dating with nucleotide substitution rates, and it is possible to confound two different duplication events that occurred at very different times, followed by massive rearrangement and gene loss. This same analysis (Jaillon et al., 2007) suggested that only one duplication event had occurred in the *Populus* genome, despite the existence of virtually unequivocal evidence for two events when relative levels of divergence are taken into account (Fig. 4). Part of the problem is the confusion about the proper relationship of grape to the Rosids. If grape is taken as on outgroup, then a clear model that incorporates both *Populus* duplications can be accommodated (Velasco et al., 2007). However, if grape is assumed to be closer to *Populus* than Arabidopsis, as sequence similarity suggests, then grape would have to share the duplication event that occurred near the time of the split with *Populus*. However, this event was not detected in grape or *Populus*, based on an analysis using reciprocal BLASTP hits, without regard to degree of divergence of putative orthologs (Jaillon et al., 2007). Fortunately, many more plant genomes are currently in sequencing pipelines, so the duplication history of the angiosperms will soon become much clearer.

3.3 Repeat Composition Compared to Other Sequenced Genomes

Repeated portions of the *Populus* genome are relatively poorly represented in the genome assembly due to the difficulty of assembling repetitive portions of the genome. As described above, repeat elements were first masked out of the assembly based on word frequency in the raw sequence reads. This word frequency also provides a convenient estimate of the repeat composition of the assembled genome. Approximately 41% of the assembled genome was repetitive based on 16mer counts greater than 34, and the frequency of 16mers was negatively correlated with the number of gaps in sequence scaffolds and the size of sequence scaffolds ($P = 0.02$; Fig. 5a). Furthermore, at the whole chromosome scale, the proportion of the linkage group that is composed of repeats is positively correlated with the proportion composed of sequence gaps (Fig. 5b), and negatively correlated with the proportion composed of predicted exons (Fig. 5c).

A more formal characterization of repeat composition reveals some interesting patterns in the *Populus* genome compared to other sequenced plants. We identified repeats by performing TBLASTN searches of the assembled genome using conserved portions of known plant repetitive elements. We also identified repeated sequences by performing all-vs.-all BLASTN searches of the assembled genome, followed by delineation of conserved repetitive elements using the RECON program (Bao and Eddy, 2002), followed by provisional annotation of the elements based on best BLASTN hits versus plant repeat databases from The Institute for Genomic

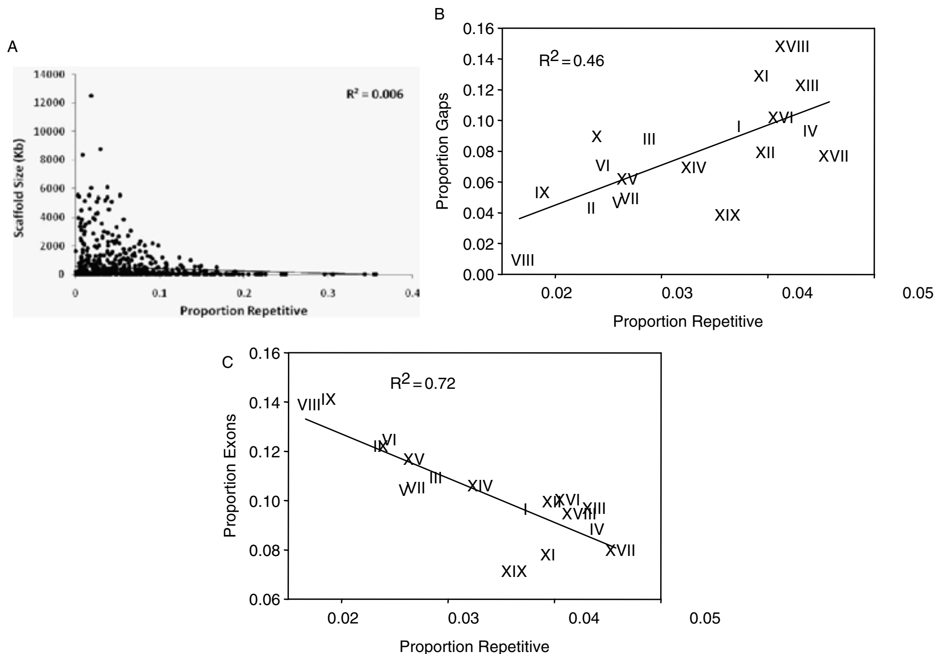

Fig. 5 Relationships between repetitive DNA and aspects of genome assembly and content. (**a**) Relationship between the proportion of a sequence scaffold that consisted of repetitive DNA (as indexed by 16mer word counts > 34) and size of the sequence scaffold. (**b**) Relationship between the proportion of a linkage group consisting of sequence gaps and the proportion of repetitive DNA, and (**c**) Proportion of the linkage group consisting of exons vs. proportion repetitive

Research. Conserved elements were then incorporated into a RepeatMasker library and the entire genome was masked using the WU-BLAST option. Individual elements were quantified by merging overlapping hits, and the relative abundance of each major class of transposable elements was quantified. In total, 181 Mb of the *Populus* genome was identified as repetitive using these methods, and over 12,000 individual repetitive elements were identified. The most abundant classes are Long Terminal Repeat (LTR) Gypsy, covering 23.9 Mb, CACTA elements, covering 5.5 Mb, and Long Interspersed Nuclear Elements (LINEs), covering 2.3 Mb. All known plant transposable element families were detected in *Populus*, but the relative abundances differed compared to other plants (Fig. 6). In particular, Type I elements (those that have an RNA intermediate for transposition) seem to be over-represented in *Populus* compared to other sequenced plants, with LINEs and Ty3/Gypsy elements particularly standing out. In contrast, Type II elements, especially the Mutator-Like Elements (MULEs) seem to be under-represented in *Populus* compared to Arabidopsis, Lotus, and Brassica. The LINEs in *Populus* are particularly interesting because there is some evidence of recent expansion, as revealed by large numbers of elements with short branch distances in Neighbor-Joining trees (Fig. 7).

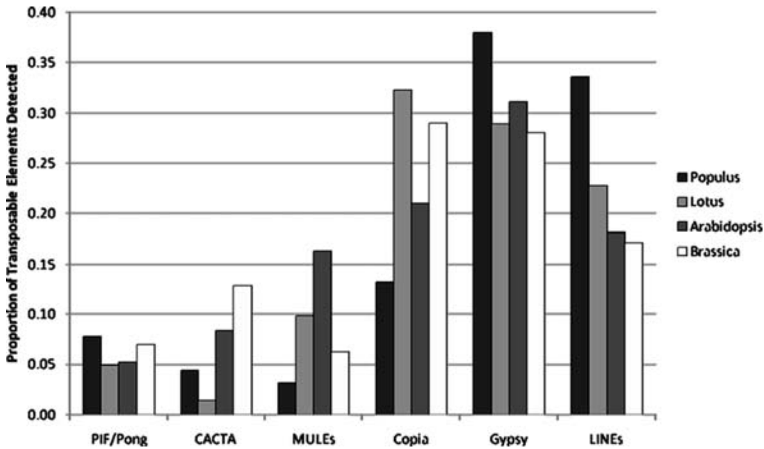

Fig. 6 Relative number of transposable elements detected in the genomes of *Populus trichocarpa*, *Lotus japonicus* (Holligan et al., 2006), *Arabidopsis thaliana* and *Brassica oleracea* (Zhang and Wessler, 2004) using methods described in Holligan et al. (2006)

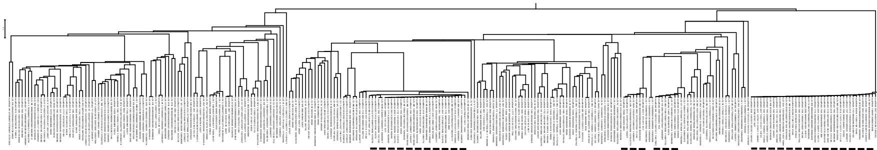

Fig. 7 Neighbor-joining tree depicting relationships among reverse transcriptase domains of LINEs in the *Populus trichocarpa* genome. Note the clusters with very short genetic distances (highlighted by *dashed lines*), suggesting recent large-scale activity in these LINE families

4 Gene Content and Comparative Genomics

4.1 Overview of Gene Prediction Methods and Gene Content

A total of 45,555 nuclear gene models ("Reference" set) were predicted in the genome assembly, and are given on the JGI P. trichocarpa v. 1.1 genome browser (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) (Tuskan et al., 2006). Automated gene calling predictions were based on based on *ab initio*, homology-based, and EST-based methods, including FgenesH Genewise, EuGène, and GrailExp6 (Birney and Durbin, 2000; Salamov and Solovyev, 2000; Xu and Uberbacher, 1997) were used independently by three different annotation groups we employed different methods. Details of the results from each method are given in Tuskan et al. (2006) Supplementary Data. As mentioned above, the annotation process was aided by the use of almost, 200,000 high quality EST sequences contributed by the *Populus* research community, and over 4,000 FL cDNA sequences

from enriched cDNA libraries prepared from Nisqually-1 that were used in training the gene-calling algorithms. Of the 45,555 genes promoted to the reference set, 4,286 models were manually annotated at a community "Jamboree". Subsequent to the publication of the genome paper (Tuskan et al., 2006) and development of the JGI v.1.1 *P. trichocarpa* genome browser, further manual annotation, genome sequencing, and sequence assembly has been carried out.

Almost 90% of the predicted *Populus* genes have similarity to Arabidopsis (Tuskan et al., 2006), and in many cases putative orthologs of *Populus* genes can be readily identified in Arabidopsis as discussed below. However, a set of almost 5,000 *Populus* genes was identified, many with evidence of expression, that do not have significant sequence similarity to any Arabidopsis gene (Tuskan et al., 2006).

Analysis of the predicted complete gene sets from *Populus* and Arabidopsis produced over 9,000 groups of inparalogs representing 14,837 *Populus* genes and 12,618 Arabidopsis genes (Tuskan et al., 2006), and the average *Populus* to Arabidopsis ratio across all orthologous groups is 1.33. However, there is great variation in the gene-to-gene ratio between the two taxa for different gene families. Paired genes at a ratio of 1:1 *Populus* to Arabidopsis, represented by over 4,600 gene pairs, is most frequent ratio, and the next most frequent ratio is 2:1. However, extreme ratios illustrate selective and large scale expansions of some gene families in both species were reported, for example F-box domain proteins have ratio of 1:40 *Populus* to Arabidopsis, while a zinc finger (B-box type) family protein/salt tolerance-like protein family has a ratio of 20:1 *Populus* to Arabidopsis (Tuskan et al., 2006). More recent analysis of the F-box protein families in *Populus*, grapevine, and papaya relative to those in Arabidopsis and rice, support the differential expansion of F-box encoding genes in herbaceous plants (Yang et al., 2008).

4.2 Retention of Duplicate Genes

In order to determine if there has been any selectivity in the retention of duplicated genes following the two recognizable whole genome duplication events in the *Populus* lineage (the Eurosid duplication and Salicoid duplication; Tuskan et al., 2006), we examined retention of genes that arose from these events, according the GoSLim (http://www.geneontology.org/GO.slims.shtm) gene ontology category (Fig. 8). Interestingly, genes in the transcription factor activity, DNA or RNA binding, and nuclear localized GoSLim categories were much more likely to have been retained (observed/expected retention much greater than 0), and there was a low likelihood that no duplicated genes in these categories were retained (observed/expected retention much lesser than 0). In contrast, this bias was not observed for genes in several other GoSLim categories, in which opposite trends were observed. These data suggest that *selection* for retention of duplicated genes potentially involved in gene regulation has helped shape the *Populus* genome, perhaps allowing new regulatory pathways important in *Populus* life history and morphology to evolve.

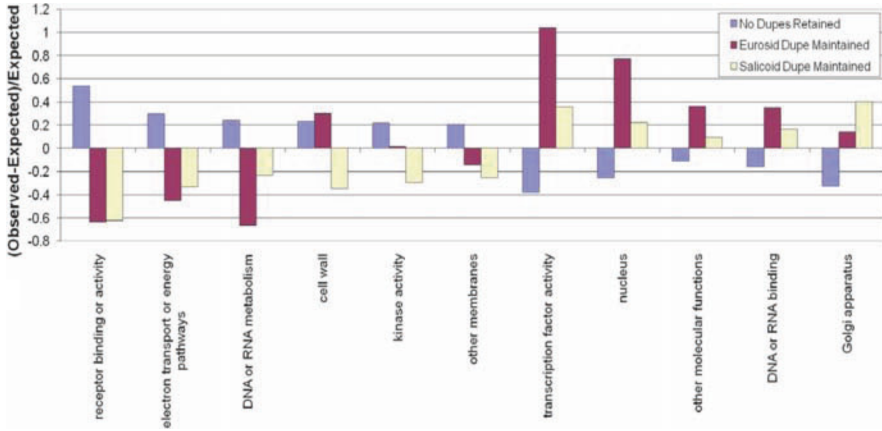

Fig. 8 Retention of duplicated *Populus* genes accoriding to functional category. GoSLim (http://www.geneontology.org/GO.slims.shtm) gene ontology categories that showed significant deviations from expected rates of loss (*blue bars*) or retention following Eurosid (*maroon*) and Salicoid (*yellow bars*) whole genome duplications are shown. Significance was determined based on Chi-squared tests

4.3 Synteny with Other Species

Availability of the *Populus* genome sequence has allowed *in silico* testing of the presence and degree of microsynteny (retention of gene order over multiple genes, but not large portions of chromosomes) between *Populus* and other species, such as Arabidopsis, for which genome sequence information is available.

Kalluri et al. (2007), when annotating and describing the *Populus* gene families encoding Auxin Response Factor (AFR) transcription factors, and their cognate AUX/IAA interacting proteins, noted significant microsynteny between the *Populus* and *Arabidopsis* regions surrounding orthologous genes. In one of the more detailed studies on microsynteny between *Populus* and Arabidopsis, Johnson and Douglas (2007) examined the comparative structure of the genomic regions surrounding the two duplicated *ARF5* (*MONOPTOROS*) genes in *Populus*, relative to the single *ARF5/MP* gene in Arabidopsis. As expected based on the salicoid genome duplication, each *Populus ARF5/MP* locus and surrounding loci show a high degree of synteny with each other over 300–400 Mb regions on LG_II and LG_V. However, tandemly duplicated cytochrome P450 (*CYP,* or *P450*) genes are found within the syntynous block on one LG, and not the other. A high degree of synteny of both *Populus* regions to loci within a >100 Mb region surrounding the Arabidopsis *ARF5/MP* gene was also documented, illustrating conservation of this contiguous block of genes since the divergence of the two lineages (Johnson and Douglas, 2007). However, some rearrangements (an inversion, gene loss/gain) had occurred, illustrating some of the events leading to the disruption of synteny as the two genomes evolve independently.

Rapid advances it the generation of BAC-based physical maps has also allowed testing of synteny between non-sequenced plant genomes and that of *Populus*. For example, based on the localization of a sample of eight paired BAC end sequences to specific LG coordinates in the *Populus* genome, (Han and Korban, 2008) concluded that apple (*Malus*) shares significant microsynenty with *Populus*, consistent with the relatively close phylogenetic relationship of the two genera within the Eurosid I clade. Similar approaches taken in tomato (Datema et al., 2008) and papaya (Lai et al., 2006), which again revealed intriguing snapshots of microsynteny between these genomes and the *Populus* genome. Completion of a draft genome sequence of papaya (Ming et al., 2008), and the analysis of synteny between papaya sequence scaffolds and the genomes of Arabidopsis, *Populus* and grape provides strong support for retention of syntenic blocks, ranging in size from 181 to 19 genes, within all four eudicot lineages (Ming et al., 2008). Clearly, with the advent of numerous sequenced plant and tree genomes in the future, such comparisons well become routine at the whole genome level, and tools for carrying out such analyses of synteny to the *Populus* and to any other fully sequenced genome are being developed (Lyons and Freeling, 2008).

4.4 Comparison of Select Gene Family Composition Between Sequenced Genomes

Based on the availability of the *Populus* genome, comparative studies of gene family evolution in *Populus* relative to other fully sequenced genomes have been published at an increasing rate. Comparisons have been primarily to Arabidopsis and in some cases to rice, with more recent studies also exploiting full genomes of grape, papaya, and the moss *Physcomitrella patens*. These studies are too numerous to fully review in this context, but some selected examples are given below. When fully sequenced genomes of trees such as *Eucalyptus*, peach, apple, and others become available, it will be interesting to search for any similarities in gene family structure that may be common to the woody perennial growth habit, which has evolved multiple times. Unfortunately, due to the large phylogenetic distance between *Populus* and conifer trees such as pine and spruce, and the relative paucity of conifer complete open reading frame data it is difficult at present to carry out meaningful comparative genomics between conifer trees and *Populus*.

Two major studies, in addition to those in the *Populus* genome paper (Tuskan et al., 2006) have surveyed the diversity of genes encoding phenylpropanoid enzymes, including those leading via branch pathways to lignin and flavonoid biosynthesis, in *Populus* relative to Arabidopsis and other fully sequenced genomes (Hamberger et al., 2007; Tsai et al., 2006). Most phenylpropanoid enzymes are encoded by multi-gene families in plants, although some, in particular P450 enzymes, are encoded by single genes in Arabidopsis and other plants. A major finding of these studies is that a core of set of phenylpropanoid and lignin biosynthetic genes is mostly conserved in the lineages examined, but that duplicated

genes encoding key phenylpropanoid P450 enzymes in *Populus* involved in monolignol biosynthesis have been retained, and that *Populus* contains overall more lignin biosynthetic genes (Tuskan et al., 2006; Hamberger et al., 2007), perhaps related to greater metabolic commitment lignin and soluble phenolic biosynthesis. Interestingly, the genome of papaya, a "semi-woody giant herb" (Ming et al., 2008), has a set of lignin biosynthetic genes intermediate in size between *Populus* and Arabidopsis.

Strikingly, however, a diversification of genes encoding enzymes in flavonoid branch pathways has occurred in *Populus* (Tsai et al., 2006), consistent with its wealth of flavonoids including condensed tannins. Genes encoding enzymes related to, but likely distinct in function from, true phenylpropanoid enzymes, are well known from the Arabidopsis genome. Hamberger et al. (2007), as well as de Azevedo Souza et al. (2008) compared structures of such "phenylpropanoid-like" genes in *Populus*, Arabidopsis and rice. Most phenylpropanoid-like clades are conserved in all three lineages, and in some cases *Physcomitrella*, suggesting conservation of metabolic function. However, clear lineage-specific amplification of certain families in each of the three lineages was observed, suggesting the evolution of lineage-specific metabolism.

Transcription factors are an attractive target for comparative genomics, since they are encoded by multi-gene families, some of which have expanded dramatically plants based on their abundance in Arabidopsis, and they are relatively easy to annotate, based on presence of conserved DNA binding domains that define families. Lineage-specific evolution of gene families has the potential to underlie changes in gene expression that may in turn underlie evolution of different developmental, life history, and adaptive traits in plants. Examples of comparative studies on transcription factor families are those on AFR and AUX/IAA regulators (Kalluri et al., 2007); MADS box transcription factors (Leseberg et al., 2006) and AP/ERF transcription factors (Zhuang et al., 2008).

In general, duplicated members of transcription factor gene families have tended to be retained in *Populus* following the eurosid and salicoid whole genome duplication (see Fig. 8). For example, the *ARF* and *AP/ERF* transcription factor families are about 1.5-fold larger in *Populus* than Arabidopsis (Kalluri et al., 2007; Zhuang et al., 2008). In other cases, *Populus*-specific expansion of transcription factor clades has occurred. For example, expansion of three ARF subgroups is evident in *Populus* (Kalluri et al., 2007). Expansion in numbers certain MADS box homologs is correlated with functions in root growth, control of flowering, and cambial activity (Leseberg et al., 2006). Clearly, functional genomic approaches will be required to sort out the functions of such *Populus* paralogs.

A preliminary comparative analysis of genes encoding transporters in *Populus* and Arabidopsis has been carried out (Tuskan et al., 2006; Supplemental Data). Overall, the total number of annotated transporters in *Populus* is about 1.7-fold higher than in Arabidopsis (1722 vs., 959; Tuskan et al., 2006). Some transporter families have undergone striking expansion in *Populus* relative to Arabidopsis, for example the ATP-dependent ATP Binding Cassette (ABC) class of transporters contains 226 members in *Populus* relative to 117 in Arabidopsis, while the smaller

Sulfate Permease (SulP) family consists of 22 members in *Populus* relative to 12 in Arabidopsis, and the Auxin Efflux Carrier family has 31 *Populus* members relative to 8 in Arabidopsis.

These and other studies, while tantalizing, offer only the first glimpses into the comparative genomics of gene family evolution in *Populus* relative to other plants and trees. *In silico* analyses based on gene content, gene orthology, and gene expression across a wide variety of plants and trees in the coming years will provide important information as to potential functions of specific gene family members. However, targeted and genome-informed experimental studies in *Populus* and other species will be necessary to provide definitive interpretations of genomic data, and will be one of the ultimate legacies of the plant and tree genome era, ushered in to a large extent by the sequencing of the *Populus* genome.

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Nucleotide Polymorphism, Linkage Disequilibrium and Complex Trait Dissection in *Populus*

Pär K. Ingvarson

Abstract Poplars and aspens often have very wide geographic distributions, large population sizes and are often highly outcrossing. These life history traits should promote the maintenance of abundant levels of genetic variation in trees and early data based on allozyme diversities also confirm these expectations. However, investigation of variation at the nucleotide level has only recently begun in Populus. Surveys of nucleotide polymorphism in Populus have shown relatively high levels of synonymous diversity, between 0.5–1%. Levels of linkage disequilibrium is also relatively low in Populus, although there seems to be large differences between species. The observations of low levels of LD in Populus are so far confined to coding regions and levels of LD non-genic regions is still uncharacterized. Nevertheless, these features suggest that very fine scale mapping is possible in Populus. In principle using recently developed methods in association mapping it should therefore be possible to map quantitative trait variation down to single causal nucleotide changes in Populus.

1 Genetic Diversity in *Populus*

Many forest trees have very wide geographic distributions and large population sizes. Forest trees are also often highly outcrossing, have long generation times and in many cases have extensive gene flow through both pollen and seeds. Taken together these life history traits should promote the maintenance of abundant levels of genetic variation in trees. Early data based on allozyme diversities in a large number of plant species also confirm these expectations; long-lived, woody perennial plants with wide geographic ranges, particularly those with a boreal-temperate distribution, generally had high levels of genetic diversity (heterozygosity) and a

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large fraction of polymorphic loci with many alleles per locus (Hamrick and Godt, 1990).

Despite the popularity of allozyme studies in plant genetics, relatively few studies have used allozymes to study variation in natural population of *Populus*, but the few studies in *Populus* do support the predictions of high levels of genetic variation. For instance, allozyme surveys in the North America species *P. tremuloides* have shown that it contain among the highest levels of genetic diversity observed in plants (Cheliak and Dancik, 1982; Jelinski and Cheliak, 1992; Liu and Furnier, 1993), despite the presumed high occurrence of clonal growth in this species (Kemperman and Barnes, 1976; Grant et al., 1992). A more comprehensive review of allozyme studies of genetic diversity in *Populus* can be found in Farmer (1996).

Recently the attention has shifted to the use of Single Sequence Repeats (SSRs), also known as microsatellites, for screening genetic variation in *Populus*. SSRs usually have mutation rates that are several orders of magnitude greater than base-pair substitutions that are the underlying cause of allozyme variation ($\mu = 10^{-2}$–10^{-4}) and SSRs are therefore usually highly variable (Estoup and Cournet, 1999). SSR markers also evolve in a step-wise manner and ancestral alleles can therefore be identified (Estoup and Cournet, 1999). Because of this SSR markers typically contain much more information per marker than other types of genetic markers do (Estoup and Cournet, 1999). These studies have shown that levels of variation at SSR loci is also high in *Populus* (Cole, 2005; Hall et al., 2007; Smulders et al., 2008). Cole (2005) compiled data from allozyme and SSR variation and showed that, as expected, levels of genetic diversity are substantially higher at SSR loci across a number of species of *Populus*.

These data also show that *Populus* species generally have low levels of population structure (Cole, 2005; Hall et al., 2007). In *P. tremuloides* genetic differentiation among eleven sites in Wisconsin, measured using F_{ST}, averaged 0.045, with a range of 0.006–0.046 across 16 SSR loci. Similarly, genetic differentiation among twelve populations of *P. tremula* sampled from across Sweden averaged $F_{ST} = 0.015$, with a range of –0.031–0.064 across 25 SSR loci. These data are entirely consistent with expectations based on the wide-spread geographic distributions of most species of *Populus* and the great dispersal capabilities of pollen and seeds, which are both wind-dispersed.

2 Nucleotide Polymorphism in *Populus*

Under the simplified scenario of a Wright-Fisher population (Ewens, 2004), expected levels of polymorphism at the nucleotide level are proportional to the effective population size, and to the mutation rate, μ. For diploid species nucleotide polymorphism is proportional to

$$\theta = 4N_e\mu \quad (1)$$

What has become apparent from the *P. trichocarpa* genome project is that the per nucleotide mutation rate is rather low in *Populus*. Tuskan et al. (2006) estimate the mutation rate in *Populus* to be six-fold lower than in *Arabidopsis*. Koch et al. (2000) estimate the mutation rate per year in *Arabidopsis* to 1.5×10^{-8} suggesting that the mutation rate in *Populus* is roughly 2.5×10^{-9}, which is low compared to most Angiosperms (Wolfe et al., 1987; Muse, 2000). This is the mutation rate *per year*; however, what matters for determining levels of intraspecific polymorphism from Equation (1) it is the mutation rate *per generation*. Since the generation time in *Populus* is at least 15 years, the *per generation* mutation rate is likely on par or even higher than in most Angiosperms.

The other important parameter determining standing levels of genetic variation in a species is the effective population size, N_e. The effective population size has traditionally been used to rescale a given population genetic model so that it behaves as a standard Wright-Fisher model of constant size (Ewens, 2004). There are several ways of defining an effective population size for a given population model; sometimes these definitions produce similar N_es whereas in other cases they do not and in yet other cases the effective population size may not even exist (Ewens, 2004). The concept of an effective population size is useful because it captures complex demographic patterns and reduce them to the standard Wright-Fisher model, thereby highlighting how these demographic patters affect rates of genetic drift (Ewens, 2004). Theory predicts that as long as the actual population size remains not too large ($N_e < 10^9$ or so), effective population size should scale well with actual population size (Gillespie, 1999).

Given the relatively high mutation rate, the expectation of a large effective population size, and based on the life history characteristics listed in the Introduction, population genetics theory suggest that *Populus* should contain abundant levels of nucleotide polymorphism. At a first glance this appears to be true, at least in *P. tremula*, where silent site nucleotide diversity average $\pi_{syn} = 0.012$ across 77 short gene fragments (Ingvarsson, 2005, 2008b). However, the stochastic nature of the coalescent process generates significant variation in nucleotide polymorphism among loci, as illustrated by Fig. 1 which documents variation in polymorphism among 131 loci from *P. tremula*.

In many other species of *Populus*, like the North American species *P. trichocarpa*, *P. balsamifera* and *P. deltoides*, levels of silent site nucleotide polymorphism appear to be substantially lower than *P. tremula* (Table 1). Based on Ecotilling, Gilchrist et al. (2006) estimate the silent site nucleotide diversity of $\pi = 0.0029$ across nine genes in *P. trichocarpa*, where as re-sequencing of ten genes by Ismail et al. (unpublished) estimate silent site diversity in *P. trichocarpa* to be $\pi = 0.0049$. Furthermore, silent site diversity estimated from the diploid genome sequence of *P. trichocarpa* averages $\pi_{syn} = 0.0035$ (Tuskan et al., 2006). Data from silent sites is thus in agreement with a four to five-fold greater effective population size in *P. tremula* compared to *P. trichocarpa* or *P. balsamifera*.

Black poplar (*P. nigra*), on the other hand, harbor similar levels of nucleotide polymorphism as *P. tremula* do, with synonymous site diversity averaging $\pi = 0.0107$ across a sample of ten genes (Chu et al., 2009) and has levels of

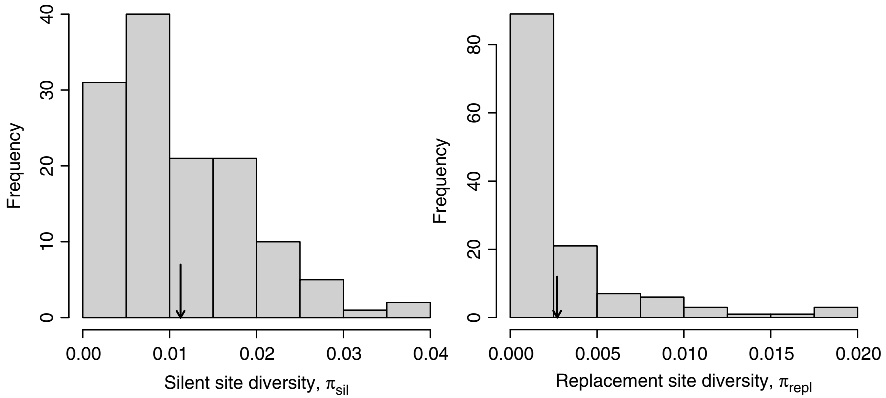

Fig. 1 The distribution of polymorphism at silent and replacement sites for 131 loci from *P. tremula*. Note that the scale of x-axis differs between the two figures. *Arrows* denote the mean polymorphism for silent and replacement sites

Table 1 Summary of surveys of nucleotide polymorphism in *Populus*

		Polymorphism			
Species	Loci	Total	Synonymous	Non-synonymous	Citation
P. trichocarpa	10	0.0037	0.0040	0.0023	Ismail et al. (unpublished)
P. trichocarpa	8	0.0018	0.0029	na	Gilchrist et al. (2006)
P. tremula	77	0.0042	0.0120	0.0017	Ingvarsson (2008b)
P. nigra	9	0.0107	0.0107	0.0046	Chu et al. (2009)
P. balsamifera	3	0.0030	0.0030	0.0023	Breen et al. (2009)
P. deltoides	2	0.0021	0.0058	0.0011	Breen et al. (2009)

non-synonymous diversity that is actually substantially higher than what is observed in *P. tremula* (Table 1). The genes surveyed by Chu et al. (2009) were selected based on the putative importance for growth, so it is possible that these genes are targets of local selection resulting in enhanced levels of non-synonymous polymorphism (Ingvarsson et al., 2006; Chu et al., 2009).

Compared to many other plants levels of nucleotide polymorphism observed in different species of *Populus* are not unusually high; if anything it is lower than in many annual and short-lived perennial plants (see Savolainen and Pyhäjärvi, 2007). Interestingly, several other forest trees, such as *Pinus* and *Picea* also show rather modest levels of nucleotide polymorphism (Savolainen and Pyhäjärvi, 2007). This lead (Savolainen and Pyhäjärvi 2007) to suggest that many tree species have not reached equilibrium levels of genetic diversity because historical changes in population size reduced nucleotide diversity an a genome-wide scale (Savolainen and Pyhäjärvi, 2007). Interestingly, several recent studies have documented historical reductions in the effective population size of many forest trees. For instance,

Heuertz et al. (2006) and Pyhäjarvi et al. (2007) both found evidence for historical bottlenecks in Norway spruce and Scots Pine, respectively. Ingvarsson (2008b) used Approximate Bayesian Computation to estimate the timing and extent of past demographic events in *P. tremula*. Several different demographic models were fit to nucleotide polymorphism data from 77 short (~500 bp) fragments and the results unequivocally indicate that *P. tremula* has gone through one (or more) bottleneck(s) in the recent past. The rather limited sequence data set used prevents a precise timing of the inferred bottleneck, but the data is consistent with a bottleneck occurring between 244 and 730 thousand years ago (KYA). Interestingly, the upper value of this estimate is close to the period in the early Quaternary (~700 KYA) that marks the beginning of the period of the strong climatic fluctuations that have continued until the present (Comes and Kadereit, 1998). It is also notable that the lower bound is substantially older than the initiation of the last full glacial period, which commenced ~100 KYA and lasted until about 10 KYA. This suggest that the demographic modelling in *P. tremula* captures the combined effects of repeated bottlenecks that have occurred during multiple glacial cycles of the Quaternary. It is thus likely that *P. tremula* has gone through repeated population size contractions and expansions over the last millennia and that periods of alternating range expansions and contractions have involved periods of population subdivision into glacial refugia, as have been shown in many other plant species (Webb and Bartlein, 1992).

The historical reductions in population size also appear to have resulted in a reduction of nucleotide diversity in *P. tremula*. The silent-site estimate of θ is 0.0129 whereas the genome-wide estimate of θ from the ABC analysis is 0.0177, suggesting that polymorphism levels in *P. tremula* have been reduced by about 30% as a result of historical bottlenecks (Ingvarsson, 2008b). Using an estimate of $\theta = 0.0177$ and a per generation mutation rate of 3.75×10^{-8} (see above) Ingvarsson (2008b) estimate the effective population size of *P. tremula* to be roughly $N_e =$ 118.000 individuals, an estimate that seem low given the large geographic distribution of *P. tremula*. An interesting perspective on the apparent discrepancy between observed and expected levels of nucleotide polymorphism in *Populus* and many other forest trees is offered by recent theoretical work by Eldon and Wakeley (2006). Eldon and Wakeley (2006) show that species, for species with overlapping generations and variation in reproductive success among individuals greatly exceeding that expected by the standard coalescent, there is a much weaker connection between actual population size N and effective population size. Overlapping generations and large variation in reproductive success are life history characteristics that describe many trees where reproductive output increase by orders of magnitude over the life span of a single tree. Interestingly, this model also predicts very different patters of linkage disequilibrium than in the standard coalescent (Eldon and Wakeley, 2008), with the possibilities of low levels of LD even if recombination rates are low (see below). The model proposed by Eldon and Wakeley (2006) captures many interesting aspects of the life history of forest trees and if this model should turn out to be a better descriptor of coalescent processes in trees than the standard coalescent, many of the inferences drawn based on the standard coalescent may be inaccurate.

3 Linkage Disequilibrium in *Populus*

Linkage disequilibrium (LD), or the non-random association of alleles, has received a great deal of attention in recent years. The primary reason is the recent advances in genomics that have enabled possibility for directly study of statistical associations between genetic markers and complex traits in natural populations, so called linkage disequilibrium mapping of association mapping (Nordborg and Tavaré (2002), see also below). Association mapping holds great promise for fine-mapping genes and variants that contribute to a trait of interest. Importantly, the utility and power of association mapping is largely dependent on genome-wide levels of LD (Nordborg and Tavaré, 2002) and this has led to the characterization of genome-wide levels of LD in a number of plant species.

Levels of LD are affected by historical demography and other evolutionary processes that affect, in a manner similar to nucleotide polymorphism. For instance, population admixture or population structure tend to increase LD just as natural selection also increase LD around a target site (Nordborg and Tavaré, 2002). Naturally LD also depends on rates recombination and even modest recombination rates are effective in rapidly (on an evolutionary timescale) eroding LD generated by natural selection or demographic processes. So far, LD has only been characterized to any great extent in European aspen (*P. tremula*) and Black poplar (*P. nigra*) (Ingvarsson, 2005, 2008b; Chu et al., 2009). Data from almost a hundred short gene fragments show a very rapid decay of LD, with LD declining to negligible levels in less than 300 bps (Ingvarsson, 2005, 2008b) and similar results was found in a small set of ten genes from *P. nigra* (Chu et al., 2009). This is not entirely unexpected. The dioecious nature of *Populus*, combined with large population sizes and little population structure due to efficient gene flow, means that there is ample of opportunities for recombination.

It is important to remember that studies of LD in *Populus* have so far only focused on exons and intervening introns. Data from other species, particularly from maize, have shown that recombination preferentially occur within genes because of large structural differences in intergenic regions (Fu et al., 2002; Fu and Dooner, 2002). The extent of structural variation in intergenic regions in *Populus* is currently unknown, but data from the physical mapping of BAC clones and comparisons with the genome sequence of *P. trichocarpa* indicate that structural variation could also be significant in *Populus* (Yin et al., 2004a; Kelleher et al., 2007). A significant fraction of the *Populus* genome sequence has been assembled into contigs smaller than one megabase, which is largely caused by hemizygosity of the diploid individual from which the genome sequence is derived (Yin et al., 2004a; Tuskan et al., 2006). Furthermore, detailed sequencing of BAC clones that co-localize to the same genome region show that the publicly available genome sequence is actually a chimeric assembly of the two different haplotypes (Kelleher et al., 2007). Also, mapping studies have identified genomic regions showing significant recombination suppression (Yin et al., 2004b, a, 2008). For instance, a region on chromosome IV shows significant recombination suppression extending across almost the entire chromosome (Stirling et al., 2001; Yin et al., 2004a) and another

region on chromosome XIX show recombination suppression across at least one third of the chromosome (Yin et al., 2004a). Interestingly, both of these regions contain major disease resistance loci; chromosome IV harbors the *MXC3* locus that confers resistance to the poplar leaf rust *Melampsora* × *columbiana* while chromosome XIX harbors the *MER* locus that confers resistance to *M. larici-populina* (Yin et al., 2004a). The limited recombination observed in these regions are not limited to a particular cross; LD extends across at least 34 kb around the *MXC3* resistance locus on chromosome IV in a sample of 82 wild-collected *P. trichocarpa* genotypes. This led Yin et al. (2004a) to suggest that the *MCX3* locus is located in a major haplotype block which experiences little or no recombination. Furthermore, Kelleher et al. (2007) found that multiple BAC contigs showed co-localization across all 19 linkage groups in *Populus*, suggesting that these represent heterozygous regions carrying haplotype-specific sequence diversity. Kelleher et al. (2007) sequenced four pairs of random BACs showing putative haplotype differences and revealed a large number of both SNPs and indels differentiating the allelic regions. Indel size ranged from a single base pair to over 11 kb and between 0.5 and 14.8% of the four BAC-pairs were made up of alignment gaps between the putative allelic copies (Kelleher et al., 2007). While these structural differences does not match those seen in maize, where even gene content differ between allelic regions (Wang and Dooner, 2006), it suggest that structural differences exist in intergenic regions and that this could affect the possibilities for recombination in these regions.

The region associated with the *MER* locus on chromosome XIX show extensive haplotype differences and is associated with both a large cluster of NBS-LLR resistance genes and a putative sex-determining locus in several species of *Populus* (Gaudet et al., 2008; Yin et al., 2008). Yin et al. (2008) found large differences in gene content between two scaffolds mapping to the same region on chromosome XIX, with over 70 unique genes on the two different contigs. These large-scale sequence differences between putative allelic regions are also associated with a suppression of recombination, an increase in levels of linkage disequilibrium and high levels of segregation distortion (Yin et al., 2008). The co-localization of a sex-determining locus with a large region showing extensive haplotype diversity and suppression of recombination led Yin et al. (2008) to suggest that this region may constitute an incipient sex-chromosome in *Populus*. This idea is intriguing because suppression of recombination and extensive sequence divergence are expected at the initial stages of sex-chromosome evolution (Charlesworth et al., 2005b). However, the interpretation is complicated by the fact that several other studies have show that recombination is suppressed in the vicinity of resistance gene clusters (e.g. Noel et al., 1999; Wei et al., 1999; Kuang et al., 2004) and that the genome organization of resistance gene clusters can be very complex. For instance, tandem duplications have resulted in large differences in gene content between different accession at the *RGC2* locus in lettuce (*Lactuca sativa*) with gene copy numbers ranging from 12 to 32 between different haplotypes (Kuang et al., 2004).

The observations of low levels of LD in *Populus* are so far confined to coding regions (Ingvarsson, 2005, 2008b), and the conclusions of low levels of LD in *Populus* must so far be regarded as tentative as virtually nothing is known about LD

in intergenic region. The results from the large regions of chromosome IV and XIX suggest that recombination rates can vary extensively, both across the genome but also between coding and intergenic regions. These results demonstrate how little we in fact know about genome-wide patters of LD in *Populus* and highlights the need for more research on how LD vary over both short and large scales in the *Populus* genome. The scale of LD across the genome will have important implications for future studies that use associations between alleles to map complex trait variation (see below).

4 Patterns of Sequence Divergence in *Populus*

The genus *Populus* is a relatively young plant genera, with the earliest fossil records dating back to the Eocene some 55 Myr ago (Eckenwalder, 1996). However, judging by the fossil record, the major radiation of the genus *Populus* appears to be quite recent and most likely occurred within the last 10 Myr (Eckenwalder, 1996). The recent radiation of the genus, combined with relatively large effective population sizes and long generation times, enhanced by clonal reproduction where individual genets may reach ages exceeding 1 Myr (Grant et al., 1992), suggests that a substantial fraction of the genetic variation present in present day species of *Populus* could be retained from a common ancestral species (Charlesworth et al., 2005a). Using data on intraspecific polymorphism and interspecific divergence it is possible to estimate the expected fraction of ancestral polymorphisms that two related species share because of their divergence from a common ancestor (Charlesworth et al., 2005a). Using polymorphism data from *P. tremula* (Ingvarsson, 2008b) and data on divergence from either *P. alba* or *P. trichocarpa* (P. K. Ingvarsson unpubl.) the estimated proportion of ancestral polymorphisms shared between these species is 10.5 and 3.9%, respectively. A substantial fraction of all segregating mutation could thus shared between even distantly related species in *Populus* simply because of their relatively recent divergence from a common ancestor. Because hybridization among different species, and even between different sections, of *Populus* are quite common (Eckenwalder, 1996), the fraction of shared polymorphism between species could be even higher than expected based on common ancestry alone. As data on nucleotide polymorphism accumulates from more species of *Populus*, it will be interesting to see how these predictions hold up.

Figure 2 show rates of sequence divergence at synonymous sites across five species of *Populus* based on 158 putative one-to-one orthologous genes. This tree topology is largely consistent with earlier phylogenetic studies of the genus *Populus* and the Salicaceae family using both morphological data (Eckenwalder, 1996) or cpDNA and ITS data (Leskinen and Alström-Rapaport, 1999; Hamzeh and Dayanandan, 2004) with the possible placement of *P. euphratica* which earlier phylogenetic studies regarded as more ancestral in the genus. However, because of the low levels of sequence divergence among the different species of *Populus* and the stochastic nature of the coalescent, it is possible that phylogenies generated from different genome segments are not congruent. In other words, individual

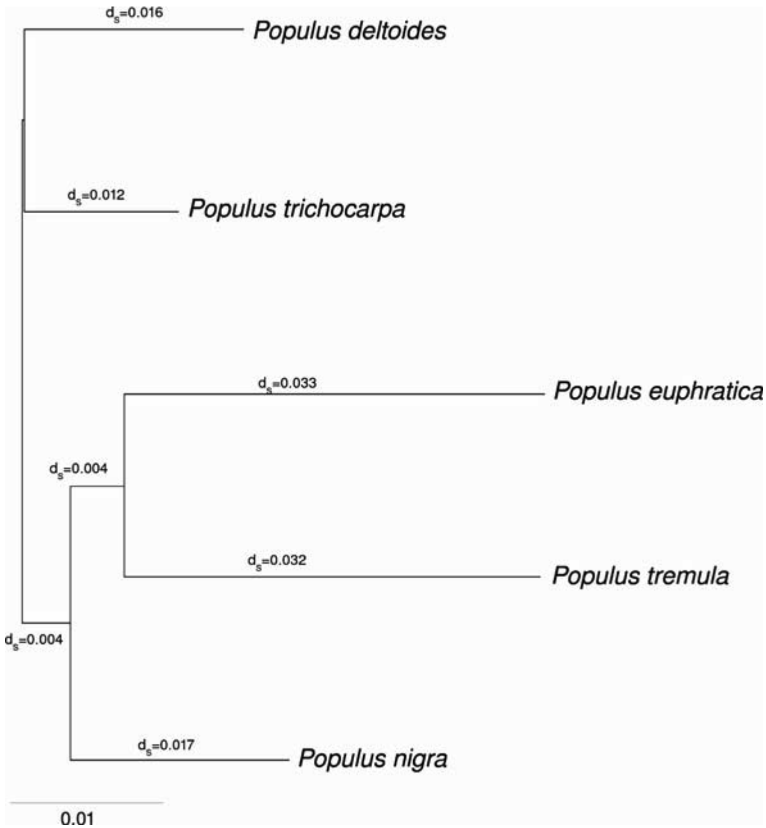

Fig. 2 Unrooted tree representing the phylogenetic relationship between six species of *Populus*. ML estimates of non-synonymous to synonymous substitution rates (d_N/d_S ratios) are shown above each branch and are calculated from a concatenated data set of 158 genes. Branch lengths of the phylogenetic tree are proportional to synonymous substitution rates. All branches have 100% bootstrap support

gene trees do not necessarily agree with the species tree (Pamilo and Nei, 1988; Rosenberg, 2002). Using arguments from coalescent theory it is possible to calculate the expected probability of topological congruence or incongruence between individual gene trees and the species tree (Pamilo and Nei, 1988; Rosenberg, 2002). For the case of three species the probability of topological congruence between a gene tree and the species tree is given by:

$$p = 1 - (2/3)e^{-T} \tag{2}$$

where T is the time between speciation events in units of $2N_e$ generations(Pamilo and Nei, 1988). These probabilities of topological congruence and incongruence are plotted in Fig. 3. It is clear from Fig. 3 that for species in the early stages of divergence ($T \leq 2N_e$ generations) there is a substantial probability for incongruence

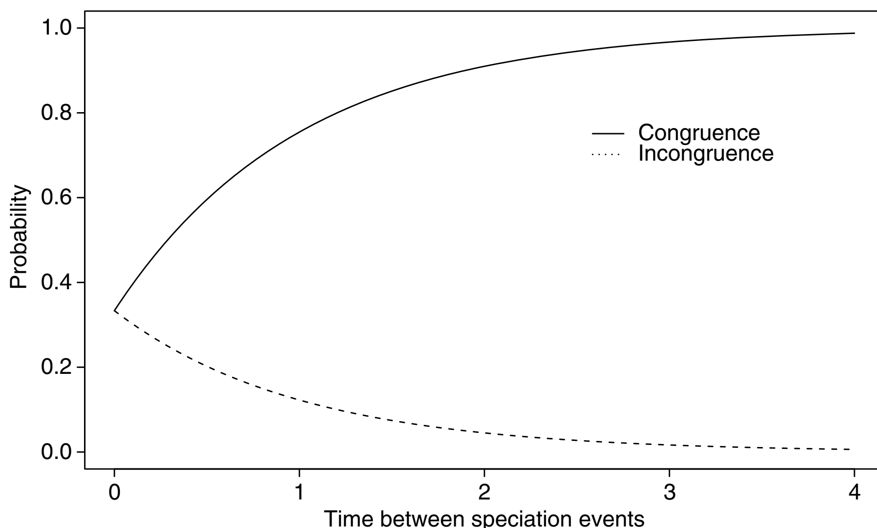

Fig. 3 The pobabilities of congruence (*dotted line*) and incongruence (*solid line*) between individual gene trees and the species tree for the case of three taxa (Pamilo and Nei, 1988)

between gene and species trees. For four or more species, the probability of congruence depends on all speciation times in the phylogeny (Pamilo and Nei, 1988). The internal branches in Fig. 2 are relatively short, indicating that there the different speciation events in *Populus* all occurred within a short period of time, thus increasing the likelihood of topological incongruences between gene and species trees.

Phylogenetic trees estimated for the 158 genes separately show that about 57% of the genes have phylogenetic trees that are congruent with the tree in Fig. 2. In *Drosophila* trees with incongruent topologies are often clustered across the genome, leading Pollard et al. (2006) to suggest incomplete lineage sorting as the likely cause for these observations. Whether this is true also in *Populus* is not clear at this time but the results suggest that incomplete lineage sorting could be a common phenomenon in the genus. This is not surprising given the recent radiation of the genus and is in line with the presence of a substantial fraction of shared ancestral polymorphisms between species. There is also evidence for significant rate heterogeneity in mutation rates across the branches in the phylogenetic tree depicted in Fig. 2 (Ingvarsson, 2008a). This demonstrates that the molecular clock hypothesis with constant substitution rates does not hold across the genus *Populus*. A close inspection of the tree in Fig. 2 show a clear increase in the synonymous substitution rate in *P. tremula* and *P. euphratica*, corresponding to the sections *Populus* and Turanga (Eckenwalder, 1996). It is not clear what is causing the observed heterogeneities in substitution rates across the genus, but differences in effective population size among species could be one potential explanation (Ingvarsson, 2008a). It is clear that these interesting patterns deserve further attention.

5 Complex Trait Dissection in *Populus*

5.1 Linkage Mapping and Genome Dynamics in Populus

The progress of dissecting of complex traits, that is traits controlled by more than a few genes, have been slow forest trees (Neale and Savolainen, 2004) and *Populus* is no exception. This can of course largely be traced to the fact that progress in elucidating the genetic architecture of complex traits using traditional quantitative genetic is slowed down by the long generation times of forest trees. The long time to maturity means that it can take a decade or longer to establish segregating mapping populations in *Populus*. While F_2 mapping populations have been established in *Populus*, the development of more advanced mapping populations, like RILs or NILs, is virtually precluded by long generation times. This has resulted in a lack of progress of positional cloning of genes in *Populus* through the traditional route from a mapped QTL to a gene underlying trait variation. An additional obstacle facing traditional QTL studies is the limited genetic variation between parental lines used to create segregating mapping population. The natural way to overcome lack of genetic diversity in mapping populations is to repeat QTL analyses in many different mapping populations. However, this approach is clearly not feasible in *Populus* because of the limited number of advanced generation mapping populations that are available. Nevertheless, the establishment of or mapping populations has yielded insights into the architecture of complex traits in *Populus*. The mapping populations have been used to investigate the genetic basis of a number of traits of both ecological and economic value such as bud phenology (Howe et al., 2000; Frewen et al., 2000; Chen et al., 2002), drought response (Street et al., 2006), response to elevated CO_2 levels (Rae et al., 2006), biomass production (Rae et al., 2007, 2008) and pathogen resistance (Stirling et al., 2001; Yin et al., 2004a; Jorge et al., 2005).

Linkage maps have been constructed for a number of different species using a variety of different techniques. Most of these maps are based on interspecific crosses and have been constructed using various pseudo-testcross strategies that utilize information from two heterozygous parents to establish two separate maps for the two species involved (Liu, 1998). However the are examples of more advanced-generation mapping populations that include parents, F_1 and F_2 individuals (Frewen et al., 2000; Chen et al., 2002). Mapping studies indicate a large degree of map co-linearity between different species of *Populus* and with a total map length of around 2500 cM (see Table 2 in Woolbright et al., 2008). Interestingly, large-scale synteny extends also to the sister genus *Salix* (Hanley et al., 2006) suggesting that genomic resources derived from *Populus* could be transferred to *Salix*.

One thing that has become apparent from the various linkage maps that have been constructed in *Populus* is that most crosses show varying degrees of segregation distortion (Bradshaw and Stettler, 1994; Yin et al., 2004b; Woolbright et al., 2008; Yin et al., 2008). Segregation distortion is usually though to result from associations between markers and recessive deleterious alleles that are present at low frequencies in outcrossing species. For instance, Bradshaw and Stettler (1994) identified

a recessive, lethal allele affecting embryo development as the cause for significant segregation distortion in a *P. trichocarpa* × *P. deltoides* mapping population. In addition, most mapping populations in *Populus* are derived from interspecific and often intersectional crosses which increase the likelihood of observing segregation distortion due to negative epistatic interactions between alleles from the two different species (Burke and Arnold, 2001; Lowry et al., 2008).

When segregation distortion occurs against heterospecific alleles this generally affects only a small chromosomal region (Rieseberg et al., 1996; Burke and Arnold, 2001). However in many *Populus* crosses the incidence of segregation distortion is not evenly spread across the genome. One region showing evidence of significant segregation distortion in a number of mapping populations is the region at the top of linkage group XIX which contains both one or more sex determining loci and a large cluster of disease resistance genes. There are several other chromosomal regions which show significant segregation distortion and which harbors disease resistance loci (Cervera et al., 2001; Yin et al., 2004b). For instance, Yin et al. (2004b) observed extensive segregation distortion covering almost the entire length of likage group IV in an interspecific backcross between an *P. trichocarpa* ×*P. deltoides* hybrid female and a pure *P. deltoides* male. Yin et al. (2004b) detected significant segregation distorting favoring heterospecific alleles, arguing against negative epistatic interactions or meiotic drive as a likely cause of the observed distortion. Interestingly, Woolbright et al. (2008) also detected significant segregation distortion across large regions on linkage groups IV and XIX in a *P. fremonti* ×*P. angustifolia*, demonstrating that these genome regions show segregation distortion in independent crosses involving completely different species.

These results highlight the involvement of regions containing clusters of disease resistance genes in hybrid incompatibilities and hybrid breakdown (Bomblies and Weigel, 2007), suggesting that these genome regions are rapidly evolving and are also associated with complex patterns of sequence evolution, including large structural differences among haplotypes (Leister, 2004).

5.2 Association Mapping and the Dissection of Complex Traits in **Populus**

Because of the slow progress of elucidating complex trait architecture using traditional quantitative genetic methods in *Populus*, hopes have been raised that the current genomics revolution will provide methods that can be used to more efficiently study the genetic basis of complex traits in *Populus* and other trees (Neale and Savolainen, 2004; Neale and Ingvarsson, 2008). The wealth of molecular markers developed over the last decade have opened up the possibility to directly study associations between markers and adaptive traits in natural populations, so called linkage disequilibrium mapping or association mapping (AM) (Balding, 2006). The utility of AM depends on genome-wide levels of linkage disequilibrium (LD) and in species like *Populus*, where LD is generally low (see Section 3) candidate gene approaches are usually adopted. A candidate gene approach relies on the application

of AM to genes that are known or suspected to be of importance for the trait(s) of interest. These can be candidate genes identified in reverse or forward genetic studies in other model plants (such as *Arabidopsis*) or candidate genes identified though QTL mapping experiments (Neale and Savolainen, 2004). A clear drawback of the candidate gene approach is that it is limited by the availability of suitable candidate genes. On the other hand, very fine scale mapping is usually possible because of the limited extent of LD. In a species like *Populus*, it is possible to map quantitative trait variation down to single causal nucleotide changes, so called *Q*uantitative *T*rait *N*ucleotides or QTNs (Neale and Ingvarsson, 2008). The first studies applying association mapping to forest trees have started to appear in the literature and so far the results appear promising (Thumma et al., 2005; González-Martínez et al., 2007; Ingvarsson et al., 2008).

Hopes have also been raised that an investigation of the genetic basis of complex traits will also provide insights in to how different evolutionary forces that have shaped both the complex traits themselves and also the underlying genes controlling these traits. Methods are now being developed that allow for direct inference of both the action of natural selection and the effects of historical demography from multi-locus DNA sequence data (Thornton et al., 2007). The combination of association genetics to dissect complex traits with population genetics methods that test for natural selection could result in diagnostic markers that could, for instance, be readily implemented in on-going breeding programs (Neale, 2007; Neale and Ingvarsson, 2008). Besides the importance in directed breeding programs, genomic information will increasingly be of value for predicting how current tree populations will respond to climate change. Such information will likely be vital for devising future management policies and programs that promote a sustainable use of forest resources (Neale, 2007).

6 Genetic Control of Bud Phenology and Dormancy

The initiation of growth cessation and dormancy represents a critical ecological and evolutionary trade-off between survival and growth in most forest trees (Howe et al., 2003; Horvath et al., 2003). Dormancy is an important adaptive strategy that enable plants to persist during periods of stressful winter conditions, and the ability to correctly time the development of dormancy determines whether perennial plants will survive winter and early spring without damage to shoot and flower buds. The developmental processes leading up to complete endodormancy takes several weeks to complete, thereby reducing the length of the season during which active growth can take place (Howe et al., 2003; Horvath et al., 2003). Evidence suggests that the most important environmental cues regulating the initiation of dormancy in perennial plants is a shortening of the photoperiod and exposure to extended periods of low, non-freezing temperatures (Horvath et al., 2003).

The genetic basis of bud phenology have been extensively studied and serves as a good model for how information from traditional QTL mapping experiments can be utilized and combined with association mapping approaches. A number of

QTL mapping experiments have performed with the aim of studying the genetic basis of bud phenology and dormancy in *Populus* (Frewen et al., 2000; Howe et al., 2000; Chen et al., 2002). The basis for these studies are two related mapping populations. This first mapping population was initially derived from a cross between a *P. trichocarpa* female from western Washington (48°N) and a *P. deltoides* male from Texas (31°N). Two offspring from this cross were subsequently crossed to yield a segregating mapping population (family 882). A second population was established using the same *P. trichocarpa* mother as in Family 822 but with a male from central Illinois (39°N). Again, two offspring from this cross were subsequently crossed to yield a segregating mapping population (family 331).

These populations have been clonally replicated and planted in a number of common gardens and phenotypic traits related to dormancy and winter survival have been scored. The results indicate show that bud flush and bud set are under moderate to strong genetic control, with clone mean heritabilities in the range 0.68–0.91 whereas winter survival and frost damage showed slightly lower heritabilities ranging from 0.52 to 0.68 (Howe et al., 2000; Chen et al., 2002). There are also strong genetic correlations between several of these traits. For instance, bud set was positively correlated with frost damage and negatively correlated with winter survival, indicating that trees setting bud late in the season suffered greater frost damage and had lower winter survival (Howe et al., 2000).

QTL mapping experiments in these populations have identified between 2 and 6 chromosome regions that influence these traits (Frewen et al., 2000; Chen et al., 2002). These experiments also show that there is a large degree of coincidence of the map positions of QTLs affecting different traits, primarily QTLs affecting bud set and bud flush (Frewen et al., 2000). For instance, Frewen et al. (2000) found that the three QTLs identified as influencing bud set had QTLs for bud flush that co-located to the same genome region, suggesting that these chromosome regions may harbor genes with pleiotropic effects on bud phenology. In an attempt to further elucidate the genetic basis of QTLs affecting bud phenology, Frewen et al. (2000) and Chen et al. (2002) also mapped candidate genes thought to be involved in regulating bud phenology. Two loci, *phyB2* and *ABI1B* were shown to map to the same intervals as QTLs for bud flush and/or bud phenology and were therefore regarded as strong candidate genes for regulating bud phenology (Frewen et al., 2000).

Luquez et al. (2008) found that bud set was also under strong genetic control in *P. tremula*, with broad-sense heritabilities of $H^2 = 0.61$ and $H^2 = 0.72$ at a southern and northern common garden site, respectively. However, the heritabilities for bud flush were substantially lower, with estimates of $H^2 = 0.44$ and $H^2 = 0.56$, respectively. Bud set showed significant differences among populations and showed strong clinal variation with latitude where as no such observations were made for bud flush (Luquez et al., 2008). There appears to be a substantially weaker genetic basis for bud flush in *P. tremula* compared to the interspecifc crosses of *P. trichocarpa* and *P. deltoides* (Frewen et al., 2000). However, the *P. trichocarpa* × *P. deltoides* mapping populations were established by interspecific crosses with the aim of maximizing segregating variation for dormancy-related traits (Chen et al., 2002). It is therefore possible that the experimental design inflated estimates of genetic contribution to

these traits because there is either more variation segregating in these populations or because genetic variation segregating in the mapping populations represents variation that is normally fixed between the two species and that hence would not be found segregating within a single species.

Ingvarsson et al. (2006) set out to evaluate the utility of association mapping in *Populus* in general and for dissecting naturally occurring variation in bud phenology in European aspen in particular (*P. tremula*). Based partly on data from Frewen et al. (2000) they choose *phyB2* as the most likely candidate for regulating dormancy-related traits in *P. tremula*. To further elucidate the genetic basis of bud phenology and its role in adaptation to the length of the growing season in *Populus*, Ingvarsson et al. (2006) characterized patterns of nucleotide polymorphism at the *phyB2* locus in samples from four populations of *P. tremula* sampled from Sweden, France and Austria. Using a sliding window approach across a 7 kb fragment including the *phyB2* gene they identified six regions showing higher than expected levels of genetic differentiation among populations. Since spatially variable selection is expected to yield peaks in the between-population component of genetic diversity, located at or close to the sites(s) that are under local selection, the identification of such regions in the *phyB2* gene are indicative of diversifying selection acting on this locus. Ingvarsson et al. (2006) also identified nine single nucleotide polymorphisms (SNPs) that were subsequently scored in an expanded set of populations collected along a latitudinal gradient in Sweden (Luquez et al., 2008). Population frequencies at three of these SNPs showed significant clinal variation with latitude thus reinforcing the notion that diversifying natural selection has acted at or in the vicinity of the *phyB2* locus (Ingvarsson et al., 2006). A subsequent association mapping study, using 41 SNPs within and around the *phyB2* gene demonstrated that two of the *phyB2* SNPs showing clinal variation were also significantly associated with naturally occurring variation in bud set (Ingvarsson et al., 2008). Interestingly, these two mutations appear to be independently associated with bud set and there is no evidence that these associations are caused by linkage disequilibrium between the two mutations (Ingvarsson et al., 2008). All SNPs were also tested for associations with natural variation in bud flush to determine whether the putative causal polymorphisms we identified above were specific to bud set or whether they are generally involved in regulating bud phenology. However, none of the SNPs showed significant associations with bud flush. This is perhaps surprising since the *phyB2*-associated QTL identified by Frewen et al. (2000) actually explain about 50% more of the variation in bud flush than the co-locating QTL for bud set.

The study by Ingvarsson et al. (2008) demonstrated that association mapping is not only a theoretical possibility in *Populus* but that it also works well in practice. The association mapping population used by Ingvarsson et al. (2008) was only 116 individuals but the small size did not preclude the identification of the two *phyB2* SNPs that were associated with bud set. However, Ingvarsson et al. (2008) used simulations to estimate the power to detect associations in mapping population consisting of c. 120 trees. They showed that less than 40% of mutations with modest effects, that is mutations explaining less than 5% of the phenotypic variation which is typical for genes influencing complex traits, will be identified in this population

(Ingvarsson et al., 2008). Furthermore, the small size of the mapping population has consequences for estimating the effect sizes of the mutations identified. Estimating the phenotypic effects of mutations from the same data that were used to establish an association leads to an ascertainment bias where the SNPs showing significant associations are also the ones that tend to be associated with the strongest phenotypic differences among genotypes (sometimes termed the "Beavis effect" (Xu, 2003) or the "winner's curse" (Zöllner and Pritchard, 2007) and this ascertainment bias is greater in small mapping populations (Ingvarsson et al., 2008). The two *phyB2* SNPs individually explain between 9.0 and 8.3% of the variation bud set. However, simulations show that these estimates are upwardly biased by 250 and 140%, respectively. This means that the true proportion of variation in bud set explained by these mutations is more likely 1.4 and 5.9%, respectively. This demonstrates the need for large mapping populations, both to have reasonable power to detect mutations with small effects and to provide relatively unbiased estimates of the effect of these mutations (Long and Langley, 1999).

Several association mapping studies are in progress in a number of different species (D. Neale, personal communication, M. Olson, personal communication, El-Kassaby, personal communication). These studies should give us more detailed insights into the utility of association mapping in *Populus* and provide a number of interesting candidate mutations for traits of both ecological importance and of economic value.

7 Perspective

Species of *Populus* often have very wide geographic distributions and are ecologically dominant species in many ecosystems (Brunner et al., 2004). Because of their perennial nature and because of the large geographic range, different species of poplars and aspens have evolved fine-tuned annual growth cycles that promote long-term survival and growth. Genetic variation among trees have also been shown to have cascading effects on the species composition of phytophagous insects, explaining up to 60% of the variation in insect diversity among different trees (Wimp et al., 2005; Shuster et al., 2006). The ecological importance of *Populus* make it an interesting model system for studying ecological and evolutionary functional genomics.

By identifying genes underlying ecologically and/or economically important traits it should be able to address many important questions regarding the genetic architecture of quantitative variation. For instance, what does the distribution of effects of mutations affecting complex trait variation look like? Theoretical results suggest that the distribution of effects of adaptive mutations fixed in a species should be roughly exponential (Orr, 1998) and data from QTL mapping experiments seem to fit this distribution (Kearsey and Farquhar, 1998). Another interesting question is whether adaptive mutations arise from de novo mutations or if they are derived from standing genetic variation. It has become clear that adaptation from standing genetic variation produce radically different patterns of nucleotide

polymorphism and linkage disequilibrium than adaptation from de novo mutations (Kim and Stephan, 2002; Przeworski et al., 2005; Hermisson and Pennings, 2005; Pennings and Hermisson, 2006). For instance, the allele frequency clines in *phyB2* found by Ingvarsson et al. (2006) must have been established within the last 7,000 years, following the post-glacial colonization of Northern Europe. If these sites were de novo mutations the rapid increase in frequency should leave a clear signature in the surrounding genome regions (Kim and Stephan, 2002) yet linked sites, separated by as little as a few base pairs, do not show clinal variation or a reduction in nucleotide polymorphism (Ingvarsson et al., 2006). This suggest that the *phyB2* sites involved in adaptation to photoperiod were derived from a heterogeneous "population" of alleles which in turn suggest that these mutations have been maintained within the species for a long time. Finally, one important question is to what degree parallel adaptations in different species are due to mutations in the same set of genes? With the availability of abundant genomic resources, an interesting ecology and abundant genetic variation, *Populus* is a promising model system for ecological and evolutionary functional genomics and have a great potential for providing interesting answers to these and many other important biological questions.

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Transformation as a Tool for Genetic Analysis in *Populus*

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Abstract We summarize the outlook for using transformation as a genetic tool in *Populus*. Transformation approaches avoid the major obstacle to performing genetics experiments in trees – namely long generation cycles and the difficulty of inbreeding to reveal loss of function alleles. Dominant transgenic alleles allow modifications in gene function to be readily observed in primary transformants. Although transformation has been mainly used for reverse genetics (where the gene sequence of interest is known), transgenic mutagenesis approaches such as activation tagging and gene/enhancer traps have also been shown to enable forward genetics (where the phenotype, not the gene, is known). We outline challenges and needs for more efficient use of transformation tools. These include expansion of the transformation toolbox (e.g., promoters, vectors, targeting), and improved ability to conduct field trials to study gene function in native and plantation environments (in spite of regulatory obstacles). Because of the power of transformation, it will remain a major genetic research tool for dissection of gene function in *Populus* for many years to come. It is the key biological attribute that makes poplar the most powerful model organism for genetic analysis of woody plant growth, adaptation, and development.

1 Introduction

Following the development of gene transfer technology for plants approximately two decades ago (De Block et al., 1984), genetic transformation has become an indispensable tool for dissection of gene function. It is used extensively in *Arabidopsis*, rice, tomato, and many other model herbaceous plant species. Its main uses are for insertional mutagenesis, complementation, ectopic gene expression and

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gene silencing. Here we review the role of transformation as a tool for functional dissection and gene discovery in *Populus* – the model taxon for woody perennial development.

Trees dominate many kinds of terrestrial ecosystems, and have a number of exaggerated developmental features that are poorly expressed in herbaceous species. These include extensive secondary meristem development, long delayed onset of reproduction, and annual cycles of dormancy acquisition and release. Despite trees' economic, ecological, and biological importance, the genetic and physiological control of these important traits remains poorly understood. This is in no small part due to the difficulties of standard genetic approaches in studying gene function. Although natural mutants have been identified for some traits (e.g. Gill et al., 2003), routine genetic segregation and inheritance studies are problematic in trees due to their long generation time and strong inbreeding depression.

Knock-out or knock-down manipulation of gene activity is widely accepted as the "gold standard" method for studying gene function. The two major approaches taken for altering gene expression are generalized mutagenesis and gene-directed transgenesis. Transformation, because it inserts DNA in an approximately random manner into euchromatic portions of the genome, is useful for both approaches. Genes can be specifically knocked-out or -down by delivering specific transgenes that trigger one or more forms of RNA interference, which act in trans to their gene targets (e.g., antisense, RNAi, synthetic microRNAs). T-DNA can also act as a random mutagen because, when inserted in the sequence of a gene, it generates lesions that usually result in a loss-of-function allele. The known sequence of the T-DNA, now present in the mutated gene, facilitates gene isolation – a method generally known as gene tagging (Parinov and Sundaresan, 2000; Sessions et al., 2002; An et al., 2005). The importance of T-DNA tagging for genetic research in model organisms is demonstrated by the generation of many collections that reach near to genome saturation (Alonso et al., 2003).

2 Value of Transformation as a Genetic Tool

Reviews of both approaches for mutagenesis that focus on trees have been published elsewhere (Fladung et al., 2004; Busov et al., 2005a, b). We will therefore only briefly highlight how transformation mitigates some of the major obstacles to performing genetics experiments in trees. A key element of these approaches is the use of transgenic methods that impart, or employ, dominant alleles. This is critical because it allows modifications in gene function to be observed in primary transformants. In contrast, most classical mutations have their manifestations via loss-of-function mechanisms, and thus show recessive inheritance. They require strong inbreeding, usually selfing, to produce homozygotes that express a physiological effect from the lesion. This makes the process of mutational analysis impractical in most tree species. Efforts to trigger precocious flowering to shorten generation cycle via transgenic approaches are underway (Bohlenius et al., 2006; Hsu et al., 2006; Flachowsky et al., 2009) (Fig. 3c, d). However, they have not led

to the production of viable gametes and seeds in many tree species tested, including poplar.

Most trees, including poplars, are largely undomesticated and carry large genetic loads (Bradshaw and Strauss 2001; Bradshaw et al., 2001). Inbreeding to reveal recessive mutations is therefore poorly tolerated, and the expression of this load among progeny would make it difficult to distinguish the effects of specific gene lesions from the large number of additional loci whose mutant alleles will also be expressed. In addition, most poplar species are dioecious, thus producing homozygous plants via inbreeding requires at least two generations of close sibling mating. This would require a decade or more for a single mutagenesis experiment.

Finally, genetic redundancy is a major problem in all plant species. Plant genomes, including *Populus*, have very large number of repeated, functional genes. The genome of the model Arabidopsis plant is significantly duplicated (Vision et al., 2000), and poplar has an additional ~30% more duplicated genes (Tuskan et al., 2006). Therefore single loss-of-function mutations often do not have obvious phenotypic effects in most experimental environments. In Arabidopsis, this problem can be circumvented by identification of individuals carrying mutations in individual genes, followed by controlled crosses bringing two or more mutations in related genes together in one individual. This strategy, which is not a trivial undertaking in Arabidopsis, is impractical in trees. Multiple gene knock-downs therefore can be only addressed by transgenic approaches (discussion below).

3 Postgenomic Challenges and Opportunities

Recent sequencing and annotation of the poplar genome sequence identified more than 45,000 gene models (Tuskan et al., 2006). Understanding even a significant fraction of these putative genes in the context of woody plant development and adaptation presents a daunting challenge. Sequence comparisons have identified a substantial number or putative homologs of previously described genes from herbaceous model systems. However the in silico predictions for many genes warrant verification as they are based on limited domain homologies. Moreover, an even larger part of the poplar gene space encodes unknown, hypothetical or putative proteins, as well as RNA regulatory molecules like microRNAs. There are also likely to be many genes, or presumed pseudogenes, that were not identified, have incorrect gene models, or have important splicing variants that were misannotated. It is therefore highly desirable that a high throughput functional transformation pipeline be developed that can evaluate the roles of the many uncharacterized, hypothetical genes in poplar.

In developing a functional genomics pipeline, the list of genes could be prioritized based on expected functions in woody plant processes, tissue level expression databases, scientific novelty, or other criteria. Genes can be fed into the pipeline efficiently through the use of recombination-based cloning systems optimized for plant expression/suppression vectors (Hilson, 2006). Among others, Gateway cloning technology allows the development of such resources (see for example

Dubin et al., 2008). The goals may include over/ectopic expresssion, RNAi, promoter studies, localization studies (GFP fusions) or *in planta* bimolecular fluorescence complementation (Karimi et al., 2002; Weiste et al., 2007; Dubin et al., 2008). Fusion proteins with GFP can be use to study precise protein localization and co-localization of potentially interacting proteins (Koroleva et al., 2005). An alternative strategy is to insert in bulk whole cDNA libraries in RNAi or ectopic expression binary vectors, transform and screen for phenotypic alterations in traits of interest. The use of normalized, full-length cDNAs libraries would help to minimize redundancy (Ichikawa et al., 2006; Weiste et al., 2007).

4 Transformation and Poplars

Populus was the first woody plant to be transformed (Parsons et al., 1986), and several genotypes – particularly in section *Populus* – can be transformed at high rates (Confalonieri et al., 2003). For transformation to be a useful tool for functional gene dissection it must be reasonably rapid, produce transgenic plants with stable transgene expression, and not induce a high frequency of transformation-associated mutation. These requirements are largely met for *Populus* (Strauss et al., 2001; Brunner et al., 2007; Li et al., 2007).

The transformation procedure, from inoculation to rooted transgenic plants carrying the transgene (see for details below), can be accomplished within 4–8 months depending on the genotype, skill of personnel and transgene used in the study. Approximately 20 independent transformation events per transgene can be generated within this period in a typical experiment.

Stable transgene expression and RNAi silencing is important if transgenic phenotypes are to be expressed over the months and years needed for phenotypic evaluation in *Populus*. Several studies have reported highly stable transgene expression in *Populus* (reviewed in Brunner et al., 2007). In a study of 40 independent transgenic hybrid cottonwood (*P. trichocarpa* × *P. deltoides*) plants that expressed herbicide resistance and GUS reporter genes and were monitored for four years under field conditions, only one showed instability of the transgene expression (Meilan et al., 2002). Similar results were obtained in a study of 20 transgenic events that expressed the GUS reporter gene under 35S and xylem-specific promoters (Hawkins et al., 2003). In the same study stable transgene expression was found under in vitro, greenhouse and field conditions and the expression was not affected by stress treatments. Although studies on efficiency and stability of gene suppression approaches in *Populus* are still scarce, in a recent study of 56 independent events, RNAi-induced gene suppression was found to be stable over 2 years and during the annual growth cycle. This occurred despite the use of an *rbcS* promoter, whose activity varied widely during transitions from active growth to dormancy, driving the RNAi transgene (Li et al., 2007). Similarly, antisense gene suppression appears also to be stable over time in transgenic *Populus* with silenced cinnamyl alcohol dehydrogenase (CAD) or caffeate/5-hydroxy-ferulate O-methyltransferase (COMT) genes (Pilate et al., 2002).

For genetic studies in species where sexual inheritance is required, single transgenic loci are greatly preferred as they give simple inheritance patterns. They are also usually less prone to co-suppression, may produce stronger gene suppression, and are less likely to cause unintended gene disruptions than multicopy insertions. However, in poplar gene copy number has no association with level of RNAi suppression (Li et al., 2007), no relationship with instability, and a positive association with level gene expression (Li et al., 2008a). Nonetheless, it is easy to recover single copy transformants in poplar. *Agrobacterium*-mediated transformation typically produces 1–2 insertions (Li et al., 2008a, b). A study of 45 independent events produced a mean of 1.5 insertions (Groover et al., 2004). Similarly among 53 independent poplar transgenic events with RNAi transgenes, 45 contained a single copy, 5 had two copies, and 3 harbored three copies (Li et al., 2007).

Morphological abnormalities induced by transgene insertion are rare for *Populus*. A main reason for this is likely that *Populus* is diploid, and gene disruption during transformation would typically allow the second allele to cover the mutation. In a large number of transgenics, morphological abnormalities were observed in only three events (~0.06%) among several thousands studied (Strauss et al., 2004). Other studies appear to have observed higher levels of somaclonal variation where highly sensitive reporter genes are employed (Wang et al., 1996; Kumar and Fladung, 2001). However, none appear to be so high as to pose a significant constraint on functional genomics studies (where several events are studied for each experimental treatment).

5 Approaches for Transgenic Modifications of Gene Function

Transgenic modifications can involve both gain- and loss-of-function like modifications. Gain-of-function is generally studied via ectopic gene expression, and usually imparts abnormal but physiologically informative phenotypic changes. Ectopic approaches are especially important in functional dissection of gene families, where functional redundancy often obscures the phenotypic effects of specific gene knock-outs.

Transgenic loss-of-function like modifications are usually achieved via post-transcriptional gene silencing (PTGS), targeting specific RNAs for degradation (Brodersen and Voinnet, 2006). PTGS is triggered by double stranded RNA (dsRNA) produced when an enzyme known as Dicer (with a RNase III domain) recognizes and cleaves the dsRNA into short 21–26-nucleotides. These fragments, termed siRNAs for "short interfering RNAs," remain in double stranded duplexes and act as templates for the RNA induced silencing complex (RISC) that targets and destroys the homologous mRNA messages. Historically, antisense technology, which represents one of the first used forms of PTGS, was successfully used in poplar to study several key enzymes in the lignin biosynthetic pathway (Baucher et al., 1996) and genes involved in control of dormancy (Rohde et al., 2002). Because of the relatively low efficiency and inability to discriminate between closely-related paralogs, antisense-mediated gene suppression as a tool for gene

suppression is rapidly being replaced by RNA interference (RNAi) (Matthew, 2004), a more potent inducer of gene silencing. Studies in plants and other eukaryotic organisms have shown that inverted-repeat transgenes (especially if they are separated by an intron) provide a reliable and highly efficient means for suppression of gene expression (Chuang and Meyerowitz, 2000; Smith et al., 2000).

The substantial gene redundancy in many plant gene families poses specific challenges to dissecting individual gene function(s) using RNAi approaches. An alternate approach is generation of "dominant negative" mutations. Typically dominant negative mutation involves modifications in the coding sequence of the protein (reviewed in Veitia, 2007). The modified gene encodes mutant polypeptides that when over-expressed will disrupt the activity of the wild-type protein. For example, mutation in proteins that oligomerize can lead to inhibition of the protein complex. Many cell surface receptors typically form di- or multimers upon binding an extracellular ligand. The dimerization leads to activation of a cytoplasmic domain (e.g., kinase). Expression of protein that lacks the cytoplasmic domain reduces or typically abolishes signal transduction by sequestering native WT proteins from productive complexes. Similarly, dominant negative mutations in the Arabidopsis actin 2 gene impaired the oligomerization of the actin fiber and allowed functional characterization of the gene with respect to growth of root and aerial organs (Nishimura et al., 2003). Another example of a dominant negative mutation is production of transcription factors with truncated activation but fully functional DNA binding domains. Such truncated proteins compete for the same DNA binding sites with WT proteins, thus reducing the activation/repression of the target gene(s) (Veitia, 2007). This strategy can also produce less severe phenotypes for regulatory genes, where loss-of-function can be lethal.

6 Other Approaches for Generation of Knock-Down Gene Modifications

Several novel approaches that show promise for generation of loss-of-function gene modifications have been recently developed. Although their utility in poplar is untested, they have been applied successfully to other non-model plants. Therefore we briefly review the technologies and their potential applications in poplar.

6.1 Artificial miRNAs and Overexpression of siRNAs

Micro RNAs (miRNAs) are non-coding small (∼20–28nt) RNA molecules that negatively regulate gene function via transcriptional or translational repression (Jones-Rhoades et al., 2006). They represent a native regulatory mechanism widely-distributed among eukaryotic organisms (Zamore et al., 2000). The mode of action and regulatory function of miRNAs very much resembles the siRNAs generated during the process of RNAi-mediated gene suppression (described above). However in contrast to RNAi, where multiple 20nt siRNAs are produced from approximately

200–300 bp sequence, miRNA biogenesis results in only one 20nt regulatory fragment. This is possible because a precursor RNA molecule forming a hairpin loop structure with a specific sequence and secondary structure features are recognized and processed by specialized recognition and nuclease machinery. The progress in identification of many precursor RNA molecules allowed generation of a "designed" miRNA precursors, now known as artificial or synthetic miRNAs (Ossowski et al., 2008).

Artificial miRNAs act in a similar manner as to RNAi molecules in causing post transcriptional gene silencing (PTGS), but are highly gene-specific – thus allowing discrimination of closely related gene family members. Genome-wide microarray analysis showed that like the native miRNAs, amiRNAs are similarly highly gene specific (Schwab et al., 2006). The utility of this approach was demonstrated in Arabidopsis, rice, tomato and tobacco (Alvarez et al., 2006; Warthmann et al., 2008). Initial reports indicate a high rate of silencing, similar to that of RNAi (∼90%.), and expression of amiRNAs can be driven by any *pol II* promoter (Ossowski et al., 2008). An interesting feature of amiRNAs is that the level of expression conferred by the promoter corresponds to the level of gene suppression (Schwab et al., 2006; Alvarez et al., 2006). Thus it is possible to generate quantitative knockouts that differ in the degree of gene suppression.

Artificial miRNAs use a functional precursor miRNA as a backbone, but the native miRNA sequence is replaced with a user-defined 20nt targeting sequence (Alvarez et al., 2006). Selection of the 20nt is important for the efficiency and specificity of gene silencing, and must follow specific guidelines to be properly processed and function (Ossowski et al., 2008). The selection of miRNAs has been automated in a web-based application that helps ensure specificity to the desired target gene and adherence to guidelines for functionality (http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl). The engineering of the selected 20nt into the precursor can be accomplished by overlap PCR where the endogenous miRNA is replaced by the selected 20nt and the resulting amplified fragment is cloned into a vector of choice. Although heterologous precursor miRNA backbones have been successfully used, to ensure correct processing it is preferable to use a native pre-miRNA (Ossowski et al., 2008). Several poplar pre-miRNAs have been already found and their processing experimentally verified (Lu et al., 2005) (Fig. 1).

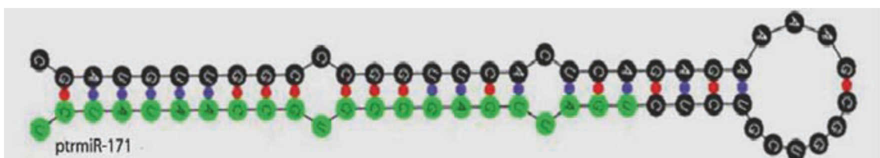

Fig. 1 Secondary structure produced by poplar *premiR171*. Sequence was amplified using RT-PCR and secondary structure generated using MFOLD The *miR171* sequence is highlighted in green. Graph courtesy of Rewati Potkar, Michigan Technological University

Small RNAs have also been used to induce transcriptional gene silencing (TGS) (Aufsatz et al., 2002). In this case, a sequence homologous to the promoter of a gene is employed in the hairpin loop structure. The derived siRNAs cause DNA

methylation and chromatin modifications. Li et al. (2007) reported a much lower rate of gene silencing with dsRNA directed at transgene promoter compared to coding sequences in poplar. We know of no systematic studies of the success rate of PGS vs. TGS in any plant species.

6.2 Zinc-Finger Nucleases (ZFN)

ZFNs have been developed to: (i) increase the extremely low rate of homologous gene targeting (GT) in plants and most mammalian systems (excluding mice) and (ii) introduce mutations in a target locus by the imperfect repair of the cleaved target DNA by the ZFNs (Durai et al., 2005). ZFNs are synthetic proteins that can introduce double-stranded breaks (DSB) in DNA. The protein consist of a DNA binding domain (DBD) composed of 3–4 (Cis2-His2) zinc fingers fused to the DNA-cleavage domain of a *FokI* restriction endonuclease (Durai et al., 2005). The zinc fingers provide targeting and binding to a particular specific sequence, and the cleavage domain introduces a DSB. The break can be repaired by either the process of homologous recombination (HR) or non-homologous end joining (NHEJ) (Ray and Langer, 2002). The HR is precise and requires extensive homology between the repaired strands. In contrast, NHEJ does not require extensive homology and the repair often results in a mutation at the repaired site. In plants NHEJ is the predominant repair mechanism, thus DSBs introduced by the ZFNs result in a high rate of mutations at the target locus (Lloyd et al., 2005).

A ZFN that will introduce DSBs in a target locus requires design of a combination of fingers that will recognize and bind to both strands of a target sequence. Each finger consists of 30aa that recognize a 3 bp nucleotide sequence. The process of matching finger-to-sequence is still imperfect, and two approaches are widely used. One is based on a selection process, where different zinc finger combinations are screened for affinity to target sequence via library (phage or bacterial two-hybrid system) screening procedures (Durai et al., 2005). An alternative strategy is using computer-generated matches that use a domain library (http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php). Delivery of ZFNs can be accomplished via genetic transformation under an inducible promoter. This strategy was demonstrated in Arabidopsis using a heat shock promoter (Lloyd et al., 2005). Alternatively, in tobacco the DNA encoding a ZFN was successfully delivered into protoplast cells via electroporation (Wright et al., 2005).

6.3 Virus Induced Gene Silencing (VIGS)

VIGS has been developed to alleviate some of the problems associated with the time required for traditional mutagenesis and transgenic approaches. By inducing gene silencing in regenerated plants, it can also bypass the problem of lethality during embryonic development (Robertson, 2004; Burch-Smith et al., 2004). VIGS provides for the rapid reduction, but not elimination, of gene expression by taking

advantage of natural PTGS mechanisms used for viral defense. It is thus fundamentally similar to RNAi technologies (Ossowski et al., 2008). Typically, VIGS vectors include the bulk of the viral genome, but where a sequence of the gene that is the target for silencing has been inserted (Robertson 2004; Burch-Smith et al., 2004). Inoculation with the recombinant virus triggers the plants' silencing machinery, to suppress both the virus and the mRNA of the gene corresponding to the inserted fragment. The silencing signal spreads systemically in RNA viruses, imparting a phenotype similar to what would have been caused by PTGS for the target gene (Robertson, 2004).

Several VIGS vectors have been developed and their utility proven in functional analyses of genes involved in diverse processes (reviewed in Robertson, 2004). The tobacco rattle virus (TRV) vector is of wide applicability in the Solanaceae, as it has a wide host range, high rate of silencing, mild viral symptoms, and acts within meristematic tissues (Ratcliff et al., 2001). In poplar, the best candidate for development of a VIGS vector is the poplar mosaic virus (*PopMV*). *PopMV* is a RNA carlavirus that naturally infects species and hybrids in the genus *Populus*. Recently a VIGS vector based on the genome sequence of *PopMV* (Smith and Campbell, 2004) was developed (Naylor et al., 2005) and successfully tested in *Nicotiana benthamiana* for suppression of a GFP reporter gene. Information on effectiveness of this vector on transgene suppression in poplar, however, is unavailable.

Delivery of VIGS vectors to plants can be accomplished in several ways (Burch-Smith et al., 2004). In vitro transcribed RNA (most VIGS vectors are RNA) or DNA can be rubbed into the leaves (Ratcliff et al., 2001). This method is labor and time intensive but yields high infections. Alternatively, the vector sequence can be cloned into a binary vector and delivered by injection of *Agrobacterium* cultures into parenchyma cells (Schob et al., 1997). Microprojectile bombardment has also been successfully used for DNA-based vectors (Redinbaugh et al., 2001).

7 Transformation Methods in *Populus*

A variety of transformation methods have been successfully used in *Populus*, including biolistic approaches (McCown et al., 1991) electroporation of protoplasts (Chupeau et al., 1994), and cocultivation with Agrobacterium. Protoplast transformation is very rarely used, and biolistics is mainly used in specialized applications such as for rapid assessment of promoter::reporter expression in different tissues, transformation of highly recalcitrant genotypes, and transformation of plastids. For routine nuclear transformation using organogenic systems – the prevalent means for poplar transformation – Agrobacterium is by far the most widely used method. Although many poplar species have been transformed at least once (discussed below), each species and genotype tends to require detailed customization of regeneration procedures for successful and efficient transformation. The reasons for the extraordinary genotype specificity of regeneration and transformation procedures remains unknown, but imposes a major obstacle to efficient transformation of all plants.

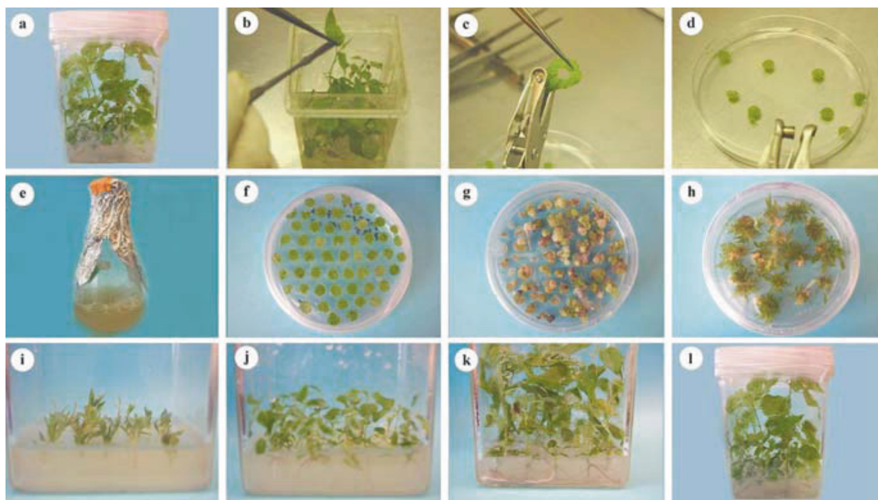

Fig. 2 Transformation in *Populus*. Explants preparation (**a–d**); co-cultivation (**e**, **f**); callus and shoot induction (**g**, **h**); shoot rooting (**i–l**). Photos courtesy of Cathleen Ma, Oregon State University

Most of transformation procedures using *A. tumefaciens* follow a classical protocol upon which there are many variations (e.g., Leple et al., 1992; Han et al., 2000; Confalonieri et al., 2003) (Fig. 2). These protocols involve the following steps in chronological order: (1) Co-cultivation of native or pre-induced tissues with Agrobacterium carrying the T-DNA of interest; (2) Callus induction if indirect shoot regeneration is employed, in the presence of a selective agent such as an antibiotic; (3) Shoot induction also in the presence of a selective agent; and (4) Rooting in the presence of a selective agent. Selective agents can be used in many ways and concentrations; they can be employed immediately after cocultivation, or their use delayed until after shoots have regenerated. Preculturing of explants before cocultivation activates cell division, and can thus increase cell competence for T-DNA integration. Three major factors determine the success of transformation procedure – genotype of the host plant, Agrobacterium strain, and the regeneration procedure (plant physiological state, in vitro medium composition, environment, selection, and hormone treatments). We will briefly discuss each of these three factors below.

Successful transformation has been reported for many poplar species, with the majority from section *Populus* (reviewed in Confalonieri et al., 2003). For example, transformation protocols have been developed for *P. alba* (Okumura et al., 2006), *P. tremula* (Fladung et al., 1997; Tzfira et al., 1997), *P. tremuloides* (Cseke et al., 2007), *P. tremula* × *P. alba* (Leple et al., 1992), *P. tremula* × *P. tremuloides* (Fladung et al., 1997), *Populus canescens* × *P. grandidentada* and *P. tremuloides* × *P. davidiana* (Dai et al., 2003). Successful transformation have been reported for species and hybrids from sections *Aigeiros* (cottonwood) and *Tacamahaca* (balsam poplar) including *P. deltoides*, *P. trichocarpa* "Nisqually-1" (Ma et al., 2004; Song et al.,

2006), *P. trichocarpa* × *P. deltoides* (De Block, 1990; Han et al., 2000), *P. deltoides* × *P. nigra* (Heuchelin et al., 1997), *P. alba* × *P. grandidentata* (Fillatti et al., 1987) and *P. nigra* × *P. trichocarpa* (McCown et al., 1991), *P. sieboldii* × *P. grandidentata* (Matsunaga et al., 2002), *P. nigra* "Italica" (Nishiguchi et al., 2006) and *P. ciliata* (Thakur et al., 2005). The most widely used transformation "laboratory rat," has been the *Populus tremula* × *alba* hybrid clone known as INRA 717-1B4 (Leple et al., 1992).

As the main vehicle for delivering the transgene, selection of an appropriate Agrobacterium strain is essential. Although both *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* have been successfully employed for poplar transformation, *A. tumefaciens* is the species of choice. The interest in *A. rhizogenes* transformation has been driven by the ease of production of transformed hairy roots, which self-select and regenerate, and those provide for more rapid generation of transgenic tissues (Cseke et al., 2007). Nopaline strains of *Agrobacterium tumefaciens* often have a higher transformation efficiency in tree species including poplars (Fladung et al., 1997). However, exceptions to that rule have been reported where octopine strains like EHA105 are more successful than nopaline strains C58 and LBA4404 (e.g., of two *Populus trichocarpa* x *Populus deltoides* clones: Han et al., 2000). Because knock-down RNAi manipulations often include sequence repeats that are prone to recombination, use of recombinant-deficient strains like AGL1 can help to avoid rearrangements during storage and transformation. AGL1 is also a hypervirulent strain and thus is believed to provide a higher level of transformation efficiency.

Most protocols in poplar transformation use direct or indirect in vitro shoot organogenesis, followed by rooting. One of the main determining factors affecting transformation efficiency is the origin and physiological condition of the explants. Although explants from leaves, petioles and stems have been successfully employed, the success rate with different types is highly variable and species-dependent. For example, transformation efficiencies with aspen are highest when leaf explants are used. In contrast petioles and stems seem to produce best results for cottonwood hybrids (Han et al., 2000). In vitro grown plants are generally used as a source for explants because they do not require sterilization prior to co-cultivation, however, protocols involving non-sterile greenhouse/growth chamber-grown plants have also been successfully used, and are sometimes found to be superior to in vitro materials (Song et al., 2006).

Co-cultivation conditions also affect transformation efficiencies. Optimum Agrobacterium concentration, precise timing, media and light conditions can affect the success of the T-DNA transfer. Callus induction usually requires presence of auxin (e.g., 2,4-D, NAA or IBA) and is performed under dark or very low-light-intensity conditions. The propensity of different types of explants (e.g., leaf, stem or petiole) to produce calli is highly genotype-specific. The callus induction step is necessary in some genotypes but not in others, and should be omitted whenever possible to reduce the risk of somaclonal variation and shorten the transformation cycle. Direct or indirect shoot organogenesis requires high levels of cytokinins such as BA, zeatin, thidiazuron (TDZ) and others. The presence of auxin in combination with

cytokinins has also proved beneficial in some species. Rooting is accomplished in auxin-dominant media and is generally a non-limiting step in poplar transformation and regeneration.

8 Using Transformation for Mutagenesis

As discussed above, insertional mutagenesis using T-DNA is widely used in plant biology to create recessive mutations revealed in homozygous individuals (Parinov and Sundaresan 2000; Sessions et al., 2002; Jeong et al., 2002; Alonso et al., 2003; An et al., 2005), but is impractical to apply in trees. However, methods that cause dominant mutations are applicable to trees. For example, activation tagging vectors allow generation of gain-of-function mutations via upregulation of genes positioned in the proximity of the insertion (Weigel et al., 2000). This is accomplished by cloning an array of strong enhancers near one of the T-DNA borders. The presence of the enhancers can cause upregulation of genes proximal to the insertion site, and thus a dominant mutation. In the majority of the cases the native expression of the gene is retained but at a higher expression level – giving a mild but clear and easily regenerated mutant phenotype. Specific gene upregulation also avoids the problem of gene redundancy that can obscure loss of function mutations.

Results from two pilot studies in *Populus* have demonstrated the effectiveness of activation tagging (Busov et al., 2003; Harrison et al., 2007). A population of 627 events was used to identify the first tagged gene in a tree (Busov et al., 2003). This population is under further investigation and results to date indicate a high rate ($\sim$7%) of mutant discovery based on (1) morphological inspection; (2) successful positioning of the tag in the genome for several dozen events; (3) activation of proximal genes up to 10 kbp; and (4) numerous successful recapitulations of the original phenotypes when the candidates are re-inserted under the control of a strong promoter. In the study reported by Harrison et al. (2007), a similar mutant discovery rate was found and different types of mutations were identified. In both cases, the majority of tagged genes are functionally novel to science, and many affect traits of particular interest for tree biology (e.g., wood quality, frost hardiness, phenology).

Enhancer and gene traps insert reporter genes that indicate the expression patterns of nearby genes. They provide high resolution (cell/tissue type) level information about the expression patterns of tagged genes (reviewed in Springer, 2000). Different reporter genes have been used in gene and enhancer traps, including the widely used GUS system. Reporters that allow imaging of live plants such as GFP (green fluorescent protein) and luciferase have also been employed (Szabados et al., 2002; Yamamoto et al., 2003). In the enhancer traps the reporter gene is preceded by a minimal promoter, which typically contains basal sequences required for transcription and translation, but is not sufficient to drive expression of the reporter gene. Insertion of the enhancer trap sequence proximal to a gene results in an activation of the reporter gene by neighboring regulatory enhancer *cis*-elements. Gene traps are variations of the enhancer traps; the reporter gene coding sequence is preceded by a splice acceptor sequence (SA). When the T-DNA sequence carrying the gene trap

inserts in the coding region of a gene, a novel splice variant between the native and reporter gene is produced that encodes a fusion protein that serves as marker of the expression pattern of the tagged gene. Enhancer/gene traps are useful in isolation of promoters and regulatory elements, as well as the identification of candidate genes that can be further manipulated by gene-silencing or ectopic expression approaches. Studies demonstrating the utility of this approach have been performed in *Populus* (Johansson et al., 2003; Groover et al., 2004; Filichkin et al., 2006b).

9 Future Prospects and Challenges

Transformation is a major tool for genetic research in poplar that, based on its frequency of use in scientific publications, is unequaled by any other taxon of trees. This valuable tool for leveraging the genome sequence, and for linking physiology to gene function, will continue to empower poplar research for many decades to come. However, there remain some substantial obstacles to the broader use of transformation and transgenic trees to improve biological research.

First, transformation remains a costly and slow procedure, requiring a laboratory with well developed means for in vitro culture, growth chambers, greenhouses, and generally more than a year from cocultivation to physiological analysis. Although there has been some progress on ex vitro methods for transformation in poplar – such as the development of a method that allows generation of transformed somatic cambium sectors (van Beveren et al., 2006) – there are no general methods that are widely used or reliable. The development of *in planta* methods, as have revolutionized Arabidopsis research, would be highly beneficial in poplar. The long, costly and facility intensive process emphasizes the need for centralized transformation facilities, as well as investment in the generation and maintenance of large transgenic collections that have broad value to the research community.

Second, transformation in poplar and many other species remains highly idiosyncratic, with its efficiency varying widely between tissues, genotypes, and species. Even in Arabidopsis, most of transgenic research is performed in a single variety (Columbia) that is highly amenable to transformation. The identification of a single reference genotype for poplar transformation would be highly desirable as it would allow more direct comparison of results across different laboratories. Advances in resequencing technologies allow near complete genome sequences for one or more standard genotypes to be readily produced. Nevertheless, in the near term the slow rate of molecular evolution, and thus the high sequence conservation found in both coding and non-coding sequences in poplar (Tuskan et al., 2006), will allow gene constructs based on the Nisqually-1 genome sequence to be productively analyzed in a variety of transformed poplar genotypes. Unfortunately, although transformation procedures have been reported for Nisqually-1 (Ma et al., 2004; Song et al., 2006) a robust technique is still not available.

In the long term, in addition to sequencing one or more transgenic models, it will be necessary to develop methods that are effective on a wide variety of genotypes chosen for their importance based on biological or breeding criteria. Breakthroughs

are needed that employ genes, such as the *rol* gene cassette from *Agrobacterium rhizogenes*, which promote the regeneration of transgenic cells (Arias et al., 2006). These genes could then be removed via methods such as recombinase mediated excision to produce normal plant phenotypes. A prototype was developed some years ago (e.g., Matsunaga et al., 2002), but does not appear to have been developed into a broadly effective method. Much more work along these lines is needed, perhaps exploring a much wider variety of types of regeneration-inducing genes.

Third, because poplar is an emerging model species, the transgenic "toolkit" is still very limited. There are only a few promoters whose tissue and cell-specific expression properties have been demonstrated in transgenic plants (e.g. Wu et al., 2000; Johansson et al., 2003; Hao et al. 2005; Filichkin et al., 2006b). Although gene excision has been used in poplar, most notably as part of the MAT system (Ebinuma et al., 1997; Matsunaga et al 2002; Zelasco et al., 2007), a number of other useful and well studied excision systems such as CRE/LOX (Marjanac et al., 2008) and FLP/FRT (Sonti et al., 1995) have not, to our knowledge, ever been characterized for their effectiveness in poplar. Nevertheless, technological advance in other species seem to be transferable to poplar (Fig. 3) (Filichkin et al., 2006a).

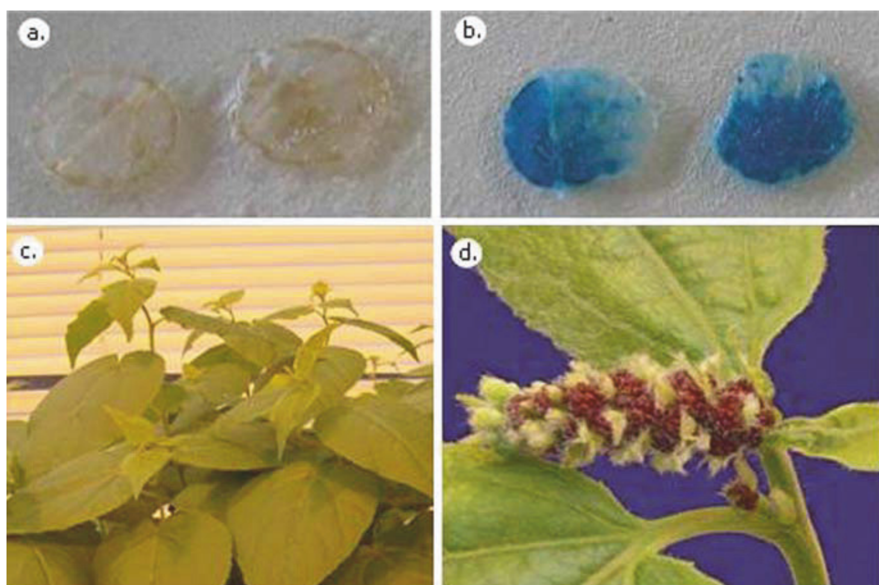

Fig. 3 Inducible expression in poplar. Photos on *left* and *right panels* represent explants and plants before and after induction respectively. *Top*: Induction of a GUS reporter gene under the control of the ecdysone induction system (construct provided by Metabolix Co. and based on the construct used in (Kourtz et al., 2007)) (**a**, **b**). Photo courtesy of Cathleen Ma, Oregon State University. Heat induction to induce flowering in poplar. *Bottom*: Plants were heat shocked by placing them in a growth chamber at 37°C for 1 h/day (2–4 weeks). Flowers were observed after 2 weeks from the initiation of the treatment. Transgenic male poplar (*P. tremula* × *P. tremuloides,* INRA 353-53) containing the Arabidopsis *FT* gene under the control of the soy heat shock promoter were employed. Photos courtesy of Huanling Zhang, Oregon State University

Fourth, the capacity for forward genetics is limited in poplars. A worldwide, public investment in high quality QTL/association mapping resources that can identify specific causative genes or polymorphisms, and a much larger investment in gene tagging populations such as the activation tagging populations discussed above, would seem to be logical avenues for progress. Such a systematic gene discovery effort for traits important to woody species is likely to identify genes that would have been entirely missed based on selective reverse genetics approaches – as work to date for activation tagging has suggested (discussed above). The high costs of phenotyping in such programs, and the lack of capacity for maintenance/sharing of these collections without costly in vitro or cryogenic facilities, are the main limitations to progress (reviewed in Tsai and Hubscher, 2004).

Fifth, the inability to efficiently target genes for mutagenesis, and the associated "position effect" variation in transgenic populations associated with individual gene insertion events, remain major obstacles to efficient dissection of gene function in poplar and all other plant species. Recent advances in zinc-finger nucleases and related technologies appear to hold significant promise (Tovkach et al., 2009), but to our knowledge have not yet been studied in poplar.

Finally, a major deterrent of using transformation as a research tool is the difficult regulatory requirements for planting of genetically modified organisms (Strauss, 2003). Tree phenotypes must ultimately be studied in the field if they are to be relevant to normal physiology and breeding. However, the wide potential dispersal of pollen and seeds pose large obstacles to field trial approval in most countries. Flowering is problematic as most research plots are within the range of wild or feral species, and the female test trees themselves could produce progeny that establish in the field as they are not fully domesticated (though some hybrids and triploids have very low fertility). However, if fully sterile trees could be produced using transgenesis or other means, and the stability of the sterility trait verified over a number of years, these genotypes might serve as hosts within which a wide variety of genes could be tested over a normal rotation length. Transgenic research toward this end will clearly take many years, but a number of strong genome-enabled options exist for producing robust sterility systems (Brunner et al., 2007). The main limitation is the regulatory system itself, which even in the USA does not allow the dispersal of genes from small research plots even if their only effect is to reduce fertility, though this would appear to pose no significant environmental risk (controls and some transgenics will not be perfectly sterile during the research phase, and it is practically very difficult to remove all flowers/fruits from large flowering trees). Unfortunately, there are no established means to accelerate the normal flowering of poplars in the greenhouse to provide an effective first screen; as discussed above the *FT* and *LEAFY* genes only appear capable of producing abnormal, partially functional flowers. We know of no public research programs anywhere that are adequately organized and funded to be able to deal with this purely regulatory hurdle to flowering and biosafety research.

Despite these challenges, transformation in its current form will clearly remain the major genetic research tool for dissection of gene function in poplar for many years to come. The natural amenability to transformation in many

genotypes remains the key biological attribute that makes poplar the most powerful model organism for genetic analysis of woody plant growth, adaptation, and development.

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Populus Resources and Bioinformatics

Nathaniel R. Street and Chung-Jui Tsai

Abstract As a model system, Populus offers the opportunity to study biological questions pertinent to perennial growth habits such as lignocellulosic cell wall biogenesis and dormancy cycles. In the past years, much has been learnt about the transcriptional control of such processes and there is an ever-growing resource of publicly available transcriptomics data and EST sequences available.

More recently greater emphasis has been placed on the study of metabolomic data and particularly in linking metabolic changes to both development and ecosystem functioning. Populus represents an ideal model system in which genetic and genomic studies can be conducted in a ecological key-stone species as well in a commercially important forest tree crop.

To facilitate biological understanding a number of bioinformatics resources have become available for Populus alongside greater integration of the species in centralized sequence data sources such as NCBI and Interpro. These developments are rapidly advancing the ability to use Populus as a model system for the study of developmental, ecological and comparative genomics questions.

Here we overview the current state of bioinformatics resources available for studies involving Populus as well as detailing the genetic material available to conduct studies on.

Since the emergence of *Populus* as the model tree species, there has been a steady and rapid development of resources enabling the use of new technologies and approaches for answering biological questions. The *Populus* genomics resources have been, and will continue to be, instrumental in addressing biological questions pertinent to perennial growth habits (e.g., lignocellulosic cell wall biogenesis and dormancy cycles). Increasingly, genomics tools are also being incorporated into ecological investigations due to the keystone role of *Populus* species in many riparian ecosystems.

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One strength of *Populus* as a model is our collective interest on not a single species but the entire genus. As a result, genomics resources have been developed from species covering a wide range of geographic distributions and phenotypic diversity. Building on the rich natural variations within the genus, the growing *Populus* genomics resources should facilitate investigations into mechanisms that underscore cross-species variation (Harding et al., 2005; Street et al., 2006; Quesada et al., 2008) with either economical or ecological significance. Currently, the use of microarrays has received the greatest attention. Transcriptomics studies have provided insight into a range of developmental processes including senescence (Andersson et al., 2004; Sjödin et al., 2008a), leaf development (Matsubara et al., 2006), wood formation (Hertzberg et al., 2001; Schrader et al., 2004a; Moreau et al., 2005) and seasonal dormancy (Schrader et al., 2004b; Druart et al., 2007; Ruttink et al., 2007). There have also been studies examining abiotic stress response and adaptation to climate change (Gupta et al., 2005; Taylor et al., 2005; Brosche et al., 2005; Druart et al., 2006; Street et al., 2006; Bogeat-Triboulet et al., 2007; Fluch et al., 2008), biotic stress response (Smith et al., 2004; Ralph et al., 2006; Miranda et al., 2007; Rinaldi et al., 2007; Frost et al., 2008) and the effects of genetic manipulation (Groover et al., 2006). Two experiments have been performed to profile gene expression in different tissue types (Qeusada et al., 2008; Sjödin et al., 2008b) with the results being compared to those of similar experiments in Arabidopsis (for example Schmid et al., 2005). These two experiments confirm the general conclusion of comparisons to Arabidopsis from other published microarray data; transcriptional control in response to environmental perturbation, biotic infection or challenge, and during tissue development is predominantly the same (i.e. evolutionarily conserved) across species. However, the *Populus* experiments have resulted in some unique results that could not easily have been obtained using Arabidopsis. Importantly, they have provided insight into many commercially and functionally important traits specific to perennial species, including winter dormancy.

1 The *Populus* Genome

The genome sequence of the female *Populus trichocarpa* “Nisqually-1” clone was published in Tuskan et al. (2006). The genome was sequenced to ∼7.5 X depth using a shotgun approach (Tuskan et al., 2006, see also Sections 1.1and 2.1 of this volume) with assembly being supported through the use of both genetic and physical maps. The assembled genome contains 450 Mb and the current annotation release contains 45,555 gene model predictions. Just under 90% of predicted gene models show homology to sequences hosted at NCBI, with ∼12% showing no homology to genes in *Arabidopsis thaliana*, which raises the interesting possibility that these may represent “tree-specific” genes. Additional gap-closing sequencing has been completed and efforts are under-way for a second round of assembly, including re-training of gene-calling algorithms (based on the ∼1% manually curated gene models from release v 1.1) to improve the quality of the predicted gene models. It

is important to mention that a significant fraction of the gene models in the v 1.1 release are incorrect, for example truncated, fused or in some cases true genes are simply missing. This is not surprising since it is the first version of the genome but it has to be kept in mind when the genome is analysed.

1.1 Genome Databases and Browsers

The Joint Genomes Initiative (JGI) web resource (http://genome.jgi-psf.org/poplar) includes a genome browser, BLAST and BLAT search tools, gene search tools, ontology browsing and a public ftp site that hosts a range of data for download (ftp://ftp.jgi-psf.org/pub/JGI_data/Poplar). In addition to the JGI genome browser, four other genome browsers are available, including the NCBI *Populus* Genome Viewer and the Plant Genome Database's *Populus* Genome Browser (described below). The extensive GRAMENE resource (http://www.gramene.org) includes a *Populus* genome browser, as does the *Populus* Integrative Genome Browser (PopGenIE, www.popgenie.db.umu.se, Sjödin et al., 2008b). PopGenIE additionally includes two dedicated synteny browsers to visualise conserved syntenic regions between a number of model plant genomes. BLAST and BLAT search tools are also provided as well as sequence and annotation extraction tools and an ftp site. GRAMENE and PopGenIE both provide a BioMart service (www.biomart.org), including web interfaces for the rapid downloading of bulk annotation information.

1.2 Expressed Sequence Tags

Expressed Sequence Tag (EST) collections were of first and foremost importance to the success of establishing *Populus* as a model system in the field of forest genomics. Large-scale *Populus* EST collections have been a rich resource for qualitative and quantitative assessment of gene expression, offering snap shots of tissue- or developmental-specific gene expression (e.g., Sterky et al., 2004; Déjardin et al., 2004; Kohler et al., 2003). EST collections from seven *Populus* species were analyzed by Sterck et al. (2005) to provide evidence for a genome-wide duplication event shared by *Populus* species, but not by Arabidopsis. ESTs have been used for examining codon usage bias (Ingvarsson, 2007). Perhaps the most important outcome of EST sequencing projects was the development of cDNA microarrays, which subsequently provided a first insight into many developmental processes, including wood formation (e.g., Hertzberg et al., 2001). The cumulative EST resources, including full-length cDNA collections (Nanjo et al., 2004; Ralph et al., 2008) later played an instrumental role in gene-calling algorithm training and annotation of the genome sequence (Tuskan et al., 2006). In the post-genomics era, EST support (especially full-length cDNAs) remains an essential tool for assessing the quality of computational gene model predictions. Segerman et al. (2007)

show that the ESTs sequences generated from different species/hybrids contained in *Populus*DB (www.populus.db.umu.se, Sterky et al., 2004) vary between species by ~2–3% on average. To date, there has not been an extensive examination of whether there is any bias in sequence homology variation between genes to identify those that may be under constrained or more rapid evolutionary change.

1.3 EST Databases

Two distinct types of databases containing *Populus* ESTs exist: those holding original EST sequences and those collating ESTs from multiple sources for secondary purposes, such as unigene prediction. The largest database with original EST collections is the *Populus*DB, which offers extensive search and query tools, including "electronic Northern" analysis (*i.e.* display of EST frequency in the various tissue sources). Table 1 details other significant EST collections.

Table 1 *Populus* databases

Database	# ESTs	Website address	Key references
EST databases			
*Populus*db	121,495	www.populus.db.umu.se/	Sterky et al. (2004)
PoplarDB	20,005	http://mycor.nancy.inra.fr/PoplarDB/	Kohler et al. (2003), Brosche et al. (2005)
AspenDB	16,718	http://aspendb.uga.edu/	Ranjan et al. (2004)
Arborea	11,591	http://www.arborea.ulaval.ca/research_results/est_sequencing_in_poplar/	
Plant Gene Database	399,405	http://www.plantgdb.org/	Dong et al. (2004)
DFCI Poplar Gene Index	420,252	http://compbio.dfci.harvard.edu/	
TIGR Plant Transcript Assemblies	354,784	http://plantta.tigr.org/	Childs et al. (2007)
NCBI	425,599	http://www.ncbi.nlm.nih.gov/	
Transcription factors			
DPTF	2,576	http://dptf.cbi.pku.edu.cn/	Zhu et al. (2007)
PTFD	2,723	http://poplartfdb.bio.uni-potsdam.de/v2.0/	Riano-Pachon et al. (2007)
DBD	1,957	www.transcriptionfactor.org	Wilson et al. (2008)
DATFAP	3,196	http://www.daimi.au.dk/cgi-chili/datfap/frontdoor.py	
miRNA			
miRBase	234	http://microrna.sanger.ac.uk/	Griffiths-Jones et al. (2008)
miPlantBrowser	2,728	http://miserver.binf.ku.dk/100/main.pl	Lindow et al. (2007)

In addition to these individual project databases, there are currently four major resources containing collations of *Populus* ESTs. All four databases make available independent unigene predictions. NCBI serves as the official repository for all ESTs and, at the time of writing, contains > 425,000 *Populus* ESTs. NCBI use the raw sequences to construct per-species (or hybrid) UniGene predictions, which include qualitative tissue distribution information, visualisation and annotation based on maximum homology to other sequences held at NCBI. There is also an NCBI *Populus trichocarpa* genome viewer that displays alignments of ESTs and UniGenes, but only those originated from *P. trichocarpa* are included at the present time. The Poplar Gene Index (PplGI), hosted by DFCI (http://compbio.dfci.harvard.edu/tgi/) uses EST sequences to construct cross-species unigene predictions (termed Tentative Consensus sequences) and includes an annotation pipeline providing EST and TC level annotation and assignment within the Gene Ontology (GO) hierarchy (www.geneontology.org/, Ashburner et al., 2000). The J. Craig Venter Institute (formerly the Institute for Genome Research, TIGR) Plant Transcript Assemblies (http://plantta.tigr.org/) provides per-species unigene predictions (termed Transcript Assembly) and annotation. Finally, the Plant Genome Database project also houses a *Populus* database (http://plantgdb.org/PtGDB/) that includes species-specific unigene predictions (termed Putative Unique Transcripts) and annotation information. Its genome browser displays aligned ESTs, PUTs and homolgous genes from other model plants, JGI Populus gene models and in-house predicted splice variants. This currently represents the most extensive collated EST resource for *Populus* other than NCBI and provides data in a highly-accessible format.

1.4 Transcription Factor Databases

Four databases (Table 1) contain predicted *Populus* transcription factors (TFs), with the number ranging from 3196 (DATFAP) to 1957 (DBD). All of the databases make predictions based on the genome sequence, except for DATFAP which utilises consensus EST information and a range of secondary external database sources. DPTF and DBD use hidden Markov Models (HMMs) of pfam (pfam.sanger.ac.uk/) domains to assign predicted transcription factor category, with DBD also using HMMs from the SUPERFAMILY (http://supfam.org/SUPERFAMILY/) database. PTFD assigns predicted transcription factor class on the basis of domain presence. DATFAP uses a largely annotation-based approach to identify transcription factors and can therefore be considered as less stringent than the other three databases, and likely to contain a higher number of false-positive entries.

1.5 Promoter Motif Analysis

The Diurnal project (http://diurnal.cgrb.oregonstate.edu/) contains a number of interrelated databases to facilitate analysis and data mining of circadian/diurnal

gene expression in Arabidopsis, rice and *Populus*, using both experimental and computational approaches. Experimentally-determined co-regulation patterns can be explored with the DIURNAL database, and a set of co-regulated genes can be used to query the ELEMENT database to identify over-represented promoter motifs. The site also provides pre-fetched promoter sequences of various lengths (500 bp–3 kb) from several sequenced plant species in downloadable format for promoter analysis. Using the promoter sequence files available from ELEMENT, a number of other motif finding tools can be used with minimal effort. For example, CLOVER (http://zlab.bu.edu/clover/, Frith et al., 2004), TOUCAN (http://homes.esat.kuleuven.be/∼saerts/software/toucan.php, Aerts et al., 2005) and the standalone tools that constitute various TOUCAN functions can easily be used.

1.6 Ortholog Prediction

A number of ortholog predictions are available, including those at the Phytozome resource (http://www.phytozome.net), the PopArray database (http://popgenome.ag.utk.edu/mdb), the Orthomap database (http://orthomap.cgrb.oregonstate.edu), the OrthologID database (http://nypg.bio.nyu.edu/orthologid), and the GRAMENE resource (www.gramene.org). A related PlantTribes database (http://fgp.bio.psu.edu/tribedb) offers objective classification of plant gene families using a graph-based clustering procedure (Wall et al., 2007). The database contains genes from five sequenced plant genomes (Arabidopsis, rice, *Populus*, *Medicago* and papaya), plus the TIGR Plant Transcript Assemblies (unigene sets) derived from ∼4 million sequences from >200 species. It is a powerful resource for comparative genomics analysis, and can also facilitate identification of species-specific gene families.

1.7 miRNA Databases

Two databases host information on *Populus* miRNAs (Table 1). miRBase (http://microrna.sanger.ac.uk/) is the official repository for experimentally-confirmed miRNA sequences. A second database, miPlantBrowser (http://miserver.binf.ku.dk/100/), contains the miRNAs from miRBase in addition to computationally predicted miRNAs and their predicted targets (Lindow et al., 2007). Somewhat unfortunately, the target predictions provided at miPlantBrowser are based on an older version (JGI v 1.0) of predicted gene models (although other resources, e.g. PopARRAY provide cross-referencing tools to overcome this problem). Ultra-high throughput (e.g., 454/Solexa/SOLiD) sequencing technologies are expected to contribute significantly to identifying the total miRNA and sRNAs population, as demonstrate by Barakat et al. (2007).

1.8 QTL Databases and Browsers

The University of Tennessee (UT) *Populus* CMap resource available at http://popgenome.ag.utk.edu/cmap/ includes QTL from two *P. deltoides* × *P. trichocarpa* populations. QTL are displayed on a genetic map of the female *P. trichocarpa*, mother of both populations. All QTL stored in this database are also displayed in the JGI genome browser, allowing easy visualisation of QTL-candidate gene co-location. There is a QTL browser available at the PopGenIE resource, which provides the majority of published QTL from the *P. deltoids* × *P. trichocarpa* inbred F_2 population, Family 331 (Wu et al., 1998; Bradshaw et al., 1994) and also includes all QTL available at the UT CMap resource. At PopGenIE, QTL are also displayed in the genome browser. Both QTL resources enable generation of gene lists within QTL confidence intervals. There are additionally two *Populus* genetic maps available at the TreeGenes database (http://dendrome.ucdavis.edu/treegenes/, Wegrzyn et al., 2008). Additional CMap resources are currently in development (G. Taylor, M. Morgante, personal communication).

2 "Omics" Methods

Methods and resources now exist for the high throughput quantification of molecules at all levels of the central dogma. First among these was the development of microarrays for the rapid quantification of gene expression at a global scale, initially using high-density EST arrays and later with whole-genome oligo arrays. Microarrays are somewhat different to other high throughput measurement techniques in that they require the development of a species (or perhaps genus) specific measurement assay and these will therefore be discussed in greater detail.

2.1 Transcriptomics

Several microarray platforms are available for *Populus*, including spotted cDNA arrays and in situ synthesized whole-genome oligo arrays (Table 2). While cDNA arrays have been the predominant platform for *Populus* transcriptomic analysis in the past decade, their use will likely diminish following the introduction of whole-genome oligo arrays. All three major commercial oligo array platforms (i.e., Affymetrix, Agilent and Nimblegen) are available for *Populus*. The transcriptome coverage ranges from ∼43,600 predicted nuclear gene models in the Agilent array to ∼56,000 in the Affymetrix and NimbleGen platforms. The Affymetrix and NimbleGen platforms also contain probes derived from EST sequences, while the Agilent and NimbleGen arrays include mitochondrial and chloroplast gene models as well. A detailed comparison of the three platforms has been discussed elsewhere (Tsai et al., 2009).

Table 2 Array platforms

Array	Type	Unique probes	Web link
Agilent	oligo (60mer)	43,795	www.agilent.com
Nimblegen	oligo (60mer)	65,911	www.nimblegen.com
Affymetrix	oligo (25mer)	61,251	www.affymetrix.com/
UPSC-POP2	cDNA	20,390	www.populus.db.umu.se/
PICME	cDNA	26,915	www.picme.at/
UBC	cDNA	15,496	www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL5921
MTU	cDNA	6,705	www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL6992

Cross-platform comparison of experimental data is likely to be challenging, because of the inherent differences in probe design and properties among the various (whole-genome or EST) array platforms. Although not ideal, the PopArray database provides tools to cross-reference between probe identifiers of the three whole-genome microarrays and JGI gene model ID. Cross-species utility of these arrays remains a concern due to the diversity of *Populus* species and hybrids used in various research. Both Agilent and NimbleGen arrays are produced via maskless in situ oligo synthesis, and therefore are more flexible platforms to accommodate future probe design change needs. The Agilent system, in particular, offers a user-friendly eArray portal for custom editing of probes, and this may prove to be a highly flexible platform for working with a range of *Populus* species.

There is currently one *Populus*-specific public database (UPSC-BASE) hosting microarray data from cDNA microarrays produced at the Umeå Plant Science Centre. The database can be accessed at http://www.upscbase.db.umu.se/ and is detailed in Sjödin et al. (2006). Two other public databases contain *Populus* expression data, the ArrayExpress database (http://www.ebi.ac.uk/microarray-as/ae/) with additional cDNA microarray data, and the NCBI Gene Expression Omnibus (GEO) repository (www.ncbi.nlm.nih.gov/geo/). The GEO repository is by far the most utilised resource for storing public microarray data, with nearly all *Populus* array platforms represented.

Until recently, microarrays were most typically used to identify genes or gene networks involved in the control of a developmental process or response to an external stimulus. Experiments of this type typically concentrate on a single genotype, and are powerful for identifying targets for functional characterisation using reverse genetics. Microarrays have also been used to study gene expression between species or genotypes (e.g., Harding et al., 2005), although data interpretation in these cases may be complicated, especially if oligo arrays are used, due to potential sequence divergence. There are now well established statistical methods for identifying differentially expressed genes and/or co-regulation gene clusters between experimental conditions or tissues, or along a temporal or spatial gradient of interest.

Advanced microarray analyses often include pathway mapping or testing for functional enrichment within identified gene sets. In the case of *Populus*, however, the supporting resources or databases for such analyses are not yet as well developed as for other model organisms. The gene ontology (GO) database is the most extensive and supported hierarchy for the classification of genes on the basis of gene function (either experimentally confirmed/identified or through homology evidence). JGI provides assignment of predicted gene models to GO classes, with this information available for download on their ftp site. http://www.geneontology.org/GO.evidence.shtmlOthers have taken the approach of assigning *Populus* genes to GO classes on the basis of homology to Arabidopsis genes, as Arabidopsis has by far the most advanced GO annotation of any plant. This approach has the obvious limitation in that it assumes the conservation of gene function and is dependent on the accuracy of ortholog assignment. A *Populus* GO structure obtained by this approach can be queried using the GO TermFinder tool at the PopGenIE resource. Finally the BLAST2GO web resource (http://www.blast2go.de/) provides GO assignment of predicted gene models and ESTs using their annotation pipeline. The PopGenIE resource additionally provides a tool (CatFisher) for identifying over-represented KEGG or KOG categories and InterPro domains. The same tool can be used to test for over-representation of transcription factor families or miRNA family target sites within a gene list.

Comparison across multiple experiments for individual genes, gene families or genes within a regulon can also provide valuable insight. The eFP browser (Winter et al., 2007), which displays gene expression data as easily-interpretable pictographs is a highly accessible route to utilising microarray data. The system requires no end-user ability to handle extensive datasets or to perform often complex analysis. Two eFP browsers are now available for *Populus*. The first displays results from whole-genome Affymetrix expression arrays and is hosted at http://bar.utoronto.ca/efppop. The second displays dual-colour ratio results from cDNA microarrays and is part of the PopGenIE resource. Such pictographs are a powerful and highly-intuitive means of displaying cross-experiment gene expression data for the inference of gene function. Both *Populus* eFP browsers offer Arabidopsis ortholog links for easy inspection of the conservation of expression patterns. In addition to the eFP browser, the PopGenIE resource includes visualisation of expression data as expression profile plots and the generation of electronic Northerns.

2.2 High Throughput Sequencing

Massively parallel sequencing offers great potential for re-sequencing projects, gene expression profiling and miRNA/sRNA profiling. The use of re-sequencing data will prove particularly important for discovering functionally-relevant polymorphisms that show evidence of selection. Sequencing can be performed using genomic DNA or cDNA. Sequencing methods can be further adapted to understand the role of

methylation (Taylor et al., 2007) and nucleosome assembly (Schones et al., 2008) in the control of gene expression. As the cost of high-throughput sequencing continues to fall, genomic comparisons between multiple species or clones has become possible and this will prove to be an important future source of data for uncovering genetic mechanisms underlying natural variation.

New sequence capture technology couples high-density oligo arrays with massively parallel sequencing to target and sequence selected regions of the genome with unprecedented enrichment, coverage and accuracy (Albert et al., 2007). The technology can capture either contiguous or dispersed regions in one single experiment, producing sequence coverage up to the megabase range. For species as diverse as *Populus*, this offers a powerful and cost-effective alternative to re-sequencing.

2.3 Metabolomics and Proteomics

Methods for quantifying metabolites and proteins and for sequencing DNA are species independent, but subsequently rely heavily on the quality of database resources for identification and annotation of measured compounds. In the case of proteomics, this is relatively (or comparatively) simple as the task is to determine the amino acid sequence represented by a set of peptide fragments. The reconstructed sequence can then be identified by comparison to the genome sequence. There are currently few examples of proteomics analysis in *Populus* but key publications include Renaut et al., (2004), Juan et al. (2006), Ferreira et al., (2006) and particularly Plomion et al. (2006).

In the case of metabolomics, the task is far more complex as the ability to identify a compound is entirely dependent on its inclusion in a reference database, such as the NIST mass spectral library, or the availability of an authentic standard for verification. *Populus* species, in particular, produce a staggering diversity of secondary metabolites (Tsai et al., 2006), many of which are species-specific and poorly represented in public chemical databases. This challenge is also platform-specific. For instance, compound matching against a public database is applicable largely for GC-MS-based approaches where retention index and mass spectral characteristics are less variable between laboratories. The chromatographic behaviour and associated characteristics of compounds separated by LC-MS-based approaches are less reproducible between instruments, making in-house reference library construction an essential requirement in LC-MS-based metabolomics efforts. Overall, metabolomics data processing pipelines, such as compound matching algorithms, data analysis and visualisation, have not been advanced as much as those of other omics to handle the large volumes of data generated from profiling analyses. To date, there is no public database of *Populus* metabolites, although a number of groups have developed their own in-house databases that may become public in the near future (T. Moritz, T. Tschaplinski, S. Harding, W. Boerjan, personal communication).

Metabolomics is rapidly gaining interest as it represents the terminal link between genotype and environment/community, and the metabolite snapshot is a

key determinant of physiological state and plant-plant and plant-pathogen/herbivore interactions. Morreel et al. (2006) used metabolite (flavonoid) profiling in an interspecific mapping population to identify significant metabolite QTL (mQTL) and were able to map candidate genes associated with flavonoid regulation. The availability of a public metabolite repository and improved metabolomics resources will be essential to the success of the systems biology approach, which will rely heavily on meta-analysis of public data. Systems biology will also require the integrated analysis of data from all omics field. Bylesjö et al. (2008) provide a first example of such an integrated analysis approach.

3 Germplasm

Although a large number of phenotypically diverse *Populus* species and hybrids are widely used by researchers to answer a range of biological questions across multiple disciplines, no central germplasm resource has yet been established. As such, obtaining plant material for experiment establishment requires personal contact with those holding germplasm of interest. One key difference when compared to many other model systems is the unsuitability of *Populus* seeds as a propagation method. From a practical point of view, this is because *Populus* seeds do not store and require effectively immediate germination. From an experimental point of view, the heterozygous, out-crossing nature of *Populus* means that seedling-derived materials would be genetically heterozygous and therefore inherently variable. Luckily, the majority of *Populus* species are easily propagated as hardwood (or in the case of aspens, root) cuttings, and can be regenerated from tissue culture. The ease of propagation combined with the strikingly rapid plant growth in a glasshouse or controlled environment make rapid establishment of large, replicated experiments highly feasible. However, clonal germplasm distribution is more complicated, as cutting material for propagation requires storage in carefully controlled, chilled and humid conditions to maintain viability. Long-term maintenance of primary germplasm collections in field or common garden settings remains a challenge (see discussion in Tsai and Hubscher, 2004). Cryopreservation-based germplasm preservation has been reported for *Populus* as a potentially long-term storage option (Jokipii, 2004; Tsai and Hubscher, 2004). However, it also requires significant upfront investment.

Although no germplasm stock centre resource exists, there are a number of key groups around the world who hold their own genetic stocks that may be available upon request. The majority of these collections were developed to supply breeding programs or for the production of QTL mapping populations. More recently, there has been a shift towards developing and extending collections of natural clones across a species range for association studies. A number of these are detailed in Table 3 and we recommend contacting the authors of relevant collections/publications for availability of material of interest. These collections of material represent a fascinating diversity of phenotypes and include clones with

Table 3 Germplasm contacts

Material	Contact name	Contact institution	Email addresss
EU			
P. nigra, *P. trichocarpa*, *P. deltoids*, hybrids and breeding material. F_1 TD.	Mark Villar	INRA Orleans	marc.villar@orleans.inra.fr
F_2 TD, access to UK breeding material incl. IEA collection.	Gail Taylor	University of Southamtpon	g.taylor@soton.ac.uk
P. nigra and breeding material.	Marijke Steenackers	Ministry of the Flemish Community Institute for Forestry and Game Management	marijke.steenackers@lin.vlaanderen.be
F_1 DN and DT	Wout Boerjan	VIB	wout.boerjan@psb.ugent.be
P. alba, *P. nigra* inc. Intra-specific F_1 and F_2 populations. Contact advice for commercial material.	Maurizio Sabatti	University of Tuscia	sabatti@unitus.it
P. tremula	Stefan Jansson	Umeå Plant Science Centre	stefan.jansson@plantphys.umu.se
North America			
P. balsamifera	William Schroeder	Agriculture and Agri-Food Canada	schroederb@agr.gc.ca
P. fremontii x *P. angustifolia*	Thomas Whitham	Northern Arizona University	thomas.whitham@nau.edu
P. trichorcapa, *P. maximowiczii*, TD and DM hybrids	Brian Stanton	Greenwood Resources	info@gwrglobal.com
P. deltoides, DM hybrids	Bill Berguson	Natural Resources Research Institute	http://www.nrri.umn.edu/
P.deltoides, *P. nigra* and *P. maximowiczii* hybrids	Don Riemenschneider	USDA Forest Service	driemenschneider@fs.fed.us
P. tremuloides	Karen Mock	Utah State University	karen. mock@usu.edu
Activation tagged lines	Steve Strauss	Oregon State University	steve.strauss@oregonstate.edu
Enhancer and gene-trap collection	Andrew Groover	USDA Forest Service	agroover@fs.fed.us
Activation tagged lines	Sharon Regon	Queens University	regans@biology.queensu.ca

highly variable apical dormancy, rooting ability, root architecture, cold tolerance, secondary chemistry and associated insect herbivores, volatile compound emissions, abiotic and biotic stress tolerance, as well as *P. trichocarpa* clones that have permanently open stomata and altered ABA insensitivity. The collections of material cover both intra- and inter-species variation, and represent a rich resource for comparative genomics and association genetics research. Another important germplasm resource is the collections of transgenic mutants, especially those derived from various insertional mutagenesis approaches (Busov et al., 2005).

Unlike the prevalence of standard, reference genotypes used by the Arabidopsis community, there is no clear standard genotype or even species used by the *Populus* community. Most typically, individual researchers select the *Populus* species, hybrid or clone most suited to answering the biological question at hand, or most suited to a particular experimental system. As tree improvement is often the ultimate goal in forest genomics and biotechnology research, it is not surprising that hybrids derived from breeding programs are among the most widely used experimental species. Two hybrid clones have been particularly commonly used for transformation studies (*P. tremula* × *P. tremuloides* “T89” and *P. tremula* × *P. alba “INRA 717 1-B”*). Potential epistatic interactions are of concern and may complicate the results, although the extent will likely vary depending on the biological process/pathway under investigation.

4 Future Directions

Considerable resources have now been developed by the *Populus* community. However significant challenges remain to ensure that maximal benefit can be made of *Populus* as a model system. This is particularly true for the use of genomics data in systems biology and ecological studies. The *Populus* community currently lacks a Model Organism Database (MOD). Such a MOD would serve to improve community coordination as well as offering a single-access portal to the range of genetic and omics data available for *Populus*. The establishment of such a resource will be essential as meta-analysis approaches (such as those employed within a systems biology context) become increasingly important. Many best-practice examples exist for other model systems and the *Populus* community can use these to guide the development of a central repository and access resource.

Although a significant genomics resource has already been established using cDNA arrays, future use of whole-genome oligo arrays that can specifically profile members of gene families, and in some cases differentiate paralogs, will become an important resource for refining gene annotations and assigning genes within a robust *Populus*-specific Gene Ontology and Plant Ontology context. The use of transcriptomics data for such purposes requires the establishment of efficient data submission, curation and automated analysis pipelines. A considerable gap remains in developing comparable bioinformatics tools for metabolomics data mining and their integration with transcriptomics data. Such efforts, coupled with increasingly

sophisticated phenotyping approaches at cellular, tissue or whole-plant levels will be an essential requirement for meaningful functional interpretation of systems biology analyses.

Populus offers great potential for extending genomics beyond the single tree to the population and ecosystem level: Although much has been learnt at the level of the individual and the ecology of insect-plant interactions, understanding of the interaction between genomes within a community is still extremely limited (Zheng and Dicke, 2008; Whitham et al., 2008), but is a fascinating area of research that will begin to form functional connections between these fairly distant biological levels. High throughput profiling techniques can be used to profile individuals within and between natural populations in order to identify metabolites, genes or regulons associated with the ecological trait of interest, such as insect herbivore preference. Combining the use of next generation sequencing methods (Wheat, 2008), differential profiling analysis, regulatory network (co-correlation) analysis and linkage or linkage disequilibrium mapping has great potential to uncover the functional mechanisms underlying adaptive natural trait variation and evolutionary interactions that serve as selective pressures influencing speciation.

There are many examples of important interactions that involve complex links between populations of genomes of different species, including mycorrhiza (Martin et al., 2008), endophyte association and plant-insect interactions. For example Evans et al. (2008) showed that genetic interactions between a specialist mite and their host hybrid *P. angustifolia* × *P. fremontii* trees drive population structure, local adaptation, and host race formation. Extension of such findings to include genetic mapping and omics profiling offers the potential of uncovering genes in trees that are driving speciation and population structure at higher trophic levels. *Populus* is ideally suited as a model system for studying such interactions. There will be many practical challenges along the way, especially as two currently disparate fields (ecology and genomics) discover the need to store data in a way that allows cross-communication and integration (Jones et al., 2006).

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Part III
Genetics and Genomics of Key *Populus* Traits

Reproductive Development in *Populus*

Amy M. Brunner

Abstract Flowering is a multistage process that includes acquiring the competency to flower, the transition to flowering in response to environmental and endogenous signals, and the development of floral organs. A non-flowering juvenile phase of several years is typical for *Populus* and most trees, and in adult *Populus,* the seasonally recurrent transition to flowering and flower development take nearly a year to complete. This chapter reviews the biology of *Populus* flowering and discuss the regulation of *Populus* flowering in a comparative context to *Arabidopsis thaliana* and other angiosperms.

1 Introduction

Due in large part to the central importance of flowering to plant fitness and crop yield, flowering is one of the best studied plant developmental processes. Particularly for the model annual plant Arabidopsis, there is now detailed knowledge of the regulatory pathways and molecular mechanisms regulating flowering (reviewed in Mutasa-Gottgens and Hedden, 2009; Michaels, 2009; Turck et al., 2008; Farrona et al., 2008). The *Populus* genome sequence has revealed the presence of orthologs of nearly all of the major genes regulating flowering in Arabidopsis (Yuceer et al., 2009; De Bodt et al., 2006; Brunner and Nilsson, 2004). Moreover, gene expression and a limited number of functional studies in *Populus* suggest that flowering regulatory pathways are broadly conserved between Arabidopsis and *Populus* (e.g., Rottmann et al., 2000; Hsu et al., 2006). The network controlling flowering in Arabidopsis is highly complex, involving a large number of genes and mechanisms. Thus, a key challenge for *Populus* researchers is uncovering

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the differences in regulatory interactions that underlie the dramatically different flowering habits of a tree and an annual plant.

A comprehensive understanding of the genes and regulatory interactions controlling initiation of flowering and flower organogenesis in *Populus* could enable development of reliable methods to manipulate flowering in this important genus and perhaps more generally for trees. This could enhance genetic improvement and plantation productivity both directly and indirectly. There are multiple aspects of flowering that would be desirable to alter temporarily or permanently, depending on the application. These features include the several year-long juvenile period when trees are incompetent to flower that greatly slows progress in tree breeding. Although dioecy facilitates controlled outcrossing, it prevents the development of inbred *Populus* genotypes for experimental uses or advanced breeding methods. From a production standpoint, it would be beneficial to prevent or delay the initiation of flowering in plantation trees in order to maximize wood growth and yield. Because of the potential for long distance gene flow by pollen and sometimes seed, and the frequent proximity of plantations to interfertile wild populations, reproductive sterility is the most effective method for genetic containment of forest tree plantations. Containment of highly domesticated, exotic or transgenic tree plantations may be desirable to minimize the risk of unintended ecological impacts, and may also be required by law or marketplace constraints (Brunner et al., 2007). Deciphering the genetic regulation of flowering would also improve our understanding of how climate change and other environmental factors affect flowering time and pollen, fruit and seed production in natural populations. Knowledge of the genes and mechanisms regulating flowering would provide an inroad to discover the nature of adaptive variation in flowering traits that could ultimately improve our ability to maintain healthy populations.

This chapter provides an overview of the biology of *Populus* flowering induction and organogenesis, including our current knowledge of the genetic regulation of *Populus* flowering.

2 Initiation of Flowering

As sessile organisms, plant reproductive success relies on mechanisms that enable plants to respond to both endogenous and environmental cues that facilitate flowering at an appropriate time in their development and at a favorable seasonal time. For a postembryonic plant to flower, it must pass through at least two developmental phase transitions (reviewed in Poethig, 2003; Baurle and Dean, 2006). Vegetative phase change, commonly referred to as maturation in trees, is the transition from the juvenile to the adult growth phase in which a plant becomes competent to flower. Changes in vegetative morphology and physiology that occur during maturation vary between species and may be prominent or subtle and gradual or discrete. When a plant has reached the adult vegetative phase, it is capable of responding to floral inductive signals and transitioning to reproductive development. Studies in Arabidopsis, maize and other plants indicate that juvenile-to-adult and floral transitions are largely independently regulated but are coordinated and share

some common regulatory elements (Willmann and Poethig, 2005; Baurle and Dean, 2006). Gibberellic acid (GA), vernalization and some genes appear to regulate both transitions, but many genes affect one but not the other. However, the relationships between vegetative phase change and flowering in *Populus* are largely unknown. In annuals, biennials, and many perennials, both vegetative phase change and the floral transition occur within a single growing season or by the second growing season (e.g., biennials require the vernalization treatment winter provides to flower). In contrast, *Populus* does not reach maturity for several years and then floral initiation occurs at a particular seasonal time in adult trees. Although an adult tree is defined as reproductively competent, it is unknown whether an adult *Populus* is continuously capable of responding to floral inductive signals. In other words, the reproductive competency of meristems may be reset seasonally in adult trees and mechanisms related to juvenility may act locally to maintain some meristems as vegetative (Battey and Tooke, 2002).

2.1 Juvenility/Maturity

For perennial plants there generally appears to be a positive correlation between length of the juvenile period and lifespan (Harper and White, 1974). Age and size tend to be strongly correlated during the juvenile phase, and size rather than age appears to be the best predictor of flowering onset in many biennials and perennials (Lacey, 1986). Size appears to be the best predictor of flowering in *Populus*, though this has not been subject to robust quantitative study. North American cottonwoods typically reach reproductive maturity at 5–10 years at heights of 8–15 m (Braatne et al., 1996). Elite cottonwood hybrids grown in plantations reach these sizes at a younger age, and flowering at age four is common and sometimes has been observed as early as the second growing season. In some cottonwoods, branch segments from mature trees root easily and then newly formed lateral organs have juvenile characteristics (Brunner et al., 2004).

Because of the polar nature of shoot growth, within-plant maturation gradients are often evident. Kearsley and Whitham (1997) studied within tree developmental changes in mature narrow leaf cottonwoods (*P. angustifolia*) that had branches distributed vertically throughout the bole. In addition to the ratio of reproductive buds to vegetative buds, they measured relative blade width (width/length) and relative petiole length (petiole length/blade length). Changes in all these traits were significantly correlated with a distance measure that incorporated vertical position of a branch on the trunk and position out on a branch. As distance from the root crown increased, leaves became wider, petioles longer, and more reproductive buds were produced. Unfortunately, not all *Populus* species and hybrids display such striking changes in leaf size/shape and maturation in vegetative characteristics, and how vegetative traits change in relation to reproduction has not been adequately studied in *Populus*. This is especially needed for aspen species and hybrids that are typically used for transgenic studies. In Arabidopsis and maize, leaf traits, such as the presence/location of trichomes distinguish the juvenile and adult phase and enable the identification of mutants that affect the juvenile-to-adult vegetative transition

but not flowering time, flowering but not vegetative phase change or both transitions. Without easily measured, unambiguous markers for vegetative phase change, we are limited to identifying genes that affect reproductive competency in *Populus*.

Temporal, spatial or both spatial and temporal signals could measure developmental time. The correlation with distance from the roots suggests the possibility of a spatial signal in *Populus*. However, studies in Arabidopsis and maize have led to models for temporal regulation of vegetative phase change (e.g., Poethig, 2003; Hunter et al., 2006). In Arabidopsis and maize, RNA silencing pathways have a key role in regulating vegetative phase change, with some components of these pathways also affecting flowering time (reviewed in Chuck et al., 2009; Willmann and Poethig, 2007). Mutations in several genes involved in biogenesis of miRNAs or siRNAs affect phase change, and specific small RNAs and their gene targets have been shown to regulate developmental transitions. In Arabidopsis, mutations disrupting the repression of the *AUXIN RESPONSE FACTOR (ARF)* family members, *ETTIN* and *ARF4* by the trans-acting (ta) siRNA, *tasiR-ARF* accelerate the juvenile-to-adult transition, but do not affect the length of the transition zone or the total number of leaves produced by the shoot (Hunter et al., 2006). Moreover, levels of *tasi-ARF* and its targets do not change during vegetative development, suggesting that *tasi-ARF* establishes the threshold at which leaf primordia respond to a temporal signal.

In both Arabidopsis and maize, the temporal signal appears to involve MIR156 and MIR172, that have reciprocal temporal expression patterns and opposite effects. MIR156 targets members of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPB)* family of transcription factors and constitutive expression prolongs juvenile vegetative traits and delays flowering (Wu and Poethig, 2006; Chuck et al., 2007). In contrast, MIR172 targets *APETALA2* (*AP2*) transcription factors and promotes transition to the adult vegetative phase in maize (Lauter et al., 2005) and the transition to flowering in Arabidopsis (Aukerman and Sakai, 2003). Taken together, these studies suggest that the relative amounts of these two miRNAs may have a key role in determining the precise timing of phase transitions. Intriguingly, both miRNAs are present in the phloem sap of *Brassica napus* (Buhtz et al., 2008), suggesting the possibility that they may regulate developmental transitions via long-distance signaling. Given the extensive regulatory conservation between a monocot and eudicot, it seems probable that small RNAs are involved in regulating maturation in *Populus*, but this remains to be determined. The proposed model for Arabidopsis that includes the setting of threshold to respond to a signal from the developmental clock would seem to offer evolutionary flexibility. For example, if the threshold-setting mechanism was spatially regulated (e.g., ability to respond to a temporal signal increased with distance from the root) in *Populus*, this could help establish the prolonged, seemingly size-related maturation in *Populus*.

Various environmental and mechanical treatments can accelerate the first onset of flowering in some tree species, but have generally not been successful in *Populus* (Meilan, 1997). A combination of the GA inhibitor paclobutrazol, root pruning, and water stress did induce floral bud formation on three month old rooted-cuttings from mature *P. deltoides* trees, but similar treatment was not successful in inducing flowering in one-year-old rooted cuttings from juvenile trees (Yuceer et al., 2003b).

Epigenetic mechanisms have long been hypothesized to regulate tree phases and in some tree species, rooted cuttings from mature trees retain mature traits (Greenwood and Hutchinson, 1993; Hackett and Murray, 1993). Although rooted cuttings from mature *Populus* trees generally appear to revert to juvenility, that they were responsive to treatments that juvenile cuttings were not (Yuceer et al., 2003b), suggests that full reversion does not occur immediately. A genotype of *P. alba* that flowered in its first growing season was discovered in a common garden study and hardwood cuttings taken from this clone flowered in the greenhouse (Meilan et al., 2004). However, subsequent in vitro propagules did not flower under greenhouse conditions, but a combination of root chilling, followed by short-day (SD) treatment to induce bud set, and chilling to release dormancy did induce flowering. These studies suggest that the acquisition of reproductive competency may be composed of a number of stages that could occur over years as well as within each seasonal cycle.

Many flowering pathway genes when ectopically overexpressed induce early flowering in Arabidopsis and other annual plants, but only a few have been shown to promote an earlier onset of flowering in *Populus*. The *CONSTANS/FLOWERING LOCUS T (CO/FT)* module is central in the photoperiodic promotion pathway in Arabidopsis, and *FT* also integrates signaling from the vernalization and autonomous pathways (reviewed in Turck et al., 2008; Michaels, 2009; Kobayashi and Weigel, 2007). *CO* encodes a B-box-type zinc finger domain protein and *FT* belongs to the phosphatidylethanolamine-binding protein (PEBP)/ Raf Kinase Inhibitor Protein (RKIP) superfamily. *CO* and *FT* are expressed in leaves and the FT protein is translocated to the shoot meristem where it promotes floral induction. Whereas overexpression of *CO* did not induce early flowering in *Populus* (Strauss et al., 2004), overexpression of the *Populus FT* orthologs *FT1* and *FT2* induced flowering within several months following transformation (Bohlenius et al., 2006; Hsu et al., 2006). *FT1* was particularly effective – some transgenic events developed flowers while still in tissue culture and larger, potted plants developed catkins similar to those on adult wild-type trees.

Expression levels of *FT1* and *FT2* around the seasonal time of floral initiation were lower in juvenile trees and gradually increased as trees matured to the adult phase, suggesting that release of *FT* repression may be involved in the juvenile to adult transition. In Arabidopsis, the MADS-box gene *FLOWERING LOCUS C* (*FLC*) represses *FT*, and vernalization releases *FLC*-mediated repression by inducing the repression of *FLC* by histone modification (reviewed in Dennis and Peacock, 2007; Amasino, 2005). *Populus* contains several *FLC* homologs (De Bodt et al., 2006), but whether they have a role in maturation is unknown, and it is also possible that they regulate the seasonal time of flower initiation in adult trees (discussed below). In addition to being transcriptionally regulated in a temporal manner, *MIR172* abundance is regulated by photoperiod via miRNA processing, and *MIR172* upregulates *FT* via a pathway independent of *CO* (Jung et al., 2007). This dual regulation could potentially provide a mechanism whereby the same factor could contribute to the control of tree maturation that occurs over years, as well as the seasonal regulation of flowering time in adult trees.

In the shoot meristem, FT interacts with the bZIP transcription factor, FLOWERING LOCUS D (FD), and the FT-FD complex is able to activate floral meristem identity genes, including the MADS-box genes *APETALA1* (*AP1*) and *FRUITFULL* (*FUL*) (Abe et al., 2005; Wigge et al., 2005). *FD* is expressed at the shoot apex before floral induction, and consistent with this expression pattern, overexpression of *FD* induces a more modest acceleration of flowering compared to *FT* overexpression (Wigge et al., 2005). However, overexpression of a *Populus FD* ortholog *PtFD1* induced extremely early flowering in *Populus* when plants were grown under LD photoperiods, but not in SD (G. Coleman, personal communication). Overexpression of *AP1* or a *Populus AP1* ortholog *PTAP1-1* does not induce early flowering in *Populus* (Strauss et al., 2004 and unpublished). Constitutive expression of the floral meristem identity gene *LEAFY* (*LFY*) does induce solitary flowers in male aspen clones and infrequently in female clones (Weigel and Nilsson, 1995; Rottmann et al., 2000). *Populus LFY* (*PTLF*) was less effective at inducing early flowering, suggesting that the orthologous proteins may differ somewhat in their activity or regulatory interactions.

All of the above studies in *Populus* have involved ectopic overexpression of floral promoters and have often been limited to greenhouse studies. In Arabidopsis, different floral promoters and repressors affect flowering time to varying degrees. Thus, more modest affects on onset of flowering may be missed if *Populus* are only studied in the greenhouse where plant size is limited. Functional study of a *Populus* homolog of Arabidopsis *TERMINAL FLOWER1(TFL1)*/snapdragon *CENTRORADIALIS (CEN)* demonstrate the value of *Populus* field studies. Loss-of-function mutations in *TFL1* induces early-flowering, but *CEN* does not affect flowering time (Shannon and Meeks-Wagner, 1991; Bradley et al., 1996, 1997). *PopCEN1*-RNAi transgenics showing strong downregulation did not show the extreme early-flowering of *PtFT* or *PtFD* overexpressors. However, a multi-year field study revealed that these RNAi transgenics transitioned to flowering during their second growing season, whereas most non-transgenic controls transitioned to flowering during their fourth growing season (Mohamed, 2006 and unpublished). In contrast to *PtFT1/2, PopCEN1* expression does not appear to differ much between juvenile and adult trees. *TFL1/CEN* and *FT* encode related proteins that can interact with the same proteins in yeast-two-hybrid studies (Pnueli et al., 2001; Abe et al., 2005; Wigge et al., 2005), leading to the hypothesis that they antagonistically regulate flowering by competing for common interacting partners (Ahn et al., 2006). Thus, one possibility is that downregulation of *PopCEN1* moderately accelerated flowering onset by reducing the amount of PtFT1/2 needed to outcompete PopCEN1.

2.2 Transition to Flowering

In monopodial annual plants such as Arabidopsis, the entire plant transitions to the reproductive phase. The vegetative shoot meristem transitions to an indeterminate inflorescence meristem that gives rise to floral meristems on its flanks. In an adult

Populus, the floral transition is integrated with the seasonal cycle of growth and dormancy. The terminal meristem of a shoot always remains a vegetative meristem. Vegetative buds contain a number of preformed leaves. Axillary vegetative meristems can form in the axils of earliest preformed leaves prior to a bud undergoing winter dormancy, and following spring bud flush, inflorescence or vegetative meristems can form in the axils of preformed leaves (Boes and Strauss, 1994; Yuceer et al., 2003a). Some shoots will also produce neoformed leaves, and buds formed in these axils are vegetative. Thus, there is a seasonal window in which inflorescences initiate. Within the bud scales, the inflorescence meristem rapidly elongates, forming lateral bracts acropetally and then in the axil of each bract a floral meristem (Fig. 1). Exactly when floral induction occurs in *Populus* and what environmental signals regulate this process are unknown. The chilling requirement to release dormancy is similar to vernalization, and the increasing photoperiods and/or increasing

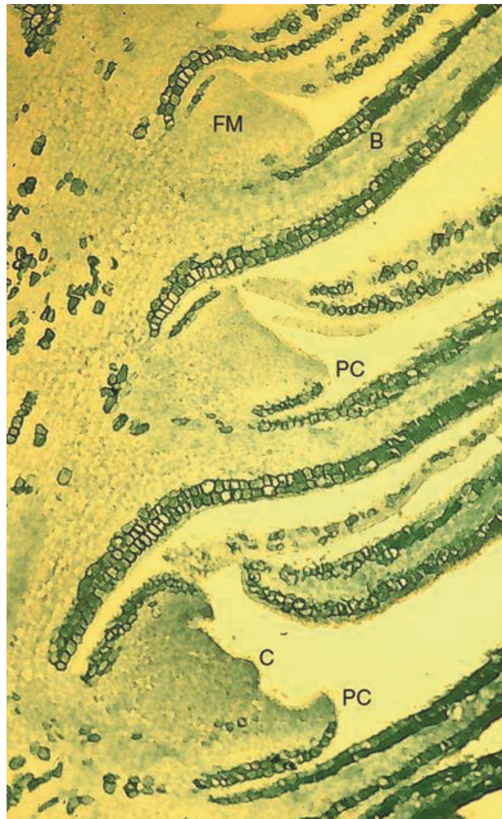

Fig. 1 Floral meristems in a developing female *P. trichocarpa* inflorescence. Longitudinal section of inflorescence bud collected in May showing three floral meristems (FM). The floral meristems display an acropetal developmental gradient of floral organ initiation. Bract, B; carpel primordium, C; perianth cup, PC

ambient temperature during spring could also play a role. Is there an endogenous developmental clock that monitors seasonal shoot growth to regulate flowering time? In Arabidopsis, the floral signal is translocated to the shoot apical meristem that converts to an inflorescence meristem, but in *Populus*, the shoot apex remains vegetative and axillary meristems transition to inflorescence meristems. Thus, is the signal for flowering translocated to the terminal shoot meristem or to axillary meristems?

Deciphering the environmental cues important for *Populus* flowering is inherently difficult because the size of adult trees prevents moving them to various controlled environments. A number of *Populus* homologs of Arabidopsis flowering time genes have distinct seasonal expression patterns, and *PtFT1* and other genes in the photoperiodic flowering pathway change expression under SD-induced budset (Bohlenius et al., 2006; Ruttink et al., 2007). Thus, another key question is how these genes and regulatory modules are able to control different developmental processes at different times. *PtFLC2* is expressed in shoot apices during LD and the early stages of SD-initiated bud development, but then declines during SD and low temperature treatment (G. Coleman, pers. comm.). In Arabidopsis, vernalization induces chromatin modifications that stably repress *FLC* transcription and all apical meristems produce flowers until the plant senesces. In *Arabis alpina*, a perennial close relative of Arabidopsis, apical meristems formed before vernalization produce flower buds during vernalization (Wang et al., 2009). In contrast, apical meristems not present or at a very early stage of development at the onset of cold treatment did not produce flowers and grew vegetatively until plants received another vernalization treatment. The *Arabis aplina FLC* ortholog *PERPETUAL FLOWERING1 (PEP1)* is only transiently repressed by cold treatment and this correlated with changes in histone modification. Thus, it is tempting to speculate that *PtFLC2* has a similar role in regulating the seasonal cycling between reproductive and vegetative phases in adult *Populus* trees.

In addition to the increased leaf expression in adult vs. juvenile trees, *FT2* showed a striking winter to summer expression pattern (Hsu et al., 2006). *FT2* expression was very low into April, but it was very strongly expressed in mid-May, and then slowly declined during the summer. *PopCEN1* also shows a striking seasonal expression pattern in shoot tips with the strongest expression around the time of bud flush (Mohamed, 2006) and also increases during the chilling treatment phase of a controlled environmental chamber dormancy cycle (unpublished). Although *PopCEN1* downregulation had a modest affect on the first onset of flowering (described above), it had a more dramatic effect on the intensity of flowering with a very large number of the lateral meristems becoming inflorescence shoots (unpublished). However, terminal meristems remained vegetative in *PopCEN1*-RNAi transgenics, suggesting that other factors maintain terminal vegetative meristems. Much remains to be determined, but taken together, these studies suggest the possibility that vernalization-induced transient repression of *PtFLC* may allow *PtFT1/2* and other genes to promote flowering at a specific seasonal time and that *PopCEN1* acts to maintain some lateral meristems as vegetative.

3 Flower Development and Sex Determination

Populus inflorescences are simple racemes (catkins) with each flower being subtended by a bract. *Populus* flowers are unusual because they are unisexual and reduced with only two whorls of floral organs: the perianth cup (floral disc) and stamens or pistil. Dioecy has evolved independently numerous times and is estimated to occur in less than 4% of the angiosperms (Ainsworth et al., 1998). In the majority of cases, both carpels and stamens are initiated and then development of female or male organs is subsequently blocked. Both detailed light and electron microscopy show that in *Populus*, only organs of a single sex are initiated. However, numerous reports over the years demonstrate that *Populus* retains the capacity to produce bisexual flowers and flowers of the alternate gender (e.g., Stettler, 1971; Slavov et al., 2009). Though the causes are unknown, these atypical flowers may occur rarely in some inflorescences, through-out some of the catkins or even throughout all catkins on the tree, and may reoccur year to year or only in particular years.

Populus flowers arise in acropetal order within the developing inflorescence bud and organ differentiation continues into fall. As bract primordia enlarge, cells in the axil form into a flattened disc-shaped structure (Boes and Strauss, 1994; Kaul, 1995; Yuceer et al., 2003a). Continued growth at the perimeter produces a raised ring of tissue that will develop into the perianth cup (Fig. 1). In females, 2–4 carpel primordia arise in the center and unite during summer to form a unilocular ovary and stigma primordia are evident in autumn (Fig. 2a, b). In the following spring, archesporial cells grow and undergo megasporogenesis and produce the embryo sac and stigma development completes (Fig. 3a, c). In males, stamen primordia first arise in the center of the meristem, and organogenesis proceeds centrifugally with stamen primordia differentiating anthers and filaments during the summer (Fig. 2c, d). In spring, the microspore mother cells undergo meiosis to form tetrads of microspores that then mature into individual pollen grains. The mature male flower consists of 5–60 stamens surrounded by a perianth cup and flowers begin to shed pollen before catkins finish elongating (Fig. 3b, d). As an adaptation to wind pollination, inflorescence buds flush before vegetative buds and rapidly elongate into pendulous catkins. Similar to vegetative bud flush, there is extensive natural variation in inflorescence phenology. Flower number per catkin varies; in a survey of a female hybrid aspen clone, flower number ranged from 75 to 147 (unpublished).

Studies of floral development in Arabidopsis, snapdragon and various other annual plants indicate broad conservation of the genes and mechanisms; however, some aspects of gene function and regulatory interaction do vary between species (reviewed in Krizek and Fletcher, 2005; Blazquez et al., 2006; Sablowski, 2007). *LFY* and *AP1* have partially overlapping roles in specifying floral meristem identity and *TFL1* acts in opposition to *LFY* and *AP1* to specify inflorescence meristem fate. The floral meristem identity genes activate an additional set of genes that specify floral organ fate. These genes were initially identified in snapdragon and Arabidopsis based on their mutant phenotypes that were characterized by homeotic transformation of one floral organ type into another. These studies led to the ABC model of

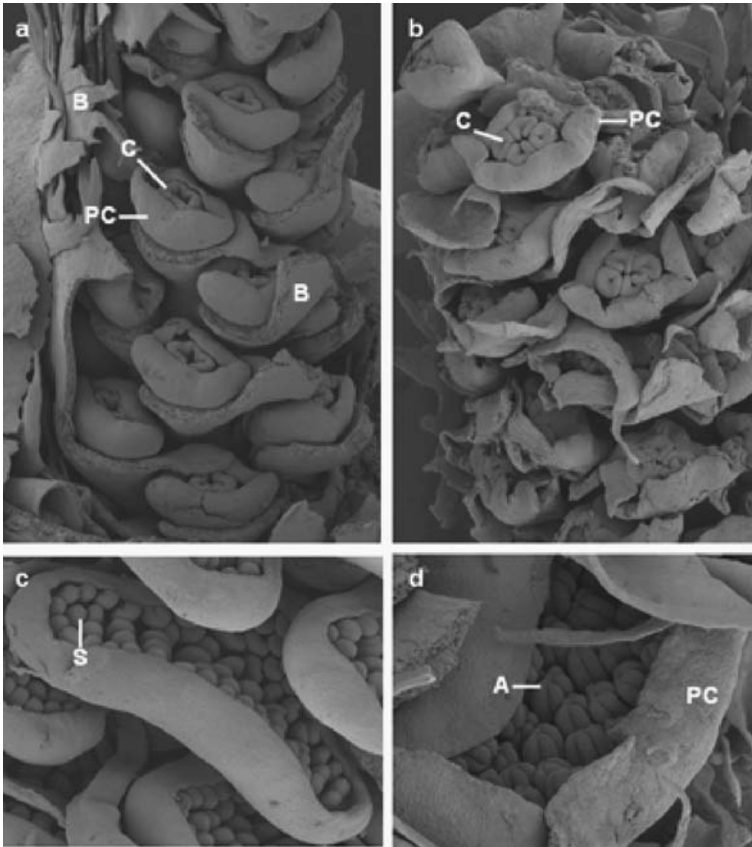

Fig. 2 *P. deltoides* female and male inflorescence development during summer and fall. Scanning electron micrographs of developing female inflorescences in (**a**) August and (**b**) October. Developing male flowers in (**c**) August and (**d**) October. Most of the bracts have been removed to reveal the developing flowers. Bract, B; fused carpels, C; perianth cup, PC; stamen primordium, S; tetrasporangiate anther, A. Photos provided by C. Yuceer

floral organ identity that describes how three regulatory functions, A, B and C, work in a combinatorial manner to specify organ identity in each whorl of the floral meristem (Coen and Meyerowitz, 1991). A function specifies sepal identity in whorl one, A plus B function confers petal identity in whorl two, B and C function specify stamen identity in whorl three and C function specifies carpel identity in whorl four and meristem determinacy.

Subsequent studies added E class genes that are required to specify petal, stamen and carpel identity (Pelaz et al., 2000). In *Arabidopsis* the A-function is defined by *AP1* and *APETALA2* (*AP2*), the B-function by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and the C-function by *AGAMOUS* (*AG*). *SEPALLATA* (*SEP*) genes act redundantly to provide E function. In the *sep1 sep2 sep3* triple mutant second and third

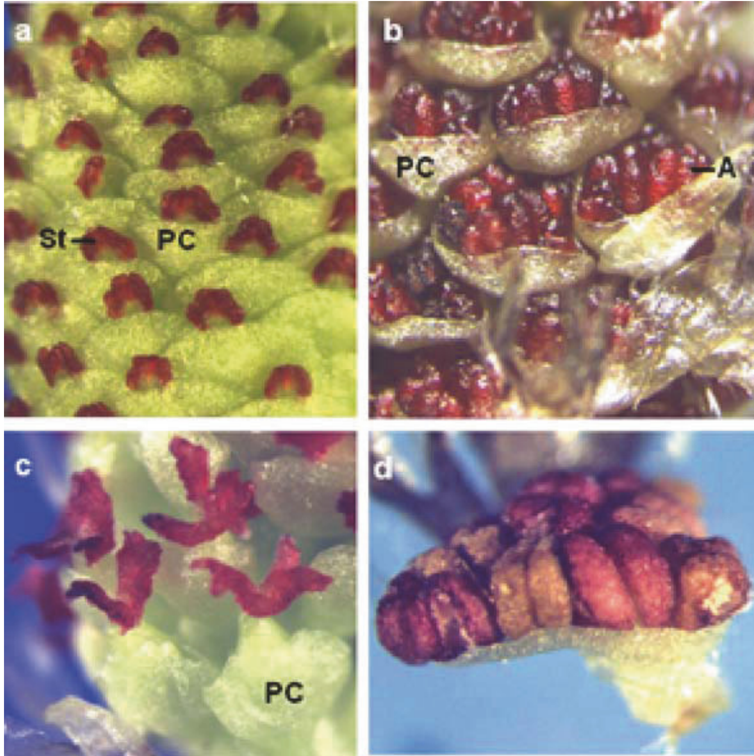

Fig. 3 Hybrid aspen flowers in March around the time of inflorescence bud flush. (**a**) and (**c**) show female flowers approximately two weeks apart. Male flowers are shown in (**b**) and (**d**). The isolated flower in (**d**) is shedding pollen. Perianth cup, PC; stigma, St: A, anther

whorl organs were converted into sepals and a new inflorescence developed from the center of the floral meristem. Mutation of a fourth *SEP* gene (*SEP4*) in a *sep1 sep2 sep3* background resulted in the production of only leaves revealing that *SEP4* functions in sepal development (Ditta et al., 2004). With the exception of *AP2*, all of the above floral homeotic genes belong to the MADS-box family.

Gene expression patterns of a number of *Populus* homologs of Arabidopsis flower development genes are consistent with a function in specifying floral meristem and reproductive organ identities. Both *PTFL* and the *Populus AP1* co-orthologs, *PTAP1-1* and *PTAP1-2*, are expressed throughout the initiating floral meristems (Rottmann et al., 2000 and unpublished). *Populus SEP1/2/3* homologs are expressed at all stages of female and male flower development (Cseke et al., 2005). *PTD*, a *Populus AP3* homolog, is initially expressed in the inner whorl of both female and male floral meristems (Sheppard et al., 2000). As reproductive primordia begin to form, *PTD* expression is maintained in stamen primordia, but excluded from carpel primordia, which is consistent with a role in specifying stamen, but not carpel identity. The *Populus AG* co-orthologs, *PTAG1* and *PTAG2*,

are expressed in the inner whorl of female and male flowers both before and after reproductive primordia emerge, indicating a conserved function in specifying both female and male reproductive identity (Brunner et al., 2000). Interestingly, *PTD*, *PTAP1-1* and *PTAP1-2* are not expressed in the initiating perianth cup, suggesting they do not specify its identity. Developmental morphology and vascular traces suggest that the perianth cup resulted from the fusion of perianth parts (e.g., Kaul, 1995), but it remains uncertain whether it is derived from sepals, petals or both. Several additional homologs of A and B-class genes as well a *SEP4* homolog are present in the *Populus* genome (De Bodt et al., 2006); thus, it is possible that one or more of these genes specify fate of the outer whorl in *Populus* flowers.

Because of the long juvenile period, there have been few functional studies of genes in *Populus* flower development. Similar to *TFL1* and *CEN*, *PopCEN1* and/or its paralog may act to maintain the indeterminacy of the inflorescence meristem (A. Brunner, unpublished data). In *PopCEN1-RNAi* transgenics, inflorescences were wild-type in appearance except that they were significantly shorter with fewer flowers than wild-type, raising the possibility that the inflorescence meristem may have converted to a terminal flower. Long-term field study of male and female aspen clones containing a *PTLF*-antisense transgene revealed striking effects on male floral development, but no effects were observed in the female clone (A. Brunner, unpublished data). Male transgenics displayed a range of altered floral phenotypes, from bisexual flowers to female flowers to a proliferation of carpelloid structures. These suggest that *PTFL* is necessary for male reproductive development, but is less important for female flower development. Co-overexpression of *LFY* and *PTAG1* in a male *Populus* induced bisexual flowers with three whorls (perianth cup, stamens and pistil), suggesting that the spatiotemporal expression patterns and/or relative expression levels of these genes affect reproductive whorl number and identity (unpublished). In some respects, these results are not surprising. In Arabidopsis, *lfy* mutants eventually produce carpelloid flowers, and any of the four floral organ types in any of the four whorls can be produced by manipulating the expression of the ABCE genes (reviewed in Krizek and Fletcher, 2005; Blazquez et al., 2006).

The *Populus* transgenic results paired with the observation that sex determination occurs before reproductive primordia are initiated raise the possibility that sex determination may involve differential regulation of floral homeotic genes. Recent studies suggest that *Populus* is in the early stages of evolving a sex chromosome. In three different *Populus* pedigrees, a sex determination locus has been mapped to the peritelomeric region of chromosome XIX (Gaudet et al., 2007; Markussen et al., 2007; Yin et al., 2008). Moreover, this chromosomal region shows suppressed recombination and high divergence between the alternate haplotypes for the maternal parent in the *P. deltoides* × *P. nigra* pedigree. This suppressed recombination would cause all genes within this region to behave as one locus; thus, the gender locus may include several gender determination genes (Yin et al., 2008). The progeny of this cross showed a strongly male-biased sex ratio, in accordance with Haldane's rule that postulates that the heterogametic sex is more likely to be absent,

rare, or sterile in interspecific crosses (Coyne and Orr, 2004). One approach to select candidate gender determination genes for further study would be to identify which genes in the XIX interval are differentially expressed in initiating female vs. male floral meristems. Furthermore, as recent studies in maize have revealed a role for miRNAs in sex determination (reviewed in Chuck et al., 2009), candidates should not be limited to genes coding for proteins.

4 Perspectives

The *Populus* genome sequence, associated genomics resources, and comparative genomics of flowering are helping advance our understanding of the regulation of flowering in *Populus*. However, studies of gene function in *Populus* maturation and reproductive development remain difficult. Long-term field study as well as studying flowering related *Populus* transgenics under extensive sets of environmental treatments are needed, but factors such as cost and governmental regulations will likely keep the number of such studies low. Thus, we need to be judicious in our choice of genes for functional study in *Populus* flowering. Various types of *Populus* 'omics resources and studies, particularly in combination, could help answer the questions of how *Populus* maturation and flowering are regulated. These include transcriptome studies at a fine scale with respect to time and location within tissues, and generating a *Populus* flowering ORFeome that would then allow protein-protein interaction networks to be mapped. In addition, careful studies are needed of maturation in geneotypes used for transgenic studies. In addition, association mapping studies should be used to identify unambiguous markers for vegetative trait maturation and establish how these change in relation to reproductive competency. Establishing these markers for Arabidopsis and maize was key to uncovering genes that regulate vegetative phase change. Similar genes and mechanisms that extensively involve small RNAs may also control the maturation that occurs over years in *Populus*. Despite the challenges, this is an exciting time to study maturation in *Populus*. Study of woody plants led the way in developing concepts of developmental phase change (Greenwood and Hutchinson, 1993; Hackett and Murray, 1993), and continued development of *Populus* omics resources and tools could lead to renewed focus on woody plants.

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Perennial Life Style of *Populus*: Dormancy Cycling and Overwintering

Päivi L.H. Rinne, Annikki Welling, and Chris van der Schoot

Abstract Deciduous trees in boreal and temperate areas are strictly conditioned by the environment, especially by photoperiod and temperature. However, it is in particular the successful submission to these conditions that has rewarded them with long-life spans. A crucial strategy to ensure growth over many seasons is to timely assume dormancy and a level of hardiness that permits survival through winter. A consensus is emerging that dormancy, although traditionally regarded as a systemic feature, is a property of the shoot apical meristem (SAM). This chapter discusses our current understanding of the regulatory mechanisms that drive the annual cycles of dormancy and acclimation.

1 Defining Perenniality

1.1 Seasonal Growth

Perennial plants are uniquely equipped with mechanisms that allow vigorous growth in the summer while preventing proliferation during the less favourable seasons. This cycle of growth and inactivity is synchronized with the seasons by sensory systems that detect alterations in environmental cues. Tree species in temperate and boreal zones rely on a declining photoperiod (SD) for the timely cessation of elongation growth and the acquisition of a dormant and freezing-tolerant state (Weiser, 1970; Welling, 2003; Welling et al., 1997, 2002). Photoperiod is not only a reliable timing signal, eliciting growth cessation at the same time every year, but it is also versatile in providing increasingly shorter critical daylengths towards the south. This permits clinal regulation of growth cessation in tree species with a wide

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distribution range. For example, southern aspen (51°N) responds with cessation of elongation growth to a photoperiod of 15 h or less, whereas for northern populations (63°N) this critical photoperiod is in excess of 20 h (Böhlenius et al., 2006). The photoperiod-specificity is heritable (Howe et al., 1995, 1996), securing an efficient use of the growth season and avoidance of potential frost damage. This would be difficult to attain when seasonal growth would be regulated by temperature alone (Howell and Weiser, 1970). Despite the predictability of photoperiodic decline, the timing of growth cessation shows some variability due to the modulating effects of other environmental conditions (Arora et al., 2003).

While photoperiod is the major factor in the initiation of dormancy and acclimation in poplar, chilling is required for the deepening of freezing tolerance, and for the release from dormancy (e.g. Weiser, 1970; Welling and Palva, 2006). In contrast, elevated temperatures are crucial for deacclimation and bud break (Sauter et al., 1996; Welling et al., 2004; Linkosalo et al., 2006). This synchronization of activities with the seasons has a high adaptive value and determines the success of perennial plant life. It is feasible that climate change might have a deregulating effect on this seasonal growth pattern through the uncoupling of temperature traits and photoperiod. Therefore continued research efforts are needed to understand at multiple levels how the environment modulates the cycles of growth and dormancy.

1.2 Meristems

Growth over many seasons gives rise to complex shoot systems with architectures that emerge from the organised activity of various types of meristems, all of which, directly or indirectly, derive from the shoot apical meristem (SAM). The SAM is situated at the shoot tip and is not only the ultimate source of the shoot system, but also contributes in varying degrees to its branching pattern by preventing activation of axillary buds lower along the stem. In addition, the SAM is a major player in dormancy cycling, during which its functional organisation undergoes dramatic but reversible alterations (Section 2.4).

The SAM of *Populus* and other dicotyledons has three sets of superimposed initials (stem cells) whose directional divisions give rise to a superficial two-layered tunica and a subjacent corpus (Newman, 1965; Steeves and Sussex, 1989). Within each layer the individual stem cells form a clonal sector consisting of a branched lineage tree that unites all descendents symplasmically to the stem cell (van der Schoot and Rinne, 1999). Sideways lineage branches and clonal sectors are united by formation of secondary plasmodesmata (PD) (van der Schoot and Rinne, 1999). The fate of SAM cells is not strictly determined by lineage but depends on their position in the SAM, as cells that deviate from the lineage path become reprogrammed (Szymkowiak and Sussex, 1996). In other words, the behaviour of individual cells is supervised by the integrated signal network of the SAM (van der Schoot, 1996; Rinne and van der Schoot, 1998). As surgically isolated SAMs can continue shoot formation (Smith and Murashige, 1970; Ball, 1980), the SAM

is also an autonomously organised unit. Thus, for SAM organization iterative communication between its cells is both necessary and sufficient (van der Schoot, 1996; Rinne and van der Schoot, 1998). For communication SAM cells utilize the cell wall space as well as PD. In recent years the importance of signalling across the extracellular divide, involving excreted ligand peptides and membrane receptor kinases, has been extensively documented (Gray et al., 2008; Chae et al., 2009). In particular, the *CLAVATA1-3* (*CLV1-3*)-*WUSCHEL* (*WUS*) system has emerged as the central regulatory loop in the SAM (Schoof et al., 2000; Tucker and Laux, 2007). Equally important, but more elusive, is signalling within the symplasmic continuum of the SAM. This continuum is functionally subdivided into symplasmic fields (SFs) that are regulated via positional closing of PD at boundaries within the SAM (Rinne and van der Schoot, 1998; Ruonala et al., 2008). Within SFs cells exchange metabolites and morphogens by diffusion (van der Schoot and Rinne, 1999). Gating of PD (Epel, 1994; McLean et al., 1997) enables the exchange of morphogens between adjacent SF (van der Schoot and Rinne, 1999), including transcription factors which might move selectively within the SAM (Carpenter and Coen, 1995; Hantke et al., 1995; Furner et al., 1996; Perbal et al., 1996; Sessions et al., 2000; Kim et al., 2003). These two signalling systems are complementary, and understanding how they work together to maintain the organization and function of the SAM is one of the biggest challenges in SAM research.

Subjacent to the SAM is the rib meristem (RM), a small meristematic area that gives rise to the inner stem (Bernier et al., 1981). Originally the SAM and the RM were recognized as morphogenetic units with different tasks (Sachs et al., 1960; Esau, 1977; Bernier et al., 1981), but presently this distinction seems often overlooked. However, the fact that SAM and RM function can be uncoupled, most visibly in rosette plants, but also in caulescent plants (Rinne et al., 2005; Ruonala et al., 2008), suggests that they are distinct in nature. This functional identity is supported by the presence of a symplasmic "barrier" between them (Ruonala et al., 2008), in which PD might function as gatable corridors for the selective entry of phloem-delivered signals from the RM into the SAM.

In poplars and other perennial dicotyledons the increase in girth of stem and branches results from the activity of the vascular cambium. Although seemingly simpler than the SAM, the cambial zone has a complexity of its own. It is composed of two types of stem cells, fusiform and ray initials, each of which in an alternating fashion produces files of derivatives that make up the axial and radial tissues of the xylem and the phloem (Esau, 1977). Stem cells of the same type might be symplasmically united (van der Schoot and van Bel, 1990). Cyto-morphologically cambium and SAM stem cells are remarkably different. Cambium cells are fusiform or brick shaped and highly vacuolated (Esau, 1977), whereas typical meristem cells possess dense cytoplasm, minute pro-vacuoles, and a nearly isodiametric shape. On the other hand, at the molecular level the cambium shows similarities with the SAM in terms of aspects of regulation, and both express *ARBORKNOX1*, a class 1 *KNOX* homeobox gene that is required to keep meristem cells in an undifferentiated state (Groover et al., 2006). An interesting genetic difference, however, is that SAM stem cells typically express the ligand encoding *CLV3* gene (Schoof et al., 2000; Tucker

and Laux, 2007), whereas the cambium of aspen expresses a gene encoding the receptor kinase CLV1 (Schrader et al., 2004).

The SAM, as the central organizer of perennial growth, is the direct target of the leaf-based mechanisms that respond to environmental cues (Rinne and van der Schoot, 2003), while the cambium is a potential second target. Considering that meristems are crucial for survival through winter this seems an appropriate strategy. However, in spite of this central role, a mechanistic understanding of dormancy cycling as a cyclic alteration in the functional organisation of the SAM is still lacking. Nontheless, a consensus is emerging that this would require both the identification of leaf-to-apex signals (inputs) and the SAM intrinsic response mechanisms that bring about and consolidate the dormant state. In practise, the uncertainty about the actual state of investigated materials – whether or not it was truly dormant – has led to ambiguity in the data and confusion about how to define the dormancy state, as pointed out by Saure (1985). As this concern remains, we will first describe the various forms of growth arrest, identify the loci of dormancy, and define some unambiguous terms.

1.3 Dormancy Concept

The term dormancy (F. *dormir*, to sleep) in its general use covers a wide spectrum of phenomena in various plant structures including seeds, tubers and buds. It is used to indicate a "resting or quiescent condition with reduced metabolism" (Usher, 1965; Allaby, 1991; Lawrence, 2005), the "suspension of visible growth" (Samish, 1954) or the "suspension of visible growth in any plant structure containing a meristem" (Lang et al., 1987). These descriptions are general and phenological, and they do not imply any specific mechanism. Moreover, the constraints that may prevent meristems from functioning are so diverse that an abundance of terminology emerged. Early efforts to come to a "universal terminology" (Lang, 1987; Lang et al., 1987) distinguished *para*-, *eco*-, and *endo*-dormancy. Although readily adopted, this terminology also raises questions. The main problem is that it addresses contexts, thereby classifying inputs rather than states. This might reflect a lack of attention for the loci of dormancy (Section 1.4) and the nature of the emerging meristem states.

A generally adopted criterion is that dormancy persists in conditions that are growth promoting. As *eco*-dormancy is due to non-permissive growth conditions, and simply removed by reintroducing permissive conditions, it does not count as true dormancy. *Para*- and *endo*-dormancy may both be regarded as true dormancy with the argument that in both cases meristems are unable to initiate growth under growth permissive conditions (Rohde and Bhalerao, 2007). Nonetheless, *para*-dormant meristems display the capacity for growth when liberated from correlative inhibition or apical dominance, or from constraints imposed by adjacent tissues (Cline, 1991; Napoli et al., 1999; Sussex and Kerk, 2001; Leyser, 2005; Ongaro and Leyser, 2007). Early on, it was already recognized that *endo*-dormancy is unique. It

is the only state that is intrinsically (*endo*) dormant, being enforced by an endogenous mechanism (Samish, 1954; Lang, 1987; Lang et al., 1987; Dennis, 1994; Seeley, 1994). In the following we will refer to this state as dormancy and, following Dennis (1994), to *eco*-dormancy as quiescence and *para*-dormancy as correlative inhibition.

1.4 Dormancy Locus

Historically, dormancy was investigated phenologically in the context of horticultural and forestry practises (Lavender, 1991). The attention gradually shifted from phenology to whole plant physiology and the controlling role of hormones and hormone balances (reviewed in Lachaud, 1989; Dennis, 1994; Arora et al., 2003; Horvath et al., 2003; Tanino, 2004). Dormancy was habitually regarded as a systemic property, or as a property of various overwintering organs or tissue systems. Awareness has been gradually growing that dormancy is not a systemic response but rather a property of the growing point (Rees, 1981), of meristematic tissues (Seeley, 1994; Rohde and Bhalerao, 2007), or of the SAM (van der Schoot, 1996; Rinne et al., 2001). The argument for the latter is based on a dynamic systems perspective, which views the SAM as self-organizing its morphogenetic activity as well as its arrest in response to input signals (van der Schoot, 1996; Rinne and van der Schoot, 1998, 2003). This view, that dormancy is a particular state of the meristem rather than a process, is currently gaining wider recognition (Schrader et al., 2004; Druart et al., 2007; Ruttink et al., 2007; Ruonala et al., 2008), and its adoption implies that gene expression studies should discriminate between the sites of signal production (source leaves), signal transduction (phloem), and responses (RM, SAM, and cambium). As dormancy is based on the execution of an intrinsic response mechanism, it is this mechanism that defines dormancy and not the input signals that arrive at the SAM (van der Schoot, 1996).

A fundamental feature of dormancy cycling is the seasonal regulation of the cellular communication network in the SAM (Rinne et al., 2001). During the dormancy cycle the SAM passes through three sequential, environmentally triggered states of symplasmic cell communication – online, offline and standby (Fig. 1). A major challenge is to map the SAM-specific changes in gene expression, and to identify genes, hormones and other signalling components that give input to the SAM and elicit cyclic alterations in its functional organisation.

2 Photoperiod Induced Priming of Overwintering

2.1 Sensing by the Leaves

Populus trees, like many other perennials and annuals, keep track of seasonal progression by monitoring photoperiodic changes with sensory mechanisms in the

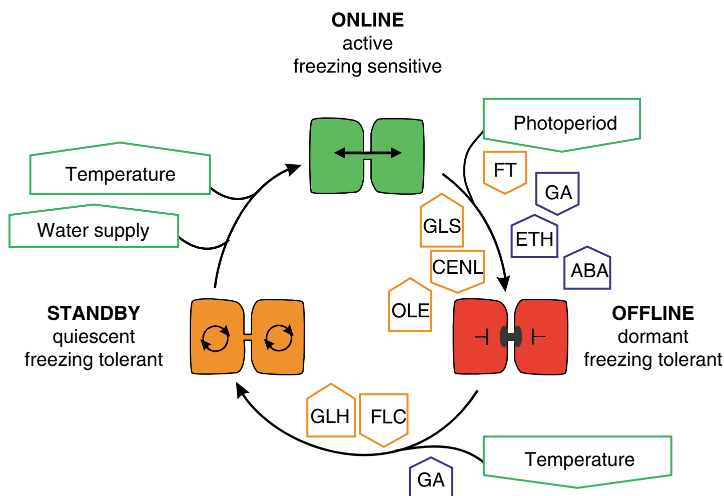

Fig. 1 Dormancy cycling in perennial trees like aspen. The SAM has repertoire of three distinct and intrinsic states of symplasmic communication – online, offline and standby – each of which has a unique responsiveness to specific environmental cues. Cellular and biochemical changes that drive or follow the cycling of the SAM are placed within the circle. Factors that indirectly influence the meristems are indicated outside the circle in an approximate temporal sequence. All environmental and endogenous factors are placed in an arrow-shaped frame indicating decrease or increase. The frames are *blue*, *orange* or *green*, representing hormonal signals, proteins/genes, and environmental factors, respectively. The proliferating and freezing sensitive SAM requires online communication via PD. At the offline dormant state cells are dehydrated, freezing-tolerant, and uncoupled by DSC and water-repellent cell walls. Chilling releases SAM dormancy by restoring the PD and the cell walls, while deepening freezing tolerance. This results in SAM that is in a standby or quiescent state. In spring, a rise in temperature and water availability enhances cellular physiology and kick-starts metabolic coupling and cell-cell signalling, resulting in SAM activation and bud burst. ABA, abscisic acid; CENL, CENTRORADIALIS-like; DSC, dormancy sphincter complexes; ETH, ethylene; FT, FLOWERING LOCUS T; FLC, FLOWERING LOCUS C; GA, gibberellin; GLH, glucan hydrolase (1,3-β-glucanase); GLS, glucan synthase; OLE, OLEOSIN; PD, plasmodesmata; SAM, shoot apical meristem; SD, short day. Adapted from Rinne et al. (2001)

leaves (Thomas and Vince-Prue, 1997). In recent years considerable progress has been made in elucidating the early signalling events that follow photoperiod sensing and eventually lead up to floral transition in Arabidopsis or transition to dormancy in poplar (Yanovsky and Kay, 2002; Böhlenius et al., 2006; Kobayashi and Weigel, 2007). Phytochromes (PHY) and other light-sensitive pigments in conjunction with physiological clocks regulate the expression of genes encoding systemic signals (Zeevaart, 1976; Bernier et al., 1993; Vince-Prue, 1994; Mouradov et al., 2002; Böhlenius et al., 2006) that target the apex.

Although end-of-season growth cessation in poplar and flowering in Arabidopsis seem very different processes, intriguing similarities exist in terms of developmental responses to SD. In *Populus*, short day (SD) induced bud set and dormancy establishment are preceded by cessation of elongation growth (Howe et al., 1995; Olsen et al., 1997; Welling et al., 2002; Böhlenius et al., 2006; Rohde and Bhalerao, 2007). The opposite process, the abrupt initiation of elongation growth during bolting is

exemplified by Arabidopsis, where LD induces the rapid emergence of an inflorescence stem in conjunction with floral transition (Koorneef et al., 1991; Yanovsky and Kay, 2002).

At the molecular level bolting requires the expression of the clock-regulated gene *CONSTANS* (*CO*) in the light to promote the expression of *FLOWERING LOCUS T* (*FT*) gene in companion cells (Kardailsky et al., 1999; Kobayashi et al., 1999; Yanovsky and Kay, 2002; An et al., 2004). FT protein is a mobile signal that moves via the phloem to the apex to induce flowering, in Arabidopsis and several other herbaceous plants (Corbesier et al., 2007; Lin et al., 2007; Tamaki et al., 2007) as well as in trees (Böhlenius et al., 2006; Hsu et al., 2006). *FT1* and *FT2* also play a role in the events leading up to the transition to dormancy in aspen (Fig. 2a) (Böhlenius et al., 2006; Ruonala et al. 2008). A crucial difference with flowering is that in the transition to dormancy transcript levels of *FT1/FT2* are rapidly and severely reduced instead of elevated. The downregulation of *FT1/FT2* occurs very early after the start of SD, about three and five weeks in advance of visible bud formation and dormancy establishment, respectively (Böhlenius et al., 2006; Hsu et al., 2006; Ruonala et al., 2008). It is unknown how the interruption of FT supply to the apex results in the cessation of elongation growth, and how it affects the downstream events of bud formation and dormancy establishment. The possibility

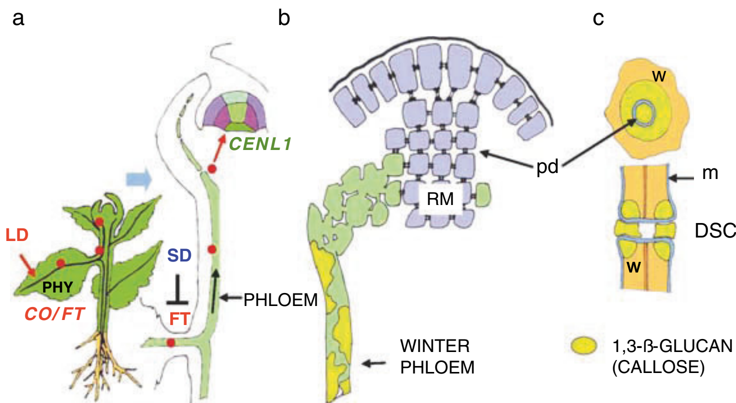

Fig. 2 Leaf-to-apex signalling during dormancy development in perennial trees like aspen. (**a**) During active growth long photoperiod facilitates the functioning of the *CO/FT* module, mediated by phytochromes and resulting in the movement of FT from leaf companion cells to the RM. The gene *CENL1* is expressed at the RM, where it is involved in regulating stem elongation. *FT* is almost instantaneously downregulated by SD, while *CENL1* is downregulated later concomitant with the cessation of elongation growth, which is followed by bud set and dormancy development. (**b**) Dormancy is arresting the SAM in a state of cellular uncoupling in which all supracellular activities are dissipated. After the storage sites in the stem are filled, winter phloem is developing. (**c**) Cellular uncoupling is enforced by installation of DSCs, which tightly seal of the ends of each plasmodesm (PD). Each DSC is composed of an extracellular ring and an intracellular plug of callose-enriched material. In the meanwhile cells dehydrate and cell walls become water repellent. *CENL*, *CENTRORADIALIS-like*; *CO, CONSTANS;* DSC, dormancy sphincter complexes; *FT, FLOWERING LOCUS T*; SAM, shoot apical meristem; SD, short day; RM, rib meristem. W, cell wall. Adapted from Rinne and van der Schoot (2003)

cannot be excluded that concomitantly a positive signal is generated in the leaves, and send out to the apex.

FT belongs to a group of proteins, including the Arabidopsis protein TERMINAL FLOWER (TFL1), that share motifs which function in ligand binding and signalling (Yeung et al., 1999; Banfield and Brady, 2000). In addition to *FT*, *TFL1* and its *Populus* ortholog *CENTRORADIALIS-LIKE1* (*CENL1*) play a role in the transition to flowering and dormancy, respectively. However, in contrast to FT both TFL1 and CENL1 are produced in the RM (Bradley et al., 1997; Ruonala et al., 2008). In flowering, the expression of *FT* and *TFL1* increase during floral induction. Although they have opposite effects on flowering time (Kobayashi et al., 1999; Koorneef et al., 1991; Shannon and Meeks-Wagner, 1991) they both promote the formation of an elongating inflorescence stem. *FT* is involved in the induction process whereas *TFL1* is implicated in enhancing elongation, as suggested elsewhere (Ruonala et al., 2008), because *tfl1* mutant have considerably shortened inflorescence stems (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1997) while overexpressors show enhanced inflorescence stem formation (Ratcliffe et al., 1998). In the transition to dormancy in aspen *CENL1* might have a similar function as *TFL1* in Arabidopsis (Ruonala et al., 2008; Section 2.2).

2.2 Events at the Apex

Signalling from leaves, whether negative or positive or both, affects the shoot system in complex ways. Global gene expression analysis of apical buds (Ruttink et al., 2007), cambium (Druart et al., 2007), and bark (Park et al., 2008) in *Populus* trees showed that under SD the expression of a large number of genes is altered. In buds major changes take place during the first three to four weeks of SD exposure, mostly concerning genes that function in metabolism and cell division, cold acclimation, and reserve accumulation (Ruttink et al., 2007). The expression analysis suggests that signalling pathways are operated in an orderly sequence, including light-, ethylene-, and abscisic acid (ABA)-signalling (Ruttink et al., 2007). Ethylene signalling precedes ABA signalling (Ruttink et al., 2007), and is probably necessary to time growth cessation and bud formation (Fig. 1) (Ruonala et al., 2006).

In buds, ABA levels peak in response to SD (Rinne et al., 1994a; Welling et al., 1997; Rinne et al., 1998; Rohde et al., 2002; Ruonala et al., 2006), and in cambium in response to SD and cold (Druart et al., 2007). Commonly, the peak in ABA corresponds with the upregulation of genes for ABA biosynthesis, ABA signal transduction, and ABA responsive transcription factors (Ruttink et al., 2007). In aspices, ABA levels do not correlate with growth cessation or dormancy development (Welling et al., 1997). However, sensitivity to ABA increases during SD and decreases during chilling (Barros and Neill, 1986; Rinne et al., 1998), indicating that changes in ABA sensitivity do play a role during dormancy development. In contrast, a correlation does exist between ABA levels and cold hardiness. ABA levels increase during acclimation, and the inability to regulate ABA levels impairs acclimation (Welling et al., 1997; Rinne et al., 1998; Welling et al., 2002).

Interestingly, *CENL1*, *Populus* ortholog of *TFL1* and of the tomato gene *SELF-PRUNING* (*SP*) (Pnueli et al., 1998), was initially strongly upregulated at the RM, one or two weeks after the reduction of *FT1*/*FT2* transcripts (Böhlenius et al., 2006; Hsu et al., 2006; Ruonala et al., 2008). However, the subsequent complete downregulation concomitant with the cessation of elongation growth indicates that *CENL1* might be required for stem elongation. This was confirmed in transgenic aspen that under SD increased elongation growth and continuously upregulated *CENL1* (Ruonala et al., 2008). In addition to its proposed role in stem elongation *CENL1* may be involved in safeguarding SAM identity during bud formation in a manner analogous to that suggested for *TFL1* in Arabidopsis (Conti and Bradley, 2007).

An early event at the apex of aspen as well as birch is the diminished symplasmic permeability of the SAM (Rinne and van der Schoot, 1998; Rinne et al., 2001; Ruonala et al., 2008). As symplasmic communication is pivotal for morphogenesis this reduction at around 10-15 SDs in aspen (Ruonala et al., 2008) might hamper the formation of new primordia. With a plastochron of one day (Ruonala et al., 2008) a mere 10-15 leaves could still be initiated from the SAM under SD. Potential symplasmic movement of signalling molecules like CENL1 between the RM and the SAM could preferentially occur in this period.

The initiation of a bud is marked by the organogenesis of scale leaves. In poplar this is suggested to start immediately (Rohde et al., 2002), probably after a single or a few SDs, involving the incipient primordia. So far no genes have been directly related to scale initiation, and all major changes in gene expression take place later (Ruttink et al., 2007), albeit scale formation might coincide roughly with the strong reduction in FT supply to the SAM (Böhlenius et al., 2006). At one week of SD the GA response modulator *GA-INSENSITIVE* and the gene *PICKLE* (*PKL*), a repressor of the Arabidopsis cell cycle regulator *AINTEGUMENTA* (*ANT*) (Mizukami and Fischer, 2000), are upregulated (Ruttink et al., 2007). These genes might be involved in scale and embryonic leaf formation by repressing normal leaf development.

2.3 Gibberellins in the Stem

In trees reduced GA levels have been thought to trigger growth cessation under SD (Junttila and Jensen, 1988; Moritz, 1995; Olsen et al., 1997; Eriksson, 2000). In aspen, this is supported by the downregulation of a gene that encodes a key GA biosynthetic enzyme in leaves, GA 20-oxidase, after just 4 SD (Eriksson and Moritz, 2002). In line with this, overexpression of GA-20 oxidase in aspen trees resulted in delayed growth cessation (Eriksson et al., 2000). Since growth cessation is not entirely prevented, other controlling factors must be involved that override the promoting effects of GA on stem elongation. Although GA levels decrease in the upper stem during SD (Olsen et al., 1995), endogenous GA levels have not been determined for the SAM, RM or even the RZ due to technical limitations.

In contrast to the upper stem parts the SAM is devoid of GA because GA 20-oxidase genes in the SAM are subject to direct transcriptional repression (Sakamoto et al., 2001). GA2-oxidase, which deactivates biologically active GAs and their precursors, is expressed at the base of the meristematic dome in Arabidopsis (Jasinski et al., 2005), and this could inactivate GAs arriving from the stem. The emerging view is that the SAM is the territory of cytokinins that in conjunction with *KNOX* genes keep it free from destructive GA influences (Shani et al., 2006).

The cambium of aspen contains low levels of bioactive GAs (Israelsson et al., 2005), whereas the major site of GA biosynthesis is the expanding xylem tissue were bioactive GA1, GA4 and GA-20 oxidase1 co-localise (Israelsson et al., 2005). In contrast to the young stem, the cambium does not downregulate GA-20-oxidase genes in autumn (Druart et al., 2007) suggesting that GA levels might not be key regulators of cambial cell division. The *Populus* homolog of *RGA* (repressor of *ga1-3*), encoding a GA-regulated nuclear DELLA protein that represses growth, was upregulated during growth cessation in the cambium (Druart et al., 2007). In buds of aspen, the DELLA gene, *GAI* was reported to be dramatically upregulated (Ruttink et al., 2007). However, in view of the recent finding that *GAI* RNA trafficks long-distance in the phloem (Huang and Yu, 2009) elevated transcript levels could also be due to import in the early stages of bud formation. This shows that even though GA biosynthesis is not altered in the autumn, the growth promoting effects of GA might be effectively diminished. DELLA proteins might therefore have some function in dormancy cycling, as proposed for seeds (Penfield et al., 2006). This is squaring with the fact that DELLAs are regulated by ethylene signalling (Achard et al., 2007), which is required for the timing of the dormancy assumption (Ruonala et al., 2006).

2.4 Dormancy at the SAM

Contrary to some general notions in the literature, a lowered metabolic state on its own is an insufficient condition for dormancy. A strong reduction in the supply of photosynthate to the apex slows down metabolism and can arrest elongation growth, but without preventing leaf development at the SAM (Rinne et al., 2005). In contrast, it is the isolation of SAM cells from each other that will halt patterning and impose dormancy. This was demonstrated for birch (van der Schoot and Rinne, 1999; Rinne et al., 2001), and aspen (Ruonala et al., 2008) in which symplasmic permeability decreased after ten to fifteen SDs (Section 2.2). This was followed by the physical isolation of SAM cells through formation of 1,3-β-glucan containing dormancy sphincter complexes (DSC) on all PD (Fig. 2c) (Rinne and van der Schoot, 2003). At a later stage, and in addition to the cellular uncoupling of cells within the SAM, the SAM as a whole becomes de facto isolated from the shoot system. This is achieved by occlusion of the supply routes to the apex by “dormancy callose” (Aloni and Peterson, 1997), a phenomenon referred to as “winter phloem” (Fig. 2b) (Aloni et al., 1991). The time lag between these events is probably used to fill the stores in the stem with photosynthetic and breakdown products from the senescing leaves.

In the SAM meanwhile, cell-cell signalling via excreted ligands and membrane-based receptor kinases is impeded by cellular dehydration and the impregnation of cell walls with water-repellent substances (Rinne et al., 2001; Welling et al., 2002). In this dormant state SAM cells have a low metabolism and membrane potentials that have decreased below the value of the diffusion potential (Rinne and van der Schoot, 1998; Ruonala et al., 2008). Altogether, the process of dormancy establishment in aspen takes six to eight weeks to develop under SD (Böhlenius et al., 2006; Ruonala et al., 2008). As the correlatively inhibited axillary buds enter dormancy in a similar time-frame, changes comparable to those in the SAM might be expected to occur.

2.5 Dormancy at the Cambium

Like the SAM, the cambium is sensitive to adverse conditions and is arrested at the end of the growing season (Schrader et al., 2004), a state traditionally referred to as "winter rest" (Esau, 1977). Although apical buds and overwintering cambium show commonalities in gene expression (Druart et al., 2007; Ruttink et al., 2007), it is as yet unclear how similar cambial and SAM dormancy are. Nonetheless, cambial arrest is real and distinct from quiescence and we will refer to it as cambial dormancy (Oribe et al., 2003; Schrader et al., 2004). As in the SAM of aspen and birch, cambial arrest involves cessation of cell proliferation, accumulation of storage products, dehydration, cold-acclimation, and cell wall modifications (Clausen and Apel, 1991; Rowland and Arora, 1997; Welling et al., 1997, 2002; Ermel et al., 2000; Rinne et al., 1998, 2001; Kozlowski and Pallardy, 2002; Schrader et al., 2004). In addition, the number of cell layers in the cambial zone is reduced from more than ten to fewer than four (Schrader et al., 2004). Concomitant changes in gene expression, investigated by analysing single cell layers (Uggla et al., 1996; Hertzberg et al., 2001), showed that major cell cycle genes are downregulated in the dormant cambium (Schrader et al., 2004). Interestingly, transcripts of some cell cycle genes remain present, perhaps facilitating reactivation of the cambium in spring and reflecting that cambial activity is regulated at the transcriptional and translational level (Schrader et al., 2004).

Although cessation of cell proliferation accompanies the establishment of dormancy, it is not a defining feature, as it is also found in case of quiescence and correlative inhibition. In the cambium the difference may reside at the level of cell cycle machinery, as key cyclin dependent kinases are lost during the transition from quiescence to the offline mode of dormancy (Espinosa-Ruiz et al., 2004). Although in the SAM of aspen and birch the dissipation of the supracellular organisation is characteristic for dormancy (Rinne and van der Schoot, 1998; Rinne et al., 2001; Ruonala et al., 2008), it remains to be seen if this is also true for cambial dormancy. The upregulation of a glucan-synthase gene in the cambial zone of aspen during dormancy establishment (Schrader et al., 2004) is suggestive of physical isolation of cambial cells by deposition of callose (1,3-β-glucan) at PD. Alternatively, the

upregulation of the glucan-synthase genes could induce the accumulation of callose in the cell walls, which would impede intercellular movement of a putative ligand for the CLV1 receptor kinase (Schrader et al., 2004).

Overall, the complexity of the cambial transcriptome is considerably reduced in the arrested state, while a number of genes involved in storage processes and defence or stress reactions are upregulated (Schrader et al., 2004; Druart et al., 2007). These include genes that are involved in drought and cold stress responses like *LEA*, *osmotin* and *ERD10*. Other strongly downregulated genes include the *Populus* homolog of *ANT* (Druart et al., 2007), which is also a putative target for cell cycle regulation in buds (Ruttink et al., 2007). Similarly, *RLK3* and *HB3*, *Populus* homologs of the Arabidopsis genes *CLV1* and *WUS*, are strongly downregulated (Schrader et al., 2004). However, expression of another *CLV1* homolog (*PttCLV1*) is maintained, probably providing some form of cambial “identity” during dormancy (Schrader et al., 2004).

Other important genes that are downregulated during cambial dormancy in aspen are *PIN1* and *PIN2*, encoding auxin efflux carriers (Schrader et al., 2004). This could explain the reduction of polar auxin transport in the dormant cambium and the desensitization to applied auxin (Schrader et al., 2004; Odani, 1975; Little and Bonga, 1974) while auxin levels in the cambium remain relatively high (Savidge and Wareing, 1982; Sundberg et al., 1991). Insensitivity to auxin is a defining property of the dormant cambium (Little and Bonga, 1974; Oribe et al., 2003) and could be used to assess its dormancy status (Aloni et al., 1991), perhaps in combination with the typical absence of specific CDKs (Espinosa-Ruiz et al., 2004). Lack of sucrose may help sustain dormancy in auxin-desensitized cambium (Little and Bonga, 1974; Oribe et al., 2003).

2.6 Events in Leaves and Stem

In some *Populus* species, elongation ceases in three to four weeks of SD (Böhlenius et al., 2006; Ruttink et al., 2007), while the leaves continue photosynthesis for at least another 4–5 weeks. Following growth arrest, the diminished demand from sinks generates a surplus of photosynthates that become re-routed to storage sinks in roots, stem and developing buds (Fege and Brown, 1984; Rinne et al., 1994b), where carbohydrate metabolism is modified to facilitate storing (Sauter et al., 1998; Schrader et al., 2004; Druart et al., 2007; Ruttink et al., 2007; Park et al., 2008). Imported photosynthates are stored as sugars, or converted to starch, fats and proteins (Sauter and van Cleve, 1991; Rinne et al., 1994b; Sauter et al., 1996). The importance of storage in the form of protein is illustrated by the fact that in aspen the gene *BARK STORAGE PROTEIN* (*BSP*), which is under strong photoperiodic regulation (Black et al., 2001), accounts for 20% of all transcripts in dormant cambium (Schrader et al., 2004). These stored proteins might be remobilised in the cambium during spring, as the increased level in amino acids appears just prior to cambial activation (Druart et al., 2007). Storage build up is important for overwintering, as hindering photosynthesis during growth cessation, for example by removal of

leaves, impairs acclimation and survival capacity (Fuchigami et al., 1971). In aspen this phase continues until dormancy has set in, and is followed by leaf senescence (Keskitalo et al., 2005).

Growth cessation is a prerequisite for leaf senescence as for example overexpression of *PHYTOCHROME A* (*PHYA*) or the *FRUITFULL-like* MADS box gene *BpMADS4* (Böhlenius et al., 2006; Hoenicka et al., 2008) prevents not only growth cessation but also leaf senescence. However, the regulation of autumnal leaf senescence is a more variable trait than end-of-season growth cessation, and it might be sensitive to both low temperatures and photoperiod (Fracheboud et al., 2009). Autumnal senescence is accompanied by a major change in gene expression reflecting a shift to mitochondrial respiration, fatty acid oxidation and nutrient mobilisation (Bhalerao et al., 2003; Andersson et al., 2004). Upregulation of genes involved in amino acid metabolism (Ruttink et al., 2007; Park et al., 2008) in bark and buds suggests that the leaf-derived mineral nutrients and nitrogenous compounds are imported by storage sites in the overwintering parts of the tree. A clear sign of advanced autumn senescence is the yellowing of leaves by carotenoids that become visible after chlorophyll breakdown, while subsequent reddening is due to anthocyans that provide protection to the ailing photosynthetic apparatus (Andersson et al., 2004; Keskitalo et al., 2005). Eventually, when phloem of both leaves and stem is occluded with callose (Aloni et al., 1991; Aloni and Peterson, 1997; Krabel et al., 1993), export ceases and the fate of the leaf is sealed.

3 Freezing Tolerance

3.1 Environmental Regulation

Cold acclimated boreal and temperate zone trees are the most freezing tolerant species on earth. Freezing tolerance is based on the capacity to endure cellular dehydration, which occurs when at sub zero temperatures water is moving along the water potential gradient to extracellular ice (Steponkus, 1984). This capacity is crucial as, due to the extremely low winter temperatures, cells have to survive almost complete dehydration for prolonged periods. Not surprisingly, successful overwintering requires numerous coordinated morphological and biochemical adaptations to dehydration, including the downregulation of metabolism and removal of reactive oxygen species and free radicals, the accumulation of protective proteins, such as dehydrins and other LEA proteins, and the maintenance of macromolecules in biologically relevant structures through accumulation of disaccharides and other glass forming compounds (Oliver et al., 2002).

The extreme cold hardiness of overwintering plants results from the additive affects of photoperiod and temperature on freezing tolerance (Weiser, 1970). Typically, SD-induced freezing tolerance starts to increase when elongation growth ceases (Welling et al., 1997, 2002; Rinne et al., 1999). SD induced acclimation is

most efficient at physiological temperatures as it involves metabolic processes that are slowed down at low temperature (Fuchigami et al., 1971). Remarkably, in trees SD exposure on its own can increase freezing tolerance beyond the level of fully acclimated herbaceous species, with a survival rate LT_{50} (50% survival) of –19 to –40°C (Junttila and Kaurin, 1990; Rinne et al., 1998, 1999). However, subsequent low and sub zero temperatures are required for a further increase of freezing tolerance. Typical for this second stage is that it is governed by alterations in ambient temperature: it increases with decreasing temperatures and vice versa (Sauter et al., 1996; Welling et al., 2004; Park et al., 2008).

3.2 Osmotic Adjustments and Dehydration

During SD induced acclimation cells and tissues adjust osmotically. This is an active metabolic process that is hampered by low temperature (Welling et al., 2002). The resulting decrease in water content and the increase in water and osmotic potential (Rinne et al., 1998; Welling et al., 1997, 2002) contribute to the cold hardiness of woody plants. These programmed events are characteristic for buds and stems, as in leaves they remain essentially unchanged (Welling et al., 1997). Collectively, the events prevent intracellular ice formation and alleviate the effects of frost-induced dehydration.

Starch breakdown provides the sugars (Sauter et al., 1996) that are required for building up freezing tolerance (Wanner and Junttila, 1999; Uemura et al., 2003), but breakdown products are also utilized for example in fatty acid metabolism (Druart et al., 2007). Highly abundant sugars might play a role in osmotic adjustments whereas less abundant sugars might also function in cryoprotection and signalling (Stitt and Hurry, 2002). Although starch hydrolysis occurs in response to low temperature during winter (Sauter et al., 1996), genes encoding the starch degrading endoamylases are expressed already under SD conditions both in buds (Ruttink et al., 2007) and in stem (Schrader et al., 2004). These endoamylases might associate with starch grains only in response to low temperature (Sauter et al., 1998), which would explain the temperature-dependence of starch hydrolysis. As hydrolysis occurs in dehydrated tissues trees might have evolved dehydrin proteins (Section 3.4) that rescue enzyme function by sequestering water at the surface of starch grains (Rinne et al., 1999). Sauter and co-workers (1996) proposed that sugars desiccate protoplasts by pulling water into the dilated tubular or vesicular ER-cisternae, where sucrose and its galactosides are sequestered after starch hydrolysis. Several studies report that sugars and other protecting molecules stabilize membranes during freeze-thaw (Close, 1996). Thus, the agents of osmotic adjustment, accumulated proteins and sugars, also provide protection against freezing for macromolecules and membranes.

Unique to woody plants is the capacity to form metastable solutions known as glasses, which make cells extremely stable and unaffected by the stresses of low temperature and ice. The formation of solid intracellular glass occurs at about −28°C in cells with high sugar concentrations and prevents further water loss to

extracellular ice at lower temperatures. Glassed solutions are not subject to ice nucleation, solute crystallization and water vapor cavitation as long as the solution remains below the melting temperature of glass (Hirsh et al., 1985; Wisniewski et al., 2003). Under severe cellular dehydration, when water is almost absent, sugars might replace water in the hydration shell of the membranes, maintaining the space between phospholipids (Hoekstra et al., 2001). Sugars are also needed for repair of freeze-induced cavitated vessels as the increase of sucrose in xylem sap results in active refilling of embolized vessels through winter stem pressure (Améglio et al., 2004; Decourteix et al., 2006).

3.3 Regulation of Gene Expression

While the phenological alterations in freezing tolerance during overwintering are well defined, the associated molecular changes have remained largely unknown. The recent transcriptional, proteomic and metabolic profiling of aspen and other boreal tree species during the annual growth cycle has provided a global map of sequential molecular changes during overwintering (Schrader et al., 2004; Druart et al., 2007; Ruttink et al., 2007; Park et al., 2008; Renaut et al., 2008). The data confirm at a molecular level the documented cellular and metabolic alterations that occur during hardiness and dormancy development. In addition, these studies suggest metabolic routes that are utilized during overwintering. For example, comparison of transcriptional analyses of stem samples from cottonwoods grown in the field and under controlled conditions confirms that genes that are predominantly expressed in autumn are responsive to SD and drought, whereas genes expressed during early and late winter respond mostly to the combination of SD and low temperature, or to low temperature alone (Park et al., 2008).

Although, in trees, the most notable alterations in SD induced gene expression relate to the cessation of growth and the development of dormancy, they are remarkably similar to the cold-induced changes in the transcriptome and metabolome of Arabidopsis (Fowler and Thomashow, 2002; Schrader et al., 2004; Druart et al., 2007; Renaut et al., 2008). SD and low temperature can induce the expression of cold-regulated genes independently, but SD can enhance cold-induced expression even in cases where on its own it does not have an effect (Park et al., 2008). Also Puhakainen et al., (2004) showed for birch that although the dehydrin gene *BpLTI36* was not expressed in leaves of SD-exposed trees, SD was “priming” the leaves for enhanced cold-induced expression of this gene. Despite its significance, the mechanism of this SD priming has remained unknown.

Several studies have shown that expression of some of the cold regulated genes is maximal during mid-winter, at the time when trees are the most freezing tolerant (Artlip et al., 1997; Rinne et al., 1998; Karlson et al., 2003; Welling et al., 2004). In that period trees are exposed to freeze-thaw conditions (Welling et al., 2004), and might need maximum protection. A number of cold regulated genes are responsive to freeze-thaw treatment (Zhu et al., 2000; Welling et al., 2004; Decourteix et al.,

2006; Tommasini et al., 2008; Welling and Palva, 2008). As the degree of freezing tolerance correlates with the expression level of cold regulated genes, sequential exposure to SD, cold and freezing might be required for a stepwise increase in cold acclimation, resulting in the extreme freezing tolerance that is typical of overwintering trees.

3.4 Dehydrins and Other Protective Proteins

Besides genes that are related to storage accumulation and membrane and cell wall modification, various genes implicated in cell defence and rescue are upregulated in *Populus* trees in response to SD and early winter (Schrader et al., 2004; Druart et al., 2007; Park et al., 2008). These include genes encoding pathogen-resistant proteins that have antifreeze-activity (Wisniewski et al., 1999) and proteins that are known to scavenge reactive oxygen species and other free radicals (Fowler and Thomashow, 2002; Schrader et al., 2004; Druart et al., 2007; Ruttink et al., 2007; Park et al., 2008). In addition, upregulated genes include those encoding dehydrins and other LEA proteins (Close, 1996) as well as a number of ABA and cold regulated proteins (Druart et al., 2007; Ruttink et al., 2007).

Dehydrins are significantly elevated during overwintering as they are produced in response to stresses that cause cellular dehydration such as low temperature, drought and high salinity (e.g. Svensson et al., 2002). In addition, dehydrins accumulate either in response to SD (Welling et al., 1997; Karlson et al., 2003), a combination of SD and cold (Welling et al., 2002, 2004) or freezing temperatures (Zhu et al., 2000; Tommasini et al., 2008; Welling and Palva, 2008). Dehydrins are stable proteins and often the total level of various dehydrins is highest during mid-winter, reflecting the need for maximum protection when temperatures are the lowest (Welling et al., 2004). Although the precise function of dehydrins has not been elucidated, their consistent presence in dehydrated tissue suggests a central role in dehydration tolerance of plants. Dehydrins have been shown to have in vitro cryo-protective (Close, 1996; Rinne et al., 1999; Wisniewski et al., 1999; Hara et al., 2001; Peng et al., 2008) and antifreeze-activity (Wisniewski et al., 1999). Dehydrins might function as compatible solutes under mild water stress, and interact under severe stress with other proteins to stabilize macromolecules and membranes (Close, 1996). Collectively, sugars, dehydrins and other LEA proteins can confer higher protection to cell membranes than sugars alone (Wolkers et al., 2001).

3.5 Transcriptional Regulation

During SD a number of transcription factors (TFs) involved in responses to dehydration, photoperiod, cold, salinity, and glucose are upregulated in poplar buds (Ruttink et al., 2007). Considering the complexity of cold acclimation, it seems likely that several of these TFs are involved in regulating different steps of the process (Druart et al., 2007). For example, in aspen two cold-induced TFs, *DRTY* and *HB12,* are expressed at different times during overwintering, possibly regulating

different target genes (Druart et al., 2007). In trees, the best characterized transcriptional regulatory pathway is the CBF/DREB1 regulon. Overexpression of the Arabidopsis *AtCBF1* in poplar induces a specific subset of cold-responsive genes and leads to an increase in freezing tolerance of both leaves and stem, suggesting that the CBF-mediated signalling pathway is conserved in perennial and annual tissues (Benedict et al., 2005). In addition, four endogenous *CBF* genes are differentially expressed in leaves and stems in response to cold, suggesting that they might regulate different CBF regulons in different tissues (Benedict et al., 2005). As *CBF* genes are expressed transiently, it has been difficult to study their role in the annual growth cycle and, for the same reason, they are usually not detected in microarray experiments (Druart et al., 2007). Ruttink and coworkers (2007) showed that numerous CBF target genes were upregulated in response to SD in bud tissues. This suggests that SD triggers the same targets as cold and drought, but that the input paths might differ. Similar to aspen, birch has at least four different *CBF* genes that are all induced in response to cold in leaves, stem and buds, with slightly different expression levels (Welling and Palva, 2008). The *CBFs* and one of the target genes, the dehydrin encoding *BpLTI36*, were expressed at a much higher level when pre-exposed to a combination of SD and cold, than when pre-exposed to cold alone. Especially freeze-thaw treatment induced very strong expression of *CBF*s and *BpLTI36*, although the transcript levels were upregulated only during the thawing-phase (Welling and Palva, 2008). These results suggest that CBFs are components of the mechanism that enables trees to cope with repeated freeze-thaw periods during winter and to maintain adequate levels of freezing-tolerance (Welling and Palva, 2008) until an increase in ambient temperature leads to the sequential deacclimation of stem and buds (Welling et al., 2004; Kalberer et al., 2006).

4 Release from Dormancy

4.1 Phenology

As much as dormancy and freezing tolerance are regulated coordinately, a relationship exists between deacclimation and dormancy, because deacclimation occurs more easily when buds are released from dormancy (Leinonen et al., 1997; Kalberer et al., 2006). Release from dormancy requires prolonged exposure to temperatures between 2 and 7°C, a process referred to as chilling (Coville, 1920; Chouard, 1960). In aspen, bud dormancy release at these temperatures takes about 6–8 weeks (Espinosa-Ruiz et al., 2004; Rinne et al., unpublished), which is fulfilled already in late autumn. In nature trees are commonly exposed longer to chilling, which might accelerate the rate of future budburst. Among species indigenous to the same latitude differences exist in chilling demands (Leinonen, 1996). Possibly, and in analogy to differences in dormancy onset among clinal ecotypes (Howe et al., 1995; Frewen et al., 2000; Luquez et al., 2008), differences in chilling demand may also exist among ecotypes, although this is little investigated (Leinonen, 1996).

Lack of sufficient chilling does not prevent eventual bud burst, but adequate chilling is important for deciduous perennials to fully develop their foliation and architectural potential. For example, in apple and beach insufficient chilling leads to a diminished bursting of buds, which will be mostly confined to the shoot tip (Arora et al., 2003). In contrast, in aspen and birch insufficient chilling promotes growth from basal buds, resulting in a bushy growth habit (Rinne et al., 1994a). In practise, inadequate chilling can be compensated partly by application of chemicals that break dormancy, such as hydrogen cyanamide (Fuchigami and Nee, 1987). This is economically important, for example in fruit tree cultivation, and insight in the mechanism may contribute to the understanding of natural dormancy release (Arora et al., 2003). Traditionally, chilling is regarded as the natural cause of dormancy release, but freezing can also release buds from dormancy (Rinne et al., 1997) and its effect is as fast as that of dormancy-release chemicals. Thus, incidental early frosts might result in premature dormancy release and bud burst, which makes them vulnerable to subsequent freezing. In general, the increase in the frequency and duration of warm spells during winter, as prognosed by climate models, might be problematic for trees which are released from dormancy through most of the winter time.

4.2 Cell Biology and Biochemistry

Traditionally dormancy has been viewed as a systemic property and investigated phenologically (Kozlowski and Pallardy, 2002). Although valuable, such approaches remain restricted and are insufficient to understand dormancy cycling in terms of a seasonal regulation of meristem activity. Observations indicate that chilling effects cannot be imported from other parts of the plant and that buds themselves need to be exposed to cold (Metzger, 1996). This is crucial to our understanding of the dormant state, as it indicates that SAM cells respond cell-autonomously to dormancy releasing signals like chilling. It is also what one would expect if all transport routes in the stem and the SAM, both symplasmic and apoplastic, are obstructed. This places the mechanism that enforces cell isolation at the centre of the dormancy phenomenon. The intrinsic mechanism that enforces the dormant state (Fig. 1) has been identified as the mechanism that obstructs PD and cell walls in the SAM (Rinne and van der Schoot, 1998; Rinne et al., 2001; Ruonala et al., 2008). Chilling therefore might impinge on cellular mechanisms which restore cell wall properties and PD functionality (van der Schoot, 1996; Rinne et al., 2001) and allow renewed exchange of peptide signals through the extracellular space and morphogens through PD (Rinne and van der Schoot, 2003). Restoration of PD might resemble the spring restoration of sieve plate pores, which originate from PD (Lucas et al., 1993), and which in winter phloem are occluded with callose deposits (Fig. 2b, c) (Aloni and Peterson, 1991, 1997). Chilling-induced removal of callose from DSC requires the targeted delivery of the callose-hydrolyzing enzyme 1,3-β-glucanase (Rinne et al.,

2001). This is a cell autonomous process as the dormant SAM is reduced to a mere collection of metabolically and physiologically uncoupled cells. After supply routes and intercellular transport paths are restored, sugars and other nutrients re-enter the SAM.

In contrast to the young stem and the pith, which during SD exposure become loaded with starch, the SAM and the RM remain free from starch, both in aspen and birch (Rinne et al., 1994b, 2001, 2008). Interestingly, the starch grains immediately subjacent to the SAM disappear during wintertime to reappear prior to bud burst in spring at the same locations (Rinne et al., 1994b). This probably contributes to freezing tolerance by providing starch-derived sugars for protection (Section 3.2). Such role for starch-derived sugars must be lacking in the starch-free SAM and RM. Instead of starch the SAM and RM, in both aspen and birch (Rinne et al., 2001, 2008), produce a remarkable number of lipid bodies (LBs). Similar LBs accumulate in large numbers in the dormant cambium of other perennials like for example *Robinia pseudoacacia* (Farrar and Evert, 1997). In Arabidopsis seeds LBs provide both cryoprotection and energy (Shimada et al., 2008), but in the dormant SAM they might additionally function in dormancy cycling (Rinne et al., 2001).

LBs contain triglycerides and are decorated with a set of proteins, including oleosins, lipases and hydrolytic enzymes (Murphy, 2001; Rinne et al., 2001). The role of oleosins is to prevent LB fusion via electrical repulsion, thereby retaining a minute size (Siloto et al., 2006). In dormant birch and other perennials the minute oleosin-decorated LBs are displaced during chilling from random positions to the peripheral cytoplasm, thereby contacting the plasmamembrane and PD (Pihakaski et al., 1987; Rinne et al., 2001). Dehydrating seeds have similarly behaving LBs (Cordova-Tellez and Burris, 2002). Isolation of LBs from birch, and analysis of their LB-proteome showed that in addition to oleosins and other peptides they contain 1,3-β-glucanase (Rinne et al., 2008). This hydrolytic enzyme might play a critical role in dormancy cycling by regulating in conjunction with 1,3-β-glucan synthase the levels of callose (1,3-β-glucan) at the PD (Rinne et al., 2001; Rinne and van der Schoot, 2003, 2004). In support of this several 1,3-β-glucanase genes and other cell wall modifying enzymes are involved in the release of seeds from primary dormancy (Leubner-Metzger and Meins, 2000; Cadman et al., 2006). In Arabidopsis seeds, these 1,3-β-glucanase genes include a member (Cadman et al., 2006) that is identified as a putative PD-associated protein (PPAP) which localizes at PD (Levy et al., 2007). In the cambial zone of *Populus* trees similar PD and wall modifications may take place considering that cold exposure upregulates a 1,3-β–glucanase as well as a receptor kinase gene which requires ligand exchange through the cell wall space (Druart et al., 2007; Park et al., 2008).

LBs originate from the outer leaflet of the endoplasmic reticulum (ER) (Napier et al., 1996; Murphy, 2001). Their production is significantly amplified during the transition to dormancy while, in contrast, cell divisions slow down and eventually cease. This suggests that the ER plays an important role in the complex array of processes that underlie growth cessation, bud formation and dormancy. Notably,

the ER is also the site of an ethylene receptor (Grefen et al., 2008), which is required for the correct timing of dormancy, both in birch (Ruonala et al., 2006) and poplar (Ruttink et al., 2007). It is tempting therefore to speculate that photoperiodic signalling somehow regulates processes at the ER of meristem cells. Ethylene biosynthesis genes are upregulated at around two weeks of SD in poplar (Ruttink et al., 2007), corresponding to the upregulation of an oleosin gene (Rinne et al., 2008). ABI3 mutants in Arabidopisis are deficient in oleosins and possess fused LBs (Parcy et al., 1994), suggesting that ABA could be required for normal LB formation. Knocking out oleosin genes similarly resulted in fused LBs, and caused a delay of dormancy release and germination (Siloto et al., 2006).

In summary, LBs are produced early during the dormancy induction period, put into action during the dormancy release phase, and decomposed during tissue activation (Riding and Little, 1984; Farrar and Evert, 1997; Rinne et al., 2001). The presence of such a pro-active system shows that temporal correlations based on transcript profiling should be regarded with some caution as cellular dormancy release mechanisms, including stored mRNAs and compartmentalized proteins, could be hold in abeyance for future use. It seems feasible that *de novo* gene expression is initially not necessary for kick-starting the dormancy release process. Together it suggests that in order to understand dormancy release more completely investigations should address the pre-instalment of the release mechanism during dormancy induction.

4.3 Gibberellins

While chilling is the natural condition that releases buds from dormancy, GA application to a dormant bud can replace chilling, suggesting that chilling effects are mediated, at least in part, by GA (Lang, 1957; Purvis, 1961). This was supported by evidence showing that chilling can activate GA-biosynthetic genes locally at the shoot tip in some flowering plants (Hazebroek et al., 1993; Zanewich and Rood, 1995). This probably involved vernalization-induced changes in methylation of the gibberellin biosynthesis genes (Burn et al., 1993; Finnegan et al., 1996, 1998). Recent studies have shown that a key biosynthesis gene for GA, GA-20-oxidase, is transiently upregulated during spring-time reactivation of the cambium in aspen Druart et al., (2007). It is uncertain if the upregulation is due to chilling or to growth induction. An exclusive role of GA in bud dormancy release is doubtful in view of the many abiotic factors that can break dormancy, such as anaerobiosis, freezing, high temperature, and a range of different chemicals (Tanino et al., 1989; Shirazi and Fuchigami, 1995; Cohn, 1996; Rinne et al., 1997; Wisniewski et al., 1997). A plausible explanation is that near-lethal factors converge on a damage-repair system, involving 1,3-β-glucanases, that kick-starts cell-cell communication between meristem cells and subsequent morphogenetic processes (Rinne et al., 2001).

The fact that the SAM might be kept free of GA (Sakamoto et al., 2001; Jasinski et al., 2005; Shani et al., 2006) suggests that the ability of exogenously applied GA to compensate chilling is due to an indirect effect on the SAM itself. It is important to mention, however, that the in situ localization of GA biosynthesis genes has so far been analysed only in active meristems of herbaceous plants. Work of Eriksson and Moritz (2002), using cDNA dot blot analysis, indicated that GA-20-oxidase levels might be higher in isolated SAMs than in the apex as a whole. Poplars differ from Arabidopsis not only because they are perennial species, but also because of their caulescent growth habit. This might set different requirements for GA biosynthesis sites compared to the rosette plant Arabidopsis. Even when GA is not produced in the SAM it could show up there if GA2-oxidases would be downregulated during chilling, as it would allow GA to diffuse into the meristem from the young stem. GA is a small molecule and can easily move via PD (Drake and Carr, 1979; Kwiatkowska, 1991; Lucas et al., 1993). GA could also function during dormancy release by promoting degradation of SD-induced DELLA proteins, thereby repressing growth repressors. Collectively, the above suggests that 1,3-β-glucanases are involved in re-establishing PD during chilling, while subsequent degradation of DELLA proteins might facilitate the liberation of meristems from growth constraints.

4.4 Chilling and Vernalization

Chilling and vernalization are both processes that are non-transferable and time-requiring, and executed under low but non-freezing temperatures. Despite these commonalities, few shared cellular or genetic features have been uncovered. In Arabidopsis winter annuals the gene *FRIGIDA (FRI)* elevates the expression of *FLOWERING LOCUS C* (*FLC*) which is predominantly expressed in the SAM, where it represses flowering (Michaels and Amasino, 1999, 2001; Sibum and Amasino, 2004). Vernalization prevents *FLC* elevation by epigenetic silencing through histone modifications (Bastow et al., 2004). However, vernalization needs *VERNALIZATION 1 (VERN1), VERN2* and *VERNALIZATION INSENSITIVE 3 (VIN3)* to stably maintain *FLC* repression (Gendall et al., 2001; De Lucia et al., 2008). Recently, differential expression of *FLC* like genes has been observed during chilling of poplar buds (Chen and Coleman, 2006), suggesting that vernalization can be a useful reference framework for the study of chilling. In addition, gene expression studies suggest the involvement of temperature-induced regulatory mechanisms, including chromatin remodelling mechanisms, both in aspen and other perennial species (Yakolev et al., 2006; Druart et al., 2007). Addressing dormancy cycling from a cell and developmental viewpoint may add context to the gene expression data and integrate them with phenology. Eventually, functional genomics studies in combination with cell biological approaches need to be performed to test candidate genes and the cellular mechanisms that drive dormancy cycling.

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Wood Formation in *Populus*

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Abstract Developmental genetic, genomic and biochemical approaches in *Populus* are providing new insights into the molecular and genetic mechanisms regulating wood formation. We discuss here wood properties, new approaches for the study of wood formation, and the genes and hormones responsible for regulating wood formation in *Populus*.

1 Biology and Salient Features of *Populus* Wood

1.1 Introduction to Wood Formation

Wood is arguably one of the most important biologically produced materials for a variety of practical reasons. The two most abundant polymers on earth, cellulose and lignin, are primary components of wood. Wood plays a major role in carbon cycles, with forests second only to oceans in terms of biological carbon sequestration. The woody bodies of trees underpin vital ecosystems and provide unique habitats and symbiotic relationships. The amazing mechanical properties of wood are exploited by carpenters and architects, and are illustrated by the massive bodies of the giant sequoia and coast redwood. Wood is the raw material supporting the forest products industry. Wood is a vital source of energy in developing countries. And wood is being developed as a source of renewable bioenergy in developed countries.

Wood also represents the culmination of a set of complex developmental and physiological events that are highly responsive to environmental cues. Wood formation provides striking examples of tissue patterning, cell differentiation, and meristematic activity that can take place over the course of thousands of years for some of the longer lived trees (e.g. bristlecone pines). Environmental cues feed into

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and modify wood development. For example, during the course of a growing season, the properties of developing cells within woody tissues can vary dramatically, as illustrated by annual rings. And wood formed in stems challenged by mechanical stress can form "reaction wood" with highly altered chemical composition.

While of high scientific and practical significance, there have been traditional barriers that have hindered understanding of the genetic and molecular regulation of wood formation. One major hindrance is the genetic properties of most tree species. Most trees have long generation times and can take many years or decades to become sexually mature. Most trees are outcrossing, highly heterozygous, and suffer from inbreeding depression. These attributes make approaches relying on multiple generations of crosses or close inbreeding (e.g. for loss of function mutagenesis) impractical for trees. However, more recently, genomic approaches that are well suited to or even exploit the genetic attributes of trees have been developed that are quickly expanding our knowledge of wood formation in trees.

Importantly, the radial organization of woody stems facilitates the harvest of large quantities of relatively homogeneous cells at specific stages of development. For example, cambial initials produce daughter cells, which in turn divide and give rise to cells that ultimately differentiate into cell types within secondary xylem and phloem. Tangential sections of stem tissues taken progressively from the bark, cambium, and through wood forming tissues thus represent a developmental series (Schrader et al., 2004). Cells can be easily harvested in large quantities during the spring when the cambium is actively dividing. The cells of the cambium have thin, fragile cell walls. An incision through the bark allows one to peel back the bark, with the tissues separating from the stem at the fragile cambium layer. Large quantities of cambium, secondary xylem, and secondary phloem can then be harvested by progressive scraping or sectioning. This feature facilitates biochemical and other approaches that are not feasible for root or shoot apical meristems.

In this chapter, we first introduce some salient features of wood formation. We then discuss approaches that have been taken to understand the genetic mechanisms regulating wood formation, including recent genomics-based approaches. Plant hormones play a major role in regulating wood formation, and their role is described in the following section.

1.2 Wood Development in Populus

To understand and discuss the molecular and genetic processes regulating wood formation in *Populus*, it is necessary first to consider some basic properties of wood. What follows is a brief description of the basic developmental processes underlying wood formation, some features of *Populus* wood anatomy and wood properties, and variation in *Populus* wood development in response to the environment.

Wood formation in *Populus* and other eudicots is a product of secondary growth – the radial growth that occurs after the elongation of stems during primary growth. Secondary growth is supported by the vascular cambium (Esau, 1977; Larson, 1994; Philipson et al., 1971). The cambial fusiform initials (oriented longitudinally to the long axis of the stem) divide periclinally to produce phloem mother

cells centrifugally and xylem mother cells centripetally. These cells in turn divide to produce daughter cells that are ultimately recruited to differentiate within secondary phloem (bark) or within secondary xylem (wood). Together, these tissues comprise the axial system with cells whose long axes are oriented vertically relative to the stem. *Populus* cambium is characterized as non-storied, meaning there is no strong tendency for cambial initials to appear in rows side-by-side when viewed in tangential section.

In addition to the axial system, the *Populus* stem also has a radial system of rays arranged horizontally relative to the long axis of the stem. The vascular rays are composed primarily of parenchyma cells and serve to transport substances including photosynthate and water across the stem between the secondary xylem and phloem. The rays also serve as a storage tissue (Esau, 1977). Rays originate from ray initials found within the vascular cambium. The ray system of *Populus* is characterized as uniserate, meaning they are one cell across in tangential cross section (Panshin, 1980).

The chemical composition of wood is a reflection of the differentiation of cells within secondary xylem. Typical *Populus* wood contains about 33% (vol/vol) tracheary (vessel) elements, 53–55% fibers, 11–14% ray parenchyma, and about 1% axial parenchyma (Mellerowicz et al., 2001). Tracheary elements and fibers both have thick, lignified secondary cell walls that impart most of the mechanical strengths to wood. Tracheary elements undergo a process of differentiation that includes construction of an elaborately patterned secondary cell wall, followed by a programmed cell death that hydrolyzes the cell contents (Groover and Jones, 1999; Moreau et al., 2005). Perforations connect neighboring tracheary elements. Perforation plates in *Populus* are "simple," meaning they have an unobstructed open hole between cells (Panshin, 1980). The final, fully differentiated cell is a hollow cell corpse which, by end to end association with other tracheary elements, forms vessels that conduct water. Fibers undergo a similar differentiation process but do not hydrolyze cell wall ends or conduct water, and serve a mechanical role. While less studied, xylem parenchyma cells generally serve primarily transport, storage, and other metabolic functions.

The vascular cambium is the ultimate source of new cells within secondary xylem. While the cambium has been the subject of numerous studies, many fundamental features of the cambium are only recently being understood at the regulatory level. One fundamental question is how the cells of the cambium are maintained in an "undifferentiated" dividing state. Elegant studies by Bannan (Bannan, 1956, 1957) demonstrated that the cambium is a dynamic tissue whose cells are likely specified by spatial cues, and can be replaced after injury. This view is in keeping with the current understanding of the more intensely studied root and shoot apical meristems, where cell to cell signaling plays a fundamental role in specifying the identity of the meristematic stem cells and regulating the size of the meristem. Daughter cells derived from the cambium must determine their position within either secondary phloem or xylem and differentiate into an appropriate cell type for their position.

In an additional analogy to the shoot apical meristem, the radial pattern of a woody stem can be viewed as a set of tissues with polarity. Just as a leaf has a top

(or adaxial) and bottom (or abaxial) surface, the woody stem has polarity. In *Populus* leaves, vascular bundles have polarity with xylem in the adaxial position in the bundle and phloem at the abaxial position. Following the vascular bundles of leaves through the petiole and into leaf traces connecting to the stem vasculature illustrates that the secondary xylem of the stem corresponds to adaxial tissues, and secondary phloem as abaxial. A fundamental question is whether similar mechanisms might determine polarity in secondary growth as determine polarity in leaves and other primary organs? As discussed in Section 3.1, there is evidence to support the general notion that some of the key mechanisms regulating shoot apical meristems, and perhaps root meristems, have been co-opted in the evolution of the cambium, but that mechanisms unique to the cambium are also likely.

1.3 Populus Wood and Its Uses

Anatomically, *Populus spp*. have whitish to cream-colored sapwood that gradually merges into the heartwood, which is light cream to light grey in color. Although there is not a distinct transition to heartwood formation, the heartwood does possess a characteristics unpleasant odor when wet (generally absent when dry). The wood has uniform texture, is soft, and generally straight-grained, displaying a gradual transition between earlywood and latewood zones of annual growth rings; the latter possessing a slightly darker color that is not always consistently visible. *Populus spp*. contains numerous small pores, which are generally concentrated in the earlywood and dissipate in size and number as latewood is formed (classified as semi-ring to ring porous). They also possess very fine, uniserate rays (Panshin and DeZeeuw, 1980). Standing poplar trees have relatively high moisture contents that do not vary significantly between the heartwood and sapwood, typically near 100% of oven-dry weight. Hybrid poplars generally have a higher standing moisture content compared to the aspens, although there is substantial seasonal and geographic variation (Kennedy, 1974).

Aspens differ slightly from hybrid poplars in that they slightly lighter in color and display a silky luster. The hybrid poplars are courser in texture – a function of the larger vessels needed for the transport of water during their rapid growth. The yield of plantation-grown hybrid poplars ranges between ~9 and 35 m^3 ha^{-1} yr^{-1}, which is substantially greater than the ~7 m^3 ha^{-1} yr^{-1} common to aspen (CCFM, 2001). At maturity, hybrid poplars have fiber lengths ranging from 0.7 to 1.35 mm (mean 1.2 mm) and fiber widths ranging between 24 and 32 μm (mean 27 μm), while the aspen are generally notably shorter 0.5–1.25 (mean 1.0 mm) and narrower: 12–25 μm in width (mean 21 μm). The xylem fiber length is shortest near the pith and progressively increases towards the cambium; increasing in overall length with age (Yanchuk et al., 1984). The fiber length then plateaus between 7 and 10 years of age, and is generally maintained for the remainder of growth. In contrast, the mean hybrid poplar vessel element length is 0.55 mm (0.4–0.84 mm range), while aspen is 0.63 mm (0.4–0.88 mm range). Vessel element width in aspen ranges between

38 and 99 μm (62 μm mean), while the range in hybrid poplars is 50–121 μm (88 μm mean).

These inherent differences in growth rates and fiber traits are reflected in the variability in overall wood density, which is generally lower than other hardwood species. Aspen's inherent small diameter and thin walled fibers are the major determinant of the variability in the difference in wood density within the genus *Populus.* Hybrid poplars wood density ranges from 285 to 390 kg/m^3, while aspen ranges from 310 to 430 kg/m^3 (Mansfield and Weineisen, 2007). The bending strength and stiffness, numerically represented by the modulus of rupture (MOR) and modulus of elasticity (MOE), are arguably the two most important mechanical properties of wood. These flexural properties have been shown to exhibit strong correlations with several inherent wood properties including intrinsic density, moisture content, and slope of grain (Mansfield et al., 2007). The poplars are generally characterized as possessing lower mechanical strength properties; however, there is a clear difference between the aspens and other *Populus spp*. For example, MOE estimates of 5.93, 5.17, 6.96 and 7.45 GPa have been reported for *P. tremuloides*, *P. balsamifera*, *P. deltoides*, *P. trichocarpa*, respectively, while the corresponding MOR estimates for these same species are 35.17, 26.89, 36.54 and 33.79 MPa, respectively (Alden, 1995).

Chemically, *Populus spp.* are generally characterized as being low in lignin content, and correspondingly high in carbohydrates. Although composition is influenced by both genetic and environmental variation, typical *Populus* wood contains roughly 43–50% glucose, 17–21% xylans 2–4% mannans and 19–24% lignin (Mansfield and Weineisen, 2007). The major form of hemicellulose in aspens is *O*-acetyl-(4-*O*-methylglucurono)-xylan, but also contains *O*-acetylated glucomannan (Gustavsson, 2001; Teleman et al., 2003). The lignin is syringyl-guaiacyl lignin, rich in syringyl units, often showing a syringyl monomer content ranging between 65 and 72 mol% syringyl. The syringyl:guaiacyl ratio, has been shown to directly correlate with ease of pulping, and therefore, the innate high-syringyl monomer composition common to *Populus spp*. imparts a low chemical load required for wood pulp production from *Populus* feedstocks (Stewart et al., 2006). The wood extractives content has been shown to range between 2 and 5% of the woody composite (Yanchuk et al., 1988; Mansfield and Weineisen, 2007). A thorough analysis of aspen wood extractives indicated that although there was little difference in the total amount of extractives, a detailed analyses of the major extractives classes (resin and fatty acid, sterols, sterol esters and triglycerides) by gas chromatography showed that there were only minor differences in the class composition as well, with an inverse relationship between content of resin and fatty acids and triglycerides (Stewart et al., 2006).

The genus *Populus* contains some of the most widespread and fastest-growing tree genus in North America and Europe. The genus is frequently the dominant broad-leaved tree species in many forested regions. *Populus* has a substantial breadth of distribution across geographic and climatic ecoregions, and are notable for their vigorous growth (Dickmann et al., 2001). *Populus* has been identified as a key genus as a fibre crop because of this rapid growth, inherent lower age of

maturity, perennial nature, and limited fertilizer requirements (Murphey et al., 1979). *Populus* has long been valued by the agroforestry industry for their use as windbreaks and shelterbelts, as well as timber belts from which farmers have derived wood resource. Environmental management applications also regularly include the use of *Populus* plantings for erosion control near streams, rivers, and reservoirs, as well as to provide for riparian buffer zones.

The utility of *Populus* as a short-rotation crop by the forest industry to provide wood-derived products is also becoming increasingly apparent. Poplar and more specifically aspen is currently employed primarily as a feedstock for pulp and paper production – the inherently small diameter fibres with thin walls are ideal for producing high-density paper sheets with very good optical properties (Mansfield and Weineisen, 2007). Furthermore, both the cottonwoods and aspens are low in lignin and high in carbohydrate, which makes them amenable to a variety of pulping regimes. In addition, *Populus spp.* are being employed as fibre resource for engineered solid wood products such as oriented strand board (OSB). The wood is well-suited for particle, flake, and strand-based composite boards because of its low density and ease of flaking, low cost, and availability (Gunn, 1963; Bendtsen et al., 1981; Dickerhoof et al., 1982). These inherent properties of *Populus spp.* medium density flake and strand boards whose strength properties are enhanced by good compaction and inter-flake contact and bonding. In contrast aspen, which is has long been a key resource for OSB production, black cottonwood (*Populus balsamifera* L.) has been shown to produce poorer quality waferboard and OSB when compared with native aspen strands (Pfaff, 1988). Recently, Semple et al. (2007) evaluated five different plantation-grown, industrially relevant hybrid poplar genotypes of the same age, grown on a common site in British Columbia Canada for their performance in strand production and properties of OSB. The results were compared against a benchmark mill-run OSB furnish derived from native aspen. Variation in solid wood density among the hybrid poplar clones was shown to influence the compaction ratio and densification of the OSB, which in-turn manifested variation in board strength properties. The lower density wood from fastest growing *P. deltoides* x *P. trichocarpa* (DTAC 7) clone resulted in better mat compaction and higher bond strength, whereas the higher density wood from a *P. trichocarpa* x *P. deltoides* (TD 50-184) clone resulted in lower compaction and bonding strength. Flexural strength (rupture and elastic moduli) and nail pull through were not as significantly affected by either board density or genotype when adjusted for density. The study clearly demonstrates that fast grown, large diameter wood of lower initial wood density from hybrid poplar is highly suited for OSB production. Despite lower clear wood strength properties, the use of intensively cultivated poplar species and hybrid poplar wood in OSB composites has yielded positive findings, and that the lower density and strength of wood from intensively managed, fast grown plantations does not, for the most part, translate into inferior composite board quality.

Populus spp. have also traditionally been used in the manufacture of specialty products such as chopsticks and pallets. *Populus* is also a source of fuel energy and an agriculture feedstock for ruminant pellet manufacture. One of the major

limitations in utilizing significant volumes of poplar wood in the secondary manufacturing sector has been the substantial degrade that results during kiln drying, and effective means of minimizing this phenomenon have been elusive. Historically *Populus spp.* have been regarded as a "low value" resource in comparison to other species (both hardwood and softwoods) and, as such, very little attention has been devoted to optimizing the processing of this wood species for the manufacturing sector. However, recently, Kang et al. (2007) demonstrated that drying schedule has a greater effect on grade recovery and degree of deformation, than the genotype of hybrid poplar. Furthermore, it was shown than many of the deformations that are inherently associated with wood derived from fast-grown poplar trees can be reduced or removed with an aggressive drying schedule. With such findings, the utility of *Populus spp.* in the manufacturing sector has the potential to increase in the near future. This is apparent from the increase use of this resource in the value-added sector, including the Italian furniture industry, which employ significant volumes of hybrid poplar as a resource supply for its value-added furniture components. The future will increasingly see this material used in several other sectors. For example, China has identified hybrid poplar as a key species for internal markets and manufacturing. As such, China has established significant hybrid poplar plantations (10 million hectares in 2002) throughout the country, and has evaluated both native and exogenous genotypes (53 species) for almost every application in both the primary and secondary manufacturing, ranging from pulp to plywood and furniture manufacture (Yukun and Xiaoyan, 2000).

1.4 Environmental Influences on Wood Formation

Wood development and resulting wood properties are highly influenced by environmental variation. One source of environmental variation is seasonal changes. The familiar annual rings seen in cross sections of *Populus* woody stems represent the rapid growth during favorable environmental conditions in spring and early summer (early wood) characterized by cells with thin secondary cell walls and larger lumens. As drought stress increases and growing conditions become less favorable during summer, tracheary elements are produced that are characterized by thicker cell walls and smaller lumens. Tracheary elements with large lumens characteristic of early wood are capable of larger capacity water conduction, but may be more prone to cavitation as compared to the tracheary elements produced in late wood.

Variation also occurs across years, as can be seen by variation in the thickness of annual rings. Years with favorable environmental conditions are associated with wider annual rings, while unfavorable years are represented by more narrow rings. The timing of transition between early wood and late wood is also influenced by environmental conditions, with unfavorable conditions leading to early transition to late wood.

Wood development is also impacted by gravity and mechanical stress. For example, a *Populus* tree that is listing at an angle after a wind storm will attempt to correct future growth to be perpendicular to the ground. This correction is achieved

by asymmetric growth characterized by preferential production of wood on the upper side of stems, referred to as tension wood (Evert, 2006). This wood has properties distinct from normally produced wood, including a low lignin content and higher cellulose content. The wood contains small vessels and fibers characterized by an inner cell wall layer (G-layer) that consists primarily of cellulose (Haygreen and Bowyer, 1996; Jourez et al., 2001; Norberg and Meier, 1996). Recent analysis has estimated that as much as 10% (mol%) of tension wood in *P. alba* is composed of non-cellulosic carbohydrates, primarily xyloglucan (Nishikubo et al., 2007).

An intriguing idea of how tension wood generates longitudinal tensile force is emerging after many years of debate and speculation. Cellulose macrofibrils (aggregates of microfibrils) in the G-layer are oriented parallel to the cell's long axis (Chaffey, 2000) and have been shown to generate tensile force, although the mechanism is unknown (Clair et al., 2006). Xyloglucan has been suggested to link cellulose macrofibrils of the G layer to adjacent cell wall layers, this serving to transfer the tensile force of the G layer macrofibrils (Long et al., 1996) to the tissue level (Mellerowicz et al., 2008; Nishikubo et al., 2007). The xyloglucan-mediated contacts between wall layers may be maintained and repaired during shrinkage even after cell death by persistent xyloglucan endo-transglycosylase and xyloglucan endo-transglycosylase/hydrolase activity (Nishikubo et al., 2007).

2 Genetic and Genomic Methods for the Study of Wood Formation in *Populus*

A number of genetic and genomic approaches have been taken to better understand the genetic regulation of wood formation in *Populus* and other tree species. These include traditional quantitative genetic approaches, molecular genetic approaches, and genomic approaches. As we will see, these approaches are different in scope and precision, but are complimentary.

2.1 Traditional Breeding and Quantitative Genetics

Traditional tree breeding and quantitative genetic approaches provide general insights into the genetic regulation of wood formation. One general question is, to what extent is variation among trees for a given wood characteristic a reflection of genetic variation, versus environmental variation? This can be estimated through measures of heritability using pedigreed material and progeny tests. Importantly, only genes with allelic variants that have large enough effects on wood properties (e.g. wood specific gravity) to be detectable using phenotypic measuring can be inferred or manipulated using traditional pedigree and progeny testing methods. Typically the segregation of individual genes cannot be detected or manipulated using these approaches. Quantitative Trait Loci (QTL) mapping can give a more

granular estimation of the number of genes responsible for variation in wood properties, and the relative magnitude of their effect. But only limited reports for QTL studies of wood properties in *Populus* have been reported (Tuskan et al., 2001) and are not discussed here.

Overall, results of quantitative genetic studies indicate that significant genetic variation exists for all wood property traits assayed, and at least some show moderate to high heritability. For example, wood specific gravity showed moderate broad sense heritability (0.41–0.46) in a test of three provenances of *Populus balsamifera*, while a test of *Populus deltoides* from the lower Mississippi valley found a high broad sense heritability of 0.62 (Olson et al., 1985). In the same study (Olson et al., 1985), holocellulose content (remaining after removal of lignin and extractives) showed low broad sense heritability (0.08), while alpha cellulose content (carbohydrate content most relevant to paper manufacture) showed moderate heritability (0.34). In general, results from breeding and quantitative genetic studies are consistent with the hypothesis that genetic variation underlying wood quality traits is a reflection of variation at several to many loci, each with modest influence on wood phenotypes.

2.2 Gene Discovery Using Gene Tagging

Molecular genetic or transgenic approaches in *Populus* that aim to identify and characterize individual genes influencing traits can be divided into two classes – forward genetic and reverse genetic. This section will examine forward genetic approaches, and the following Section 2.3 will examine reverse genetic approaches. Examples of forward molecular genetic approaches in *Populus* include the use of gene and enhancer trap tagging, and activation tagging. Activation tagging has not resulted in published identification of wood development genes in *Populus* and thus will not be further discussed, but has been used to identify development-related genes in *Populus* (Bush et al., 2007). For gene trap tagging, a construct is introduced into the *Populus* genome at random that contains a reporter gene such as GUS proceeded by splice acceptor sites. If the construct lands in the expressed portion of a gene in the same reading orientation of that gene, the result is a translational fusion of the GUS protein to the interrupted gene's protein product. Enhancer traps are similar but contain a marker gene with a minimal promoter, and results in marker expression when it lands in or near an expressed gene. In both cases marker gene expression typically reflects the normal expression pattern of the tagged gene (Groover et al., 2004; Groover et al., 2003).

Populus gene and enhancer traps have been used to identify genes expressed during vascular development, including genes expressed in the cambial zone and secondary xylem (Groover et al., 2004). Because the expression patterns of genes is revealed in detail by marker gene expression, genes expressed within specific tissues or even cell types within complex vascular tissues were discovered (Fig. 1). Gene and enhancer traps revealed that 40% of genes expressed in leaves were expressed

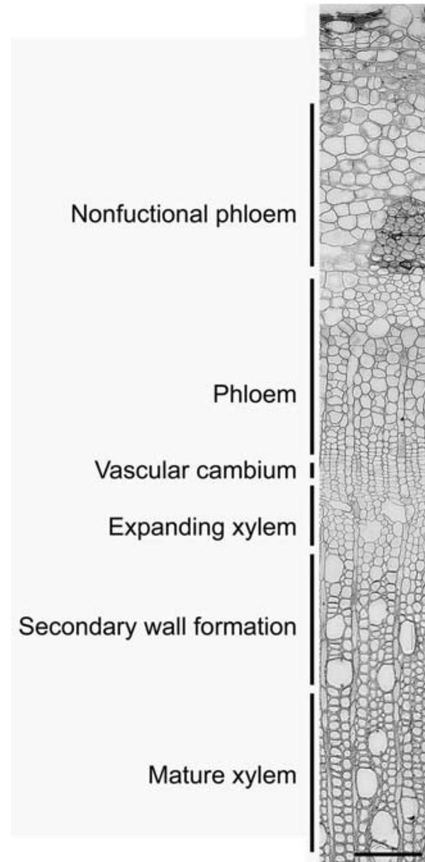

Fig. 1 Transverse section through a *Populus* stem

exclusively within the veins, showing that a large number of genes are involved in primary vascular development and function (Groover et al., 2004). In addition, vascular-expressed genes were commonly found to be expressed in both primary and secondary vascular tissues, likely reflecting the similar cell types found in both tissues, and a shared evolutionary origin for primary and secondary vascular tissues (Groover, 2005). For genes expressed in the cambium, a relatively broad expression was found and there were no genes identified whose expression was uniquely associated with the presumptive cambial initials. This could reflect an artifact of expanded GUS staining, insufficient sampling, or could indicate that there are few if any genes whose expression tightly defines narrow regions or boundaries within the cambial zone. Indeed, for a gene expression pattern to be tightly associated with the initials would require not only expression in those cells, but also rapid degradation of the transcript as the cell is displaced out of the initial position and into a mother cell position.

2.3 Determining Gene Function Using Transgenics

The function of individual genes can be studied in detail using a transgenic approach, even in the absence of natural allelic variation for the gene of interest. In *Populus*, the primary strategy is to introduce a transgene that produces a dominant phenotype that can be scored in primary transformants. This general strategy obviates the need for lengthy rounds of sexual reproduction to produce mutants that can be studied (e.g. loss of function homozygous mutants). The primary approaches reported to date for *Populus* have involved use of constructs for overexpression or knock down of target gene expression using RNA interference (RNAi). Resources have also been developed for using synthetic miRNAs in *Populus*. A web-based tool is now available for the identification of optimized sequences for targeting transcripts of individual or multiple genes using these short interfering RNAs (http://wmd.weigelworld.org/cgi-bin/mirnatools.pl). Currently, promoters for driving transgene expression in specific tissues of the stem are largely lacking.

While a powerful approach for determining gene function, as in other species, the production and thorough characterization of transgenics in *Populus* is laborious and time consuming. The number of published reports using this approach is thus relatively low, yet provides some of the most insightful and detailed information about wood development. However, ambitious projects are underway for *Populus* using a reverse genetics approach paired with higher throughput screening for wood properties (notably, in Sweden and the United States). Findings from some individual studies are incorporated in Section 3.1 below.

2.4 Gene Discovery Using Microarray Profiling of Gene Expression

Microarray profiling of global gene expression across wood forming tissues has produced the most comprehensive view of the genes and mechanisms regulating wood formation. A highly informative strategy has been to harvest serial longitudinal sections across the cambium and wood forming tissues. Because of the radial structure of woody stems, the collection of such sections represent a developmental gradient including cambial initials, cambium daughter cells, and early through late xylem development. Comprehensive gene expression profiling of each section allows reconstruction of the changes in gene expression through development and wood formation. Experiments to date have used both sequencing of ESTs and microarray profiling to catalogue gene expression in wood forming tissue of *Populus* (Schrader et al., 2004; Sterky et al., 1998). Current microarray platforms available for *Populus* include both "spotted" arrays and synthesized oligo arrays that include probes interrogating all available gene models from the *Populus* genome (Affymetrix, NimbleGen, and Agilent). The most comprehensive microarray analysis of the cambium and wood forming tissues to date was performed by Schrader et al. (2004). Their results are discussed below in Section 3.1.

2.5 Proteomic and Other "Omic" Approaches

Wood is a chemically complex but anatomically simple tissue that can be harvested in large quantities, and can be subjected to a wide array of biochemical, metabolomic, and proteomic techniques. This stands in contrast to many other plant tissues that are intermingled with other tissue types (for example, vascular tissues embedded within leaf mesophyll) and are thus difficult to harvest in quantities easily amenable to biochemical techniques requiring relatively large inputs of material.

Proteomic approaches extend and are highly complementary to gene and transcript-based approaches (Finnie, 2006). Transcript levels do not always correlate well with protein levels, and protein activity can be regulated by post-translational modifications. Proteomic methods that can survey proteins and protein modifications are being extended to *Populus* research. Similarly, methods for metabolomic profiling and determining wood quality parameters are available that are quickly being extended to *Populus*. Currently, *Populus* projects are underway in Sweden, Canada, France, and the United States that have major proteomic and metabolomic components.

An example of a proteomics approach applied to wood formation in *Populus* is given by Du et al. (2006), who identified proteins expressed during regeneration of cambium and secondary growth after girdling of Chinese white poplar. Proteins were isolated from stems at different stages of regeneration and subjected to 2-D gel electrophoresis. Protein spots of interest were then excised from gels and sequenced after trypsin digestion using a MALDI-TOF Mass Spectrometer. Increased expression of proteins associated with wood formation and secondary cell wall formation was noted during regeneration and resumption of normal growth progressed.

3 Genetic Regulation of Cambium Functions and Wood Formation

The development and application of genomic technologies have rapidly increased our understanding of the regulatory and biochemical processes regulating wood formation in *Populus*. In this section, we discuss some of the biological principles of wood formation emerging from genomic and related studies.

3.1 Global Gene Expression Across Cambium and Wood Forming Tissues

Studies of global gene expression using EST sequencing (Sterky et al., 1998) and microarrays (Schrader et al., 2004) have produced a nearly comprehensive catalogue of genes involved in cambium regulation and different stages of wood formation. Results from expression profiling of tissue layers, combined with anatomical

studies and measures of cell division from the cambium across wood forming tissues is consistent with the hypothesis that there is a developmental gradient that ranges from meristematic cells within the cambium through progressive, irreversible cell differentiation in wood forming tissues. Strict developmental boundaries are not identified. This could reflect a limitation of the tissue sectioning method, which results in samples 20 μm thick (corresponding to about three cell layers) and containing many thousands of cells. But for the relatively small number of meristem-related genes expressed in the cambium for which in situ hybridizations have been performed, these genes show relatively broad expression patterns. One biological explanation for this result would be that gene expression with tight developmental boundaries would require not only cell-specific expression, but also rapid degradation of transcripts in daughter cells.

However, clustering of genes based on peak expression levels across the cambial zone and wood forming tissues reflects developmental processes and correlates with anatomical features. This type of analysis has been extremely informative in defining the cambium and wood forming tissues in terms of gene expression. Regions of the cambium and wood forming tissues can be defined based on gene expression, and include a region of cambial initials, region of cell division, region of cell expansion, and region of terminal cell differentiation.

In the cambial zone, a layer of cells can be identified that show a small number of uniquely expressed genes, and which do not show high expression of genes associated with cell division. These cells likely represent the "stem cells" or initials of the cambium, having relatively low rates of cell division consistent with other stem cell populations in plants and animals. In the shoot apical meristem, distinct parts of the meristem are identified by specific genes regulating the meristem. For example, the Arabidopsis Class I KNOX transcription factor, *SHOOTMERISTEMLESS* (*STM*), is required for meristem maintenance, and is expressed broadly in the meristem but downregulated in leaf primordia (Long et al., 1996). The *Populus* ortholog of *STM*, *ARBORKNOX1*(*ARK1*), is expressed both in the shoot apical meristem and also in the cambial zone (Groover et al., 2006). Overexpression of *ARK1* in *Populus* results in inhibition of the differentiation of lignified, xylem and phloem fibers (Groover et al., 2006), consistent with the notion that, as for *STM* in the shoot meristem, *ARK1* functions to support meristematic cell fate and inhibit differentiation. Microarray analysis of these mutants shows misregulation of genes associated with cell wall formation and lignin biosynthesis, consistent with the hypothesis that *ARK1* negatively regulates genes associated with terminal cell differentiation. A related Arabidopsis gene, *KNAT1*, is also broadly expressed in the shoot apical meristem and serves to inhibit terminal cell differentiation. The *Populus* ortholog of *KNAT1*, *ARBORKNOX2* (*ARK2*), is expressed in both the shoot apical meristem and the cambial zone (A. Groover and Juan Du, unpublished). Overexpression of *ARK2* in *Populus* results in the expansion of the cambial zone and inhibition of terminal cell differentiation of both tracheary elements and fibers in secondary xylem, and of phloem fibers (Fig. 2). Knock-down of *ARK2* results in early appearance of lignified secondary xylem and thicker secondary cell walls. Together these results support the hypothesis that at least some critical cambium genetic regulatory mechanisms were

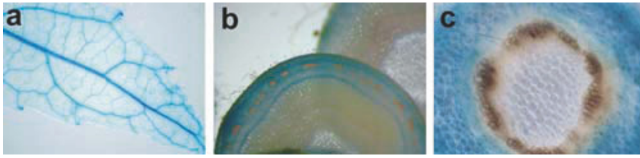

Fig. 2 Examples of *Populus* enhancer trap staining patterns. An enhancer trap construct carrying the GUS reporter was introduced at random into *Populus*. Individual transformants, each with a unique insertion, were then screened for GUS expression. Patterns of GUS expression reflect the normal expression pattern of a nearby gene. (**a**) Enhancer trap 4-109 with GUS expression in the vasculature of leaves. (**b**) Enhancer trap 4-110 with expression in the cambium region through the cortex in a stem cross section. (**c**) Enhancer trap 4-558 with expression throughout the cortex in a stem cross section

co-opted from the shoot apical meristem during the evolution of secondary growth (Groover, 2005).

Not all shoot meristem genes are expressed in the cambium, however. Other key shoot meristem regulatory genes include the transcription factor *WUSCHEL* (*WUS*), which is expressed in a small number of cells in the stem cell-organizing center that underlies the stem cell population of the meristem (Scheres, 2007). The *Populus WUS* ortholog is not expressed in the cambium, although another *WUS*-like gene is (Schrader et al., 2004). This could indicate that *WUS* related functions are present in the cambium and the duplication of an ancestral *WUS* gene allowed for new expression and function of a duplicated gene in the cambium. Alternatively, it could indicate that *WUS*-mediated signaling does not occur in the cambium. Unfortunately functional analysis of *WUS*-like and other genes regulating shoot meristem maintenance and size (e.g. CLAVATA genes) have not yet been reported in *Populus*. As more genes are characterized a more complete picture of cambium regulation and evolution will emerge, including the relationship to the root and shoot apical meristems.

Progressing inwards (towards the xylem) across the cambial zone, a distinct increase in the expression of genes associated with cell division is observed (Schrader et al., 2004). This region includes the xylem mother cells, which are responsible for the bulk of cell divisions that produce cells that will ultimately differentiate in secondary xylem. Cell division genes upregulated in this region include *Populus* orthologs of *Cyclin A1*, *Cyclin D3*, cyclin-dependent kinase *CDKB2*, *CKS1*, and a *DP-E2F*-like (*DEL*) gene. Variation in expression among cell cycle genes was also noted, including maintenance of the *Populus Cyclin A2* (*CYCA2*) well into the region of secondary cell wall formation. Because CYCA2 expression is believed to be associated with the competence to divide, this could reflect that xylem cells maintain the ability to divide until late in their development.

The expression of several genes associated with cell expansion increase across the cambial zone, and then show relatively consistent expression across the zones of division and expansion (Schrader et al., 2004). These genes include expansins, pectin methyltransferase, and xyloglucan endotransglycosylase that are variously involved in cell wall modification, loosening, and intrusive growth (Nishikubo et al., 2007; Siedlecka et al., 2008). Aquoporin-encoding genes are also expressed in a

similar pattern. Aquaporins could potentially be involved in the uptake of water, which increases turgor pressure and drives cell expansion, although this has not been shown experimentally.

Not surprisingly, genes involved in the synthesis and lignification of secondary cell walls show increased expression in progressively older tissue of secondary xylem. Genes associated with programmed cell death are expressed slightly later, during the cell death and lysis of tracheary elements whose hollow cell corpses conduct water within secondary xylem. Genes regulating the balance of meristematic cell fate versus cell differentiation include the Class I KNOX transcription factors, as previously mentioned. Additional genes regulating cell differentiation include the NAC transcription factors. While not yet characterized in *Populus*, NAC family members have been shown to be primary regulators of tracheary element differentiation in Arabidopsis, and overexpression can result in ectopic tracheary element differentiation (Masatoshi Yamaguchi et al., 2008; Zhong et al., 2006). Other genes identified in Arabidopsis but not yet characterized in *Populus* include genes regulating the patterning of secondary cell wall. These include genes encoding proteins directing the position of cellulose-synthase rosettes by the cytoskeleton.

3.2 Regulation of Tissue Patterning

Secondary vascular tissues in poplar are regularly patterned with xylem to the inside and phloem to the outside of the stem. The microarray profiling by Schrader et al. (2004) found that genes known to regulate polarity of leaves derived from the shoot apical meristem are also expressed in the cambial zone and early differentiating secondary vascular tissues. YABBY and KANADI gene families in Arabidopsis function to promote an abaxial fate in leaves. No evidence for differential expression was found for three *Populus* YABBY genes across the cambial zone, suggesting these genes may regulate polarity exclusively in leaves. In contrast, an ortholog of the Arabidopsis *KANADI1* showed differential expression in the phloem side of the cambial zone. Secondary phloem (bark) represents an abaxial tissue, consistent with the idea that the *Populus KANADI1* ortholog could function to promote abaxial fate both in lateral organs derived from the shoot apical meristem and in secondary vascular tissues.

Five genes comprise the Class III HD-ZIPs gene family in Arabidopsis. These genes have various functions in both meristem regulation and vascular development, including promoting adaxial fate in leaves. Interestingly, these genes are all negatively regulated post-transcriptionally by microRNAs. Schrader et al. (2004) found that the poplar orthologs of *PHAVOLUTA/PHABULOSA*, *ATHB8*, and *ATHB15/CORONA* all showed differential expression on the xylem (adaxial) side of the cambial zone. A *Populus* ortholog of the fifth member of this gene family in Arabidopsis, *REVOLUTA*, has recently been characterized using transgenics. Interestingly, *Populus* overexpressing a micro-RNA resistant *Populus REVOLUTA* ortholog showed defects in pattering secondary vascular tissues that are consistent with this gene regulating polarity in *Populus* stems (A. Groover and M. Robischon,

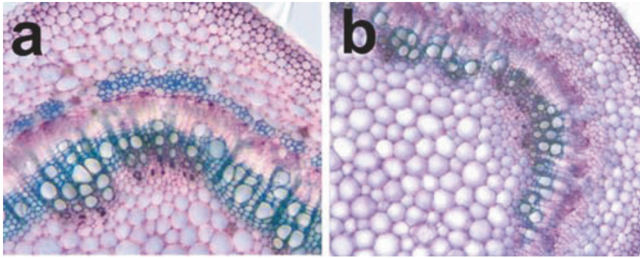

Fig. 3 Cross sections stained with TBO from matched internodes from a transgenic hybrid aspen overexpressing the *Populus* ortholog of *KNAT 1, ARBORKNOX2,* and wildtype control. (**a**) Overexpression plants have a wider cambium region, copious procambium, reduced phloem fibers, and reduced lignified xylem. (**b**) Wildtype control (Images from A. Groover and J. Du, unpublished)

unpublished). Plants showing extreme defects include the formation of a second cambium which produces secondary xylem towards the outside of the stem (Fig. 3). This and other defects suggest a fundamental role for Class III HD ZIPs in regulating how woody tissues are patterned.

4 Hormonal Control of Wood Formation

4.1 Introduction

Most, if not all, plant hormones have been implicated in the regulation of wood formation. Classical hormone treatment studies have shown that several plant hormones can stimulate radial growth through induction of cambial cell proliferation or affect xylem cell differentiation when applied to tree stems. Recently, through the sequencing of the *Populus trichocarpa* genome and the development of various genetic, genomic and biochemical tools for *Populus*, it has become possible to move beyond exogenous hormone treatments and more directly address the function of hormones in wood formation. Importantly, recent studies using transgenic *Populus* trees with modified hormone responses and determination of hormone concentrations and hormone-related gene expression across wood forming tissues have greatly extended our understanding of hormonal regulation of wood formation. However, hormone signaling pathways regulating the cambium and secondary growth are highly complex and require new approaches to be fully resolved. We describe below the current understanding of the role of specific hormones in secondary growth and wood formation in *Populus*.

4.2 Auxin

Auxin is the best known hormonal regulator of wood formation. Classical hormone treatment studies implicated auxin as a stimulator of cambial activity, and apically

applied exogenous auxin can reactivate cambium in decapitated shoots (Snow, 1935; Digby and Wareing, 1966; Björklund et al., 2007; reviewed by Savidge, 1988). The shoot apex is a major source of auxin (Sundberg and Uggla, 1998), and auxin is hypothesized to be transported in a polar fashion through the cambial zone down the stem. Furthermore, a radial gradient of auxin (indole-3-acetic acid, IAA) has detected across the cambial zone of both *Populus* and *Pinus* trees (Uggla et al., 1996, 1998; Tuominen et al., 1997). The level of IAA peaks in the dividing cambial cells, from which it decreases steeply towards differentiating phloem and more gradually towards the differentiating xylem. This gradient is assumed to be formed by auxin transported downwards from the stem apex being distributed radially across the cambial zone (Schrader et al., 2003). Significantly, differential radial expression of various genes encoding auxin influx and efflux carrier genes has been found across the cambial zone in *Populus* (Schrader et al., 2003).

The cambial auxin gradient correlates with an expression peak of auxin signaling genes in the cambial cells (Moyle et al., 2002). However, recently studies identified auxin responsive genes from hormone treated *Populus* whole stem samples and compared their expression patterns across the cambial zone to the auxin gradient (Nilsson et al., 2008). They observed that a large portion of the identified auxin-responsive genes was expressed at a higher level in the differentiating xylem cells than in the dividing cells where the auxin concentration is at its highest (Nilsson et al., 2008). The reason for this difference between the auxin signaling and auxin response gene expression patterns remains to be clarified.

During transition to cambial dormancy, polar auxin transport is severely reduced, as is expression of genes encoding auxin transporters (Schrader et al., 2003). This is reflected by the observation that the cambial activity can not be reactivated when auxin is applied to decapitated stems in a dormant state (Little and Bonga, 1974). Also the expression of auxin inducible *AUX/IAA* transcriptional repressor *PttIAA* genes is reduced during transition of the active cambium into dormancy, consistent with a down-regulated status of auxin signaling (Moyle et al., 2002). However, it has been shown in *Pinus* that the cambial IAA concentration does not decrease upon the cessation of cambial growth during the induction of dormancy (Uggla et al., 1996, 2001). Taken together, the results indicate that the level of auxin transport, responsiveness and signalling, but not the auxin concentration itself, may link the status of cambial activity to seasonal changes.

Recently, functional studies using transgenic *Populus* trees have further described the role of auxin in the regulation of wood formation (Nilsson et al., 2008). Nilsson et al. (2008) engineered transgenic *Populus* trees to ectopically express a stabilized form of a *Populus AUX/IAA* transcriptional repressor gene (*PttIAA3*), leading to reduced auxin responsiveness. In these trees, a reduced number of both periclinal and anticlinal cell divisions was observed in the vascular cambium, resulting in the compromised radial growth of the stems. A definition of the cambial initials by Larson (1994) and Schrader et al. (2004) states that they are the only cells in radial files that are able to produce both phloem and xylem mother cells through periclinal cell divisions and to initiate new cell files by anticlinal divisions. Schrader et al. (2004) observed that in *Populus* the anticlinal divisions appeared to be restricted on the phloem side of the cambial zone, whereas in the *PttIAA3*

overexpressing trees the anticlinal divisions were spread across a wider zone, occurring also in the middle of the cambium (Nilsson et al., 2008). This observation indicates that auxin signaling may regulate the position of cambial initials in the vascular cambium, or at least the position where their anticlinal divisions take place. Additionally, the transgenic trees with reduced auxin responsiveness had reduced width and length of xylem fiber and vessels, indicating that auxin is involved in regulating the anatomy of developing xylem cells (Nilsson et al., 2008).

4.3 Gibberellin

Gibberellin (GA) has been implicated in cambial growth due to its stimulatory effect on cambial activity upon hormone treatments on tree stems (Digby and Wareing, 1966; Wang et al., 1997; Björklund et al., 2007), and its synergistic action with auxin. As described above, auxin (IAA) is able to induce cell divisions in the vascular cambium when applied to decapitated tree stems (Little and Bonga, 1974). Application of gibberellin to decapitated *Populus* stems stimulates cell divisions in the cambial zone, but the identity of the newly formed cells remains unresolved (Björklund et al., 2007). The morphology of the GA-induced cells is somewhat abnormal; in a cross-section they look more spherical than the flat, thin-walled cells of the normal cambium. Furthermore, instead of differentiating into xylem cells on the xylem side of the cambial zone, they seem to remain in the parechymous state. As a result the GA treatment leads to the loss of an easily distinguishable vascular cambium (Björklund et al., 2007). These observations indicate that GA alone is not sufficient to maintain and stimulate cambial activity (Björklund et al., 2007). However, application of IAA together with GA enhances cambial cell divisions more than either hormone alone, indicating that these two hormones have a synergistic effect on cambial growth (Digby and Wareing, 1966; Björklund et al., 2007). Björklund et al. (2007) also showed that IAA concentration in stem tissues is higher when IAA is applied in combination with GA than when IAA is applied alone, indicating that GA action promotes cambial auxin transport. Furthermore, GA treatment induces expression of a cambial abundant *Populus* auxin transport protein gene, *PttPIN1*. Auxin treatment also stimulates expression of GA biosynthesis genes, inhibits expression of GA degradation genes, and GA and auxin treatments induce similar transcriptional changes (Björklund et al., 2007). The stimulating effect of GA on plant growth was demonstrated by increased shoot size after ectopic overexpression of a GA biosynthetic enzyme (*AtGA20ox1*) in transgenic *Populus* trees (Eriksson et al., 2000).

Only trace amounts of GAs have been detected in the dividing cambial cells, whereas they peaked in the differentiating xylem cells (Israelsson et al., 2005). Correspondingly, genes coding for GA biosynthetic enzymes and GA signaling pathway genes have low cambial expression, whereas they are higher expressed in both differentiating phloem and xylem cells (Israelsson et al., 2005). A transient

induction of a GA biosynthetic enzyme gene (*PttGA20ox*) was observed in spring during the cambial reactivation from dormancy (Druart et al., 2007). It is possible that the GA stimulates cambial activity mostly through promoting polar auxin transport into the cambial cells.

Gbberellin has also been indicated to function in control of xylem development during wood formation. Both analyses of transgenic trees overproducing GAs and hormone application experiments have shown that GAs stimulate xylem fiber elongation (Digby and Wareing, 1966; Eriksson et al., 2000). Furthermore, tissue specific distribution pattern of GAs across the wood-forming tissues in *Populus* show that bioactive GAs peak in the expanding xylem cells coincident with expression of GA biosynthetic and signaling genes, indicating a role for GAs in xylem differentiation (Israelsson et al., 2005). Functional studies with transgenic *Populus* trees having modified cambial auxin and GA responses are required to clarify the function of these two hormones between the regulation of cambial cell divisions and xylem differentiation during wood development.

4.4 Cytokinin

Since their discovery as regulators of plant cell division (Miller et al., 1955), cytokinins have been assumed to function in the control of cambial activity. Evidence for this action was deduced from hormone treatment experiments, where exogenously applied cytokinin was shown to act synergistically with auxin to enhance cambial cell divisions in diverse plant organs and species (Loomis and Torrey, 1964; Saks et al., 1984).

However, until recently the role of cytokinin in cambial development remained uncertain. Nieminen et al. (2008) found that both a cytokinin primary response gene (*PtRR7*) and genes encoding cytokinin receptors are expressed in the cambial zone of *Populus* stems. Transgenic *Populus* trees with repressed cambial cytokinin signaling were created by expressing an Arabidopsis cytokinin degrading enzyme (*AtCKX2*) under the promoter for a birch cytokinin receptor gene which drives high cambial expression. The transgenic trees have reduced cytokinin content and responsiveness in the cambial zone. They also have significantly impaired radial growth caused by a reduced number of cell divisions in the vascular cambium. Together, these data suggest that cytokinin is required for vascular cambium function by influencing cell division.

Cytokinin also affects xylem cell dimensions, as xylem fiber length and vessel width are slightly reduced in transgenic trees with reduced cytokinin signalling (Nieminen et al., 2008). However, it is possible that these differences were caused by the altered rate of cell proliferation in the cambial zone rather than by the compromised cytokinin signalling. The interaction between cytokinin and other hormone signalling pathways during wood formation is an important area requiring further study.

4.5 Ethylene

Ethylene has been implicated to affect wood formation based on hormone treatment studies (Telewski and Jaffe, 1986; Junghans et al., 2004; reviewed by Little and Savidge, 1987). The application of an ethylene releasing compound on *Populus* stems can induce radial swelling (Junghans et al., 2004). The organization of the ethylene-induced xylem tissue was disturbed, however. The axis of the xylem cells was not strictly vertical; the cells had instead grown into a tilted orientation. It thus remains unresolved in which extend the ethylene-induced cell proliferation resembles normal cambial development (Junghans et al., 2004). However, the expression of an ethylene biosynthetic gene from *Populus*, *PttACO1*, peaks in the developing xylem cells in the cambial zone, implying a possible function for ethylene in xylem differentiation (Andersson-Gunnerås et al., 2003). Furthermore, ethylene treatments reduce xylem fiber and vessel length (Junghans et al., 2004), indicating a possible function for ethylene in regulation of xylem cell morphogenesis.

Further data supporting a role for ethylene in the regulation of wood formation have been provided by studies examining ethylene signalling during tension wood development. In *Populus*, expression of both ethylene biosynthetic and signalling genes, together with auxin signalling genes, has been shown to be increased in developing tension wood (Andersson-Gunnerås et al., 2003, 2006). Recent studies using both pharmacological treatments and ethylene overproducing *Populus* transgenics correlated endogenous ethylene production with increases in cell division and differential growth during tension wood formation (Love et al., 2009). The interaction of IAA and ethylene during tension wood formation is less clear, and induction of tension wood formation did not result in an increase in IAA concentration in the upper side of a bent *Populus* shoot, whereas the IAA level in the lower side of the shoot was reduced (Hellgren et al., 2004). On the other hand, support for the interaction between ethylene and auxin signalling pathways in wood formation has been provided by gene expression studies which found that several ethylene biosynthesis genes are induced by auxin in wood-forming tissues of *Populus* (Nilsson et al., 2008).

4.6 Other Hormones

In addition to auxin, GA, cytokinin and ethylene, other hormones have been detected in cambial cells, including abscisic acid (ABA). Recently, Druart et al. (2007) demonstrated that the growth cessation of cambial cells occurs before cambial ABA levels are increased, and that high levels of ABA are present in the cambial cells at the time of cambial reactivation in spring. ABA levels are lower during the active cambial growth phase in summer. As ABA is known to regulate cold acclimation (Welling et al., 2002), these results suggest that ABA could function in the regulation of cold hardiness of cambial cells, but may not have a direct role in the regulation of cambial activity.

Little is currently known about any possible function of other plant hormones during wood formation. Brassinosteroids have been identified from the cambial zone of *Pinus* trees, indicating that they may have a role in regulation of this meristem function (Kim et al., 1990), and have also been implicated in the differentiation of tracheary elements (Yamamoto et al., 1997). In the future, a major challenge will be to determine the role both of individual hormones in regulating secondary growth, and also the interaction and cross-talk between different hormones.

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Populus Responses to Abiotic Stress

Urs Fischer and Andrea Polle

Abstract In their natural habitats *Populus* trees often face rapidly as well as seasonally changing climatic conditions and especially drought and other osmotic stresses contribute globally to loss of productivity in *Populus* stands. Rich genetic variation in drought sensitivity and response make *Populus* to a valuable model genus in order to study adaptation to water stress. Here, we outline tree specific responses and the underlying hormonal signaling in response to drought stress.

1 Introduction

The adaptation and acclimation to changing environmental conditions is crucial for survival and productivity of trees. In addition to seasonal changes, trees also have to cope with unfavorable, acute weather conditions such as extended drought periods, late frost events or with gradually changing environmental conditions imposed by environmental pollution.

In most areas world-wide, water shortage is the major limiting factor for plant productivity (Chaves et al., 2009). To afford agricultural production land is being irrigated, frequently by usage of so called "grey water", which is waste water still containing relatively high concentrations of ions, or by utilizing slightly saline water in coastal areas. These irrigation practices lead to secondary soil salinization, successively rendering the land unsuitable for agriculture. Currently about 6% of the world's land area are salt affected (Munns and Tester, 2008). The devastated areas are abandoned and new land has to be gained for food production. Since this usually involves burning or cutting of natural forests, problems related to global change are increasing.

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Drought spells and their direct and indirect consequences for silviculture are not only a growing problem in arid areas but are also likely to affect temperate and boreal forests. Climate forecasts for the northern hemisphere predict significant changes in precipitation patterns with less rain in summer (Meehl and Tebaldi, 2004, Schär et al., 2004). Species in the genus *Populus* are among the most desiccation-susceptible woody taxa, but nevertheless significant genotypic variability exists, which renders them suitable to explore the molecular basis of drought tolerance (Ceulemans et al., 1978; Pallardy and Kozlowski, 1981; Gebre and Kuhns, 1991; Liu and Dickmann, 1996; Chen et al., 1997; Marron et al., 2002, 2003; Hukin et al., 2005; Monclus et al., 2006).Therefore, the focus of this overview is on drought and other osmotic stresses in *Populus*. Basic molecular and physiological signaling and defense responses will be covered briefly. Emphasis is on tree-specific responses such as restructuring of the hydraulic system and on leaf abscission and branch sacrifice as important adaptation measures to drought stress that have only received little attention to date.

2 Stress Signaling and Responses at the Cellular and Tissue Level

2.1 Cellular Consequences of Water Shortage

Water is the transport medium for nutrients and metabolites and the solvent, in which all biochemical reactions of the living cell take place. Water deficits result in dehydration and turgor loss with negative consequences for cellular functions such as membrane integrity and enzyme activities. Physiological water deficits are caused by drought stress, but also by freezing or soil salinity. Freezing often starts in the extracellular compartment where apoplastic crystallization of ice has the same effect as dry air: the water vapour is reduced and consequently, water is removed from the protoplast, which will shrink accordingly. This results in increasing cellular concentrations of salts and other solutes. Similarly, high soil salinity may also impede water uptake or even reverse water flux during events of sudden salt shock, hence, requiring osmotic adjustment to maintain water transport to the leaves. Long-term salt exposure results in excessive accumulation of Na and Cl, which require specific compartmentation to avoid ion-induced injury. We refer to recent reviews that treat these effects of salt toxicity (Munns and Tester, 2008; Chen and Polle, 2010) and focus here on the implications of the osmotic component common to drought, salinity and cold stress.

Since different stresses have similar effects at the cellular level, it is not surprising that they lead to activation of overlapping signaling and response networks to mediate acclimation (Yamagushi-Shinozaki and Shinozaki, 2006). Knowledge of the cellular pathways involved in osmotic stress signaling in *Populus* is however still very fragmentary. But – as outlined below – the basic components are conserved in land plants.

2.2 Signaling Water Limitations

An important mediator of stress responses in plants with some tree-specific features is the phytohormone abscisic acid (ABA) (Popko et al., 2010). Recently, a putative sensor of ABA has been identified in the plasma membrane that has high homology with a gene encoding Bet V allergen protein that is very abundant in trees (Ma et al., 2009). Furthermore, two homologs of the putative osmosensor *HK1* of *Arabidopsis* (Urao et al., 1999) have been identified in eucalypt and seem to play roles in transduction of dehydration signals in trees (Liu et al., 2001) (Fig. 1).

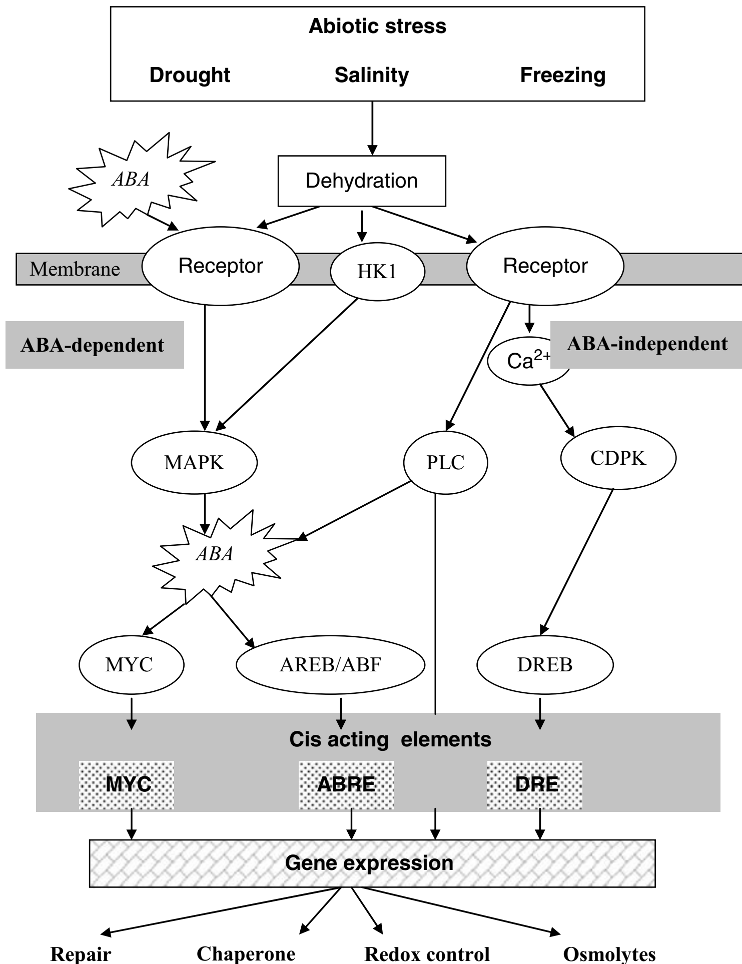

Fig. 1 Molecular responses to osmotic stress. Upon dehydration the cell activates an array of responses, starting with signaling cascades and leading to the activation of gene transcription and stress adaptation to acquire a new level of homeostasis (for details see text)

Subsequent signaling pathways involve several MAP kinases (mitogen activated like protein), phosphatases, and Ca-dependent protein kinases CDPK (Luan, 1998; Lee et al., 1999; Knight and Knight, 2001) whose activation is mediated through ABA responsive elements (ABRE) and ABA-independent motives (DRE/CRT) in their promoters (Bartels and Souer, 2004, Yamagushi-Shinozaki and Shinozaki, 2006). It has been suggested that sensitivity of these pathways may differ in tree species with different stress responsiveness. For example, different *Populus* species as well as different ecotypes of the same species originating from different climatic conditions varied in their stomatal responsiveness to ABA (Chen et al., 2002a; Yin et al., 2004; Zhang et al., 2004). Correlative evidence indicated that polyamines and ethylene acted as such modulators of the intensity of the ABA signal (Chen et al., 2002b). Hence, fine-tuning of the ABA signal leads to differences in acclimatory responses to drought in *Populus*.

2.3 Activation of Cellular Defences

Stress signaling pathways activate down-stream genes encoding enzymes for detoxification and synthesis of osmoprotectants as well as chaperones that rescue cellular proteins from deactivation (Fig. 1). "Osmoprotectants" encompass diverse chemical components such as mannitol, raffinose, galactinol, trehalose, glycine betaine, proline, etc. (Rathinasabapathi, 2000). However, in most cases their mode of action is unclear. For example, in various tree species drought or salt stress caused pronounced increases in proline (Arndt et al., 2001; Watanabe et al., 2001; Peuke et al., 2002; Sofo et al., 2004; Ottow et al., 2005). Transgenic approaches in herbaceous plants that led to increased proline concentrations enhanced their resistance against drought, salt, and cold (Kishor et al., 1995; Yoo et al., 2005). In *P. euphratica*, however, calculations showed that the accumulation of proline was insufficient to contribute substantially to the adjustment of the osmotic pressure (Ottow et al., 2005). By contrast, *P. tomentosa* overexpressing a gene encoding mannitol-1-phosphate dehydrogenase leading to elevated mannitol levels were more stress-resistant (Hu et al., 2005). Albeit, analysis of primary carbohydrates such as glucose, fructose and sucrose suggested that their accumulation in response to drought was by far more important for osmotic adjustment (Luo et al., 2009). Since compatible solutes also have free radical scavenging capacities (Shen et al., 1997a, b), they may prevent oxidative injury by osmotic stress (Shen et al., 1999). Another explanation for the protective function of these compounds is their ability to act as molecular chaperones stabilizing proteins by preventing unfolding and loss of function (Vinocur and Altman, 2005).

Transcriptional profiling has been used as another strategy to characterize osmotic stress responses. Genes encoding proteins with chaperone-like activities (dehydrins, LEA proteins, HSP proteins), repair enzymes (aldehyde and alcohol dehydrogenases), water and solute transport (aquaporins) and enzymes involved in phenylpropanoid and polyamines biosynthesis were up-regulated (Brosché et al., 2005; Ottow et al., 2005; Bogeat-Triboulot et al., 2007; Street et al., 2006). However,

it should be emphasized that transcriptional responses strongly depend on the duration and severity of stress, which is difficult to control in drought stress treatments. Proteomic analyses showed that both the dark and light reaction of photosynthesis were particularly sensitive to water limitation (Bonhomme et al., 2009). Combined proteome and transcriptome studies in trees are scarce. In *Populus*, no overlap was found for differentially drought-regulated proteins and genes (Bogeat-Triboulot et al., 2007). This might have partially been a technical problem since a 6.4 K microarray was used covering only part of the *Populus* genome. However, the result may also reflect differences in time courses of protein and RNA biosynthesis, an experience also gained with systems approaches of other model species (Gygi et al., 1999). Despite these limitations, the analysis showed that proteins of the photosynthetic apparatus were most sensitive to changes in plant water status, whereas asparagine synthetase, cold-regulated *LTCOR12*, thioredoxin H, and alcohol dehydrogenase were early drought-responsive genes (Bogeat-Triboulot et al., 2007). This suggests that adjustment of photosynthesis, regulation of the redox balance and protection of membranes are of prime importance.

Up-regulation of asparagine synthase (synonymous with glutamine dehydrogenase) was repeatedly found in salt or drought-stressed *Populus* (Brosché et al., 2005; Ottow et al., 2005; Bogeat-Triboulot et al., 2007) but also in drought-stressed *Arabidopsis* (Rizhsky et al., 2004). This observation points to an unexpected co-regulation of drought acclimation and nitrogen metabolism. Accordingly, El-Khatib et al. (2004) showed that *Populus* trees overexpressing glutamine synthetase were more drought-resistant and maintained higher photosynthetic electron transport and Rubisco activity than wild type plants. Increased stress persistence was probably caused by increased capacity for photorespiration, which might have served as a protective sink for electrons from photosynthetic reaction centres (El-Khatib et al., 2004).

Although these results shed only spotlights on cellular signaling and activation of defenses in response to osmotic stress, they underpin that fundamental patterns of stress sensing and acclimation in trees and herbaceous plants are similar and that drought tolerance has unexpected links with primary nitrogen metabolism. The few studies currently available that compared stress tolerant and sensitive *Populus* species indicate that differences in regulation of transcriptional networks exist. Our current knowledge on these differences and their relevance for stress tolerance at the cellular levels is still patchy because dehydration is a dynamic process modulated within the environmental and physiological context of the cell.

3 Tree-Specific Adaptation Measures

3.1 Growth Responses to Drought

When trees are subjected to osmotic stress, a general chronology of growth responses can be observed: usually roots continue to grow, whereas shoot growth stops (Wilkinson and Davies, 2002). Radial growth is generally more sensitive

to drought stress than shoot elongation growth, a feature observed in juvenile as well as in adult trees (Breda and Granier, 1996). Water deficit tolerance has been defined operationally as the ability to limit the decrease of biomass production in response to moderate water deficits (Passioura, 2002). Detailed analysis of the time course of drought responses in a susceptible *Populus* species showed that radial stem growth was most sensitive declining before any ecophysiological measurements indicated water deficit (Bogeat-Triboulot et al., 2007). When drought stress increased further, shoot elongation growth stopped whereas root growth continued until a severe decline in photosynthesis was found, which probably limited the supply with assimilates (Bogeat-Triboulot et al., 2007). The decline in photosynthesis is initially caused by ABA mediated stomatal closure, upon progression of desiccation stress by degradation of the photosynthetic apparatus and finally by shedding of leaves. The latter process, discussed later in this chapter, has not received much attention although it can be regarded as an adaptation measure that reduces water loss by transpiration.

It is obvious that soil water deficits have diverse effects on different tree organs. The drought responses follow a strict orchestration, whose underlying mechanisms are not yet fully understood. However, they are more diverse than reactions observed in small herbaceous plants. As they constitute important adaptation measures to maintain and regulate water uptake, transport and transpirational loss, their morphological, physiological and molecular basis will be considered in greater detail.

3.2 Adaption of the Hydraulic Architecture to Drought

With respect to the ecology of woody species, the hydraulic architecture is particularly important following climatic gradients from wet to dry conditions (Westoby and Wright, 2006; Swenson and Enquist, 2007). In angiosperms, vessel lumina are most relevant for the hydraulic characteristics because conductivity increases with the fourth power of radius and only linearly with vessel number (Tyree and Zimmermann, 2002). Smaller vessels are usually less susceptible to drought-induced xylem embolism than those with large volumes (Sperry and Tyree, 1988; Sperry and Saliendra, 1994; Hargrave et al., 1994; Lo Gullo et al., 1995; Hacke and Sauter, 1996). Species surveys show that cavitation resistance is usually correlated with wood density (Hacke and Sperry, 2001; Hacke et al., 2001, 2006). Since the transport features of embolized vessels can be restored by re-filling, drought tolerance also depends on species-specific repair capabilities (Hacke et al., 2001). Interestingly, repeated cycles of cavitation and re-filling caused cavitation fatigue in some species including *Populus*; this phenomenon probably results from rupture or loosening of the cellulosic mesh of interconduit pit membranes during the water stress and cavitation treatment (Hacke et al., 2001). Indeed, it was not possible to increase cavitation resistance of *P. trichocarpa* clones by hardening through preceding drought cycles (Harvey and van den Driessche, 1997), whereas usually hardening enhances tolerance to subsequent stress events.

Wood formation involves a strongly orchestrated sequence of molecular and physiological processes (Mellerowicz et al., 2001; Mellerowicz and Sundberg, 2008). Anatomical features and growth potential of the xylogenic cambium are genetically determined (Chen et al., 1997; Monclus et al., 2006, Novaes et al., 2009). However, the realized radial growth is highly plastic and strongly affected by environmental conditions such as nutrient availability and weather conditions (Wind et al., 2004; Luo et al., 2005; Lautner et al., 2007; Luo and Polle, 2009; Novaes et al., 2009). In particular, interannual variability in precipitation strongly influences wood formation of temperate trees and results in a close correlation between wood production and water availability (Sass and Eckstein, 1995; Garcia-Gonzalez and Eckstein, 2003; Fonti and Garcia-Gonzalez, 2004).

Wood formation is a highly carbon costly process. Therefore, drought-induced decline in photosynthesis and consequently diminished assimilate supply have negative effects on wood formation (Sauter, 2000). However, it is necessary to distinguish between long and short term effects of osmotic stress since the initial decline in radial growth precedes decreases in photosynthesis (Bogeat-Triboulot et al., 2007). Under mild water deficit cell division continues, but the expansion of vessels is strongly diminished (Arend and Fromm, 2007; Bogeat-Triboulot et al., 2007). Cell expansion depends on turgor, which is achieved by solute accumulation and cellular water uptake (Passioura and Fry, 1992; Langer et al., 2002). Therefore, already in initial stages, signals that lead to osmotic changes regulating vessel size must be important for the adaptation of the wood anatomy. This is probably also true for salt exposed trees (Junghans et al., 2006; Escalente-Perez et al., 2009). Both drought as well as salt stress result in smaller vessels but increased vessel frequencies compared with non-stressed trees (Junghans et al., 2006; Arend and Fromm, 2007). Actually, the sum of vessel lumen area remained unchanged under mild salt stress compared with non-stressed Populus because an increased vessel frequency compensated the decreased size of individual vessels (Fig. 2). Furthermore, cell wall thickness was increased leading to higher resilience of the conductive system to decreasing turgor pressure (Junghans et al., 2006). Similar structural changes were also reported for water-limited *Eucalyptus* (February et al., 1995; Searson et al., 2004) underlining the ability of trees to adapt their wood anatomy flexibly to environmental requirements.

Currently, we have only little information which molecular events and signals mediate adjustment of wood structures to external conditions. Phytohormones such as ABA and auxin play important roles in seasonal variation of wood formation (Lachaud, 1989; Aloni et al., 2000; Mellerowicz et al., 2001). Since they are also involved in mediating osmotic stresses (see above), they most likely are key components for structural flexibility of wood formation. For example, Arend and Fromm (2007) showed that drought induced changes were only significant during early but not during late wood formation, i.e., only when wide vessels and not when small vessels are formed. They speculated that the apparent differences in seasonal desiccation sensitivity might have been related to seasonal variation in sensitivity of cambial cells to ABA. Such seasonal shifts in sensitivity to ABA and also to auxin have been postulated for a long time (Lachaud, 1989).

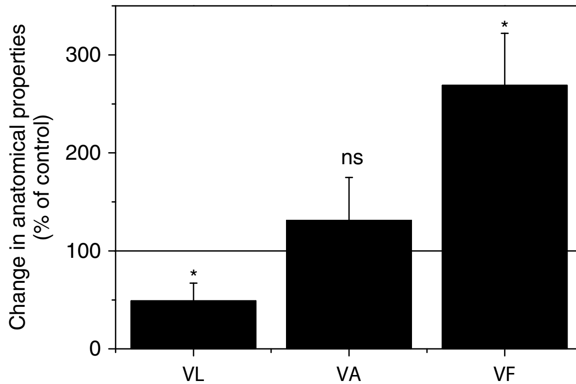

Fig. 2 Changes in wood anatomical properties in *P.* x *canescens* in response to osmotic stress. Stress was imposed by exposure by stepwise increase of NaCl to 75 mM in hydroponic solution (details, see Ehlting et al., 2007). Cross sections of xylem developed during the experimental stress exposure were analysed for luminal area of individual vessels (VL), sum of all vessel lumina per area unit (VA) and vessel frequency (VF). Changes induced by salt are shown relative to controls, which were set as 100% (*horizontal line*). *Stars* indicate significant differences from controls

In *Populus* as in other vascular plants most auxin is present in conjugated forms with amino acids or peptides that serve as a storage form (Cohen and Bandurski, 1982). Auxin amidohydrolases release active auxin from these storage conjugates. In *Populus*, moderate salt stress that led to smaller vessels resulted in decreased concentrations of auxin conjugates in the developing xylem of sensitive but not in that of salt tolerant *Populus* species (Junghans et al., 2006). Furthermore, reporter gene constructs with the auxin sensitive *GH3* promoter were activated in the cambium of salt stressed *P.* x *canescens* but not in non-stressed trees, despite high auxin concentrations in the latter tissues (Teichmann et al., 2008). These observations suggest that dynamic changes in auxin levels and changes in auxin-responsiveness and not the amount of auxin per se are part of the molecular net required to adjust wood anatomy to environmental cues. Indeed, Nilsson et al. (2008) recently showed that perturbations in auxin-responsiveness affected wood anatomy. It is obvious that future studies will require systems approaches at cell, tissue and plant level to understand the integration of environmental input signals and their transduction into adjustment of wood formation to osmotic stresses. This knowledge may aid selection programs for drought and salt tolerant tree species.

4 Abscission as a Drought Stress Avoidance Strategy

Leaf and branch abscission offer deciduous trees an additional strategy to face drought stress, compared to *Arabidopsis* and other rosette plants. Whereas *Arabidopsis* does not develop functional abscission zones, neither on petioles,

pedicels nor inflorescence stems (Patterson, 2001), many other plants have the possibility to shed organs either upon stress or seasonal signals. Leaf shedding as an adaptive measure is well known in tropical trees but has also been observed in temperate and boreal tree species (Bochert, 1998; Fort et al., 1998; Gindaba et al., 2004; Ogaya and Penuelas, 2006; Castro-Diez and Navarro, 2007). Theoretical considerations suggest that it is next to low epidermal conductance a major factor to prolong plant survival under severe drought (Sinclair, 2000).

By separating lateral organs form the hydraulic continuum, the overall transpiration of the main plant body is greatly lowered and therefore reduced xylem cavitation can be expected. Since lateral meristems are proximal to the leaf abscission zone, they remain on the growth axis after leaf shedding. In other words, lateral buds can potentially resume growth after stress induced or seasonal leaf abscission. Depending on the degree of loss of lateral organs this stress avoidance strategy is costly; however given re-establishment of growth at protected meristematic sites after a dry period, survival is achieved. Especially in trees, avoidance of xylem cavitation is of importance since water has to be transported to greater heights, and damage might not easily be overcome by root sap pressure or capillary forces as in annual plants.

In addition to seasonal leaf abscission, which depends on the perception of decreasing day-length and temperature, other environmental cues can induce leaf abscission. Exogenous application of various plant hormones indicates that downstream of such environmental cues several plant hormones have either a promoting (ethylene, ABA) or inhibiting (auxin) effect on leaf abscission (Taylor and Whitelaw, 2001; Roberts et al., 2002). An attractive model based on experiments employing explants of various herbaceous species states that an auxin gradient is spanning the abscission zone, with a maximum distally (faced to the leaf) to the abscission zone. When the auxin production of the leaf is decreasing, the gradient in the abscission zone flattens or reverses, leading to higher ethylene sensitivity of the cells in the abscission zone (Addicott et al., 1955). Ethylene is then supposed to trigger the induction or the secretion of hydrolytic enzymes, which are responsible for the spatial separation. Although this model never has been proven, it became well accepted and represents nowadays text-book knowledge (*e.g.* Taiz and Zeiger, 2002). Despite a formal proof for an instructive auxin gradient as a regulator of leaf abscission is lacking, the involvement of ethylene as a downstream signaling compound seems to be granted also for trees (Ruonala et al., 2006).

Despite ABA was discovered as an abscission inducing agent in cotton (Liu and Carns, 1961; Ohkuma et al., 1963; Addicott et al., 1968) its role during leaf abscission is still a matter of debate (Taylor and Whitelaw, 2001; Roberts et al., 2002) and the current consensus is that it affects leaf abscission indirectly by enhancing senescence. However, there is no doubt that exogenous application of ABA in many experimental systems can promote abscission (Roberts et al., 2002) and there is also support for a more direct role of ABA in cell separation processes in seeds (Sargent et al., 1984) and floral organs (Aneja et al., 1999).

4.1 Stress Induced Abscission

Chen et al. (1997, 2002b) showed that drought stress induces rapidly leaf abscission in *P.* x *euamericana* (cv. Italica) but not in *P. popularis* and that the application of ABA to the xylem sap is sufficient to induce leaf shedding in well watered plants of both species, with *P. popularis* being the less sensitive one. In *P.* x *euamericana* a sharp increase of ABA concentration in the xylem sap, which peaked 3 days after the drought stress had been initiated, preceded leaf abscission. After leaf abscission the water status of *P.* x *euamericana* plants rapidly recovered. By contrast, in *P. popularis* leaves remained on the stem during the water stress and the ABA concentration increased during the first day of treatment but rapidly reached normal levels thereafter. The authors argue that ABA levels had not reached a certain threshold or that rapid re-establishment of normal ABA levels was the cause for retention of those leaves. Since ABA application is sufficient to induce leaf abscission, ABA seems not only to be important as a short-term signal in drought stress but also directly be involved in drought induced leaf abscission for long-term adaptation.

In order to uncover molecular players Street and coworkers (2006) elegantly made use of two *Populus* species different in their reaction to drought stress. *P. trichocarpa* showed quick and dramatic necrosis 14 days after the water regime had started, whereas in *P. deltoides* necrosis proceeded slowly and leaves were not shed in consequence of drought stress. Global transcriptome analysis under drought stress revealed that differentially expressed genes are involved in ABA (one EST matching to *AREB1*) and ethylene signaling and biosynthesis (*EIN3* and an *ACC oxidase*). AREB1 is a leucine zipper transcription factor, which binds to ABA responsive elements of ABA inducible genes (Uno et al., 2000; Fujita et al., 2005). ACC oxidases (ACO) are required for the biosynthesis of ethylene and have been found to be strongly induced during ethylene burst in *Populus* (Andersson-Gunnerås et al., 2003). Under drought stress two ESTs matching to one *Populus ACO* gene model were drought-induced in the leaf-shedding species *P. trichocarpa* but not in the leaf retaining species *P. deltoides*. Even though ACC synthase (ACS) is considered as the rate limiting step in ethylene biosynthesis in *Arabidopsis* (Chae and Kieber, 2005) the above summarized data suggest that under drought stress *ACO* induction leads to increased ethylene biosynthesis and subsequent leaf abscission.

In addition to the separation of single leaves from the hydraulic continuum several *Populus* species show premature senescence of leaves and leaf abscission on whole branches and subsequently necrosis and loss of these branches (Tyree et al., 1993); a process, which is referred to as “branch sacrifice” (Rood et al., 2000). In contrast to simple leaf abscission, growth of the proximal buds can not be resumed by entering more favorable conditions. Hence, branch dieback can be considered as a more extreme form of stress avoidance than leaf abscission. Unlike during abscission removal of the dead branch at a histological separation zone is not involved. Dead branches will be retained in the crown or eventually be separated from the tree body by mechanical forces. Interestingly, the phenomenon of branch sacrifice can be observed in *P. deltoides* and *P. fremantii*, two species, which are native to

semi-arid areas and therefore supposedly well adapted to drought stress; whereas in *P. trichocarpa*, *P. balsamifolia* and *P. augustifolia* branch dieback only rarely was observed during drought periods (Rood et al., 2000). Branch dieback in *P. deltoides* and *P. fremantii* correlates with higher xylem vulnerability compared to *P. trichocarpa*, which is adapted to more humid areas (Rood et al., 2000). The paradoxon of higher xylem vulnerability in species of arid compared to humid regions indicates the importance of abscission as a mechanism for survival during drought periods.

Regulation of the water balance of a tree via branch sacrifice goes at the expense of not only annual but also perennial parts of the tree. However, one should note that the process involves leaf senescence as observed on the basis of leaf yellowing (Rood et al., 2000), and therefore a substantial amount of the leaf structure might be recycled. Branch sacrifice only is an efficient mechanism if dieback proceeds from younger, more apical to older, more basal parts of a branch. If dieback is not coordinated nutrient remobilization during the initial phase of branch sacrifice will not be yielding. Indeed it was reported that xylem vulnerability is gradually decreasing from petioles over young branches to less peripheral regions (Tyree et al., 1993).

In comparison to stomatal closure, which reduces the transpiration rate per unit leaf area, abscission or branch sacrifice might leave this rate constant. The water balance is in this case regulated by reducing the surface area of the canopy and hence the total water demand of a tree is lowered.

4.2 Abscission Induced by Seasonal Drought Stress

Leaf abscission in deciduous tress is considered as an adaptive advantage to seasonally reoccurring drought (Addicott, 1968). In temperate regions the winters are dry and with low temperature the viscosity of water in the soil increases and therefore its availability decreases. In such a niche seasonal leaf shedding is seen as a strategy to reduce leaf surface area and consequently the overall transpiration of a tree. Reduction of such an annually reoccurring drought stress goes along with carbon loss and subsequently rich stands of deciduous trees are expected to occur on nutritious soil. Indeed, geographic dominance of deciduous over evergreen stands strongly correlates with winter drought stress and rich soils (DeFries et al., 2000; Hansen et al., 2000; Givnish, 2002). The ability of shedding leaves may have evolved when trees manifested dry niches during the Cretacous (Axelrod, 1966; Addicott, 1968). Although there is considerable criticism on the relevance of seasonal drought stress as a selective force in the evolution of the deciduous character (Givnish, 2002), there is no doubt that at least coincidently leaf shedding alleviates drought stress during winter. In contrast to trees, which commonly only flower after several growth periods, annual plants can overcome seasonal dry periods by setting seeds that endure drought periods.

Primary induction of seasonal leaf abscission is given by decreasing day length and possibly decreasing temperature (Keskitalo et al., 2005; Fracheboud et al., 2009). Downstream of this primary, environmental cues, hormonal signals come into play. In birch, overexpression of the dominant allele *etr1-1* of the *Arabidopsis* ethylene receptor leads to ethylene insensitivity and strongly delayed seasonal leaf abscission (Ruonala et al., 2006). Hence, ethylene signaling seems to be required for seasonal leaf abscission. Evidence that ethylene is involved in seasonal leaf abscission also in *Populus* comes from a global gene expression analysis during autumn (Andersson et al., 2004).The expression levels of five 1-aminocyclopropane-1-carboxylate oxidase genes (*ACO*) gradually increased during natural autumnal senescence in *P. tremula* (Andersson et al., 2003). The increase negatively correlates with the temperature and precipitation; two factors, which reduce water availability and therefore contribute to seasonal drought stress.

From microarray data a hint can be gotten if during autumnal senescence *Populus* trees are indeed experiencing drought stress. Tables 1 & 2 show a comparison of the above mentioned experiments of Andersson et al. (2004) and Street et al. (2006), listing the overlap of significantly regulated ESTs during autumnal senescence in *P. tremula* and of regulated ESTs during drought stress in *P. deltoides* and *P. trichocarpa*. The overlap between the seasonal and drought stress regulon is surprisingly strong (Tables 1 & 2), i.e. 229 and 383 ESTs of a total of 13490 were up-regulated during drought and season, respectively; of which 31 ESTs were up-regulated in both experiments (Table 1). The expected random overlap for the same number of regulated genes is only 6.5 ESTs (13490*(229/13490)*(383/13490)), while for the down-regulated ESTs 2.4 hits are expected and 22 ESTs were indeed identified (Table 2). Most of the down-regulated ESTs of both experiments match to genes involved in both the light and dark reaction of photosynthesis. While this outcome is expected for the seasonal regulation, other microarray experiments and proteomics studies support the reduction of components of photosynthesis under drought stress (Bogeat-Triboulot et al., 2007; Plomion et al., 2006; Xiao et al., 2009). Interestingly, 13 of the 22 commonly down-regulated EST encode for proteins, which will be transported to the plastid suggesting intense communication between plastids and nucleus during drought as well as autumnal senescence.

Among the ESTs induced during both drought stress and seasonal senescence several map to *Arabidopsis* gene models, which have been evidentially associated to drought stress. The two transcription factors *AtAF1* and *AtHB-12* have been described as inducible by ABA and drought stress (Lu et al., 2007; Olsson et al., 2004). Interestingly single mutants in these loci render the plants hyposensitive towards ABA, indicating that they function in the ABA dependent drought response (Yamaguchi-Shinozaki and Shinozaki, 2006). Also *HISTONE1-3* (*HIS1-3*), a divergent but in plants conserved *HISTONE1*, is in *Arabidopsis* inducible by drought and ABA (Ascenzi and Gantt, 1997 and 1999). These findings strongly suggest that *Populus* trees experience drought stress via ABA signaling during natural seasonal senescence.

Most prominently represented among the co-regulated genes during drought stress and seasonal senescence are the metallothionein (MT) proteins, which have

Table 1 ESTs significantly up-regulated during autumnal senescence (*P. tremula*; Andersson et al., 2004) and drought stress (*P. trichocarpa* and *P. deltoides*; Street et al., 2006)

EST[a]	Arabidopsis gene model[b]	Description/Function
Regulation		
PU00285	At1g51200	zinc finger (AN1-like) family protein
PU10362	*AtAF1*	NAC DNA binding domain; negative regulation of abscisic acid mediated signaling
PU09347	*AtHB-12*	homeodomain leucine zipper class I; response to ABA stimulus and drought stress
PU02852	At3g61260	DNA-binding family protein/remorin family protein
PU03690	*HIS1-3*	structurally divergent linker histone, induced by dehydration and ABA.
PU10820	*CIPK10*	CBL interacting kinase
PU03431	*AtCBL9*	Calcineurin B-like calcium sensor protein; drought stress induced
PU03538	*STRS1*	RNA helicase; suppression of stress response
PU09968	At4g32250	protein kinase family protein
Cytoskeleton		
PU10724	*AtMAP70-4*	microtubule associated protein
PU04900	*AtG8F*	autophagy; microtuble binding
PU08221	*ERD10*	actin binding; response to dehydration
Metabolism		
PU02043	*AtNADP-ME4*	malate oxidoreductase
PU05029	*AtNADP-ME4*	malate oxidoreductase
PU05169	*AtNADP-ME4*	malate oxidoreductase
PU01406	*AtUGT85A4*	UDP-glucosyl transferase
PU03376	*AtSRG1*	member of the Fe(II)/ascorbate oxidase gene family; senescence-related gene
PU11611	*LKR/SDH*	lysine-ketoglutarate reductase
Other		
PU08542	*GUT15*	non-coding RNA
PU03101	At5g65520	unknown
PU10963	*ELIP1*	chlorophyll binding
PU10994	At2g04620	cation efflux family protein
PU10380	*AtMT-1*	metallothionein protein; limiting oxidative damage
PU10607	*AtMT-1*	metallothionein protein; limiting oxidative damage
PU03887	*AtMT-1*	metallothionein protein; limiting oxidative damage
PU11789	*AtMT-1*	metallothionein protein; limiting oxidative damage
PU10863	*AtMT-3*	metallothionein protein; limiting oxidative damage
PU05599	*AtMT-3*	metallothionein protein; limiting oxidative damage

[a]ESTs refer to POP1 arrays and the corresponding *Populus* and *Arabidopsis* gene models can be retrieved from the PopulusDB, http://www.populus.db.umu.se/.
[b]Information about *Arabidopsis* gene models retrieved from *Arabidopsis* Information Resource (TAIR), www.arabidopsis.org/.

Table 2 ESTs significantly down-regulated during autumnal senescence (*P. tremula*; Andersson et al., 2004) and drought stress (*P. trichocarpa* and *P. deltoides*; Street et al., 2006)

EST[a]	Arabidopsis gene model[b]	Description/Function
Photosynthesis		
PU00298	*LHCB3*	light-harvesting chlorophyll b binding protein
PU08604	*PSBQ-2*	PsbQ subunit of PSII
PU08719	*LHB1B1*	light-harvesting chlorophyll-protein complex II subunit B1
PU09731	*LHCA1*	light harvesting complex of PSI
PU10089	*LHCB6*	light harvesting complex of photosystem II
PU10383	*PSAO*	subunit O of photosystem I
PU08681	*HEMA1*	glutamyl-tRNA reductase; chlorophyll biosynthesis
PU09533	*CH-42*	subunit of a magnesium chetalase; chlorophyll biosynthesis
PU10714	*RBCS1A*	ribulose bisphosphate carboxylase small chain 1A
PU11249	*RCA*	rubisco activase
PU08539	*RBCS1A*	ribulose bisphosphate carboxylase small chain 1A
PU11749	*RPL4*	plastid ribosomal protein L4
PU12504	*ATPD*	chloroplast ATPase delta-subunit
Regulation		
PU08670	At5g04840	bZIP protein
PU09075	At1g08010	zinc finger (GATA type) family protein
PU09378	At1g55150	DEAD box RNA helicase
PU09599	*RBR*	retinoblastoma homologue
PU08668	*UBP8*	ubiquitin specific protease
PU08561	*CRT1*	calreticulin
PU02810	At3g13470	chaperonin
Other		
PU08461	At4g19160	unknown protein
PU08710	At2g34430	unknown protein

[a]ESTs refer to POP1 arrays and the corresponding *Populus* and *Arabidopsis* gene models can be retrieved from the PopulusDB, http://www.populus.db.umu.se/.
[b]Information about *Arabidopsis* gene models retrieved from *Arabidopsis* Information Resource (TAIR), www.arabidopsis.org/.

already been linked to drought stress in *Populus* (Brosché et al., 2005; Plomion et al., 2006; Bogeat-Triboulot et al., 2007) and furthermore to senescing tissue in various species (Buchanan-Wollaston, 1994; Butt et al., 1998; Mir et al., 2004). MTs limit oxidative damage to DNA and other molecules and are supposed to be involved in the translocation of heavy metals in senescing tissue (Mir et al., 2004). Both roles seem to be relevant in the proposed mechanism of leaf shedding as a drought stress avoidance mechanism.

Beyond all questions the cytoskeleton plays an important function during drought stress (e.g. change in cell turgor) and autumnal senescence (e.g. breakdown of cellular structure). Indeed Xiao et al. (2009) identified lower abundance of an alpha-tubulin during drought stress. The comparison of the drought stress and the autumnal senescence regulon (Table 1) reveals further overlap of ESTs linked to

the cytoskeleton. Two ESTs for microtubule binding proteins and also one EST for an actin binding protein are co-regulated during drought stress and autumnal senescence (Table 1). One of these ESTs matches with the microtubule binding protein AtG8F, which is involved in autophagy, a process, which ensures recycling of cytoplasmic components via transport in membrane surrounded compartments into vacuoles or lysosomes (Contento et al., 2005). The other microtubule binding protein is of unknown function in *Arabidopsis*, while the actin binding protein ERD10 is a member of the divergent protein families of dehydrins and late embryogenesis-abundant (LEA) proteins, which are induced by various dehydration stressors via ABA signaling (Rorat, 2006).

Co-regulated ESTs for homologs of *AtCBL9* (*CALCINEURIN B-LIKE PROTEIN 9*), *CIPK10* (*CBL-INTERACTING PROTEIN KINASE 10*) and the calreticulin *AtCRT1* indicate an involvement of calcium signaling during autumnal senescence and drought response. Calreticulin is involved in homeostasis of Ca^{2+} (Persson et al., 2001) and protein folding (Christensen et al., 2008), whereas the calcium sensor protein AtCBL9 and CIPK10 are involved in calcium signaling.

In summary, co-regulation of EST during drought and autumnal senescence can be abundantly identified. Major groups of this co-regulon are genes involved in photosynthesis, regulation of cytoskeleton, calcium signaling and various transcription factors (Fig. 3). The surprisingly high number of genes, which are co-regulated during seasonal senescence and drought stress, indicates that during these processes similar biochemical events and hormonal signaling take place. Interestingly, many ABA-regulated drought specific genes are up-regulated during seasonal senescence, revealing drought stress during this period and a central role for ABA signaling during seasonal senescence.

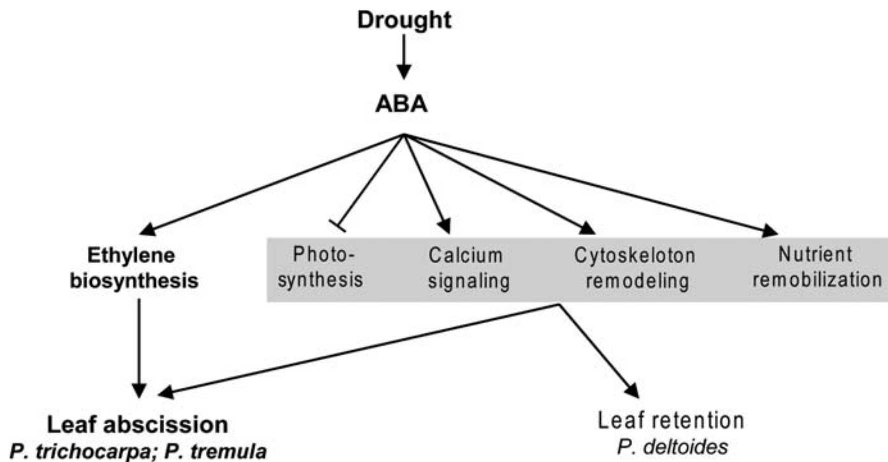

Fig. 3 Model of hormonal signaling during drought stress in leaf shedding and leaf retaining *Populus* species

5 Conclusions

Increasing demand on sustainable wood biomass requires that the yield of existing stands is improved and that new, less favorable areas are exploited in order to grow trees. Loss due to abiotic stress, especially osmotic stress, counters these strategies and therefore the study of responses to abiotic stressors is essential. Although valuable information from the study of annual species can be obtained, tree models need to account for responses specific to perennials. Among the perennial-specific responses leaf abscission stands out as efficient strategy for survival under severe drought stress. In climates with occasional severe droughts, trees sensitive to abscission could be beneficial. For less extreme climates however, engineering or breeding of trees with increased drought tolerance, but not involving abscission, is more promising. Molecular targets are plentiful and have been listed in this review.

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Defense and Nutrient Mutualisms in *Populus*

George Newcombe, Francis Martin, and Annegret Kohler

Abstract The effects of symbionts on plant defense and nutrition are increasingly seen as significant but neglected factors in plant ecology. The roles of some endophytic and mycorrhizal fungi associated with *Populus* are beginning to be addressed with advanced tools of genetics and genomics. At the same time previously unknown *Populus* symbionts are still being discovered and described. In this chapter, defense and nutrient mutualists of *Populus* are reviewed in the context of the wider literature, and with an eye towards potential applications to poplar culture.

1 Introduction

Interactions between plants and fungi are now studied at expanding scales from molecular mechanisms to the ecosystem level (Glazebrook and Ton, 2007). In this review we will highlight recent research on two groups of symbionts that appear to be able to increase host plant growth and tolerance of biotic and abiotic stress: endophytic and mycorrhizal fungi. Both are relevant to discussion of *Populus* as a model with expanding implications for woody plant biology (Bradshaw et al., 2000; Bradshaw and Strauss, 2001; Brunner et al., 2004).

Populus and *Salix* diverged 60–65 mya (Tuskan et al., 2006), but there are still many parallels between them in their fungal symbionts and much of what follows will likely apply quite well to *Salix*. Although some genera of pathogenic fungi are exclusive to either *Populus* or *Salix* (e.g., the specialization of *Rhytisma* on *Salix* but not *Populus*, or the specialization of *Taphrina* on *Populus* but not *Salix*), commonalities are likely to emerge from comparative studies of the symbiont communities of each. A further disclaimer is that this chapter will not reiterate fairly

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recent reviews of *Melampsora* (Pei and McCracken, 2005) and other pathogens of *Populus* (Newcombe, 1996; Newcombe et al., 2001). Our focus here is instead on mycorrhizal and endophytic fungi that function as nutrient and defense mutualists or that play still undetermined roles in the ecology of species of *Populus*. Rather than starting with the symbionts of *Populus*, we will start with the wider literature because it reveals the many directions that *Populus* symbiont research might take.

A striking example of fresh momentum in endophyte research involves infection with a specific fungus that can make the difference between life and death for its host plant attempting to grow in geothermal soils (Redman et al., 2002). Endophytes may assume roles as defense or protection mutualists by deterring insect pests (Clark et al., 1989; Calhoun et al., 1992; Wilson and Carrol, 1997; Wilkinson et al., 2000; Miller et al., 2002) and by reducing disease severity (Clay, 1988; Liu et al., 2001; Arnold et al., 2003; Wicklow et al., 2005; Clarke et al., 2006; Ganley et al., 2008). Well known endophytes in grasses deter mammalian herbivores (Clay and Schardl, 2002), but this deterrence is now known to operate in other plants as well (Valdez Barillas et al., 2007). *Muscodor albus*, an endophyte of *Cinnamomum zeylanicum*, provides perhaps the most dramatic example of a defense mutualist by killing most fungal and bacterial pathogens of its host with a mixture of volatile compounds (Strobel et al., 2001).

Even though some endophytes may be mutualists that confer protection or some other benefit on their hosts in exchange for photosynthates and microhabitat (Kogel et al., 2006), others may be parasites or commensalists (Stone et al., 2000), and this idea of a continuum in interactive ‘lifestyles’ has been applied to mycorrhizas as well (Jones and Smith, 2004). Endophytes have been variously defined, but most commonly they are symbionts colonizing and living within an apparently healthy plant (Stone et al., 2000). Of course, candidate mutualists such as endophytic and mycorrhizal fungi are by no means the only classes of symbionts to interact with a plant. Pathogens, insect herbivores, pollinators, rhizobacteria, and bacterial shoot endophytes also affect host growth and fitness. Symbiont classes not only interact indirectly with one another via induced defense signaling that will be discussed at greater length, but they may also interact directly. Interactions of the symbiont community of a plant that are mediated entirely or in part by the plant immune system have been termed the ‘interactome’ (Glazebrook and Ton, 2007), and we will use this lense to review what we know currently of the community of fungal symbionts of *Populus*.

2 Ecological Relevance

Interactions between plants and symbionts are now studied primarily at two levels: (1) the molecular level of signaling networks and gene expression related to defense and nutrient transfer, and (2) the ecological level of pot and field experiments. It has to be said at the outset that the ecological relevance of endophytes and even mycorrhizal fungi at the field level is somewhat contentious. On the one hand, in asking

whether endophytes of forest trees are mutualists, one recent reviewer emphasizes the impossibility of producing the adult, endophyte-free trees that are needed for comparative purposes (Sieber, 2007); he regards this difficulty as one that blocks proof of mutualism. "Mutualism of tree endophytes is often assumed, but evidence is mostly circumstantial" (Sieber, 2007). Exactly the same point about controls is made in a review focused on the question of whether mycorrhizas are always mutualists (Jones and Smith, 2004). In reviewing the literature on the question of whether arbuscular mycorrhizal (AM) fungi alter plant-pathogen interactions, another author laments the dominance of studies with few, economically important plant species growing in low-phosphorus soil in the greenhouse (Borowicz, 2001). Even with simplified, model systems, it has proven difficult to generalize about the effects of AM fungi on interactors such as pathogens because host genotype, the species of pathogen, and AM species and strain are all significant variables that have not been fully explored (Borowicz, 2001). Thus, these authors and others are concluding that mycorrhizas may actually vary "along the continuum from parasitism to mutualism" (Jones and Smith, 2004), and the same conclusion, albeit with less supporting evidence, has been posited to apply to endophytes (Schulz and Boyle, 2005).

On the other hand, a strikingly divergent view highlighting the importance of plant-fungal symbiosis is apparent in the literature. At this end of the spectrum of interpretation, endophytes can be seen as determinants of plant community structure (Clay and Holah, 1999), or as controlling elements in insect host-parasite interaction webs (Omacini et al., 2001). According to the habitat-adapted symbiosis hypothesis, endophytes are essential to plant establishment in high-stress habitats (Rodriguez and Redman, 2008). Mycorrhizal fungal diversity may determine "plant biodiversity, ecosystem variability and productivity" (van der Heijden et al., 1998). The extraradical mycelial networks of mycorrhizal fungi have been described as "networks of power and influence" and supporting evidence for their role in "controlling plant communities and agroecosystem functioning" is considerable (Leake et al., 2004). Further, the importance of the mycorrhizal symbiosis has been integrated into one widely accepted definition of mycorrhizas: as symbiotic associations "essential for one or both partners, between a fungus (specialized for life in soils and plants) and a root (or other substrate-contacting organ) of a living plant, that is primarily responsible for nutrient transfer" (Brundrett, 2004).

Diametrically opposed to the views in the preceding paragraph is the position that plant symbionts are essentially irrelevant. For instance, symbiotic contributions to the discipline of plant stress physiology have largely been ignored by plant biologists (Rodriguez and Redman, 2008). Similarly, symbiotic contributions to resistance to plant pathogens and other biotic stresses are frequently lacking in reviews of this subject. Most research in plant ecology is conducted as if endophytes did not exist, and frequently, mycorrhizal fungi are ignored as well. So, the middle-ground view of the ecological relevance of endophytic and mycorrhizal symbionts is actually held by those symbiologists who maintain that ecological relevance is still a good question without a convincing answer (Jones and Smith, 2004; Sieber, 2007).

In contrast, the ecological relevance of other plant symbionts such as pathogens and insect herbivores is unquestioned. Not only is "40% of all world food production" lost to pathogens, insects and competing vegetation (Pimentel, 2008), but crop losses due to plant pathogens in the United States alone are valued at $21 billion annually (Rossman, 2009). Moreover, losses due to pathogens are certainly not restricted to agricultural ecosystems. Forests in the original range of the American chestnut, *Castanea dentata*, were notoriously transformed by an introduced pathogen (Paillet, 2002) with huge, collateral effects on other tree species (McGormick and Platt, 1980) and even extinctions of chestnut-dependent organisms (Opler, 1978). Concerns about pathogens in forest tree plantations are based on everything from decreased volume growth (Bendz-Hellgren and Stenlid, 1997) to mortality (Newcombe et al., 1994). We could go on but our point here is to emphasize that balanced mutualisms can be based on defense against organisms that are already known to seriously reduce the fitness of the host.

3 Defense Mutualisms

So, what could symbionts contribute to plant defense? Whereas nutrient mutualisms between plants and fungi may be confined to mycorrhizas (Brundrett, 2004) with notable endophytic exceptions (Doty et al., 2005), both endophytes and mycorrhizas may potentially be involved in plant defense mutualisms. By priming plants for rapid response to pathogens or herbivores (Beckers and Conrath, 2007), and by activating defense signaling pathways, both endophytes and mycorrhizas may improve host fitness in exchange for photosynthates.

Transmission of defense mutualists. Symbiont-mediated protection is not necessarily restricted to vertically transmitted organisms (Haine, 2008), at least in plants where such transmission from one generation to the next is through seed. Examples of mycorrhiza- and endophyte-mediated protection abound for fungi that are horizontally transmitted and must therefore infect each new plant generation (Arnold et al., 2003; Waller et al., 2005; Newcombe et al., 2009). We also know that the line between defense mutualists and pathogens may be thin, as mutation at a single locus converted a pathogenic *Colletotrichum* into a defense mutualist (Freeman and Rodriguez, 1993; Redman et al., 1999). And conversely, an endophytic mutualist in grasses, *Epichloë festucae*, was converted into a pathogen by a single-copy plasmid insertion in the NADPH oxidase gene, *noxA* (Tanaka et al., 2006).

Piriformospora indica. A model defense mutualist has emerged in the last decade, in part because it interacts with *Arabidopsis*. This fungus, *Piriformospora indica*, has been called a root endophyte (Varma et al., 1999; Waller et al., 2005; Deshmukh et al., 2006) but *P. indica* actually belongs to a broadly mycorrhizal order, the Sebacinales (Weiss et al., 2004). This root-colonizing mutualist can function as a symbiont of *Populus* as well as *Arabidopsis* and a wide range of other plants that host arbuscular mycorrhizal fungi. This native of the Thar desert of Rajasthan in India (Verma et al., 1998) was shown early to have attributes beyond defense; it

doubled root and shoot biomasses of *Populus tremula* as well as maize, tobacco and other plants (Varma et al., 1999). *Piriformospora indica* also promoted adventitious root formation of cuttings (Druege et al., 2007), and amazingly it reprogrammed barley to salt-stress tolerance, disease resistance and higher yield (Waller et al., 2005). Not only was growth of *Arabidopsis* improved (Peskan-Berghofer et al., 2004), perhaps through production by *P. indica* of auxin (Sirrenberg et al., 2007), but its seed production (i.e., its fecundity) was increased as well (Shahollari et al., 2007). Flowering of yet other plants was enhanced (Rai et al., 2001). As a defense mutualist, *P. indica* induced systemic resistance in *Arabidopsis*, by activating the defense pathway characterized by jasmonic acid signaling (i.e., induced systemic resistance or ISR) (Stein et al., 2008).

Perhaps the most provocative result thus far in this research is the discovery that hosts of *P. indica* may need leucine-rich repeat proteins to recognize this fungus (Shahollari et al., 2007). The most closely related homologue of the protein in question, pii-2, is a protein thought to be associated with disease resistance in rice. Major genes for resistance to pathogens, or *R* genes, frequently encode protein products characterized by nucleotide binding site (NBS) and leucine-rich repeat (LRR) domains associated with recognition of pathogen effectors (Jones and Dangl, 2006). Prior to the research with *P. indica* we had learned that genes for resistance to powdery mildew modulated mycorrhization by *Glomus mosseae* (Ruiz-Lozano et al., 1999). With *Populus* we also know that quantitative trait loci related to ectomycorrhiza formation can at least be linked to genes associated with resistance to *Melampsora* (Tagu et al., 2005). So, although it might thus be tempting to think that *R* genes are broadly involved in recognition of mutualists as well as parasites (i.e., symbiont recognition genes), given the topic of this chapter and the fact that *Populus* possesses more than 400 NBS genes (Tuskan et al., 2006; Kohler et al., 2008), that tentative inference has to be considered in the context of what is called the plant immune system. The latter is presumably the central mechanism of the interactome, and the primary means by which plant symbionts interact with one another.

The plant immune system. Though they both interact with the plant immune system, defense mutualists and pathogens do somehow bring about different symbiotic outcomes within plants (i.e., balanced and unbalanced, respectively) (Jones and Dangl, 2006). The reason for the different outcomes will hopefully emerge in time with an improved understanding of the plant immune system. Currently, we know that plants first respond to pathogen-associated molecular patterns, or PAMPs, that are generated by pathogens attempting to colonize plants (Bittel and Robatzek, 2007). It appears that MAMPs, or microbe-associated molecular patterns, of defense mutualists are essentially the same as PAMPs (Van Wees et al., 2008) in that they may be cell wall constituents common to all microbes. Or, MAMPs may be small, secreted proteins, or antibiotics. Pathogens may then produce effectors that can suppress a second, stronger immune response from the plant that will follow the first unless the plant recognizes the effector or its activities. The first, most basic, immune response is termed pathogen-triggered immunity whereas the second, stronger response is effector-triggered immunity (Jones and Dangl, 2006), and

it is the latter that can result in the programmed cell death that is known as the hypersensitive response, that is traditionally associated with *R* genes.

Defense signaling pathways. Perception of the intruder meanwhile activates defense signaling pathways leading to different sets of defense genes (Pozo and Azcon-Aguilar, 2007) that may modulate one another via 'cross-talk'. If expressed systemically, defense reponses may link aboveground symbionts with belowground counterparts (Erb et al., 2008). Salicylate-dependent, systemic acquired resistance (SAR) tends to be effective against biotrophic pathogens (i.e., obligate parasites of living host cells and tissues) whereas jasmonate-dependent, induced systemic resistance (ISR) tends to work against necrotrophs (i.e., parasites that kill and then consume host cells and tissues). As biotrophs, arbuscular mycorrhizal (AM) fungi may repress SAR while enhancing ISR, and this modulation of these two pathways could possibly explain both greater susceptibility of aboveground tissues of AM plants to biotrophs and reduced susceptibility to necrotrophs and generalist insects (Pozo and Azcon-Aguilar, 2007). Variations on this type of symbiont interaction are common however. It is now known that specific AM fungi are regulated differentially (Scervino et al., 2005). It is also important to bear in mind that signaling pathways are by no means restricted to the two that we have mentioned thus far (Glazebrook and Ton, 2007). Volatiles may even prime neighboring plants for faster defense responses (Pieterse and Dicke, 2007). In particular, it is becoming apparent that the reactive oxygen gene network of plants is extensive (i.e., 152 genes in *Arabidopsis*), and that reactive oxygen species (ROS) may be key regulators of mutualistic interactions (Tanaka et al., 2006; Rodriguez and Redman, 2008).

Commonalities among symbioses may include the *in planta* secretion of effectors or virulence factors by pathogenic and mutualistic fungi alike. The ectomycorrhizal fungus, *Laccaria bicolor*, produces myorrhiza-induced, cysteine-rich, small, secreted proteins (MISSPs) that might act as "effector proteins to manipulate host cell signaling or to suppress defense pathways during infection" (Martin et al., 2008). The ascomycetous genus *Fusarium* also possesses genes with similarities to "known virulence factors" (Cuomo et al., 2007). Of course, this might seem unsurprising because *Fusarium* is known primarily for its pathogenic members. However, endophytic isolates of *Fusarium* are quite common as well (Kuldau and Yates, 2000), so it is possible that all three groups (i.e., pathogens, mycorrhizal fungi and endophytes) produce effectors.

Symbiont interactions. Symbiont interactions can be direct. An obvious example is the mycoparasitism of *Melampsora* rust fungi on *Populus* by another fungus, *Eudarluca caricis* (Nischwitz et al., 2004), and many others undoubtedly remain undiscovered. But symbiont interactions mediated by the host plant must be much more common in nature than direct interactions, and few examples and no generalizations can be cited at this point in time. Grass endophytes, for example, are known to reduce mycorrhization by AM fungi (Omacini et al., 2006; Antunes et al., 2008) and this, in turn, would affect additional symbionts, perhaps via modulation of signaling pathways as just outlined. Endophyte-infected plants differ biochemically in many ways from endophyte-free plants (Rasmussen et al., 2007; Rasmussen et al.,

2008) so the mechanism by which endophytes reduce mycorrhization is not clear although it is assumed to involve allelochemicals (Antunes et al., 2008).

Life cycles of defense mutualists. Horizontally transmitted symbionts may spend part of their own life cycles outside the tissues of their hosts. Many fungi that are isolated as endophytes of root and/or shoot tissues are also found in the rhizosphere (Koike et al., 2001; Djonovic et al., 2006), and some of them promote growth and also induce resistance. For example, species of *Trichoderma* with known potential to reduce the effects of plant diseases (Harman et al., 2004) are often isolated either as endophytes or as rhizosphere inhabitants. *Trichoderma virens* can play a variety of roles in a plant symbiont community because it can act as a mycoparasite, induce systemic resistance, and combat other fungi at a distance with antibiotics (Djonovic et al., 2006). A small protein, designated as Sm1, is produced by *T. virens*. Sm1 induces expression of defense-related genes and it also stimulates production of ROS in a diverse array of plants (Djonovic et al., 2006). As such, Sm1 appears to be more than a MAMP. Yet other endophytic *Trichoderma* isolates also appear to be able to induce defense responses that then inhibit pathogens of cacao (Bailey et al., 2006).

A web of symbiont interactions. Trichoderma species are also known to interact with arbuscular mycorrhizal fungi that can themselves induce resistance to pathogens (Pozo and Azcon-Aguilar, 2007). Interactions in root-free soil compartments between *Glomus intraradices* and *Trichoderma harzianum* resulted in suppression of the latter (Green et al., 1999), which is a reversal of suppression of *Glomus* by grass endophytes in the first interaction mentioned in this section. It appears possible that the same symbiont could interact with a plant and the rest of its symbiont community in one manner as a rhizosphere inhabitant and in another as an endophyte.

Similarly, both microbial products and symbiont-induced, plant products may serve more than one role in symbiont interactions. 2,4-diacetylphloroglucinol (DAPG) is an antibiotic produced by *Pseudomonas fluorescens*. An antibiotic, of course, may be used by one symbiont against another, but in this case DAPG also induces ISR that is dependent on both jasmonic acid and ethylene (Iavicoli et al., 2003). In the grass endophyte system in which endophyte-produced alkaloids are key (Clay and Schardl, 2002), damage to simulate vertebrate grazing reduces resistance to aphids, but a *Neotyphodium* endophyte in *Festuca arundinacea* can increase resistance following damage (Bultman et al., 2004). Chitinase production is induced in pine by endophytes (Pirttila et al., 2003) and by ectomycorrhizal fungi (Sauter and Hager, 1989) but the possibility that chitinase might play differential roles has not been explored.

Eventual applications of defense mutualists in *Populus* will likely affect transcriptional responses to *Melampsora* that have thus far been determined in their absence (Miranda et al., 2007; Rinaldi et al., 2007). The tools to test this hypothesis are in place as some naturally occurring endophytes from *P. trichocarpa* that can reduce rust severity have now been isolated (Fig. 1; Raghavendra and Newcombe, unpublished). Populus mutualists that activate jasmonate-dependent signaling are

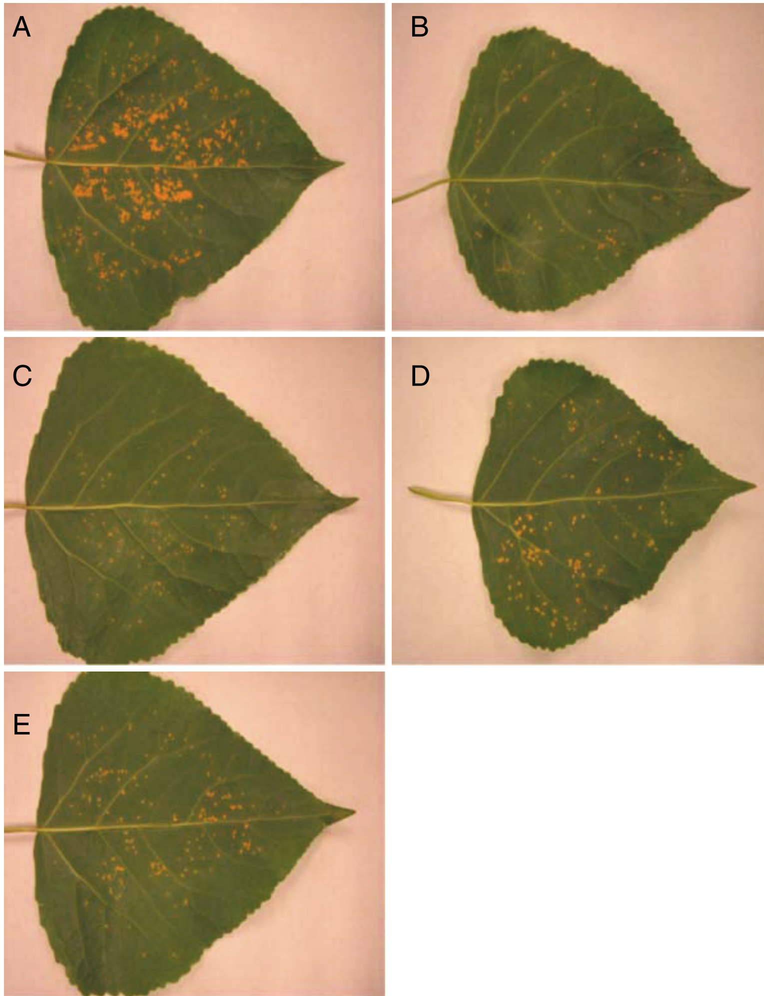

Fig. 1 Endophyte-mediated resistance to *Melampsora* leaf rust in a hybrid poplar clone. (**a**) Control leaf that was inoculated with *Melampsora* x c*olumbiana* without having first been inoculated with one of four endophytes (**b-e**) that reduced rust severity (i.e., uredinial density) (**b**) *Stachybotrys* sp (**c**) *Trichoderma* sp. (**d**) *Truncatella* sp. (**e**) *Ulocladium* sp

likely to induce changes in carbon transport and partitioning that could not only directly affect herbivory but many other interactions (Babst et al., 2005). Systemic defense signaling is known to occur in Populus (Major and Constabel, 2007).

To re-emphasize the complexity of symbiont interactions, it is now clear that specific organismal and functional groups are not homogeneous with respect to their interactions with plants. For instance, all plant growth-promoting rhizobacteria do not induce resistance to pathogens in the same manner (Wang et al., 2005).

Rhizosphere bacteria also do not all affect metal uptake by plants in the same manner; only specific bacteria in the rhizosphere of *Salix caprea* improved metal uptake (Kuffner et al., 2008), an important issue in phytoremediation. Or, whereas it was once thought that necrosis-inducing pathogens generally trigger SAR (Felton et al., 1999), it is now known that *Piriformospora indica* induces necrosis (Deshmukh et al., 2006) but not SAR. Instead, the resistance to pathogens that *P. indica* confers to its hosts is jasmonate-dependent and thus an example of ISR (Schafer et al., 2007). So, rather than generalizing from single examples from model systems, one might justifiably question, for instance, whether yeast increases resistance to bacterial diseases and necrotrophic fungal diseases in general, or whether this effect is seen in *Arabidopsis* alone versus specific bacteria and fungi (Raacke et al., 2006). Since virtually all plants in nature are infected with both endophytic and mycorrhizal symbionts, it will be important in coming years to determine under realistic conditions the net effects on host fitness of the entire complex of interactions among endophytes, mycorrhizas, pathogens and other symbionts.

4 Mycorrhizal Fungi

Ectomycorrhizal versus arbuscular mycorrhizal symbionts. *Populus* is among the woody plant lineages that form both ectomycorrhizae (EM) (Figs. 2, 3 and 4) and arbuscular mycorrhizae (AM) in "tripartite" associations (Lodge, 2000). *Populus* was thus a good candidate for sequencing of both its own genome (Tuskan et al., 2006) and the genomes of *Glomus intraradices* and *Laccaria bicolor*, representing the AM and EM fungi, respectively (Martin et al., 2004). Coincidentally, many plant taxa that can form tripartite associations are commercially important for forestry: *Salix*, *Eucalyptus*, *Alnus*, *Acacia*, and *Casuarina* (Molina et al., 1992; Brundrett et al., 1996; Lodge, 2000). Now we also know that a third mycorrhizal interaction type is also possible for *Populus* (Kaldorf et al., 2005) although interactions with *Piriformospora indica* have not yet been observed in nature. Future surveys might reveal other members of the Sebacinales that do naturally form mycorrhizae with *Populus*. Broad receptivity to mycorrhizal associations increases the number of symbionts that associate with *Populus*. Furthermore, the dark septate endophytes of the *Phialocephala fortinii* s.l. – *Acephala applanata* species complex are common in many woody plants of the northern hemisphere (Grünig et al., 2008), yet still largely unexplored in *Populus*.

The capacity of *Populus* species to simultaneously interact with several symbiotic partners from both groups of AM and EM fungi introduces a unique dilemma; detecting and punishing those exploiting the mutualism becomes increasingly difficult if these individuals can continue to access resources from alternative sources. Although both environment and genetics have been shown to affect the mycorrhizal colonization of host plants, the impacts of these factors on hosts that can be dually colonized by both EM and AM fungi are less understood. Gehring et al. (2006)

Fig. 2 Seedlings of *Populus tremula x alba* colonized by the ectomycorrhizal fungus *Pisolithus tinctorius* in vitro. Lateral roots ensheathed by the mycobiont are *boxed*. Photos courtesy of A Jambois (© INRA)

examined the influence of environment and host crosstype on the EM and AM colonization of cottonwoods (*Populus angustifolia* and natural hybrids) by comparing levels of colonization of trees growing in common gardens that differed in elevation and soil type. Environment, particularly soil moisture, has a larger influence on colonization by AM versus EM fungi than host genetics, and suggest that environmental stress may be a major determinant of mycorrhizal colonization in dually colonized host plants. A shift in abundance between AM and EM associations has been observed during floodplain succession along river shores (Piotrowski et al., 2008). The peak in AM fungi infectivity and hyphal length during early succession suggests that regular flooding and establishment of new sites promotes AMF abundance in this ecosystem. Similarly, AMF surveys in *Populus-Salix* stands along complex floodplain gradients of the Verde River (Arizona, USA) (Beauchamp et al., 2006) showed that AM species richness declined with stand age and distance from and elevation above the channel and was positively related to perennial species cover and richness and gravimetric soil moisture. Distance from and elevation above the active channel, forest age, annual species cover, perennial species richness, and exchangeable potassium concentration all played a role in structuring the AMF community in this riparian area. Regulation of rivers that eliminates creation of new sites may reduce contributions of AMF to riparian areas.

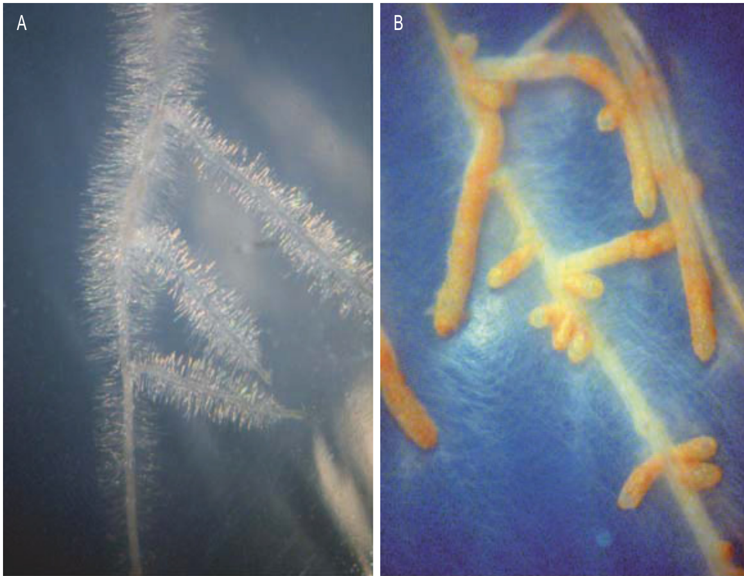

Fig. 3 The ectomycorrhizal symbiosis and its impact on root morphology **(a)** Nonmycorrhizal roots of *Populus tremula x alba* with abundant root hairs (**b**) Lateral roots of *P. tremula x alba* colonized by the ectomycorrhizal fungus *Laccaria bicolor*; a thick mantle of fungal hyphae is ensheathing the root tips. Clusters of roots are also induced by the mycobiont colonisation. Photos courtesy of J Richter (© INRA)

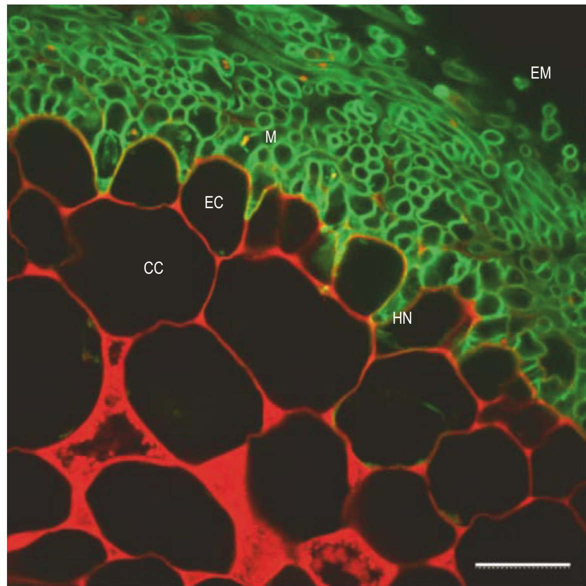

Fig. 4 Microscopy image of a transverse section of a *P. tremula x alba* – *Laccaria bicolor* ectomycorrhiza showing extramatrical mycelium (EM), the mantle (M) and Hartig net hyphae (HN) between epidermal cells (EC); cortical cells (CC); scale, 10 μm. Photos courtesy of J Richter and V Legué (© INRA)

Ectomycorrhizal species associated with Populus. Like most trees, *Populus* species host a wide diversity of species of EM fungi. Symbionts in seedling roots from *Populus maximowiczii, Salix sachalinensis* and *S. hultenii* var. *angustifolia* were identified based on their morphotypes and ribosomal DNA internal transcribed spacer (ITS) sequences (Obase et al., 2007). *Laccaria amethystea, Inocybe lacera, Hebeloma mesophaeum, Scleroderma bovista, Thelephora terrestris, Thelephoraceae* spp., *Tuber* sp. and unidentified fungi were the most abundant symbionts. The ectomycorrhizal community under trembling aspen (*Populus tremuloides* Michx.), white birch (*Betula papyrifera* Marsh.) or white spruce [*Picea glauca* (Moench) Voss], and balsam fir [*Abies balsamea* (L.) Mill.] is influenced by the relative proportions of host tree species. *Piloderma* sp., *Russula* sp., *Cortinarius* sp., and *Lactarius* sp. were the most common ectomycorrhizas. Interestingly, tomentelloid EM are constant, diverse and abundant members of the EM communities in temperate-continental broad-leaved forests.

Impact of elevated CO_2. The effects of elevated atmospheric CO_2 and subsequent plant responses on the soil microbial community composition associated with trembling aspen was assessed using Free Air Carbon Dioxide Enrichment (FACE) systems. Comparative analysis of the microbial community metagenome of trembing aspen grown in free-air CO_2 and O_3 enrichment FACE experiments (Lesaulnier et al., 2008) provided a detailed and deep branching profile of population changes incurred as a response to this environmental perturbation. Total bacterial and eukaryotic abundance did not change; however, an increase in heterotrophic decomposers and EM fungi was observed. These changes in soil biota are evidence for altered interactions between trembling aspen and the microorganisms in its surrounding soil, and support the theory that greater plant detritus production under elevated CO_2 significantly alters soil microbial community composition. These detritus from aboveground and belowground plant parts constitutes the primary source of C for soil organic matter (SOM), and accumulation of SOM in forests may provide a significant mechanism to mitigate increasing atmospheric CO_2 concentrations. The mycorrhizal external mycelium of Populus is the dominant pathway (62%) through which carbon entered the SOM pool, exceeding the input via leaf litter and fine root turnover (Godbold et al., 2006). In this FACE experiment, the input via the mycorrhizal external mycelium was not influenced by elevated CO_2, but elevated atmospheric CO_2 enhanced soil C inputs via fine root turnover. The turnover of the mycorrhizal external mycelium may be a fundamental mechanism for the transfer of root-derived C to SOM.

Mycorrhizas in plantation forestry. Production of native and hybridized varieties of *Populus* has received considerable interest in temperate regions as an alternative to agricultural crops and an additional wood source, while acting as a potential carbon (C) sink to offset emissions of fossil fuel-based greenhouse gases. Several large-scale inoculation programmes have shown that mycorrhizal associations are essential in the establishment and growth of poplar and aspens. *Populus* clones showed variable degrees of colonization by both ectomycorrhizal and arbuscular mycorrhizal fungi, suggesting differential host susceptibility (Khasa et al., 2002; Quoreshi and Khasa, 2008). Mycorrhizal inoculation increases the resistance of the

cuttings to soil-borne pathogens and their competitiveness for nutrients and space against weeds (Baum et al., 2002). Colonisation by AM fungi could influence the longevity of roots, an important element determining the fluxes of nutrients and carbon within terrestrial ecosystems (Hooker et al., 1995).

Phytoremediation. Poplars are suitable for phytoremediation purposes, confirming that mycorrhizal fungi can be useful for phytoremediation. This fact underscores the importance of appropriate combinations of plant genotypes and fungal symbionts (Lingua et al., 2008). Trembling aspen (*Populus tremuloides* Michx.) and paper birch (*Betula papyrifera* Marsh.) seedlings inoculated with *Hebeloma crustuliniforme* or *Laccaria bicolor* appeared to be relatively tolerant of the applied NaCl treatment and did not develop visible leaf symptoms that are characteristic of salt injury (Yi et al., 2008).

Symbiosis development & functioning. There is a striking variability in the ability to form ectomycorrhizas among *P. trichocarpa* x *P. deltoides* progenies, including individual genotypes which are different to either parent confirming a genetic basis for mycorrhiza formation (Tagu et al., 2001; Tagu et al., 2005). For optimal development of the symbiosis, ectomycorrhizal partners evolve complex coordinated developmental processes and at the same time, sense and respond to physiological factors and environmental cues (Martin et al., 2001; Martin et al., 2007; Martin et al., 2008). The establishment of the ectomycorrhiza is a coordinated process of cross-talk between plant and fungus, followed by metabolic, developmental, and structural changes in the fungus, resulting in its growth toward the root. Special interest has been paid for many years to the role of auxins in the formation of the symbiosis. *Populus* inoculated with the ectomycorrhizal *Laccaria bicolor* (Fig. 2) produced an increased number of ectomycorrhizal roots confirming that some morphogenetic steps controlling the mycorrhiza development are regulated by fungal auxins (Richter et al., 2009) (Fig. 3). This involves an alteration in the accumulation of transcripts coding for auxin transporters and auxin/ethylene-responsive genes (Richter et al., 2009). On the other hand, development of *Amanita muscaria* ectomycorrhiza was not affected by the transformation of aspen (*Populus tremula x P. tremuloides*), expressing the IAA biosynthetic genes in roots (Hampp et al., 1996). Coordinated expression of malate synthase and other lipid metabolism genes along with acetyl-CoA acetyltransferase, suggest that alteration in lipid metabolism could be an important part of the preinfection process in ectomycorrhizal symbiosis and in the transfer and utilization of the carbon in the fungus (Hiremath et al., 2006). It has also been suggested that haemoglobin genes may play a role during ectomycorrhiza development in hybrid aspen (*Populus tremula x tremuloides*) (Jokipii et al., 2008).

Transcript profiling. Transcript profiling of *Populus* ectomycorrhizas revealed upregulation of genes coding for proteins involved in stress and defense responses as well as for proteins involved in cell wall modification (Kohler et al., unpublished data). Transcripts coding for regulators of root development, such as a NAC domain-containing protein, an AP2-TINY-like transcription factor, a SCARECROW like modulator and an auxin-binding protein are up-regulated in the root tips in contact with *Pisolithus* mycelium (Kohler et al., unpublished data) (Table 1). The first

Table 1 Poplar transcripts showing the highest induction during the colonisation of roots by the ectomycorrhizal basidiomycete *Pisolithus microcarpus*

Putative function	4d of contact (fold)	14d of contact (fold)	GenBank#	*P. trichocarpa* Protein ID
Primary metabolism				
UDP-glucose dehydrogenase	2.0	**18.6**	CA825241	556397
Cytosolic malate dehydrogenase	**2.4**	–1.2	CA820897	564942
Glyceraldehyde-3-phosphate dehydrogenase	**3.4**	–1.2	CA821253	575307
S-adenosylmethionine synthetase	**3.6**	–1.4	CA824979	644907
Phenylpropanoid pathway				
Trans-caffeoyl-CoA 3-O-methyltransferase	–1.2	**7.3**	CA824791	829835
4-coumarate-CoA ligase	–1.1	**4.6**	CA825131	662119
Protein metabolism				
60S ribosomal protein	**6.4**	–1.2	CA824447	813651
Peptidyl-prolyl cis-trans isomerise	**4.1**	–1.2	CA825316	818813
Cysteine peptidase	**3.6**	1.3	CA824069	822036
U-Box protein	1.8	**3.8**	CA823526	564694
Signaling/hormone related				
WD-40 repeat protein	1.1	**17.4**	CA826076	592758
IAA-amino acid hydrolase	**4.4**	**10.4**	CA823558	551444
Myo-inositol-1-phosphate synthase	**4.7**	**9.5**	CA821145	831371
EF-Hand containing calcium-binding protein	1.8	**9.4**	CA825399	645031
Auxin-binding protein	**2.8**	**6.0**	CA821074	180742
Calmodulin-related protein	–1.7	**5.4**	CA825093	717167
Serine/threonine protein kinase	1.1	**5.1**	CA826022	592708
Guanine nucleotide-binding protein, beta subunit	–1.1	**4.9**	CA825225	834267
Serine/threonine protein kinase	–1.1	**4.7**	CA825903	834835
NAC-domain protein	1.4	**2.8**	CA821225	654710
Calreticulin	**2.9**	–1.3	CA822515	729432
Stress/defense related				
Pathogenesis-related thaumatin	1.4	**9.0**	CA821226	180318
Cytochrome P450	**6.5**	**8.7**	CA821073	645827
Glutaredoxin-like protein	*1.1*	***6.6***	*CA825390*	586055
Proteinase inhibitor	1.8	**3.1**	CA825852	739058
Heat shock protein 70	**3.2**	–1.0	CA822754	563894
Xyloglucan endo-1,4-beta-d-glucanase	**2.6**	–1.6	CA824320	658681
Transcription factor				
GRAS family transcription factor	–1.1	**5.1**	CA824677	654429
MYB-related protein	2.9	**4.6**	CA821175	784079
AP2/ERF domain-containing transcription factor	1.4	**2.4**	CA822110	739204

Table 1 (continued)

Putative function	4d of contact (fold)	14d of contact (fold)	GenBank#	*P. trichocarpa* Protein ID
RNA silencing				
Argonaute-like protein	1.2	**3.3**	CA824775	709669
Membrane transporter				
Lys/His-specific transporter	**2.7**	**3.2**	CA822109	815560

Poplar plantlets (*P. tremula x alba*, INRA clone 717-1B4) were grown together with *Pisolithus microcarpus* 441 mycelium in an in vitro system described in (Luster and Finlay, 2006) for 4 days and 14 days, respectively. After 4 days the fungal hyphae are attached to the root and after 14 days mycorrhizal tissues are differentiating. Three biological replicates of these samples as well as controls from non-inoculated poplar plantlets were used to hybridize a Populus 4.6 K cDNA macroarray. This array is described as platform GPL4887 at GEO (http://www.ncbi.nlm.nih.gov/geo/index.cgi). Representative differentially-expressed transcripts compared to the control roots (>2fold, p-value<0.05, values in bold) are listed by functional categories. For each transcript, its putative function, the Genbank accession number of the cDNA clone spotted on the macroarray and the JGI protein ID of the corresponding *Populus trichocarpa* gene are given.

genome-wide transcript profiling of *L. bicolor* interacting with *Populus* roots has revealed several genes with a striking upregulation in symbiotic tissues (>100-fold) (Martin et al., 2008). One of the more exciting findings has been the observation that many of the mycorrhiza-upregulated transcripts are coding for small cysteine-rich proteins that eventually find their way out of the colonizing hyphae and onto the surface of the fungal and root cells (Fig. 5). These proteins may play a role in the construction of the novel symbiotic apoplastic interface, act as decoys to help the mycobiont evades the host defense system, and/or may be directed into the root cells. Understanding the molecular function of these secreted effectors became widely accepted as essential for a mechanistic understanding of the processes underlying plant colonization by filamentous pathogens.

Carbohydrates. A major function of the ectomycorrhizal symbiosis is the exchange of fungus-derived nutrients for plant-derived carbohydrates, enabling the colonization of mineral nutrient-poor environments. As shown in various *Populus* ectomycorrhizal associations, the creation of such a strong sink is directly related to the efficiency of fungal hexose uptake at the plant/fungus interface, the conversion of plant-derived hexoses into fungus-specific compounds, and the export of carbohydrates towards soil growing hyphae (Nehls et al., 2007). Tracer experiments have shown (Bucking and Heyser, 2001): (1) the fungal partner has a high capacity to attract photosynthates; (2) the main transfer of carbohydrates is localized in the median zone of a mycorrhizal root; (3) carbohydrates that are absorbed by the mycorrhizal fungus are translocated to the fungal sheath and are homogeneously distributed. In *Amanita muscaria-Populus* (*P. tremula x tremuloides*) ectomycorrhizas, the transcript abundances of genes encoding key enzymes of fungal trehalose

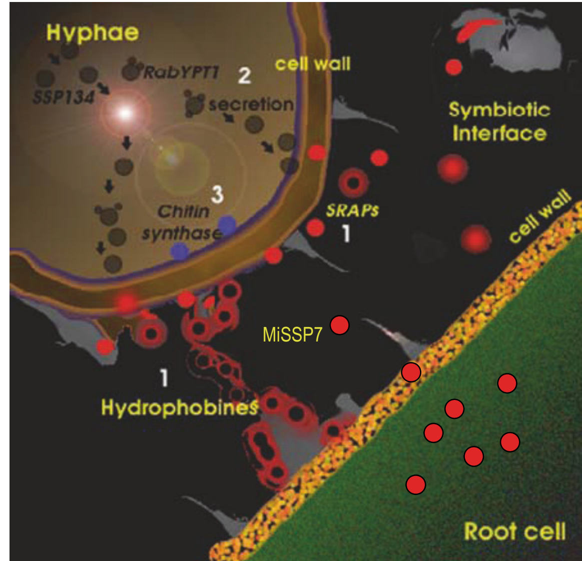

Fig. 5 A hypothetical model indicating the presence of mycorrhiza-induced secreted proteins (*red circles*), such as the small secreted protein MiSSP7, in the symbiotic apoplastic space (*1*) and within the host cells. Expression of hydrophobins and the symbiosis-regulated acidic polypeptides SRAP32 is also induced by ectomycorrhizal development and are likely involved in the hyphae attachment on the root surface. High expression of the transcripts coding for the secretory pathways (*2*) and cell wall synthesis (*3*) is also observed during the interaction

biosynthesis, namely trehalose-6-phosphate synthase (TPS), trehalose-6-phosphate phosphatase (TPP) and trehalose phosphorylase (TP), were increased (Lopez et al., 2007). The enhanced trehalose biosynthesis at the plant-fungus interface indicates that trehalose is a relevant carbohydrate sink in symbiosis. However, not only the fungus but also the plant partner increase its expression of hexose importer genes at the plant/fungus interface. This increase in hexose uptake capacity of plant roots in combination with an increase in photosynthesis may explain how the plant deals with the growing fungal carbohydrate demand in symbiosis and how it can restrict this loss of carbohydrates under certain conditions to avoid fungal parasitism.

Nitrogen. Ectomycorrhizal symbionts play a key role in the nitrogen metabolism of their host plants. The high-affinity ammonium importer from *P. tremula x tremuloides* (*PttAMT1.2*) is strongly increased in a N-independent manner upon ectomycorrhiza formation (Selle et al., 2005) indicating an increased ammonium uptake capacity of mycorrhizal *Populus* roots and suggest, together with the expression of putative ammonium exporter genes in the ectomycorrhizal fungus *Amanita muscaria* and *L. bicolor* (Selle et al., 2005; Martin et al., 2008) that ammonium could be a major N source delivered from the fungus towards the plant in symbiosis.

Drought stress. The formation of ectomycorrhizas may also increase plant survival under drought stress conditions. The water transport capacity of the plasma membrane of root cells is strongly increased in mycorrhizal *Populus* (Marjanovic et al., 2005). On the other hand, Siemens and Zwiazek (2008) suggested that hyphal penetration of the root cortex in itself may have little influence on root hydraulic properties.

5 Plant Symbiont Communities – Diversity

Part of the difficulty in determining net effects of symbiont interactions on host plants is that the diversity of symbiont communities is poorly known. And the longer-lived and larger the plant, the more complex its community of fungal symbionts is likely to be. But even for relatively short-lived plants of small stature our knowledge of symbionts is unlikely to be complete even with ambitious sampling (Shipunov et al., 2008). For perspective, the human mouth, or 'oral microbiome', hosts hundreds of microbes that are still unknown to science (Mlot, 2004). The microbes that populate specific niches, such as the human mouth, have been called the 'uncultured majority' (Mlot, 2004), and unculturable bacteria and fungi (Arnold et al., 2007) are fairly common endophytes of plants. But culturability is hardly the main issue; endophytes in particular are mostly rare in overall frequency at the host plant population and species levels, with largely unknown geographic distributions (Shipunov et al., 2008). A rare endophyte that could potentially perform some valuable service to *Populus* culture might never be naturally recruited into hybrid poplar plantations. Instead, a human-aided introduction would be needed.

Since most readers of this chapter will be plant biologists it is worth emphasizing here a key shortcoming in mycology vis-à-vis botany: today, 83% of vascular plants are known to science, whereas, at best, from 7% to perhaps 20% of the fungi are presently described (Hawksworth, 2001; Cox and Moore, 2005; Rossman, 2009). Diversity matters if symbionts play specific and significant roles in plant ecology. This is doubly true if their unknown distributions across the landscape were discovered to be confounded with variables that are more commonly measured by ecologists (e.g., plant traits or abiotic, environmental variables).

The sampling problem for symbionts, and endophytes in particular, is formidable. By way of example, some of the most extensive, modern studies of endophyte communities of trees have involved *Theobroma cacao*. Even with many studies (Arnold and Herre, 2003; Arnold et al., 2003; Evans et al., 2003; Rubini et al., 2005; Bailey et al., 2008), "it is likely that only a small part of the vast microbial diversity associated with cacao has been described" (Bailey et al., 2008). Ironically, the endophyte communities of our model *Populus* are much more obscure at this point than those of cacao.

Existing records of fungi on *Populus* do not help us make up this deficit of knowledge of its endophytes for they emphasize its pathogens (i.e., fungi that cause diseases). For example, for *P. trichocarpa*, the fungal databases of the USDA Systematic Mycology and Microbiology Laboratory list 363 host-pathogen combinations, 567 records, and 850 specimens (Farr et al., n.d.). For *P. deltoides*, the numbers are 146, 299, and 526, respectively. Similar numbers obtain for other commercially important species of *Populus*, but the fungi of non-commercial species have, of course, only been studied incidentally (e.g., the numbers for *P. heterophylla* are 5, 6 and 26, and for *P. ciliata* 15, 23, and 2).

Furthermore, the lifetime associations with fungi of a single *Populus* tree have never been recorded in their entirety, but it is safe to say that they would be diverse and that they would change with the age of the tree and with local availability of inoculum. Decay fungi, isolated as quiescent endophytes in the young tree

(Hutchinson, 1999) for example, can become active in decay of the older tree. A *Populus* tree also experiences continuous or episodic battles with pathogens (Newcombe, 1996; Newcombe et al., 2001) and herbivores (Whitham et al., 1996; Mattson et al., 2001); not only can symbionts affect the outcomes of these battles but it is likely that the battles themselves affect recruitment into ever-changing, symbiont communities.

6 Endophytes Associated with *Populus*

Endophyte studies in *Populus* lag behind those of many other taxa of woody plants. "Members of the Betulaceae, Fagaceae, Cupressaceae and Pinaceae have been most intensively examined for the presence of endophytic fungi" (Sieber, 2007). In Sieber's review of studies of the endophytes of woody plants, the only species of *Populus* that received mention was *P. tremula*, based on a study of its endophytes in Spain (Santamaria and Diez, 2005). An earlier review of studies of endophytic mycobiota of nearly 80 plant hosts did not include a single species of *Populus* (Stone et al., 2000).

There are, however, a few studies that might be compared to that of the endophytes in leaves, twigs, and stem bark of *P. tremula* in Spain (Santamaria and Diez, 2005). Comparisons provide a sense of how much of the endophytic diversity of *Populus* remains to be discovered. The first that we will consider is one of fungi in bark and wood of *P. tremuloides* in western Canada (Hutchinson, 1999). These two aspen species, *P. tremula* and *P. tremuloides*, are similar ecologically and closely related by descent from a common ancestor (Eckenwalder, 1996). Although Hutchison's was not explicitly an endophyte study, great care was taken to avoid surface contaminants so that it does appear comparable to the Spanish study. Conducted in a small area of Spain, a total of 48 fungal species were isolated from 960 plant fragments. From twigs specifically, isolates belonged to a total of 26 genera, whereas in western Canada isolates belonged to 37 genera. Only nine genera (i.e., 35% of the Spanish community and 24% of the western Canadian community) were common to both wood-inhabiting, fungal communities.

Wood-inhabiting, or xylotropic, endophytes of *P. tremuloides* have also been sampled in eastern North America (Chapela, 1989). Chapela isolated ten genera that were not subsequently found in either western Canada or Spain: *Daldinia*, *Herpotrichia*, *Melanconium*, *Pestalotia*, *Coniophora*, *Peniophora*, *Sistotrema*, *Ozonium*, *Phaeococcum* and *Cylindrocarpon*. Hutchison also notes in his study the "conspicuous absence of the microfungus *Trichocladium canadense* despite the frequent isolation of this species from aspen wood in Quebec (Laflamme and Lortie, 1973), Ontario (Weingartner and Basham, 1985) and New Hampshire (Shigo, 1963)."

These comparisons simply illustrate that *Populus*-wide inventories of fungal endophytes remain largely unknown, especially if it is true in a general sense that

endophytes may be hyperdiverse in plants (Arnold et al., 2000; Ganley et al., 2004; Arnold et al., 2007). Because we know so few of its endophytes, it is not surprising that new species of fungi associated with *Populus* have continued to be described in the last decade: *Endoconidioma populi* gen. et sp. nov. (Tsuneda et al., 2004), or *Knufia endospora* sp. nov. (Tsuneda and Currah, 2005). But, currently, bacterial endophytes of *Populus* appear to be receiving more study than its fungal endophytes. For example, new species of bacteria have been described (Van Aken et al., 2004), ecologically important species have been reported (Doty et al., 2005) and sequence-based surveys and community-level work have also been reported (Germaine et al., 2004; Ulrich et al., 2008).

If endophytes of *Populus* are like its pathogens (Newcombe, 1996), then they are likely to play specific roles in the symbiont community. This has been suggested by studies of natural hybrid zones of *Populus* (Bailey et al., 2005). This was also certainly the case for the recently discovered mycoparasite, *Hydropisphaera fungicola*, that is endophytic in *Populus trichocarpa* and that specifically parasitizes another endophyte in the community (Rossman et al., 2008).

7 Plant Symbiont Communities – Assembly Rules

The assembly rules of plant communities are still hotly debated after nearly a century of research initiated by Clements and Gleason (Chase, 2003). We do know that plant communities tend to reach single stable equilibria when prevailing conditions include “small regional species pools, high rates of connectance, low productivity and high disturbance”(Chase, 2003). We also know that when conditions differ from the latter, the history of community assembly may itself become an important factor leading to multiple stable equilibria.

Rules governing assembly of the symbiont communities of plants are even murkier. We do know that specific groups of fungi succeed one another during decomposition of wood (Boddy, 2000), and plant litter generally (Thormann et al., 2003). We know that competition to capture resources is fierce, ranging from antagonism at a distance through to mycoparasitism (Boddy, 2000). Competition and mycoparasitism may also be expected among fungi in symbiont communities of living plants. But, in contrast to a dead substrate, the immune system of a living plant might be modulated with each new microbial arrival. Sequential arrival (i.e., assembly history) is itself a highly significant factor in structuring microbial communities in freshwater ecosystems (Fukami and Morin, 2003), and the same may turn out to be true of plant symbiont communities. Insights thus far into the workings of the interactome of *Populus* have mostly been derived from studies of single microbial species under controlled conditions. But, in nature, hundreds or thousands of microbes are likely members of the fungal symbiont community of a single *Populus* at any given time. In this sense, the *Populus* interactome is still a blank slate.

8 Applications of Fungal Symbionts of *Populus*

Domesticating *Populus* to help meet the "wood, fiber, energy, and environmental needs of the rapidly growing world" (Bradshaw and Strauss, 2001) is a broad and worthwhile challenge. The primary objective of *Populus* domestication is increased yield, but leaf rust caused by *Melampsora*, leaf spot (*Marssonina*), leaf and shoot blight (*Venturia*), and leaf spot and stem canker (*Septoria*) limit the productivity of *Populus* (Newcombe et al., 2001). To combat these diseases, *Populus* breeders and pathologists have followed an expensive and constraining 'agricultural model' involving cultural practices, pesticides, and genetically resistant clones selected in a traditional manner. This work has often had to proceed without full understanding of defense mechanisms at the molecular level in *Populus*.

Genomics and genetic engineering have been seen as the principal, novel means by which *Populus* might be rapidly improved for characters associated with growth and pest resistance (Bradshaw and Strauss, 2001; Brunner et al., 2004). Application of select symbionts has been suggested as another novel means to improve *Populus* (Doty, 2008), in addition to conventional breeding and selection (Riemenschneider et al., 2001). However, it has not really been clear how best to proceed to select symbionts in a genus that collectively might host thousands of unique microbes. Symbiont selection assays would need to be developed, and the potential for these assays to be based on underlying genetic mechanisms is still untapped although it will no doubt be facilitated by ongoing symbiotic sequencing efforts (Martin et al., 2004).

By way of example, another undomesticated tree, coffee, is in the process of being improved via symbiont selection (Vega et al., 2008); two entomopathogenic fungal endophytes, *Beauveria bassiana* and *Clonostachys rosea*, have been isolated from coffee itself and are thought to be able to provide continuous protection against the coffee berry borer and other insects. Endophytes of cacao, including new species of *Trichoderma* (Hanada et al., 2008), have shown potential for biological control of the diseases of cacao (Bailey et al., 2008), as discussed above. Rugulosin-producing endophytes are currently being inoculated into spruce seedlings on a commercial basis in Canada (Miller et al., 2002) to protect the outplanted trees from spruce budworm. Finally, isolates of *Muscodor albus* are being selected so that they could be inoculated into forest and orchard trees for control of pathogens and insects (Strobel, 2006). In fact, symbiont selection is potentially rewarding with any wild woody plant in which traditional improvement via breeding might be perceived as slow, or where biotechnological progress might be impeded by regulation (Strauss et al., 2004), or where resistance to disease of transgenic trees might be less than optimal (Mohamed et al., 2001).

Combatting the eventual loss of enemy release in Populus culture. It is important to remember that many species and hybrids of *Populus* have been introduced far outside their native ranges where they likely benefit both from enemy release (Mitchell and Power, 2003), and from positive feedback with mycorrhizal fungi (Klironomos, 2002). However, enemy release has typically been temporary for crop plants, and it is likely to be that way for *Populus* also. For example, after being

introduced into North America, *Populus nigra* cv. 'Italica' has been reunited with at least two of its pathogens that it had temporarily left behind in its native range in Eurasia: *Melampsora larici-populina* (Newcombe and Chastagner, 1993) and *Venturia populina* (Newcombe, 2003). As the benefits of enemy release are lost it will be necessary to respond to a growing list of pathogens. It is in that context that defense mutualists with a broad spectrum of activity will be most useful to save *Populus* breeders from having to respond to each 'new' pathogen. Restoring defense mutualists to 'exotic' *Populus* clones might actually be interpreted as classical biological control. Some even see agriculture and production forestry as disruptive of beneficial symbiotic systems that then need to be fixed (Tikhonovich and Provorov, 2007).

At this point, it is difficult to judge the potential of symbiont selection with *Populus* in part because we know few of its symbionts, let alone which might be most efficacious in host defense. Ironically, an endophyte isolated from *Populus tomentosa*, *Chaetomium spirale*, is being applied in the field in China but not in *Populus* plantations (Xin and Shang, 2005). Instead, *C. spirale* is being applied to control of apple canker.

Historically, J.E. Bier was one of the first to take an interest in endophyte-pathogen interactions in *Populus* (Bier and Rowat, 1962). Bier demonstrated that naturally occurring saprophytes could prevent the development of *Hypoxylon* canker, but this promising line of research was never developed into an application. Strains of *Streptomyces* have since been used to reduce infection rates of *Septoria musiva* (Gyenis et al., 2003) but it remains to be seen whether these soil-borne actinomycetes could become stable members of the symbiont community of *Populus*.

9 Evolutionary History and Fossil Record

The arbuscular mycorrhiza is the ancestral type of mycorrhiza as suggested by its distribution among extant land plants, the fossil record, and mapping of mycorrhizal types onto a land plant phylogeny (Remy et al., 1994; Wang and Qiu, 2006). The ancestors of the modern-day Glomeromycota (SchÜßLer et al., 2002) formed AM-like symbioses with Devonian plants 400 million years ago, but AM have since been replaced in many plant lineages with other types of mycorrhizal relationship.

Populus appears to be among the latter that is currently transitioning from AM to EM; the Salicaceae are regarded as 15% AM, 49% EM, 34% AM+EM, and 2% ectendomycorrhizal (Wang and Qiu, 2006). Although the mycorrhizae of only 41 species of the Salicaceae have been examined it appears likely that this formula captures the trend. *Populus trichocarpa* may be relatively advanced in its evolutionary transitioning in that it is reported as EM only (Wang and Qiu, 2006), and even its young seedlings are rapidly colonized by EM fungi (Helm et al., 1996).

Foliar fossil fungi have tended to be interpreted as parasites or pathogens but they could also have been endophytes. The record of fossil fungi specifically associated

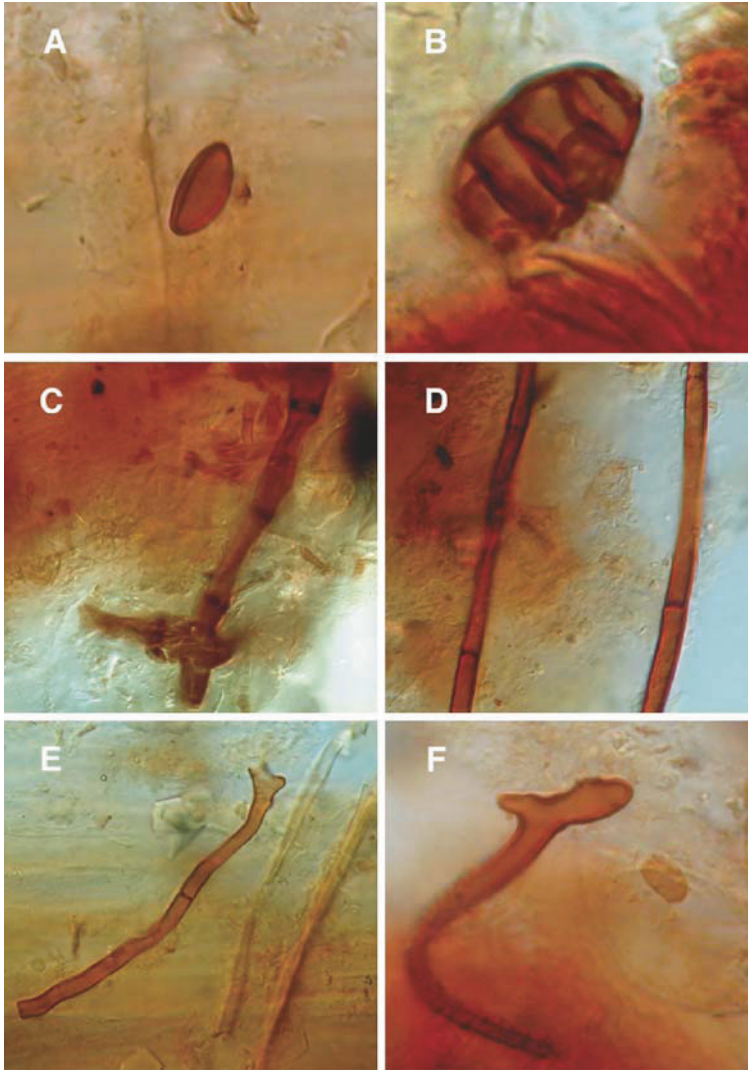

Fig. 6 Images of dispersed spores (**a** and **b**), hyphal fragments (**c** and **d**) and conidiophores (**e** and **f**) obtained from a Miocene leaf fossil of *Populus* from the Clarkia deposits of north central Idaho. The leaves were separated from their rock matrix with hydrofluoric acid (Rember, 1991) and cleared in KOH (Phipps, 2007) before being mounted in lactoglycerol on glass slides. (**a**) Spore 9.5 μm in length. (**b**) *Alternaria*-like spore 18 μm in length. (**c**) Hypha 13 μm in width with verticillate branching. (**d**) Hypha 5 μm in width. (**e** & **f**) Conidiophores that are *Cladosporium*-like. E: 3.5 μm wide. F: 5 μm wide and warty below

with *Populus* has received little attention to date, yet it might eventually shed light on coevolutionary relationships and paleoclimatic adaptations of *Populus* and its symbionts. For example, the dispersed spores, hyphal fragments, and conidiophores of

fossil fungi found in Miocene leaves of *Populus* (Fig. 6) are all reminiscent of modern fungi (Pirozynski, 1976). All of the images are from a single leaf fossil obtained from the Clarkia deposits of Idaho (Rember, 1991). Seen in this single leaf were some ten spore types varying in length from 5 to 98 μm. Hyphae varied in width from 2.5 to 15 μm. This morphological variation, and the absence of spores typical of modern pathogens, suggest a community of endophytes that might have had time to sporulate after their host leaf was shed and before they themselves became metabolically inactive. Further study is underway.

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The Impact of Genomics on Advances in Herbivore Defense and Secondary Metabolism in *Populus*

C. Peter Constabel and Richard L. Lindroth

Abstract The genus *Populus* is ideally suited for applying the tools of genomics to plant-herbivore interactions and secondary metabolism. *Populus* is rich in phenolic secondary metabolites including condensed tannins and salicylate-based glycosides; these and related-compounds strongly shape the interactions of *Populus* with a host of invertebrate and vertebrate herbivores in diverse natural environments and commercial plantations. Microarray studies have been instrumental in delineating the induced defense response to herbivore damage and in identifying defense-related genes in *Populus*. These can now be functionally tested in vitro as recombinant proteins as well as in vivo in transgenic plants. Analysis of the *P. trichocarpa* genome has provided access to candidate genes likely to be important for the synthesis of phenolic secondary metabolites, thereby accelerating progress in understanding the ecological functions of these compounds. Combining genomics with improved metabolite profiling will lead to a deeper understanding of how the substantial variation in phenolics among *Populus* species and genotypes is generated, as well as the ecological consequences of this variation.

1 Introduction

The motivation for elucidating the genome of *Populus trichocarpa* is rooted in widespread interest in understanding the biology of a woody perennial plant – i.e., processes of growth and development in a perennial context, including wood formation, dormancy, and adaptations to seasonal changes. In addition, however, *Populus* genomics research affords the potential to address questions of fundamental interest to ecologists and evolutionary biologists interested in plant-animal interactions. *Populus* species are quintessential "foundation species" (Ellison et al., 2005) in

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many ecosystems, and it is the interactions between *Populus* and their herbivores that largely shape the ecological and evolutionary dynamics of those ecosystems (Whitham et al., 2006; Schweitzer et al., 2008). Moreover, plant defense traits are key mediators of community- and ecosystem-level interactions, and thus are of principal interest in emerging "genes to ecosystems" research.

Populus makes a broad array of phenolic secondary metabolites, with considerable qualitative and quantitative variation among species and ecotypes. Since secondary metabolites have strong ecological and evolutionary impacts on herbivores, the *Populus* system provides excellent opportunities for investigating the interactions of plants, their defensive secondary metabolites, and herbivores, in both managed and natural environments. *Populus* has also been the subject of growing interest for biofuel production and carbon sequestration, both of which are intimately linked to plant secondary metabolism. Genomic tools and resources applied to *Populus* have already yielded significant insights into woody plant defense. Genome-level information can also be directly applied to studies of genetic variation and diversity within and between species of *Populus*, for example identifying rapidly evolving genes that may be under positive selection for pest resistance. In short, the intersection of chemical ecology with genomics will make for a fruitful area of research for many years to come.

Our objectives in this chapter are: (i) to provide a brief summary of current knowledge of the interrelated areas of *Populus* secondary metabolism and herbivore defense, (ii) to review the impact of the *P. trichocarpa* genome sequence and genomics-based approaches on our understanding of these areas, and (iii) to indicate promising directions for future research. We will not review all aspects of *Populus* defense and secondary metabolism; rather, we will focus on those where specific genomic approaches are having significant impact, or have the potential to do so. Other overviews of *Populus* herbivore defense and secondary metabolism are available in recent reviews (Lindroth, 2001; Constabel and Major, 2005; Phillipe and Bohlmann, 2007), whereas the genes related to phenolic metabolism of *Populus* were recently described by Tsai et al. (2006).

2 Overview of *Populus* Defense and Secondary Metabolism

2.1 Populus *as the "Ecogenomics" Model Plant*

The genus *Populus*, comprised of about 30 species, has emerged as *the* model system for studies of the ecological genomics of woody plant defense and secondary metabolism. *Populus* species and hybrids exhibit extensive geographical distribution in the northern hemisphere and are widely planted in the southern hemisphere. Distributional ranges of several species (e.g., *Populus tremuloides* and *P. tremula*) are among the largest of any plant species on Earth. *Populus* is a major food source for hundreds of species of arthropods, birds and mammals, and is a host plant for many insect pests (Baker, 1972; Furniss and Carolin, 1977; Dickmann and Stuart, 1983; Perala, 1990). Several *Populus* species exhibit extraordinary genetic variation,

including traits important for interactions with herbivores (Lindroth and Hwang, 1996a). Furthermore, *Populus* species synthesize a diversity of both phenolic-based secondary metabolites and protein-based defenses, subject to variable production, induction by herbivores, and systemic signalling. These attributes of *Populus* make this genus ideal for application of genomic tools to the central question of how-plant insect interactions are mediated at the chemical and gene level.

2.2 **Populus** ***Secondary Metabolites and Their Effects on Herbivores***

The most abundant secondary metabolites of poplars and aspens of relevance to herbivory are phenolics, synthesized via the shikimic acid pathway. Salicin-based phenolic glycosides are the signature compounds of the family, and found only in the Salicaceae. To date, at least 20 structurally-diverse salicylates have been identified in *Populus* (Pearl and Darling, 1968; Palo, 1984; Tsai et al., 2006; Fig. 1). Some, such

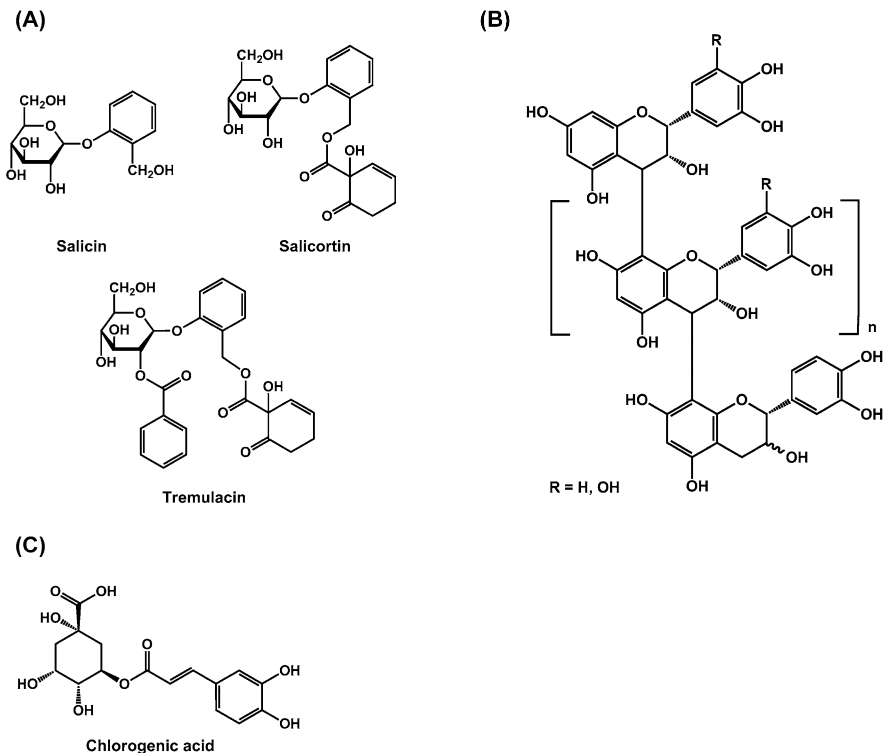

Fig. 1 Major classes of phenylpropanoid-derived phenolics in *Populus* species (**a**) Representative phenolic glycosides (salicylates) (**b**) General structure of polymeric condensed tannins (n = 0–~20). (**c**) Representative hydroxycinnamate derivative

as salicin and salicortin, are widespread throughout the genus. Others, such as HCH-salicortin, have a restricted distribution. Chemical structure strongly influences the biological activity of *Populus* salicylates. For example, compounds containing a hydroxycyclohexen-on-oyl functional group (e.g., salicortin, tremulacin) are especially toxic to insect herbivores (Lindroth and Peterson, 1988; Lindroth et al., 1988). The biosynthetic pathways leading to production of salicylate phenolic glycosides remain largely unresolved, but likely include either isochorismate or benzoate intermediates (Tsai et al., 2006).

A second abundant class of phenolic secondary metabolites in *Populus* comprises the condensed tannins (proanthocyanidins), polymers of flavan-3-ols derived via the flavonoid pathway (Fig. 1). Tannin polymer size and composition vary among *Populus* species. The extender units are comprised of epicatechin, gallocatechin, or epigallocatechin monomers, and range in number from 0–5 (*P. fremontii*), to 8–11 (*P. tremuloides*) or 15–20 (*P. angustifolia*) (Schweitzer et al., 2008). The ecological implications of variation in extender composition and number are likely significant, but remain unexplored. Other products of the flavonoid pathway that accumulate in leaves are the flavonoids kaempferol and quercetin, which have been studied in the context of their UV screening abilities (Warren et al., 2003). Because of multiple glycosylations with different sugars, the flavonol profiles of poplar and aspen leaves appear to be complex.

Hydroxycinnamate derivatives (HCDs) define a third major class of phenolic constituents in *Populus*. Hydroxycinnamates are synthesized from cinnamate via hydroxylation and *O*-methylation of the aromatic ring, and undergo additional quinate ester conjugations to produce HCDs (e.g., chlorogenic acid; Tsai et al., 2006). *Populus* expresses considerable diversity of HCDs both among and within species, although their role as defenses against herbivory or other stresses is poorly understood in *Populus*. However, caffeic acid derivatives such as chlorogenic acid are excellent substrates for polyphenol oxidase (PPO), itself implicated in herbivore defense (Constabel et al., 2000).

In addition to these relatively abundant phenolic metabolites, however, the list of secondary compounds described from the genus is extensive. Comprehensive studies by W. Greenaway and collaborators on the bud exudate from several *Populus* species (i.e. English et al., 1991; Greenaway and Whatley, 1990, 1991) identified a myriad of phenolics including almost all classes of chalcones and flavonoids, a variety of substituted benzoic acids and their esters, and various hydroxycinnamates and their esters. Remarkably, representative species from different sections of *Populus* showed very different profiles and constituents. While these are purely analytical studies, they demonstrate the tremendous biosynthetic potential of the genus, in particular in the phenylpropanoid pathway. Terpenoids are also present in bud exudates (English et al., 1991). Volatile leaf terpenes have also been investigated, principally as potential signalling molecules, as they may be used as cues by predators to find their hosts or to prime subsequent defense reactions. The sesquiterpenes (-)germacrene D, E-ß-ocimene, linalool, (E,)-alpha-farnesene, ß-caryophyllene are released from hybrid poplar leaves in response to caterpillar feeding (Arimura et al., 2004; Frost et al., 2007). Sterols and triterpenes are components of wood extractives in *P. tremuloides* (Fernandez et al., 2002).

Secondary metabolites strongly influence interactions between *Populus* trees and both specialist and generalist insect herbivores. High levels of phenolic glycosides in *P. tremuloides* correlate with reduced feeding, altered food preferences, decreased development, growth and reproduction, and increased mortality in a variety of aspen-feeding Lepidoptera (Lindroth and Hwang, 1996; Lindroth, 2001). Phenolic glycosides have been documented to influence the distributional patterns of gypsy moth (*Lymantria dispar*) larvae and reduce rates of tree defoliation (Donaldson and Lindroth, 2007). In contrast, phenolic glycosides are feeding stimulants for specialized chrysomelid beetles that employ the compounds to manufacture their own salicylate-based defense (Donaldson and Lindroth, 2004; Vigue and Lindroth, 2008). Surprisingly, high concentrations of condensed tannins do not appear to negatively affect major lepidopteran defoliators of aspen (Hwang and Lindroth, 1997; Osier and Lindroth, 2004; Schweitzer et al., 2008), although they may negatively impact chrysomelid beetles (Donaldson and Lindroth, 2004).

Populus secondary metabolites also contribute to defense against mammalian herbivores. Wooley et al. (2008) found that elk preference for trembling aspen (*P. tremuloides*) genotypes was inversely correlated with phenolic glycoside concentrations, but not tannin levels. Moreover, preferential feeding by elk can lead to an increase in phenolic glycoside expression in aspen populations (Bailey et al., 2007). Results from these studies are consistent with other work on cervids, showing that nontannin phenolics are more important than tannins in determining diet selection (McArthur et al., 1993). Similarly, Diner et al. (2009) reported that use of aspen trees by porcupines was influenced by levels of phenolic glycosides, but not tannins. However, Bailey et al. (2004) reported that tannins influence foraging by beavers among riparian cottonwood species and hybrids.

In short, phenolic glycoside levels are clearly correlated with reduced herbivory, while the evidence for condensed tannins is mixed. In the future, it should be possible to directly test the roles of these compounds and learn more about their mechanisms through the use of transgenics in which levels of one or more of the compounds have been altered. In order to achieve this goal, however, we need an improved understanding of the metabolic pathways and their regulation at the gene level.

2.3 Herbivory and Induction of Defenses in Populus

Plant resistance is determined not only by the constitutive secondary metabolites encountered by the herbivore, but by the response of the plant to the attack. Havill and Raffa (1999) found that leaf damage in hybrid poplars reduced subsequent growth rates of gypsy moths by up to 71%. This protection could also be induced by wounding or jasmonic acid. Hale et al. (2005) also found that feeding damage by gypsy moths on hybrid poplar reduced subsequent growth of gypsy moth, but not white-marked tussock moth, larvae. Similar induced resistance was demonstrated with the forest tent caterpillar (*Malacasoma disstria)* (Robison and Raffa, 1997). Furthermore, the extent of induced resistance varied among hybrid poplar clones, similar to the highly variable chemistry (see Section 5). Although

the chemical basis for the enhanced resistance was not identified in these studies, the effect was systemic, i.e., observed in undamaged portions of the damaged saplings.

These reports provide a biological context for the observation that herbivore damage can itself influence the expression of phenolic-based chemical defenses in *Populus*. Phenolic glycoside concentrations in trembling aspen increase substantially in new leaves produced immediately after a defoliation event (Stevens and Lindroth, 2005; Donaldson and Lindroth, 2008). However, leaf damage does not induce accumulation of phenolic glycosides in either remaining leaf fragments (rapid induced response; Osier and Lindroth, 2001; Stevens and Lindroth, 2005) or in leaves produced a year after defoliation (delayed induced response; Osier and Lindroth, 2004). Responses of condensed tannins are opposite those of phenolic glycosides. Tannin levels increase in damaged leaves as well as in foliage produced a year after defoliation, but decrease in leaves that flush immediately after a defoliation event (Osier and Lindroth, 2001, 2004; Stevens and Lindroth, 2005; Donaldson and Lindroth, 2008). The rapidly induced tannin accumulation is systemic in existing plant organs and mediated by increased transcription of genes encoding the tannin biosynthetic pathway, with enhanced accumulation of key transcripts coordinate with other inducible defense genes (Peters and Constabel, 2002; Tsai et al., 2006). This suggests that studies of gene expression can provide important insight into defensive secondary metabolism in *Populus*.

At the molecular level, pioneering work in *P. trichocarpa x P. deltoides* (TD) hybrid poplar by Milton Gordon's laboratory established that simulated leaf herbivory leads to the rapid induction of specific defense-related genes (Parsons et al., 1989; Bradshaw et al., 1991). Importantly, this transcriptional response was observed within 8 h in both the wounded and unwounded leaves on wounded plants, indicating long-distance signalling and a systemic signal (Davis et al., 1991). The systemic nature of the response suggests that the changes in gene expression are important for defense against later herbivory, rather than simply contributing to wound-repair, and provide a potential mechanism for the observed systemic induction of resistance (Havill and Raffa, 1999). Strongly responding genes were identified as encoding a Kunitz trypsin inhibitor (KTI) win3, a putative vegetative storage protein (VSP) related to poplar bark storage protein, and chitinases win6 and win8 (Bradshaw et al., 1991). KTIs and other protease inhibitors are common anti-herbivore defenses known from seeds and vegetative tissues of many plants, and can interfere with digestive processes in herbivore guts (Ryan, 1990). Protease inhibitors are classic inducible defense proteins and their efficacy in reducing pest insect performance when overexpressed in transgenic plants has been shown (Ryan, 1990). The effectiveness of at least some chitinases as anti-herbivore proteins has also been documented (Ding et al., 1998; Lawrence and Novak, 2006). By contrast, the function or biochemical activity of the VSP-like protein is not clear, despite its consistent and strong induction by leaf damage in several later studies (Ralph et al., 2006; Christopher et al., 2004).

Pre-genomics studies of *Populus* defense also led to the cloning and characterization of a wound and herbivore-inducible polyphenol oxidase (PPO) in TD

hybrid poplar and *P. tremuloides* (Constabel et al., 2000; Haruta et al., 2001). The wound- and herbivore-inducible expression suggested a defensive role for this enzyme. PPOs oxidize common phenolic compounds to quinones that alkylate and cross-link other phenolics or proteins, leading to the browning of plant extracts. When such reactions occur in insect guts or mouthparts, they may lead to alkylation of dietary protein, reducing its quality for the insect. Low oxygen levels, however, may limit its effectiveness in some insects (Constabel and Barbehenn, 2008).

The observation that strongly induced genes encode proteins with adaptive or defensive potential is a basic principle with broad application in stress physiology. The early molecular work in *Populus* confirmed that trees, much like herbaceous plants, respond to herbivore attack with rapid transcriptional changes and induction of defense-related genes. This research provided the basis for the genomics and microarray studies of poplar defense that followed.

3 Impact of *Populus* Genomics on Knowledge of Inducible Defense Proteins

3.1 Discovery of Defense-Related Genes via ESTs and Gene Arrays

The application of genomic tools such as ESTs, micro- and macro-arrays, and differential display to poplar defense was a natural development given the strong inducible defense response identified earlier. A series of such studies from three different research groups provided two fundamental insights (Christopher et al., 2004; Ralph et al., 2006, 2008; Major and Constabel, 2006, 2007a, b, Lawrence et al., 2006). First, they made available a catalogue of candidate genes with potential roles in defense ("marker discovery"). Second, the expression profiles of a large number of genes allowed inferences to be drawn regarding signalling and physiological aspects of the response ("biology discovery").

An initial "snapshot" of broader gene expression and transcriptional changes in systemically induced TD poplar leaves was provided by a small-scale EST sequencing project and corresponding macroarray (Christopher et al., 2004). Among the 103 upregulated genes with functions in metabolism and defense, several new and highly divergent KTI genes were discovered. Likewise, a differential display experiment identified a suite of 57 caterpillar and wound-induced genes (Lawrence et al., 2006), while exploitation of a more comprehensive EST project and a resulting 15,500 gene microarray (representing approximately one quarter of the annotated genome) found that 1,191 genes were upregulated by forest tent caterpillar damage (Ralph et al., 2006). Genes affected by herbivory have putative functions in defense, signalling, transport, transcription, and secondary metabolism. Together, these studies have provided a substantial list of candidate genes from both primary and stress metabolism that need to be characterized further. Interestingly, genes belonging to the KTI, chitinase, pop3, nucleotide phosphohydrolase (apyrase), and

VSP families were identified among the most strongly induced genes in differential screening experiments by several different laboratories. This underscores their importance for the poplar defense response, even though a clear defensive role for the corresponding gene product may not be apparent.

The suite of poplar herbivore-induced genes should provide many opportunities for discovering novel herbivore defense mechanisms. For example, the pop3 gene encodes the ortholog of *P. tremula* Sp1, a previously studied boiling stable protein that may act as a chaperone (Wang et al., 2002; Dgany et al., 2004). It would be interesting to study the effects of such a protein on insect digestive systems. Other candidate genes for more detailed analysis encode esterase- and lipase-like proteins, the VSP-like proteins, and the apyrase and acid phosphatase. At present, functional studies can proceed on only one or a few genes at a time; thus the functional analysis of novel genes is clearly a bottleneck to progress in this area. To date, only for a handful of genes have roles with respect to herbivory been directly demonstrated (see Section 4).

Genes encoding proteins with possible signalling functions in defense have been of great interest to researchers in induced defense. For example, Ralph et al. (2006) identified 40 transcription factors whose abundance increased following feeding by forest tent caterpillar (FTC). Other inducible genes encode the octadecanoid pathway enzymes, for example, lipoxygenase and allene oxide cyclase. These enzymes participate in the synthesis of jasmonic acid and related molecules called jasmonates, key regulators of defense responses in herbaceous plants (Schaller and Stinzi, 2008). Since methyl jasmonate and jasmonic acid are highly effective inducers of the defense response in *Populus* (Constabel and Ryan, 1998; Havill and Raffa, 1999), it is likely that the *Populus* defense signalling mechanisms are similar to those in other model plants. Grafting experiments using defense response mutants suggested that the systemically mobile signal in tomato is jasmonic acid or a derivative (Li et al., 2002). The poplar systemic defense signal is likely also a jasmonate, but this has yet to be proven. Major and Constabel (2006) reported that several genes encoding ZIM motif proteins were wound- and caterpillar regurgitant-induced on macroarrays. Surprisingly, in tomato and *Arabidopsis*, a ZIM motif protein was found to be a key regulator of jasmonate signalling, and is hypothesized to be part of the complex that acts as a site of jasmonate perception (Thines et al., 2007).

An important caveat to the cumulative findings on herbivore-induced defense in poplar is the reliance of these studies on the TD hybrid H11-11, the genotype in which induction was first observed in M. Gordon's laboratory (Bradshaw et al., 1991). Whether the induced defense genes identified here are equally important in other *Populus* hybrids or species remains an open question. All *Populus* share very similar genomes, and as such have the same defense "toolbox"; however they may differ in how these are utilized. For example, PPO is strongly inducible in TD hybrids, less so in *P. tremuloides*, and virtually not induced in *P. tremula x alba* hybrid 717 (Wang and Constabel, 2004; Haruta et al., 2001; unpublished observations). Such differences are consistent with the high level of intraspecific and interspecific variation observed in defense compounds within *Populus* (see Section 5).

3.2 Global Gene Expression Patterns and Insights into the **Populus** Defense Response

Genome-level expression profiling has allowed for an analysis of transcriptional changes from a global perspective, leading to insight into broader adaptive patterns of the defense response. The induction by forest tent caterpillar of more than one thousand genes on a cDNA array emphasizes the complexity of the herbivore defense response. The large number of genes responding to this stress is similar to that described for other model plants (reviewed by Zheng and Dicke, 2008). At the level of transcriptional remodelling, real or simulated herbivory are clearly strong stressors.

A major question in the field of plant-herbivore interactions is whether the plant response is specific to different types of damage, and to what extent plants can differentiate between mechanical damage and herbivore feeding. Using a 569-element macroarray in poplar, Major and Constabel (2006) showed that the transcriptional response to wounding did not differ substantially from the response to forest tent caterpillar (FTC) regurgitant applied to small leaf punctures. While FTC regurgitant in the absence of significant wounding was a potent inducer of defense genes, crushing leaf margins with pliers elicited essentially the same set of genes. These results suggest that both inducing signals are transduced by a similar pathway. Like other lepidopteran herbivores, FTC regurgitant contains the fatty acid amino acid conjugate volicitin (Major and Constabel, 2006), a known defense elicitor in other plants (Felton and Tumlinson, 2008). For the highly induced genes of poplar, FTC regurgitant may act essentially as an amplifier of the wound response, perhaps by the insect's extracting and processing of plant-derived "self-damage" signals (Major and Constabel, 2007b). By contrast, much greater specificity in the response to caterpillar feeding on hybrid poplar was documented by Arimura et al. (2004). Here, FTC feeding, but not mechanical wounding, resulted in a dramatic release of terpenoid volatiles from hybrid poplar leaves, indicating that the plants are differentiating between simulated and actual herbivory (Arimura et al., 2004). In other experimental systems such as *Nicotiana attenuata* and *Arabidopsis thaliana,* clear differences in the induction profile of expressed genes in insect-damaged vs. mechanically-damaged plants have been observed (Roda et al., 2004; Reymond et al., 2004; Zheng and Dicke, 2008). However, the timing, extent, and repetition of mechanical damage can all modulate defense reactions, and responses previously thought to be insect-specific could be induced by a mechanical caterpillar that mimics feeding behaviour (Mithofer et al., 2005). These observations emphasize that can be difficult to compare transcriptomic responses between very different types of damage.

The question of defense response specificity should be addressed with herbivores from different feeding guilds, as has been done with *Arabidopsis* (de Vos et al., 2005; Kempema et al., 2007). *Populus*, with its diversity of natural pests, will be a natural system in which to do so. However, it will be essential to do comparisons within the same poplar genotype to avoid possible differences in the defense programs. Comparative experiments can now be carried out with standard *Populus* microarray platforms that are available, and have been used to compare

pathogen and herbivore defense response in poplar (Miranda et al., 2007). Although these defense reactions are generally thought to be distinct, a comparison of tent caterpillar damage and *Melampsora medusae* (poplar rust) infection using the same TD hybrid and 15,500 gene microarray found that many herbivore-induced defense genes are also induced 24h after *Melampsora* infection. However, the genes are then dramatically down-regulated (Miranda et al., 2007). This study illustrates the power of large scale transcriptomics for identifying larger trends of relevance for poplar defense, an approach can also be applied to study reactions to other poplar pests.

Expression profiling is also beginning to provide insight into the systemic and whole-plant effects of the induced defense response. Phillippe and Bohlmann (2007) reported that when sugar-exporting source leaves are wounded, the sugar-importing sink leaves develop a transcriptional response that includes activation of sugar metabolism genes. This observation is significant because the systemic signal activating defense genes first follows the strongest source-sink phloem connections defined by the vasculature of the sapling (Davis et al., 1991), although intraplant signaling via released volatiles may also be involved (Frost et al., 2007). Furthermore, jasmonate treatment of poplar saplings leads to repartitioning of the plant's carbon budget and stimulates increased export of carbon into sink leaves and roots (Arnold et al., 2004; Babst et al., 2005). How altered sink strength could contribute to systemic defense signalling, what signal molecules are involved, and how whole-plant responses are modulated, are all exciting questions for future research. In addition, since herbivory also leads to a down-regulation of transcripts encoding primary metabolic functions such as photosynthesis (Ralph et al., 2006), we can now begin to observe the effects of herbivore stress on primary metabolic processes. How the plant balances growth with defense, how carbon allocation is altered by herbivore stress, and how such metabolic reprogramming is regulated, can now be investigated (see Schwachtje and Baldwin, 2008). As a perennial, *Populus* may have evolved adaptive strategies different from those of other model plants.

3.3 Whole Genome Perspectives on Populus *Defense*

The elucidation of the *Populus* genome has facilitated evaluation of the size of gene families in this plant relative to herbaceous plants. One expectation has been that in perennials such as *Populus,* extended life span would lead to elaboration of the stress adaptive genes. While the comparative analysis of defense gene families is only just beginning, a first scan of the complete *P. trichocarpa* genome indicates an apparent over-representation of defense genes relative to the *Arabidopsis* genome (Tuskan et al., 2006; Kohler et al., 2008). This includes both the R-genes that are involved in recognition of pathogens, as well as classic pathogen defense genes such as the thaumatins (55 in *Populus* vs 24 in *Arabidopsis*), ß-glucanases and chitinases (together 131 vs 73). These are also of interest to herbivore defense, as some members of these gene families are induced by FTC (Ralph et al., 2006). Chitinases have been found among the most strongly and consistently herbivore-induced genes, and at least one such gene encodes a protein with direct negative impacts on lepidopteran pests (Lawrence and Novak, 2006).

Of the known herbivore defense response genes, the KTI gene family has been studied in the most detail. Analyses suggest that the KTI gene family contains at least 22 members (perhaps as many as 30) in *P. trichocarpa*, but their sequences diverge so that some genes in the family share only 25% sequence identity (Major and Constabel, 2008). Expression patterns as visualized by digital northerns and microarrays are also divergent. The KTIs are classified as inhibitors of serine proteases, but their low sequence similarity suggests that they could have distinct target specificities, and any one KTI is likely to inhibit only a subset of serine proteases. Differences in target protease specificity could thus explain the multiplicity of this group of genes in *Populus*. This hypothesis has been tested and corroborated by in vitro experiments (Major and Constabel, 2008). Representative KTIs taken from the major wound-inducible KTI clades had both different target preferences and 50% inhibitory concentrations (IC_{50}) when tested with commercial proteases trypsin, chymotrypsin, and elastase. Differences were also clearly apparent when tested with total proteases in crude midgut preparations from two different lepidopteran herbivores, FTC and bertha army worm (Major and Constabel, 2008). These data clearly support the idea that different members of the KTI gene family are specialized against different proteases, and perhaps pest insects. Significantly, several orthologous KTIs in natural populations of *P. tremula* in Europe have molecular signatures that indicate they are evolving rapidly and may be under positive selection (Section 5). Thus, the observed diversity of KTI genes in the *P. trichocarpa* genome reflects biochemical diversity and appears to be strongly influenced by selection. Such population-level studies need to be conducted with other defense gene families, for example the chitinases and polyphenol oxidases.

Proteomic-based studies of *Populus* herbivore defense also benefit from the availability of the *P. trichocarpa* genome, as such studies rely on genome-derived protein databases. A recent proteomic analysis focused on poplar phloem exudate, to determine if phloem protein profiles are altered by damage to leaves (Dafoe et al., 2009). Very few phloem exudate proteins appeared to change in abundance in response to wounding. However, two stress-related proteins, a pop3 and a thaumatin-like protein, are induced, and their presence in sieve elements has been corroborated via immunological methods (Dafoe et al., 2009). Their role in defense or in phloem physiology still requires investigation, however.

4 The Impact of Genomic Analysis on Advances in Phenylpropanoid and Other Secondary Metabolite Pathways

4.1 Genomic Analysis of Known Secondary Pathways – Insights into Flavonoid Biosynthesis and Regulation

As outlined above, *Populus* genomics has been very successful in identifying induced genes encoding proteins with potential direct defensive roles, such as protease inhibitors, enzymes, and a variety of proteins implicated in the regulation of the defense response. Such studies also discovered herbivore-upregulated genes

with similarity to enzymes of secondary metabolism, but which could not be easily linked to specific pathways or biochemical reactions. For example, cytochrome P450 and isoflavone reductase-like genes were identified as highly wound-inducible transcripts (Christopher et al., 2004; Ralph et al., 2006), but without deeper functional analysis or clearly co-expressed marker genes, their functions could not be readily determined. With the complete *P. trichocarpa* genome available, more systematic studies on *Populus* secondary pathways have been undertaken (Tuskan et al., 2006). The phenolic glycoside biosynthetic pathway has not yet been elucidated; by contrast, the flavonoid and condensed tannin pathway make an interesting target for comparative genomics studies, as this is well-characterized from work in *Arabidopsis* (Fig. 2). As a result, a catalogue of *Populus* genes required for basic flavonoid structures, including flavonols, flavones, chalcones, anthocyanidins, and condensed tannins, is now available (Tsai et al., 2006). Flavonoid phytochemicals can have many ecological functions, including ultraviolet and visible light screens, pigmentation, or signals (Harborne and Williams, 2000). For herbivore defense, the condensed tannins (proanthocyanidins) are of greatest importance. Genes relevant for the general phenylpropanoid pathway and lignin biosynthesis are described elsewhere in this volume (Chapter10), so are not discussed here. Nevertheless, some of the monolignol pathway-like genes are likely involved in other secondary metabolite pathways.

Many of the gene families dedicated to secondary metabolism appear to be expanded in the *Populus* genome, relative to *Arabidopsis* (Tuskan et al., 2006; Tsai et al., 2006). In the flavonoid pathway, for example, many of the steps are encoded by at least two genes in *Populus,* while *Arabidopsis* has only single-copy genes; this includes the late flavonoid pathway enzymes dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), and anthocyanidin reductase (ANR), as well as flavone synthase and the flavonoid 3′, 5′-hydroxylase (Fig. 2). *Populus* also contains three different genes for LAR (leucoanthocyanidin reductase), one of the last known steps in tannin synthesis. LAR is not present in *Arabidopsi*s, which unlike most species, produces only anthocyanidin reductase (ANR)-derived tannins. For the chalcone isomerase (CHI), flavanone 3 hydroxylase (F3H) and flavonoid 3′ hydroxylase (F3′H) steps, only single genes are present in both *Populus* and *Arabidopsis*. By contrast, *Populus* contains six chalcone synthase (CHS) genes, compared to a single gene in *Arabidopsis* (Tuskan et al., 2006). Remarkably, *Populus* is reported to contain 11 genes identified as flavonoid *O*-methyltransferases (FOMTs), compared to one in *Arabidopsis*. Conversely, *Arabidopsis* has six flavonol synthase (FLS) genes vs. four in *Populus.* Functional analysis was able to detect FLS activity for only one of the *Arabidopsis* FLS genes (Owens et al., 2008), however, a reminder that functional prediction for enzymatic activities based on only sequence similarity must be approached with caution.

The functional significance of the additional copies of flavonoid-related genes in *Populus* is as yet unclear. The large diversity of flavonoids of poplars and aspens is likely an important factor, as is the large flux of carbon into the major flavonoid end product, condensed tannin. Some of the additional genes may represent tissue-specific isoforms that facilitate complex patterns of accumulation. RT-PCR analysis

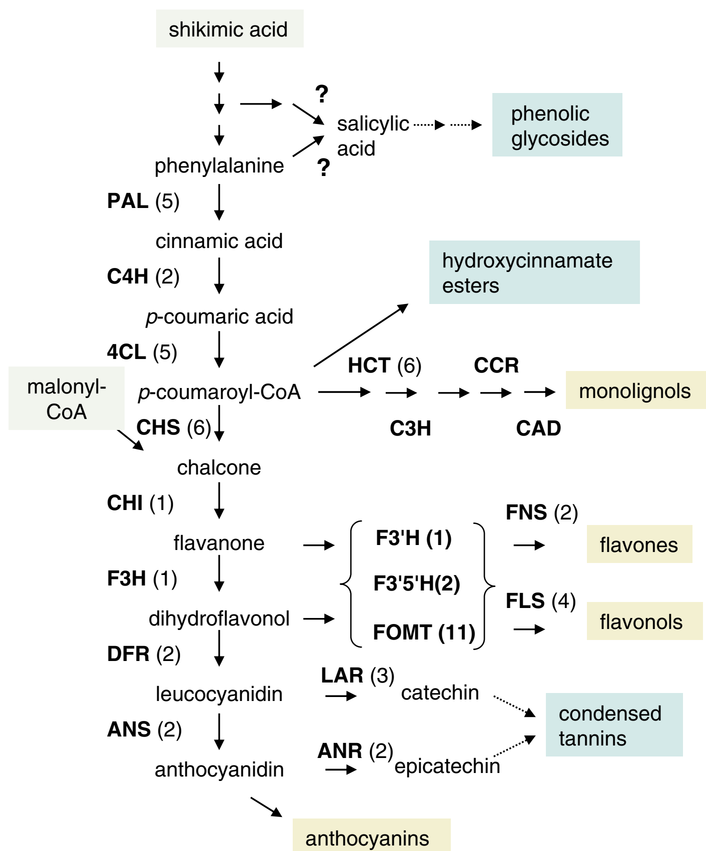

Fig. 2 Overview of biosynthetic pathways leading to major groups of phenolic compounds in *Populus*. Phytochemicals of primary relevance to plant defense are indicated on *blue panels*. *Dashed arrows* indicate unresolved biosynthetic steps, and the *brackets* indicate enzymes with uncertain position in the pathways. Numbers in parenthesis indicate the number of genes for that enzyme identified in the poplar genome. Abbreviations are as follows: PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate CoA Ligase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl-transferase, C3H, *p*-coumaroyl CoA 3-hydroxylase, CCR, cinnamoyl-CoA reductase; CAD, cinnamoyl alcohol dehydrogenase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FFLS, flavonol synthase; FNS, flavone synthase II; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase

of the CHS family, for example, does indicate some differential expression between members of this expanded gene family (Tsai et al., 2006). However, many flavonoid-related genes appear to be constitutively expressed in the same tissues, such as root tips or young leaves (Tsai et al., 2006). More precise localization of gene expression may clarify if there is differential expression of isoforms within tissues or cell types. For example, Kao et al. (2002) used in situ hybridisation to localize the expression of PAL1 and 4CL2 isoforms to areas of tannin accumulation, clearly distinct from

expression of lignin-related PAL and 4CL isoforms. Expanded gene families could also reflect functional specialization of isoforms to specific environmental conditions or stresses such as wounding, pathogen attack, or UV light stress. LAR3 and ANR1 isoforms, for example, are more wound-responsive than other members of the respective gene families, although all members responded to some extent (Tsai et al., 2006).

Alternatively, the additional copies of enzymes may display altered substrate preferences and lead to different end products. The large number of O-methyl transferase genes could be required to methylate various *Populus* flavonoids, for example. For the CHS gene family, Tsai et al. (2006) speculate that the additional isoforms may use cinnamoyl-CoA, caffeoyl-CoA, or feruloyl-CoA rather than the "standard" *p*-coumaroyl-CoA as substrates. Each isoform may thus be required to produce the respective chalcones pinocembrin, eriodictyol, and homoeriodictyol, rather than naringenin. These chalcones have all been identified in poplar bud exudate, but the in vitro specificity of the enzymes is not known.

A powerful approach for determining the functions of specific members of flavonoid enzyme gene families, or for identifying additional genes in flavonoid biosynthesis, is co-expression analysis. In *Arabidopsis*, Yonekura-Sakakibara et al. (2007) have shown how gene expression profiling and co-expression with known anthocyanin genes has helped to identify the rhamnosyl transferase gene acting on flavonoids. Their strategy was particularly successful because the co-expression approach was applied to transgenic plants overexpressing a specific transcription factor, the *Arabidopsis* PAP1 anthocyanin regulator (Tohge et al., 2005; Saito et al., 2008). A similar approach could be useful for identifying the genes involved with late enzymatic steps in tannin synthesis, in particular the condensation steps, and *Populus* is a good system to tackle this. The recent identification of the *Populus* MYB134 gene, which drives condensed tannin accumulation when overexpressed in transgenic aspen (Mellway et al., 2009), should facilitate progress in this area. The publication of the genome sequence of grapevine (*Vitis vinifera*) (Jaillon et al., 2007) will also provide additional opportunities for using comparative genomics to help elucidate secondary pathways, since grape accumulates many flavonoids and phenolics also found in *Populus.*

It will be interesting to characterize the transcription factors regulating flavonoid pathways in *Populus* using a co-expression approach. Potential candidates are the MYB, bHLH, and WD40 transcription factor genes, which are required for tannin biosynthesis in *Arabidopsis* seed coats (Lepiniec et al., 2006) and thus likely important for *Populus* tannin biochemistry as well. For example, the MYB134 gene was first identified by similarity to the *Arabidopsis TT2* gene and co-expression with tannin biosynthetic genes after stress treatments, and shown to be a key regulator of condensed tannin synthesis in vegetative tissues of *Populus*. Other transcription factor genes will undoubtedly be identified using this strategy, so that the regulatory network controlling flavonoid synthesis can ultimately be dissected. Unlike their distribution in *Arabidopsis*, in *Populus* condensed tannins are synthesized in most tissues and organs including leaves, roots, and bark (Lindroth and Hwang, 1996); thus it will be possible to compare the regulatory mechanisms for tannin synthesis

in different developmental and environmental contexts. Such transcription factor genes will provide important tools for manipulating entire secondary pathways in transgenic plants, and for facilitating the testing of defensive and other ecological functions of these secondary metabolites.

The regulatory genes that control flavonoid biosynthesis may provide clues as to how trees can integrate the different environmental signals and parameters that collectively influence phenolic profile and content. Many environmental factors, including the availability of resources (e.g., CO_2, light, nutrients, water) and diverse stresses (herbivores, pathogens, UV light), shape the quantitative profiles of secondary compounds in *Populus*. While in general, levels of phenolic glycosides show only a minimal response to differential resource availability (Kinney et al., 1997; Hemming and Lindroth, 1999; Hale et al., 2005), levels of condensed tannins are strongly responsive to resource availability and stress (Miranda et al., 2007; Peters and Constabel, 2002; Harding et al., 2005; Osier and Lindroth, 2006). Consistent with the growth-differentiation balance hypothesis (Herms and Mattson, 1992), concentrations of tannins typically increase under conditions of high light and high CO_2 availability, but decrease under conditions of high nutrient availability (Kinney et al., 1997; Hemming and Lindroth, 1999; Osier et al., 2006). Increasingly, genomic tools will afford insight into the regulation of differential responses of biosynthetic pathways to these environmental factors.

4.2 Genomic Analysis and Discovery of Novel Pathways and Metabolites

The portions of the phenylpropanoid pathway dedicated to lignin synthesis in *Populus* have been under intense investigation (Hamberger et al., 2007) and are described elsewhere (Chapter 10). For the major biosynthetic steps, the *bona fide* lignin biosynthetic genes are clearly delineated phylogenetically, and in many cases validated with biochemical assays of gene products. For several "lignin" gene families, however, the *P. trichocarpa* genome contains additional genes, often in expanded and distinct clades; these gene products are believed to catalyse related reactions but using different phenolic or phytochemical substrates (Tuskan et al., 2006; Hamberger et al., 2007). For example, the cinnamoyl dehydrogenase (CAD) gene family is greatly expanded, with 15 CAD-like genes in two subclades in the *Populus* genome. Both subclades show lineage-specific expansion (Hamberger et al., 2007), suggesting that these genes function in *Populus*-specific secondary phenolic pathways. Since CAD catalyses the NADP-dependent reduction of cinnamaldehyde to cinnamyl alcohol, one can speculate that the additional genes reduce other types of phenolic substrates. Similar ideas apply to the cinnmoyl-CoA reductase (CCR) gene family, which contains a large number of genes in several clades that appear to function outside of lignin synthesis (Hamberger et al., 2007). Two other gene families, the caffeoyl CoA O-methyl transferases (CCOMTs) and the caffeic acid O-methyl transferases (COMTs) also have non-lignin subclades, but

in *Populus* these subclades are smaller than in *Arabidopsis* and contain four and six members, respectively (Hamberger et al., 2007).

In the case of the hydroxycinnamoyl transferase (HCT) gene family, *Populus* contains five additional genes not found in *Arabidopsis*. HCT is a recently discovered enzyme of lignin biosynthesis (Hoffman et al., 2003) catalysing the reversible transfer of *p*-coumaroyl-CoA to shikimate to generate *p*-coumaroyl shikimate. This protein is very similar to the enzyme identified in tobacco that makes chlorogenic acid (caffeoyl-quinate) (Niggeweg et al., 2004), itself not a direct lignin intermediate. Since *Populus* trees can accumulate substantial amounts of chlorogenic acid and other quinate and shikimate HCDs that vary with species and section of the genus (Tsai et al., 2006), there is likely a relation between the diversity of these chemicals and the number of HCT-like genes in the genome. While the function of the HCDs has not been demonstrated, caffeic acid derivatives are excellent substrates for the herbivore-induced poplar PPO, and could thus contribute directly to defense against insects (Constabel et al.2000; Wang and Constabel, 2004). HCDs may also be important as UV light screens or antioxidants.

The discovery of lignin-related enzymes is a rich ground for functional genomics, as the reactions catalysed are likely conserved. Delineation of the gene families can now be followed up with gene-specific expression profiling that may allow for more precise association of these genes with tissues or cells of known metabolic and physiological function, or with stress responses. Hamberger et al. (2007) used expression data from poplar microarray experiments (Ralph et al., 2006) to demonstrate the response of several members of the CAD-like (CADL) gene families to real or simulated herbivory. The results validate the co-expression approach and provide leads for further analysis; for example, CADL10 responded most strongly to herbivory, while CADL3, 9, and 10 responded to artificial wounding or methyl jasmonate. One can speculate that these genes are involved in unknown defense-related secondary metabolite reactions or pathways. Greater knowledge of the diversity of secondary metabolites and pathways of various *Populus* species is now required to move this type of work forwards. Phytochemical databases for *Populus* secondary metabolites as have been generated for *Arabidopsis* flavonoids (Yonekura-Sakakibara et al., 2008) would be immensely useful. As more whole-genome experiments are available, the power of expression profiling in combination with metabolomic and phytochemical profiles will become apparent.

The salicylate-derived phenolic glycosides comprise a significant amount of fixed carbon in *Populus*, yet very little is known about their biosynthesis beyond the shikimic acid pathway (Tsai et al., 2006). We anticipate that genomics approaches will facilitate identification of relevant enzymes via identification of the genes involved. Indeed, several research groups are currently investigating phenolic glycoside biosynthesis via candidate gene (e.g., QTL) or other genomics-informed approaches. Unlike the tannins, and despite strong evidence indicating a function in defense against insects, the salicylate PGs appear not to be strongly wound-induced in most *Populus* species and genotypes, limiting the usefulness of the expression profiling approach.

5 Functional Analysis of Genes Important for *Populus* Defense and Secondary Metabolism

Transcriptional profiling and genome mining have proven useful for identifying genes implicated in defense and secondary metabolism of *Populus.* However, candidate genes must still be functionally tested to define their roles in plant or insect fitness, or as components of secondary metabolite pathways. In the post-genomic era, these are typically the limiting steps, and methods facilitating higher throughput are needed. The Gateway cloning system, with its diversity of available destination vectors for protein expression in trasngenic plants or other organisms, greatly facilitates functional studies (Curtis and Grossniklaus, 2003; Karimi et al., 2007). Genetic transformation of *Populus,* while relatively efficient, is still time-consuming, and only practical with a restricted set of genotypes (Han et al., 2000).

The direct testing of defense genes in transgenic *Populus* has been most extensively applied to PPO, in a *P. tremula x alba* background with low endogenous PPO activity. Early instar FTC feeding on PPO-enhanced leaves of this aspen hybrid grew at reduced rates and experienced higher mortality compared with insects fed control leaves. However, this response was observed only late in the season, using egg masses of reduced vigour (Wang and Constabel, 2004). Other results with these plants were less clear: fourth instar *Lymantria dispar* had decreased growth rates on high PPO foliage, but experiments with *Orgyia leucostigma* showed contradictory results (Barbehenn et al., 2007). Nevertheless, PPO could contribute to defense against poplar and aspen pests in conjunction with other defenses. In transgenic tomato plants overexpressing PPO, enhanced resistance against leaf eating caterpillars has been demonstrated (Constabel and Barbehenn, 2008).

Direct anti-herbivore effects of putative poplar defense proteins were also shown using a transient infection of tomato and *Nicotiana benthamiana* with potato virus X (PVX) (Lawrence and Novak, 2001, 2006). This heterologous system permits the expression and functional testing of genes within 3–4 weeks. One disadvantage is that *Populus* pests cannot be tested on tomato or *Nicotiana* leaves. Nevertheless, the activity of the win3 KTI against a lepidopteran (*Heliothis virescens*), and the activity of the win6 chitinase against a coleopteran (*Leptinotarsa decemlineata*), were established in this manner. For gene products with direct effects on insects, defense proteins or enzymes can be tested as recombinant proteins rather than in transgenics, for example in in vitro assays as described for the KTIs (Major and Constabel, 2008). It should be noted that the inhibition of gut proteases in vitro does not in itself demonstrate that this gene product will impact the herbivore. To test for an effect in vivo, the most active recombinant KTI was produced in large *E. coli* cultures, purified, and incorporated into artificial diets for bioassays. When consumed by FTC larvae at physiologically realistic levels, the recombinant KTI resulted in a significant reduction in pupal weight compared to controls (I. Major, E. Despland, C.P. Constabel, unpublished results).

For *Populus* secondary metabolism, few genes have been tested directly, though there are many interesting candidates for functional analysis. Where mutants for

equivalent steps are known in *Arabidopsis*, complementation will be a useful tool to establish biochemical functions, i.e. to define substrates and products. Roles for specific gene family members in particular pathways can be established using RNAi to downregulate individual genes in transgenic *Populus*, combined with phytochemical profiling to confirm the predicted alteration. The availability of metabolite databases and standards to facilitate the identification of altered metabolites will be essential for this strategy to be effective and facilitate rapid screening. A recent RNAi experiment to down-regulate the *p*-coumaroyl CoA 3′-hydroxylase for lignin synthesis provides an instructive example. The downregulation of this gene demonstrated its central function for monolignol synthesis, but it also led to unexpected accumulation of secondary compounds including *p*-coumaroyl glycosides and esters, as well some salicylate-derived phenolic glycosides (Coleman et al., 2008). Likewise, transgenic modification of *gai* and *rgl1*, genes involved in gibberellin-mediated growth physiology, simultaneously altered secondary metabolite profiles in *Populus* (Busov et al., 2006). These experiments show how reduced carbon flux through the lignin biosynthetic pathway in leaves can lead to an increase in phenylpropanoid-derived storage and defense compounds. Likewise, the role of the MYB134 transcription factor in condensed tannin synthesis was shown in the dramatic accumulation of condensed tannins in MYB134-overexpressing transgenic *Populus* (Mellway et al., 2009) Such transgenic experiments are essential for uncovering metabolic interactions of primary and secondary pathways, and ultimately enhance our understanding of metabolism and control of secondary chemistry. Plants with modified secondary metabolite profiles will also be of tremendous importance in refining our knowledge of defensive and other ecological functions of secondary metabolites. In particular, transgenic poplar or aspen with altered salicylate phenolic glycosides or condensed tannins will permit the direct testing of their defensive roles against different pests or stresses, and are already showing unexpected results in greenhouse experiments (Mellway and Constabel, 2009). Ultimately, experiments in natural settings can be also be carried out. The importance of testing transgenic plants in realisitic ecological situations has been convincingly demonstrated in the *Nicotiana attenuata* system developed by Baldwin and colleagues (i.e., Kessler et al., 2004).

6 The Importance of Variation in Plant Defense and Secondary Metabolite Profiles in *Populus*

Populus species often exhibit striking genetic variation, and in most cases, substantial phenotypic plasticity (which itself is genetically determined). These characteristics have been fundamentally important to the evolutionary success of *Populus*, and provide both challenges and opportunities for understanding interactions of these trees with the environment. The ecological importance of variation in chemical profiles and the value of presenting a “moving target” in terms of delaying the evolution of resistance in insects, are widely accepted (Adler and Karban, 1994). Genotype-dependent variation in levels of resistance to insect herbivores has been

shown for native aspen genotypes (Lindroth and Hwang, 1996) as well as different poplar hybrids (Robison and Raffa, 1994; 1997).

In *Populus*, foliar levels of the phenolic glycosides and condensed tannins, exhibit extraordinary quantitative variation (1–25% dry weight), both among and within species. Such variation is the consequence of multiple, interacting factors, including genetics, ontogeny, as well as environment. Striking intraspecific (clonal) variation in concentrations of both phenolic glycosides and condensed tannins has been reported in trembling aspen (e.g., Lindroth and Hwang, 1996; Donaldson et al., 2006; Osier and Lindroth, 2006). Concentrations of these phenolic metabolites are generally much more variable among genotypes than are those of primary metabolites such as carbohydrates. Interspecific genetic variation can also strongly influence types and amounts of phenolic glycosides and condensed tannins in *Populus* species. For example, in the hybridising complex *P. fremontii* x *P. angustifolia*, *P. fremontii* produces very low levels of tannins and appreciable levels of the salicylate HCH-salicortin, whereas *P. angustifolia* produces substantial levels of tannins and little to no HCH-salicortin (Rehill et al., 2006). Both species, however, produce salicortin.

An additional source of variability in secondary chemistry is a consequence of strong ontogenetic variation (genetically-determined developmental patterns), as individual trees of some species, such as cottonwoods, have discrete juvenile and mature developmental zones with different chemical patterns. Foliar phenolic glycoside concentrations decrease, while tannins increase, with developmental age, and the magnitude of developmental trajectory varies among species (Rehill et al., 2006). Other *Populus* species, such as aspens, have less clear-cut developmental zones within individual trees, but nonetheless show strong developmental shifts in chemical composition with tree (or ramet) age (Donaldson et al., 2006). Marked ontogenetic variation in biochemical traits emphasizes the importance of making inferences about gene expression within appropriate developmental contexts, as the influence of various environmental factors on gene expression may differ between juvenile and mature trees. Variation due to environmental conditions, stresses, and nutrient availability was discussed previously, but it must be noted that responsiveness to the environment itself varies widely within different clones and genotypes (Harding et al., 2005; Havill and Raffa, 1999).

To determine how this broad variation at the phenotypic level is encoded by variation in the genome at the DNA sequence level will be the next great challenge. For the chemical variation described above, the characterization of pathways and regulatory genes will be a necessary first step, and must take into account the integration of developmental and environmental signals. However, rapid and economical resequencing and single nucleotide repeat (SNP) detection methods may permit association mapping to identify loci relevant to the generation of chemical variability (Neale and Savolainen, 2004; Whitham et al., 2008). For defense gene products with clearly defined functions and direct ecological effects, the pattern of variation of nucleotide sequences in different populations and species is already very informative. The KTI genes encode inhibitor proteins that interact directly with herbivore proteins (gut proteases), and thus provide a direct read-out of ecologically

relevant nucleotide sequence variation. A recent study compared synonymous vs. non-synonymous nucleotide substitution patterns in the *P. tremula* wound-induced KTIs, from four different European populations (Ingvarsson, 2005). Two of the KTI gene show signs of long-term adaptive evolution, possibly due to herbivore pressure. Several other KTI genes also show elevated rates of non-synonymous substitutions, implying non-neutral evolution. Given that the KTIs exhibit divergent biochemical specificities in vitro which indicated that any one KTI is effective against only a subset of proteases (Major and Constabel, 2008), it is tempting to speculate that these increased non-synoymous substitution rates are the result of selection for new inhibitor specificities.

Variation in *Populus* defense genes and secondary metabolite profiles affect entire complexes of organisms, and thus are key determinants of the community and ecosystem "phenotypes" of *Populus* (Whitham et al., 2006, 2008). When a foundation species' genotype influences the abundance and composition of associated species (e.g., microbes, insects) to the extent that discrete communities develop, community phenotypes result. Similarly, when such genetic variation governs ecosystem structure or function (e.g., nutrient cycling), ecosystem phenotypes result. Of all plant systems, community and ecosystem phenotypes have been most thoroughly documented in *Populus* and *Eucalyptus*. In the riparian hybridizing complex *P. fremontii* x *P. angustifolia* in the western U.S.A., cottonwood genotype influences tannin production, which in turn influences the community composition of folivorous insects as well as aquatic macroinvertebrates (Whitham et al., 2006). Tannin concentration also affects terrestrial and aquatic litter decomposition, and terrestrial nitrogen mineralization (Whitham et al., 2006, 2008). Significant heritabilities of canopy insect communities, insect-bird interactions, soil microbial communities, and soil nutrient pools reveal that community and ecosystem phenotypes are based on genetic variation in cottonwood chemistry (Whitham et al., 2008). In a similar manner, aspen tannin concentrations influence litter decomposition (Madritch et al., 2006). Consequently, aspen clones create spatial mosaics of genetically-mediated ecosystem functioning across natural landscapes (Madritch et al., 2009). In short, secondary chemistry and its variation have proven to be key intermediates in efforts to link ecological structure and function with underlying genomics.

7 Key Issues and Future Directions

1. *Populus* adaptation and responses to herbivory clearly involve large numbers of genes, and to focus on the most important "candidate genes" will require additional experimental approaches. In addition to high-throughput functional analysis and detailed coexpression studies, strategies to exploit the high degree of natural genetic diversity in *Populus* populations, for example, using association mapping to determine genes of adaptive value, hold much promise. Similarly, defense genes could be screened in wild populations for evidence of strong selection in their evolutionary history. The identification of genes determining

success in defense against herbivory will contribute to our understanding of mechanisms of resistance, provide markers for future breeding programs, and facilitate development of a toolbox of genes for potential genetic engineering of resistant *Populus* genotypes.

2. The size and architecture of *Populus* make it a useful model for investigating whole plant aspects of induced defense responses. The systemic nature of defense signalling and how this interacts with leaf vascular connections and source-sink dynamics needs to be studied further, as well as the potential contributions of volatiles to intra-plant signalling (Frost et al., 2007). Furthermore, the importance of the juvenile growth phase, compared to the mature tissues, in induced defense has not been approached at the molecular level. Likewise, how above- and below-ground portions of the plant interact during stress responses and pest resistance is only beginning to be investigated (Major and Constabel, 2007a), but are key elements of the ecological roles and interactions of *Populus* species with the environment.
3. Phenolic constituents, particularly phenolic glycosides and tannins, play singularly important roles in *Populus* biology, and future genome-informed research must focus on further elucidation of the biochemical pathways and genes involved in their synthesis and regulation. This work will require more complete phytochemical profiles for different *Populus* species and hybrids of interest. The future availability of phytochemical and metabolomic databases for *Populus* would greatly accelerate work in this area, as will access to gene expression data in standardized platforms (i.e. Affymetrix *Populus* arrays). When metabolomic and gene expression data are integrated, coexpression analysis becomes a powerful tool for functional genomics that is particularily suited for analysis of secondary metabolism and its regulation (Saito et al., 2008). Dissecting the regulatory mechanisms that control phenolic metabolism will be the first step in understanding how the variable phytochemistry within the genus is generated, and how the diverse developmental and environmental signals that influence phenolic metabolism are integrated within the plant.
4. Because *Populus* includes numerous foundation species, functional genomic studies of plant defense and secondary chemistry will play important roles in the newly emerging field of landscape genetics, which addresses spatial patterns in population genetic structure and evolutionary processes. Future research should seek to understand the molecular underpinnings of adaptive variation in *Populus* secondary chemistry, and how they play out over large spatial and temporal scales to influence the structure of, and dynamic processes within, entire landscapes. The application of newly developed, high-throughput genome sequencing and genotyping technologies to natural populations (e.g., Whitham et al., 2008) will facilitate identification of genetic polymorphisms associated with complex adaptive traits (Neale and Savolainen, 2004).
5. *Populus* has emerged as the most important genus of woody plants for biofuel production in North America (DOE 2006). Lignin, a product of the phenylpropanoid pathway, poses a significant barrier to use of *Populus* as feedstock for the production of cellulosic ethanol. Alternatively, however, lignified

tissues provide a reservoir for biomass sequestration of atmospheric carbon. Multiple research projects, relying heavily on genomic approaches, are currently underway to engineer poplar with altered lignin quality and quantity. The interconnectedness of the lignin and phenylpropanoid pathways necessitates a much better understanding of how modulation of flux in one area of metabolism can influence associated pathways. The unexpected consequences of genetic engineering on metabolic pathways emphasize that our knowledge of the regulation of carbon flux through secondary pathways is still incomplete.

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Part IV
Populus for the Future

Populus Breeding: From the Classical to the Genomic Approach

Brian J. Stanton, David B. Neale, and Shanwen Li

Abstract *Populus* breeding is distinguished by a long history in forest tree improvement and its frequent dual reliance on inter-specific hybridization and varietal selection as the prominent domestication strategy. This chapter presents a review of the genecology and the principal long-term improvement approaches considered in the manipulation of the genus' key taxa, the pertinent experimental design features of worldwide varietal evaluation programs, and the current understanding of the morphological, physiological, and pathology components of yield and the physical and chemical components of wood quality. The chapter concludes with an assessment of the molecular tools being developed for an integrated translational genomics program to improve upon present breeding and selection methodologies.

1 Introduction

Populus was the first woody perennial to gain recognition as a model for worldwide tree breeding programs because of the groundbreaking work in species hybridization, polyploid breeding, and investigations into pathogen resistance during the early part of the twentieth century (Pauley, 1949). More recently, the success that *Populus* clonal testing, selection, and deployment has achieved in boosting the trend toward worldwide varietal forestry over the last 20 years cannot be overestimated. Although tree improvement work in *Populus* may be surpassed in sophistication by today's *Pinus* and *Eucalyptus* breeding programs, the model designation remains deserved in view of the sequencing of the *Populus* genome – the first of any tree in 2006 – and the subsequent investigations into genotype-phenotype associations. This chapter presents an overview of the traditional approach to applied *Populus* breeding and the advent of translational genomics, surely the next stage in a truly fascinating story.

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Domestication of the genus began in Europe, perhaps as a consequence of the introduction of eastern cottonwood (*P. deltoides*) in the late eighteenth century and the frequency with which spontaneous – and at times valuable – hybrids with the native black poplar (*P. nigra*) (hybrid binomial – *P.* ×*canadensis*) occurred under natural conditions. That led to their cultivation for timber production to forestall widespread wood shortages, especially after the Second World War (Schreiner, 1959). During his tour of European *Populus* culture in 1952, American poplar pioneer Ernst Schreiner reported that 11 countries were heavily invested in *Populus* controlled breeding programs, saying, *"...poplar specialists and growers... generally recognize that there is an essential and continuing job to obtain better clones for future use and to replace those that may fall prey to unusual environmental conditions or to new diseases and insects."* His recognition was taken to heart in Europe and elsewhere, for over the next half century catalogues of superior cultivars complete with photographs and growth and form metrics were published for four of the five continents where *Populus* culture had spread. This acknowledgement included Europe (Koster, 1972; van Broekhuizen, 1972), North America (Roller, 1984), South America (Arreghini et al., 2000), and Asia (Chen, 2005). At the 23rd Session of the International Poplar Commission held in Beijing in 2008, it was reported that over 125 elite *Populus* cultivars were globally in use (FAO 2008).

Populus management is unique in that its markets include a wide range of forest products, including energy feedstock, wood chips for pulping fibers and composite panels, saw- and veneer logs, agro-forestry, and phyto-remediation as well as several other environmental applications. Presently, the worldwide *Populus* estate encompasses over 5,255,000 hectares of plantations and 3,867,000 hectares of agroforestry and environmental plantings (FAO, 2008). The management of this estate continues to emphasize the breeding of improved cultivars. This domestication activity, in turn, relies upon the accumulated knowledge of *Populus* genecology, the physiological and morphological components of yield, the genetics of pathogen resistance, and the inheritance of quantitative and qualitative traits (Stanton, 2009). While work in each of these areas has provided insights into the genetics of adaptation and wood production, the identification of controlling genes and the characterization of selectable markers is now forging new breeding approaches that will extend *Populus*' claim as the model woody perennial (Bradshaw et al., 2000).

2 Genecology

Geographic Distribution – The genus *Populus* is made up of six sections, three of which – *Aigeiros* (cottonwoods), *Tacamahaca* (balsam poplars), and *Populus* (white poplars and aspens) – account for nearly the world's entire applied breeding work. Recent taxonomies published in the West closely agree on the total number of species, which range from 29 (Eckenwalder, 1996) to 32 (Dickmann and Kuzovkina, 2008). But in Asia a more liberal classificaion is the rule, with 47–50 species recognized in China alone (Wu and Raven, 1999; Zheng, 1985). Taxonomic rank has,

at times, been extended below the species level to geographic varieties to recognize entities with distinct morphological or physiological features. Good examples include the xeromorphic *P. nigra* var. *caudina* and *P. tremula* var. *davidiana* and *P. deltoides* var. *monilifera* that at times have been used in breeding and selection programs (Kajba et al., 2004).

Directed manipulation of the genus started with an understanding of population variation patterns in adaptive and commercial traits within each of the genus' key species that, as a rule, cover expansive geographic areas (Fig. 1). In section *Populus*, for instance, the transcontinental range of quaking aspen (*P. tremuloides*) covers approximately 110° of longitude and over 50° of latitude in North America, from Alaska's sub-arctic region and Canada's Northwest Territory to disjunct populations in central Mexico (Perala, 1990). Likewise, common aspen (*P. tremula*), its sibling species, has the most expansive range in the genus and is found throughout most of Europe and a substantial part of Asia. It spans 155° of longitude from Europe's Iberian Peninsula east to Asia's Kamchatka Peninsula, and 55° of latitude from Scandinavia to southeastern China (Boratynska and Boratynski, 1977). White poplar (*P. alba)* is also found over a large expanse of Eurasia. It is spread across a longitudinal range of approximately 115° from Spain's Atlantic Coast eastward to China's Xinjiang Uigur Autonomous Region, Afghanistan, Iran, Iraq, and Pakistan. North-to-south, *P. alba* covers approximately 30° of latitude found as far north as 54–58° N latitude in Germany, Poland, and Russia and as far south as 30° N latitude in North Africa.

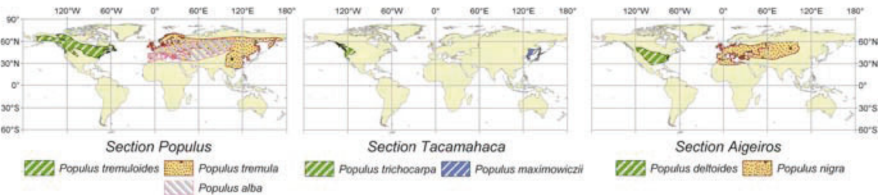

Fig. 1 World distribution of *Populus* species most commonly used in controlled breeding programs

Extensive distributions also characterize *P. nigra* and *P. deltoides* of section *Aigeiros*. The former is found over a large portion of Europe, the Mediterranean basin, Central Asia, the Ukraine, Russia, and the northwest of China spanning more than 40° of latitude and 90° of longitude (Boratynska and Boratynski, 1977). Distribution of *P. deltoides* covers over 20° of latitude in North America between the Canadian prairie and the Gulf of Mexico and over 40° of longitude between the Atlantic seaboard and the Great Plains (Cooper, 1990). The distribution of species in section *Tacamahaca* is also substantial: Black cottonwood (*P. trichocarpa*) spans approximately 35° of latitude from Cook Inlet along the Alaskan Coast southward to the outlying populations of Mexico's Baja Peninsula, and 45° of longitude from the Rocky Mountains to the coast of southeast Alaska (DeBell, 1990). Finally, Japanese poplar (*P. maximowiczii*) ranges throughout eastern Asia, including Russia's Kamchatka Peninsula and the Kuril Islands, the Provinces of Liaoning,

Jilin, and Heilongjuang in the northeast of China, the Korean peninsula, and the Japanese Islands of Sakhalin, Hokkaido, and Honshu, covering 25° of longitude and 20° of latitude (Chiba, 1984).[1]

Variation Patterns – Genetic variation in adaptive traits for each of these species is commonplace and often associated with latitude as clines. Phenology is perhaps the best example, and an understanding of its variation pattern has long been a foundation of well-designed *Populus* breeding programs. Generally, southerly seed sources initiate growth later in the spring and initiate the onset of dormancy later in the fall and, as a consequence, are often less tolerant of winter temperature extremes when compared to more northerly sources in common garden experiments. A range-wide study of autumnal phenology in *P. deltoides* provenances from 30° to 45° N latitude and planted at 40° N latitude demonstrated this phenomenon. The date of leaf abscission was closely associated with seed source latitude in a north-to-south trend over which the date of leaf abscission grew progressively later (Ying and Bagley, 1976). The timing of autumnal leaf abscission was also observed to vary in a northwest-to-southeast direction within the southwestern portion of the *P. deltoides* range between 28° 51′ and 38° 25′ N latitude, in which southeastern seed sources exhibited a later date of abscission than northwestern ones (Nelson and Tauer, 1987). A similar trend was observed in the date of autumnal bud set in an investigation of *P. tremula* seed sources sampled from 56° to 66° N latitude in Sweden where a later terminal bud set date was associated with southerly sources (Luquez et al., 2008). The photoperiod of the genotype's provenance has been implicated as the controlling environmental factor. This was evident in studies of northern and southern sources of *P. trichocarpa* and *P. tremula* (∼ 34–53° N latitude for the former and ∼56–66° N latitude for the latter) where the onset of the dormancy process of the southern sources responded to a shorter day length compared with more northerly sources (Howe et al., 1995; Ingvarsson et al., 2006).

Temperature replaces photoperiod as the controlling mechanism that triggers spring phenological events. Seed sources originating at low latitudes require either a more stringent chilling requirement or higher heat sums before growth is initiated when compared with their counterparts from higher latitudes (Farmer, 1993). Farmer and Reinholt (1986) illustrated the trend in a controlled chilling study of balsam poplar (*P. balsamifera*) populations originating from 45° to 55° N latitude where the length of time to initiate shoot growth decreased with increases in seed source latitude.

The upshot of spring and autumnal adaptive patterns for controlled breeding programs is that selections moved south of their provenance – either as clones or as breeding stock – may not perform as well as local sources due to the inability to take full advantage of the growing season, while selections moved north of their

[1] Several authors in this text follow Eckenwalder's (1996) taxonomy that considers *P. maximowiczii* as a variety of Siberian poplar (*P. suaveolens*). We, however, treat *P. maximowiczii* as a distinct species following the reasoning of Dickmann and Kuzvokina (2008), because it is commonly known as such by *Populus* breeders worldwide.

provenance often exceed the performance of local selections within the constraints imposed by temperature extremes (Farmer, 1993). This holds special importance for breeding the all-important *P. deltoides* for lower latitudes of the world where inter-specific crosses with other species endemic to low latitudes, such as Himalayan poplar (*P. ciliata)* and Yunnan poplar (*P. yunnanensis*), may result in inter-specific heterosis while maintaining adaptation to local photoperiods.

Intra-specific population differences are also encountered on a more limited geographic scale as the following demonstrates: (1) across 3.80° of latitude on the Japanese island of Hokkaido (approximately 41° 36′–45° 24′ N) southern sources of *P. maximowiczii* initiate growth cessation later than northern sources (Chiba, 1984); (2) over 4.35° of latitude across a southwest to northeast gradient in the Pacific Northwest (44° 44′ N–49° 05′ N) the growth of southwesterly sources of *P. trichocarpa* remains active longer into the fall than northeastern sources (Weber et al., 1985); (3) across 3.50° of latitude in the north central region of the United States *P. balsamifera* populations from the southeast grow faster in height and set terminal buds later than those from the central and northwest sectors of the region (Riemenschneider and McMahon, 1993). Local population variation in the timing of spring growth initiation in *P. trichocarpa* is tied to changes in temperature gradients within river drainages, while autumnal events are associated with both temperature gradients and/or disease pressure dependent on the specific individual drainages (Dunlap and Stettler, 1996). These finer expressions of population variation are as important as the broader, range-wide ones in the design of *Populus* breeding programs.

Beyond phenology, genetic variation among populations within species has been reported for a variety of growth, eco-physiological, and morphological traits that impact *Populus* breeding programs. In *P. trichocarpa*, for example, a latitudinal cline in the rate of photosynthesis has been reported among coastal populations sampled between 44° and 56° N latitude where more northerly sources display a greater capacity to assimilate carbon dioxide as, perhaps, a compensatory strategy for their earlier curtailment of the growing season (Gornall and Guy, 2007). Conversely, a strong differentiation of populations was not evident in the assimilation rate of *P. balsamifera* provenances across a comparable range of latitude (43–53° N) (Schnekenburger and Farmer, 1989). The photosynthetic rate of *P. trichocarpa* also varies on a more local geographic scale with populations endemic to xeric environments of higher light intensity capable of superior rates compared with those from mesic environments of lower light intensity (Dunlap et al., 1993). An eco-physiological trait of equal importance – water use efficiency – also exhibits population variation in: (1) *P. trichocarpa*, e. g. populations from arid, continental climates possess higher efficiencies than those from moist coastal environments of mild climate (Bassman and Zwier, 1991) and (2) *P. deltoides*, e. g. clones selected from dry sites exhibit lower stomatal resistances and the ability to prolong growth under drought conditions compared to those from sites of higher moisture availability (Kelliher and Tauer, 1980). Tolerance of autumnal frosts and winter injury is a third example of an eco-physiological trait where local population variation has been studied: For example, inland sources of *P. trichocarpa*

have developed higher tolerances to both factors compared to their coastal counterparts in the Pacific Northwest (McCamant and Black, 2000). Population variation in eco-morphological traits has similarly been reported: Crown morphology of *P. trichocarpa* populations from xeric sites differs from those from mesic sites in terms of individual leaf size, crown architecture, and leaf area indices. These, too, have been exploited in selective breeding strategies (Dunlap et al., 1995).

However, eco-physiological trait differentiation may not always reflect local climatic or edaphic selection pressures, as observed in the appreciable variation in both photosynthetic and transpirational rates and tolerance of soil salinity among four populations of *P. deltoides* var. *wislizenii* from a relatively restricted part of the southwestern United States (33° 55′–36° 12′ N latitude) (Rowland, 2001; Rowland et al., 2004). Likewise, significant genotypic variation in the growth response of *P. trichocarpa* to seasonal flooding is not associated with the population of origin (Smit, 1988).

Adaptive variation in disease resistance, historically of high importance in *Populus* breeding, has been demonstrated in studies of environmental conditions conducive to pathogen selection pressure. For example, populations of *P. trichocarpa* from mesic environments are now known to be characterized by significantly higher levels of *Melampsora* leaf rust resistance compared with populations native to arid regions (Dunlap and Stettler, 1996). *P. deltoides* populations sampled from humid, wet sites in the southwestern portion of its range were shown to exhibit heightened levels of *Melampsora* rust resistance compared to populations from drier environments that evolved with less exposure to the pathogen (Nelson and Tauer, 1987).

Despite the oftentimes definitive effect of source location on such a wide range of phenological, physiological, and pathology traits, studies of the manner in which genetic resources are organized within the genus have usually shown that a sizable component of variation in each of these characteristics resides within divergent populations (Fig. 2). To illustrate, whereas variation among populations of *P. deltoides* between 30° 30′ and 34° 55′ N latitude in the lower Mississippi River Valley accounted for 5% of total phenotypic variation in growth rate, 30% of that total was attributed to variation at the level of clones-within-populations (Foster, 1986). Greater within- than among-population variation has also been noted in studies of juvenile growth in *P. tremuloides* (Thomas et al., 1997) and in those addressing winter dormancy and spring phenology in *P. balsamifera* (Farmer, 1993; Farmer and Reinholt, 1986). Molecular data also suggested a weak differentiation among North American populations of *P. tremuloides* (Cole, 2005; Yeh et al., 1995) and Italian *P. tremula* populations (Salvini et al., 2001). A study of nucleotide sequence variation at three loci in *P. balsamifera* further reinforces the finding that the majority of genetic diversity resides within populations (Breen et al., 2009). It is believed that ample gene flow partially counters the effects of natural selection that would otherwise allow populations to diverge (Weber and Stettler, 1981). However, there are exceptions. Coastal *P. trichocarpa* populations are strongly differentiated in photosynthetic rate across a latitudinal transect with little inherent residual variation (Gornall and Guy, 2007). Cathay poplar (*P. cathayana*) populations from the

Fig. 2 A stand of *P. trichocarpa* in which individual trees exhibit varying stages of spring vegetative shoot development. Such within-population variation may be an adaptation to yearly variation in the timing of spring frosts

Qinghai-Tibetan Plateau of southeastern China show strong differentiation in microsatellite markers due to the topography of the region that creates distinct selection pressures while precluding gene flow (Peng et al., 2005). The manner in which variation is distributed among the hierarchy of genetic organization is an important consideration to building first generation breeding populations (Breen et al., 2009).

3 Controlled Breeding

Reproductive Biology – *Populus* species are dioecious, although reports of hermaphroditism have been filed for each of the three major sections (Fig. 3). Sex appears to be determined by a single locus or a group of tightly linked genes on chromosome XIX (Yin et al., 2008). The bisexual condition may result from a relaxation of the mechanism that suppresses recombination at this locus that otherwise keeps sex-determining multigenes intact during reduction division. Male and female reproductive structures in *P. trichocarpa* in the northern hemisphere are formed April through June of the year preceding reproduction (Boes and Strauss, 1994). Reproduction involves wind pollination of inflorescences that contain approximately 60 staminate flowers or 35 pistillate flowers (Boes and Strauss, 1994)

Fig. 3 Sub-gynoecious *P. trichocarpa* variety 'PS-53-97'. This condition produces predominantly pistillate inflorescences with occasional staminate ones borne on the same shoot (*left* photo). Additionally, pistillate inflorescences may contain hermaphroditic flowers bearing a pistil and stamens as shown in the photo on the *right*

(Fig. 3). The process of controlled reproduction is well understood but requires varying techniques and approaches for each of the genus' major sections (Stanton and Villar, 1996, Fig. 4). Artificial crosses are made in greenhouses using pollen extracted from floral cuttings of male selections maintained in water culture (Seitz, 1958). Pollen mother cells of Simon poplar (*P. simonii*) begin meiosis within 72 h of being forced in greenhouses and complete the process at the time that one-quarter of the length of a staminate inflorescence has emerged from the bud (Wang et al., 2009). Megagametophytes of *P. tremuloides* develop to the two- to four-nucleate stage during the winter and move to the eight-nucleate stage 18 h following spring pollination under greenhouse conditions (Fechner, 1972). Seed is produced on pistillate cuttings that are set in water, grafted on to potted under-stock (Farmer and Nance, 1968), or rooted in soil (Joennoz and Vallee, 1974). In China, controlled breeding techniques for seed orchard trees using scaffolding or partially dislodged and guyed trees exceed greenhouse-based techniques in cost-savings and ease of fruit production (Zhou et al., 2008). Seed matures in one growing season, does not undergo physiological dormancy, and germinates readily under adequate temperature and moisture conditions without stratification. Breeding populations typically

Fig. 4 An indoor *P. deltoides* female breeding orchard. In addition to the use of a rooting hormone, soil-warming pads attached to the propagation buckets speed the development of an adventitious root system necessary to sustain development of the seed crop for 8–20 weeks

achieve a level of flowering that is sufficient to initiate selective breeding within 10 years. Asexual reproduction is quite advanced within the genus; this is exploited using either adventitious field rooting of 1-year-old hardwood cuttings or 2-year-old poles (section *Aigeiros* and *Tacamahaca*), or greenhouse rooting of succulent shoots under mist propagation (section *Populus*).

Inter-specific hybridization has figured prominently in poplar breeding from its inception. Inter-sectional crosses between *Aigeiros* and *Tacamahaca* are compatible for the most part although reciprocal crossing effects can be problematic at times. For example, both the *P. deltoides* × *P. trichocarpa* and the *P. deltoides* × *P. maximowiczii* cross combinations are far more productive than crosses in which the *Tacamahaca* parent is used as the female parent (Stanton, 2005; Zsuffa et al., 1999). The same effect has been encountered in breeding the intra-sectional *P.* ×*canadensis* taxon: The *P. deltoides* × *P. nigra* cross is highly fertile but the cross is wholly ineffective when attempted in the reverse, *P. nigra* × *P. deltoides* direction, (Melchior and Seitz, 1968). Section *Populus* is, for all practical breeding purposes, reproductively isolated from *Aigeiros* and *Tacamahaca* due to incompatibility in the pollen-stigma recognition process (Gaget et al., 1984; Villar et al., 1987). However, the use of complex hybrids of section *Populus* (e.g. *P.* ×*canescens* × [*P. alba* × *P. grandidentata*]) as female parents has shown promise in effecting inter-sectional crosses with sections *Aigeiros* and *Tacamahaca* (Ronald, 1982). Species within section *Populus* are freely crossable under artificial conditions, however.

Although arguable, most of the applied breeding work is concentrated on seven species based on investment in controlled hybridization, the number of commercial cultivars in use, and the area under production plantation management. These are *P. deltoides* and *P. nigra* of section *Aigeiros*, *P. maximowiczii* and *P. trichocarpa* of section *Tacamahaca*, and *P. tremula*, *P. tremuloides*, and *P. alba* of section

Populus. *Populus* breeders worldwide have used them to develop the following commercial taxa: (1) *P.* ×*wettsteinii*, the intra-sectional combination of *P. tremula* and *P. tremuloides*, (2) *P.* ×*canadensis*, the intra-sectional hybrid of *P. deltoides* and *P. nigra*, (3) *P.* ×*generosa* the inter-sectional hybrid of *P. deltoides* and *P. trichocarpa*, (4) Chinese white poplar (*P.* ×*tomentosa*), the intra-sectional combination of *P. alba* and *P. tremula* var. *davidiana*[2], and (5) intra-specific hybrids of *P. deltoides* (Table 1). Two other taxa lacking assigned hybrid binomials – *P. nigra* × *P. maximowiczii* and *P. deltoides* × *P. maximowiczii* – are currently not as prevalent as the other five but are likely to soon achieve parity in terms of breeding, cultivar development, and the significance of their contribution to *Populus* cultivation.

Breeding Strategies – First generation (F_1) inter-specific hybridization combined with reciprocal recurrent selection (RRS) of the parental species is the most frequently recommended long-term improvement approach. Today RRS is being used to develop the *P.* ×*wettsteinii*, *P.* ×*canadensis*, and *P.* ×*generosa* taxa. As an alternative to F_1 hybridization, F_2 *P.* ×*canadensis* breeding is used to develop a synthetic hybrid species. This is noteworthy in view of computer simulations that suggest the advanced generation approach is a more cost-efficient route to genetic improvement than a RRS – F_1 program (Kerr et al., 2004). This assumes, however, that there is no breakdown of hybrid vigor in the F_2 generation as appears to be true of *P.* ×*canadensis*, a cross between species of the same section, *P. deltoides* and *P. nigra*. Advanced generation breeding of more distantly-related species may experience diminished hybrid performance owing to a number of causes, including the disruption of co-adapted or species-specific linkages within an otherwise integrated genome (Lester, 1973; Stettler et al., 1996).

Other breeding strategies – backcrossing, multiple-species hybridization, polyploidy, somaclonal variation – are not frequently pursued as mainline, long-term breeding approaches although they are used in short-term programs. Examples include: (1) backcrossing F_1 *P.* ×*generosa* hybrids to *P. deltoides* for increased resistance to *Melampsora* leaf rust (Pinon et al., 2006), (2) multiple species hybridization of the cross (*P. laurifolia* × *P. nigra*) × *P. maximowiczii* for increased site adaptability (Cagelli and Lefevre, 1995), (3) screening triploid *P.* ×*canadensis* clones for increased growth rate and fiber production (Zhang et al., 2004), and (4) induction of somaclonal variation in a *P. nigra* var. *betulifolia* × *P. trichocarpa* genotype through callus culture, followed by field evaluation and selection for *Septoria* canker resistance (Ostry and Ward, 2003). An amalgam of some of these approaches is employed in developing the *P.* ×*tomentosa* taxon in China.

The popularity of F_1 inter-specific hybridization is a result of the predominance of heterosis and the ease with which it can be economically exploited by vegetative propagation: Clonal selection captures the advantages of inter-specific hybrid vigor once the laborious process of controlled hybridization has been completed, while

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[2]The origin of *P.* ×*tomentosa* may be in dispute; we consider it as a first-generation hybrid of *P. alba* and *P. tremula* var. *davidiana* following the analysis of Zhang et al. (1995). We assume this taxon is distinct from the *P.* ×*canescens* taxon, itself an inter-specific combination of *P. alba* and *P. tremula*.

Table 1 Examples of commercial *Populus* cultivars of select taxa registered with FAO's International Poplar Commission

Taxon	Cultivar	Country of origin
P. ×*canadensis* (Moench)	*P. deltoides* × *P. nigra* 'Blanc du Poitou'	France
	P. deltoides × *P. nigra* 'Koltay'	Hungary
	P. deltoides × *P. nigra* 'Luisa Avanzo'	Italy
	P. deltoides × *P. nigra* 'Manawatu Gold'	New Zealand
P. ×*generosa* (Henry)	*P. trichocarpa* × *P. deltoides* 'Beaupre'	Belgium
	P. deltoides × *P. trichocarpa* 'Donk'	The Netherlands
	P. deltoides × *P. trichocarpa* 'Generosa'	United Kingdom
	P. trichocarpa × *P. deltoides* 'Hoogvorst'	Belgium
P. ×*tomentosa* (Carriere)	*P. alba* × *P. tremula* var. *davidiana* 'Dapikongi'	China
	P. alba × *P. tremula* var. *davidiana* 'Jingxi'	China
	P. alba × *P. tremula* var. *davidiana* 'Yixiancizhu'	China
	P. alba × *P. tremula* var. *davidiana* 'Xizhi Xiaiye'	China
P. ×*wettsteinii* (Hamet-Ahti)	*P. tremula* × *P. tremuloides* 'Astria'	Germany
	P. tremula × *P. tremuloides* 'Grosshansdorf'[a]	Germany
	P. tremula × *P. tremuloides* 'Vorwerksbusch'	Germany
P. deltoides (Bartram ex Marsh.)	*P. deltoides* 'Delta Gold'	United States
	P. deltoides 'Dunav'	Serbia
	P. deltoides 'Harvard'	Italy
	P. deltoides 'Jagdish'	India
No hybrid binomial assigned	*P. maximowiczii* × *P. nigra* 'Geyles'[b]	New Zealand
	P. nigra × *P. maximowiczii* 'Maxfunf'	Germany
	P. maximowiczii × *P. nigra* 'Rochester'	United States
No hybrid binomial assigned	*P. deltoides* × *P. maximowiczii* 'Eridano'	Italy
	P. maximowiczii × *P. deltoides* 'Suwon'	The Netherlands

[a]*P. tremula* × *P. tremuloides* 'Grosshansdorf' is a new varietal mixture of 14 individual clonal selections not yet registered with IPC.
[b]Newly-selected cultivar of The New Zealand Institute for Plant and Food Research that will be released in the spring of 2010.

allowing within-family selection with a high degree of precision. While heterosis has been widely assumed in *Populus* breeding, it has not always been substantiated with rigorous controlled studies; oftentimes conclusions are based on a limited sampling of first-generation families or comparisons involving only one parent. Nonetheless, good examples of first-generation inter-specific heterosis, presented as the mean of the F_1 family as a percentage of the bi-parental mean or the value of just one parent, are:

1. Increases of 75 and 177% in 2-year-old stem volume in *P.* ×*canadensis* and *P.* ×*generosa*, respectively (Dillen et al., 2009),
2. Ninety percent increase in 3-year-old stem volume of *P.* ×*wettsteinii* relative to *P. tremuloides* (Li et al., 1998; Li and Wu, 1996),

3. Fourty four percent increase in 2nd-year stem volume of *P.* ×*canadensis* (Marron and Ceulemans, 2006), and
4. Fifty percent increase in 4th-year stem volume of *P.* ×*generosa* relative to *P. trichocarpa* (Ceulemans et al., 1992).

Other reports of F_1 superiority are based on the performance of individual clones and have been referred to as "clonal heterosis." These, it could be argued, may be equally attributable to transgressive segregation and not hybrid vigor. Even so, Wu et al. (1992) reported clonal heterosis values of 107–123% in stem volume of *P. deltoides* × *P. simonii* clones relative to their *P. deltoides* female parent. And, Yu et al. (2001) reported a 290% increase in 5th year stem volume of four *P.* ×*wettsteinii* hybrid selections compared to local *P. tremula* selections.

Regardless of the strength of the quantitative evidence for hybrid vigor, experimentation into the morphological and physiological components of hybrid growth and development substantiates such claims. For instance, it has been accepted for some time that the inheritance of large leaf cells from *P. trichocarpa* and the greater density of leaf cells from *P. deltoides* result in their F_1 progeny's increased leaf area, which allows for greater light capture and superior yield in the *P.* ×*generosa* taxon (Ridge et al., 1986). In the *P.* ×*canadensis* taxon, increases in leaf increment rate and leaf area are similarly important in explaining the superiority of the F_1 generation (Marron and Ceulemans, 2006), although increased production of gibberellins may also be a controlling factor in the *P.* ×*canadensis* heterotic growth response (Bate et al., 1988). Differences between *P.* ×*wettsteinii* and *P. tremula* genotypes in the size of guard cells has been documented but is not clearly related to hybrid vigor in the taxon (Yu, 2001).

The financial impact of hybrid vigor is, as would be expected, of notable effect in *Populus*: Economic analyses of *Populus* genetic improvement have demonstrated internal rates of return approximating 13% for plantation operations with yield increases of 10–15%, which supports using advanced generation *Populus* breeding programs to achieve sustained advancement in heterosis (Van der Meiden, 1977). Five such long-term improvement programs illustrate the range of breeding strategies in use throughout the world today. Three are based on F_1 inter-specific hybridization coupled with a variety of recurrent parental species selection programs; a fourth includes a combination of advanced generation techniques and polyploid breeding; and the fifth is an intra-specific recurrent breeding program. These are: (1) the University of Minnesota's Aspen-Larch Cooperative's *P.* ×*wettsteinii* program, (2) the Italian *P.* ×*canadensis* program led by the Poplar Research Institute at Casale Monferrato, (3) GreenWood Resources' *P.* ×*generosa* breeding program, (4) Beijing Forestry University's *P.* ×*tomentosa's* breeding effort, and (5) the U.S. Forest Service-Industrial *P. deltoides* program for the southeastern United States. A critical element of all long-term breeding programs is the prediction of parental breeding values. This is especially challenging when breeding F_1 inter-specific hybrids where the expense of managing multiple parental species via a reciprocal recurrent breeding program may be prohibitive. To overcome this hurdle, a simple recurrent breeding program can be substituted for the more complicated and

involved reciprocal recurrent programs, especially if pure-species general combining ability estimates are a reliable gauge for general hybridizing ability (see Dungey, 2001; Nikles,1993 for a review in *Pinus*).

3.1 Examples of Long-Term Breeding Programs

P. ×wettsteinii – Modified Reciprocal Recurrent Selection – The *P. ×wettsteinii* taxon is being developed for European plantations in Scandinavia and the Baltic States (Rytter and Stener, 2003; Tullis et al., 2007). This taxon is also viewed favorably in North America where the University of Minnesota has been hybridizing *P. ×wettsteinii* since 1952 through its Aspen and Larch Genetics Cooperative in support of the pulp and paper and the oriented strand board industries. Long-term improvement is based upon full-sib reciprocal recurrent selection of *P. tremuloides* and *P. tremula* for inter-specific heterosis in yield, in addition to improvements in wood quality and *Hypoxylon mammatum* canker resistance (Li and Wyckoff, 1991). Breeding populations are sized at 150 individuals for each parental species utilizing *P. tremula* selections from Poland, Germany, and The Netherlands, and *P. tremuloides* selections from Wisconsin, Michigan, Minnesota, and Saskatchewan. Reciprocal crossing effects are non-existent and hybrid crosses are made in both directions. The 300 genotypes of the two breeding populations are assigned to 25, 6 × 6 disconnected factorials to develop 900 full-sib F_1 families for the first cycle of inter-specific progeny tests. Evaluation is conducted at age five leading to the identification of 100 parents of each species that are mated intra-specifically using a circular mating design. A second evaluation of the inter-specific population conducted at age 10 is then used to choose the top 75 parents whose intra-specific progeny enter field trials. (The purpose of the two-stage selection is to accelerate the initiation of the intra-specific crosses to shorten the generation intervals; in this sense the reciprocal recurrent selection program is modified.) One hundred and fifty selections of each species are grafted into clonal breeding orchards for use as parenting stock for the second cycle of first generation inter-specific hybridization. Disconnected factorials are again used to generate 900 inter-specific families; the best 45 individuals are selected for clone deployment to commercial plantations.

P. ×canadensis – Semi-Reciprocal Recurrent Selection – The *P. ×canadensis* inter-specific hybrid taxon is perhaps the world's most widely planted, used in operations on all five continents where *Populus* is grown. The most advanced *P. ×canadensis* breeding program is conducted in Italy by the Poplar Research Institute at Casale Monferrato in the Po River Valley (Bisoffi and Gullberg, 1996). Traditionally bred for the veneer industry, development of the taxon now also targets renewable energy feedstock. The main selection targets are growth rate and *Marssonina* leaf spot resistance. The reciprocal crossing effect in *P. × canadensis* has defined the Italian strategy to the extent that parental inter-specific hybridizing values can only be estimated for *P. deltoides* females and *P. nigra* males (Bisoffi, 1990). Thus, the program is known as semi-reciprocal recurrent selection.

The program began with 150 selections of *P. deltoides* and an equivalent number of *P. nigra* selections that are managed as single breeding units, because interactions between planting site and parental breeding values appear unimportant. Female *P. deltoides* selections are evaluated for general hybridizing ability (GHA) in a *P. nigra* polycross mating design. But because of the inability to reproduce the *P. nigra* × *P. deltoides* cross, general combining ability (GCA) estimates are relied upon in the evaluation of *P. nigra* females using the same *P. nigra* pollen mix used in the inter-specific polycross evaluation of female *P. deltoides*. Breeding value estimation of *P. deltoides* (GCA) and *P. nigra* (GHA) males is based upon the common use of a tester mating design of six *P. deltoides* females. As an alternative to conventional polycross breeding, Wheeler et al. (2006) proposed combining this method with paternity analysis for a *P. deltoides* × *P. nigra* reciprocal recurrent selection program as a way to manage inbreeding in each of the parental species when individual components of the pollen mixes vary insubstantially in reproductive success.

One noteworthy research finding of the Italian program has been the reasonable correlation between parental genotypic values and GHA values for several traits (Bisoffi, 1990). Genotypic values are now partially relied upon as surrogates for breeding value estimates, because of the inability to reproduce the reciprocal cross. As such, clonal trials of parenting stock are important adjuncts in the management of recurrent breeding populations, although conventional inter-specific progeny tests still figure into the estimation of GHA values for female *P. deltoides* and male *P. nigra* parents. The semi-reciprocal recurrent selection program renews each generation with 300 selections of each species and a balanced sex ratio. Improvement for growth traits emphasizes selection within full-sib families while among-family selection is emphasized for *Marssonina* resistance. The Italian program also includes a simple recurrent *P.* ×*canadensis* selection program to develop a synthetic inter-specific hybrid species using the rationale that an additive model may be a more appropriate explanation for heterosis than one based on overdominance.

P. ×*generosa – Multiple Population Breeding* – The *P.* ×*generosa* taxon has been a staple of *Populus* culture in western Europe since the 1960s. In North America, development of clonal plantations and controlled breeding began in the Pacific Northwest in the 1980s in response to a shortage of hardwood fiber for the manufacture of high-quality communications-grade paper (Stettler et al., 1988). Drs. Reinhard Stettler of the University of Washington and Paul Heilman of Washington State University worked together on developing the region's initial hybrid varieties during the 1970–1980s. Today, GreenWood Resources manages 14,000 hectares of *Populus* operations along the lower Columbia River floodplain on the windward side of the Cascade Mountains and, on the leeward side, in the arid mid-Columbia River basin for the production of quality saw logs on 12–15 year rotations using varietal selections of the F_1 *P.* ×*generosa* taxon. Long-term improvement began with the assembly of breeding populations of both *P. trichocarpa* and *P. deltoides*. The *P. trichocarpa* effort began with replicated clonal testing of 1,428 genotypes assembled from 67 provenances along the windward slope of the Cascade Mountains between 48° 56′ and 42° 56′ N latitude in Washington and Oregon leading to the identification of 328 superior genotypes. Paralleling this, a *P. deltoides* breeding

population was comprised of 204 second-generation clonal selections from 104 full-sib families bred from superior first generation clonal selections originating between 35° 14′ and 30° 36′ N latitude in Tennessee, Mississippi, and Louisiana. Genotypes of each species are being re-tested to arrive at a final breeding population of 144 individuals, the upper one-third of which will be assigned to one of three multilines designed for the improvement of solidwood, bio-energy, and pulping applications. Each multiline is managed with eight female and eight male genotypes that are crossed intra-specifically using a circular mating design to develop superior selections – based on GCA estimates as well as genotypic values – for the second cycle of F_1 inter-specific hybridization. Parental genotypes not assigned to one of the three multilines are managed as a single unit to allow for the creation of trait combinations that would not occur otherwise.

P. ×tomentosa – Backcross, multi-species and polyploid breeding – *P. ×tomentosa* is a naturally-occurring hybrid of *P. alba* and *P. tremula* var. *davidiana* (Zhang et al., 1995), although some consider it a hybrid of *P. alba* and *P. adenopoda.* It is China's most valuable native *Populus* planted throughout the Yellow River drainage from Shanxi Province in the interior eastward to the coastal province of Shandong. It is managed for the plywood and pulp and paper industries and is valued for its growth rate, pest resistance, and wood quality. Few native stands remain and today's hybridization work relies upon backcross varieties developed over 50 years ago. However, a concerted effort of phenotypic selection, provenance evaluation, and family evaluation was initiated nearly 20 years ago for one of the hybrid's parental species, *P. tremula* var. *davidiana* (Li et al., 1999). Promising new crosses with *P. alba* also have been accomplished, foreshadowing the possible production of a second cycle of F_1 *P. ×tomentosa* hybridization.

A national tree improvement program was launched in 1983 by Beijing Forestry University. Initially 1,047 superior phenotypes were selected throughout the hybrid's natural range and established in provenance-clonal trials. This led to the release of 12 genotypes for the commercial plywood and construction industry with yield improvements of 40–50% (Zhu and Zhang, 1997). Continued breeding relies on the original germplasm collection now established in breeding arboreta and used in support of: (1) backcross hybridization of [*P. ×tomentosa* × *P. alba* 'Bolleana'] × *P. ×tomentosa* primarily for the pulp and paper industry, and (2) multi-species hybridization involving *P. ×tomentosa* × *P. alba* 'Bolleana' hybrids in crosses with *P. ×canescens*, bigtooth aspen (*P. grandidentata*), Chinese aspen (*P. adenopoda*), and *P. tremuloides* for the colder, arid region of northeastern China and Inner Mongolia. A third approach – allotriploid breeding – is conducted using diploid *P. ×tomentosa* pollen in crosses with normal haploid gametes of *P. ×tomentosa* ×*P. alba* 'Bolleana' female selections (Zhu et al., 1995). Triploids have also lately been bred using diploid *P. ×tomentosa* pollen in crosses with *P. alba* × *P. glandulosa* F_1 hybrid females (Wang et al., 2008). Polyploids are valued for pulp and paper applications, because of their high cellulose-to-lignin ratio, increased fiber length, and superior growth rate.

P. deltoides – Recurrent Selection for General Combining Ability – P. deltoides is the genus' most important species used as breeding and/or propagation stock in North and South America, Europe, Asia, and Australia. In North America, the

decline in natural regeneration along the Mississippi River in the 1960s gave rise to a *P. deltoides* improvement program initiated by the U. S. Forest Service's Stoneville, Mississippi Experimental Station. (Inter-specific hybrids have not performed well in this region, because of their susceptibility to *Septoria* stem canker.) The Stoneville project began with a collection of 3,700 genotypes from provenances of the Mississippi River between Memphis, Tennessee (35° 14′ N latitude) and Baton Rouge, Louisiana (30° 36′ N latitude) and from the Brazos, Trinity, and Red Rivers of east Texas and Oklahoma between 35° 57′ N and 30° 03′ N latitude. These were screened in a number of replicated clonal field trials culminating in the identification of 197 genotypes that were brought together and retested in 1980 in a series of cooperative industrial trials at Wickliffe, Kentucky (Westvaco Corporation), Fitler, Mississippi (Crown Zellerbach Corporation), and Profit Island, Louisiana (Trans Match Corporation). The objective of these so-called advanced clone trials was to determine the extent of genotype-by-environmental interactions throughout the lower Mississippi River Valley when composing breeding populations for long-term, recurrent selection and breeding (Cooper, 1980).

The goal has been a recurrent selection program for general combining ability for the production of multiple-purpose clones for pulpwood, saw timber, and veneer (Cooper, 1976). Priority selection criteria have included adventitious rooting, growth rate, resistance to *Melampsora* leaf rust and *Marssonina* leaf spot, and wood specific gravity (Robison et al., 2006). Because of an imbalanced sex ratio in the first-generation clonal collection, a male-in-female nested mating design was proposed to develop the second-generation (Foster, 1984); 50 male and 25 female selections theoretically could be mated hierarchically to generate 50 full-sib families, each represented by seven full sibs. From the resultant population of 350 second-generation genotypes, combined selection would identify 36 genotypes for assignment to four, 4×5 disconnected factorials to generate 80 third-generation families of seven sibs (560 in total). Yield improvements through the third generation were projected at 49% relative to unimproved base population stock.

In 2000, Mississippi State University undertook a large-scale sampling of 64 provenances throughout the southeastern United States approximately between 75° and 90° W longitude and 30° and 36° N latitude. Preliminary evaluations for leaf rust and growth rate at multiple sites has identified new selections that compete with the best commercial standards from the original U. S. Forest Service project (Jeffreys et al., 2006). Furthermore, a significant source of *Melampsora* rust resistance has been identified within populations residing in the southeast Atlantic and east Gulf regions (Land and Jeffreys, 2006).

4 Testing and Selection

Experimental Techniques – Populus genetic evaluation field trials are, in nearly all cases, clonally replicated in view of: (1) the added precision of estimating genetic parameters and increases in genetic gain during recurrent selection (Shaw and Hood,

1985), and (2) the need to expedite the selection of new varieties required by commercial ventures to substitute for the lowest-ranking varieties presently in use (Foster and Shaw, 1988). Replication of individual genotypes has the potential for creating very large test populations and, as a consequence, a multiple-stage evaluation process has been recommended to accommodate such sizable experiments (Libby, 1987). The number of genotypes is sequentially reduced between test stages with attendant increases in replication, spacing, test locations, and rotation (Cooper, 1976). Typically, testing begins with seedling populations that undergo combined family and within-family selection. Selected genotypes are then clonally replicated in first-stage field trials; these evaluate clones grown in small-sized plots where inter-genotypic competition is unchecked and are usually established at multiple sites (Riemenschneider et al., 2001). The final stage involves a limited number of highly-selected clones that are established in monoclonal plots of sufficient size to provide reliable growth and yield estimates.

Assuming that a fixed amount of resources place a limit on the number of experimental plants, the optimum combination of clones and ramets in preliminary screening trials is based upon the desired degree of genetic gain, expected level of clonal variance, and the required precision of selection. The most frequently chosen experimental design approximates dozens to several hundred clones tested in multiple, three- to five-tree row plots, and planted using a randomized complete block design of four to12 replicates (Ares, 2002; Hansen et al., 1992; Isik and Toplu, 2004; Koo et al., 2007; Riemenschneider et al., 2001; Yu and Pulkkinen, 2003; Zhang et al., 2008). Cooper (1976) reported the success of a variety of incomplete block designs, e.g. triple, balanced, and cubic lattices, in accounting for edaphic variation in *P. deltoides* clonal tests. Today, row-column designs[3] are considered preferable to the randomized complete block design for clonal evaluations based on simulation studies that show increases in precision of up to 10% (Gezan et al., 2006a). Related to the issue of optimum experimental designs is the correct number of replications and plot structure for clonal testing: Replication rates of two-to-six ramets per genotype as single-tree plots are considered to provide the greatest experimental efficiency dependent on micro-site variability and clonal repeatability (Gezan et al., 2006b; Isik et al., 2005; Russell and Libby, 1986, Fig. 5). Statistical efficiencies have also been achieved in experiments of *Populus* clonal adaptability to varying nutrition and salinity levels through the use of analyses of covariance that account for variation in the dimension of the propagation stock used to initiate field trials (Fung et al., 1998; van den Driessche, 1999). More recently, mixed model techniques have been developed to account for the covariance of within-clone residual effects to further increase the precision of *Populus* clonal comparisons (Zamudio et al., 2008).

A mid-rotation or later schedule has been the longstanding recommendation for the timing of varietal selections for growth and yield improvement in *Populus* (Mohrdiek, 1979; Zsuffa, 1975). An early report by Cooper and Ferguson (1979)

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[3]Row-column designs are experimental designs that control or block site heterogeneity that occurs in two directions.

Fig. 5 Multiple-tree plots may not lend as much experimental efficiency as single-tree plots assuming the same number of ramets per clone. In this photograph of one replicate of a five-tree row plot, little micro-environmental variation is detected among the first four ramets in the plot. This provides little additional accuracy compared to an independent randomization of the five trees as single-ramet plots over a wider area

showed that rotation-age selection for height and diameter in *P. deltoides* resulted in two to three times the magnitude of improvement when selections were made at approximately one-third of a rotation length. Yet within the context of Libby's (1987) multiple-stage test protocol, truncation of base populations using a relatively low level of selection intensity at preliminary stages to identify a subset that undergoes more concentrated testing now is in vogue in many *Populus* testing programs. Kumar and Singh (2001) suggested using a 60% truncation rate for *P. deltoides* clonal test populations at age two to maintain a reasonable certainty that the top clones are included in the final age-four evaluation. Yet when viewed from the perspective of gain-per-unit time, early selections of individual genotypes for rotation performance may stand on their own merits: An evaluation of *P.* ×*wettsteinii* demonstrated that selection of cloned genotypes for stem volume at age three was two and one-half times as efficient as selection at age nine, based on an annualized rate of improvement (Stener and Karlsson, 2004). However, these authors also point out that early selection in *P.* ×*wettsteinii* is compromised by the inability to evaluate *Hypoxylon* canker resistance that does not express itself until much later during stand development. Furthermore, inspection of *P. deltoides* clone trials in Argentina in which one-third of the clonal distribution underwent significant changes in growth ranking between the third and 9th years concluded, as did Kumar and Singh (2001), that early selections are best focused on groups of clones rather than individual genotypes (Ares, 2002). Thus the mid-rotation recommendation generally remains

valid for growth and yield traits. Matyas and Peszlen (1997) further extended this recommendation to the selection of wood quality traits where early stage selections for specific gravity, modulus of rupture, and modulus of elasticity proved unreliable.

Statistical techniques employed for *Populus* varietal evaluation have focused on three areas: (1) simultaneous selection for multiple traits, (2) classification of clonal phenotypes for extended testing, and (3) accounting for the effects of genotype-by-environment interaction. Initially, the need to select *Populus* cultivars for a multiplicity of traits was accommodated by the construction of independent culling levels. A good example is the use of selection thresholds for growth rate, resistance to *Marssonina brunnea*, and *Melampsora* spp. leaf diseases, stem form, and wood quality in the selection of clones for quality veneer log production for Portugal's match stick industry (Monteiro, 1988). Alternatively, index selection is now more frequently used in the evaluation of multiple criteria, especially in view of the developing definition of *Populus* ideotypes that has extended the range of target traits to several morphological and physiological variables. Riemenschneider et al. (1992) utilized this technique where restricted indices were used to check unfavorable correlations between growth and pathogen susceptibility that would otherwise compromise overall genetic gains in growth rate.

The need to group *Populus* genotypes for extended testing has relied upon multivariate analysis techniques. For example, Tharakan et al. (2001) used cluster analysis to select the best subset of 38 *Populus* and willow clones for yield trials based on covariances in stem volume, growing season length, and survival. Abrahamson et al. (1990) used the same multivariate technique to categorize the performance of 54 clones of diverse taxa to identify an optimum class for immediate operational use in coppice rotations, as well as a separate class for extensive site adaptability testing. Riemenschneider et al. (2001) used principal component analysis to categorize best-adapted sets of clones for regional deployment. Isik and Toplu (2004) also used principal component analysis to distinguish genotypes within a clonal collection of *P. nigra* and *P.* ×*canadensis* based on co-variation in growth, stem form, apical dominance, branching, spring and autumnal phenology.

Genotype-by-environment interactions have been reported as variously significant in *Populus* clonal testing but include notable occurrences where the interaction component of variance for growth exceeded the clonal component by 85% in one *P.* ×*wettsteinii* study (Yu and Pulkkinen, 2003), and by 51% in a study of predominately *P. deltoides* intra- and inter-specific varieties (Riemenschneider et al., 2001). Yet, it has been shown several times that individual genotypes of good commercial appeal can be found that perform well across a range of environments with striking contrasts in climate and soils (Wu and Stettler, 1997). Broadly-adapted varieties are often the selection objective. Analysis techniques to identify such genotypes have made use of standard phenotypic stability parameters – slope coefficients and mean square deviations from the linear regression of genotypic means on environmental means. This approach has been used to evaluate *P. tremula* var. *davidiana* clone trials in Korea (Koo et al., 2007) and in the analysis of *P.* ×*wettsteinii* clonal performance in Finland (Yu and Pulkkinen, 2003). Similar to genotype-by-environmental

interaction analyses, has been the treatment of *P.* ×*generosa* genotype-by-time interactions in periodic growth increments that were analyzed using a spit-plot design for repeated measurements to partition the interaction variance into its linear and quadratic components to identify selections suited to short rotation management (Stanton, 2001).

Selection Criteria – Reflecting the suitability of its wood for a wide diversity of markets, *Populus* breeding has focused on an equally wide array of improvement criteria. However, improvement in agronomic characteristics – yield, climatic adaptability, adventitious rooting, disease resistance, etc. – invariably has been the priority in breeding programs. Wood quality also has received attention, mostly focused on improvements in specific gravity. This focus is expanding now to include more routine evaluations of physical and chemical wood components in light of the escalating interest in *Populus* biomass energy feedstock and the advent of increasingly affordable and reliable assessment methods.

Growth rate variation in preliminary stage clone trials is usually quantified in terms of stem height and diameter as surrogates for individual tree volume, itself an indicator of stand yield. Between the two, stem diameter has proven over a lengthy period to be the more important, because it is more easily and more accurately measured and has a much larger impact on the determination of stem volume (Mohn and Randall, 1971). Clonal repeatabilities typically fall within a range of 0.40–0.70 depending whether they are calculated on an individual tree, or on a clone mean basis and whether they are reported for an entire population or a within-family basis. Predicted levels of gain are often substantial as illustrated in the following:

1. Selection of the top 10% of a *P.* ×*wettsteinii* clonal distribution evaluated across multiple sites at age nine predicted a 45% increase in stem volume based on a broad-sense heritability of 0.39 (individual tree basis) (Stener and Karlsson, 2004);
2. Selection for site-specific clone performance in *P.* ×*tomentosa* predicted a 34% improvement in 5th-year stem volume based on the use of the topmost one-third of the clonal distribution and a clone mean repeatability of 0.90 (Zhang et al., 2008);
3. Selection of the uppermost 5% of the distribution of a *P. deltoides* × *P. simonii* clonal population was associated with an estimated 51% improvement in 6th-year volume with repeatabilities of clone mean height and diameter of 0.93 and 0.95, respectively (Wu et al., 1992);
4. Selection of the top 12% of the multiple-site clone ranking in a *P.* ×*wettsteinii* experiment in which genotype-by-site interactions were highly significant led to predicted increases in 3rd-year stem diameter growth of 15% associated with a clonal repeatability of 0.52 (clone mean basis) (Yu and Pulkkinen, 2003);
5. Selection of approximately the best 8% of the clone distribution at each of three sites resulted in an estimated improvement in age six yield of 23–89% (Riemenschneider et al., 2001); and,
6. Selection of approximately the top 12% of the genotype distribution in a multi-site *P. tremula* var. *davidiana* clone trial equated to a selection differential in 12th-year stem volume of 19% (Koo et al., 2007).

Survival as a component of yield in short-rotation clonal plantations has a greater impact on productivity than individual stem volume when survival rates fall below 90% (Chambers and Borralho, 1997). Thus clonal evaluation for the ability to undergo vegetative propagation at consistently high establishment rates is quite important and, dependent upon the taxon in question, is an afterthought, challenging, or frustratingly elusive. For instance, section *Tacamahaca* and their inter-sectional hybrids propagate easily from un-rooted, 1-year-old hardwood cuttings when compared with species and intra-sectional hybrids of section *Aigeiros* (Zalesny et al., 2005). On the other hand, hardwood cutting propagation of *P. deltoides* is often quite variable, though good selection opportunities exist within southerly populations with clonal repeatabilities for number of roots (individual tree basis) varying between 0.33 and 0.53 dependent on soil type (Wilcox and Farmer, 1968). However C-effects often associated with the position from which cuttings are taken from stock plants, will potentially confound genetic effects in any clonal evaluation of adventitious rooting ability (Farmer et al., 1989; Zalesny at el. 2003). Schroeder and Walker (1991) showed, for example, that cuttings taken from the basal portion of 1-year-old nursery whips rooted with an increased frequency of 30% and produced sprouts that were 16% taller at the end of the first season when compared with cuttings of the same genotype cut from the distal portion of the whip. In section *Populus*, vegetative propagation from hardwood stem cuttings is extremely problematic, and cloning has resorted to either root cuttings or succulent stem cuttings propagated under mist as containerized, greenhouse-grown stock (Haapala et al., 2004; Stenvall et al., 2006). The genotype factor is again of critical importance. Stenvall et al. (2006) reported more than a fivefold difference in rooting percentage among *P.* ×*wettsteinii* clones grown from root cuttings. Haapala et al. (2004) reported similar findings in *P.* ×*wettsteinii* using succulent cuttings where survival varied among clones between 36 and 85% when using a hedge propagation system and between 0 and 89% when using a serial propagation method. Selection opportunities to improve the success of succulent stem cutting propagation of *P. alba* have been reported where the clonal repeatability for adventitious root initiation and establishment is 0.34 (Harfouche et al., 2007).

Another class of biomass productivity components – morphological and physiological characteristics – has also been extensively studied in *Populus*. Most notably, these are associated with leaf area, both on an individual leaf basis as well as a whole crown basis (Harrington et al., 1997; Li et al., 1997; Marron et al., 2007). The hope has been that such characteristics used as indirect selection criteria might provide for greater selection efficiency compared with clonal evaluations based solely on yield, dependent on the degree of genetic control, genotypic co-variances, and the age and cost with which they can be assessed. While an active area of research, morphological and physiological traits have not yet played a role in applied *Populus* breeding; estimates of genetic parameters will partially determine how well they fulfill their potential role. To illustrate, Monclus et al. (2005) calculated a broad-sense heritability of 0.63 for maximum seasonal leaf area in a clonal *P.* ×*canadensis* population where variation was associated with biomass production with a linear correlation coefficient of 0.74. A similar correlation coefficient of 0.84 was observed for the

linear relationship between individual leaf area and biomass production in a high-density coppice system of mostly *P.* ×*generosa* genotypes (Bunn et al., 2004). Rae et al. (2004) also demonstrated that biomass production in a *P.* ×*generosa* coppice system was associated with clonal variation in individual leaf area, the number of leaves on the dominant sprout, and leaf plastochron index that, in turn, exhibited within-family, broad-sense heritabilities varying between 0.37 and 0.62. In a limited number of *P. deltoides* and *P.* ×*canadensis* genotypes, Dowell et al. (2009) presented evidence that the more productive ones were also those that could be characterized by higher levels of cumulative leaf area indices throughout the course of the growing season. Finally, Bonhomme et al. (2008) determined linear correlations for the *P.* ×*canadensis* taxon between stem growth and several leaf traits that were non-significant in some instances, e. g. specific leaf area, $r = 0.35$, while significant in others, e.g. foliar nutrient contents, $r = 0.62–0.50$.

Orlovic et al. (1998) extended the list of potential indicators of biomass productivity to thickness of the palisade parenchyma leaf tissue and the number of stomata on the adaxial leaf surface in *P.* ×*canadensis* both of which exhibit moderately strong broad sense heritabilities, e.g. 0.52 – 0.62, and reasonable phenotypic correlations with yield based on genotypic means, e.g. 0.66–0.84. Regardless of whether morphological and physiological selection criteria fulfill a role as indirect selection criteria for segregating populations under evaluation they will be, nonetheless, important when choosing species for inter-specific hybridization programs based on trait complementation (Marron et al., 2007) or when defining selection objectives for specific markets or end products (Scarascia-Mugnozza et al., 1997).

Selection for pest resistance has been of great importance since the inception of *Populus* breeding due to the challenge posed primarily by a range of pathogens that infect both crown and stem. The genetics of pathogen resistance has been most extensively studied in *Melampsora* leaf rust and involves both qualitative and quantitative components (Dowkiw and Bastien, 2007; Lefevre et al., 1998; Newcombe, 1998; Newcombe and Ostry, 2001). Qualitative resistance is expressed in the host's hypersensitive response to infection conditioned by major genes whose effect is mediated by modifying genes. When the hypersensitive response is lacking, polygenic resistance comes into play as a quantitative expression of variation in latent period, sporulation rate, and the number and size of uredina. Although a number of laboratory procedures have been developed to assess the components of leaf rust resistance, genotype selection is regularly evaluated in the field using a categorical scoring method (Fig. 6). Although not measured as a true metric character, resistance has been invariably treated as such in quantitative analyses that report clonal repeatabilities of 0.50–0.80 (Jokela, 1966; Thielges and Adams, 1975). Stem-cankering pathogens are also important in *Populus* breeding, with *Septoria musiva* and *Hypoxylon mammatum* recognized as the most serious threats. Resistance evaluation involves field trials, but oftentimes incorporates artificial stem inoculations to increase the reliability of genetic selections that differentiate pathogen resistance from pathogen escape (Enebak et al., 1999; Weiland et al., 2003). Resistance to *Septoria* canker may be conditioned by the thickness of the periderm and the formation of necrophylactic periderm as a pathogen-containing response following

Fig. 6 Selection for field resistance to *Melampsora* leaf rust can be decidedly effective. Pictured are 2-year-old test ramets of a highly susceptible clone in the foreground growing alongside a highly resistant variety in the background. The taxon is *P.* ×*generosa* and the tree spacing is 1.9 × 1.9 m

infection (Weiland and Stanosz, 2007). *Hypoxylon* canker resistance in *P. tremuloides* involves the development of a lignified response zone within proximity of the infection followed by rapid development of callus tissue (Bucciarelli et al., 1999). Unlike the *Melampsora* system where testing for resistance to a range of pathotypes is important, the same consideration seems less critical in screening for *Septoria* canker resistance where a more stable pathosystem may exist (LeBoldus et al., 2008; Ward and Ostry, 2005).

Specific gravity has been the most commonly evaluated wood quality trait included in *Populus* breeding programs, usually as a component of biomass yield (Farmer and Wilcox, 1968; Olson et al., 1985) but also as an attribute of wood quality (Mutibaric, 1971). It normally shows moderate to strong levels of clonal repeatability that can reach as high as 0.90 (Song et al., 1997; Zhang et al., 2003) but often is coupled with a limited range of variation relative to growth variables, e.g. coefficient of genotypic variation less than 10% (Pliura et al., 2007; Song et al., 1997). The relationship between specific gravity and radial growth is of keen interest and has varied from non-significant (Zhang et al., 2003) to weakly negative

(Ivkovich, 1996; Song et al., 1997), leading to the recommendation that simultaneous selection for clones of above average growth and specific gravity is possible. However, negative genotypic correlations between stem volume and wood specific gravity that are of moderate or moderately-strong effect (e. g. –0.59 to –0.74) have led to the opposing recommendation that selection should be based on an integrated measure of the two – dry fiber weight – as opposed to selection to improve both simultaneously (Olsen et al., 1985; Pliura et al., 2007). Genetic improvement opportunities for *Populus* renewable energy feedstock – specific gravity, lignin content, etc. – are now of growing interest (Davis, 2008). Frequently, these traits are being quantified for large populations under selection using near infrared spectrometry. Maranan and Laborie (2007 and 2008) reported correlations between near infrared spectral data and selected wood chemical and physical properties within a range of 0.80–0.95. Schimleck et al. (2005) also reported a similarly strong predictive relationship, i.e. $r = 0.94$, for cellulose content in *Populus* using near infrared spectroscopy.

5 Translational Genomics

We define translational genomics as the research and development process that bridges the basic discovery phase and the application phase in commercial breeding programs. This has often been neglected, because the motivation for application is sometimes lacking among academic researchers, and the development of genomic tools is too basic for applied programs with little or no research budgets. Translational research is an important and active area in human medical research and is increasingly seen as very important in agriculture and forestry. In traditional forest tree breeding there has been little, if any, gap between the basic and applied phases. As we saw earlier in this chapter, it was often academic researchers who developed *Populus* hybrids that were quickly used in plantations. However, with the emergence of biotechnologies and genomics sciences, it can be a long way from the discovery of a gene to the release of a new variety or improved populations. In the United States and elsewhere, research cooperatives have taken a quasi translational role, but these cooperatives have had difficulty sustaining financial support and many have disappeared. Notably in the United States, the Poplar Molecular Genetics Cooperative led by Dr. Toby Bradshaw at the University of Washington in Seattle, Washington sought to bring genomics-based breeding to application in *Populus* breeding. This program was probably too early for its time and is badly needed now that massive genomics resources are available. However there is no infrastructure currently in place to translate this resource and knowledge base into application.

In response to this widening gap between basic genomic discovery and direct application in plant breeding, the United States Department of Agriculture developed the Plant Genome Coordinated Agricultural Product (CAP) as a publicly-funded program to bring discovery to application in crop and forest tree breeding in

the United States. Each year, a research group focused on a single crop species is awarded a grant of five million dollars for four years. Awards have been made for rice, wheat, barley, Solanaceae and conifers. The conifer CAP is called the Conifer Translational Genomics Network (www.pinegenome.org/ctgn) and is a consortium of six universities, four tree breeding cooperatives, and the United States Forest Service. A similar forest tree translational genomics project also was funded in Europe. It is called NovelTree (www.noveltree.eu). A translational genomics project for *Populus* has not yet been funded but planning is underway for a *Populus* CAP in the United States.

Broadly defined, translational genomics might include all biotechnological approaches to tree improvement. These include traditional breeding, marker-based breeding, and transgenic or genetically modified trees (White et al., 2007). In this chapter, we focus entirely on marker-based breeding (see Chapter 19, White et al., 2007). Readers are referred to a number of excellent reviews on transgenic approaches (Strauss et al., 2004). Before marker-based breeding can be applied, associations between genetic markers and traits of interest must be discovered. We will first briefly review how marker-trait associations are found and summarize the state of this knowledge in *Populus*. A more detailed review of this topic can be found in Section 2.2 of this volume.

5.1 Discovery of Marker-Trait Associations

There are many approaches to discovering marker-trait associations (see Chapter 18, White et al., 2007). These include: (1) two-point linkage analysis between a marker and a qualitatively inherited trait such as a disease resistance gene, (2) quantitative trait locus (QTL) mapping, and (3) association genetics. All three approaches have been used in several *Populus* species and for a variety of traits, e.g. bud phenology, water use efficiency, disease resistance, biomass production and others; however, the catalog of validated marker-trait associations is very limited (see Section 2.2). The two-point linkage approach has been used successfully a number of times to map single genes coding for resistance to *Populus* leaf rust (*Melampsora* spp.) (reviewed by Feau et al., 2007). QTL mapping has been used to identify map locations of QTLs for a number of quantitative traits but, in fact, the number of validated marker-trait associations found through QTL mapping is so limited that this knowledge base could never support a marker-based breeding program of any kind in any species. For the reasons described in Section 2.2, the QTL approach is rarely used in *Populus*.

The association genetics approach holds much more promise for identifying marker-trait associations in *Populus* and other trees (Neale and Savolainen, 2004; Neale and Ingvarsson, 2008) and is now the method of choice in *Populus* (Section 2.2). There are several steps in the implementation of an association genetics research program that we will review for *Populus*. We will also identify gaps in the *Populus* discovery stream that will need to be filled before genomic-based breeding can be fully realized. The steps in the discovery process include: (1) population

development, (2) test site establishment, (3) candidate gene identification, (4) resequencing and SNP discovery, (5) genotyping platform development, (6) phenotypic evaluation, and (7) tests for association and validation. The status of each of these steps is summarized for several species and association genetics studies around the world in Table 2.

Population development and test site establishment – To begin, association genetics studies have been initiated in just a few *Populus* species (*P. deltoides, P. nigra, P. tremula* and *P. trichocarpa*). Earlier in this chapter we described four inter-specific taxa that are of most interest (*P.* ×*canadensis*, *P.* ×*generosa*, *P.* ×*tomentosa,* and *P.* ×*wettsteinii*). Perhaps not so coincidentally, the parentage of these hybrids frequently involves all of the species listed above for which association genetics studies have been initiated. However, association genetic studies are lacking in three other similarly important species – *P. alba*, *P. maximowiczii,* and *P. tremuloides*. A resequencing and SNP discovery project is underway in *P. alba* as will be discussed in the next section.

Forest tree association genetics populations can be either clonal, family-based or both (Neale and Savolainen, 2004). There are relative merits to each population type (Gonzalez-Martinez et al., 2007). In *Populus*, clonal propagation is easy and is used in all of the populations listed in Table 2. This not only increases heritability but also allows for establishment of multiple test sites and estimation of genotype-by-environment interactions, something that nearly all studies have employed. Population sizes vary from 350 (*P. tremula*) to 1,100 (*P. trichocarpa*) in the ongoing United States Department of Energy's Bioenergy Science Center[4] – University of British Columbia joint study. More is always better, but these population sizes are probably of adequate power to detect associations.

Candidate gene selection, resequencing and SNP identification – Resequencing and SNP discovery using first generation sequencing technology dictated that a candidate gene-based approach be used, because genome-wide resequencing was not feasible. This situation has changed dramatically with the completion of the *P. trichocarpa* reference genome sequence (Tuskan et al., 2006) and the release of second generation sequencing platforms. All projects listed in Table 2 will employ the genome-wide resequencing approach as resources become available and costs go down. At this time, however, most are still using a candidate gene approach employing either first or second generation sequencing platforms. The *Populus* genome contains ~45,000 genes, so ideally all genes will ultimately be resequenced. In practicality, a fewer number of genes have been or will be resequenced in these projects simply due to limited resources. It is expected that as second generation sequencing platforms are used and as third generation platforms become available, all projects will resequence all 45,000 genes if not entire genomes.

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[4]The Bioenergy Science Center is a research affiliation of 10 organizations operating under the auspices of the United States Department of Energy and the leadership of the Oak Ridge National Laboratory.

Table 2 Summary of worldwide *Populus* association genetics projects

Species	Clones	Test sites	Genes	SNPs	Phenotypes	Positive associations	Reference	Lab
P. trichocarpa	457	3	40	1,486	Lignin content, S/G ratio	37	N/A	University California, Davis, USA, David Neale
P. trichocarpa	1,100	3	7,000	N/A	Lignin/cellulose, water-use-efficiency, phenology, wood properties	N/A	N/A	University of British Columbia, Canada, Carl Douglas
P. trichocarpa	1,100	3	1,000	N/A	Lignin/cellulose	N/A	N/A	Department of Energy, Bioenergy Science Center, USA, Gerald Tuskan
P. deltoides	815	1	N/A	N/A	N/A	N/A	N/A	University of Florida, USA, Matias Kirst
P. nigra	612	2	40	1,237	Lignin/cellulose	N/A	N/A	University of California, Davis, USA, David Neale
P. nigra	398	1	20	53	Phenology, biomass, leaf development	3	N/A	University Southampton, U.K., Gail Taylor
P. tremula	350	2	40	100	Phenology, senescence, herbivory	4	Ingvarsson et al. (2008)	Umea Plant Science Center, Sweden Stefan Jansson, Par Ingvarsson,

Candidate gene selection is determined by a *priori* or suggestive knowledge of which genes control certain phenotypes. Three approaches are generally used for candidate gene identification: (1) known function in model systems, (2) gene expression analysis, or (3) map co-location with QTLs. For some traits, pathways and genes within pathways are quite well known, e.g. lignin and cellulose biosynthesis. This approach was used in the joint University of California, Davis – GreenWood Resource *P. trichocarpa* and *P. nigra* projects and the Umea Plant Science Center[5] *P. tremula* project because resources allowed only a few candidate genes to be sequenced using first generation sequencing platforms. The *P. trichocarpa* projects of the Bioenergy Science Center and the University of British Columbia were more recently started and both are using second generation sequencing platforms. These projects will sequence many more genes so candidate selection does not have to be so restrictive.

Once genes or genomes are resequenced in a diversity panel of some kind, it is then necessary to identify the polymorphic nucleotide position – the so-called single nucleotide polymorphisms (SNPs). Resequencing can also identify insertion/deletion (indel) polymorphism but the bioinformatics associated with indels is much more complex thus indels are often ignored. This will certainly change in the future as human geneticists are finding that indel variation can be responsible for much of the phenotypic variation in populations. An example of indel variation having a large effect on the phenotype is the *CAD* null indel that was discovered in loblolly pine that affects lignin properties (Gill et al., 2003). Presently, SNPs have been reported for just three of the active association genetics projects: the joint University of California, Davis – GreenWood Resource *P. trichocarpa* and *P. nigra* projects, and the Umea Plant Science Center *P. tremula* project. The frequency of the SNPs uncovered in these projects is roughly one in 100 base pairs. Modern population genomic methods can then be applied to estimate measures of nucleotide diversity and divergence as well as test for selection (see Section 2.2 for a comprehensive treatment of these methods and reports in *Populus*).

SNP genotyping platforms – Once the SNPs have been discovered in a diversity panel, each SNP can then be typed for each individual of the association population. Full genome sequences or even candidate gene sequences, for every member of an association population of 500–1,000 individuals has, to date, been prohibitively expensive. Because there are a large number of high throughput genotyping platforms on the market and these technologies change rapidly, SNP genotyping platforms will not be discussed in detail here. The only *Populus* association genetics project to date that has used one of these platforms is the University of California, Davis – GreenWood Resource *P. trichocarpa*. This project used the Illumina Golden Gate assay to type 1,536 SNPs in the full association population

[5]The Umea Plant Science Center located in Umea, Sweden is a research center in plant biology formed in 1999 from the Department of Plant Physiology, Umea University and the Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences.

of 457 clones. The other projects listed in Table 2 will use the same or similar technology. These SNP genotyping platforms can type up to 50,000 or more SNPs in parallel at a cost less than $0.01 per data point. So it is clear that *Populus* association populations will be genotyped for tens of thousands of SNPs from nearly all *Populus* genes in the next couple of years. Once this task is completed, the SNP genotype database will be somewhat complete and then all the attention focuses on the phenotype side of the process.

Trait phenotyping – The challenges and issues associated with trait phenotyping in association studies are not different from those in normal breeding programs, so will not be discussed in great detail here. The trait types being evaluated in association studies (Table 2) are much the same as have always been of interest (growth, biomass, wood properties, disease resistance, adaptation, etc). With the increasing interest in *Populus* as a feedstock for cellulosic ethanol, many studies are putting a high priority on lignin and cellulose quantity and quality (Davis, 2008). Other traits related to the decomposition of cellulose to simple sugars and fermentation will also be of great interest. The molecular phenotypes, e.g. transcriptome, proteome and metabolome, are also target phenotypes in association studies. By taking an integrated "omic" approach all the way through to complex, whole plant phenotypes, it becomes possible to take a network approach to association genetics and begin understanding epistatic interactions at the molecular level.

Tests of association – Once genotypic and phenotypic data are in hand for all members of an association population, it is possible to test for associations. The type of test performed depends on the population type. For example, the transmission disequilibrium test (TDT) has often been used with human parent-offspring data but such a test would not likely be applied in *Populus*. Rather, a variation on this test, the quantitative transmission disequilibrium test (QTDT) might be used where there is a family structure in the population (Gonzalez-Martinez et al., 2007). The populations given in Table 2 generally lack a family structure so simple regression (GLM) based methods are possible. However, the confounding effects of family and population structure must be taken in to account and corrected. The other issue that must be addressed is that of multiple testing. Generally, some type of false discovery rate probability is determined. New methods of association testing are being developed continuously and will certainly add to the power and precision of association testing. But, as always, advanced statistical approaches can never make up for poor experimental design and/or poor quality genotyping and phenotyping.

5.2 Marker-Assisted Selection

Once a large number of marker-trait associations are discovered, it will be possible to develop marker-assisted selection programs in *Populus* species. White et al. (2007) distinguish between marker-assisted selection (MAS) and marker-assisted breeding (MAB). MAS is defined as the selection of superior trees based on their

molecular genotype, whereas MAB includes broader applications of markers, such as quality control and breeding designs, in tree improvement programs. We will only consider MAS in this chapter. White et al. (2007) further distinguish two general approaches to MAS: (1) indirect selection based on genetic markers linked to desirable QTLs, and (2) direct selection based on desirable alleles at genetic loci controlling target traits. Grattapaglia (2007) defines approaches (1) and (2) as *linkage equilibrium* and *linkage disequilibrium* approaches, respectively. Grattapaglia (2007) further defines *direct selection* when the functional mutation (quantitative trait nucleotide, QTN) itself can be directly selected upon. Again, we will only consider the MAS linkage disequilibrium approach in this chapter. Another approach that is being developed for application in dairy cattle and other livestock species is called *genomic selection* (Dekkers and Hospital, 2002). The general idea behind genomic selection is that a very large number of markers can be used to predict breeding values, even if marker-trait associations are not known. This approach is dependent on the extent and distribution of linkage disequilibrium in the genome.

In Section 2.2, Ingvarsson presented the concept of linkage disequilibrium and its relevance to detecting marker-trait associations in *Populus*. Furthermore, Ingvarsson describes the extremely low level of linkage disequilibrium found in *Populus* populations such that any marker (generally a SNP) associating with or controlling a phenotype would likely be within the genetic locus, or at an extremely close physical distance. If haplotypes can be established for genetic loci associating with phenotypes, full allelic discrimination is expressed at that genetic locus. Furthermore, it is possible using standard quantitative genetic methods to estimate the size and direction of effects of haplotypes (alleles). If haplotypes and haplotype effects are established for large numbers of genetic loci such that a large portion of the phenotype variance for a target trait can be accounted for, the reagents are now in place to practice direct MAS.

MAS will undoubtedly be combined with some form of phenotypic selection in either a sequential and/or combined manner. In a sequential approach, a large amount of breeding material might be screened with markers only to identify the smaller amount of material that would be field tested and phenotypically evaluated. Final selections might be made using index selection with multiple traits and markers. The exact way in which MAS will be applied in *Populus* breeding is yet to be developed, but it is quite certain that marker data will be abundant and inexpensive to obtain. The challenge remains as to how to fully capture the value of nearly complete information on a tree's genotype.

6 Conclusion

One significant advantage enjoyed by *Populus* breeders is the extensive knowledge of natural variation in phenological and eco-physiological traits. Complimenting this is the insight into the physiological and morphological determinants of yield and pathogen resistance that characterize segregating populations. Armed with such

extensive knowledge, *Populus* breeders can look forward to widespread application to selection programs offering greater precision and earlier schedules. However, this expectation has not yet been fully realized in most operational breeding programs, where evaluation and selection remains focused on integrated traits, e. g. climatic adaptability, biomass production and pest resistance. Although this has worked extraordinarily well to advance the number of productive and well-adapted genotypes now used commercially, the reality is that as productive genotypes continue to materialize future selection thresholds will increase. This presents a greater challenge to sustained genetic advancement. New methodologies are warranted, then, if this challenge is to be surmounted, and molecular tools are rapidly approaching their practical utility in dissecting the inheritance of complex traits that will lead to more effective manipulation and evaluation techniques (Stanton, 2009). At the same time, the field of plant phenomics is developing a rich assortment of imaging techniques to improve analyses of plant growth and performance, which should accelerate the use of molecular tools and translational genomics programs. A recent article in *Science* assessed the importance of phenomics, stating that it allows "*... plant physiologists to "catch up" with genomics...*" and plant breeders to "*... shift breeding into overdrive*."[6] This emphasis on phenotyping capability could propel *Populus* breeding to the next chapter of its successful story. To take full advantage of this, comparative resequencing studies of the most important species – *P. deltoides*, *P. maximowiczii*, *P. nigra*, and *P. trichocarpa* – should now become a research imperative. Such exploration will promote a superior understanding of between- and within-species allelic variation and how best to recombine the variation in both intra- and inter-specific breeding programs.

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Conservation Genomics

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Abstract Conservation of *Populus* genetic resources can be defined at three levels: conservation of all *Populus* species, conservation of the variation present within species, and the conservation of the key evolutionary processes that will enhance further evolution of *Populus* species. In this context, genomics, the study of an organism' entire genome, can be considered as a powerful tool that would allow us to reach an integrated view of the genetic variation to be conserved. In particular, the application of principles and methodologies of population genomics to *Populus* hold great potential to answer some of the most relevant questions in poplar, aspen and cottonwood conservation genetics, such as how much functional variation is currently present and how to conserve it. Here, we review recent progress in applied conservation genetics in *Populus* and we sketch the potential for conservation genomics to transform this field in the very near future.

1 Introduction

The increasing loss of biological diversity – biodiversity for short – is a globally discussed fact. This loss manifests itself not only in terms of species numbers, but also on the level of *ecosystem diversity* (diversity of habitats) and *genetic diversity* (diversity of genes and genotypes within populations and individuals of the same species). The level of biodiversity is often used as a measure of the health of a biological system. Consequently, the loss of genetic variability (also called *genetic erosion*) is generally expected to reduce the ability of species to adapt to changing environments. Increased inbreeding depression and reduced fitness may also limit their evolutionary potential. The reasons why biodiversity decreases on all levels are multiple, but human-associated factors such as habitat loss, introduced species, overexploitation or environmental pollution are often cited. There are also stochastic

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factors of demographic, catastrophic, or environmental origins (for instance, forest fires or river floods) that reduce genetic biodiversity. To minimize the risks of genetic erosion, it is often necessary to counteract actively. The science of finding the factors that result in a decline in fitness or that cause extinction (at the species or population levels) and of establishing and executing management regimes aimed at diversity preservation is called "conservation genetics".

The first step in conservation genetics is to characterize the remaining diversity. From a methodological point of view, molecular characterization of individuals, populations and species, evolutionary genetics (studying the mechanisms of evolution), population and quantitative genetics, breeding science, phylogeny and systematics converge with the single goal of delineating measures for preserving the evolutionary potential of organisms.

The main fields of activity and questions in conservation genetics (Pearse and Crandall, 2004) are listed below. We will try to give answers to these for *Populus* species towards the end of this chapter:

- How many species? A clear taxonomic treatment is the basis of any conservation measure. Molecular methods can help in defining genera, species and subspecies, but not on a stand-alone basis – traditional taxonomy and close interdisciplinary interaction are necessary.
- How many populations? A population is usually defined as an independent breeding unit, a group of individuals that mainly interbreed among themselves over many generations, with only occasional contributions from other populations (immigration). It is necessary to know where to draw a line between populations in order to tackle conservation measures population by population.
- What are the relevant life-history traits and the reproductive biology of the species (mating systems, reproductive success, pollen and seed dispersal, migration patterns), including any reproductive barriers between species and natural and anthropogenic hybridization (*genetic contamination*)?
- Which populations to conserve? Measuring the levels of genetic diversity (defined as number and distribution of alleles and genotypes) within and between populations to identify threatened populations (those with a low genetic diversity, or with special adaptations) as conservation targets, also taking into account the genetic basis of adaptive traits.
- How to deal with them? Establishing and executing a viable management plan for maintaining the genetic variability of the species is often the most difficult task, as socio-economic factors can often override scientific evidence. The usual approaches are selection of *"in situ"* genetic reserves, sampling for the establishment of *"ex-situ"* germplasm collections, development of breeding work within the *"ex-situ"* collections, and restoration of populations in the wild.

There is growing awareness that genomics, the study of an organism's entire genome, and functional genomics, the study of the transcribed and/or translated portion of the genome, have the potential to transform conservation biology (Ryder, 2005; Kohn et al., 2006). In this context, we may define conservation genomics

as a tool box that allows us to extend traditional conservation genetics to reach an integrated view of the genetic variation to be conserved, at different scales of organization. Whereas traditional molecular conservation genetics often stopped at the point where neutral genetic variability was studied and conserved (Frankham et al., 2004), as a supposed surrogate for genome-wide variation, the promise of "conservation genomics" is to identify the molecular determinants of detrimental and adaptive variation (Kohn et al., 2006) and on this basis improve management strategies. In long-lived species such as poplars, aspens, and cottonwoods, this can be achieved most easily by taking advantage of the suite of concepts and methodologies circumscribed as *population genomics* (Luikart et al., 2003; Storz, 2005; Stinchcombe and Hoekstra, 2008), which we shall outline further below. By drawing from these methodologies, conservation genomics can bring conservation genetics closer to understanding genetic determinants of relevance for an organism's phenotype and fitness, and closer to useful options for managing populations in order to conserve their evolutionary potential.

To further circumscribe "conservation genomics", a definition analogous to the one for biological diversity, as outlined above, applied to the particular case of poplars, aspens and cottonwoods, may be useful. Likewise, conservation genomics of *Populus* would be concerned with the conservation of the genomes of all *Populus* species, conservation of the variation present in the individual species' genomes, and conservation of the potential for further evolution of genomes in *Populus* species by maintaining opportunity for evolutionary processes to act on these genomes.

Special features of the *Populus* genome that deserve attention in this respect are its particular architecture, characterized, for instance, by many duplications (Tuskan et al., 2006), the high level of heterozygosity and nucleotide diversity (much higher than other forest trees; Ingvarsson et al., 2008), and the functional diversity presented by many species that have special adaptations to a great variety of environments. Indeed, the species from this genus have been selected as tree models not only because of their genetic characteristics (see preface and previous chapters), but also because within the genus there is a wide degree of variation regarding adaptation to different environmental conditions (e.g. tolerance to drought, salt, and other abiotic stresses, or resistance to pests and pathogens), and in their habit of growth. This variability offers interesting possibilities to study evolution in response to new environmental conditions (increased temperature, drought, or CO_2) and for the isolation of genes of ecological or economical importance. In addition, population processes that are thought to maintain the evolutionary potential in poplars, aspens and cottonwoods are the predominant dioecious mating system (thus, they are often obligate outcrossers), the persistence of large and continuous populations in some species vs. fragmented and isolated ones in others (metapopulation structure), the large pollen and seed dispersal distances, their exposure to shifting patterns of climate and other environmental factors, and a relatively rapid population turnover (compared to many other tree species).

Here, we review recent progress in applied conservation genetics in *Populus*, and we sketch the potential for conservation genomics to transform this field in the very near future.

2 Conservation of Genetic Diversity in *Populus* – Traditional Approaches

Populus is a tremendously diverse genus, including over 30 species of aspens, poplars and cottonwoods for some authors (Taylor, 2002) or many more for others – for example, the Flora of China (http://flora.huh.harvard.edu/china/mss/intindex.htm) lists 134 species for China only -, divided into six sections (Eckenwalder, 1996) and distributed throughout the Northern Hemisphere. Taxonomic issues are apparent in this genus, as no full molecular phylogeny of *Populus* has been published yet, despite several attempts at partial phylogenies in the past. This is discussed in more detail in Section 1.2 of this volume.

Molecular markers provide a convenient tool to study genetic variability in organisms with such long generation times, which make common garden testing unpractical and tedious (although in this respect, *Populus* sp. are still at an advantage against other tree species due to their faster turnover and cloning capability). A wide range of molecular markers are normally employed (variation in cpDNA and mtDNA, often found in gene spacers and introns, anonymous markers like AFLPs and others, RFLPs, nuclear and cytoplasmic SSRs, and SNPs, see reviews in Heinze, 1998a; Krutovsky and Neale, 2006). Although the application of new generations of markers may even be seen as a fashion (Schlötterer, 2004), isoenzymes, microsatellites or SSRs and SNPs are currently regarded as the most useful ones, as they are widespread in the nuclear genome and codominant. In *Populus*, a database that includes thousands of SSR loci has been developed by the International Populus Genome Consortium (www.ornl.gov/ipgc). Other genetic tools that are currently under development in the framework of various international consortia include resources for ESTs (Sterky et al., 2004), genome-wide sequencing or SNP genotyping (see, for instance, www.evoltree.eu or dendrome.ucdavis.edu/pbgp/).

Isoenzyme studies in *Populus* are numerous (e.g. Culot et al., 1995; Legionnet and Lefèvre, 1996; see review in Heinze, 1998a and in previous chapters of this volume). RAPDs and other anonymous markers have also been adopted readily by researchers studying *Populus* populations (RAPDs: e.g. Chong et al., 1994; Yeh et al., 1995; Su et al., 1996; Rajagopal et al., 2000; Sánchez et al., 2000; Saito et al., 2002; AFLPs: e.g. Arens et al., 1998; Winfield et al., 1998; Smulders et al., 2008b). Most of these studies were concerned with the study of population genetic structure and diversity and the identification of related clusters of populations. However, clustering based on dominant markers is only a very coarse tool; it performs poorly in many situations, as, for instance, when a population is recently admixed from two equally distant sources. Microsatellites have allowed a better assessment of relationships among populations (e.g. Krystufek, 2001; Cole, 2005; Lexer et al., 2005; Namroud et al., 2005; Pospíšková and Šálková, 2006, van Loo et al., 2008; Smulders et al., 2008b). As microsatellites are codominant and often highly polymorphic, they are also amenable to new approaches for the identification of origin and assignment of individuals to populations (see further below).

The relevance of studies of genetic variation for conservation has been emphasized especially in Europe, where the native black poplar (*Populus*

nigra) has been the focus of a network of experts in the European Forest Genetic Resources Programme (EUFORGEN, Bioversity International, www.bioversityinternational.org) for more than a decade (Cagelli and Lefèvre, 1995; Frison et al., 1995; Lefèvre et al., 1998; Lefèvre et al., 2001a, b, Kajba et al., 2002; Heinze, 2005). Research using molecular markers in this species has shown: (a) high differentiation among river basins but often some similarity along river courses, (b) large gene flow and dispersal distances, with populations from different catchments but relatively close to each other often showing similar allele frequencies, (c) no significant difference in pollen (wind-) or seed (wind and waterflow-) mediated gene flow, and (d) notable levels of genetic diversity and no or very little introgression from non-native poplars (Imbert and Lefèvre, 2003; Smulders et al., 2008a,b, Heinze, 2008). The European black poplar is therefore a paradigmatic example of a species whose conservation depends more on socio-economic factors, such as use and management of riparian systems, than on its standing genetic resources.

One fact of relevance to genetic conservation in diploid organisms, such as poplars, aspens, and cottonwoods, is the distinction between allelic and genotypic diversity. Allelic diversity can be biased by linked alleles within individuals (genotypes) due to vegetative propagation. Poplars often propagate asexually (vegetatively) from dormant primordia (roots and shoots) or by means of cladoptosis (abscission of shoots that can be carried on watercourses and subsequently root elsewhere). Several authors have studied the extent of clones in various *Populus* species (e.g. Legionnet et al., 1997; Rottenberg et al., 2000; Barsoum, 2001; Peltzer, 2002; Barsoum et al., 2004; Suvanto and Latva-Karjanmaa, 2005, van Loo et al., 2008, and most recently Brundu et al., 2008; Ally et al., 2008 and Mock et al., 2008), which often ranges from some tens (maximum distance of ~60 m in aspen; Suvanto and Latva-Karjanmaa, 2005) or hundreds of metres (~100–200 m in grey and white poplar from Central Europe; van Loo et al., 2008) up to a few kilometres (in white poplar from the Iberian Peninsula; our own unpublished results).

Finally, although the genetic diversity in plants is located mainly in nuclear DNA, there is also some variation in cytoplasmic DNA, plastids and mitochondria, which are maternally inherited (Keim et al., 1989; Radetzky, 1990; Mejnartowicz, 1991; Paige et al., 1991; Rajora et al., 1992; Rajora and Dancik, 1992). This variation can be exploited for species identification (Vornam et al., 1994; Heinze, 1998b) and also to reconstruct maternal (i.e. seed dispersed) lineages. Thus, cytoplasmic DNA markers, alone or combined with nuclear markers, can be used to indicate the amount and the direction of introgression between species (see further below).

3 Hybridization and Genetic Conservation

Many *Populus* species hybridise under natural conditions, or when female flowers are pollinated with pollen collected from another species (see review by Stettler et al., 1996 and Section 1.2 of this volume). In fact, the cases where species within a section would *not* hybridise spontaneously when brought into contact are rather the exception than the rule. Hybridization across section boundaries, e.g. between members of Aigeiros and Tacamahaca, is commonly observed in nature, and exploited

in breeding (Stettler et al., 1996). This is the case despite a series of reproductive barriers that have been described in *Populus*, both at prezygotic or postzygotic stages. The main mechanisms described as prezygotic barriers are flowering asynchrony, pollen competition, or interspecific prefertilisation incompatibility (Stanton and Villar, 1996). For instance, a study in Belgium has shown that functional traits such as flowering phenology are unlikely to introgress into native *P. nigra* from the cultivar, *P. nigra* "Italica", simply because asynchronous flowering cannot lead to "massive" introgression (Vanden Broeck et al., 2003a, b). Among postzygotic barriers, the most important ones reported are hybrid inviability and poor survival, hybrid breakdown and hybrid sterility (see Vanden Broeck et al., 2005, for a review). Hamzeh et al. (2007) have described a seemingly similar case of asymmetrical introgression in North America that does not involve any apparent reproductive barriers though. Hybrid breakdown appears to affect fitness traits due to linked gene complexes, which are disrupted in further generations of hybrids. This would explain the fact that some parts of the genomes are more permeable than others to introgression. In this way, introgression can be an important mechanism for the transmission of functional traits and *collective evolution* (as described by Morjan and Rieseberg, 2004). Determining whether cases of natural introgression are detrimental or beneficial for the evolutionary potential and integrity of *Populus* species is crucial for their conservation.

Introgressive hybridization and gene flow from domesticated species into their wild relatives is often regarded as detrimental for the persistence and evolution of wild populations. The availability of species-specific markers has allowed to asses the level of introgression for the European black poplar, *Populus nigra*. Introgression of concern to conservationists occurs between black poplar and commercial hybrids, e.g. *P.* x *canadensis*. The main concern regarding this source of introgression is the potential introduction of maladapted genes into the native populations. More important, however, may be that the restricted number of genotypes represented by exotic clones will, through interbreeding, become over-represented in the genepool of wild *P. nigra*. Such an outcome would represent a reduction in effective population size with an associated loss of diversity and probably, a loss of adaptive potential. Molecular marker studies have shown that in situations where female black poplars grow in close proximity to both hybrid and non-hybrid males, little evidence of introgression from *P.* x *canadensis* is detected (Fossati et al., 2003; Heinze, 2008; but see Benetka et al., 1999, 2002). However, if there are no male *P. nigra* trees within pollinating distance, the female black poplars will cross with the male *P.* x *canadensis* in the vicinity (Vanden Broeck et al., 2004; Ziegenhagen et al., 2008). "Lonely females" seem to pose an extra risk for introgression (Eckenwalder, 1982). The introduced hybrid poplars do not therefore pose a major threat to native black poplars provided there is enough pollen available from male black poplars. Thus, every situation has to be judged on a case-by-case basis.

There are a number of studies that have tried to assess the magnitude of anthropogenic hybridization and introgression in *Populus* species or hybrid combinations (e.g. Heinze, 1997; Benetka et al., 1999; Vanden Broeck et al., 2004;

Meirmans et al., 2007; Ziegenhagen et al., 2008; Heinze, 2008), but only with a sufficiently high number of diagnostic markers, introgression in generations further beyond F2 and BC1 can be reliably assessed. Such high numbers of markers are only now becoming available (Meirmans et al., 2007), and it will be interesting to see whether the long history of clonal cultivation has left such a low-level imprint in the genomes of native species as recent studies, using a low number of markers, suggest. The current evidence indicates that although only low levels of introgression are detected in natural poplar populations, cultivated poplars are reproductively active along several river systems in Europe and that they may compete with the native species in establishment in riverine habitats (Vanden Broeck et al., 2005). This fact may pose a greater concern to conservation of native stocks than the potential impoverishment of gene pools by introgression into the native species.

Following Allendorf et al. (2001), conservation programmes for *Populus* species should consider the following questions:

- Are the "threats" that introduced cultivars (among them, hybrids) pose for natural populations relevant in any case? The examples of *P. nigra* cited above have not yet given direct evidence for the maladaptation of introgressed trees.
- Is there an acceptable low proportion of admixture/hybridization? We have argued into this direction in the past (Heinze and Lickl, 2002), but the evolutionary consequences of even "a few percent" of introgression are largely unknown. Genomics offers a toolbox to assess the effects of introgression of a few genomic regions into the genetic background of another species. By comparisons with natural hybrid zones, where such later introgressants may be common (see further below for the *P. tremula*/*P. alba* example in Europe), phenotypic and functional tests will inform us about the magnitude (if any) of this "low-level" introgression (Lexer et al., 2007).
- Can parental individuals be rescued from admixed populations? The efforts by EUFORGEN and its networks for *P. nigra* (1994–2005) and later, for Social Broadleaves (2005–2009), have actually shown that this is a viable option, as parents can be tested for their genetic "purity" and collected by vegetative propagation in gene banks (Storme et al., 2004). Trees raised by vegetative propagation from such collections have now been widely planted in restoration projects across Europe.

4 Population Genomics Provides New Tools for *Populus* Conservation

The genomic resources now available in *Populus* species hold the potential to transform conservation genetics in poplars, aspens and cottonwoods. We shall now focus on the power of population genomics to identify and manage functionally relevant

variation in *Populus*. Conservation genomics of *Populus* can profit greatly from concepts and methodologies recently developed and tested in other groups of animals and plants (e.g. Luikart et al., 2003; Campbell and Bernatchez, 2004; Beaumont, 2005; Storz, 2005; Stinchcombe and Hoekstra, 2008), including application of the population genomics approach to natural interspecific hybrid populations (Buerkle and Lexer, 2008). Most population genomics research carried out so far was motivated by an interest to understand basic principles in evolutionary genetics rather than advancing conservation management (but see González-Martínez et al., 2006; Namroud et al., 2008). A particularly exciting aspect of applying these technologies to forest trees such as *Populus* is that in forestry there is a long tradition of translating conservation genetics into management practice. Variation in functionally and ecologically relevant, fitness-related traits and genes is of direct relevance to forest management (White et al., 2007). Thus, forest trees such as *Populus* have a potential to become text book examples for translating population genomics research into forest tree conservation practice.

Population genomics refers to the use of a large number of molecular genetic markers, preferably with known genomic locations, to disentangle two different sources of present-day patterns of genetic variability in natural populations: demographic history on one hand, and different forms of selection on the other (Luikart et al., 2003). Separating the effects of demographic history from those of selection is possible because the former will affect all or most loci in the genome, whereas the latter will only affect loci and genomic regions that are direct or indirect targets of selection (Luikart et al., 2003; Schlötterer, 2003; Storz, 2005; but see Hahn's, 2008 commentary to Begun et al., 2007, the first population genomic study based on whole-genome analysis). Different approaches have been developed to identify loci with genealogies that depart from genome-wide neutral expectations, indicative of recent positive selection. For instance, measures of population divergence such as F_{ST} can be estimated for each locus in a population genomic dataset, and comparison of such single-locus F_{ST} estimates to genome-wide neutral expectations can then reveal "outlier" loci or genomic regions potentially subject to divergent or balancing selection (Beaumont, 2005). Non-anonymous, codominant genetic markers such as microsatellites and SNPs (i.e. markers based on DNA sequence data) are ideal to develop approaches based on detection of "outlier" loci (e.g. Namroud et al., 2008; Eveno et al., 2008). This type of work is often carried out with dominant genetic markers such as AFLPs (e.g. Wilding et al., 2001; Campbell and Bernatchez, 2004), or with datasets combining both dominant and codominant markers (Scotti-Saintagne et al., 2004), but AFLP fragment homoplasy can produce a reduction of up to 15% in the power to detect selective loci using these approaches and caution is recommended with this type of markers (Caballero et al., 2008). In respect to statistical methodologies, detection of outlier loci can be carried out using either frequentist or Bayesian analysis of F_{ST} (Beaumont, 2005), or using population split models that allow a more refined modelling of population history and demography, e.g. different population divergence times and population bottlenecks of different strengths (Vitalis et al., 2001).

In addition to F_{ST}-based approaches, outlier loci under positive selection can also be detected by a *hitchhiking mapping* of recent selective sweeps, which involves the

detection of loci or genome regions with greatly reduced genetic diversity in particular local populations (Schlötterer, 2003). An important aspect of this approach is that, by restricting the analysis of diversity to *pairs* of populations, between-locus differences in mutation rates are taken into account; these differences among loci will be cancelled out because the *ratio* of diversity between two local populations is used in the tests, rather than diversity in one local population per se (Schlötterer, 2003).

A different but related population genomic approach makes use of the increased genetic and phenotypic variation often found in interspecific hybrid populations (Lexer et al., 2004). Just as loci or genome regions under selection are expected to exhibit genealogies that differ from the bulk of the genome in pairwise population comparisons (see above), loci under selection are also expected to exhibit unusual behaviour in admixed populations or interspecific *hybrid zones* (Barton and Gale, 1993; Rieseberg et al., 1999). These differences among loci can be detected simply by comparing introgression frequencies at each locus to genome-wide expectations (Rieseberg et al., 1999). A more accurate approach suitable for codominant markers such as microsatellites or SNPs, recently applied to *Populus*, involves the comparison of genotypic clines to neutral expectations calculated specifically for *each* locus (Lexer et al., 2007; Buerkle and Lexer, 2008). By obtaining neutral expectations for each locus, differences in mutation rates and diversities among loci can be taken into account in the analysis. Also, by estimating genotypic clines separately for homozygotes and heterozygotes it becomes possible to examine the genetic basis of fitness differences, e.g. simple dominance relationships among alleles at loci under selection (Lexer et al., 2007). This suite of methodologies is curently being developed further, e.g. to account for "multiple testing" issues (Gompert and Buerkle, unpublished results).

Studies of admixed populations or hybrid zones typically operate in settings with high linkage disquilibrium (LD) – it takes several generations of recombination to break up the chromosomal blocks derived from each parental population in natural hybrids (Buerkle and Lexer, 2008). The hybrid zone approach is of special interest in hybridizing species pairs that are ecologically divergent. In such cases, a proportion of loci or genome regions that deviate from neutrality in natural hybrid populations will do so because of ecological selection (Lexer et al., 2004), hence such "natural interspecific mapping populations" may be used to identify loci of ecological relevance (Buerkle and Lexer, 2008). As demonstrated with experimental hybrid lineages in *Helianthus* (wild annual sunflowers), a fair proportion of the quantitative trait loci (QTL) that are polymorphic between species can be expected to be polymorphic also at the within-species level (Lexer et al., 2005). Thus, studies of interspecific hybrid zones can potentially reveal candidate regions or loci under selection in natural populations that can then be followed up by studies at the intraspecific level.

An important aspect of all of these approaches is that they can easily be combined with QTL and association mapping studies (dealt with in Section 2.2 in this volume). Indeed, it is often desirable to combine population genomics and association mapping work because population genomic analysis can detect candidate loci under selection but does not reveal the traits affected by them, whereas

association mapping can reveal linkage of the same loci or genome regions to specific phenotypes (González-Martínez et al., 2006; Stinchcombe and Hoekstra, 2008). The intimate relationship between population genomics and association mapping becomes intuitively obvious in genomic scans of interspecific hybrid zones, where loci experiencing strongly reduced interspecific gene flow (=loci under negative selection) will also tend to be in *linkage disequilibrium* (LD) with genomic regions controlling interspecific phenotypic differences (Kim and Rieseberg, 1999; Buerkle and Lexer, 2008). Nevertheless, population genomics work can also yield valuable insights even in the absence of information on genotype-phenotype associations, despite a recognized higher power of the latter to detect loci underlying adaptive traits. In addition, the costs of population genomics projects are likely to decrease at a more rapid pace in the future than the costs of association mapping (which involves extensive genetic testing in common garden experiments).

First indications in *Populus* for the potential of conservation and population genomics came from experimental crosses. Indeed, QTL mapping studies (reviewed in Section 2.2) indicate the presence of extensive genetic correlations among growth, developmental, and ecological traits in the form of linkage and/or pleiotropy. Specific regions of the *Populus* genome seem to control (at least in hybrids) multiple phenotypic traits of potential relevance for conservation management, including traits that may be expected to be targets of selection in natural populations. Indeed, a large linkage mapping study in a cross between *P. trichocarpa* and *P. deltoides* revealed large-scale heterospecific segregation distortions of molecular genetic markers, suggestive of positive selection (Yin et al., 2004b). One question relevant for *Populus* conservation genomics now is whether the genetic signature of positive selection on specific loci or genome regions is also detectable in natural populations.

We suggest that *Populus* population and conservation genomics will greatly benefit from three ongoing developments: (a) the increase in throughput and reduction in costs of sequencing and genotyping brought about by novel, non-Sanger based DNA sequencing approaches, (b) the increasing ease with which expressed sequence tags (ESTs) can be generated and utilized in genomic scans for local adaptation (e.g. Vasemägi et al., 2005; Namroud et al., 2008), which facilitates candidate gene approaches with very large numbers of loci (i.e. genome-wide) and (c) the increasing knowledge of variation in recombination rates and levels of LD across the genomes of wild *Populus* species. With respect to the latter, we expect that not all regions of the *Populus* genome will be equally difficult to scan for the genetic signature of local adaptation. Indeed, LD has recently been shown to be greatly increased in regions of the *Populus* genome containing resistance genes to *Melampsora* rust (Yin et al., 2004a). A more recent study indicates strongly suppressed recombination across more than 700 kb of DNA surrounding multiple nucleotide-binding site/leucine rich repeat (*NBS-LRR*) type resistance genes at the proximal end of chromosome 19 (Yin et al., 2008). We suspect that more discoveries such as this are waiting to be made in the genomes of wild *Populus* species. Such high-LD regions could represent ideal starting points for pilot projects into the population and conservation genomics of *Populus* species. Increased LD as found on chromosome 19 of *Populus* should facilitate the detection of the signature of

local adaptation because *genetic hitchhiking* between markers and selected loci will extend across larger chromosomal distances there (Suter, 2008). Of course, increased LD will make it more difficult to identify the exact genes or nucleotide positions under selection. Nevertheless, increased LD in particular genome regions should greatly facilitate initial screens for non-neutral variation in natural populations, which we believe to be important at this early stage of *Populus* conservation genomics.

5 Conservation Genetics and Genomics in Forest Management and Restoration Ecology

Thanks to recent progress and on-going work in the field, the questions put forward by Pearse and Crandall (2004) in conservation biology and genetics, introduced earlier in this chapter, can now be readily tackled for many *Populus* species:

- How many species? A number of useful tools are available to tackle this question; most of them concern chloroplast DNA. The full chloroplast DNA sequences of two *Populus* species, *P. alba* (Okumura et al., 2004; Ueda et al., 2007) and *P. trichocarpa* (Tuskan et al., 2006) can be downloaded from GenBank and used for identifying regions of sequence variation useful for phylogenies; the latter chloroplast sequence may turn out as a future "gold standard" in chloroplast sequencing, given its high coverage of 400 x (Tuskan et al., 2006, supplementary material). We have recently discovered a highly variable intron in a chloroplast gene that helps in defining species (B. Fussi and B. Heinze, unpublished results).
- How many populations? Microsatellite analysis, coupled with assignment and structuring algorithms like those implemented in STRUCTURE (Falush et al., 2007), is straightforward in many *Populus* species. There is a tremendous amount of microsatellite loci available (*International Populus Genome Consortium*, www.ornl.gov/ipgc), and useful results have recently been obtained e.g. with *P. nigra* in large parts of its European range (Smulders et al., 2008b), seven microsatellite loci). A related program, NEWHYBRIDS (Anderson and Thompson, 2002) is very useful for identifying introgressed individuals and assessing their status viz. F1, F2 or backcross (Smulders et al., 2008a).
- Which population does an individual come from? This is a very interesting question in *Populus* conservation and breeding. Chloroplast and assignment methods as those mentioned above may help in finding the source populations for many of the widespread or interesting clones, e.g. the long-disputed origin of the Lombardy poplar, *P. nigra* "Italica", the also column-shaped and widespread female *P. nigra* "Thevestina", the only *P. simonii* clone in central Europe (a male), the only historic introduction of a (male) *P trichocarpa* clone, called "Senior", in Germany (Müller and Sauer, 1974), and other examples. Furthermore, it would be extremely interesting to somehow "dissect" the genetic make-up of historic *P.* x *canadensis* varieties and analyse their maternal (*P. deltoides*-derived) ancestry

for their geographic origin. This may help in defining zones within the natural distribution range of *P. deltoides* of special interest to breeders in certain countries, possibly reducing the need for full tests of many geographic zones for suitability to their special climatic conditions. The crucial missing factor in such ideas, however, is that full-range molecular surveys are only in their infancy in many *Populus* species (but see Smulders et al., 2008b, Cottrell et al., 2005).

- How much functional variation? Kohn et al. (2006) argue that the use of enhanced surveys of genomic variation in endangered species will serve to better manage their functional genetic variation. Surveys of functional genetic variation have a long tradition in *Populus*, though molecular analyses have only come into the picture more recently. Phenolic compounds have long been studied in various *Populus* species (e.g. Böritz, 1962; Greenaway, 1991). Lindroth and Hwang (1996), Hwang and Lindroth (1997) and Osier et al. (2000) have shown that some of these compounds – which are under strong genetic control – interact with insect herbivores. Philippe and Bohlmann (2007) have recently reviewed the molecular and genomic side of this field. Ingvarsson (2005) has analysed variation in protease inhibitor genes in *P. tremula*. Legionnet et al. (1999) have studied resistance levels in *P. nigra* against leaf rust fungi, and found that a large part of the variation was between regions and stands, in contrast to patterns observed with neutral (isozyme) genetic markers (Legionnet and Lefèvre, 1996). Stevens et al. (2007) argue that although there is genetic variation in both resistance and tolerance, there is no evidence for a trade-off between these two “strategies”, in their analysis of foliar chemistry in trembling aspen (*P. tremuloides*). Such a trade-off would have been predicted by many as one possible explanation for the maintenance of genetic variation in these functional traits.

The genus *Populus* offers a wide range of opportunities for the development of conservation genetics and genomics approaches, from narrow endemics – e.g. *P. ilicifolia* in Southern Kenia and *P. monticola* in Southern Baja California and East-Central Sonora (Mexico) – to species with highly fragmented populations, e.g. *P. euphratica* across Northern Africa and South-Eastern and Central Asia. On the other hand, species with large distribution ranges and wide ecological amplitudes may be, at present, undergoing adaptive differentiation or even ecological speciation. This is possibly the case of *P. alba*, where there are emerging subspecies all around the Mediterranean (B. Fussi and B. Heinze, unpublished results) that deserve in-depth study.

A logical sequence of priorities to be addressed for any poplar, aspen or cottonwood endangered population/species emerges:

1. Determine the level of clonality in populations (and maybe even between them, as dispersal can be large in *Populus*). Rottenberg et al. (2000) have described unisexual populations of *Populus euphratica*, a widespread but highly fragmented species, that probably originate from founder effects by a few, or maybe even a single individual. Smulders et al. (2008b) have found many clonal duplications in *P. nigra* in Great Britain and Belgium. In natural stands of several

Populus species, intricate patterns of interspersed clones were found, and there was evidence for somatic mutations to accumulate (Heinze and Fussi, 2008).

2. Determine the risk of hybridization and introgression from non-native sources. Thorough population surveys are needed to assess this risk; flowering phenology observations will greatly enhance them. DNA techniques will play best in detecting "low-level" or cryptic hybridization and introgression, as phenotypic differences are often obvious in first generation hybrids, but not necessarily beyond the first generation.
3. Determine the uniqueness of the populations in terms of neutral genetic distance and variation, as well as in terms of adaptive and detrimental functional variation. Common garden tests (and in the near future adaptive markers, such as SNPs) will serve to assess the functional variation, as *Populus* species can efficiently be propagated vegetatively. Farmer (1970), Dunlap and Stettler (1996), and more recently Luquez et al. (2008) provide examples of such collections. In the absence of common garden (or provenance trial) studies, field observations can help in quantifying functional variation, but it is difficult to imagine achieving the same level of accuracy in the field, where information about population history is often the crucial missing item and the effects of genetic and environmental variation cannot be separated.
4. Identify the molecular genetic basis of non-neutral population variation in endangered species and populations. This whole book is concerned with this topic, although we are not aware of any example yet where the question has been answered satisfactorily in a conservation setting. We are hopeful however to see many such examples in the near future.

Traditional and newly-developed genetic tools could give answers, in the near future, to questions of major importance for poplars, aspens and cottonwoods such as: (a) How great are levels of standing variation and how is genome-wide genetic variation distributed within and among populations? (b) What are the adaptive consequences of natural and anthropogenic introgression? (c) How should natural hybrid zones be conserved in a shifting climate? Is it necessary to conserve several hybrid swarms, e.g. in *P.* x *canescens*, or would they be re-created instantly once the parental species come into contact? (d) Can rates of evolution and mutation be estimated or directly be calculated from the increasing body of molecular genetic data? However, as the final goal of conservation is to conserve the evolutionary potential of species, any static approach will be fundamentally flawed, and conservation goals can only be achieved for a given timeline target, but never once and for all time.

Glossary

- *Adaptative genetic variation:* genetic variation that affects fitness.
- *Admixture:* the mixing of genomes of divergent parental taxa; usually quantified by an admixture proportion or "hybrid index".

- *Biodiversity:* the variation of life at all levels of biological organization.
- *Collective evolution*: Evolution mediated by the spread across hybridizing species of globally advantageous alleles.
- *Comparative genomics*: evolutionary relationship between the genes and proteins of different species, and its applications to infer their structure and functions.
- *Ecosystems diversity*: diversity in a given unit area, ranging from a particular ecosystem to the entire Earth.
- *Functional diversity:* variation of genes and proteins with respect to function, often a consequence of environmental heterogeneity.
- *Genetic diversity*: diversity of genes within a species, i.e. the genetic variability among the populations and the individuals of the same species.
- *Genetic erosion:* loss of genetic diversity in small and/or isolated populations.
- *Genetic hitchhiking:* the process by which an evolutionarily neutral or in some cases deleterious allele or mutation may spread through the gene pool by virtue of being linked to a beneficial mutation.
- *Hitchhiking mapping:* a population genetics approach for the identification of genomic regions carrying a favorable mutation.
- *Hybrid zones*: regions where the ranges of two interbreeding species meet. For a hybrid zone to be stable, the offspring produced by the cross (the hybrids) have to be less fit than members of the parent species.
- *Introgression:* movement of alleles of one taxon into the genetic background of another through reproduction between hybrids and members of one or both parental species,
- *Linkage disequilibrium:* statistical association between genotypes at different loci, or between a phenotype and a focal locus, such that one can predict probabilistically the genotype of the second locus (or phenotype) on the basis of the genotype at the first (from Buerkle and Lexer, 2008)
- *Species diversity*: diversity among species in an ecosystem.

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Index

A
ABA responsive elements (ABRE), 227
Abaso, 21
Abies, 4
Abiotic stress responses
  drought stress avoidance strategy, 232–239
  hormonal signaling during drought stress, model, 239
  signaling at cellular and tissue level
    cellular defences activation, 228–229
    water limitations, 226–228
    water shortage, cellular consequences, 226
  tree-specific adaptation measures
    drought, growth responses, 229–230
    hydraulic architecture changes, 230–232
Abscisic acid (ABA)
  as abscission inducing agent, 233
  in *Populus* wood formation, 220–221
  signaling water limitations, 226–228
  See also Wood
Abscission
  as drought stress avoidance, 232–233
    by seasonal drought stress, 235–239
    stress induced, 234–235
  See also Abiotic stress responses
Acephala applanata, 255
Activation tagging effectiveness, in *Populus,* 124
Adaptive traits, 29–30
Affymetrix platforms, 141, 143
AFR regulators, 85
AGAMOUS (AG) gene, 164
AG co-orthologs, expression, 165
Agilent platforms, 141
Agrobacterium rhizogenes, 121
Agrobacterium strains
  biology, pioneering advances in, 5
  transformation procedures and protocol, 121
Agrobacterium tumefaciens, 120–121
  AGL1 strain, 123
  nopaline strains,C58 and LBA4404, 120–123
  octopine strains, like EHA105, 123
Aigeiros, 45
AINTEGUMENTA (ANT) gene, 179
Allocation patterns, 46–47
Allometric relationships, analysis, 47–48
Allozyme
  diversities, 91
  markers, 22–26
Amanita muscaria, 259
1-Aminocyclopropane-1-carboxylate oxidase genes *(ACO),* 236
Angiosperms, 4
  tree model system, 1990s, 68
Anthocyanidin reductase (ANR), 291
Anthocyanidin synthase (ANS), 291
Antisense-mediated gene suppression, 117
Antisense technology, 117
AP/ERF transcription factor families, 85
AP/ERF transcription factors, 85
APETALA1 (AP1) gene, 159
APETALA2 (AP2) gene, 158
Aquaporins, 55
Arabidopsis spp.
  A/B/C-function, defined, 164
  FLC gene, 159
  floral signal, 161–162
  LBs in, 189
  RNA silencing pathways, 157–158
  SEP gene, 164
  temporal signal, 158
  See also Perennial life

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Arabidopsis thaliana
as alternative model system, 68
analysis of gene sets from *Populus* and, 81–82
genome
comparison, 78
duplicated, 114
sequence, 5–6
as model plant, 3
not good for arboreal traits study, 4
mutation rate, 92
phenylpropanoid enzymes in, 84
in planta methods, 125
See also Populus
Arabis aplina, 162
Arborea (Laval University, Quebec), 6
ARBORKNOX1 (ARK1), 213
ARBORKNOX2 (ARK2), 213
ARBORKNOX1 gene, 174
Arbuscular mycorrhizal (AM) fungi, 248
and *Populus,* association, 255–258
ARF5 (MONOPTOROS) genes, 83
ARF5/MP gene, 83
ARF transcription factor families, 85
ARK2 overexpression, 213
ArrayExpress database, 142
Array platforms, 141
and cross-species utility, 141
Artificial or synthetic miRNAs, role in PTGS, 118
Aspens
Arabidopsis *AtCBF1,* overexpression, 186
difference from hybrid poplars, 204–205
gene expression analysis, 178
genes in cambial dormancy, 182
and hybrid poplars, difference, 204–205
See also perennial life; *Populus*
Association mapping (AM), 95, 102–103
and candidate gene approach, 102
ATP-dependent ATP Binding Cassette (ABC) class, 85
Autumnal leaf senescence, regulation, 182–183
Autumnal senescence
ESTs up-regulation/down-regulation, 237–238
AUX/IAA interacting proteins, 83
AUX/IAA regulators, 85
Auxin Efflux Carrier family, 85
Auxins, in wood formation, 55, 216–218
See also Wood
Auxin Response Factor (AFR) transcription factors, 83
AUXIN RESPONSE FACTOR (ARF) gene, 158
Axillary vegetative meristems, formation, 160

B
BAC-based physical maps advances and synteny test, 84
BAC clones, comparison of sequences, 71
BAC end sequences (BES), 72
Bacillus subtilis model*,* bacterial spore formation, 3
BAC minimum tiling path, 11
BAC physical map, 69
See also Populus trichocarpa genome project
Bacterial artificial chromosome (BAC) library, 71
BARK STORAGE PROTEIN (BSP) gene, 182
Beauveria bassiana, 264
Beavis effect, 102
Beijing Forestry University's *P.* ×*tomentosa's* breeding program, 318
Betula, 4
Betula papyrifera Marsh., 259
Biennials, vernalization treatment, 156
Biolistic approaches, 121
Biomass equations, for clones, 48
Biomass estimations, 47
Biomass yields and growth, 39–44
Birch, *see Betula*
Black cottonwood, *see Populus balsamifera* L.
BLAST2GO web resource, 142
BLASTN hits, 74
BLASTN searches, 80
BLAST search tools, 136
BLAT search tools, 136
BpMADS4 gene, 182–183
Brassica napus, 158
Breeding and selection for pest resistance, 328–329
Breeding program and approaches
biomass productivity, 327
controlled breeding, 313–322
F1 inter-specific hybridization, 317, 318
genecology, 308–313
genetic and varietal evaluation, 322–325
hybrid vigor impact, 318
intra-specific recurrent breeding program, 318
long-term breeding programs, 318–322
Populus cultivars, registered with FAO's International Poplar Commission, 313

specific gravity, evaluated wood quality trait, 328
testing and selection, 322–329
translational genomics, 329–336
See also Reproductive development, in *Populus*
Breeding strategies, of *Populus,* 313, 315–318
Bud dormancy release, in *Populus,* 187
Bud phenology, genetic control, 103–106
Burkholderia cepacia Bu72, 8
bZIP transcription factor, (FLOWERING LOCUS D (FD), 159

C
CACTA elements, 80
Caffeic acid O-methyl transferases (COMTs), 293
Caffeoyl CoA O-methyl transferases (CCOMTs), 293
CALCINEURIN B-LIKE PROTEIN 9 (AtCBL9), 239
Cambium
dormancy, 181–182
features, 202
function, genetic regulation, 212
global gene expression across, 212–215
See also Perennial life
Canadian forestry, focus on conifers, 6
Candidate gene approach, 102
Canopy establishment, influence of competition, 45–46
Carbon neutral renewable energy source, 40
Cartagena Protocol on Biodiversity, 12
Castanea dentata, 249
CatFisher, 142
CBF genes, 187
CBL-INTERACTING PROTEIN KINASE 10 (CIPK10), 239
CDNA arrays, 141
Cellular defences, activation, 228–229
Cellulose macrofibrils, 208
CENTRORADIALIS (CEN) gene, 160
CENTRORADIALIS-LIKE1 (CENL1) gene, 177–178
Chaetomium spirale, 265
Chalcone isomerase (CHI), 291
Chalcone synthase (CHS), 291
Chilling, 191
Chilling-induced removal, of callose, 188
China, controlled breeding techniques of *Populus,* 314
Cinnamomum zeylanicum, 248
Cinnamoyl dehydrogenase (CAD), 293
Cinnmoyl-CoA reductase (CCR), 293
Classifications, *Populus,* 18, 21–22
and inconsistencies, 21–22
CLAVATA1-3 (CLV1-3)-WUSCHEL (WUS) system, 172
Clonal selection, for improvement, 45
Clone number 383-2499, 69
Clonostachys rosea, 264
CLOVER, 139
CLV3 gene, 174
CMap resources, 140
Coalescent theory, 98
Commercial purposes usage, 40
Complementation, 113
Complex traits dissection
association mapping and, 102–103
incidence of segregation distortion, 101–102
linkage mapping and genome dynamics, 100–102
Conifers, 4
Conservation genomics, 347–349
genetic diversity conservation, 350–351
hybridization and genetic conservation, 351–353
population genomics for, 353–357
Conservation programmes for *Populus* sp., considerations, 353
CONSTANS (CO) gene, 177
CONSTANS/FLOWERING LOCUS T (CO/FT) gene, 159
Coordinated Agricultural Product (CAP), 330
Coppicing, impact on growth, 44
Cortinarius sp., 258
Cottonwoods, *see Populus angustifolia*
CTAB-based protocol, 70
Cyclin A2 (CYCA2), 214
Cytokinins, 55, 123
in wood formation, 219
See also Wood
Cytoskeleton, role, 238

D
DATFAP, annotation-based approach to identify TFs, 139
Defense, in *Populus*
mutualisms, 250–255
endophyte association, 262–263
evolutionary history and fossil record, 265–268
fungal symbionts, 264–265
mycorrhizal fungi association, 255–261
plant symbiont communities, 261–264

Defense, in *Populus* (*cont.*)
proteins and genomics impact, 288–289
defense-related genes discovery, 285–286
gene expression patterns and defense responses, 286–288
and secondary metabolism, 280–284
application of genomic tools, 285–289
biosynthetic pathways, to phenolic compounds, 290
functional analysis of genes, 294–296
future perspectives, 298–299
importance of variation, 296–298
and phenylpropanoid, genomic analysis, 289–294
variations and secondary metabolite profiles, 296–298
Dehydrins, in overwintering, 186
See also Freezing tolerance, in *Populus*
DELLA proteins, role, 180
2,4-Diacetylphloroglucinol (DAPG), 253
Dicer, 117
Dihydroflavonol reductase (DFR), 291
Diploidization process, 76
Diploid species, nucleotide polymorphism, 92
DIURNAL database, 139
Diurnal project, 139
DNA-based vectors, microprojectile bombardment use, 121
DNA extraction protocol, 70
Domestication, 46, 307
Dominant negative mutations, 117
Dormancy
in cambium, 181–182
cycles, 135, 175
in Aspen, 176
feature, 176
definition, 174–175
genetic control, 103–106
locus, 175–176
release
cell biology and biochemistry, 188–190
chilling and vernalization, 191
gibberellins, 190–191
phenology, 187–188
in SAM, 180–181
See also Perennial life
Dormancy sphincter complexes (DSC), 180
DP-E2F-like *(DEL)* gene, 214
Drought stress avoidance, abscission, 232–233
by seasonal drought stress, 235–239
stress induced abscission, 234–235
See also Populus

E

Eco-dormancy, definition, 175
Ecogenomics model plant, *Populus* as, 280
Eco-TILLING approach, 67
Ectomycorrhizal species, associated with *Populus,* 258
Ectomycorrhizal spp. and *Populus,* association, 258
Ectomycorrhizal symbiosis, 259–260
Ectopic approaches, 117
Ectopic gene expression, 113
Ectopic overexpression, in *Populus,* 160
Effective population size, concept, 92
EFP browser, 142–143
ELEMENT database, 139
Elite cottonwood hybrids, 157
Endoconidioma populi, 263
Endo-dormancy, definition, 175
Endophyte and *Populus spp.,* association, 262–263
Endoplasmic reticulum (ER), role, 189
Enhancer and gene traps, 124
Enterobacter sp. 638, 8
Epichloë festucae, 250
Epigenetic mechanisms, role, 158
Escherichia coli, as model prokaryotic microbe, 3
EST databases, *Populus*
launch, 6
types, 137–139
Ethylene
inhibitory substances, 55
in wood formation, 220
See also Wood
Eucalyptus
breeding programs, 307
genome, 68
species, 4, 5
Eudarluca caricis, 252
Eudicot genome sequence, 68
EUGENE, 8
EuGène, 81
Euphrates poplar, *see Populus euphratica*
European black poplar, *see Populus nigra*
European FACE experiment, 44
Exogenous application, of plant hormones, 233
Expressed Sequence Tag (EST), *Populus,* 137, 356

F

FD ortholog *PtFD1* overexpression, 159–160
Festuca arundinacea, 253
FgenesH, 8

FgenesH Genewise, 81
Fine roots, defined, 44
Fingerprinted contigs (FPC), 72
Flacourtiaceae family, 20
Flavanone 3 hydroxylase (F3H), 291
Flavonoid *O*-methyltransferases (FOMTs), 291
Flavonol synthase (FLS), 291
Floral meristems, in *P. trichocarpa* inflorescence, 161
Flowering
  acropetal order, 163
  development and sex determination, 162–166
  E class genes study, 164
  initiation, 156–162
  See also Reproductive development, in *Populus*
FLOWERING LOCUS C (FLC) gene, 159, 191
FLOWERING LOCUS D (FD) gene, 159
FLOWERING LOCUS T (FT) gene, 177
Forest management and restoration ecology
  conservation genetics, 357–359
  See also Conservation genomics
Forest tent caterpillar, *see Malacasoma disstria*
Forest tree genomics projects, 6
Fossil records, *Populus,* 20
Four-fold degenerate nucleotide sites (4DTV), 76
Free Air Carbon Dioxide Enrichment (FACE), 258
Freezing tolerance, in *Populus*
  dehydrins and proteins, 186
  environmental regulation, 183–184
  gene expression regulation, 185
  osmotic adjustments and dehydration, 184–185
  transcriptional regulation, 186–187
Fremont cottonwood, *see Populus fremontii*
FRIGIDA (FRI) gene, 191
FRUITFULL (FUL) gene, 159
FT and *LEAFY* genes, flowering, 127
FT overexpression, 159
Full-length (FL) cDNA information, 73
Fungal symbionts, of *Populus* spp., 264–265

G
Gain-of-function mutations, 123
Gateway cloning technology, 115
Genecology, *Populus,* 308
  species distribution, 308–313
Gene content and genomics, 81–82
  duplicate genes retention, 82–83
  prediction methods, 81–82
  synteny with other species, 83
Gene-directed transgenesis, 113
Gene discovery
  gene tagging in, 209–210
  microarray profiling of gene expression, 211
Gene expression regulation, in freezing tolerance, 185
  See also Perennial life
Gene function determination, transgenics approach, 210–211
Gene Ontology (GO)
  database, 142
  hierarchy, 138
General combining ability (GCA), 319
General hybridizing ability (GHA), 319
Generalized mutagenesis, 113
Gene redundancy, 117
Gene silencing, 113
Gene tagging, 114
Genetic and quantitative trait loci (QTLs) studies, 47
Genetic diversity, 91–92
  conservation, 350–351
  See also Conservation genomics
Genetic improvement of *Populus,* economic analyses, 318
Genetic redundancy, 114
Genetic variation, of *Populus,* 309–313
Gene trap tagging, in gene discovery, 209–210
Genome Canada, 6
Genome mining, in *Populus* defense and secondary metabolism, 294–295
Genome sequence and shotgun results
  BAC based MAP, 71–73
  genotype selection, 69–70
  map-based assembly, 74–75
    peritelomeric portion of LG_XIX, 74–75
    scaffolds, 74–75
  sequence assembly, 70–71
  template preparation and contamination, 70–71
  See also Populus trichocarpa genome project
Genome sequence, *Populus,* 155
  sequencing efforts, 68
    sequencing strategy, 69
    use EST and full-length (FL) cDNA information, 73
  significance, 67–68

Genome structure, 76–77
comparison with grape genomes, 77–78
repetitive portions and comparison with other genomes, 79–81
Geographic distribution, of *Populus,* 308–309
GEO repository, 142
Gibberellins (GA), 55, 156, 190–191
alteration, 56
chilling releasing effects, 190–191
in *Populus*
stem, 179–180
wood formation, 216–218
role in wood formation, 218–219
Global gene expression patterns, in *Populus* defense response, 286–288
Global transcriptome analysis, 234
Glomus intraradices, 253, 255
Glomus mosseae, 251
Glucan-synthase gene, upregulation, 181
Gold standard method, for gene function study, 113
GoSLim gene ontology categories, 82
GRAIL, 8
GrailExp6, 81
GRAMENE, provide BioMart service, 137
GRAMENE resource, 136, 139
Grapevine, *see Vitis vinifera*
GreenWood Resources' *P.* ×*generosa* breeding program, 318
Growth, 56–57
and allocation patterns, 46–47
average above-ground biomass yields, 42–43
biomass yields, higher in coppiced *Populus,* 44
canopy establishment, influence of competition, 45–46
clonal selection, for improvement, 45
coppicing impact on, 44
hybridization, 22
interspecific, for improvement, 45
objectives of, 45
hybrid vigor or heterosis, 45–46
relationships between yield *vs* tree dimensions/canopy/ leaf traits, 47
roots, 44–45
species variety illustration, different ages/stages of development, 39
weed control
and *Populus* plantations, 41
risks from diseases and pests, 44
weed control and *Populus* plantations, 41
See also Reproductive development, in *Populus*
Gymnosperms, and experimental disadvantages, 4
Gypsy moth, *see Lymantria dispar*

H

Habitat, *Populus,* 16–18
Haldane's rule, 165, 166
Hardy-Weinberg equilibrium, 23, 26
Hebeloma crustuliniforme, 259
Hebeloma mesophaeum, 258
Herbivory and defense induction, in *Populus,* 283–284
See also Defense, in *Populus*
High-density *Populus* plantations, in different parts of world, 41
HISTONE1-3 (HIS1-3), 236
Homologous recombination (HR), 120
Hybridization, 22
and genetic conservation, of *Populus,* 351–353
interspecific, for improvement, 45
objectives of, 45
See also Conservation genomics
Hybrid poplars and aspen, difference, 204–205
Hybrid *Populus* clones, drought-response characteristics, 54
Hybrid vigor or heterosis, 45–46
Hydraulic architecture adaption, to drought, 230–232
Hydropisphaera fungicola, 263
Hydroxycinnamate derivatives (HCDs), 282
Hydroxycinnamoyl transferase (HCT), 293
Hypoxylon mammatum, 318, 328

I

Induced systemic resistance (ISR), 251
Inducible expression, in poplar, 126
Inocybe lacera, 258
Insertional mutagenesis, 113, 123
Internal transcribed spacer (ITS), 258
Italian *P.* ×*canadensis* program, 318

J

JAZZ assembler, 70
J. Craig Venter Institute (formerly the Institute for Genome Research, TIGR), 138
JGI gene model ID, 141
JGI genome browser, 140
JGI Populus gene models, 139
Joint Genomes Initiative (JGI) web resource, 136

K
KANADI gene, 215
Knock-out or knock-down manipulation, genes, 113
KNOX gene, 179
Knufia endospora, 263
KTI gene, 288–289
Kunitz trypsin inhibitor (KTI), 284

L
Laccaria amethystea, 258
Laccaria bicolor, 252, 255
Lactarius sp., 258
Larix, 4
Late embryogenesis abundant (LEA), 239
Leaf abscission, 233, 235–236, 239
  See also Abscission
Leaf rust, by *Melampsora* spp., 41
Leaf spot, by *Marssonina* spp., 41
Leaf-to-apex signalling
  in dormancy development, 173
  role, 178–179
  See also Perennial life
Leaf traits, and productivity, 50–51
LEAFY (LFY) gene, 160
Leucine-rich repeat (LRR), 251
Life history, *Populus,* 18–20
  asexual reproduction, 19–20
  flowering and fertilization, 19
  site-specific biases in sex ratio, 19
  See also Reproductive development, in *Populus*
Lignin biosynthetic pathway, 117
Lignocellulosic cell wall biogenesis, 135
Linkage disequilibrium (LD), 71, 95–97
  BAC contigs showed co-localization, 96
Linkage disequilibrium mapping, 102
Linkage groups, genes comparison, with significant alignments, 77
Linkage maping, 100–102
Lipid bodies (LBs), 189–190
Long Interspersed Nuclear Elements (LINEs), 80
Long Terminal Repeat (LTR) Gypsy, 80
LTCOR12 gene, 229
Lymantria dispar, 282, 295

M
MADS box transcription factors, 85
Maize
  RNA silencing pathways, 157–158
  temporal signal, 158
Malacasoma disstria, 283
Management, *Populus,* 308
Map assembly, 72
Map-based assembly of *Populus* genome, portion representation, 75
Map co-linearity, species of *Populus,* 100
Mapping populations, in *Populus,* 100–101
Marker-assisted breeding (MAB), 335
Marker-assisted selection (MAS) programs, in *Populus* sp., 335
Marker-trait associations, discovery, 330–331
Marssonina brunnea, 324
“Maturation effects,” 5
Medicago truncatula, for symbiotic nitrogen fixation, 3
Melampsora larici-populina, 48, 265
Melampsora medusae, 287
Melampsora spp., 324
Meristems, in *Populus,* 172–174
  See also Perennial life
Meselson effect, 23
Metabolomics, in *Populus,* 143–144
Microarray platforms, for *Populus,* 141–142
Microarray profiling of gene expression, in gene discovery, 211
Microsatellites, 91
  availability, *populus,* 26
  diversity and differentiation in, 27–29
MiPlantBrowser, 140
MiRBase, 140
MiRNA databases, 140
MiRNA precursors, designed, 118
MiRNAs biogenesis, genes mutations, 158
Model Organism Database (MOD), 147
Model or reference organisms, defined
  criteria for selection of, 3
Modulus of elasticity (MOE), 205
Modulus of rupture (MOR), 205
Molecular biology, *Populus,* 68
Molecular clock hypothesis, 100
Monoclonal plantations, 41, 44
Morphology, *Populus,* 15–16
Motif finding tools, 139
M. populi BJ001, 8
Multiple gene knock-downs, transgenic approaches, 114–115
Muscodor albus, 248, 264
Mutator-Like Elements (MULEs), 80
MYB134 transcription factor, role, 296
Mycorrhizal fungal diversity, 249
Mycorrhizal fungi, 255
  ectomycorrhizal symbiosis and impact on root morphology, 256
  history and fossil record, 265–268

Mycorrhizal fungi (*cont.*)
mycorrhiza-induced secreted proteins, model, 257
Mycorrhizas, in plantation forestry, 258–259
Myorrhiza-induced, cysteine-rich, small, secreted proteins (MISSPs), 252

N
"Narrow-leaved, thin-twigged" species, 21
Native species, 39
Natural genetic diversity, clones/species/hybrids, 39
NBS-LLR resistance genes, 97
NCBI Gene Expression Omnibus (GEO) repository, 142
NCBI, official repository for ESTs, 137
NCBI *Populus* Genome Viewer, 136
NCBI *Populus trichocarpa* genome viewer, 138
NEWHYBRIDS program, 357
See also Conservation genomics
Nicotiana benthamiana, 121, 295
NimbleGen platforms, 141
Nisqually-1, 7
clone number 383-2499, 69
genotype, ironies with, 89–70
haploid genotypes, 71
Nisqually River, 7
NIST mass spectral library, 143
Non-homologous end joining (NHEJ), 120
North-American cottonwoods, 20
North-American plains cottonwood, *see Populus deltoides*
North-American quaking aspen, *see Populus tremuloides*
Nuclear transformation, using organogenic systems, 121
Nucleotide-binding site/leucine rich repeat (NBS-LRR), 356
Nucleotide binding site (NBS), 251
Nucleotide diversity, in *Populus,* 29
Nucleotide polymorphism, 92–95
reductions in population size, 95
surveys, 94

O
Oleosins, role, 189
Oligo arrays platforms, 141
Orgyia leucostigma, 295
Oriented strand board (OSB), 206
OrthologID database, 139
Ortholog predictions, 139–140
Orthomap database, 139
Oryza sativa, for monocot biology, 3
Osmotic adjustments, in freezing tolerance, 184–185
See also Perennial life
Overwintering initiation, in *Populus,* 171–172
See also Freezing tolerance, in *Populus;* Perennial life

P
Paper birch, *see Betula papyrifera* Marsh.
Para-dormancy, definition, 175
Pedigrees, sex determination locus, 166
Perenniality, defined, 171
Perennial life
dormancy, 174–175
locus, 175–176
freezing tolerance
dehydrins and protective proteins, 186
environmental regulation, 183–184
gene expression regulation, 185
osmotic adjustments and dehydration, 184–185
transcriptional regulation, 186–187
meristems, 172–174
photoperiod induced overwintering, 177–183
release from dormancy
cell biology and biochemistry, 188–190
chilling and vernalization, 191
gibberellins, 190–191
phenology, 187–188
seasonal growth, 171–172
PERPETUAL FLOWERING1 (PEP1) gene, 162
Phenolic secondary metabolites, 281–283
Phenology, refers, 52–53, 187
See also Dormancy
Phenylpropanoid-derived phenolics, 281
Phenylpropanoid metabolism, 68
Phialocephala fortinii, 255
Phosphatidylethanolamine-binding protein (PEBP), 159
Photoperiod, overwintering initiation, 171, 177
apex events, 178–179
dormancy at SAM and cambium, 180–182
GA levels in stem, 179–180
leaves and stem events, 182–183
sensing, by leaves, 177–178
PhyB2 gene, 101–102
PhyB2 SNPs, 102
Physcomitrella patens, 84
Physcomitrella patens genome, 68
Physiological growth factors, 49
PHYTOCHROME A (PHYA) gene, 182

Phytochromes (PHY), 177
Phytohormone abscisic acid (ABA)
  inhibitory substances, 55
  roles to regulate plant growth, 56
  transgenic strategies, 56
Phytoremediation, 259
Picea, 4
PICKLE (PKL) gene, 179
Piloderma sp., 258
Pinus, 4
Pinus breeding programs, 307
Piriformospora indica, 250–251
Pisolithus microcarpus, 266
Plant-fungal symbiosis, 248–250
Plant Genome Database project, 138
Plant Genome Database's *Populus* Genome Browser, 136
Plant repetitive elements, 80
Plant reproductive success, mechanisms, 156
Plants distributions, determinants, 39
Plant Transcript Assemblies, 138
PlantTribes database, 139
Polymorphism and heterozygosity, in *Populus,* 23
PopARRAY, 140
PopArray database, 139, 141
PopCEN1-RNAi transgenics, 160
  inflorescences in, 166
PopGenIE, provide BioMart service, 137
PopGenIE resource, 143
  GO TermFinder tool at, 142
  tool for InterPro domains, 142
  tool for KEGG or KOG categories identification, 142
Poplar Gene Index (PplGI), 138
Poplar Genome Steering Committee, 6
Poplar mosaic virus *(PopMV),* 120–121
Poplars, *see Populus*
Population genomics, for *Populus* conservation, 353–357
  See also Conservation genomics
Population structure, low levels in *Populus,* 92
Populus alba, 158
Populus angustifolia, 20, 21, 157
Populus balsamifera, 20, 45, 55
Populus, as model tree, 3, 31
  accepted by committee in 2002, 7
  advantages, 4–5
  breeding and technology, 12–13
  characteristics, 205
  classes of phenylpropanoid-derived phenolics in, 281
  classifications, 18, 21–22
  consensus cladogram, 18, 21–22
class of phenolic secondary metabolites, 281
cryopreservation-based germplasm, 145
databases, 138
diverse habitats, 16–18
as "Ecogenomics" model plant, 280
ectomycorrhizas, transcript profiling, 259–260
efforts in early 1990s, 6
EST resource, in Sweden, 6
EST sequencing, performed on *P. tremula,* 6
events responsible for selection, 5
experiments to profile gene expression and results, 135–136
fossil records of, 20
future research, 12–13
gene model choice, 8–9
genetics, 68
genetic variations
  adaptive genetic variation, 29–30
  deviations caused by heterozygote deficiency, 23
  markers and RFLP, diversity and differentiation, 22–26
  nucleotide diversity, 29
  occurrence of triploids, 26
  polymorphism and heterozygosity, 23
  tri-nucleotide microsatellites, 29
  variable microsatellites, diversity and differentiation, 26–29
genome browser, 136
genome program, 6
genome sequencing, female clone *Nisqually-1*
  ab initio gene prediction algorithms, 8
genomics resources, 135–136
  databases and browsers, 136–137
  EST collections and databases, 137–139
  germplasm contacts, 146
  miRNAs databases, 140
  ortholog predictions, 139–140
  promoter motif analysis, 139
  QTL databases and browsers, 140
  resequenced by JGI, 11
  sequencing, female clone *Nisqually-1,* 8, 136
  transcription factors (TFs), databases, 139
germplasm, 144–145
herbivory and defense induction, 283–284

Populus, as model tree (*cont.*)
hybridization role, naturally occurring hybrids, 22
hybrid used for transformation (T89), 6
hydroxycinnamate derivatives (HCDs), 282
"Jamboree set" of genes, 10
LC-MS-based approaches, 144
life history, reproduction, 18–20
mapping populations, derived from interspecific intersectional crosses, 101
metabolomics, 143–144
microarrays use, 135
morphology, 15–16
neutral genetic markers, differentiation among populations, 30
Nisqually-1 planting, 10
per generation mutation, 92
proteomics, 143–144
repeated, functional genes, 114
role in riparian ecosystems, 135
scientific interest in, 10
secondary metabolites of *Populus,* effects on herbivores, 280–283
sequencing, 143
similarity with orthologous *Arabidopsis* genes, 8
sister lineage, *Salix* (willows) relationships, 20–21
transcriptomics studies, 135, 141–143
Populus association genetics projects, 332
Populus BAC library, 69
Populus balsamifera, 206, 209
Populus deltoides, 20, 45, 51, 158, 209, 307
Populus euphratica, 16, 358
Populus fremontii, 20
Populus Genome Annotation Jamboree in Walnut Creek, 10
Populus Integrative Genome Browser, 136
Populus lasiocarpa, 16
Populus LFY (PTLF) gene, 160
Populus maximowiczii, 258
Populus mexicana, 21
Populus nigra, 20, 45, 307
Populus simonii, 21
Populus-specific public database (UPSC-BASE), 142
Populus tomentosa, 228, 265
Populus tremula, 6, 250, 262
Populus tremuloides, 6, 17, 20*,* 262
Populus trichocarpa (black cottonwood), 6, 15, 20, 29, 45, 51, 55, 216, 230
Populus trichocarpa genome project, 67
and Arabidopsis as representative eudicots, 68
association genetics and QTL mapping, 67
Auxin Response Factor (AFR) transcription factors, 83
characterization of repeat composition, 79
diversification of genes encoding enzymes, 84
gene content, and prediction methods for
homology-based, and EST-based methods, 81
genes encoding transporters, comparison, 85
genome sequencing efforts and strategy, 68, 69
genome shotgun results, 69, 71–75
BAC-based physical map, 71–73
genotype selection, *Populus,* 69–70
map-based assembly, 74–75
sequence assembly, 70–71
sequencing strategy, 69
sequencing template preparation and contamination, 70–71
genome structure, 76–77
grape genomes comparison with, 77–78
LG_II genome assembly, show overlap BACFPC map to same site, 72
LINEs in, 80
Nisqually-1 sequence, as reference, 67
data for SNP discovery, 67
pedigrees for, 69
phenylpropanoid enzymes, 84
repeat composition, and sequenced genomes comparison, 79–81
retention of duplicated genes, 82–83
sequenced genomes, and gene family composition comparison, 84–85
synteny with other species, 83
TBLASTN searches, 79
transformation systems, 68
transposable elements in, 80
See also Populus
Populus varietal evaluation, statistical techniques, 324
*Populu*s x *euamericana,* 234
Positional cloning of genes, lack of progress, 100
Posttranscriptional gene silencing (PTGS), 117
Potato virus X (PVX), 295
P. putida W619, 8
Productivity, determinants, 48, 56–57
abiotic and biotic factors role, 49–50
branch orientation role, 52

gas exchange role, 48–49
leaf area, size and traits role, 50–51
light interception and CO_2 assimilation, 48
phenology, 52–53
phytohormone ABA role, 55–56
relationship between photosynthesis and biomass yield, 49
role of branching pattern, 51–52
stomata role, 54–55
water relations and, 53–54
See also Growth
Proleptic branches, 51
Protease inhibitors, 284
Proteomic approaches, in *Populus* wood formation study, 211–212
Proteomics and metabolomics developed, for *Populus,* 6
Protoplast transformation, 121
Pseudomonas fluorescens, 253
PTFD, 139
PtFLC2 expression, 162
PttACO1 gene, 220
Putative PD-associated protein (PPAP), 189

Q
QTL tracks, 11
Quantitative genetic approaches, in *Populus* wood formation study, 208–209
Quantitative Trait Loci (QTL), 208, 331
*Q*uantitative *T*rait *N*ucleotides (QTNs), in *Populus,* 102
Quantitative transmission disequilibrium test (QTDT), 334–335

R
Radial growth response to drought stress, 229–230
Radial growth response, to drought stress
Raf Kinase Inhibitor Protein (RKIP), 159
Reciprocal recurrent selection (RRS), 315
Recombination-based cloning systems, 115
RECON program, 80
Regression coefficients, 48
RepeatMasker library, 80
Reporter gene, 124
Repressor of *ga1-3* (RGA), 180
Reproductive biology, of *Populus,* 313–315
Reproductive development, in *Populus*
AG co-orthologs, *PTAG1* and *PTAG2,* 165
AP3 homolog, 165
epigenetic mechanisms, role, 158
FLC homologs, 159
flowering
induction and organogenesis, 156
initiation, 156–157
pathway genes, 159
gene expression patterns, 165
gibberellins (GA), 155
juvenility/maturity, 157–160
maize
RNA silencing pathways, 157–158
temporal signal, 158
multiple aspects, 155
paclobutrazol role, 158
plant reproductive success, mechanisms, 156
RNA silencing pathways, 157–158
SEP1/2/3 homologs, 165
sex determination and flower development, 162–166
temporal signal, 158
transitions to reproductive phase, 160–162
vegetative phase change, in plants, 156
Re-sequencing data, usage, 143
Responses to abiotic stress, 225–226
Restriction Fragment Length Polymorphisms (RFLP), 23
Rib meristem (RM), 173
Riparian species, phreatophytic, 16
RNA induced silencing complex (RISC), 117
RNA interference (RNAi), 211
RNAi technologies, 120, 123
Rol gene cassette, 125
Root systems, role in growth, 44–45
Royal Institute of Technology, KTH, 6
Russula sp., 258

S
Salix caprea, 253
Salix hultenii var. *angustifolia,* 258
Salix sachalinensis, 258
Salix (willows), relation with *Populus,* 4, 20–21
Scleroderma bovista, 258
Secondary metabolites of *Populus,* effects on herbivores
SELF PRUNING (SP) gene, 178
SEPALLATA (SEP) genes, 164
SEP1/2/3 homologs, expression, 165
Septoria musiva, 328
Sequence capture technology, 143
Sequence divergence patterns, 97–100
phylogenetic trees, relationship between species, 99
probability of congruence and incongruence, 98, 100
at synonymous sites, 98

Sequencing technologies, 140
  for understanding methylation role, 143
Sex determination, in *Populus,* 162–166
Shoot apical meristem (SAM), 172
Shoot apical meristem (SAM), dormancy, 180–181
SHOOTMERISTEMLESS (STM), 213
Short-day (SD), 159
  dehydrins accumulation, 186
  induced acclimation, 183
  induced gene expression, 185
  osmotic adjustments, 184–185
Short interfering RNAs (siRNAs), 117
Shotgun sequencing
  assembly, 70
  " mega-genome," 7–8
Simple sequence repeat (SSR) markers, 74
Single nucleotide polymorphisms (SNPs), 297, 333–334
Single Sequence Repeats (SSRs), 91
SiRNAs biogenesis, genes mutations, 158
S. maltophilia, 8
Smith-Waterman alignments, 76
SNP polymorphisms, 71
Soil organic matter (SOM), 258
Solexa short-read platform, 11
Species variety illustration, different ages/stages of development, 39
Specific leaf area (SLA), 51
Splice acceptor sequence (SA), 124
S. proteamaculans, 8
SQUAMOSA PROMOTER BINDING PROTEINLIKE (SPB) gene, 158
SSR markers, 91
Stem canker, by *Septoria* spp., 41
Stomata, role in CO_2 uptake and water relations, 54–55
Sulfate Permease (SulP) family, 85
SUPERFAMILY database, 139
Swedish University of Agricultural Sciences in Umeå, 6
Sylleptic branches
  difference between proleptic and, 52
  plasticity of, 51
  role in growth, 51
Symplasmic fields (SFs), 172
Synteny, 83
Systemic acquired resistance (SAR), 251

T
Tacamahaca, 21, 45
TBLASTN searches, 79
T-DNA tagging, 114
TERMINAL FLOWER1(TFL1) gene, 160, 177
The Institute for Genomic Research, 80
Thelephoraceae spp., 258
Thelephora terrestris, 258
Theobroma cacao, 261
Thidiazuron (TDZ), 123
TIGR Plant Transcript Assemblies, 140
Tissue patterning, regulation, 215–216
  See also Wood
Tobacco rattle virus (TRV) vector, 120
TOUCAN, 139
Transcriptional gene silencing (TGS), small RNAs induce, 119
Transcriptional profiling, usage, 228, 294–295
Transcription factor families, 85
Transcript profiling, of *Populus* ectomycorrhizas, 259
Transformation, for genetic analysis, 113–114
  callus induction step, 123
  co-cultivation conditions role, 123
  factors affecting efficiency, 123
  importance of, 114
  knock-down gene modifications generation, approaches
    micro RNAs (miRNAs) and siRNAs overexpression, 118–119
    Virus Induced Gene Silencing (VIGS), 120–121
    Zinc-Finger Nucleases (ZFN), 119–120
  morphological abnormalities, 116
  obstacles to, 125–127
  in poplar
    with *Agrobacterium,* procedure and methods, 115–117, 121–123
    protocols, in vitro shoot organogenesis, 123
  postgenomic challenges, 115
  role in mitigating obstacles in experiments, 114
  transgenic modifications approaches, 117–118
  use for mutagenesis, 123–124
  See also Populus
Transgenic approaches, in *Populus* wood formation study, 209–211
Transgenic loss-of-function gene modification, 117, 118
Transgenic modification, of trees, 56
  approaches, 117–118
Translational genomics, definition, 329–330
Transmission disequilibrium test (TDT), 334
Tree breeding, in *Populus* wood formation study, 208–209

TreeGenes database, 140
Treenomix (University of British Columbia, Vancouver), 6
Trembling aspen (*Populus tremuloides* Michx.), 259
Trichocladium canadense, 263
Trichoderma harzianum, 253
Trichoderma virens, 252
Tuber sp., 258
Turanga, 21
Ty3/Gypsy elements, 80
Typical balsam poplars
  P. balsamifera, 21
  P. trichocarpa, 21

U
Umeå University, 6
University of Minnesota's Aspen-Larch Cooperative's *P. ×wettsteinii* program, 318
U.S. Forest Service-Industrial *P. deltoides* program, 318
Utility, *Populus,* 204–207

V
Vascular cambium, role, 174, 203
Vegetative phase change, in plants, 156
Vegetative storage protein (VSP), 284
Venturia populina, 265
VERANALIZATION (VERN1) gene, 191
Vernalization, 191
VERNALIZATION INSENSITIVE 3 (VIN3) gene, 191
VIGS vectors, 120
Virus Induced Gene Silencing (VIGS), 120–121
Vitis vinifea, 68, 292

W
Water
  deficit tolerance, definition, 230
  requirements and growth, 53–54, 57
  shortage, cellular consequences, 226
  See also Abiotic stress responses
Weed control
  and *Populus* plantations, 41
  risks from diseases and pests, 44
Wetlands, 16
White poplars, 16
Willow*, see Salix*
Wine grape *(Vitus)* genome, compared with *Populus,* 77–78
Winner's curse, 102
Wood, 201
  chemical composition, 203
  features, 201
  formation, 201–202, 231
    auxin role, 216–218
    development, 202–204
    environmental affects, 207–208
    and genetic regulation, 212–216
    gibberellin (GA) role, 218–219
    hormonal regulation, 216–221
    study, genetic and genomic approaches, 208–212
  role, 201
  tissue patterning, regulation, 215–216
  uses, 204–207
"Word" length, 70
Wright-Fisher model, 92–93
WU-BLASTN, 71
WU-BLAST option, 80
WUSCHEL (WUS) gene, 213

Y
YABBY gene, 215

Z
Zeatin, 123
Zinc-Finger Nucleases (ZFN), 119–120