agronomy

Genetics and Breeding for Productivity Traits in Forage and Bioenergy Grasses

Edited by
John W. Forster and Kevin F. Smith

Printed Edition of the Special Issue Published in *Agronomy*

www.mdpi.com/journal/agronomy

MDPI

Genetics and Breeding for Productivity Traits in Forage and Bioenergy Grasses

Special Issue Editors
John W. Forster
Kevin F. Smith

MDPI • Basel • Beijing • Wuhan • Barcelona • Belgrade

Special Issue Editors
John W. Forster
The Centre for AgriBioscience
Australia

Kevin F. Smith
The University of Melbourne
Australia

Editorial Office
MDPI AG
St. Alban-Anlage 66
Basel, Switzerland

This edition is a reprint of the Special Issue published online in the open access journal *Agronomy* (ISSN 2073-4395) from 2016–2017 (available at: http://www.mdpi.com/journal/agronomy/special_issues/forage_grasses_genetics).

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Author 1; Author 2. Article title. *Journal Name* **Year**, *Article number*, page range.

First Edition 2017

ISBN 978-3-03842-542-7 (Pbk)
ISBN 978-3-03842-543-4 (PDF)

Table of Contents

About the Special Issue Editors

John W. Forster is currently Leader and Principal Research Scientist in Molecular Genetics for Agriculture Victoria Research and Professor of Molecular Genetics at La Trobe University in Melbourne, Victoria, Australia. He is a graduate of the University of Cambridge, UK and completed a Ph.D. in microbial genetics at the University of Liverpool, UK before teaching and researching for 14 years at Aberystwyth University, UK. At Agriculture Victoria's Centre for AgriBiosciences (AgriBio) on La Trobe University's Bundoora Campus, he leads a group of 30 staff and students who apply DNA sequencing and genotyping technologies for improvement of a broad range of domestic animals (e.g., dairy cattle), agricultural microbes (e.g., fungal endophytes) and crop species (e.g., pasture grasses, cereals, oilseeds, pulses and horticultural crops), using state-of-the-art equipment platforms. His personal research work has focused on developing and implementing genomics-assisted tools for breeding improvement of forage grasses and legumes (including the pasture grass-endophyte combination). This has involved the development of DNA-based selection tools, identification of genomic regions controlling key agricultural characteristics, and development of novel strategies for use in breeding programs.

Preface to "Genetics and Breeding for Productivity Traits in Forage and Bioenergy Grasses"

Forage crops provide the major components of pasture-based livestock production systems in temperate regions, and are also important for feeding of animals in developing countries of the tropics and sub-tropics. Productivity traits for forage crops include biomass, seasonality of yield, vegetative persistence, influence of flowering time, digestibility and nutritive quality. Traditionally, vegetative biomass yield has been the major breeding objective, but nutritive quality traits (such as content of sugars, proteins and lipids) have become increasingly important. Accurate measurement of productivity characters has been difficult, limiting genetic gain in traditional breeding. The increasing availability of genomic and phenomic data for a range of forage crop species now allows characterisation and selection at the individual plant level.

In parallel, a number of grass species (such as switchgrass and miscanthus) have been developed for bioenergy purposes, and productivity characters, including biomass delivery and biochemical content, are also critical for improvement of these crops. While some of these traits are shared priorities with forage grasses, others are of particular interest for bioenergy production. This increased specialisation of grass cultivars creates both challenges and opportunities for grass breeders.

The aim of the Special Issue on Genetics and Breeding for Productivity Traits in Forage Crops was to provide a forum for contemporary studies of the genetics, genomics and phenomics of productivity traits in forage and bioenergy grasses, along with the application of such data to breeding practices and cultivar development. A total of 15 papers have been published, including review and original research articles and are here collated into a single volume. Aspects of both transgenic- and sequence polymorphism-based approaches to crop improvement are included, along with classical breeding methods (such as doubled haploid production). The range of target species described in these articles includes perennial and Italian ryegrass, red clover, alfalfa, brachiaria, napier grass. Collectively these studies demonstrate that forage crops, which have historically lagged behind many major row crops in terms of development and implementation of molecular breeding systems and strategies, are rapidly benefitting from the implementation of such technologies.

John W. Forster and Kevin F. Smith

Special Issue Editors

agronomy

MDPI

Article

Re-Programming Photosynthetic Cells of Perennial Ryegrass (*Lolium perenne* L) for Fructan Biosynthesis through Transgenic Expression of Fructan Biosynthetic Genes under the Control of Photosynthetic Promoters

Stephen Panter [1], Aidyn Mouradov [1,2], Pieter Badenhorst [3], Luciano Martelotto [1,4], Megan Griffith [1], Kevin F. Smith [5] and German Spangenberg [1,6,*]

1 Agriculture Victoria Research, AgriBio, The Centre for AgriBiosciences, Bundoora, Melbourne, Victoria 3083, Australia; stephen.panter@ecodev.vic.gov.au (S.P.); aidyn.mouradov@rmith.edu.au (A.M.); luciano.martelotto@ecodev.vic.gov.au (L.M.); megan.griffith@ecodev.vic.gov.au (M.G.)
2 RMIT University, Bundoora, Melbourne, Victoria 3083, Australia
3 Agriculture Victoria Research, Hamilton, Victoria 3300, Australia; pieter.badenhorst@ecodev.vic.gov.au
4 Monash University, Clayton, Melbourne, Victoria 3168, Australia
5 The University of Melbourne, Faculty of Veterinary and Agricultural Sciences, Hamilton, Victoria 3300, Australia; kfsmith@unimelb.edu.au
6 School of Applied Systems Biology, La Trobe University, AgriBio, The Centre for AgriBiosciences, Bundoora, Melbourne, Victoria 3083, Australia
* Correspondence: german.spangenberg@ecodev.vic.gov.au; Tel.: +61-03-9032-7165

Academic Editor: Peter Langridge
Received: 16 February 2017; Accepted: 10 May 2017; Published: 25 May 2017

Abstract: High molecular weight fructans are the main class of water-soluble carbohydrate used for energy storage in many temperate grass species including perennial ryegrass (*Lolium perenne* L.). As well as being important readily mobilisable energy reserves for the plant, fructans are also involved in stress tolerance. Fructans are also readily digested by grazing ruminants and hence are a valuable source of energy for sheep, beef and dairy production systems in temperate regions. This paper describes the re-programming of the expression of fructan biosynthesis genes through the transgenic manipulation of 6-glucose fructosyltransferase (6G-FFT) and sucrose:sucrose 1-fructosyl-transferase (1-SST) in perennial ryegrass. Transgenic events were developed with altered fructan accumulation patterns with increases in fructan accumulation and greatly increased accumulation of fructan in leaf blades as opposed to the traditional site of fructan accumulation in the pseudostem. This altered site of fructan accumulation has potential benefits for animal production as leaf blades form the major part of the diet of grazing ruminants. Some of the transgenic events also exhibited enhanced biomass production. This combination of high quality and enhanced yield is of great interest to forage plant breeders and whilst the expression of these phenotypes needs to be confirmed under field conditions, the identification and characterisation of the transgenic events described in this paper validate the potential for the manipulation of fructan biosynthesis in perennial ryegrass.

Keywords: fructan; ryegrass; *Lolium perenne*; transgenic; cisgenic

1. Introduction

Fructans are a class of water soluble carbohydrate whose primary function is to provide a readily accessible energy reserve for plant growth. Fructans are associated with various advantageous characters in grasses, such as cold and drought tolerance [1,2], increased tiller survival, good regrowth after

cutting or grazing, improved recovery from stress, early spring growth and increased nutritional quality. Fructans represent the major non-structural carbohydrate store in 15% of plant species [3] and play a key role in forage quality. Ruminant livestock grazing on high fructan diets shows improved animal performance including increased mass and milk production, and increases ammonia assimilation [4–6].

Fructan synthesis and metabolism in grasses and cereals are complex. Fructans consist of linear or branched fructose chains attached to sucrose. The chain length of plant fructans ranges from three up to a few hundred fructose units. Different types of fructans can be distinguished based on the linkage types present. In perennial ryegrass, three types of fructans have been identified: inulins, inulin neoseries and levan neoseries with four fructosyltransferse (FT) enzymes involved in this fructan profile. The enzyme 1-SST (sucrose: sucrose 1-fructosyltransferase) catalyses the first step in fructan biosynthesis while the remaining enzymes elongate the growing fructose chain (1-FFT: fructan: fructan 1-fructosyltransferase, 6G-FFT: 6-glucose fructosyltransferase, and 6-SFT: sucrose: fructose 6-fructosyltransferase). The enzymes 1-FEH or 6-FEH (fructoexohydrolase) reduce fructan chain length by releasing fructose molecules.

Fructans accumulate in the stems and leaf sheaths with the majority of the accumulation in the leaf sheaths or pseudostem at the base of the tillers. This has driven research and development efforts towards increasing water soluble carbohydrate levels in grasses used in improved pastures, both through molecular breeding and biotechnology. Fructan synthesis and metabolism is complex. In grasses, the level and composition of fructans can be increased in stems and leaf sheaths through the engineered expression of fructosyltransferase (FT) genes [7–9]. This, however, does not increase significantly the level of high degree of polymerization fructans (high DP fructans) in leaf blades, the tissues which are normally eaten by the large grazing animals in the field. In leaf blades, the expression of members of FT family genes involved in fructan polymerization is generally low, but can be induced by a number of abiotic stresses. Thus, accumulation of high DP fructans in leaves could provide more accessible nutrition for grazing animals.

Therefore, fructans in mature leaf blades accumulate less than in leaf sheaths and stems [10]. In order to specifically increase the level of fructans in leaf blades, a strategic approach was devised that coordinately expresses fructan biosynthesis genes in photosynthetic tissues. This involved metabolic re-programming of photosynthetic cells for enhanced sucrose and fructan production.

2. Results

2.1. Production of Transgenic Plants

The results of the biolistic transformation of embryogenic calli of perennial ryegrass are illustrated in Figure 1. The bombardment of 500 calli for each of the transformation vectors led to the recovery of between nine and 44 transgenic plants per vector that were confirmed to be positive for both the selectable marker and gene of interest (Table 1).

Table 1. Summary of the transformation progress for perennial ryegrass with wheat photosynthetic-regulated expression of *Lp1-SST* and *Lp6G-FFT* and fusion open reading frames (ORFs).

Species	Genotype	Transforming DNA	No Plates Bombarded	No calli Bombarded	No +ve Transgenics	No Plates Analysed	*hph* +ve Plants	GOI +ve Plants
L. perenne	FLP 418-20	*TaRbcS::Lp1-SST::Tarbcs +pACH1*	10	500	46	46	37	32
L. perenne	FLP 418-20	*TaRbcS::Lp6G-FFT::Tarbcs +pACH1*	10	500	50	50	48	38
L. perenne	FLP 418-20	*TaRbcS::Lp1-SST-Lp6G-FFT::Tarbcs (1) + pACH1*	10	500	47	47	47	44
L. perenne	FLP 418-20	*TaRbcS::Lp1-SST-Lp6G-FFT::Tarbcs (3) + pACH1*	10	500	26	26	26	23
L. perenne	FLP 418-20	*TaRbcS::GUS::Tarbcs +pACH1*	10	500	13	13	11	9

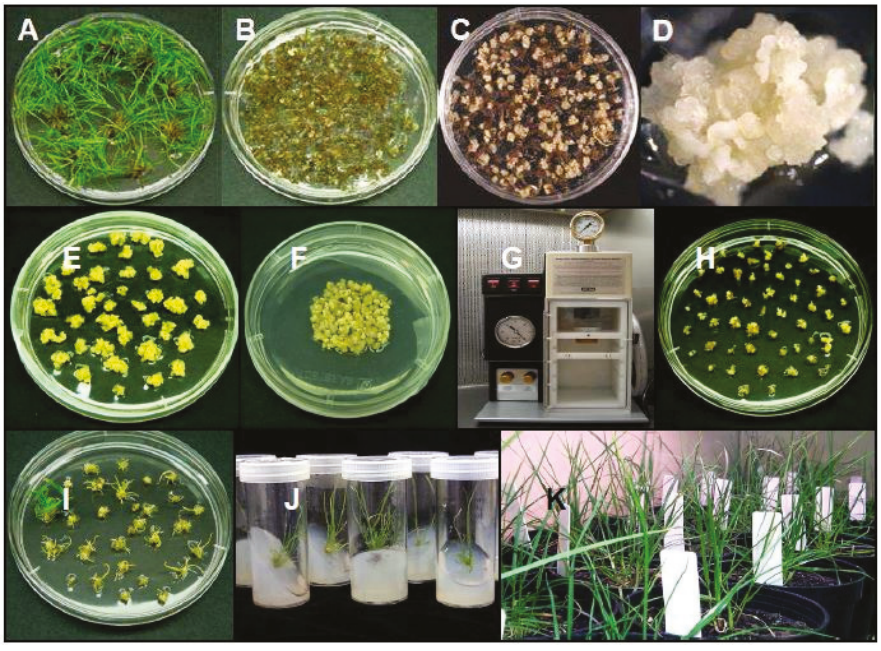


Figure 1. Production of transgenic perennial ryegrass plants from microprojectile bombardment of shoot meristem-derived calli. (**A**) Donor material for shoot meristems; high vegetative biomass, nil-to-low root development; (**B**) Distribution of basal meristematic material on callus initiation medium; (**C**) Proliferation of callus from basal meristematic regions; (**D**,**E**) Proliferation of embryogenic callus derived from basal meristems; (**F**) Distribution of calli of high osmotic medium prior to biolistic transformation; (**G**) Biolistic transformation device, PDS-1000/He; (**H**,**I**) Growth and development of hygromycin-resistant shoots, 30–75 days after bombardment; (**J**) Growth and development of hygromycin-resistant shoots in vitro; (**K**) Hygromycin-resistant plants established in soil and grown under glasshouse containment conditions.

2.2. Biochemical and Morphological Characterisation of Transgenic Plants

During the regeneration of the putative transgenic perennial ryegrass lines, differences in growth phenotypes were noticed between the lines. Both the tissue culture regenerants and corresponding soil grown plants from both of the fusion-1 and fusion-3 transgenic lines showed a superior vigour phenotype compared to the transgenic plants containing either *TaRbcS*::*Lp1-SST*, *TaRbcS*::*Lp6G-FFT*, *TaRbcS*::*GUS* or control plants containing only the selectable marker, *hph* (Figure 2).

The plants showing the phenotype were confirmed to contain the gene-of-interest (GOI) using real-time PCR and Southern hybridization analysis [11]. The superior growth phenotype of the transgenic fusion-1 and fusion-3 lines was first observed during the early stages of plant regeneration conducted on plates. Specifically, just 12 days after incubation under lights, the calli showed further developed green shoots. The fast growth rate of the fusion transgenic plants became more evident 22 days after transferring to rooting media. Transgenic plants containing either fusion-1 or fusion-3 constructs showed an obvious increase in tiller number. In addition, the fusion transgenics consistently showed a higher tiller density per plant compared to control lines (Figure 2).

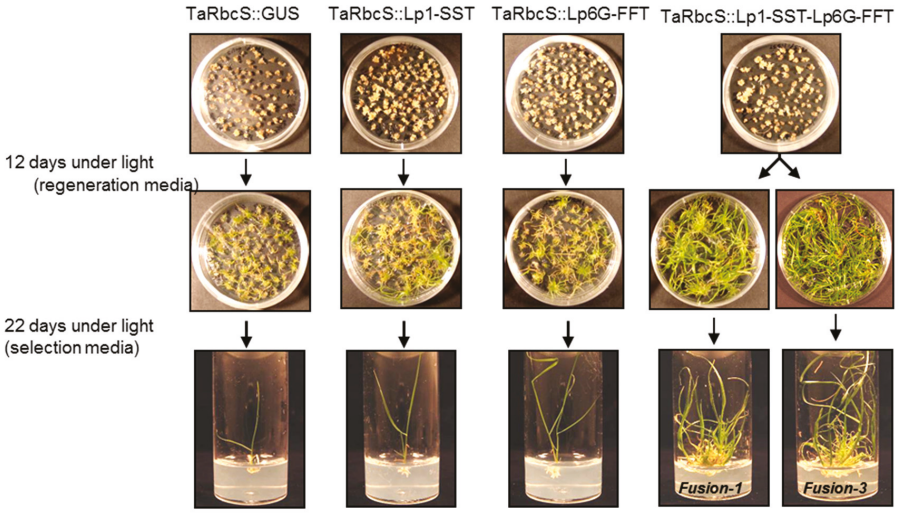


Figure 2. Plant regeneration phenotypes of transgenic perennial ryegrass (FLP-418-1) after co-transformation with the light-regulated gene constructs (Table 1) and the *pAcH*1 vector, with selection on hygromycin. The plants that contain either of the *Lp1-SST-Lp6G-FFT* fusion constructs consistently perform better in tissue culture (far right column).

Following transfer to soil and propagation under glasshouse conditions, more specific differences were observed between the fusion-1 and fusion-3 lines. Even though both fusion plants displayed more vigorous growth, fusion-1 lines had longer, thicker and slightly darker green leaf blades. Also, the plants were physically more robust with thicker leaf sheaths and leaf blades. Fusion-3 lines continued to grow faster than the other control plants with longer leaf blades and more vigorous tiller growth, but the leaf morphology was more similar to wild-type. An increase in root biomass was also observed in both fusion-1 and fusion-3 soil grown plants (Figure 3).

The control transgenic lines harbouring either the *Lp1-SST* or *Lp6G-FFT* as single genes did not show the increased growth rate that was observed in the fusion-1 and -3 lines. Their appearance is similar to each other, although some developed more vigorously than the transgenic plants containing either Gus or *hph* (Figure 3).

Leaf blades from individual plants were cut and hand sectioned and viewed under a microscope (Figure 4). There were apparent differences in the number of cells with chloroplasts: being more in both of the transgenic fusion lines than in the control plants. In addition, chloroplasts were present in cells located on the abaxial side (lower part of the leaf) of transgenic plants, despite both plants being grown under the same light conditions in the growth room. Sometimes it was observed that control plants produced more chloroplasts in mesophyll cells located on the adaxial side (upper side which faces the light source) than on the abaxial side, whereas the transgenic plants most often produced a near-equal number of chloroplasts on both sides. It was not possible to determine difference in cell size or overall cell numbers from hand-made sections.

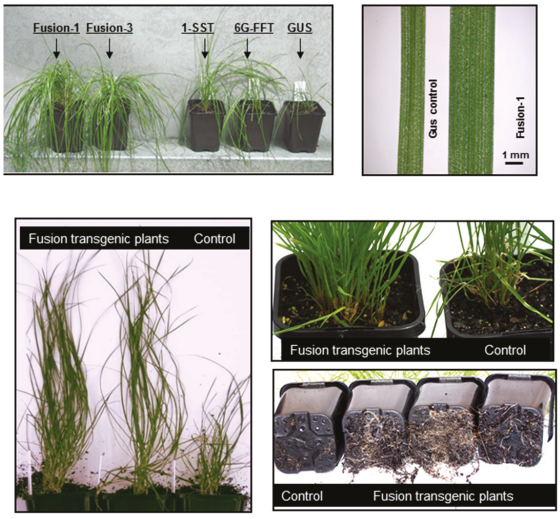


Figure 3. Mature plant phenotypes. Representative samples of transgenic plants at vegetative stage. The plants were trimmed equally three weeks earlier. Close-up micrographs of the leaf blades (right). The fusion consistently has wider blades compared to control plants.

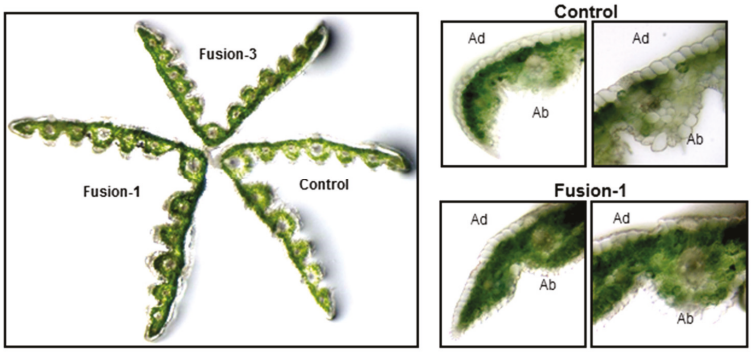


Figure 4. Leaf phenotypes. Representative samples of hand sections of leaf blades at the vegetative stage. Left shows a comparison of whole leaf sections; right shows magnified areas of leaf sections. Ad-Adaxial, Ab-abaxial.

Biochemical analysis by HPAEC of water soluble carbohydrates extracted from independent transformants harbouring the *TaRbcs::FT-Fusion-1*, *TaRbcs::FT-Fusion-3*, *TaRbcs::Lp1-SST*, *TaRbcS::Lp6G-FFT*, and two control lines (*hph* only) showed that the fusion-1 and fusion-3 transgenic plants contained significantly higher levels of total fructans (Figure 5), showing up to a 2.5-fold increase over the control lines (Figure 6).

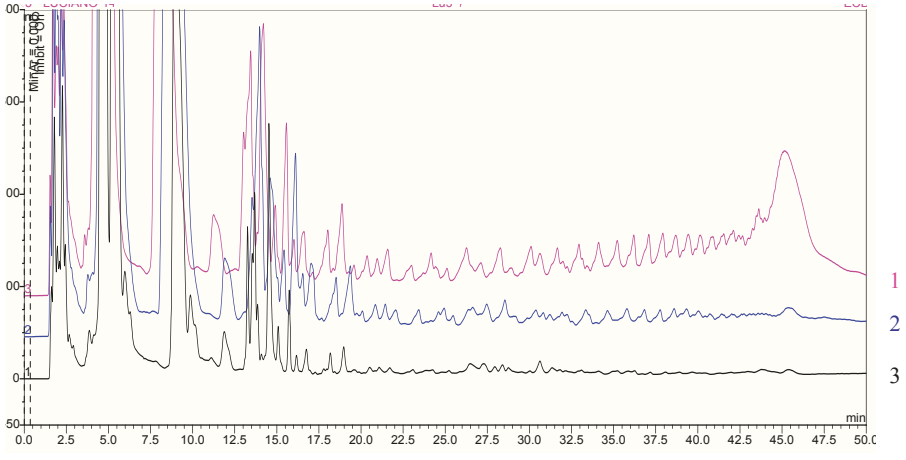


Figure 5. Biochemical analysis (HPAEC) of fructan level and composition present in transgenic TaRbcs::Fusion-3 (1), TaRbcs::Fusion-1 (2) and control plants (3) harbouring only the selectable marker (*hph* gene).

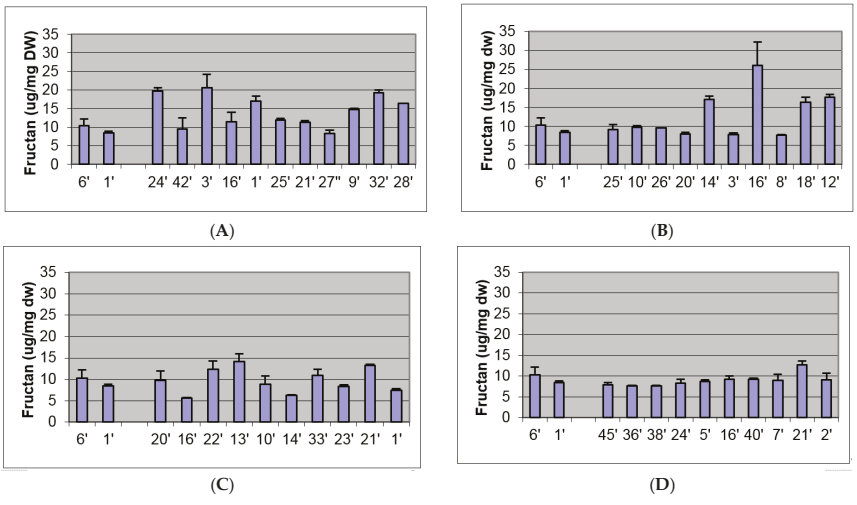


Figure 6. Biochemical analysis (HPAEC) of total fructans present in whole tillers of (**A**) *TaRbcs*::*Lp1-SST-Lp6G-FFT* fusion-1, (**B**) *TaRbcs*::*Lp1-SST-Lp6G-FFT* fusion-3, (**C**) *TarbcS*::*Lp1-SST*, and (**D**) *TarbcS*::*6G-FFT* transgenic lines compared to control lines (lanes 6′ and 1′), harbouring only the selectable marker (*hph* gene).

In addition, the levels of 1-kestose were up to four times higher in fusion-1 lines, and three times higher in fusion-3 lines compared to the *hph* controls (Figure 7). In the *TaRbcs*::*Lp1-SST* plants, 1-Kestose increased up to three-fold whereas total fructan content only increased 0.5-fold. In contrast, 1-kestose levels in the *TaRbcS*::*Lp6G-FFT* transgenic plant lines showed marginal increases in 1-kestose (up to 0.5-fold) and only one line showed a small increase in total fructans (Figure 7). Analysis of sucrose contents of all the lines revealed that some of the high fructan lines also showed an increase in total sucrose content (Figure 8).

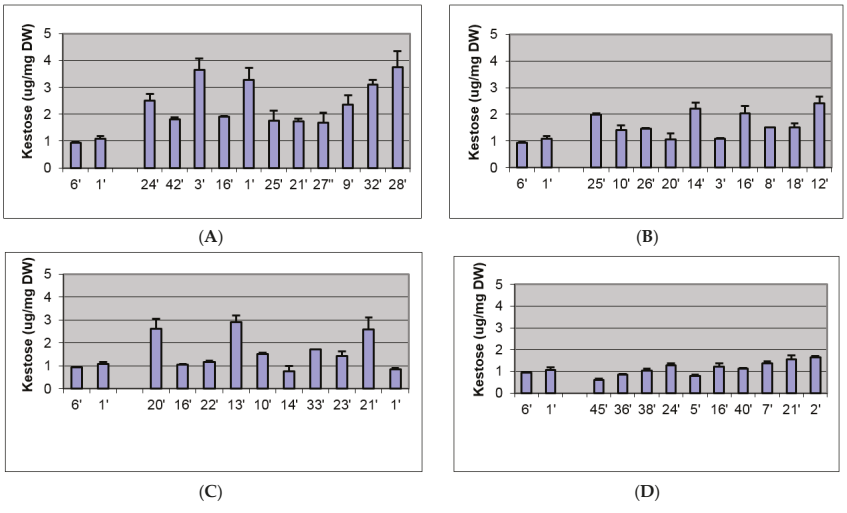


Figure 7. Biochemical analysis (HPAEC) of 1-kestose present in whole tillers of (**A**) *TaRbcs*::*Lp1-SST-Lp6G-FFT fusion-1*, (**B**) *TaRbcs*::*Lp1-SST-Lp6G-FFT* fusion-3, (**C**) *TarbcS*::*Lp1-SST*, and (**D**) *TarbcS*::6*G-FFT* transgenic lines compared to control lines (lanes 6′ and 1′), harbouring only the selectable marker (*hph* gene).

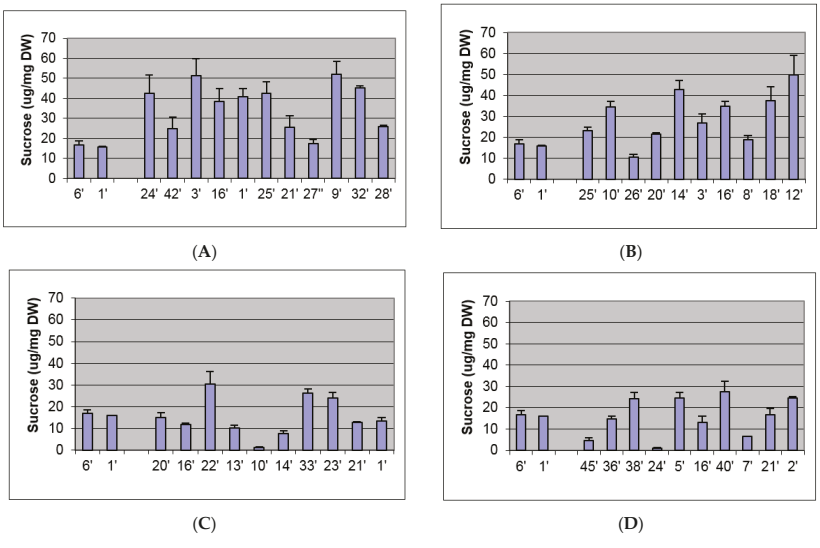


Figure 8. Biochemical analysis (HPAEC) of sucrose present in whole tillers of (**A**) *TaRbcs*::*Lp1-SST-Lp6G-FFT fusion-1*, (**B**) *TaRbcs*::*Lp1-SST-Lp6G-FFT fusion-3*, (**C**) *TarbcS*::*Lp1-SST*, and (**D**) *TarbcS*::6*G-FFT* transgenic lines compared to control lines (lanes 6′ and 1′), harbouring only the selectable marker (*hph* gene).

3. Discussion

The main storage carbohydrates in perennial ryegrass are high molecular weight fructans with a prevalence of β(2-6) linkages [10,12,13]. The expression of enzymes involved with fructan biosynthesis (*6G-FFT, 1-SST and 6-SFT*) [12,13] is highly tissue-specific and tends to be associated with the base of leaves and leaf sheaths/pseudostems which are also the site of fructan accumulation in perennial ryegrass which is consistent with the role of fructans as storage carbohydrates to support the regrowth of leaves [14] and their potential role in osmoregulation and membrane protection during drought and water stress [15].

In this research, we have demonstrated that through the development of transgenic plants with fructan biosynthetic genes under the control of photosynthetic promotors, not only have concentrations of fructan, kestose and sucrose been increased overall but also in the leaf blades. This is of key importance if the aim of increasing fructan concentrations is to improve the nutritive value of perennial ryegrass through increasing the concentration of water-soluble carbohydrates [16]. Increasing the concentration of water-soluble carbohydrates is important for grazing ruminants as it serves to increase both the energy concentration of the herbage but also plays a role in improving the synchrony of fermentation in the rumen and hence the efficiency of conversion of ingested grass into animal products [17]. Comprehensive bioeconomic modelling of the impact of an increase of 1 MJ/kg in dairy pasture has been shown to have the potential to increase the profitability of dairy farms both directly through the increased nutritive value of the herbage and indirectly through the reduction in the use of purchased supplementary sources of energy such as grain or pellets [18].

The mechanism of the high biomass phenotype observed in some transgenic events in this study is unknown and it is acknowledged that the phenotype was measured in primary transformants but it is clear that the combination of increased productivity combined with increased concentrations could lead to benefits over and above those described by Ludemann et al. [18].

Previous work has shown that it is important to combine high energy traits with good agronomic adaption to the target environment, otherwise the trait is not always expressed or the seasonal growth pattern may not suit the needs of the grazing enterprise [17,19]. The transgenic events described in this paper are in a tissue culture-responsive genotype from an elite perennial ryegrass breeding line and further work is planned to evaluate these events under field conditions and to further cross them into a broader range of backgrounds to investigate the effects of genotype x environment interactions on the expression of the transgene.

4. Materials and Methods

4.1. Identification and Cloning of Photosynthetic Promoters from Perennial Ryegrass

The expression of *RbcS* and Chlorophyll a/b Binding Protein (*CAB*) is well characterised by light-regulated genes in higher plants.

Both *LpRbcS* and *LpCAB* genes were chosen for promoter discovery and isolation in perennial ryegrass. Publicly available cDNA sequences (*LpRbcS*, *EC*778430 and *LpCAB*, *EC*778438) were used as query sequences in a BLAST search of the perennial ryegrass EST database in our in-house database. As both genes are members of multigene families, several contigs (each contig represents an individual gene) were identified in our perennial ryegrass EST collection. Nine contigs were identified to be homologous to the published *LpRbcS* cDNA sequence and thirteen contigs were found to be homologous to the *LpCAB* cDNA sequence. Two contigs, *LpRbcS* (*LPCL9_C*359) and *LpCAB* (*LpCL*1112_C12), representing the genes of the promoters to be isolated, contained (47) and (19) EST sequences, respectively. These sequences came from a variety of libraries representing a range of different tissues. This data was used for in silico expression analysis and indicated that both genes are only expressed in photosynthetic tissues.

DNA sequence alignments for each of the gene family members were performed, and gene-specific primers were designed for contigs *LpRbcS_C*359 and *LpCAB_C*12 and used to screen perennial ryegrass

BAC DNA pools by PCR. The BAC clones were identified and sequenced. Primers were designed and the *Lolium perenne*-specific promoter regulatory sequences were cloned, sequenced and the *cis*-regulatory sequences specific for photosynthetic promoters were identified by PLACE (Table 2). The sequences included the I-Box motif and the GT1 box for RbcS [19,20]. In addition, 16/19 nucleotides of the LpRbcS sequence shared homology with the monocot Rbs Consenesus sequence [21]. The I-Core box and SORLIPs *cis*-regulatory sequences were present in the CAB promoter. SORLIPs were found to be over-represented in light-induced promoters in Arabidopsis [22].

These *L. perenne*-specific promoter regulatory sequences were subsequently used in the construction of backbone-free expression cassettes with fructan biosynthesis genes.

Table 2. The position of the *cis*-regulatory sequences identified by PLACE. Common *cis*-acting regulatory sequences are listed [19,21,22,24]. Positions noted are the first nucleotide in the sequence relative to the ATG. (n.p.—not present).

cis-Acting Regulatory seq.	Accession	Position *LpRbcS*	Position *LpCAB*
I-Box Core	S000199	−184	−137
I-Box	S000124	−311	−137
GT1 consensus	S000198	−304	n.p.
RbcS monocot seq	Schaffner et al., 1991	−173 to −151	n.p.
SORLIPs	S000482	n.p.	−58, −217, −647, −695

4.2. Isolation of Fructan Biosynthesis Genes

The *Lolium perenne* cDNA clones encoding sequences for *Lp1-SST* and *Lp6G-FFT* have previously been isolated from a perennial ryegrass cDNA library [12,23]. The complete gene sequences of the isolated perennial ryegrass fructosyltransferase homologues are publicly available.

4.3. Cloning of FT Translational Fusion

It has been proposed that FT proteins may physically associate with each other to facilitate the efficient biosynthesis of fructans. Therefore, a genetic fusion was created between the open reading frames for *Lp1-SST* and *Lp6G-FFT*.

The *Lp1-SST* gene was PCR-amplified with a GATEWAY recombination site incorporated in the forward primer. A sequence that codes for three glycine residues followed by a *Hind* III site was incorporated into the reverse primer, with the stop codon removed. The *Lp6G-FFT* gene was PCR-amplified with a *Hind* III site followed by a sequence that codes for three glycine residues and the gene-specific sequence without the ATG. The reverse primer for the *Lp6G-FFT* gene was flanked by a second GATEWAY recombination site. The primer sequences are provided in Table 3. The purified fragments were digested with *Hind* III and the ligated product was cloned into the Invitrogen GATEWAY pDONR221 entry vector. When the resultant pENTRY1-*Lp1-SST*-*Lp6G-FFT*-2 entry clones were sequenced, one sequence (fusion-1) was confirmed to be the predicted product, with eight amino acids in the linker joining the two genes. However, another sequence (fusion-3) contained two consecutive *Hind* III sites, which would result in the addition of another two amino acids, giving a total of ten amino acids between the two FT genes upon translation.

Table 3. Primer sequences used to amplify the PCR fragments used to generate the translational fusion of the *Lp1-SST* and *Lp6G-FFT* fructosyl transferase genes (*FT Lp1-SST*::*Lp6G-FFT*). Black sequences are gene-specific, blue and red (*Hind* III RE site) sequences are nucleotides introduced to generate the linker region, and green nucleotides represent the recombination-specific sequences.

Gene	Forward Primer	Reverse Primer
Lp1-SST	GGGGACAAGTTTGTACAAAAAAGCAGG CTTCATGGAGTCCCCAAGCGCCGTC	TCTAAGCCTTTCCTCCTCCCAAGTCG TCGTTCGTG
Lp6G-FFT	ACTAAGCTTGGAGGAGGAGAGTCCAG CGCCG	GGGGACCACTTTGTACAAGAAAGCTGGG TCCTACATGTCGTCAGCCAAGAAGGCC

4.4. Generation of Vectors for Transgenic Assays

A number of vectors were constructed using Invitrogen Multisite Gateway™ technology based on recombinational cloning. This methodology relies on the generation of individual entry plasmids containing either the promoter, gene of interest (GOI), or terminator sequences flanked by recombination sites. The recombination sites facilitate the directional triple insertion of each of the entry plasmids into a gateway-enabled destination vector, by recombination. The final vector is then sequenced and used directly for plant co-transformation with a plasmid for expression of a plant selectable marker.

In order to test the function of the fusion protein, the FT fusion-1 and FT fusion-3 ORFs were cloned under the control of the enhanced cauliflower mosaic virus (CAMV)35S^2 promoter [25], using the Invitrogen Multisite Gateway™ Technology recombination into *Agrobacterium* binary vector [26].

Gateway entry vectors were constructed for the (CAMV)35S^2 promoter, the *TaRbcS* terminator sequence, as well as FT fusion-1 and FT fusion-3 ORFs. The cloned fragments were sequence-verified and used for three-way recombination cloning with the cloned GOI cDNA sequences into the *pPZP*200*ubi*:*bar-nos* R4 R3 destination vector. In addition, constructs also included the *Lp6G-FFT* and *Lp1-SST* single ORF driven by the (CAMV)35S^2 promoter as controls. The *Lp*1-*FFT* single ORF will also be cloned in the same manner. As a control, the GUS ORF was used for confirmation of expression. The following constructs were made.

- pPZP200: 35S^2::*Lp6G-FFT*::*TaRbcS*
- pPZP200: 35S^2::*Lp1-SST*::*TaRbcS*
- pPZP200: 35S^2::*Lp*1-*FFT*::*TaRbcS*
- pPZP200: 35S^2::*Lp1-SST*::6*G-FFT*::*TaRbcS*
- pPZP200: 35S^2::*GUS*::*TaRbcS*

4.5. Function of Lp1-SST, Lp6G-FFT and FT-Fusion Protein in Transient Transgenic Assays

For proof-of-function, transient expression of the 35S promoter constructs was conducted in tobacco plants, as they do not naturally store fructans. The method involved agro-infiltration of the individual constructs into *N. benthamiana* leaves [27,28] followed by biochemical analysis by anion exchange. Three days after the injection, the plant material was harvested and the water-soluble carbohydrates were extracted using a hot water extraction method. The extracts were separated using high performance anion exchange chromatography (HPAEC). The results showed production of fructans, with the increased production of both 1-kestose and 6G-kestose by the fusion protein data (not shown) so vectors were designed for the stable transformation of perennial ryegrass.

4.6. Generating Vectors for Stable Transformation

A 695 kb promoter fragment from a previously published sequence containing the TATA signal from the *TaRbcS* gene (NCBI accession number AB042069) was PCR-amplified with Gateway™ (Invitrogen) recombination sites at the primer flanks. The fragment was cloned into the Invitrogen pDONRP4-P1R entry vector using Gateway™ recombination technology. The 696 bp *TarbcS* gene termination signal sequence [29] was also PCR-amplified using primers with recombination sites and cloned into the Invitrogen pDONRP2-P3R entry vector. The cloned fragments were sequence-verified and used for three-way recombination cloning with the cloned GOI cDNA sequences into the pDEST-R4R3 destination vector: pDESTR1-R2R-*Lp1-SST*, pDESTR1-R2-*Lp6G-FFT*, and pDESTP1-P2R-*Lp1-SST-Lp6G-FFT* gene fusion expression vectors. The following constructs for photosynthetic-regulation of expression of fructosyltransferases by the *TarbcS* promoter to be used are outlined below.

- pDEST-*TaRbcS*::*Lp1-SST*::*TaRbcS*
- pDEST-*TaRbcS*::*Lp6G-FFT*::*TaRbcS*
- pDEST-*TaRbcS*::*Lp1-SST-Lp6G-FFT*::*TaRbcS* (fusion-1)

- pDEST-*TaRbcS*::*Lp1-SST-Lp6G-FFT*::*TaRbcS* (fusion-3)
- pDEST-*TaRbcS*::*GUS*::*TaRbcS*

4.7. Production of Transgenic Ryegrass Plants

Biolistic co-transformation of perennial ryegrass with the vectors containing the *TaRbcS* regulatory sequences, driving the expression of individual fructan genes or as a translational fusion, and the *pAcH*1 vector for hygromycin resistance was conducted on embryogenic calli for perennial ryegrass using the method of Spangenberg et al. [30]. The pAcH1 vector was previously constructed and has been used successfully in plant transformation experiments [30–34]. The GUS marker gene was also cloned as a positive control. Table 2 summarises the transformation and molecular analysis for the generation of these lines. Following agarose gel electrophoresis, the resulting DNA fragment was purified from the agarose gel prior to being used for plant transformation to produce DNA without vector backbone sequences. The pAcH1 vector previously constructed and used successfully in plant transformation experiments was also digested with restriction enzymes to produce a DNA fragment for the expression of the selectable marker only [30–34].

A tissue culture responsive genotype, FLp418-20, was selected for use in these experiments on the basis of observed shoot regeneration from embryogenic callus. Clonal replicates of this genotype were used to provide the material for biolistic transformation using the vectors described above and following the method of Spangenberg et al. [30] and illustrated in Figure 1.

4.8. Transgene Detection

The presence of the transgene and selectable markers were confirmed using the methods described in detail by Badenhorst [11]. In summary, following growth in selectable media during tissue culture, the presence of the transgene was confirmed using real-time PCR and Southern hybridisation. Real-time PCR results were scored in comparison to positive (plasmid DNA) and negative (non-transgenic plant DNA, no-template) control templates with the endogenous histone H3 gene (*LpHisH*3) included as a control [11]. Southern hybridization of genomic DNA with chemiluminescent detection was used to visualize the results of probes designed for the *RBcS* or *LpFT*1 promoter, the *LpFT*4 terminator and the *hph* selection cassette [11] (results not shown).

4.9. Quantification of Carbohydrates

Concentrations of monosaccharides and fructan in plant tissue samples were quantified using a high performance-anion exchange chromatography (HP-AEC) method described by Liu et al. [35].

5. Conclusions

Transgenic events were developed with altered fructan accumulation patterns with increases in fructan accumulation and greatly increased accumulation of fructan in leaf blades as opposed to the traditional site of fructan accumulation in the pseudostem. This altered site of fructan accumulation has potential benefits for animal production as leaf blades form the major part of the diet of grazing ruminants. Some of the transgenic events also exhibited enhanced biomass production. This combination of high quality and enhanced yield is of great interest to forage plant breeders and whilst the expression of these phenotypes needs to be confirmed under field conditions, the identification and characterisation of the transgenic events described in this paper validate the potential for the manipulation of fructan biosynthesis in perennial ryegrass.

Acknowledgments: This work was carried out as part of the Molecular Plant Breeding Co-operative Research Centre and the Dairy Futures Co-operative Research Centre.

Author Contributions: G.S., A.M., M.G., P.B., K.F.S. and S.P. conceived and designed the experiments; M.L., S.P. and P.B. performed the experiments; A.M., L.M., M.G., S.P., P.B. and G.S. analyzed the data; K.F.S. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Guerrand, D.; Prud'homme, M.; Boucaud, J. Fructan metabolism in expanding leaves, mature leaf sheaths and mature leaf blades of *Lolium perenne*. Fructan synthesis, fructosyltransferase and invertase activities. *New Phytol.* **1996**, *134*, 205–214. [CrossRef]
2. Thomas, H.; James, A. Partitioning of sugars in *Lolium perenne* (perennial ryegrass) during drought and on rewatering. *New Phytol.* **1999**, *142*, 295–305. [CrossRef]
3. Hendry, G.; Wallace, R. The origin, distribution and evolutionary significance of fructans. In *Science and Technology of Fructans*; Suzuki, M., Chatterton, N.J., Eds.; CRC Press: Baton Rouge, FL, USA, 1993; pp. 119–139.
4. Nocek, J.; Russell, J. Protein and energy as in integrated system. Relationship of ruminal protein and carbohydrate availability to microbial synthesis and milk production. *J. Dairy Sci.* **1988**, *70*, 2070–2107. [CrossRef]
5. Biggs, D.; Hancock, K. In vitro digestion of bacterial and plant fructans and effects on ammonia accumulation in cow and sheep rumen fluids. *J. Gen. Appl. Microbiol.* **1998**, *44*, 167–171. [CrossRef]
6. Taweel, H.Z.; Tas, B.M.; Smit, H.J.; Elgersma, A.; Dijkstra, J.; Tamminga, S. Effects of feeding perennial ryegrass with an elevated concentration of water-soluble carbohydrates on intake, rumen function and performance of dairy cows. *Anim. Feed Sci. Technol.* **2005**, *121*, 243–256. [CrossRef]
7. Ye, X.; Wu, X.; Zhao, H.; Frehner, M.; Nosberger, J.; Potrykus, I.; Spangenberg, G. Altered fructan accumulation in transgenic *Lolium multiflorum* plants expressing a *Bacillus subtilis sacB* gene. *Plant Cell Rep.* **2001**, *20*, 205–212. [CrossRef]
8. Hisano, H.; Kanazawa, A.; Kawakami, A.; Yoshida, M.; Shimamoto, Y.; Yamada, T. Transgenic perennial ryegrass plants expressing wheat fructosyltransferase genes accumulate increased amounts of fructan and acquire increased tolerance on a cellular level to freezing. *Plant Sci.* **2004**, *167*, 861–868. [CrossRef]
9. Gadegaard, G.; Didion, T.; Folling, M.; Storgaard, M.; Andersen, C.H.; Nielsen, K.K. Improved fructan accumulation in perennial ryegrass transformed with the onion fructosyltransferase genes 1-SST and 6G-FFT. *J. Plant Physiol.* **2008**, *165*, 1214–1225. [CrossRef] [PubMed]
10. Pollock, C.; Jones, T. Seasonal patterns of fructan metabolism in forage grasses. *New Phytol.* **1979**, *83*, 9–15. [CrossRef]
11. Badenhorst, P.E. 2014. Phenomic Evaluation and Molecular Breeding of Field-Grown Transgenic Perennial Ryegrass (*Lolium perenne*) with Altered Fructan Biosynthesis. Ph.D. Thesis, La Trobe University Bundoora, Melbourne, Australia, 2014.
12. Chalmers, J.; Lidgett, A.; Cummings, N.; Cao, Y.; Forster, J.; Spangenberg, G. Functional genomics of fructan metabolism in temperate grasses. *Plant Biotechnol. J.* **2005**, *3*, 459–474. [CrossRef] [PubMed]
13. Lasseur, B.; Lothier, J.; Wiemken, A.; van Laere, A.; Morvan-Bertrand, A.; Van den Ende, W.; Prudhomme, M.P. Towards a better understanding of the generation of fructan structure diversity in plants: Molecular and functional characterization of a sucrose:fructan 6-fructosyltransferase (6-SFT) cDNA from perennial ryegrass (*Lolium perenne*). *J. Exp. Bot.* **2011**, *62*, 1871–1885. [CrossRef] [PubMed]
14. Morvan-Bertrand, A.; Boucaud, J.; Le Saos, J.; Prud'homme, M.P. Roles of fructans from leaf sheaths and from the elongating leaf bases in the regrowth following defoliation of *Lolium perenne* L. *Planta* **2001**, *213*, 109–120. [CrossRef] [PubMed]
15. Livingston, D.P.; Hincha, D.K.; Heyer, A.G. Fructan and its relationship to abiotic stress tolerance in plants. *Cell. Mol. Life Sci.* **2009**, *66*, 2007–2023. [CrossRef] [PubMed]
16. Smith, K.F.; Simpson, R.J.; Oram, R.N.; Lowe, K.F.; Kelly, K.B.; Evans, P.M.; Humphreys, M.O. Seasonal variation in the herbage yield and nutritive value of perennial ryegrass (*Lolium perenne* L.) cultivars with high or normal herbage water soluble carbohydrate concentrations grown in three contrasting Australian dairy environments. *Aust. J. Exp. Agric.* **1998**, *38*, 821–830. [CrossRef]
17. Smith, K.F.; Reed, K.F.M.; Foot, J.Z. An assessment of the relative importance of specific traits for the genetic improvement of nutritive value in dairy pasture. *Grass Forage Sci.* **1997**, *52*, 167–175. [CrossRef]
18. Ludemann, C.I.; Eckard, R.J.; Cullen, B.R.; Jacobs, J.L.; Malcolm, B.; Smith, K.F. Higher energy concentration traits in perennial ryegrass (*Lolium perenne* L.) may increase profitability and improve energy conversion on dairy farms. *Agric. Syst.* **2015**, *137*, 89–100. [CrossRef]
19. Terzaghi, W.B.; Cashmore, A.R. Light-regulated transcription. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1995**, *46*, 445–474. [CrossRef]

20. Francis, S.A.; Chapman, D.F.; Doyle, P.T.; Leury, B.J. Dietary preferences of cows offered choices between white clover and "high sugar" and "typical" perennial ryegrass cultivars. *Aust. J. Exp. Agric.* **2006**, *46*, 1579–1587. [CrossRef]
21. Schaffner, A.R.; Sheen, J. Maize rbcS Promoter Activity Depends on Sequence Elements Not Found in Dicot rbcS Promoters. *Plant Cell* **1991**, *3*, 997–1012. [CrossRef] [PubMed]
22. Hudson, M.E.; Quail, P.H. Identification of promoter motifs involved in the network of phytochrome A-regulated gene expression by combined analysis of genomic sequence and microarray data. *Plant Physiol.* **2003**, *133*, 1605–1616. [CrossRef] [PubMed]
23. Chalmers, J.; Johnson, X.; Lidgett, A. Isolation and characterisation of a sucrose:sucrose 1-fructosyltransferase gene from perennial ryegrass (*Lolium perenne*). *J. Plant Physiol.* **2003**, *160*, 1385–1391. [CrossRef] [PubMed]
24. Martinez-Hernandez, A.; Lopez-Ochoa, L.; Arguello-Astorga, G.; Herrara-Estrella, L. Functional properties and regulatory complexity of a minimal RBCS light-responsive unit activated by phytochrome, cryptochrome, and plastid signals. *Plant Physiol.* **2002**, *128*, 1223–1233. [CrossRef] [PubMed]
25. Kay, R.; Chan, A.; Daly, M.; McPherson, J. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science* **1987**, *236*, 1299–1302. [CrossRef] [PubMed]
26. Hajdukiewicz, P.; Svab, Z.; Maliga, P. The small, versatile pPZP family of Agrobacterium binary vectors RT for plant transformation. *Plant Mol. Biol.* **1994**, *25*, 989–994. [CrossRef] [PubMed]
27. Kapila, J.; De Rycke, R.; van Montagu, M.; Angenon, G. An Agrobacterium-mediated transient gene expression system for intact leaves. *Plant Sci.* **1997**, *124*, 227. [CrossRef]
28. Wydro, M.; Kozubek, E.; Lehmann, P. Optimization of transient Agrobacterium-mediated gene expression system in leaves of *Nicotiana benthamiana*. *Acta Biochim. Pol.* **2006**, *53*, 289–298. [CrossRef] [PubMed]
29. Sasanuma, T. Characterization of the rbcS multigene family in wheat: Subfamily classification, determination of chromosomal location and evolutionary analysis. *Mol. Genet. Genom.* **2001**, *265*, 161–171. [CrossRef]
30. Spangenberg, G.; Wang, Z.Y.; Wu, X.; Nagel, J.; Potrykus, I. Transgenic perennial ryegrass (*Lolium perenne*) plants from microprojectile bombardment of embryogenic suspension cells. *Plant Sci.* **1995**, *108*, 209–217. [CrossRef]
31. Bilang, R.; Shigeru, I.; Peterhans, A.; Potrykus, I.; Paszkowski, J. The 3′-terminal region of the hygromycin-B-resistance gene is important for its activity in *Escherichia coli* and *Nicotiana tabacum*. *Gene* **1991**, *100*, 247–250. [CrossRef]
32. Spangenberg, G.; Wang, Z.Y.; Wu, X.L.; Nagel, J.; Iglesias, V.A.; Potrykus, I. Transgenic tall fescue and red fescue plants from microprojectile bombardment of embryogenic suspension cells. *J. Plant Physiol.* **1995**, *145*, 693–701. [CrossRef]
33. Ye, X.; Wang, Z.Y.; Potrykus, I.; Spangenberg, G. Transgenic Italian ryegrass (*Lolium multiflorum*) plants from microprojectile bombardment of embryogenic suspension cells. *Plant Cell Rep.* **1997**, *16*, 379–384. [CrossRef]
34. Bai, Y.; Qu, R. Genetic transformation of elite turf-type cultivars of Tall Fescue. *Int. Turfgrass Soc. Res. J.* **2001**, *9*, 129–136.
35. Liu, Z.; Mouradov, A.; Smith, K.F.; Spangenberg, G. An improved method for quantitative analysis of total fructans in plant tissues. *Anal. Biochem.* **2011**, *418*, 253–259. [CrossRef] [PubMed]

agronomy

MDPI

Article

Variation for Concentrations of Various Phytoestrogens and Agronomic Traits among a Broad Range of Red Clover (*Trifolium pratense*) Cultivars and Accessions

Valerie Little [1], Kevin F.M. Reed [1,3] and Kevin F. Smith [2,*]

1 Agriculture Victoria, Private Bag 105 Hamilton, Victoria 3300, Australia; valerie.little@ecodev.vic.gov.au
2 Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Private Bag 105 Hamilton, Victoria 3300, Australia
3 Reed Pasture Science, Brighton East, Victoria, Australia; rps@eftel.net.au
* Correspondence: kfsmith@unimelb.edu.au

Academic Editor: John W. Forster
Received: 15 September 2016; Accepted: 2 May 2017; Published: 5 May 2017

Abstract: Agronomic characteristics and phytoestrogen concentrations were measured on 17 cultivars and 47 accessions of red clover (*Trifolium pratense*). These accessions included a range of currently recommended cultivars—from Australia and overseas—and germplasm accessed from genetic resource collections. All lines were grown in the field at Hamilton Vic in 2000 and 2001. Significant genetic variation was detected for key agronomic parameters such as growth habit, leaf shape and markings, leaf area, herbage yield, flowering time, and prolificacy. Significant variation in the concentration of the four main phytoestrogens was found; total isoflavone concentration ranged from 0.14–1.45% DM. Maximum concentrations of daidzein, genistein, formononetin, and biochanin were 0.06, 0.08, 0.86, and 0.91% DM respectively. Multivariate analysis showed that the accessions grouped into 10 distinct clusters that had between 1 and 10 members. Several accessions were superior to existing cultivars—notably Mediterranean accessions with regard to cool season vigour—and valuable for breeding programs to develop high yielding cultivars with either high (for possible medicinal purposes) or low (for grazing) phytoestrogen concentrations.

Keywords: red clover; variability; phytoestrogen

1. Introduction

Red clover (*Trifolium pratense* L.) is a short-lived perennial legume [1] with a strong tap root. The optimum temperatures for productivity in red clover are between 20 °C and 25 °C [2] and has been widely sown in temperate regions of the former Soviet Union, Argentina, Chile, Columbia, Mexico, USA, Canada, Japan, Australia, and New Zealand [2]. Generally, although the plant is quite frost tolerant, herbage production in winter is poor. However, some cultivars that have been bred by crossing superior New Zealand varieties with Mediterranean genotypes have shown improved winter production [3]. The species is seen as an alternative to the more widely sown white clover and lucerne in Australia and New Zealand but the need exists for further breeding and development of adapted cultivars [4,5].

When consumed by ruminants, oestrogenic isoflavones in red clover can result in the production of equol, an oestrogenic substance that may impair reproductive processes. If exposed to Hamua red clover, ewe weaners rapidly exhibit teat elongation [6] and ewes grazing red clover exhibit irregular oestrus, cystic ovaries, and persistent glandular cysts affecting both the cervix and the endometrium. Prolonged grazing on oestrogenic pastures for several years may result in permanent and progressive infertility in ruminants [7]. To this end, low phytooestrogen cultivars have been developed in Australia to provide safe grazing for sheep—e.g., Redwest and Redquin, selected from Hamua and Quinequeli, respectively [8]. In Australia, the cultivars Genstar and Genstar Null have been selected for high isoflavone concentrations for pharmaceutical use [9].

While the effects of red clover oestrogens on the reproduction of sheep and cattle are largely negative, red clover extracts have been the subject of research for their application in human medicine, especially in the treatment of menopausal symptoms in women [10]. Oestrogen plays an important role in the human body and when oestrogen levels decrease with the onset of menopause, negative physical effects can occur. Phytoestrogens are considered promising for preventing degenerative diseases such as osteoporosis that are associated with oestrogen deficiency [11] although further research is required to validate the role of red clover and red clover-derived phytoestrogens in the treatment of a range of menopausal symptoms [12–14].

There is significant inter-varietal variation for oestrogenic isoflavone content in red clover [15]. Identification of material with a high concentration of phytoestrogens or desirable ratios of specific oestrogens, combined with good agronomic performance, could progress the genetic improvement of red clover for pharmaceuticals.

Various genotypes of red clover have been grouped by the use of cluster analysis for quantitative and qualitative characters. Kouame and Quesenberry [16] evaluated more than 800 accessions of red clover that represented 41 countries of origin. Great diversity was detected among accessions from Eastern and Northern Europe, providing a structure for identifying a limited number of core accessions in order to represent a large collection [16].

A set of red clover cultivars and accessions was grown out to characterise the accessions for agronomic performance and biochemical characters. The material was selected to represent a broad range of geographic originswith the aim of identifying new sources of germplasm for hybridization to existing cultivars for future varietal development.

2. Results

2.1. Range and Variance of Agronomic Traits

There were significant genetic effects for all traits, for example visual assessment of winter dry matter yield varied from 2.0 to 4.9 (on a 1–10 scale) and leaf width varied from 13.7 mm to 22.2 mm (Table 1). The proportion of variance expressed by genetic effects was large, ranging from 0.97 for flowering time to 0.45 for stem colour. Variance components for all traits were significant (greater than twice the standard error) except in the case of leaf shape (Table 1). Therefore, a large proportion of variation observed in this experiment was due to genetic effects. The mean and range of characteristics for each trait across all entries are presented in Table 1. There was a range for leaf markings present in the accessions that varied from no markings to a full, intermediate V mark [17] —data not shown.

Table 1. Mean and range of characterisation measurements of red clover accessions. (σ^2_g = genetic variation, s.e = standard error; R = repeatability).

Trait	Range	Mean	$\sigma^2_g \pm$ s.e	R
Autumn Recovery (Aut rec)	0.95–2.68	1.704	0.214 ± 0.044	0.694
Flowering Time (FT)	28.0–83.2	50.320	136.9 ± 25.60	0.877
Growth Habit, Spring/Summer (gh.ss)	2.19–5.84	3.976	1.580 ± 0.290	0.967
Leaf Shape (leaf sh)	1.10–2.40	2.166	0.035 ± 0.018	0.467
Flowering Prolificacy (prolificacy)	3.02–5.92	4.789	0.520 ± 0.104	0.820
Stem Colour (stem)	1.10–2.53	1.780	0.089 ± 0.033	0.447
Stipule Pigmentation (stipule)	1.50–3.73	2.278	0.314 ± 0.088	0.632
Cool Season Yield (DM, g/plant) (winter)	1.97–4.94	2.938	0.411 ± 0.086	0.734
Leaf Width (mm) (leaf w)	13.66–22.16	17.085	4.08 ± 1.11	0.658
Leaf Length (mm) (leaf l)	17.87–28.85	22.397	9.20 ± 2.66	0.638
Leaf Area (cm^2) (leaf a)	1.56–2.44	1.944	0.063 ± 0.018	0.589
Biochanin (g/kg DM) (Bioch)	0.09–0.91	0.480	0.021 ± 0.01	0.656
Daidzein (g/kg DM) (Diadz)	0.003–0.060	0.010	0.0001 ± 0.0	0.791
Formononetin (g/kg DM) (Form)	0.06–0.86	0.354	0.011 ± 0.01	0.588
Genistein (g/kg DM) (Genist)	0.001–0.076	0.017	0.0001 ± 0.001	0.374
Total Isoflavones (g/kg DM)	0.14–1.45	0.865	0.051 ± 0.028	0.670

The BLUP estimates for agronomic and biochemical traits across red clover lines are presented in Tables 2 and 3. The data demonstrate considerable variation for most traits. For example, the BLUP estimates for flowering time ranged from 28 days from the commencement of data collection (SA 19676) to 83.2 days after the commencement (M 98) ($p < 0.05$). Variation in leaf width was also evident with the smallest (SA 20017) being 38 % smaller at 13.7 mm than the largest (A 9086) at 22.2 mm ($p < 0.05$). Recovery of plants in autumn was closely correlated with cool season vigour which varied markedly; 17 accessions ranked ahead of the best cultivar (PAC 19, Chile). Accessions from Morocco, Tunisia, Sardinia, and Turkey were superior for cool season vigour with yield increases up to 55% above the best cultivar. Variation was also present for biochemical traits with BLUP estimates for biochanin content ranging from 0.09% (SA 32.374) to 0.91% DM (S 36) and total isoflavone content ranging from 0.14% to 1.45% DM.

Table 2. Mean effects of agronomic traits across red clover accessions.

Line No.	Cultivar/ Accession	Origin	Autumn Recovery	Flowering Time (days)	Growth Habit Spring/Summer	Leaf Shape	Prolificacy of Flowering	Stem Colour	Winter DM Yield (kg)	Leaf Width (mm)	Leaf Length (mm)	Total Leaf Area (log_e cm^2)	Stipule Pigmentation
1	PAC 19	Chile	1.94	71.9	2.47	2.12	4.12	1.53	6.58	19.6	24.3	2.19	2.04
2	A9807	Unknown	1.71	49.1	2.54	1.89	4.86	1.68	5.23	17.8	24.6	2.19	1.76
3	Rajah	Denmark	1.51	71.7	4.73	2.24	4.79	1.59	3.90	14.6	17.9	1.61	1.62
4	SA 26.449	Turkey	2.13	45.3	4.95	2.24	4.73	1.59	7.41	15.8	19.6	1.78	2.09
5	PI 4379	Unknown	1.60	43.3	3.09	2.16	5.41	1.87	5.78	17.3	23.7	2.11	2.33
6	SA 20.017	Turkey	1.86	30.4	5.45	2.16	4.65	1.72	6.10	13.7	18.0	1.58	2.92
7	PI 4377	Unknown	1.56	56.6	3.03	2.28	5.10	1.66	5.08	16.5	21.6	1.88	1.87
8	S48/9732	Turkey	1.75	45.4	5.61	2.15	4.65	1.75	6.07	16.1	21.3	1.89	2.67
9	El Sureno	Argentina	0.95	43.2	2.76	2.15	5.31	1.66	4.36	19.5	25.8	2.23	2.41
10	SA 758	Morocco	2.68	69.7	5.34	2.12	3.24	1.72	7.95	17.6	22.8	2.14	3.37
11	Renegade	USA	1.67	43.7	2.53	2.11	4.93	1.93	5.37	17.6	24.9	2.05	2.04
12	H: 4949	Unknown	1.67	51.2	3.06	2.24	5.76	1.55	6.33	17.6	22.5	2.09	1.76
13	Krano	Denmark	1.82	46.8	2.81	2.06	5.27	1.62	5.67	17.5	22.8	1.99	1.82
14	PI 5290	Unknown	1.47	52.7	2.95	2.14	5.35	2.02	5.92	18.3	23.6	1.91	2.39
15	T 106	Tunisia	2.54	46.4	4.91	2.06	4.71	2.53	9.88	17.6	23.8	2.15	3.08
16	SA 15.891	Turkey	1.74	71.5	4.45	2.14	3.43	1.89	5.29	14.2	19.1	1.56	2.43
17	H: 7565	Unknown	1.25	56.5	3.46	2.35	5.11	1.73	5.41	17.4	21.2	1.97	2.13
18	SA 22.825	UK	1.25	42.2	4.22	2.17	5.53	1.63	5.11	15.5	21.7	1.85	1.78
19	Cherokee	USA	1.42	38.3	2.90	2.11	5.14	1.62	5.89	17.5	24.1	2.05	1.84
20	SA 32.382	Turkey	1.92	34.7	4.99	2.13	4.45	1.80	6.42	15.7	20.2	1.73	2.61
21	S42/9635	Turkey	2.27	66.2	5.64	2.02	4.37	2.34	6.91	16.1	22.0	1.94	3.19
22	M 98	Morocco	2.75	83.2	5.65	2.11	3.39	1.73	8.64	17.2	22.1	2.05	2.93
23	SA 32.377	Turkey	2.02	48.3	5.23	2.28	4.17	1.85	6.87	17.4	22.5	2.05	2.02
24	SA 21.963	Uruguay	1.40	36.9	2.63	2.04	5.35	1.76	5.75	17.5	25.7	2.16	2.20
25	S36	Sardinia	2.48	55.2	5.71	2.10	3.79	1.97	8.53	15.9	20.5	1.82	2.48
26	SA 901	UK	1.61	49.3	3.36	2.34	5.19	1.71	5.54	17.0	21.1	1.84	2.20
27	SA 8.440	Russia	1.48	44.3	3.17	2.33	4.60	1.57	5.39	15.5	19.7	1.72	2.20
28	Colenso	New Zealand	1.34	58.0	2.89	2.28	5.31	1.59	5.08	17.5	22.2	2.08	1.91
29	SA 32.376	Turkey	2.16	53.5	5.05	2.22	4.77	1.76	6.93	16.8	22.0	1.96	2.05
30	SA 26.500	Turkey	2.05	46.0	4.93	2.15	4.66	2.08	6.72	16.3	24.3	2.05	2.66
31	Hamidori	Japan	1.50	60.4	2.78	2.26	4.54	1.71	4.60	15.7	18.9	1.67	1.50
32	M 80	Morocco	2.64	66.9	5.48	2.10	3.75	1.70	9.17	16.7	21.8	2.12	3.73
33	S44/9664	Turkey	1.93	40.9	5.54	2.08	5.31	2.29	6.68	16.1	22.2	1.88	2.89
34	Quinqueli	Chile	1.16	56.9	3.47	2.18	4.87	2.00	5.61	19.0	24.7	2.15	2.14
35	SA 18.686	Australia	1.47	43.5	3.29	2.22	5.56	1.67	6.15	18.0	23.2	2.04	2.12
36	SwisSelectn	Switzerland	1.63	49.4	2.19	2.22	4.74	1.45	5.51	19.4	24.8	2.23	1.78
37	S46/9679	Turkey	1.93	53.2	5.79	2.12	4.61	1.76	5.88	16.5	20.9	1.86	2.35
38	M 154	Morocco	2.54	69.8	5.74	2.46	3.16	1.75	8.10	16.8	21.2	1.98	2.32
39	A9806	Turkey	1.26	52.8	2.80	2.19	5.36	1.67	5.11	22.2	28.9	2.44	1.80
40	Redquin	Australia	1.45	45.1	2.82	2.22	5.42	1.71	4.91	19.8	24.4	2.14	1.79

Table 2. *Cont.*

Line No.	Cultivar/ Accession	Origin	Autumn Recovery	Flowering Time (days)	Growth Habit Spring/Summer	Leaf Shape	Prolificacy of Flowering	Stem Colour	Winter DM Yield (kg)	Leaf Width (mm)	Leaf Length (mm)	Total Leaf Area (log_e cm^2)	Stipule Pigmentation
41	Makimidori	Japan	1.51	57.2	2.76	2.18	4.75	1.96	5.17	15.4	18.6	1.70	2.24
42	SA 32.381	Turkey	2.10	49.8	5.18	2.18	4.05	1.55	7.32	17.7	24.2	2.20	2.46
43	S47/9706	Turkey	1.87	57.3	5.54	2.16	4.69	2.31	6.20	14.1	18.3	1.59	2.64
44	Concorde	Unknown	1.52	53.6	2.99	2.21	5.23	1.82	4.56	15.5	20.0	1.80	1.78
45	Maneta	France	1.69	68.3	2.57	2.24	3.92	1.71	5.65	17.8	22.2	1.92	2.13
46	SA 19676	Afghanistan	1.12	28.0	2.90	2.13	4.89	1.71	4.07	16.1	21.8	1.89	1.91
47	Hamua	New Zealand	1.21	53.0	2.92	2.07	5.92	1.59	4.95	16.7	22.7	1.95	2.36
48	PI 4383	Unknown	2.10	64.6	3.53	2.00	3.02	1.86	6.38	17.4	23.5	2.07	2.38
49	PI 4378	Unknown	1.14	51.6	2.85	2.32	5.28	1.83	5.39	16.9	22.2	1.99	1.94
50	SA 22.000	USA	1.14	43.6	2.57	2.24	5.51	1.66	4.59	18.7	23.8	2.00	2.36
51	SA 32.380	Turkey	1.19	41.6	3.83	2.07	5.19	1.59	4.93	17.2	24.3	2.09	2.03
52	SA 32.374	Turkey	2.09	29.5	5.29	1.88	4.36	1.84	6.91	16.8	22.2	1.90	2.11
53	S59/9895	Turkey	1.56	34.2	5.73	2.12	4.35	1.70	5.97	15.5	19.8	1.71	2.40
54	SA 32.061	Russia	2.16	51.0	5.85	2.22	4.28	1.91	6.83	16.5	21.0	1.88	2.23
55	FLMR7	USA	1.53	48.3	4.02	2.20	5.38	1.74	4.62	15.6	20.1	1.66	2.64
56	LE 116	Uruguay	1.63	33.2	2.49	2.15	5.75	1.56	6.61	21.0	27.6	2.36	1.95
57	PI 6435	Unknown	1.32	41.7	2.77	2.06	5.30	1.72	5.12	20.2	27.5	2.32	2.73
58	Kenland	USA	2.04	51.4	5.64	2.30	4.07	1.77	6.72	16.3	21.2	1.94	2.70
59	Astred	Portugal	1.28	43.2	2.70	2.40	5.30	1.74	5.12	18.0	22.7	2.04	1.78
60	S49/9742	Turkey	0.99	40.9	2.88	2.01	5.46	2.05	4.89	17.4	24.9	2.16	2.33
61	SA 12.274	Turkey	1.19	50.6	3.91	2.34	5.74	1.62	5.03	15.9	20.5	1.84	2.10
62	P42	Unknown	1.90	55.2	5.51	2.08	5.00	2.03	5.95	15.4	20.1	1.84	3.13
63	T 98	Tunisia	1.50	40.0	5.63	2.15	4.79	1.60	5.65	16.3	20.6	1.91	2.52
64	37796	Unknown	1.81	42.2	4.04	2.06	4.84	1.83	7.08	20.5	27.1	2.33	1.73
S.e.d			0.229	3.47	0.287	0.216	0.460	0.319	0.871	1.77	2.78	0.233	0.504

Table 3. Mean effects of isoflavone concentrations (%DM) across red clover accessions.

Cultivar/Line No.	Cultivar/Accession	Origin	Daidzein	Genistein	Formononetin	Biochanin	Total Isoflavone
1	PAC 19	Chile	0.011	0.04	0.59	0.62	1.26
3	Rajah	Denmark	0.009	0.01	0.67	0.53	1.25
4	SA 26.449	Turkey	0.006	0.00	0.28	0.59	0.88
5	PI 4379	Unknown	0.019	0.04	0.47	0.57	1.10
6	SA 20.017	Turkey	0.007	0.03	0.31	0.62	0.97
7	PI 4377	Unknown	0.014	0.02	0.68	0.63	1.34
8	S48/9732	Turkey	0.008	0.05	0.33	0.33	0.71
9	El Sureno	Argentina	0.005	0.08	0.59	0.78	1.45
10	SA 758	Morocco	0.008	0.03	0.13	0.10	0.27
11	Renegade	USA	0.014	0.02	0.48	0.50	1.01
13	Krano	Denmark	0.007	0.01	0.27	0.78	1.06
14	PI 5290	Unknown	0.008	0.01	0.51	0.67	1.20
15	T 106	Tunisia	0.060	0.01	0.29	0.17	0.53
16	SA 15.891	Turkey	0.005	0.03	0.41	0.56	0.99
18	SA 22.825	UK	0.010	0.01	0.32	0.82	1.16
19	Cherokee	USA	0.010	0.01	0.37	0.74	1.13
20	SA 32.382	Turkey	0.012	0.00	0.34	0.47	0.82
21	S42/9635	Turkey	0.007	0.01	0.44	0.54	0.99
22	M 98	Morocco	0.006	0.00	0.02	0.13	0.17
23	SA 32.377	Turkey	0.013	0.03	0.22	0.31	0.58
24	SA 21.963	Uruguay	0.009	0.01	0.57	0.62	1.20
25	S36	Sardinia	0.009	0.02	0.16	0.91	1.09
26	SA 901	UK	0.003	0.01	0.29	0.76	1.06
27	SA 8.440	Russia	0.008	0.01	0.23	0.45	0.68
28	Colenso	New Zealand	0.007	0.02	0.56	0.71	1.30
29	SA 32.376	Turkey	0.012	0.01	0.39	0.39	0.80
30	SA 26.500	Turkey	0.014	0.01	0.28	0.27	0.57
32	M 80	Morocco	0.006	0.00	0.58	0.07	0.14
33	S44/9664	Turkey	0.008	0.05	0.19	0.37	0.62
34	Quinqueli	Chile	0.005	0.01	0.62	0.66	1.23
35	SA 18.686	Australia	0.004	0.01	0.25	0.78	1.05
37	S46/9679	Turkey	0.006	0.01	0.35	0.44	0.80
38	M 154	Morocco	0.008	0.00	0.07	0.18	0.27
42	SA 32.381	Turkey	0.009	0.01	0.31	0.35	0.67
43	S47/9706	Turkey	0.018	0.03	0.37	0.23	0.65
46	SA 19676	Afghanistan	0.005	0.00	0.08	0.23	0.31
47	Hamua	New Zealand	0.005	0.01	0.52	0.70	1.23
49	PI 4378	Unknown	0.026	0.03	0.55	0.57	1.17
51	SA 32.380	Turkey	0.007	0.02	0.17	0.34	0.53
52	SA 32.374	Turkey	0.013	0.05	0.30	0.21	0.57
53	S59/9895	Turkey	0.004	0.01	0.09	0.09	0.19
54	SA 32.061	Russia	0.010	0.01	0.27	0.49	0.78
55	FLMR7	USA	0.006	0.00	0.28	0.50	0.79
56	LE 116	Uruguay	0.009	0.01	0.86	0.51	1.39
58	Kenland	USA	0.007	0.04	0.12	0.19	0.36
59	Astred	Portugal	0.018	0.02	0.55	0.68	1.26
61	SA 12.274	Turkey	0.004	0.01	0.56	0.72	1.29
62	P42	Unknown	0.010	0.01	0.38	0.66	1.06
63	T 98	Tunisia	0.013	0.00	0.25	0.16	0.43
S.e.d			0.011	0.340	0.231	0.271	0.408

2.2. Principal Component Analysis of Phenotypic and Biochemical Variance among Red Clover Accessions

The principal component plot (Figure 1) shows correlations between the various measured traits. Leaf length and width measurements tended to be positively correlated with leaf area. Lines such as number 44 (cv Concorde), which had long and wide leaves, had high prolificacy of flowering scores and higher total isoflavone concentrations. The biplot shows that there is the potential to identify lines with high concentrations of individual isoflavones and also with contrasting agronomic attributes.

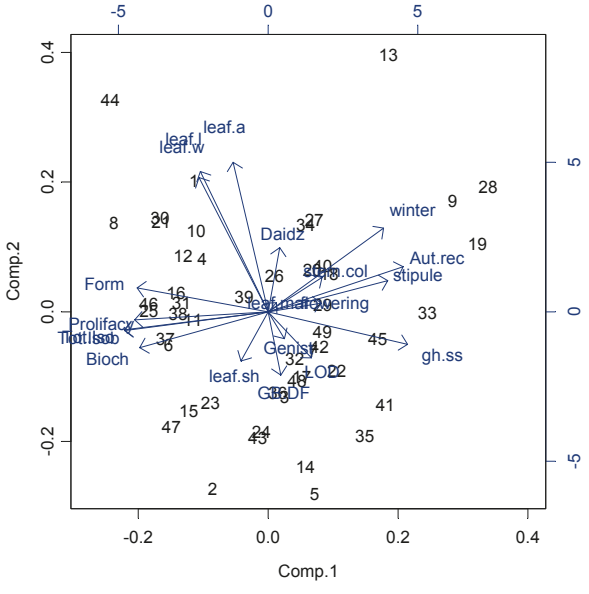


Figure 1. Principal component plot of biochemical and agronomic traits measured in 49 red clover cultivars/accessions. A description of the trait abbreviations used in this figure is given in Table 1.

2.3. Cluster Analysis of Red Clover Accessions

Clustering of the data grouped the 64 accessions into ten distinct clusters that had between one and twenty accessions (Figure 2). There were three accessions (M 98, A 9806, FLMR 7) that did not satisfactorily cluster with any other accessions and therefore needed to be classed as separate clusters.

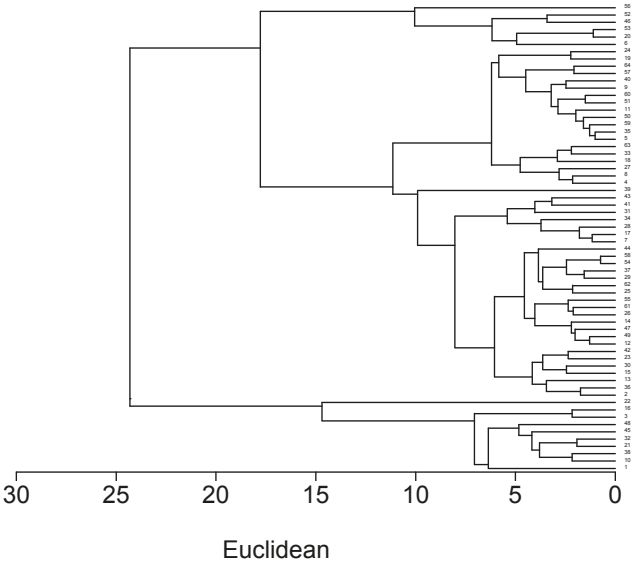


Figure 2. Clustering of 64 red clover accessions based on agronomic and phytoestrogen data.

3. Discussion

The traits measured in this experiment were similar to those descriptors used by Kouame and Quesenberry [12]. However, the addition of phytoestrogens provides valuable new insight into genetic variation for this important trait in the growth of red clover for pharmaceutical purposes. Many of the traits were highly correlated (Figure 1). For example, those accessions with long leaves such as A 9806, FLMR 7, LE 116, and SA 16658 tended also to have wide leaves and consequently had increased leaf area. However, there were some accessions such as SA 21963 and Renegade that had long but narrow leaves. Mediterranean accessions which displayed good autumn recovery in this experiment (e.g., S 42 and M 98) tended to have decreased flowering prolificacy. Plant breeders need to be mindful of this information when selecting material based on its autumn yield performance in isolation from other characteristics such as prolificacy of flowering.

There were several instances where accessions were found to have herbage yields that were superior to Astred, a popular cultivar commonly used in Australia and New Zealand. Astred is a stoloniferous cultivar selected from a Portuguese accession. It can reproduce through seed or vegetatively through stolons and daughter plants. In the absence of root diseases, Astred can be a most persistent variety; 55% ground cover has been measured after 16 years in Tasmanian pasture trials [18].

There are several different clustering approaches that have been successfully used to characterise plant accessions into similarity groups. Kouame and Quesenberry [16] used germplasm evaluation data from 800 accessions of red clover, which represented 41 countries of origin, and discovered large variations for most characters over all origins. They clustered the accessions using standardised values of 15 morphological and physiological descriptors and were able to identify three distinct groups that corresponded to early, medium, and late maturity groups. Different similarity groups were then identified within each of these three groups. This analysis revealed a large range of diversity among the red clover accessions over all origins, with the most diversity occurring in accessions collected from eastern and northern Europe, perhaps reflecting the history of breeding the species in those regions.

In this experiment, the 64 accessions evaluated fit into 10 distinct clusters. The accessions M 98, A9806, and FLMR 7 were not clustered to any other accessions and therefore were regarded as single clusters. There were several clusters however, where accessions were tightly linked to the commercial cultivars Astred, Colenso, and PAC 19. Accessions SA 23061 and SA 901 were tightly clustered together with Astred, while P1 4377 and P 42 were closely linked to Colenso, illustrating that these lines should be good sources of genetic variation similar to these commercially successful cultivars. The accessions linked closely with PAC 19 formed a cluster that was quite distinct from all other clusters. The mid-winter yield assessment confirmed the importance of Mediterranean material as a source of outstanding cool season vigour as has previously been emphasised in studies on white clover [19] and perennial grasses [20].

The fact that accessions clustered closely with the main cultivars, Astred and Colenso, shows that there are plants with similar characteristics that may serve as potential sources of resistance to diseases such as the root rot diseases that have limited the commercial use of red clover in Australia [21]. The study reported in this paper reflects a major characterization of red clover genetic resources under Australian conditions and may serve as a source of novel germplasm for future breeding and evaluation programs for either grazing or phytoestrogen production.

4. Materials and Methods

4.1. Germplasm

Over two years, 17 cultivars and 47 accessions were planted and characterized in the field. The cultivars and accessions and their country of origin are listed in Table 2.

4.2. Establishment

Seed of each of the 64 lines was sown in seedling trays filled with seed raising media. Seedlings were cultivated in unheated glasshouse with natural lighting for eight weeks and then removed from the glasshouse to harden outside prior to transplanting.

Seedlings were transplanted into a prepared seedbed in the field at Hamilton, Victoria (37°44′ S, 142°01′ E, alt. 200 m) in autumn 1999. Hamilton has a mean average annual rainfall of 703 mm. The site was finely cultivated with power harrows and fertilized with 200 kg/ha of a compound (NPK) fertilizer. The basalt-derived, duplex soil type at the experimental site was a clay-loam overlying heavy clay. Weed mat was laid over the prepared site and holes made at appropriate intervals for the plants.

4.3. Trial Design

The seedlings were transplanted into the field experiment as a row column design, latinised within columns. There were eight replicate blocks within which each entry was represented by a cell of four plants, randomised within the block design.

4.4. Plant Measurements

Plant measurements were taken over a two-year period for the following characters: growth habit (rating score 1 = erect, score 6 = prostrate), leaf markings and shape [22], leaf length, width and area, flowering time (days after November 1st 1999 when the plant had three or more fully opened flower heads, prolificacy of flowering (number of flowers per plant, rating score 1 = few, 9 = many), stem colour (score 1 = green, score 5 = red) and stipule pigmentation [22]. Recovery after summer drought was rated in autumn 2000 (1 = poor, 3 = good). Cool season vigour was assessed in July 2000; all plants were rated (1 = poor, 10 = good) and 10 plants (1 for each rating) were randomly selected from each block. These plants were cut at 50 mm above ground level and the harvested material dried and weighed in order to calibrate the visual score and so provide an estimate of the yield of dry matter for all plants. Samples for biochemical analysis were collected in October 2000 at hay cutting time. Two young, fully developed leaves were harvested from each plant and were bulked into three replicates.

4.5. Biochemical Analysis

The concentration of the phytoestrogens, daidzein, genistein, formononetin, and biochanin in leaves were determined for 49 of the cultivars/accessions in the analytical laboratories of Novogen Ltd., North Ryde, NSW. Isoflavones were analyzed using a modification of previously published methods [23,24]. Aliquots (10 mL) of alcohol extract from leaf material were mixed with 100 mL of glucuronidase. The mixture was incubated for 24 h at 37 °C after which it was extracted on a C-18 solid phase extraction column (Waters Pty. Ltd., Sydney, Australia). Isoflavones were eluted with 3 mL of methanol and 10 mL of the extract was injected into the high-performance liquid chromatography system. The high-performance liquid chromatography system consisted of a 25-cm, 5 nM, C-18 stationary phase column (Symmetry, Waters Pty. Ltd.) and a gradient acetonitrile/water mobile phase. The limit of detection of the assay for each of the isoflavones measured was 5 ng/mL. The interassay coefficient of variation (CV) was <15%.

4.6. Statistical Analysis

Data from the experiment were analysed using the method of residual maximum-likelihood (REML) [25] to derive best linear unbiased predictor (BLUP) estimates for each of the characteristics measured for each entry. To display the relationship among accessions and traits in the data (using BLUP estimates), a biplot graphical representation was used. For this a principal component analysis (PCA) was performed using S-Plus 2000 (MathSoft, Inc., Cambridge, MA, USA). The relationship between the accessions and the traits were displayed using a point-vector plot, with points representing

accessions, and directional vectors representing traits. The angles between the vectors reflected the correlation structure among traits. By drawing a perpendicular line from the treatment points to the trait vectors, the trait measurements for the accessions can be compared with the average, which is represented by the origin. Equal scaling of the component axes was needed for accurate projection of the points onto the trait vectors. Finally, an agglomerative, hierarchical grouping technique was used with squared Euclidean distance as the dissimilarity measure, based on the BLUP estimates, was carried out using S-Plus 2000 (MathSoft, Inc. Cambridge, MA, USA).

5. Conclusions

Significant variation for agronomic and biochemical traits was found amongst accessions. In several instances, their yield attributes were superior to the current major cultivar used in Australia and New Zealand, Astred. Clustering helped to identify several red clover accessions that were tightly linked to commercial cultivars (viz. Astred, Colenso, and PAC 19) and a valuable source of variation for a genetic improvement program.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/7/2/34/s1.

Acknowledgments: The authors wish to thank L. Huang and A.J. Husband from Novogen Ltd. for assistance with biochemical analyses.

Author Contributions: K.R. and K.S. conceived and designed the experiments; V.L. performed the experiments; K.S. and V.L. analyzed the data; K.S. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Smith, R.S. Red Clover (*Trifolium pratense*). In *Alternative Pasture Legumes 1993, Proceedings of the 2nd National Alternative Pasture Legumes Workshop, Coonawarra, Australia, 25–28 July 1993*; Michalk, D.L., Craig, A.D., Collins, W.J., Eds.; South Australian Research and Development Institute: Urrbrae, Australia, 1994.
2. Fergus, E.N.; Hollowell, E.A. Red Clover. *Adv. Agron.* **1960**, *12*, 365–436.
3. Clark, S.G.; Reed, K.F.M. Performance of Grassland Hamua × Moroccan red clover crosses in a marginal environment. In Proceedings of the 4th Australian Society of Agronomy Conference, Melbourne, Australia, August 1987; Australian Society of Agrononoy: Melbourne, Australia, 1987.
4. Moot, D.J. An overview of dryland legume research in New Zealand. *Crop Pasture Sci.* **2012**, *63*, 726–733. [CrossRef]
5. Nichols, P.G.H.; Revell, C.K.; Humphries, A.W.; Howie, J.H.; Hall, E.J.; Sandral, G.A.; Ghamkhar, K.; Harris, C.A. Temperate pasture legumes in Australia—Their history, current use, and future prospects. *Crop Pasture Sci.* **2012**, *63*, 691–725. [CrossRef]
6. Kenny, P.T.; Reed, K.F.M. Effects of pasture type on the growth and wool production of weaner sheep during summer and autumn. *Aust. J. Exp. Agric. Anim. Husb.* **1984**, *24*, 322–331. [CrossRef]
7. McDonald, M.F. Effects of plant oestrogens in ruminants. *Proc. Nutr. Soc.* **1995**, *NZ 20*, 43–51. [PubMed]
8. Oram, R.N. *Register of Australian Herbage Plant Cultivars*; CSIRO: Melbourne, Australia, 1990.
9. IP Australia. Plant Breeders Rights Database. IP Australia: Australian Government: Canberra. Available online: www.ipaustralia.gov.au/get-the-right-ip/plant-breeders-rights (accessed on 1 May 2017).
10. Dornstauder, E.; Jisa, E.; Unterrieder, I.; Krenn, L.; Kubelka, W.; Jungbauer, A. Estrogenic activity of two standardized red clover extracts (Menoflavon®) intended for large scale use in hormone replacement therapy. *J. Steroid Biochem. Mol. Biol.* **2001**, *78*, 67–75. [CrossRef]
11. Alimoradi, A.; Mahdizad, H.F. Effects of phytoestrogens on bone mineral density during the menopause transition: A systematic review of randomized controlled trials. *Climacteric* **2016**, *19*, 535–545.
12. Ghazanfarpour, M.; Sadeghi, R.; Roudsari, R.L.; Khorsand, I.; KhadivZedah, T.; Muoio, B. Red clover for treatment of hot flashes and menopausal symptoms: A systematic review and meta-analysis. *J. Obstet. Gynaecol.* **2016**, *36*, 301–311. [CrossRef] [PubMed]
13. Chen, M.N.; Lin, C.C.; Liu, C.F. Efficacy of phytoestrogens for menopausal symptoms: A meta-analysis and systematic review. *Climacteric* **2016**, *18*, 260–269. [CrossRef] [PubMed]

14. Clifton-Bligh, P.B.; Nery, M.L.; Clifton-Bligh, R.J.; Visvalingam, S.; Fulcher, G.R.; Byth, K.; Baber, R. Red clover isoflavones enriched with formonetin lower serum LDL cholesterol—A randomized, double-blind, placebo-controlled study. *Eur. J. Clin. Nutr.* **2015**, *69*, 134–142. [CrossRef] [PubMed]
15. Francis, C.M.; Quinlivan, B.J. Selection for formononetin content in red clover (Trifolium pratense). In *Plant Introduction, Breeding and Seed Production, Proceedings of the XII International Grassland Congress, Moscow, Soviet Union, 11–20 June 1974*; Organizing Committee of the International Grassland Congress: Moscow, Soviet Union, 1974; pp. 84–88.
16. Kouame, C.N.; Quesenberry, K.H. Cluster analysis of a collection of red clover germplasm. *Genet. Resour. Crop Evol.* **1993**, *40*, 39–47. [CrossRef]
17. Williams, W.M. Genetics and Breeding. In *White Clover*; Barker, M.J., Williams, W.M., Eds.; CAB International: Wallingford, UK, 1987.
18. Smith, R.S.; Bishop, D.J. Astred—A stoloniferous red clover. In Proceedings of the 17th International Grassland Congress, Palmerston North, New Zealand, 8–21 February 1993; Organising Committee International Grassland Congress: Palmerston North, New Zealand, 1993; pp. 421–423.
19. Reed, K.F.M.; Cade, J.W.; Williams, A.E. The significance of Mediterranean plant introductions for increasing the winter growth of pasture in the high rainfall areas of Victoria. In Proceedings of the 1st Australian Agronomy Conference, Lawes, Australia, April 1980; Wood, I.M., Ed.; Australian Society of Agronomy: Sydney, Australia, 1980; p. 238.
20. Reed, K.F.M.; Clement, S.L.; Feely, W.F.; Clark, B. Improving tall fescue for cool season vigour. *Aust. J. Exp. Agric.* **2004**, *44*, 873–881. [CrossRef]
21. Dear, B.S.; Moore, G.A.; Hughes, S.J. Adaptation and potential contribution of temperate perennial legumes to the southern Australian wheatebelt: A review. *Aust. J. Exp. Agric.* **2003**, *52*, 973–983. [CrossRef]
22. Cade, J.; Stapleton, P. *How to Identify Sub Clover Varieties. Agricultural Notes—Agdex 137/33*; Natural Resources and Environment: Victoria, Australia, 1984; pp. 1–4.
23. Setchell, K.D.; Welsh, M.B.; Lim, C.K. High-performance liquid chromatographic analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical and thermospray mass spectrometric detection. *J. Chromatogr.* **1987**, *386*, 315–323. [CrossRef]
24. Franke, A.A.; Custer, L.J.; Cerna, C.M.; Narala, K. Rapid HPLC analysis of dietary phytoestrogens from legumes and from human urine. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 18–26. [CrossRef]
25. GenStat Committee. *GenStat®Release 7.1*; VSN International Ltd.: Oxford, UK, 2003.

agronomy

MDPI

Article

Molecular Characterizations of Kenyan Brachiaria Grass Ecotypes with Microsatellite (SSR) Markers

Naftali Ondabu [1,2], Solomon Maina [1], Wilson Kimani [1], Donald Njarui [2], Appolinaire Djikeng [1] and Sita Ghimire [1,*]

[1] Biosciences Eastern and Central Africa-International Livestock Research Institute (BecA-ILRI) Hub, P.O. Box 30709, Nairobi 00100, Kenya; gondabu@yahoo.com (N.O.); solomon.dna.maina@gmail.com (S.M.); wilkims2005@yahoo.com (W.K.); A.Djikeng@cgiar.org (A.D.)

[2] Kenya Agricultural and Livestock Research Organization (KALRO), P.O. Box 57811, Nairobi 00200, Kenya; donaldnjarui@yahoo.com (D.N.)

* Correspondence: s.ghimire@cgiar.org; Tel.: +254-20-422-3820

Academic Editors: John W. Forster and Kevin F. Smith
Received: 24 October 2016; Accepted: 18 January 2017; Published: 9 February 2017

Abstract: Brachiaria grass is an emerging forage option for livestock production in Kenya. Kenya lies within the center of diversity for *Brachiaria* species, thus a high genetic variation in natural populations of *Brachiaria* is expected. Overgrazing and clearing of natural vegetation for crop production and nonagricultural uses and climate change continue to threaten the natural biodiversity. In this study, we collected 79 *Brachiaria* ecotypes from different parts of Kenya and examined them for genetic variations and their relatedness with 8 commercial varieties. A total of 120 different alleles were detected by 22 markers in the 79 ecotypes. Markers were highly informative in differentiating ecotypes with average diversity and polymorphic information content of 0.623 and 0.583, respectively. Five subpopulations: International Livestock Research Institute (ILRI), Kitui, Kisii, Alupe, and Kiminini differed in sample size, number of alleles, number of private alleles, diversity index, and percentage polymorphic loci. The contribution of within-the-individual difference to total genetic variation of Kenyan ecotype population was 81%, and the fixation index (F_{ST} = 0.021) and number of migrant per generation (Nm = 11.58) showed low genetic differentiation among the populations. The genetic distance was highest between Alupe and Kisii populations (0.510) and the lowest between ILRI and Kiminini populations (0.307). The unweighted neighbor-joining (NJ) tree showed test ecotypes grouped into three major clusters: ILRI ecotypes were present in all clusters; Kisii and Alupe ecotypes and improved varieties grouped in clusters I and II; and ecotypes from Kitui and Kiminini grouped in cluster I. This study confirms higher genetic diversity in Kenyan ecotypes than eight commercial varieties (Basilisk, Humidicola, Llanero, Marandú, MG4, Mulato II, Piatá and Xaraés) that represent three species and one three-way cross-hybrid Mulato II. There is a need for further collection of local ecotypes and their morphological, agronomical, and genetic characterizations to support Brachiaria grass breeding and conservation programs.

Keywords: analysis of molecular variance; breeding; fixation index; genetic conservation; private allele

1. Introduction

Brachiaria grass is one of the most important tropical grasses distributed throughout the tropics, especially in Africa [1]. The genus *Brachiaria* consists of about 100 documented species of which 7 perennial species of African origin have been used for pasture production in South America, Asia, South Pacific, and Australia [2]. It has high biomass production potential and produces nutritious herbage resulting in increased livestock productivity [3,4]. *Brachiaria* is adapted to drought and

low-fertility soils, sequesters carbon through its large root system, enhances nitrogen use efficiency, and subsequently minimizes eutrophication and greenhouse gas emissions [5–8]. *Brachiaria* plays important roles in soil erosion control and ecological restoration. *Brachiaria* species have been an important component of sown pastures in humid lowlands and savannas of tropical America, with current estimated acreage of 99 million hectares in Brazil alone [9].

In Africa, the evaluations of *Brachiaria* species for pasture improvement started during the 1950s. These researches focused on *B. brizantha*, *B. decumbens*, *B. mutica*, and *B. ruziziensis* for forage production, agronomy (establishment, drought, cutting intervals, and fertilizers), compatibility with herbaceous and tree legumes, nutritive values, and their benefit to ruminant production. These studies concluded the suitability and broader adaptation of several *Brachiaria* species to different agroecological zones in Africa [10]. However, these practices were not widespread because of ample communal grazing lands, limited realization on roles of sown pasture in the livestock production, subsistence animal farming, and low government priority to pasture development. Recently, the mounting demand for livestock products in Africa has renewed interest of farmers, researchers, development agencies, and government organizations on forages, particularly in species with good adaptability to climate change such as Brachiaria grass. Therefore, there has been multiple repatriations of Brachiaria grass to Africa in the form of hybrids and improved landraces [11,12]. These materials have shown positive performance in terms of biomass production, improved forage availability and livestock productivity in Kenya and Rwanda. These results have revealed *Brachiaria* as an ideal forage option for the livestock farmers in East Africa.

Despite high popularity, the Brachiaria acreage in Africa is low and relies on a few varieties that were developed for tropical Americas and Australia. Within a short period of introduction, some of these varieties have shown susceptibility to pests and diseases, raising question on the expansion of Brachiaria acreage in Africa with these varieties. There is therefore a need for an Africa-based *Brachiaria* improvement program to develop varieties that are tolerant to biotic and abiotic stress for different environmental conditions. Germplasms of broad genetic base is the prerequisite for any crop improvement. The best approach to increase genetic variations in apomictic species such as *Brachiaria* is tapping natural variations from the center of diversity. Since the 1950s, multiple missions were undertaken in Africa to collect *Brachiaria* germplasms, with a current inventory of 987 accessions of 33 known *Brachiaria* species [13]. Considering distribution of *Brachiaria* in Africa and size of the continent, the number of samples available in collection is definitely non-exhaustive and warrants further collection efforts. However, the existence of these genetic resources in Africa is continuously threatened by overgrazing and clearing of vegetation for crop production and nonagricultural uses and adverse effects of climate change.

Kenya is located within a region that represents a center of diversity for genus *Brachiaria*. Therefore, high natural variation is expected among *Brachiaria* populations in Kenya. This study aimed to create a collection of local *Brachiaria* ecotypes in Kenya, assess their genetic diversity using microsatellite markers, and examine their genetic relationships with eight commercial cultivars. The study will broaden geographical coverage and/or genetic base of the global *Brachiaria* collection and provide invaluable information for *Brachiaria* improvement and conservation programs.

2. Results

2.1. Descriptive Statistics for Simple Sequence Repeat (SSR) Markers

Descriptive statistics for all marker sets were computed (Table 1). The major allele frequency ranged from 0.2405 (Brz3002) to 0.8228 (Brz0076) with a mean of 0.5184. The number of different alleles ranged from 3 (Brz0029) to 10 (Brz0130) with a mean of 5.45. The genetic diversity averaged to 0.6225 with a range of 0.3169–0.8021. Similarly, the polymorphic information content (PIC) ranged from 0.3087 (Brz0076) to 0.8384 (Brz3002) with a mean of 0.5825.

Table 1. Descriptive statistics for microsatellite markers.

Marker	MAF	N_{DA}	*I*	*PIC*
Brz0012	0.4304	5	0.7101	0.6670
Brz0028	0.4304	5	0.6521	0.5892
Brz0029	0.6203	3	0.5124	0.4327
Brz0067	0.4051	5	0.7419	0.7061
Brz0076	0.8228	3	0.3169	0.3087
Brz0087	0.481	8	0.6983	0.6649
Brz0092	0.8101	5	0.3352	0.3240
Brz0100	0.4684	4	0.6614	0.6052
Brz0115	0.3671	7	0.8021	0.7829
Brz0117	0.6076	6	0.5371	0.4676
Brz0118	0.5063	4	0.5573	0.4613
Brz0122	0.4557	6	0.6739	0.6225
Brz0130	0.3418	10	0.7947	0.7706
Brz0149	0.7722	5	0.3874	0.3679
Brz0156	0.6456	4	0.5365	0.497
Brz0203	0.3671	7	0.7685	0.7379
Brz0212	0.5823	8	0.6195	0.5906
Brz0213	0.7468	4	0.4192	0.3932
Brz0214	0.4304	7	0.7432	0.7138
Brz0235	0.4051	4	0.7438	0.709
Brz3002	0.2405	5	0.854	0.8384
Brz3009	0.4684	5	0.6313	0.5643
Mean	0.5184	5.45	0.6225	0.5825

MAF = minor allele frequency, N_{DA} = number of different alleles, *I* = Shannon's genetic diversity, and *PIC* = polymorphic information content.

2.2. Population Diversity Indices

The population diversity indices for five ecotype populations from Kenya were summarized (Table 2). The International Livestock Research Institute (ILRI) population had highest number of different alleles, and the Alupe population had the least. The number of private alleles was highest for the ILRI population and the lowest for Kisii population. The information index ranged from 0.408 to 0.887 with a mean of 0.599. The observed heterozygosity was higher than expected for all populations. The percentage polymorphic loci ranged from 46.47% (Kitui) to 86.87% (ILRI).

Table 2. Summary of population diversity indices averaged over 22 simple sequence repeat (SSR) markers.

Population	*N*	*Na*	*Np*	*Ae*	*I*	*Ho*	*He*	*PL* (%)
ILRI	60	3.633	0.833	2.21	0.887	0.76	0.499	86.67
KITUI	3	1.233	0.133	1.171	0.408	0.417	0.261	46.67
KISII	5	1.567	0.067	1.396	0.498	0.537	0.315	56.67
ALUPE	4	1.6	0.0133	1.486	0.524	0.544	0.333	60.00
KIMIN	7	2.133	0.1	1.833	0.678	0.647	0.41	70.00
Mean	15.8	2.033	0.22926	1.619	0.599	0.581	0.364	64.00

N = number of samples, *N*a = number of different Alleles, *N*p = number of private alleles, *A*e = number of effective alleles, *I* = Shannon's information Index, *H*o = observed heterozygosity, *H*e = expected heterozygosity and *PL* = percentage polymorphic loci.

2.3. Genetic Diversity and Relationships

The pairwise genetic distance and population matrix of Nie genetic identity were calculated (Table 3). The genetic distance was highest between Alupe and Kitui populations (0.510), whereas it was the lowest between ILRI and Kiminini populations (0.307). Similarly, genetic identity was the highest between ILRI and Kiminini populations (0.636) and the lowest between Alupe and Kitui populations (0.235). The principal coordinate analysis (PCoA) plot of ecotypes from five populations showed no distinct clustering pattern (Figure 1). The first two principal coordinates explained 18.27% of the total genetic variation within the test ecotypes. Specifically, the first and second coordinates explained 10.85% and 7.42% of the total genetic variation, respectively. However, an unweighted neighbor-joining tree of 79 ecotypes and 8 commercial cultivars showed them grouped into three distinct clusters (Figure 2). Cluster I included 50 ecotypes from all five populations and six cultivars,

cluster II included 21 ecotypes from three populations (Alupe, ILRI, and Kisii) and two cultivars, and cluster III included 8 ecotypes, all from the ILRI population.

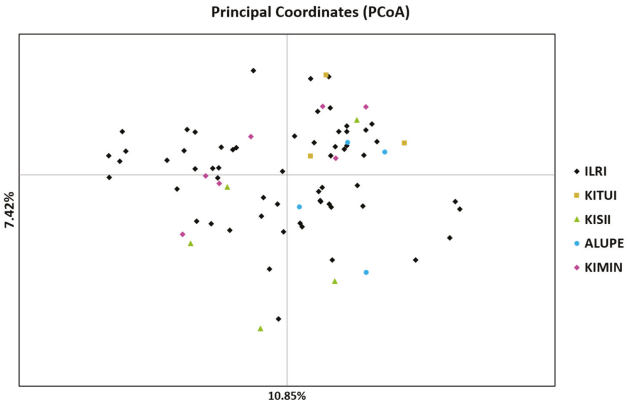


Figure 1. Principal coordinates analysis (PCoA) biplot showing the clustering of the 79 *Brachiaria* ecotypes from different parts of Kenya (orange = Kitui, black = International Livestock Research Institute (ILRI) Farm, green = Kisii, blue = Alupe, and purple = Kiminini).

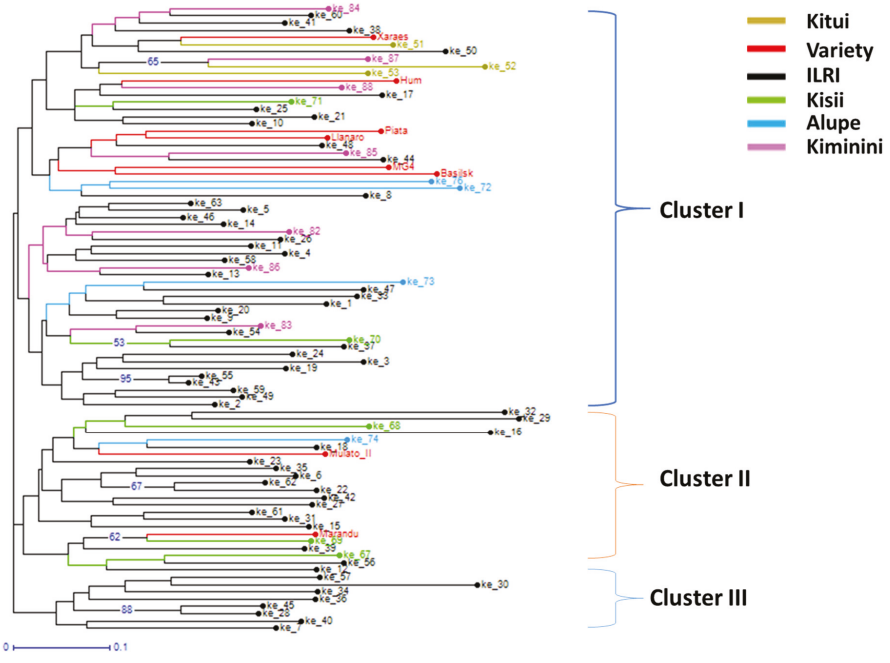


Figure 2. Unweighted neighbor-joining tree using the simple matching dissimilarity coefficient based on 22 microsatellite loci for all collected 79 *Brachiaria* ecotypes (ke_1 to ke_88) collected from different parts of Kenya (orange = Kitui, red = commercial cultivars, black = ILRI Farm, green = Kisii, blue = Alupe, and purple = Kiminini), and 8 commercial cultivars (*B. brizantha* cvs. Marandú, MG4, Piatá, and Xaraés; *B. decumbens* cv. Basilisk; *B. humidicola* cvs. Humidicola and Llanero; and three-species-ways cross-hybrid Mulato II.

Table 3. Pairwise genetic distance based on shared allele (left) and population matrix of Nie genetic identity (right) among the *Brachiaria* ecotype population from Kenya.

Population	Alupe	ILRI	Kiminini	Kisii	Kitui
Alupe	-	0.462	0.388	0.323	0.235
ILRI	0.393	-	0.636	0.440	0.327
Kiminini	0.448	0.307	-	0.399	0.299
Kisii	0.467	0.392	0.446	-	0.247
Kitui	0.510	0.441	0.413	0.503	-

2.4. Analysis of Molecular Variance (AMOVA)

The partitioning of the total variation in population at different levels was estimated with AMOVA (Table 4). Within-the-individual difference contributed highest (81%) to total variation followed by among-individual difference (17%) and among-population differences (2%). The fixation index (F_{ST}) and number of immigration per generation (Nm) for study populations were 0.021 and 11.585, respectively.

Table 4. Analysis of molecular variance among and within populations, and within individuals for *Brachiaria* accessions based on 22 SSR loci.

Source	Degree of Freedom	Sum of Squares	Mean Squares	Estimated Variance	Variation (%)	*p* Values
Among Populations	4	43.440	10.860	0.155	2%	0.023
Among Individual	74	619.649	8.374	1.215	17%	0.001
Within Individual	79	469.500	5.943	5.943	81%	0.001
Total	157	1132.589		7.313	100%	
F_{ST} = 0.021 and Nm = 11.580						

F_{ST} = Fixation index; Nm = Number of migration per generation.

3. Discussion

The genetic complexity, primarily apomictic mode of reproduction, and abundant natural variations in Africa urge for a two-pronged approach (selection and breeding) for improving Brachiaria grass in Africa. All-inclusive germplasm base with documented variations are prerequisite for the effective breeding programs. This study collected 79 *Brachiaria* ecotypes in Kenya and documented their genetic variations using microsatellite markers.

The PIC values for 22 SSR markers averaged to 0.5825, suggesting markers were capable of differentiating 79 Kenyan *Brachiaria* ecotypes. The PIC value in this study is within the range reported by Silva et al. [14], Jungmann et al. [15], and Vigan et al. [16], but was lower than that found by Jungmann et al. [17] and Pessoa-Filho et al. [18]. Similarly, the average numbers of allele detected per loci (5.45) was in the range reported by Silva et al. [14], Jungmann et al. [15], and Vigan et al. [16], but was about half and one-third of that reported by Jungmann et al. [17] and Pessoa-Filho et al. [18], respectively. However, these comparisons between studies may not be conclusive due to differences in types and number of germplasms and markers used among studies.

The analysis of the distributions of alleles across populations is important for explaining genetic diversity and population relationships [19]. Private alleles are important in plant breeding and conservation as they are present only in a single population among a broader collection of populations [20]. Five ecotypes populations of Kenya were different for private alleles, with the highest number of private alleles in the ILRI population and the least in the Kiminini population. Such variations in the private alleles among populations most likely was the effect of the number of individuals per population, which ranged from 3 to 60 individuals. Although no information was available on species composition of each population, it is likely the presence of multiple species resulting in a high number of private alleles in some populations. Irrespective of populations, H_O was higher than H_E, indicating mixing of previously isolated populations. This is consistent with the human involvement in moving planting materials and outcrossing nature of some *Brachiaria* species, for example, *B. ruziziensis*.

The study population varied in genetic distance and genetic identity coefficients. The highest genetic distance between Alupe and Kitui populations can be explained by the wider geographical distance between these two locations (675 km), but the genetic distance between other populations could not be associated to geographical proximity. Reports are available on forage research, including seed production of *B. ruziziensis* in Kitale, Kenya [21,22], and involvement of Kenya Agricultural Research Institute and Kenya Seed Company in the past in production and trading of *B. ruziziensis* seeds [23]. It is likely that some of these *Brachiaria* seeds might have reached farmers' fields and other research stations in Kenya, including the ILRI, and afterwards naturalized in the wild. If this hold true, a low genetic distance (0.307) between the ILRI and Kiminini (20 km away from Kitale) populations could be because of shared genetic materials in early days.

The contribution of within-individual difference to total variation was 81%, whereas among-the-individual and among-populations differences contributed 17% and 2%, respectively (Table 5). These observations were in agreement with Vigna et al. [16] and Pessoa-Filho et al. [18], who reported high contributions of within-the-accession/individual differences to total variation in *B. brizantha* (84%) and *B. ruzizi*ensis (88%) populations. Similarly, Garcia et al. [24] and Azevedo et al. [25] reported 73% and 65% of total variation attributed to within species or cluster, respectively. However, Jungmann et al. [26] reported 44% of the variation in *B. humidicola* accessions as being due to the subdivision of the germplasms into five groups. The F_{ST} and effective number of migrants per generation (*N*m) values of 0.021 and 11.580 indicated a relatively low genetic differentiation among populations [27] and relatively high level of gene flow among the Kenyan ecotypes populations [28], respectively. A low genetic differentiation among the study populations could be associated with apomictic mode of reproduction, variable ploidy causing meiotic anomalies leading to reduced pollen fertility, and dispersal of seeds by migratory herbivorous and human activities such as hay transportation for feeding animals [16,26,29–32]. Polyploid plants are effective colonizers that can occupy pioneer habitats and generate individuals that are able to exploit new niches or outcompete progenitor species, whereas apomictic polyploid plants can fix heterosis [16,26,30].

Table 5. Microsatellite markers, primer sequences, annealing temperature (*T*a), allele sizes, and number of repeat motifs (adapted from Silva et al. [14]).

Marker	Forward Primer	Reverse Primer	*Ta* (°C)	Allele Size (bp)	Repeat Motif
Brz0012	ACTCAAACAATCTCCAACACG	CCCACAAATGGTGAATGTAAC	59	160	$(AT)^{8}$
Brz0028	CATGGACAAGGAGAAGATTGA	TGGGAGTTAACATTAGTGTTTTT	57	158	$(TA)^{8}$
Brz0029	TTTGTGCCAAAGTCCAAATAG	TATTCCAGCTTCTTCTGCCTA	56	150	$(AG)^{14}$
Brz0067	TTAGATTCCTCAGGACATTGG	TCCTATATGCCGTCGTACTCA	51	156	$(AT)^{9}$
Brz0076	CCTAGAATGCGGAAGTAGTGA	TTACGTGTTCCTCGACTCAAC	58	151	$(AT)^{7}$
Brz0087	TTCCCCCACTACTCATCTCA	AACAGCACACCGTAGCAAGT	60	243	$(GA)^{9}$
Brz0092	TTGATCAGTGGGAGGTAGGA	TGAAACTTGTCCCTTTTTCG	54	251	$(AT)^{6}$
Brz0100	CCATCTGCAATTATTCAGGAAA	GTTCTTGGTGCTTGACCATT	56	256	$(AT)^{11}$
Brz0115	AATTCATGATCGGAGCACAT	TGAACAATGGCTTTGAATGA	59	252	$(AT)^{6}$
Brz0117	AGCTAAGGGGCTACTGTTGG	CGCGATCTCCAAAATGTAAT	60	260	$(TA)^{5}$
Brz0118	AGGAGGTCCAAATCACCAAT	CGTCAGCAATTCGTACCAC	57	252	$(CT)^{11}$
Brz0122	CATTGCTCCTCTCGCACTAT	CTGCAGTTAGCAGGTTGGTT	57	253	$(CA)^{6}$
Brz0130	TCCTTTCATGAACCCCTGTA	CATCGCACGCTTATATGACA	57	248	$(CT)^{14}$
Brz0149	GCAAGACCGCTGTTAGAGAA	CTAACATGGACACCGCTCTT	57	245	$(AT)^{11}$
Brz0156	GCCATGATGTTTCATTGGTT	TTTTGCACCTTTCATTGCTT	58	260	$(AC)^{7}$
Brz0203	CGCTTGAGAAGCTAGCAAGT	TAGCCTTTTGCATGGGTTAG	57	301	$(GA)^{8}$
Brz0212	ACTCATTTTCACACGCACAA	CGAAGAATTGCAGCAGAAGT	57	301	$(CA)^{5}$
Brz0213	TGAAGCCCTTTCTAAATGATG	GAACTAGGAAGCCATGGACA	57	296	$(CA)^{7}$
Brz0214	TCTGGTGTCTCTTTGCTCCT	TCCATGGTACCTGAATGACA	57	309	$(AT)^{8}$
Brz0235	CACACTCACACACGGAGAGA	CATCCAGAGCCTGATGAAGT	57	298	$(TC)^{9}$
Brz3002	GCTGGAATCAGAATCGATGA	GAACTGCAGTGGCTGATCTT	57	160	$(AAT)^{7}$
Brz3009	AGACTCTGTGCGGGAAATTA	ACTTCGCTTGTCCTACTTGG	55	151	$(AAT)^{10}$

This study is an effort to build a collection of *Brachiaria* ecotypes in Kenya and identify the potential values of these genetic resources in the *Brachiaria* breeding program. It is important to acknowledge some methodological limitations of this study while inferring population genetic parameters such as unequal and/or small sample sizes (3–60 individuals per population), unknown species and ploidy status of ecotypes, and dominant scoring scheme used in recording SSR fragments. The current *Brachiaria* taxonomy is far from satisfactory and the problem of generic identity, and species composition across entire taxa needs to be undertaken [1,22]. Application of robust genetic markers and bioinformatics procedures in genetic analysis of *Brachiaria* spp. have been constrained by a limited understanding of *Brachiaria* genetics, cytogenetic and reproductive biology, and unavailability of reference genome. The agricultural and environmental importance of *Brachiaria* has recently spurred several studies on *Brachiaria*, including sequencing of *B. ruziziensi*s genome. Therefore, Kenyan ecotypes collected in this study should be conserved and characterized further with the advent of new genomics and bioinformatics tools developed for species with complex genome.

This is among the very first studies of this century in sub-Saharan Africa that involved collection of local *Brachiaria* ecotypes from different parts of Kenya and examination of their genetic differences using microsatellite markers. The genetic diversity data revealed that ecotypes, though representing a few locations of Kenya, contained much more diversity than currently available 8 improved *Brachiaria* varieties, which represent three species (*B. brizantha*, *B. decumbens*, and *B. humidicola*) and three-way cross-hybrid Mulato II (*B. brizantha* × *B. decumbens* × *B. ruziziensis*). These results clearly indicate a need for (I) further collection of local ecotypes in Kenya and other east and central African countries that represent center of diversity of *Brachiaria* species to enrich the *Brachiaria* genepool in the gene bank collections; (II) genetic characterization of local ecotypes and currently available gene bank materials to understand diversity and ascertain the need for further collection; and (III) morphological characterization of available genetic resource to identify/develop varieties suitable for different production environments.

4. Experimental Section

4.1. Source of Plant Materials

Whole plant sample of 79 *Brachiaria* ecotypes were collected from five different parts of Kenya: Alupe (n = 4), ILRI Farm (n = 60), Kiminini (n = 7) Kisii (n = 5), and Kitui (n = 3) in 2013 and 2014, and maintained in field at forage research plots of International Livestock Research Institute (ILRI), Headquarters, Nairobi, Kenya. Seeds of eight improved varieties—*B. decumbens* cv. Basilisk, *B. brizantha* cvs. Marandú, Xaraés, Piatá, and MG4, *B. humidicola* cvs. Humidicola and Llanero (Marangatu Sementes, Ribeirão Preto, São Paulo, Brazil), and Mulato II (Tropical Seeds, Coral Springs, FL, USA)—were planted in a variety demonstration plot at the ILRI Campus. About 4-week-old leaves were harvested from all 79 ecotypes and 8 varieties (one sample/variety), freeze-dried, and stored at −80° prior to DNA extraction. Ecotypes from all location but the ILRI Campus were collected jointly by Biosciences eastern and central and Africa-International Livestock Research Institute (BecA-ILRI) Hub and Kenya Agricultural and Livestock Research Organization (KALRO). The collection details are summarized in Table 6.

Table 6. Collection details of Kenyan *Brachiaria* ecotypes included in the diversity assessment.

Ecotype	Species	Status	Location	Alt. (m a.s.l.)	Lat. (S)	Lon. (E)	Collection Year
ke_1	*Brachiaria* spp.	Wild	ILRI Farm	1761	1.27085	36.72204	2013
ke_2	*Brachiaria* spp.	Wild	ILRI Farm	1783	1.27091	36.72200	2013
ke_3	*Brachiaria* spp.	Wild	ILRI Farm	1787	1.27117	36.72206	2013
ke_4	*Brachiaria* spp.	Wild	ILRI Farm	1805	1.27152	36.72212	2013
ke_5	*Brachiaria* spp.	Wild	ILRI Farm	1798	1.27306	36.72255	2013

Table 6. *Cont.*

Ecotype	Species	Status	Location	Alt. (m a.s.l.)	Lat. (S)	Lon. (E)	Collection Year
ke_6	*Brachiaria* spp.	Wild	ILRI Farm	1804	1.27307	36.72384	2013
ke_7	*Brachiaria* spp.	Wild	ILRI Farm	1810	1.27292	36.72390	2013
ke_8	*Brachiaria* spp.	Wild	ILRI Farm	1813	1.27281	36.72404	2013
ke_9	*Brachiaria* spp.	Wild	ILRI Farm	1815	1.27269	36.72436	2013
ke_10	*Brachiaria* spp.	Wild	ILRI Farm	1814	1.27262	36.72483	2013
ke_11	*Brachiaria* spp.	Wild	ILRI Farm	1808	1.27275	36.72517	2013
ke_12	*Brachiaria* spp.	Wild	ILRI Farm	1871	1.27077	36.72224	2013
ke_13	*Brachiaria* spp.	Wild	ILRI Farm	1814	1.27076	36.72532	2013
ke_14	*Brachiaria* spp.	Wild	ILRI Farm	1870	1.27073	36.72562	2013
ke_15	*Brachiaria* spp.	Wild	ILRI Farm	1852	1.27088	36.72697	2013
ke_16	*Brachiaria* spp.	Wild	ILRI Farm	1851	1.27091	36.72702	2013
ke_17	*Brachiaria* spp.	Wild	ILRI Farm	1840	1.27135	36.72716	2013
ke_18	*Brachiaria* spp.	Wild	ILRI Farm	1836	1.27152	36.72699	2013
ke_19	*Brachiaria* spp.	Wild	ILRI Farm	1832	1.27214	36.72649	2013
ke_20	*Brachiaria* spp.	Wild	ILRI Farm	1830	1.27236	36.72605	2013
ke_21	*Brachiaria* spp.	Wild	ILRI Farm	1828	1.2725	36.72592	2013
ke_22	*Brachiaria* spp.	Wild	ILRI Farm	1823	1.27268	36.72547	2013
ke_23	*Brachiaria* spp.	Wild	ILRI Farm	1825	1.27263	36.72520	2013
ke_24	*Brachiaria* spp.	Wild	ILRI Farm	1825	1.27273	36.72519	2013
ke_25	*Brachiaria* spp.	Wild	ILRI Farm	1825	1.27261	36.72560	2013
ke_26	*Brachiaria* spp.	Wild	ILRI Farm	1833	1.27213	36.72660	2013
ke_27	*Brachiaria* spp.	Wild	ILRI Farm	1835	1.27196	36.72673	2013
ke_28	*Brachiaria* spp.	Wild	ILRI Farm	1843	1.27144	36.72709	2013
ke_29	*Brachiaria* spp.	Wild	ILRI Farm	1852	1.27109	36.72713	2013
ke_30	*Brachiaria* spp.	Wild	ILRI Farm	1876	1.27067	36.72585	2013
ke_31	*Brachiaria* spp.	Wild	ILRI Farm	1837	1.27086	36.72210	2014
ke_32	*Brachiaria* spp.	Wild	ILRI Farm	1882	1.27084	36.72208	2014
ke_33	*Brachiaria* spp.	Wild	ILRI Farm	1854	1.27252	36.72235	2014
ke_34	*Brachiaria* spp.	Wild	ILRI Farm	1839	1.27264	36.72424	2014
ke_35	*Brachiaria* spp.	Wild	ILRI Farm	1826	1.27274	36.72518	2014
ke_36	*Brachiaria* spp.	Wild	ILRI Farm	1824	1.27233	36.72612	2014
ke_37	*Brachiaria* spp.	Wild	ILRI Farm	1830	1.27257	36.72567	2014
ke_38	*Brachiaria* spp.	Wild	ILRI Farm	1835	1.27165	36.72692	2014
ke_39	*Brachiaria* spp.	Wild	ILRI Farm	1847	1.27101	36.72718	2014
ke_40	*Brachiaria* spp.	Wild	ILRI Farm	1871	1.27077	36.72536	2014
ke_41	*Brachiaria* spp.	Wild	ILRI Farm	1866	1.2708	36.72210	2014
ke_42	*Brachiaria* spp.	Wild	ILRI Farm	1859	1.27134	36.72213	2014
ke_43	*Brachiaria* spp.	Wild	ILRI Farm	1842	1.27285	36.72249	2014
ke_44	*Brachiaria* spp.	Wild	ILRI Farm	1835	1.27242	36.72230	2014
ke_45	*Brachiaria* spp.	Wild	ILRI Farm	1829	1.2734	36.72302	2014
ke_46	*Brachiaria* spp.	Wild	ILRI Farm	1828	1.27315	36.72381	2014
ke_47	*Brachiaria* spp.	Wild	ILRI Farm	1829	1.27271	36.72427	2014
ke_48	*Brachiaria* spp.	Wild	ILRI Farm	1828	1.27269	36.72454	2014
ke_49	*Brachiaria* spp.	Wild	ILRI Farm	1816	1.27261	36.72550	2014
ke_50	*Brachiaria* spp.	Wild	ILRI Farm	1829	1.2717	36.72688	2014
ke_51	*Brachiaria* spp.	Wild	Kitui	1163	NA	NA	2014
ke_52	*Brachiaria* spp.	Wild	Kitui	1163	NA	NA	2014
ke_53	*Brachiaria* spp.	Wild	Kitui	1163	NA	NA	2014
ke_54	*Brachiaria* spp.	Wild	ILRI Farm	1754	1.27778	36.38821	2014
ke_55	*Brachiaria* spp.	Wild	ILRI Farm	1857	1.2708	36.72206	2014
ke_56	*Brachiaria* spp.	Wild	ILRI Farm	1856	1.27284	36.72204	2014
ke_57	*Brachiaria* spp.	Wild	ILRI Farm	1844	1.27162	36.72208	2014
ke_58	*Brachiaria* spp.	Wild	ILRI Farm	1840	1.27203	36.72217	2014
ke_59	*Brachiaria* spp.	Wild	ILRI Farm	1822	1.2732	36.72357	2014
ke_60	*Brachiaria* spp.	Wild	ILRI Farm	1822	1.27321	36.72358	2014
ke_61	*Brachiaria* spp.	Wild	ILRI Farm	1810	1.27281	36.72506	2014
ke_62	*Brachiaria* spp.	Wild	ILRI Farm	1821	1.27176	36.72678	2014
ke_63	*Brachiaria* spp.	Wild	ILRI Farm	1824	1.27155	36.72697	2014
ke_67	*Brachiaria* spp.	Wild	Kisii	1750	0.68575	34.78978	2014
ke_68	*Brachiaria* spp.	Wild	Kisii	1750	0.68486	34.78914	2014
ke_69	*Brachiaria* spp.	Wild	Kisii	1750	0.68484	34.78910	2014

Table 6. *Cont.*

Ecotype	Species	Status	Location	Alt. (m a.s.l.)	Lat. (S)	Lon. (E)	Collection Year
ke_70	*Brachiaria* spp.	Wild	Kisii	1750	0.68471	34.78896	2014
ke_71	*Brachiaria* spp.	Wild	Kisii	1750	0.68473	34.78884	2014
ke_72	*Brachiaria* spp.	Wild	Alupe	1200	0.49766	34.12480	2014
ke_73	*Brachiaria* spp.	Wild	Alupe	1200	0.49781	34.12480	2014
ke_74	*Brachiaria* spp.	Wild	Alupe	1200	0.49847	34.12319	2014
ke_76	*Brachiaria* spp.	Wild	Alupe	1200	0.49855	34.12284	2014
ke_82	*Brachiaria* spp.	Wild	Kiminini	1750	0.89104	34.91368	2014
ke_83	*Brachiaria* spp.	Wild	Kiminini	1750	0.89102	34.91378	2014
ke_84	*Brachiaria* spp.	Wild	Kiminini	1750	0.89126	34.91338	2014
ke_85	*Brachiaria* spp.	Wild	Kiminini	1750	0.89144	34.91310	2014
ke_86	*Brachiaria* spp.	Wild	Kiminini	1750	0.89139	34.91302	2014
ke_87	*Brachiaria* spp.	Wild	Kiminini	1750	0.8913	34.91272	2014
ke_88	*Brachiaria* spp.	Wild	Kiminini	1750	0.89131	34.91264	2014

4.2. Genomic DNA Extraction

The DNA was extracted using the cetyl-trimethyl ammonium bromide (CTAB) [33] method with slight modifications. About 150 mg of the young leaves were cut into small pieces, ground in liquid nitrogen, and added with 800 µL of 2% CTAB buffer. The suspension was transferred into clean microfuge tubes and incubated at 65 °C for 30 min, followed by incubation at room temperature for 5 min and centrifuged at 3500 rpm for 10 min. After centrifugation, 400 µL of supernatant was transferred into new microfuge tubes and 400 µL of chloroform iso-amyl alcohol (24:1) was added to each tube and mixed by inversion for 10 min. Tubes were spun at 3500 rpm for 10 min, aqueous phase was transferred to clean microfuge tubes, and 400 µL of chloroform iso-amyl alcohol (24:1) was added again to each tube and spun for 10 min at 1100 rpm; this process was repeated twice. After the final centrifugation, the DNA was precipitated in 300 µL of cold isopropanol (100%) and inverted about 50 times to facilitate the mixing and precipitation, and incubated overnight at −20 °C. The following day, the microfuge tubes were removed from the freezer, thawed and spun at 3500 rpm at 4 °C for 20 min. The isopropanol was decanted and the genomic DNA pellet was air-dried. The DNA pellet was rinsed with 300 µL of 70% (*w*/*v*) ethanol and dissolved in 100 µL of low-salt TE buffer containing 3 µL of 10 mg/mL of 1% RNase solution and incubated in a water bath at 45 °C for 90 min. DNA quality and quantity were checked by 0.8% agarose gel (*w*/*v*) and NanoDrop Spectrophotometer. The genomic DNA was adjusted to the final concentration of 20 ng/µL and stored at 4 °C for PCR amplification.

4.3. PCR Amplification and Genotyping

The genomic DNA was amplified using AccuPower®PCRPreMix with Bioneer negative dye (Bioneer, Alameda, CA, USA). A reaction volume of 10 µL containing 0.4 µL $MgCl_2$ (final concentration of 2 mM $MgCl_2$), 0.4 µL each of forward and reverse primers labeled with different fluorescent dyes (6-FAM (blue), VIC (green), NED (black), and PET(red)), 2 µL template DNA (20 ng/µL), and 6.8 µL of sterile distilled water was used for PCR amplification. A total of 22 SSR markers (Table 5) initially developed for *B. ruziziensis* with the proven transferability to other species were used in this study [14]. The PCR conditions were: initial denaturation for 5 min at 94 °C followed by 35 cycles at 94 °C for 30 s, 57 °C for 60 s, 72 °C for 2 min, and final extension at 72 °C for 10 min. The amplicons' integrity was checked using agarose gel electrophoresis in 2% agarose gel (*w*/*v*) stained with 2.5 µL of GelRed solution. The agarose gel images were visualized under Ultra-Violet and the digital image was captured. The size of amplified fragments was estimated comparing with 1 kb DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA). The SSR fragment sizes and allele variations in the repeats were assessed by capillary electrophoresis of amplicons and sequencing of the amplified loci. The multiplexed PCR products were mixed with 8.87 µL Hi-Di-formamide and 0.135 µL fluorescent-labeled GeneScan™ LIZ size standard (Applied Biosystems, Foster City, CA, USA) in a 96-well microtiter plate. The mixed products were denatured at 95 °C for 3 min and snap-chilled on

ice for 5 min to avoid the formation of double-strand DNA. The products were loaded to Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

4.4. Data Analysis

The allele sizes generated by all 22 SSR markers on 79 ecotypes and 8 commercial varieties were scored using GeneMapper v4.1 software (Applied Biosystems, Foster City, CA, USA). Since the information on ploidy levels of test ecotypes was not available, SSR fragments were analyzed following a dominant scoring scheme, as used for other polyploidy species [34–37]. ALS-Binary and Allelobin software [38,39] were used to convert allelic data to binary data (0, 1) where 0 and 1 represent absence and presence of an allele, respectively. Statistical analysis of allelic and binary data was performed using PowerMarker v.3.25 [40] to obtain total number of alleles per locus, allele size range, genetic diversity and heterozygosity, and frequency-based genetic distances were calculated using shared alleles distance matrix. The population diversity indices (e.g., number of alleles, private alleles, and effective alleles per locus, Shannon Information index, and observed and expected heterozygosity) were calculated using GenAIEx v.6.5 [41]. The same software was used to compute analysis of molecular variance (AMOVA), principal coordinate analysis (PCoA), and matrix of genetic distance. The Dice binary similarity coefficient [42] was used to generate the unweighted neighbor-joining tree (NJT) showing relationships among test genotypes in Darwin Software v6.0 [43].

5. Conclusions

Brachiaria is a native African grass which is widely distributed in Kenya. It is one of the most extensively cultivated forages in tropical Americas, Australia and East Asia. However the cultivation of Brachiaria for pasture production in Kenya and Africa in general has been recently initiated through the repatriation of Brachiaria in the form of hybrids and improved landraces from South America. Despite excellent herbage production performance and benefits to livestock productivity, some of these introduced materials have shown susceptibility to pests and diseases within a short period of establishment. It has raised serious concern on the expansion of Brachiaria acreage in Kenya urging the needs for the Africa based *Brachiaria* improvement program. This study with collection of 79 Brachiaria ecotypes from a few locations of Kenya and their genetic diversity analyses revealed the presence of substantial genetic variations among Kenyan ecotypes, and close genetic relationships among improved landraces and Hybrid Mulato II. This study suggests need for collecting more ecotypes from different agroecological regions of Kenya to broaden genetic bases of existing genebank collections, and their morphological, agronomical, and genetic characterizations to support Brachiaria grass breeding and conservation programs.

Acknowledgments: This project was supported by the BecA-ILRI Hub through the Africa Biosciences Challenge Fund (ABCF) program. The ABCF Program is funded by the Australian Department for Foreign Affairs and Trade (DFAT) through the BecA-CSIRO partnership; the Syngenta Foundation for Sustainable Agriculture (SFSA); the Bill & Melinda Gates Foundation (BMGF); the UK Department for International Development (DFID) and; the Swedish International Development Cooperation Agency (Sida). We are grateful to Marie Christine Dusingize and Jean de dieu Ayabagabo for their assistance in experiment and data analysis, respectively. Thanks are due to Monday Ahonsi for assistance in research planning and implementation.

Author Contributions: N.O., D.N., A.D. and S.G. conceived and designed the experiments; N.O., S.M. and W.K. performed experiment; N.O. and S.G. performed data analysis; N.O. and S.G. wrote manuscript, D.N., S.M., W.K. and A.D. reviewed manuscript and S.G. supervised all research work.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Renvoize, S.A.; Clayton, W.D.; Kabuye, C.H.S. Morphology, taxonomy and natural distribution of *Brachiaria* (Trin.) Griseb. In *Brachiaria: Biology, Agronomy, and Improvement*; Miles, J.W., Maass, B.L., do Valle, C.B., Eds.; International Centre for Tropical Agriculture: Cali, Colombia, 1996; pp. 1–15.

2. Miles, J.W.; Maass, B.L.; do Valle, C.B. *Brachiaria: Biology, Agronomy, and Improvement*; International Centre for Tropical Agriculture: Cali, Colombia, 1996; p. 288.
3. Holmann, F.; Rivas, L.; Argel, P.J.; Perez, E. Impact of the adoption of Brachiaria grasses: Central America and Mexico. *Livest. Res. Rural Dev.* **2004**, *16*. Available online: http://www.lrrd.org/lrrd16/12/holm16098.htm (accessed on 21 January 2017).
4. Jotee, D. Evaluation of the potential of some common forage germplasms in Mauritius. In Proceedings of the Third PANESA Workshop, Arusha, Tanzania, 27–30 April 1987; Dzowela, B.H., Ed.; ILCA: Addis Ababa, Ethiopia, 1988; pp. 81–90.
5. Subbarao, G.V.; Nakahara, K.; Hurtado, M.P.; Ono, H.; Moreta, D.E.; Salcedo, A.F.; Yoshihashi, A.T.; Ishikawa, T.; Ishitani, M.; Ohnishi-Kameyama, M.; et al. Evidence for biological nitrification inhibition in *Brachiaria* pastures. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 17302–17307. [CrossRef] [PubMed]
6. Arango, J.; Moreta, D.; Núñez, J.; Hartmann, K.; Domínguez, M.; Ishitani, M.; Miles, J.; Subbarao, G.; Peters, M.; Rao, I. Developing methods to evaluate phenotypic variability in biological nitrification inhibition (BNI) capacity of Brachiaria grasses. *Trop. Grassl.-Forrajes Trop.* **2014**, *2*, 6–8. [CrossRef]
7. Moreta, D.E.; Arango, J.; Sotelo, M.; Vergara, D.; Rincón, A.; Ishitani, N.; Castro, A.; Miles, J.; Peters, M.; Tohme, J.; et al. Biological nitrification inhibition (BNI) in *Brachiaria* pastures: A novel strategy to improve eco-efficiency of crop-livestock systems and to mitigate climate change. *Trop. Grassl.-Forrajes Trop.* **2014**, *2*, 88–91. [CrossRef]
8. Rao, I.; Ishitani, M.; Miles, J.; Peters, M.; Tohme, J.; Arango, J.; Moreta, D.E.; Lopez, H.; Castro, A.; Hoek, R.V.D.; et al. Climate-smart crop-livestock systems for smallholders in the tropics: Integration of new forage hybrids to intensify agriculture and to mitigate climate change through regulation of nitrification in soil. *Trop. Grassl.-Forrajes Trop.* **2014**, *2*, 130–132. [CrossRef]
9. Jank, L.; Barrios, S.C.; do Valle, C.B.; Simeão, R.M.; Alves, G.F. The value of improved pastures to Brazilian beef production. *Crop Pasture Sci.* **2014**, *65*, 1132–1137. [CrossRef]
10. Ndikumana, J.; de Leeuw, P.N. Regional experiences with *Brachiaria*: Sub-Saharan Africa. In *Brachiaria: Biology, Agronomy, and Improvement*; Miles, J.W., Maass, B.L., do Valle, C.B., Eds.; International Centre for Tropical Agriculture: Cali, Colombia, 1996; pp. 247–257.
11. Maass, B.L.; Midega, A.O.; Mutimura, M.; Rahetlah, V.B.; Salgado, P.; Kabirizi, J.M.; Khan, Z.R.; Ghimire, S.; Rao, I.M. Homecoming of *Brachiaria*: Improved hybrids prove useful for African animal agriculture. *E. Afr. Agric. For. J.* **2015**, *81*, 1–78.
12. Ghimire, S.; Njarui, D.; Mutimura, M.; Cardoso, J.; Johnson, L.; Gichangi, E.; Teasdale, S.; Odokonyero, K.; Caradus, J.; Rao, I.; et al. Climate-smart *Brachiaria* for improving livestock production in East Africa: Emerging opportunities. In *Sustainable Use of Grassland Resources for Forage Production, Biodiversity and Environmental Protection*; Vijaya, D., Srivastava, M., Gupta, C., Malaviya, D., Roy, M., Mahanta, S., Singh, J., Maity, A., Ghos, P., Eds.; Range Management Society of India; ICAR-Indian Grassland and Fodder Research Institute: New Delhi, India, 2015; pp. 361–370.
13. Keller-Grein, G.; Maass, B.L.; Hanson, J. Natural variation in *Brachiaria* and existing germplasm collection. In *Brachiaria: Biology, Agronomy, and Improvement*; Miles, J.W., Maass, B.L., do Valle, C.B., Eds.; International Centre for Tropical Agriculture: Cali, Colombia, 1996; pp. 16–42.
14. Silva, P.I.; Martins, A.M.; Gouvea, E.G.; Pessoa-Filho, M.; Ferreira, M.E. Development and validation of microsatellite markers for *Brachiaria ruziziensis* obtained by partial genome assembly of Illumina single-end reads. *BMC Genom.* **2013**, *14*, 17. [CrossRef] [PubMed]
15. Jungmann, L.; Vigna, B.B.Z.; Paiva, J.; Sousa, A.C.B.; do Valle, C.B.; Laborda, P.R. Development of microsatellite markers for *Brachiaria humidicola* (Rendle) Schweick. *Conserv. Genet. Resour.* **2009**, *1*, 475–479. [CrossRef]
16. Vigna, B.B.Z.; Jungmann, L.; Francisco, P.M.; Zucchi, M.I.; do Valle, C.B.; Souza, A.P. Genetic diversity and population structure of the *Brachiaria brizantha* germplasm. *Trop. Plant Biol.* **2011**, *4*, 157–169. [CrossRef]
17. Jungmann, L.; Sousa, A.C.B.; Paiva, J.; Francisco, P.M.; Vigna, B.B.Z.; do Valle, C.B. Isolation and characterization of microsatellite markers for *Brachiaria brizantha* (Hochst. ex A. Rich.) Stap. *Conserv. Genet.* **2009**, *10*, 1873–1876. [CrossRef]
18. Pessoa-Filho, M.; Azevedo, A.L.S.; Sobrinho, F.S.; Gouvea, E.G.; Martins, A.M.; Ferreira, M.E. Genetic diversity and structure of Ruzigrass germplasm collected in Africa and Brazil. *Crop Sci.* **2015**, *55*, 2736–2745. [CrossRef]

19. Szpiech, Z.A.; Jakobsson, M.; Rosenberg, N.A. ADZE: A rarefaction approach for counting alleles private to combinations of populations. *Bioinformatics* **2008**, *24*, 2498–2504. [CrossRef] [PubMed]
20. Kalinowski, S.T. Counting alleles with rarefaction: Private alleles and hierarchical sampling designs. *Conserv. Genet.* **2004**, *5*, 539–543. [CrossRef]
21. Boonman, J.G. Experimental studies on seed production of tropical grasses in Kenya part 2 tillering and heading on in seed crops of eight grasses. *Neth. J. Agric. Sci.* **1971**, *19*, 237–249.
22. Boonman, J.G. *East Africa's Grasses and Fodders: Their Ecology and Husbandry*; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1993; p. 343.
23. Wandera, J.L. Forage research and production in western Kenya. In *A Review of Agricultural Practices and Constraints in the North Rift Valley Province*; Rees, D.J., Nkonge, C., Wandera, J.L., Eds.; Kenya Agricultural Research Institute: Nairobi, Kenya, 1997; pp. 169–187.
24. Garcia, M.; Vigna, B.B.Z.; Sousa, A.C.B.; Jungmann, L.; Cidade, F.W.; Toledo-Silva, G.; Francisco, P.M.; Chiari, L.; Carvalho, M.A.; Karia, C.T.; et al. Molecular genetic variability, population structure and mating system in tropical forages. *Trop. Grassl.-Forrajes Trop.* **2013**, *1*, 25–30. [CrossRef]
25. Azevedo, A.L.S.; Costa, P.P.; Machado, M.A.; de Paula, C.M.P.; Sobrinho, F.S. High degree of genetic diversity among genotypes of the forage grass *Brachiaria ruziziensis* (Poaceae) detected with ISSR markers. *Genet. Mol. Res.* **2011**, *10*, 3530–3538. [CrossRef] [PubMed]
26. Jungmann, L.; Vigna, B.B.Z.; Boldrini, K.R.; Sousa, A.C.B.; do Valle, C.B.; Resende, R.M.S.; Pagliarini, M.S.; Zucchi, M.I.; de Souza, A.P. Genetic diversity and population structure analysis of the tropical pasture grass *Brachiaria humidicola* based on microsatellites, cytogenetics, morphological traits, and geographical origin. *Genome* **2010**, *53*, 698–709. [PubMed]
27. Wright, S. The genetical structure of populations. *Ann Eugen* **1951**, *15*, 323–354. [CrossRef] [PubMed]
28. Vucetich, J.A.; Waite, T.A. Is one migrant per generation sufficient for the genetic management of fluctuating populations? *Anim. Conserv.* **2000**, *3*, 261–266. [CrossRef]
29. Do Valle, C.B.; Savidan, Y.H. Genetics, cytogenetic and reproductive biology of *Brachiaria*. In *Brachiaria: Biology, Agronomy, and Improvement*; Miles, J.W., Maass, B.L., do Valle, C.B., Eds.; International Centre for Tropical Agriculture: Cali, Colombia, 1996; pp. 147–163.
30. Harrington, K.C.; Beskow, W.B.; Hodgson, J. Recovery and viability of seeds ingested by goats. *N. Z. Plant Prot.* **2011**, *64*, 75–80.
31. Leitch, A.R.; Leitch, I.J. Genomic plasticity and the diversity of polyploid plants. *Science* **2008**, *320*, 481–483. [CrossRef] [PubMed]
32. Malo, J.E.; Suárez, F. Herbivorous mammals as seed dispersers in a Mediterranean *dehesa*. *Oecologia* **1995**, *104*, 246–255. [CrossRef]
33. Doyle, J.J.; Doyle, J.L. Isolation of plant DNA from fresh tissue. *Focus* **1990**, *12*, 13–15.
34. Cordeiro, G.M.; Pan, Y.; Henry, R.J. Sugarcane microsatellites for the assessments of genetic diversity in sugarcane germplasm. *Plant Sci.* **2003**, *165*, 181–189. [CrossRef]
35. Cidade, F.W.; Vigna, B.B.Z.; de Sauza, F.H.D.; Valls, J.F.M.; Dall'Agnol, M.; Zucchi, M.I.; de Sauza-Chies, T.T.; Sauza, A.P. Genetic variation in polyploid forage grass: Assessing the molecular genetic variability in the Paspalum genus. *BMC Genet.* **2013**, *14*, 50. [CrossRef] [PubMed]
36. Lu, X.; Zhou, H.; Pan, Y.B.; Chen, C.Y.; Zhu, J.R.; Chen, P.H.; Li, Y.R.; Cai, Q.; Chen, R.K. Seggregation analysis of microsatellite (SSR) markers in sugarcane polyploids. *Genet. Mol. Res.* **2015**, *14*, 18384–18395. [CrossRef] [PubMed]
37. Schie, S.; Chaudhary, R.; Debener, T. Analysis of a complex polyploid plant genome using molecular markers: Strong evidence for segmental allooctoploidy in garden Dahalias. *Plant Genome* **2014**, *7*, 3. [CrossRef]
38. Prasanth, V.P.; Chandra, S. *ALS-Binary. A Program for Converting Allele Size in Microsatellite Markers into 0–1 (Binary) Data*; ICRISAT: Hyderabad, India, 1997.
39. Prasanth, V.P.; Chandra, S.; Jayashree, B.; Hoisington, D. *AlleloBin. A Software for Allele Binning of Microsatellite Markers Based on the Algorithms of Idury and Cardon*; ICRISAT: Hyderabad, India, 1997.
40. Liu, K.; Muse, S.V. PowerMarker: An integrated analysis environment for genetic marker analysis. *Bioinformatics* **2005**, *21*, 2128–2129. [CrossRef] [PubMed]
41. Peakall, R.; Smouse, P.E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—An update. *Bioinformatics* **2012**, *28*, 2537–2539. [CrossRef] [PubMed]

42. Perrier, X.; Jacquemoud-Collet, J.P. DARwin: Dissimilarity Analysis and Representation for Windows, Version 5.0.157. Computer Program. 2006. Available online: http://darwin.cirad.fr/darwin (accessed on 21 January 2017).
43. Dice, LR. Measures of the amount of ecologic association between species. *Ecology* **1945**, *26*, 297–302. [CrossRef]

agronomy

MDPI

Article

Development and Testing of Cool-Season Grass Species, Varieties and Hybrids for Biomass Feedstock Production in Western North America

Steven R. Larson [1,*], Calvin H. Pearson [2], Kevin B. Jensen [1], Thomas A. Jones [1], Ivan W. Mott [1], Matthew D. Robbins [1], Jack E. Staub [1] and Blair L. Waldron [1]

[1] USDA-ARS, Forage and Range Research, Utah State University, Logan, UT 84322, USA; Kevin.Jensen@ars.usda.gov (K.B.J.); Thomas.Jones@ars.usda.gov (T.A.J.); Ivan.Mott@ars.usda.gov (I.W.M.); Matthew.Robbins@ars.usda.gov (M.D.R.); Jack.Staub@ars.usda.gov (J.E.S.); Blair.Waldron@ars.usda.gov (B.L.W.)

[2] Agriculture Experiment Station, Department of Soil and Crop Sciences, Colorado State University, Fruita, CO 81521, USA; Calvin.Pearson@colostate.edu

* Correspondence: Steve.Larson@ars.usda.gov; Tel.: +1-436-5-797-1703

Academic Editors: John W. Forster and Smith Kevin F. Smith
Received: 29 September 2016; Accepted: 14 December 2016; Published: 1 January 2017

Abstract: Breeding of native cool-season grasses has the potential to improve forage production and expand the range of bioenergy feedstocks throughout western North America. Basin wildrye (*Leymus cinereus*) and creeping wildrye (*Leymus triticoides*) rank among the tallest and most rhizomatous grasses of this region, respectively. The objectives of this study were to develop interspecific creeping wildrye (CWR) × basin wildrye (BWR) hybrids and evaluate their biomass yield relative to tetraploid 'Trailhead', octoploid 'Magnar' and interploidy-hybrid 'Continental' BWR cultivars in comparison with other perennial grasses across diverse single-harvest dryland range sites and a two-harvest irrigated production system. Two half-sib hybrid populations were produced by harvesting seed from the tetraploid self-incompatible Acc:641.T CWR genet, which was clonally propagated by rhizomes into isolated hybridization blocks with two tetraploid BWR pollen parents: Acc:636 and 'Trailhead'. Full-sib hybrid seed was also produced from a controlled cross of tetraploid 'Rio' CWR and 'Trailhead' BWR plants. In space-planted range plots, the 'Rio' CWR × 'Trailhead' BWR and Acc:641.T CWR × Acc:636 BWR hybrids displayed high-parent heterosis with 75% and 36% yield advantages, respectively, but the Acc:641.T CWR × 'Trailhead' BWR hybrid yielded significantly less than its BWR high-parent in this evaluation. Half-sib CWR × BWR hybrids of Acc:636 and 'Trailhead' both yielded as good as or better than available BWR cultivars, with yields similar to switchgrass (*Panicum virgatum*), in the irrigated sward plots. These results elucidate opportunity to harness genetic variation among native grass species for the development of forage and bioenergy feedstocks in western North America.

Keywords: arid ecosystems; cold-desert; high-elevation; interspecific hybrids; self-incompatibility

1. Introduction

Development of biofuel feedstocks in the United States has been focused on switchgrass (*Panicum virgatum*) as a model crop system in part because of its performance in herbaceous crop screening trials conducted across Alabama, Iowa, Indiana, New York, North Dakota, Ohio and Virginia; and also because decision makers recognized the strategic importance to demonstrate the feasibility of developing a cellulosic biofuel crop with limited funding [1–4]. Switchgrass is a warm-season rhizomatous perennial grass endemic to southeastern North America encompassing parts of Cuba,

southeastern Canada, northeastern Mexico, and most the United States east of the Rocky Mountains [5] including all of the states where it was tested during the initial phase of biofuel feedstock screening in the United States [1–4]. However, with the exception of western North Dakota, located near the geographic center of North America, most of the western United States was excluded from the initial herbaceous crop screening process [1–4]. Studies have shown that cool-season perennial Triticeae grasses including crested wheatgrass (*Agropyron desertorum*), intermediate wheatgrass (*Thinopyrum intermedium*), mammoth wildrye (*Leymus racemosus*), tall wheatgrass (*Thinopyrum ponticum*), and native western wheatgrass (*Pascopyrum smithii*) may be useful for forage and bioenergy feedstock production across the prairie provinces of Canada and the central United States including Alberta, Kansas, Manitoba, North Dakota, Saskatchewan, and South Dakota [1,4,6–10]. These cool-season perennial Triticeae grasses are also well adapted to the high-elevation cold-desert Great Basin region of the western United States including large regions of Nevada and Utah [11–13]. Intermediate wheatgrass and tall wheatgrass produced substantially more biomass than warm-season grasses, including five varieties of switchgrass, in a five-harvest irrigated production system in northern Utah [11]. Basin wildrye (*Leymus cinereus*) performed relatively well in low-irrigation single-harvest management systems in the cold-desert environments of the Idaho, Nevada, and Utah [12,14], where this species is native. However, efforts to improve biomass productivity of these cool-season perennial grasses lag behind switchgrass or miscanthus.

The development and testing of intraspecific and interspecific hybrids has been a focal point of breeding and genetic research in bioenergy grasses [15], including switchgrass and miscanthus. One of the most productive forms of miscanthus (*Miscanthus* × *giganteus*) is a sterile hybrid between *Miscanthus sacchriflorus* and *Miscanthus sinensis* that is clonally propagated by rhizomes [1,16–19]. Some hybrids of *M. sacchriflorus* and *M. sinensis* display up to 30%–35% F_1 high-parent heterosis without selection for specific combining ability [20], and there has been considerable effort to identify, develop, and test new miscanthus hybrids [19,21–24]. Switchgrass hybrids also show evidence of heterosis. Full-sib hybrid families, population hybrids, and advanced generation synthetic hybrid populations of upland and lowland switchgrass ecotypes showed evidence of mid-parent heterosis in spaced-plant plots [25] and high-parent heterosis in sward plots [26]. Extensive testing of reciprocal crosses within and between the upland and lowland ecotypes detected no evidence of high-parent heterosis and mid-parent heterosis was limited to a small fraction of the hybrids [27]. Nevertheless, the identification of complementary gene pools is expected to help produce useful hybrids and hybrid-derived populations for switchgrass biomass production [5,28,29]. As a native plant species, there is a wealth of regionally adapted genetic resources available for the development of switchgrass varieties and hybrids in North America [1,5,29,30]. Likewise, development and testing of native cool-season grasses has the potential to diversify cropping systems and expand the expected range of adaptation of bioenergy feedstocks throughout western North America.

Basin wildrye (BWR) is considered one of the largest and most conspicuous native bunchgrasses in western North America, with aerial stems in excess of 2 m [31] and a deep fibrous root system [12,32]. Although the native range of BWR is quite large, its distribution is restricted to sites where water and soil accumulate, which includes road sides and field margins [33]. Three genetically distinct races, including allotetraploid (2n = 4*x* = 28) and auto-duplicated octoploid (2n = 8x = 56) cytotypes, have been identified and named based on their corresponding distributions across the Columbia, Rocky Mountain, and Great Basin ecogeographic regions [33]. The octoploid (8X) cytotypes have significantly larger leaves, longer culms, and greater crown circumference compared to tetraploid (4X) cytotypes, and there is evidence of climatic adaptations within BWR [34]. However, it is not known how these differences related to biomass productivity under cultivation. The two standard BWR varieties—octoploid (8X) 'Magnar' and tetraploid (4X) 'Trailhead'—were collected directly from wild natural populations in southeastern British Columbia (Columbia race) and southcentral Montana (Rocky Mountain race), respectively [33], without subsequent selection. A new synthetic BWR variety, 'Continental', was selected from a hybrid population of chromosome-doubled (4X + 4X) 'Trailhead'

and 8X 'Magnar' [35]. 'Continental' was released based on its superior stand establishment in range seeding evaluations [35], but the biomass-related traits of 'Continental' have not been compared to 'Trailhead', 'Magnar', or any other grass. Although BWR shows potentially useful biomass yields in a single-harvest management systems [12,14], none of the BWR cultivars were bred for biomass yield and they may lack the defoliation tolerance needed for more intensive multiple-harvest production systems [14].

Creeping wildrye (*Leymus triticoides*) is closely related to BWR and has a similar range of distribution throughout western North America, but it is usually found in different habitats such as saline meadows and harsh alkaline sites in [33]. Creeping wildrye (CWR) is also different from BWR in that it has extensive rhizomes, typically grows much shorter than BWR, and it had significantly lower biomass yield, by about 50%, in comparisons with BWR [14,31]. The only available CWR cultivar, 'Rio', was collected from a natural population in Kings Valley of California [36] and has been evaluated as a forage and biomass crop in the western San Joaquin Valley using saline-sodic drainage water for irrigation [37,38].

Interspecific hybrids of CWR and BWR have been developed and tested for biomass yield and other agronomic traits [14,31]. In a single-harvest management system, two CWR × BWR single-cross hybrid genets showed indications of mid-parent heterosis for dry matter yield (DMY), with both hybrids showing substantially better yields than the lower-yielding CWR parent, and one of the single-cross hybrid genets showed significantly ($p < 0.001$) greater yields than the higher-yield BWR parent [14]. It was also postulated that increased rhizomatousness in the CWR × BWR hybrids may provide a mechanism of defoliation tolerance and regrowth not present in BWR [14,39], which may in turn facilitate management in multiple-harvest production systems. However, these previous studies [14,39] were based on evaluations of clonal propagules from two single-cross hybrid genets. The difficulty of controlling pollination and producing substantial quantities of hybrid seed has limited the testing of CWR × BWR hybrids on the field-scale level [14], which has also been a challenge with switchgrass and other allogamous perennial grasses [26]. Thus, for this study, we have proposed a strategy to produce larger volumes of hybrid seed for different CWR × BWR hybrids by growing rhizome propagules from one self-incompatible CWR genet, Acc:641.T, in isolated field hybridization plots with different varieties or natural populations of BWR as the only available pollen source. Using this approach, we anticipated several possible outcomes: (1) distinct half-sib hybrid seed populations resulting from crosses of Acc:641.T CWR pollinated by different BWR populations; (2) no seed set due to the lack of compatible pollen; and (3) seeds resulting from self-fertilization of the Acc:641.T CWR genet.

The overall purpose of the research reported herein was to develop and test BWR and CWR × BWR hybrids for efficient biomass feedstock production in western North America. This study addresses several questions:

- Is it possible to utilize the gametophytic self-incompatibility mechanism of perennial grasses combined with the highly rhizomatous nature of CWR to mass produce half-sib hybrid seed for different 4X CWR × 4X BWR or 4X CWR × 8X BWR crosses on a field scale level?
- How do the relative biomass yields of CWR × BWR half-sib hybrids and higher-yielding BWR parent varieties compare to relative yields of previously tested CWR × BWR hybrid genets and their BWR parent varieties [14], in single-harvest dryland range production systems?
- What is the relative performance of CWR × BWR hybrids, interploidy BWR hybrid, standard BWR varieties, and other bioenergy candidate species in terms of DMY in a two-harvest irrigated production system of western North America?
- Do the CWR × BWR hybrids show evidence of biomass heterosis?

Specific experimental objectives that were formulated to address these questions were to (1) compare the yield, average seed size, and percent germination of seed harvested from one 4X CWR genet (Acc:641.T) grown in combination with different 4X or 8X BWR populations in isolated

hybridization plots; (2) examine the genetic identity of seed harvested from the 4X Acc:641.T CWR genet, in different hybridization plots, relative to CWR and BWR parental genotypes, using DNA markers; (3) compare biomass accumulation potential of half-sib CWR × BWR hybrids and the interploidy BWR hybrid to the high-parent BWR reference populations in dryland range environments, and; (4) compare early-season, late-season, and average yearly biomass yields of half-sib CWR × BWR hybrids, the interploidy BWR hybrid, two standard BWR varieties, intermediate wheatgrass, switchgrass, and tall wheatgrass in a two-harvest irrigated management system designed for efficient biomass production in this region.

2. Results

2.1. Yield and Quality of Half-Sib Hybrid Seed Production

Yield, average seed weights, and percent germination of seed harvested from hybridization plots were significantly influenced ($p < 0.001$) by the presence of different BWR pollen populations (Table 1). Seed yield and average seed weights also showed significant variation ($p < 0.001$) over years. Seed yield in the second year was significantly higher ($p < 0.05$) than other years (Table 1) but seed weights were significantly greater ($p < 0.001$) in the first year compared to other years (results not shown). Seed yields in the fourth year were significantly lower than the second year ($p < 0.001$) or third year ($p < 0.05$) and seed weights in the fourth year were significantly lower than all other years ($p < 0.001$). The average seed yields in hybridization plots containing 4X BWR pollen parents (5.9 $g \cdot m^{-2}$) was significantly greater ($p < 0.001$) than the average seed yields in hybridization plots containing 4X BWR pollen parents (1.0 $g \cdot m^{-2}$). The average seed weights in hybridization plots containing 4X BWR pollen parents (2.68 mg) was significantly greater ($p < 0.001$) than the average seed yields in hybridization plots containing 4X BWR pollen parents (1.74 mg). Finally, the percent germination in hybridization plots containing 4X BWR pollen parents (56.3%) was significantly greater ($p < 0.001$) than the average seed yields in hybridization plots containing 4X BWR pollen parents (11.0%).

Table 1. Yield, average seed weight, and percent germination of seed harvested from the tetraploid (4X) Acc:641.T creeping wildrye genet grown in hybridization plots containing octoploid (8X) or tetraploid (4X) basin wildrye (BWR) pollen-parent populations. Significant differences among groups of entry means are indicated by lettered ranks, within table columns, based on least significant differences ($p < 0.05$).

BWR Pollen-Parent Population	Year 1 Yield ($g \cdot m^{-2}$)	Year 2 Yield ($g \cdot m^{-2}$)	Year 3 Yield ($g \cdot m^{-2}$)	Year 4 Yield ($g \cdot m^{-2}$)	Avg. Yield ($g \cdot m^{-2}$)	Seed Weight (mg)	Percent Germination
4X Acc:636 BWR	3.8 a	10.2 a	4.5 b	1.9 ab	5.1 b	2.70 a	54.1 a
8X 'Continental' BWR	0.6 b	1.8 b	1.0 c	0.2 c	0.9 c	1.72 b	12.5 b
8X 'Magnar' BWR	0.4 b	2.1 b	1.3 c	0.5 bc	1.1 c	1.75 b	9.6 b
4X 'Trailhead' BWR	3.7 a	12.1 a	7.7 a	3.1 a	6.6 a	2.67 a	58.5 a
Standard error	0.6	2.5	0.6	0.5	0.6	0.05	2.8
Average	2.1	6.6	3.6	1.4	3.4	2.21	33.7

2.2. Genetic Identity and Genetic Diversity of Seed Harvested from CWR × BWR Hybridization Plots

Principle coordinates analysis of DNA profiles from individual plants elucidated genetic diversity within and between three major groups comprised of CWR, BWR, and apparent half-sib hybrids of CWR and BWR (Figure 1). Moreover, genetically distinct subgroups corresponding to the 4X Acc:636, 4X 'Trailhead', and 8X 'Magnar' populations were also detectable within the highly-diverse BWR group (Figure 1). The 8X 'Continental' BWR interploidy hybrid population showed considerable overlap with its 8X 'Magnar' BWR parent population, but it did not overlap with its other 4X 'Trailhead' BWR parent population (Figure 1). Most of the progeny sampled from the hybridization plots appeared to be hybrids of CWR and BWR, similar to previously described TC1 and TC2 single-cross hybrid genets of the 4X Acc:641 CWR × 4X Acc:636 BWR cross (Figure 1). In fact, the TC1 and TC2 hybrids were indistinguishable from progeny sampled from hybridization plots containing 4X Acc:636 BWR

(Figure 1), which all have a very similar genetic background except that the TC1 and TC2 single-cross hybrids originated from 4X Acc:641 CWR plants that were not genetically identical to the 4X Acc:641.T CWR genet.

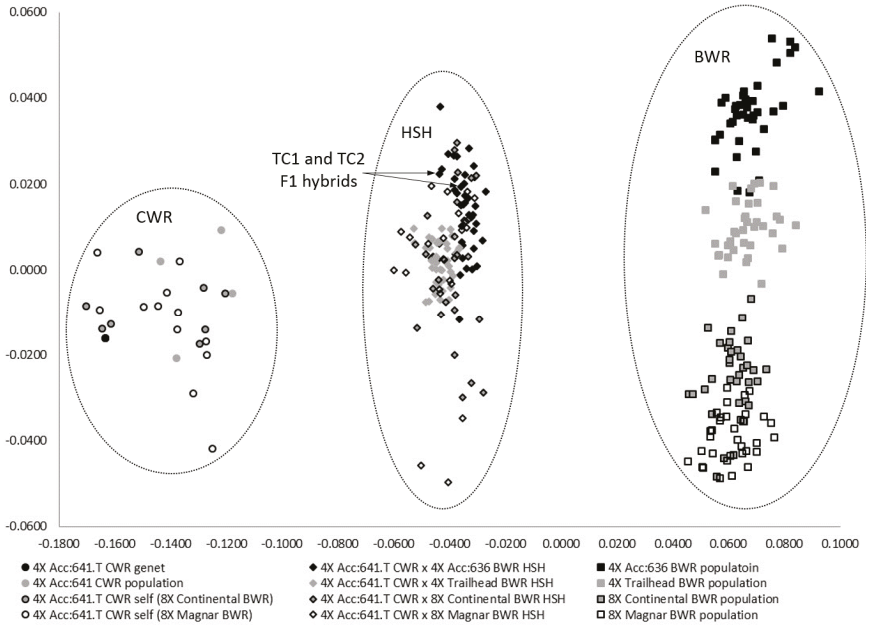


Figure 1. Principle coordinates analysis of genetic similarity coefficients among DNA genotypes from 314 individual plants of creeping wildrye (CWR), basin wildrye (BWR), and half-sib hybrid (HSH) populations harvested from the 4X Acc:641.T CWR genet in hybridization plots containing tetraploid (4X) or octoploid (8X) pollen-parent populations of 4X Acc:636 BWR, 4X 'Trailhead' BWR, 8X 'Continental' BWR, and 8X 'Magnar' BWR. Population identifiers include presumed self-progeny of the 4X Acc:641.T CWR genet (from hybridization plots containing 8X 'Continental' BWR or 8X 'Magnar' BWR). The identity of two 4x Acc:641 CWR × 4X Acc:636 BWR single-cross hybrids, TC1 and TC2, are also identified.

Not all of the progeny harvested from hybridization plots containing 8X BWR pollen sources appeared to be CWR × BWR hybrids. Eight of the 33 progeny sampled from hybridization plots containing the 8X 'Continental' BWR pollen source grouped with other 4X Acc:641 CWR plants, indicating that approximately 24% of these progeny resulted from self-pollination of the 4X Acc:641.T CWR genet (Figure 1). Likewise, 12 of the 38 progeny sampled from hybridization plots containing the 8X 'Magnar' BWR pollen source grouped with other 4X Acc:641 CWR plants, indicating that approximately 32% of these progeny resulted from self-pollination of the 4X Acc:641.T CWR genet (Figure 1). All of the remaining progeny sampled from hybridization plots containing 4X or 8X BWR pollen parents were CWR × BWR hybrids, but many of these apparent hybrids did not separate into genetically distinct groups corresponding to the BWR pollen parents (Figure 1).

Bayesian cluster analysis (Figure 2) provided further insights into the ancestry of hybrid progeny. Four nearly-pure ancestry groups corresponding to 4X Acc:641 CWR, 4X Acc:636 BWR, 4X 'Trailhead' BWR, and 8X 'Magnar' were identified using an a priori model of K = 4 Bayesian groups (Figure 2). As might be expected, plant samples from the 8X 'Continental' interploidy BWR hybrid showed mixed ancestry coefficients ranging from about 0.25 to 0.75 'Trailhead' BWR and 0.75 to 0.25 'Magnar' BWR. As expected, all of the other progeny sampled from the 4X Acc:641.T CWR genet in hybridization plots

containing BWR pollen sources, had nearly pure CWR ancestry or mixed ancestry coefficients of about 0.65 CWR and 0.35 BWR (Figure 2). All of the progeny sampled from hybridization plots containing the 4X 'Trailhead' BWR pollen source appeared to be half-sib hybrids of 4X Acc:641.T CWR genet × 4X 'Trailhead' BWR (Figure 2). Likewise, most of the progeny sampled from hybridization plots containing the 4X Acc636 BWR pollen source appeared to be half-sib hybrids of 4X Acc:641.T CWR genet × 4X Acc636 BWR except that a few (at least six) appeared to be half-sib hybrids of 4X Acc:641.T CWR genet × 4X 'Trailhead' BWR (Figure 2). Conversely, most of the progeny sampled from hybridization plots containing 8X BWR pollen sources, 'Magnar' or 'Continental', appeared to be half-sib hybrids of 4X BWR (either 'Trailhead' or Acc:636) or self-progeny of the 4X Acc:641.T CWR genet (Figure 2). Only three progeny from hybridization plots containing 8X 'Magnar' BWR as the pollen source actually contain 'Magnar' ancestry (Figure 2). Likewise, only three of the sampled progeny from hybridization plots containing 8X 'Continental' BWR as the pollen source actually contained portions of 4X Acc:641 CWR, 'Trailhead' BWR, and 'Magnar' ancestry expected from a "three-way hybrid" of the 4X Acc:641.T CWR genet and the 8X 'Continental' interploidy BWR hybrid (Figure 2). Interestingly, most of the apparent hybrids of 4X Acc:641.T CWR genet × 8X BWR or self-pollinated 4X Acc:641.T CWR progeny occurred in the second evaluation year.

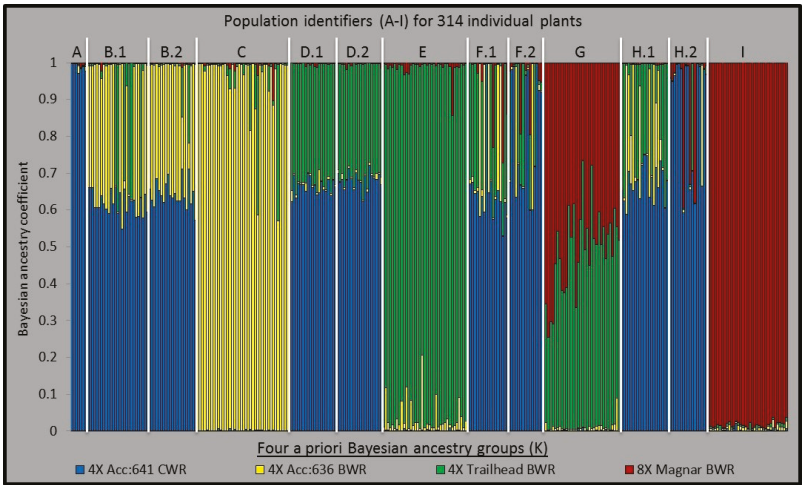


Figure 2. Bayesian cluster analysis of DNA genotypes from 314 individual plants of creeping wildrye (CWR), basin wildrye (BWR), and half-sib hybrid (HSH) populations harvested from the 4X Acc:641.T CWR genet in hybridization plots (HPs) containing tetraploid (4X) or octoploid (8X) pollen-parent populations of 4X Acc:636 BWR, 4X 'Trailhead' BWR, 8X 'Continental' BWR, and 8X 'Magnar' BWR. Population identifiers include (**A**) 4X Acc:641 CWR (including the 4X Acc:641.T CWR genet); (**B**) progeny of the 4X Acc:641.T CWR genet in HPs containing 4X Acc:646 BWR harvested in years 1 and 2; (**C**) 4X Acc:646 BWR; (**D**) progeny of the 4X Acc:641.T CWR genet in HPs containing 4X 'Trailhead' BWR harvested in years 1 and 2; (**E**) 4X 'Trailhead' BWR; (**F**) progeny of the 4X Acc:641.T CWR genet in HPs containing 8X 'Continental' BWR harvested in years 1 and 2; (**G**) 8X 'Continental' BWR; (**H**) progeny of the 4X Acc:641.T CWR genet in HPs containing 8X 'Magnar' BWR harvested in years 1 and 2; and (**I**) 8X 'Magnar' BWR.

The average genetic similarity coefficients (S ± SE) varied from lowest to highest within populations of 4X 'Trailhead' BWR (0.641 ± 0.005), 8X 'Continental' BWR (0.676 ± 0.005), 8X 'Magnar' BWR (0.707 ± 0.006), 4X Acc:636 BWR (0.710 ± 0.005), 4X Acc:641.T CWR × 4X 'Trailhead' BWR half-sib hybrid (0.732 ± 0.004), 4X Acc:641.T CWR × 4X Acc:636 BWR half-sib hybrid (0.742 ± 0.004), and 4X

Acc:641.T CWR (0.746 ± 0.012). The average genetic similarity coefficients within the two half-sib hybrid families were both significantly higher ($p < 0.001$) than their respective BWR pollen-parent populations. The average genetic similarity coefficient with the 8X 'Continental' BWR synthetic interploidy hybrid was significantly ($p < 0.001$) higher than its 4X 'Trailhead' BWR parent and significantly ($p < 0.001$) lower than its 8X 'Magnar' BWR parent.

2.3. Biomass Yields in Dryland Range Environments

Dry matter yield of two half-sib hybrid populations was evaluated relative to four BWR reference populations in spaced-plant plots over two years and two dryland range environments: Providence and Tintic (Table S1). A full-sib hybrid population of 4X 'Rio' CWR × 4X 'Trailhead' BWR was also evaluated at the Providence site (Table 2) but there was insufficient seed to test this hybrid population at Tintic or any of the other sites. Variation in DMY was significantly influenced ($p < 0.001$) by main effects of population, location, and year. There was a significant interaction of year × location ($p < 0.001$), but there were no significant interactions of population × year or population × location. Values of DMY for each population were compared by location, in part because the full-sib hybrid population was not planted at the Tintic location (Table 2). The 4X 'Rio' CWR × 4X 'Trailhead' BWR full-sib hybrid population showed significantly ($p < 0.05$) greater DMY, compared to any other BWR or CWR × BWR population, at the Providence location (Table 2), which was true in the first and second year. The 4X Acc:641.T CWR × 4X Acc:636 BWR half-sib hybrid population showed significantly ($p < 0.05$) greater DMY than its 4X Acc:636 BWR parent population, averaged over both years and both locations (Table 2). Conversely, DMY of the 4X 'Trailhead' CWR × 4X Acc:636 BWR half-sib hybrid population was significantly ($p < 0.05$) lower than its 4X 'Trailhead' BWR parent population (Table 2). Dry matter yield of the 4X Acc:641.T CWR × 4X Acc:636 BWR half-sib hybrid population was not significantly different ($p < 0.05$) from the three release varieties of BWR at Providence but it was significantly lower ($p < 0.05$) than 'Continental' and 'Trailhead' at the Tintic location. Moreover, DMY of the synthetic-hybrid 8X 'Continental' BWR population was equal to or significantly ($p < 0.05$) greater than either of its parental populations, 'Trailhead' or 'Magnar', at the Tintic location and ranked highest based on the overall averages (Table 2).

Table 2. Dry matter yield (Mg·ha^{-1}) for tetraploid (4X) and octoploid (8X) populations of basin wildrye (BWR), creeping wildrye (CWR) × BWR half-sib hybrids (HSH), and CWR × BWR full-sib hybrids (FSH) in spaced-plant dryland range plots evaluated over two years and two locations (Providence and Tintic) in Utah. Significant differences among groups of entry means are indicated by lettered ranks, within table columns, based on least significant difference.

Population	Providence	Tintic	Avg.
4X Acc:636 BWR	0.9 c	0.8 de	0.9 d
4X 'Trailhead' BWR	1.6 b	1.3 ab	1.5 b
8X 'Continental' BWR	1.5 b	1.6 a	1.5 b
8X 'Magnar' BWR	1.4 b	1.2 bc	1.3 bc
4X Acc:641.T CWR × 4X Acc:636 HSH	1.4 b	1.0 cd	1.2 c
4X Acc:641.T CWR × 4X 'Trailhead' HSH	0.9 c	0.7 e	0.8 d
4X 'Rio' CWR × 'Trailhead' BWR FSH	2.8 a	—	2.8 a
Standard error	0.1	0.1	0.1
Average	1.5	1.3	1.4

For comparison, the relative dry matter yields among two 4X Acc:641 CWR × 4X Acc:636 BWR single-cross hybrid genets (TC1 and TC2) and their parent populations (4X Acc:641 CWR and 4X Acc:636 BWR) from other experiments conducted over four years at Hyde Park and Tetonia (Table S1), were summarized here (Table 3). Moreover, field evaluations at the Hyde Park and Tetonia sites also included other important reference populations such as 'Rio' CWR, 'Trailhead' BWR, 'Continental' BWR, 'Magnar' BWR, 'Alkar' tall wheatgrass, 'Mustang' Altai wildrye (*Leymus angustus*), and four

switchgrass cultivars ('Alamo', 'Dacotah', 'Falcon' and 'Sunburst'). 'Falcon' was the only switchgrass cultivar that successfully established and persisted at both locations, Hyde Park and Tetonia, thus it was the only one reported (Table 3). Dry matter yield variation among CWR × BWR s hybrid genets and other reference populations, at Hyde Park and Tetonia, was significantly influenced ($p < 0.001$) by population and there was a significant interaction of population × location ($p < 0.01$). Yields of the 4X Acc:641 CWR × 4X Acc:636 TC1 and TC2 single-cross hybrid genets were significantly higher than their 4X Acc:641 CWR parent population and yields of the TC1 hybrid were significantly greater than the 4X Acc:636 BWR high-parent. However, yields of the TC1 single-cross hybrid genet were not significantly different than the other BWR cultivars (Table 3). Moreover, it was also important to note that the 4X Acc:641.T CWR genet and 4X Acc:641 CWR reference population displayed substantially lower yields compared to all other populations including the 4X 'Rio' CWR cultivar (Table 3). These CWR reference populations were not included in other field experiments reported herein, in part because their aggressive rhizomes are difficult to manage and also because initial hybrid field evaluations, conducted at Hyde Park and Tetonia, showed that BWR is the higher-yielding reference parent (Table 3).

Table 3. Dry matter yield (Mg·ha^{-1}) for tetraploid (4X) and octoploid (8X) forms of basin wildrye (BWR), creeping wildrye (CWR), CWR × BWR single-cross hybrids (SCH), Altai wildrye (AWR), switchgrass (SG), and tall wheagrass (TWG) in dryland range plots evaluated over four years and two locations (Hyde Park, Utah and Tetonia, Idaho). Differences among groups of entry means are indicated by lettered ranks, within table columns, based on least significant difference.

Population	Hyde Park	Tetonia	Avg.
4X Acc:636 BWR	5.1 d	4.8 ab	5.7 cd
4X 'Trailhead' BWR	7.8 ab	4.6 ab	7.1 ab
8X 'Continental' BWR	7.1 ab	5.9 a	7.5 ab
8X 'Magnar' BWR	6.7 bc	6.1 a	7.1 ab
4X Acc:641 CWR	2.5 e	1.5 de	2.3 f
4X Acc:641 CWR.T genet	1.9 e	1.0 e	1.7 f
4X 'Rio' CWR	4.5 d	4.2 abc	4.8 de
4X Acc:641 CWR × 4X Acc:636 BWR TC1 SCH	7.2 ab	6.0 a	7.8 a
4X Acc:641 CWR × 4X Acc:636 BWR TC2 SCH	5.6 cd	4.1 bc	5.4 cd
'Alkar' TWG	8.2 a	4.5 abc	6.4 bc
'Falcon' SG	4.6 d	2.1 de	3.8 e
'Mustang' AWR	4.8 d	2.9 cd	4.0 e
Standard error	0.5	0.6	0.4
Average	5.5	4.0	5.3

2.4. Biomass Yields in an Irrigated Production System

Dry matter yield of two CWR × BWR half-sib hybrid populations was evaluated relative to other perennial grasses, including three released varieties of BWR, and two species mixtures, in a two-harvest irrigated production system at the Western Colorado Agriculture Experiment Station, Fruita location (Table S1).

Dry matter yield showed significant effects ($p < 0.001$) for population, year, harvest, and relative regrowth (difference between late- and early-season harvests). All two-way interactions and the three-way interactions among these four fixed effects were also significant ($p < 0.001$). Combined overall years and harvests, 'Alkar' tall wheatgrass produced significantly ($p < 0.05$) more dry matter than any other population (Table 4), but it also had significantly lower relative regrowth values indicating that it showed the greatest DMY decline from early-season to late-season harvests. Moreover, DMY of 'Alkar' tall wheatgrass was not significantly greater than the switchgrass variety mixture in the third and fourth evaluation years. Switchgrass consistently produced significantly ($p < 0.05$) more dry matter in the late-season harvest, overall years, compared to any of the other perennial grasses evaluated in this study (Table 4). Switchgrass also had significantly greater relative regrowth (Table 4), meaning that it produced relatively low DMY in the early-season harvest and relatively high DMY in the late-season

harvest compared to the cool-season grasses. The overall DMY of the 'Trailhead' BWR variety was significantly greater ($p < 0.05$) than the 'Continental' and 'Magnar' BWR populations, and it was also significantly greater ($p < 0.05$) than the 4X Acc:641.T CWR × 4X Acc:636 BWR half-sib hybrid population. However, the overall DMY of the 'Trailhead' BWR variety was not significantly greater than the 4X Acc:641.T CWR × 4X 'Trailhead' BWR half-sib hybrid population. The 4X Acc:641.T CWR × 4X 'Trailhead' BWR half-sib hybrid population showed significantly greater ($p < 0.05$) relative regrowth than its 'Trailhead' BWR parent population, which in this case indicated that the hybrid showed less DMY decline between the early- and late-season harvests (Table 4).

Table 4. Dry matter yield (Mg·ha^{-1}) for basin wildrye (BWR), creeping wildrye (CWR) × BWR half-sib hybrids (HSH), Altai wildrye (AWR), intermediate wheatgrass (IWG), switchgrass (SG), and tall wheatgrass (TWG) in a two-harvest, irrigated production experiment at the Western Colorado Agriculture Experiment Station. Significant differences among groups of entry means are indicated by lettered ranks, within table columns, based on least significant differences.

Population	Year 1	Year 2	Year 3	Year 4	Early Cut	Late Cut	%Δ [1]	Avg. Total
4X 'Trailhead' BWR	9.9 c–e	20.2 b	9.4 b	17.9 bc	9.4 b	4.9 de	−47 d	14.3 b
8X 'Continental' BWR	9.0 ef	18.3 bc	7.5 cd	16.5 c	7.8 cd	5 de	−36 c	12.8 c–e
8X 'Magnar' BWR	9.2 d–f	18.4 bc	7.5 cd	13.7 d	7.6 cd	4.7 e	−38 c	12.2 e
4X Acc:641.T CWR × 4X Acc:636 BWR HSH	10.0 c–e	17.3 cd	7.2 d	15.8 cd	7.8 cd	4.8 de	−37 c	12.6 de
4X Acc:641.T CWR × 4X 'Trailhead' BWR HSH	10.3 cd	17.6 cd	7.9 cd	17.5 c	8.0 c	5.4 cd	−33 c	13.3 b–e
'Mustang' AWR	10.4 c	18.0 b–d	9.7 b	16.8 c	7.2 d	6.5 b	−5 b	13.7 b–d
'Rush' IWG	12.7 b	18.3 bc	8.5 bc	16.5 c	9.1 b	4.9 de	−46 d	14.0 bc
'Oahe' IWG	12.9 b	17.6 cd	7.9 cd	17.7 bc	8.9 b	5.1 c–e	−42 d	14.0 bc
'Blackwell' and 'Dacotah' SG	8.5 f	15.6 d	10.9 a	22.0 a	6.4 e	7.9 a	+23 a	14.3 b
'Alkar' TWG	14.8 a	23.0 a	11.1 a	20.1 ab	11.6 a	5.7 c	−51 e	17.3 a
Standar error	0.4	0.8	0.5	1.0	0.3	0.3	0.3	0.4
average	10.8	18.4	8.8	17.4	8.4	5.5	−35	13.9

[1] Percent change (%Δ) calculated as (early cut yield—late cut yield)·100/(early cut yield).

2.5. Summarized Analysis of Biomass Yield from Dryland and Irrigated Testing Sites

A summarized analysis of overall years and testing sites was performed by nesting populations within groups including the 8X BWR, 4X BWR, 4X CWR, 4X CWR × 4X BWR hybrids (Table 5). Significant variation ($p < 0.0001$) was detected for fixed effects of locations, years, population within group, and group. However, these results (Table 5) should be viewed cautiously because not all populations were tested at all sites. In fact, the only populations that were tested across all sites were 'Trailhead' BWR, 'Continental' BWR, 'Magnar' BWR, and the 4X Acc:641 CWR × 4X Acc:636 BWR hybrids (as single-cross or half-sib hybrids). This meta-analysis shows that BWR and 4X CWR × 4X BWR hybrids displayed significantly more dry matter yield than CWR at dryland sites and suggests that this could be extrapolated to other sites, even though CWR was only tested at two of the four dryland testing sites (Table 5). This meta-analysis also shows that 8X BWR performed better than 4X BWR in dryland environments, whereas 4X BWR performed better than 8X BWR in the irrigated environment (Table 5). Estimated yields of the 4X CWR × 4X BWR hybrids including all half-sib hybrids, full-sib hybrids, and single-cross hybrids were significantly less than 8X BWR in dryland environments, significantly less than 4X BWR in the irrigated environment, but not significantly different from 4X or 8X BWR overall (Table 5). The estimated yield of switchgrass in dryland environments was based only on the best of four populations, where three populations failed to flourish, at only two of the four sites. Thus, estimated yield of switchgrass in dryland environments should also be viewed cautiously.

Table 5. Dry matter yield estimates for tetraploid (4X) and octoploid (8X) forms of basin wildrye (BWR), creeping wildrye (CWR), CWR × BWR hybrids, Altai wildyre (AWR), intermediate wheatgrass (IWG), switchgrass (SG), and tall wheatgrass (TWG) across four dryland ranges and one irrigated testing environment. Significant differences among groups of entry means are indicated by lettered ranks based on least significant difference.

Population	Dryland Range	Irrigated	Overall
4X BWR	4.0 b	14.3 b	5.6 b
8X BWR	4.4 a	12.5 d	5.5 b
4X CWR	0.6 d	—	2.7 c
4X CWR × 4X BWR	4.2 ab	13.0 cd	5.8 b
AWR	2.0 c	13.7 bc	5.2 b
IWG	—	14.0 b	6.1 b
SG	1.6 c	14.3 b	5.0 b
TWG	4.7 ab	17.3 a	8.3 a
Standard error	0.2	0.5	0.3
Average	3.1	14.1	5.5

3. Discussion

3.1. Development and Testing of CWR × BWR Hybrids

Our study demonstrated useful methods of producing hybrid seed for allogamous plants on a field scale, which has been a considerable challenge in perennial grass breeding [15,26]. Our approach worked effectively for crosses of 4X CWR and 4X BWR, but it did not work in crosses of 4X CWR and 8X BWR. One possible reason for low production of 4X CWR × 8X BWR hybrids may be delayed flowering of the 8X BWR populations. However, other intrinsic fertility barriers such as abnormal endosperm development are expected from interploidy crosses of 4X CWR and 8X BWR, 4X and 8X BWR, or 4X and 8X switchgrass interploidy hybrids [40,41]. This is why chromosome doubling of 4X 'Trailhead' BWR was required to make the 8X 'Continental' interploidy hybrid of 4X 'Trailhead' and 8X 'Magnar' BWR cultivars. Thus, reproductive incompatibility mechanisms such as the endosperm genic balance number requirement [41] explain the prevalence of relatively light seed and poor germination of seed obtained from the 4X CWR × 8X BWR hybridization plots (Table 1). Most of the seed from the 4X CWR × 8X BWR hybridization plots that did germinate was derived from self-pollination or pollination by 4X BWR from other hybridization plots. Conversely, all of the seed sampled from 4X CWR × 4X BWR hybridization plots was of hybrid origin with relatively high seed weights and germination rates. The average estimated seed yields of the 4X Acc:641.T CWR genet within hybridization plots containing 4X BWR (Table 1) were about five-fold lower than the reported seed yield averages of about 336 kg·ha^{-1} (33.6 g·m^{-2}) from 'Rio' CWR [42]. However, it is impossible to say whether our yields were limited by inherent limitations of this technique, such as gamete incompatibility, or other factors such as differences in the productivity of CWR genotypes or environments.

The half-sib 4X CWR × 4X BWR hybrid populations are similar to that of semi-hybrids of grass or alfalfa [43–46] in the sense that the hybrids are comprised of genetically heterogeneous individuals, but different in that virtually all of the seeds harvested from the 4X CWR seed parent were hybrids. The semi-hybrids, in contrast, contain an equal mixture hybrid and parental populations [43–46]. With prolific production of rhizomes and tillers, clonal production of the CWR seed parent is feasible. This approach of half-sib hybrid seed production, using one clonally propagated seed parent, creates opportunity to select individual genets that have good combining ability and also provides a rapid way to introduce novel genes into hybrid populations. Although similar approaches of producing full-sib single-cross hybrids has been proposed by clonal propagation of two self-incompatible genets, the difficulty of clonal propagation limits the application of this approach in perennial ryegrass (*Lolium perenne*) or switchgrass [15].

As a group, 4X CWR × 4X BWR hybrids showed yields that were at least equal to their 4X BWR parental populations, overall sites, and significantly higher than CWR yields in dryland environments (Table 5). Moreover, 4X BWR populations displayed substantially and statistically greater yields than 4X CWR in dryland environments (Tables 3 and 5). Thus, we cautiously assume that our 4X CWR × 4X BWR hybrid populations show indication of mid-parent heterosis if their yields are equivalent to their 4X BWR parent populations and show indication of high-parent heterosis if their yields are significantly greater than their 4X BWR parent populations in dryland range growing environments. The 4X Acc:641.T CWR × 4X Acc:636 BWR half-sib hybrid population showed a significant 33% advantage over its 4X Acc:636 BWR parent population at the Providence and Tintic testing sites (Table 2), indicating possible high-parent heterosis of this half-sib hybrid. One of the 4X Acc:641 CWR × 4X Acc:636 BWR single-cross hybrid genets also displayed a significant 36% yield advantage over the 4X Acc:636 BWR population at the Hyde Park and Tetonia testing sites (Table 3), which may indicate high-parent heterosis, but we did not have the actual 4X Acc:636 parent plant that was needed as a reference for this single-cross hybrid. Nevertheless, the 4X Acc:636 BWR parent population and both 4X Acc:641 CWR × 4X Acc:636 BWR single-cross hybrids all showed at least two-to three-fold greater yields than the 4X Acc:641.T CWR genet or 4X Acc:641 CWR population at the Hyde Park and Tetonia testing sites (Table 3). Thus, we conclude that yields of 4X Acc:641.T CWR × 4X Acc:636 BWR hybrids show indications of high-parent heterosis at the Providence and Tintic testing sites (Table 2) and that the 4X Acc:641 CWR × 4X Acc:636 BWR single-cross hybrids showed indications of mid-parent and possible high-parent heterosis at the Hyde Park and Tetonia testing sites (Table 3). Yields of the 4X 'Rio' CWR × 4X 'Trailhead' BWR full-sib hybrid population were 75% greater than its 4X 'Trailhead' BWR parent population at the Providence site (Table 2) and the 4X 'Trailhead' BWR population showed yields that were nearly 50% greater than the 4X 'Rio' CWR at the Hyde Park and Tetonia sites (Table 3). Therefore, the 4X 'Rio' CWR × 4X 'Trailhead' BWR full-sib hybrid population also showed indications of high-parent heterosis by outperforming its 4X 'Trailhead' BWR parent population by a 75% margin. Although yields of 4X CWR × 4X BWR hybrid populations were slightly but not significantly greater than 4X BWR populations' overall dryland environments (Table 5), the 4X Acc:641 CWR.T × 4X 'Trailhead' BWR half-sib hybrid performed much worse than its 4X 'Trailhead' BWR population at the Providence and Tintic dryland testing sites (Table 2), indicating that performance of this hybrid may be more similar to its presumed low-parent, 4X Acc:641 CWR.T.. As a group, yields of the 4X CWR × 4X BWR hybrids were significantly less than estimated yields of the 4X BWR populations in the irrigated testing site (Table 5), but yields of the 4X Acc:641.T CWR × 4X 'Trailhead' BWR half-sib hybrid were significantly less than its 4X 'Trailhead' BWR parent population in this two-harvest irrigated management system (Table 4). Thus, we conclude that some but not all 4X CWR × 4X BWR hybrids show indications of high-parent heterosis in dryland environments. Although performance of 4X CWR × 4X BWR half-sib hybrids was comparable to 4X BWR in the two-harvest irrigated production system, there were no indications of high-parent heterosis in this type of management system. Thus, it seems that heterosis in the 4X CWR × 4X BWR hybrids showed stronger indications of expression in the less competitive conditions of space-planted plots, which was also true for population hybrids of perennial ryegrass [46].

Dryland biomass yields of the 4X 'Rio' CWR × 4X 'Trailhead' BWR single-cross hybrid population were at least two-fold greater than biomass yields of either of the two half-sib hybrid populations produced by the 4X Acc:641.T CWR genet. One factor that may explain higher yields of the 4X 'Rio' CWR × 4X 'Trailhead' BWR hybrid is that the 4X 'Rio' CWR showed about two- to three-fold greater yields than 4X Acc:641 CWR population or 4X Acc:641.T CWR genet, respectively, in dryland yield trials conducted at Hyde Park and Tetonia. Also, the 4X 'Trailhead' BWR showed significantly greater yields than the other 4X BWR parent, Acc:636, used in this study (Tables 2 and 3). Delayed flowering may be another factor that may have contributed to relatively strong heterosis expressed in the 4X 'Rio' CWR × 4X 'Trailhead' BWR hybrid. It has been shown that delayed flowering can be a strong driver of biomass yield in switchgrass hybrids [27]. In our observations, 'Rio' CWR and the 4X 'Rio'

CWR × 4X 'Trailhead' BWR hybrid flowered about one week later than the 4X BWR, 4X Acc:641 CWR, or 4X Acc:641 CWR × 4X BWR populations. Biomass yields up to 11.5 Mg·ha^{-1} per year with saline irrigation water and repeated harvesting have been reported for 'Rio' CWR in the San Joaquin Valley of California [37]. Thus, an important future goal will be to generate half-sib hybrid seed for the cross of 4X 'Rio' CWR × 4X 'Trailhead' BWR and test these hybrids in multiple dryland and irrigated environments of California, Colorado, Nevada, and Utah where BWR and CWR may be useful as biomass feedstocks [12,37]. In any case, comparisons of hybrids made using two different CWR genets—4X Acc:641.T and one 4X 'Rio' plant—indicate that the identification and selection of superior CWR seed-parents, as well as superior BWR pollen parents, may be the fastest and most promising approach to develop higher-yielding CWR × BWR hybrids and native grass bioenergy feedstocks for western North America.

3.2. Dryland Yield Potential of Perennial Grasses in Cold-Desert Environments

Average yearly dryland biomass yields ranging from 2.2 to 9.6 Mg·ha^{-1} for the 4X Acc:636 BWR population were previously reported based on testing over four years and two locations near Hyde Park, Utah and Tetonia, Idaho [14] but otherwise we are not aware of any other published reports on non-irrigated biomass yields of BWR in its native range environments. The average yearly dryland biomass yields of the 4x Acc:636, 4X 'Trailhead', 8X 'Continental', and 8X 'Magnar' BWR populations ranged from 0.5 to 2.1 Mg·ha^{-1} at Providence and Tintic (Table 2), whereas the average yearly biomass yields of these same four populations ranged from 2.2 to 13.3 Mg·ha^{-1} at the Hyde Park and Tetonia testing sites (Table 3). Thus, dryland biomass yields from experiments conducted at the Providence and Tintic sites were lower than previously described experiments conducted at the Hyde Park and Tetonia testing sites [14]. One possible explanation for these differences is that plant densities in the Hyde Park and Tetonia sites were twice as high as the Providence and Tintic sites. The plants at Providence and Tintic were, however, simply less vigorous. Under these conditions, we would expect BWR plants to grow larger, not smaller, at the lower plant densities such as those used at Providence and Tintic (1 plant·m^{-2}). Although the Tetonia and Tintic testing sites are located in dissimilar environments (Table S1), the Hyde Park and Providence sites were located at nearly identical elevations in the same valley, only 12.8 km apart, with the same soil types (Nibley silty clay loam). The average yearly biomass yields at Hyde Park and Tetonia varied nearly three-fold, between 3.1 and 8.6 Mg·ha^{-1}, over four harvest years (Table 3), indicating that seasonal variations in climate can have dramatic effects on perennial grass productivity especially under low-input management. However, other factors probably contributed to differences between evaluations conducted at the Providence and Tintic sites (Table 2) versus the Hyde Park and Tetonia sites (Table 3). Nevertheless, the 4X 'Trailhead' BWR population ranked relatively high and the 4X Acc:636 BWR population ranked relatively low across all dryland environments (Tables 2 and 3). Moreover, 8X BWR populations performed significantly better across all dryland range testing sites (Table 5). These results demonstrate significant genetic variation for BWR biomass yields within its native growing environments. Wide variation in the average yields among sites, and significant genotype by environment interactions, observed across our dryland testing sites also demonstrate the importance of testing plant materials over multiple locations and years.

The average total biomass yields of all four BWR populations, two CWR × BWR single-cross hybrid genets, and tall wheatgrass were all significantly greater than the best of four switchgrass varieties tested in non-irrigated experiments conducted at Hyde Park and Tetonia (Table 3). This was not surprising considering that these relatively high elevation, cold-desert testing sites are not located within the native range of switchgrass [1,5]. However, there did appear to be some variation for adaptation to these environments, among the four switchgrass varieties tested, and it was interesting that the best adapted variety, Falcon, was an upland ecotype originating from relatively high-elevations (1517 m) in New Mexico on the western range of switchgrass distribution [5]. Falcon did not perform as well as Dacotah in high-latitude regions of Europe [47], but it did perform better than Dacotah in our high-elevation cold-desert testing sites (Table S1). Additional screening and breeding research

may help identify and develop switchgrass populations that perform better in cold high-latitude or high-elevation growing environments [48], such as those found in western North America.

Although 'Alkar' tall wheatgrass was clearly the best yielding population in the irrigated testing environment, it is interesting that overall dryland biomass yields of BWR cultivars and the 4X Acc:641 CWR × 4X Acc:636 BWR TC2 single-cross hybrid genet were equal to or significantly greater than 'Alkar' tall wheatgrass (Table 3). Tall wheatgrass was introduced and widely naturalized throughout North America [13], and has proven to be a high yielding cool-season grass in other non-irrigated field evaluations in studies conducted in western North Dakota [6] and Kansas [9].

3.3. Irrigated Yield Potential of Perennial Grasses in Cold-Desert Environments

The early-season biomass yields and average yearly biomass yields of perennial grasses including 'Trailhead' BWR, 'Alkar' tall wheatgrass, and switchgrass evaluated in our two-harvest irrigated production experiment in western Colorado can be compared to the total annual yields in a single-harvest irrigation system in western Nevada [12]. In the single-harvest evaluation conducted in western Nevada, DMY values of 'Trailhead' BWR and 'Alkar' tall wheatgrass averaged 7.2 and 7.3 Mg·ha^{-1} under low-water treatments (71 cm water annually) or 8.7 and 9.9 Mg·ha^{-1} in the high irrigation (120 cm water annually) treatment, respectively [12]. These values from single-harvest irrigated field trial [12] were similar but slightly lower than the average early-season harvest yields of 'Trailhead' BWR and 'Alkar' tall wheatgrass from western Colorado (Table 4). Since all of the cool-season grasses evaluated in the two-harvest irrigated production experiment were in the anthesis or post-anthesis stage of development on the early-harvest dates, we believe that they would have reached maximum or near-maximum annual yields in a single-harvest system. However, this was not true for switchgrass, which was not yet flowering and did not have a chance to reach maximum biomass accumulation values on the early-harvest dates in this two-harvest experiment (Table 4). Nevertheless, the switchgrass yields from both the early- and late-harvests in western Colorado (Table 4) were similar to the observed DMY of 7.8 Mg·ha^{-1} in the high-water treatment in western Nevada and substantially greater than 2.8 Mg·ha^{-1} observed in the low-water treatment [12]. Although the timing of our early-season harvest was optimized for cool-season grasses, it provided time for all of the perennial grasses, including switchgrass, to regrow and produce significantly more yearly total biomass (Table 4) than the single-harvest irrigated production system used in western Nevada [12]. The overall biomass yields showed nearly three-fold variation, from 5 to 15 Mg·ha^{-1}, over three years in Nevada [12] and over two-fold variation over four years in Colorado (Table 4).

The average yearly total yields of 'Alkar' tall wheatgrass, Rush intermediate wheatgrass, and switchgrass from our two-harvest irrigated evaluation in western Colorado (Table 4) can be compared to the average yearly total yields from another multiple-harvest irrigated study, conducted in northern Utah, containing these same species [11]. The average yearly total yields of 'Alkar' tall wheatgrass and Rush intermediate wheatgrass from Colorado (Table 4) were substantially lower than the maximum DMY values of 27.6 and 26.8 Mg·ha^{-1} observed for these same varieties, respectively, at the optimum water level of a five-harvest irrigated production evaluation in northern Utah [11]. Yet, the average yearly total DMY of one switchgrass variety mixture tested in the two-harvest irrigated production system (Table 4) was very similar to the average yearly total of about 14.0 Mg·ha^{-1} observed for the four switchgrass varieties tested in the five-harvest irrigated production system [11]. Thus, with adequate irrigation, switchgrass was competitive with cool-season grasses in one-harvest [12] and two-harvest management systems (Table 4), but substantially greater yields can be attained from repeated harvesting of cool-season grasses including tall wheatgrass and intermediate wheatgrass [11]. Comparisons of these three studies suggest that something, perhaps cool-nights, may be impeding regrowth of warm-season grasses with repeated harvests in these high-elevation cold-desert environments. The early-season DMY values of tall wheatgrass and intermediate wheatgrass were greater than switchgrass in multiple-harvest studies conducted in Utah [11] and Colorado (Table 4), whereas the late-season yields of switchgrass were always greater

than cool-season grasses in both studies, over all irrigation levels [11]. Presumably this difference in yield phenology is related to the C4 photosynthesis system of switchgrass and the low-temperature growth potential of cool-season grasses.

Although early-season yields of 'Trailhead' BWR were substantially greater than switchgrass, the average total yields were not significantly different in the two-harvest irrigated experiment in Colorado (Table 4) or either of the two irrigation treatment levels tested in Nevada [12]. The early-season and average total biomass yields of 'Alkar' tall wheatgrass were both significantly greater than switchgrass in the two-harvest irrigated experiment in Colorado (Table 4) and the low-water treatment in the one-harvest experiment conducted in Nevada [12]. The average total biomass yields of 'Alkar' tall wheatgrass was nearly two-fold higher than the best of four switchgrass varieties at the highest water levels and more than three-fold higher at the lowest water levels in the five-harvest irrigated experiment in Utah [11]. Thus, results from irrigated production systems in Colorado (Table 4), Nevada [12] and Utah [11] indicate that cool-season grasses such as BWR, intermediate wheatgrass, and tall wheatgrass could be better than switchgrass when irrigation water supplies are limited in the cold-desert environments of western North America.

Significant variance among the relative regrowth of cool-season grasses (Table 4) may reflect dissimilarities in phenology or defoliation tolerance. Tall wheatgrass displayed significantly greater yield declines from early- to late-season harvests, compared to other grasses (Table 4), which might be related to its relatively late flowering times. Yield declines of the 4X 'Trailhead' BWR population were significantly greater than the 4X Acc:641.T CWR × 4X 'Trailhead' BWR hybrid. The most obvious explanation for this is that CWR and the interspecific hybrids are much more rhizomatous and produce more tillers than BWR, which may provide mechanisms for better defoliation tolerance and regrowth potential [14,39]. Expression of rhizomatousness in the CWR × BWR hybrids is largely controlled by a combination of two recessive genes: one dominant gene, and one partially-dominant gene with major effects [14,39]. We are not aware of any other differences in phenology or physiology that would likely explain differences in the regrowth potential between BWR and the CWR × BWR hybrids. However, it was somewhat surprising that most of the BWR and CWR × BWR plant materials displayed significantly less yield declines, from early- to late-season harvests, compared to tall wheatgrass or intermediate wheatgrass. Native range grasses, such as BWR, are usually not included in multiple-harvest production systems, such as the five-harvest irrigated production trial conducted in northern Utah [11], in part because they do not have the forage quality of conventional pasture grasses and also because it has been generally presumed that they do not have significant regrowth potential. In fact, to our knowledge, this is the first documented experiment involving seasonal regrowth of BWR in an irrigated production system. Thus, improved regrowth of the 4X Acc:641.T CWR × 4X 'Trailhead' BWR hybrid could be a potentially useful attribute of the CWR × BWR hybrids [14,39].

Although yields of switchgrass from the two-harvest irrigated production study in Colorado (Table 4) were good, these yields have been surpassed in other environments. Average annual yields of upland and lowland switchgrass ecotypes ranged from 4 to 21 Mg·ha^{-1} in single-harvest production experiments conducted across various latitudes of central North America, from 36 to 46° N, including Kansas, Nebraska, Oklahoma, and Wisconsin where switchgrass is well adapted [28]. Switchgrass yields up to 26 and 33 Mg·ha^{-1} have been reported in one- and two-harvest production systems in the southeastern parts of North America [1].

4. Materials and Methods

4.1. Plant Materials Used for Making CWR × BWR Hybrids

The term "population" was used herein as a generic term for individuals sampled from genetically heterogeneous cultivars, hybrids, or natural germplasm accessions. Plant materials used for making and testing experimental CWR × BWR hybrids included two tetraploid (4X) BWR populations,

two octoploid (8X) BWR populations, and two 4X CWR populations. The BWR populations included 8X 'Continental', 4X 'Trailhead', and 8X 'Magnar' in addition to one natural 4X population Acc:636 collected near Lethbridge, Alberta sometime prior to 1974 by Sylvester Smoliak. 'Trailhead' originated from a natural population near Roundup, Montana and was released in 1991 [49]. 'Magnar' originated from a natural population in south-eastern British Columbia and was released sometime before 1995 [50]. The 4X CWR populations included a natural germplasm accession Acc:641 originally collected near Jamieson, Oregon in 1975 by Kay Asay and the cultivar 'Rio', which was originally collected from Kings Valley, California and released in 1991 [36].

The term "genet" was used herein to identify and reference clonally propagated individuals such as the two single-cross hybrids—TC1 and TC2—of 4X Acc:641 CWR × 4X Acc:636 BWR and the 4X Acc:641.T CWR plant used to make the full-sib pseudo-backcross families [51] and identify chromosome regions controlling biomass yield [14]. Both BWR and CWR are considered highly self-incompatible [52] and previous studies showed that the 4X Acc:641.T CWR genet was receptive to pollen from the TC1 and TC2 single-cross hybrids with less than 4% selfing using paper bags to cover its spike-inflorescences [51]. However, the quantities of seed produced by these techniques is insufficient for extensive testing in seeded field trials. Thus, we hypothesized that it would be possible to produce half-sib hybrid seed by growing the highly rhizomatous 4X Acc:641.T CWR genet in isolated "hybridization plots" with different 4X or 8X BWR populations, described above, as the only available pollen source. For purposes of testing other sources of CWR in hybrids with BWR, a new full-sib hybrid population was developed using paper bags to cover the spike-inflorescences of one 4X 'Rio' CWR plant and manually shaking pollen from extruded anthers of spikes from one 4X 'Trailhead' BWR genet.

4.2. Description of Locations Used to Develop and Test CWR × BWR Hybrids

All testing sites (Table S1) were located on properties of the Utah State University, Utah Agriculture Experiment Station; the Colorado State University, Western Colorado Research Center; or the University of Idaho, Eastern Idaho Agriculture Experiment Station. The average precipitation and temperature data was obtained from the Prism Climate Group [53] using geographic coordinates (Table S1) and 30-year normals (1981–2010) data setting at 800 m resolution. Precipitation data was summarized as seasonal averages for winter (December, January and February), spring (March, April and May), summer (June, July and August), and fall (September, October and November). The average, minimum, and maximum number of freeze-free days above 0 °C were determined using data available from the nearest station of similar elevation available from the Utah Climate Center [54].

4.3. Evaluation of Half-Sib Hybrid Seed Production

This study included an experiment designed to compare the yield and quality of seed from the 4X Acc:641.T CWR genet pollinated using the two 4X (Acc:636 and 'Trailhead') and the two 8X (Continental and 'Magnar') BWR populations as different fixed effects. The 4X Acc:641.T CWR genet was grown in isolated hybridization plots with each of the four different BWR pollen parent populations in a randomized complete block (RCB) design with four different hybridization plots (four different BWR pollen parents) within each block and three replicated blocks for a total of 12 hybridization plots. Beginning in December of 2007, rhizome propagules from the 4X Acc:641.T CWR genet and seedlings of the four BWR pollen parents were grown in a sufficient number of racked soil containers (4-cm diameter) to produce 972 clones of the 4X Acc:641.T CWR genet and 408 individual seedlings from each of the four BWR pollen parents. These plant materials were transplanted to a testing site near Richmond, Utah in the spring of 2008. Plants were irrigated after planting, but not irrigated during subsequent years of seed production. Each hybridization plot had three rows of the 4X Acc:641.T CWR genet flanked by two rows of the BWR pollen parent on each side. Rows were 13 m long with 0.91 m (36 inch) spacing between rows with plants spaced 0.5 m apart within rows. The hybridization plots were cultivated between rows and fertilized once per year with approximately

56 kg N ha^{-1} in the fall (October) and 34 kg N ha^{-1} in the spring (May) in the form of urea. Thus, the harvested CWR plot size was approximately 37 m^2, not including space used by the BWR pollen parents. The 12 hybridization plots were tandemly arranged in seven parallel rows, with 13 m space between test plots, for a total length of 323 m and total width of 5.5 m. The randomization scheme was restricted such that hybridization plots containing the 4X or 8X BWR pollen parents were alternated.

The total seed yield, average seed weight, and seed germination rates were measured using seed harvested from the 4X Acc:641.T CWR genet in each of the 12 hybridization plots over the course of four years, from 2009 to 2012, using a plot combine (Wintersteiger Inc., Salt Lake City, Utah, USA). Additional fine threshing and cleaning was performed manually before the seed yield ($g \cdot m^{-2}$) was determined for the harvested plot area (37 m^2). Three sets of 100 seed were counted and weighed from each plot, each year, to determine the average seed weight ($mg \cdot seed^{-1}$) and germination rates (%). Germination rates were determined by treating each 100-seed sample with tetramethythiuram disulfide, and then spreading each sample on blue germination blotter paper (Anchor Paper, St. Paul, Minnesota, USA) moistened with distilled water in 11 × 11 × 3.5-cm Cont 156C germination boxes (Hoffman Manufacturing, Inc., Jefferson, Oregon, USA). Using standard seed testing procedures for CWR and BWR, seeds were imbibed for 3 d at 25 °C, stratified at 4 °C for 14 d in dark chambers, and then tested for germination in dark plant-growth chambers maintained on a diurnal cycle of 16 h at 15 °C and 8 h at 25 °C. Germinated seeds were counted each week and until the final 28 d count.

4.4. Genetic Testing of Half-Sib Hybrid Seed

The genetic identity of seed produced by the 4X Acc:641.T CWR genet, from the first and second years of seed production, was tested by comparing it with representative genotypes of the CWR and BWR parents using a principle coordinate analysis of genetic distances based on comparisons of DNA profiles. Fresh leaf samples were lypholized and milled using a MM300 (Retsch Inc., Newtown, Pennsylvania, USA) mixer mill for DNA extraction using the DNeasy 96 Plant Kit (Qiagen, Germantown, Maryland, USA). Multi-locus DNA profiles of each DNA sample were developed using the AFLP technique [55] with modifications for detection using fluorescent labels and capillary electrophoresis. Briefly, the selective *Eco*RI primers were fluorescent labeled with 6-carboxyfluorescein and fractionated by capillary electrophoresis on an ABI3100 instrument (PE Applied Biosystems, Foster City, California, USA) with internal GS-500 size standards (PE Applied Biosystems) for each sample in each channel. The *Eco*RI and *Mse*I preamplification primers both included one selective nucleotide A and C, respectively. The selective amplification primers included two additional selective nucleotides in four different combinations including E36(ACC)//M61(CTG), E37(ACG)//M60(CTC), E38(ACT)//M60(CTC) and E41(AGG)//M47(CAA). Different AFLP markers were identified and scored for the presence or absence of bands (DNA amplicons) based on the relative mobility corresponding to about 1 bp using Genographer open source software. A principle coordinates analysis of DNA profiles was performed using NTSYSpc, Numerical Taxonomy System version 2.21 (Exeter Software, Setauket, New York, USA) based on pairwise comparisons of genetic similarity among individual plants computed using the similarity index (S) formula $S_{XY} = 2N_{XY}/(N_X + N_Y)$, where N_{XY} is the number of shared bands and where N_X and N_Y are the numbers of bands in plants X and Y. Population structure and ancestry coefficients of seed harvested from the 4X Acc:641.T CWR genet, in different hybridization plots, were also compared to BWR and CWR reference samples using Bayesian clustering [56,57] modeled a priori for possible admixture among the four expected base populations of 4X Acc:641 CWR, 4X Acc:636 BWR, 4X 'Trailhead' BWR, and 8X 'Magnar' BWR. All other genotypes were anticipated to be hybrid- or direct-descendants of these four base populations. Finally, statistical comparisons of genetic diversity within populations were based on the similarity index (S), based on unbiased estimates of the variance for S corrected for covariance [58].

4.5. Evaluation of DMY in Dryland Range Environments

In many cases, natural stands of BWR and other caespitose grasses grow with open spaces between plants rather than solid swards in the Great Basin region of western North America. Dry matter yields of experimental half-sib hybrid and full-sib hybrid populations were compared to the four BWR reference populations (Acc:636, 'Continental', 'Magnar', and 'Trailhead') in simulated range plots established using seedlings transplanted from soil containers at Tintic, Utah and Providence, Utah testing sites (Table S1). Germinated seedlings were planted into soil containers (4-cm diameter) in December, 2010 and then transplanted into a cultivated soil with dibbled holes in May, 2010 so that each plot was comprised of seven plants in a row, with 1 m between plants and 1 m between rows for a total plot size of 7 m^2 (1 plant·m^{-2}). Plots were replicated in a randomized complete block (RCB) design with six replications at each testing site. Plots were maintained with light cultivation to remove weeds as needed, with no fertilizer or irrigation. Once established, these plots have plant densities similar to typical natural stands of BWR in its native range environments. The plots were harvested to a height of 8 cm and weighed using a Swift Forage Harvester (Swift Machine and Welding LTD, Swift Current, Saskatchewan, Canada) once each year on 9 August 2011 and 28 August 2012 at the Providence site and 25 August 2011 and 29 August 2012 at the Tintic site. Results were reported on a DMY per unit area basis (Mg·ha^{-1}). Subsamples of harvested plant material were weighed, at the time of harvest, and dried at 60 °C in a forced-air oven to constant weights that were used to convert fresh harvest weights to DMY.

Prior to development of the half-sib hybrids, biomass yields of two single-cross 4X Acc:641 CWR × 4X Acc:636 BWR hybrid genets—TC1 and TC2—were also evaluated relative to the 4X Acc:641.T CWR genet, 4X Acc:641 CWR population, 4X Acc:636 , 'Magnar' BWR, 'Continental' BWR, 'Trailhead' BWR, 'Rio' CWR, 'Mustang' Altai wildrye, 'Alkar' tall wheatgrass, and four varieties of switchgrass ('Alamo', 'Dacotah', 'Falcon', and 'Sunburst') in space-planted dryland range plots over four years (2008–2011) and two locations near Hyde Park, Utah and Tetonia, Idaho (Table S1). The management of the experiments conducted at the Hyde Park and Tetonia sites was similar to the dryland range evaluations at the Providence and Tintic sites, except that plants were spaced 0.5 m within rows [14] and cultivated at least once each year, deeply, to maintain separation between highly rhizomatous plots containing CWR and CWR backcross lines [14].

4.6. Evaluation of Biomass-Related Traits in an Irrigated Production System

Dry matter yield BWR, CWR × BWR half-sib hybrid populations, and other large-statured perennial grasses were evaluated in a two-harvest irrigated production system over four years at the Western Colorado Research Center located in the Grand Valley of the Colorado River near Fruita (Table S1).

A seedbed was prepared for furrow irrigation using equipment commonly used in Grand Valley region, which resulted in a smooth, flat-bed surface with V-shaped furrows approximately 10-cm deep with 76-cm space between the bottom of each furrow. The experiment was planted on 20 October 2011 using a cone plot planter. Two seed rows were sown about 2-cm deep with 30-cm spacing within each bed and about 46-cm spacing between beds. The plot size was 3.05 m wide (four seed beds with a total of eight seed rows) and 4.57 m long for a total area of 13.94 m^2. The soil was a Glenton very fine sandy loam (coarse-loamy, mixed (calcareous), mesic family of Typic Torrifluvents). The plot area was soil sampled prior to planting, which was performed on 20 October 2011. The results of the soil test analysis were: pH 7.7, 0.4 mmhos/cm, 1.4% organic matter, 8 ppm NO_3-N, 13 ppm P, and 62 ppm K.

This experiment included two CWR × BWR half-sib hybrid populations plus selected reference populations of Altai wildrye (*Leymus angustus*); intermediate wheatgrass (*Thinopyrum intermedium*), switchgrass (*Panicum virgatum*), tall wheatgrass (*Thinopyrum intermedium*), and two species mixtures. 'Mustang' Altai wildrye; four BWR populations 4X Acc:636, 8X 'Continental', 8X 'Magnar', and 4X 'Trailhead'; 'Oahe' and 'Rush' intermediate wheatgrass; 'Alkar' tall wheatgrass; and two CWR × BWR half-sib hybrid populations were all seeded at a rate of 16.8 kg·ha^{-1} for each population in each plot.

A switchgrass variety mixture, comprised of cultivars 'Blackwell' and 'Dacotah', was seeded at rates of 3.36 kg·ha^{-1} for each variety with an overall seeding rate of 6.72 kg·ha^{-1}.

Plots were harvested twice each year, over a period of four years, with an automated forage plot harvester [59]. In the first harvest year of 2012, while plants were in the juvenile phase of development, plots were harvested on 24 July and 11 October with an application of 56 kg N ha^{-1} on 27 July. In the second harvest year of 2013, plots were harvested on 20 June and 22 October with an application of 56 kg N ha^{-1} on 24 June. In the third harvest year of 2014, plots were harvested on 19 June and 9 October, with an application of 56 kg N ha^{-1} + 122 kg P_2O_5 ha^{-1} on 20 June. In the fourth harvest year of 2015, plots were harvested on 19 June and 8 October, with applications of 56 kg N ha^{-1} on 10 April and 56 kg N ha^{-1} + 40 kg P_2O_5 ha^{-1} + 13 kg K_2O ha^{-1} on 21 June. During harvest, a small forage sample was obtained from each plot and oven-dried at 50 °C to a constant weight that was used to convert fresh weight yields to DMY. The experiment was furrow-irrigated each year with irrigation water from the Colorado River delivered through a canal system. No herbicides were applied at any time to the plots during the study.

4.7. Statistical Analysis of Trait Data

Field trait data were analyzed within and among years and locations using the MIXED procedure of SAS version 9.4 with the repeated option to model covariance structure between years (SAS Institute, Cary, North Carolina, USA). Population, years, and locations were assumed to be fixed effects with replications as random effects. Mean comparisons were made among populations using Fisher Protected Least Significant Difference tests at the $p = 0.05$ level of probability.

5. Conclusions

This study demonstrated a useful approach to produce hybrid seed for allogamous perennial grasses, which has been a long-standing problem in perennial grass improvement. Our study also provided new information on the biomass yield accumulation potential of BWR and other cool-season grasses in a two-harvest irrigated production system in a high-elevation, cold-desert environment of the western United States. Performance of BWR and other grasses in a two-harvest irrigated production system demonstrated significant regrowth potential of native grasses such as BWR. Comparisons of relative biomass yields across a wide range of irrigated and dryland environments of western North America, including new testing sites utilized in this study, indicate that cool-season grasses may have significant advantages over switchgrass under non-irrigated or limited-irrigation systems in this region. Although tall wheatgrass was clearly the best yielding population in this irrigated testing environment, BWR and CWR × BWR hybrids performed as good as or better than tall wheatgrass and other introduced species in the dryland range environments. Results the two-harvest irrigated field experiment demonstrated that switchgrass can also do well in a cold-desert environment if irrigation supplies are sufficient throughout the entire growing season. However, development and testing of cool-season perennial grass species, hybrids, and varieties across a wide range of growing environments is needed to expand the range of adaptation of grasses used for low-input bioenergy and forage production in the western United States and other parts of North America. Although biomass yields of BWR and other perennial grasses varied greatly over different environments, we detected significant genetic variation for this trait across widely various conditions. Some hybrids of CWR × BWR exhibited indications of mid- or high-parent heterosis and there were very large differences between the biomass yields obtained using a small sample of only two different CWR genets as seed parents and two BWR populations as pollen parents. Future efforts to develop CWR × BWR hybrids should focus on the identification and utilization of superior CWR genets as half-sib hybrid seed parents in addition to superior BWR pollen parents.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/7/1/3/s1.

Author Contributions: "S.R.L., C.H.P., K.B.J., and I.W.M. conceived and designed experiments; T.A.J. developed the TC1 and TC2 F_1 hybrids and the interploidy hybrid 'Continental'; S.R.L., M.D.R., and B.L.W. analyzed data; S.R.L. and J.E.S. wrote the paper." All authors read and approved the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Lewandowski, I.; Scurlock, J.M.O.; Lindvall, E.; Christou, M. The development and current status of perennial rhizomatous grasses as energy crops in the U.S. and Europe. *Biomass Bioenergy* **2003**, *25*, 335–361. [CrossRef]
2. McLaughlin, S.B.; Kszos, L.A. Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. *Biomass Bioenergy* **2005**, *28*, 515–535. [CrossRef]
3. Parrish, D.J.; Fike, J.H. The biology and agronomy of switchgrass for biofuels. *Crit. Rev. Plant Sci.* **2005**, *24*, 423–459. [CrossRef]
4. Wright, L.; Turhollow, A. Switchgrass selection as a "model" bioenergy crop: A history of the process. *Biomass Bioenergy* **2010**, *34*, 851–868. [CrossRef]
5. Casler, M.D.; Vogel, K.P.; Harrison, M. Switchgrass germplasm resources. *Crop Sci.* **2015**, *55*, 2463–2478. [CrossRef]
6. Monono, E.M.; Nyren, P.E.; Berti, M.T.; Pryor, S.W. Variability in biomass yield, chemical composition, and ethanol potential of individual and mixed herbaceous biomass species grown in North Dakota. *Ind. Crops Prod.* **2013**, *41*, 331–339. [CrossRef]
7. Wang, G.J.; Nyren, P.; Xue, Q.W.; Aberle, E.; Eriksmoen, E.; Tjelde, T.; Liebig, M.; Nichols, K.; Nyren, A. Establishment and yield of perennial grass monocultures and binary mixtures for bioenergy in North Dakota. *Agron. J.* **2014**, *106*, 1605–1613. [CrossRef]
8. Lee, D.; Owens, V.N.; Boe, A.; Koo, B.C. Biomass and seed yields of big bluestem, switchgrass, and intermediate wheatgrass in response to manure and harvest timing at two topographic positions. *CB Bioenergy* **2009**, *1*, 171–179. [CrossRef]
9. Harmoney, K.R. Cool-season grass biomass in the southern mixed-grass prairie region of the USA. *Bioenergy Res.* **2015**, *8*, 203–210. [CrossRef]
10. Jefferson, P.G.; McCaughey, W.P.; May, K.; Wosaree, J.; MacFarlane, L.; Wright, S.M.B. Performance of American native grass cultivars in the Canadian Prairie Provinces. *Nativ. Plants J.* **2002**, *3*, 24–33. [CrossRef]
11. Robins, J.G. Cool-season grasses produce more total biomass across the growing season than do warm-season grasses when managed with an applied irrigation gradient. *Biomass Bioenergy* **2010**, *34*, 500–505. [CrossRef]
12. Porensky, L.M.; Davison, J.; Leger, E.A.; Miller, W.W.; Goergen, E.M.; Espeland, E.K.; Carroll-Moore, E.M. Grasses for biofuels: A low water-use alternative for cold desert agriculture? *Biomass Bioenergy* **2014**, *66*, 133–142. [CrossRef]
13. Pearson, C.H.; Larson, S.R.; Keske, C.M.H.; Jensen, K.B. Native grasss for biomass production at high elevations. In *Industrial Crops: Breeding for Bioenergy and Bioproducts*; Cruz, M.V., Dierig, D.A., Eds.; Springer: New York, NY, USA, 2015; pp. 101–132.
14. Larson, S.R.; Jensen, K.B.; Robins, J.G.; Waldron, B.L. Genes and quantitative trait loci controlling biomass yield and forage quality traits in perennial wildrye. *Crop Sci.* **2014**, *54*, 111–126. [CrossRef]
15. Aguirre, A.A.; Studer, B.; Frei, U.; Lubberstedt, T. Prospects for hybrid breeding in bioenergy grasses. *Bioenergy Res.* **2012**, *5*, 10–19. [CrossRef]
16. Heaton, E.A.; Dohleman, F.G.; Miguez, A.F.; Juvik, J.A.; Lozovaya, V.; Widholm, J.; Zabotina, O.A.; Mcisaac, G.F.; David, M.B.; Voigt, T.B.; et al. *Miscanthus*: A promising biomass crop. *Adv. Bot. Res.* **2010**, *56*, 75–137.
17. Linde-Laursen, I. Cytogenetic analysis of *Miscanthus* 'Giganteus', an interspecific hybrid. *Hereditas* **1993**, *119*, 297–300. [CrossRef]
18. Hodkinson, T.R.; Chase, M.W.; Takahashi, C.; Leitch, I.J.; Bennett, M.D.; Renvoize, S.A. The use of DNA sequencing (ITS and *trn*l-f), AFLP, and fluorescent in situ hybridization to study allopolyploid *Miscanthus* (Poaceae). *Am. J. Bot.* **2002**, *89*, 279–286. [CrossRef] [PubMed]
19. Yamada, T. *Miscanthus*. In *Industrial Crops: Breeding for Bioenergy and Bioproducts*; Cruz, M.V., Dierig, D.A., Eds.; Springer: New York, NY, USA, 2015; pp. 43–66.

20. Robson, P.; Jensen, E.; Hawkins, S.; White, S.R.; Kenobi, K.; Clifton-Brown, J.; Donnison, I.; Farrar, K. Accelerating the domestication of a bioenergy crop: Identifying and modelling morphological targets for sustainable yield increase in *Miscanthus*. *J. Exp. Bot.* **2013**, *64*, 4143–4155. [CrossRef] [PubMed]
21. Nishiwaki, A.; Mizuguti, A.; Kuwabara, S.; Toma, Y.; Ishigaki, G.; Miyashita, T.; Yamada, T.; Matuura, H.; Yamaguchi, S.; Rayburn, A.L.; et al. Discovery of natural *Miscanthus* (Poaceae) triploid plants in sympatric populations of *Miscanthus sacchariflorus* and *Miscanthus sinensis* in southern Japan. *Am. J. Bot.* **2011**, *98*, 154–159. [CrossRef] [PubMed]
22. Dwiyanti, M.S.; Rudolph, A.; Swaminathan, K.; Nishiwaki, A.; Shimono, Y.; Kuwabara, S.; Matuura, H.; Nadir, M.; Moose, S.; Stewart, J.R.; et al. Genetic analysis of putative triploid *Miscanthus* hybrids and tetraploid *M. sacchariflorus* collected from sympatric populations of Kushima, Japan. *Bioenergy Res.* **2013**, *6*, 486–493. [CrossRef]
23. Tamura, K.; Sanada, Y.; Shoji, A.; Okumura, K.; Uwatoko, N.; Anzoua, K.G.; Sacks, E.J.; Yamada, T. DNA markers for identifying interspecific hybrids between *Miscanthus sacchariflorus* and *Miscanthus sinensis*. *Grassl. Sci.* **2015**, *61*, 160–166. [CrossRef]
24. Hodkinson, T.R.; Klaas, M.; Jones, M.B.; Prickett, R.; Barth, S. *Miscanthus*: A case study for the utilization of natural genetic variation. *Plant Genet. Resour. C* **2015**, *13*, 219–237. [CrossRef]
25. Martinez-Reyna, J.M.; Vogel, K.P. Heterosis in switchgrass: Spaced plants. *Crop Sci.* **2008**, *48*, 1312–1320. [CrossRef]
26. Vogel, K.P.; Mitchell, K.B. Heterosis in switchgrass: Biomass yield in swards. *Crop Sci.* **2008**, *48*, 2159–2164. [CrossRef]
27. Casler, M.D. Heterosis and reciprocal-cross effects in tetraploid switchgrass. *Crop Sci.* **2014**, *54*, 2063–2069. [CrossRef]
28. Casler, M.D.; Vogel, K.P. Selection for biomass yield in upland, lowland, and hybrid switchgrass. *Crop Sci.* **2014**, *54*, 626–636. [CrossRef]
29. Zalapa, J.E.; Price, D.L.; Kaeppler, S.M.; Tobias, C.M.; Okada, M.; Casler, M.D. Hierarchical classification of switchgrass genotypes using SSR and chloroplast sequences: Ecotypes, ploidies, gene pools, and cultivars. *Theor. Appl. Genet.* **2011**, *122*, 805–817. [CrossRef] [PubMed]
30. Lu, F.; Lipka, A.E.; Glaubitz, J.; Elshire, R.; Cherney, J.H.; Casler, M.D.; Buckler, E.S.; Costich, D.E. Switchgrass genomic diversity, ploidy, and evolution: Novel insights from a network-based SNP discovery protocol. *PLoS Genet.* **2013**, *9*, 139–147. [CrossRef] [PubMed]
31. Larson, S.R.; Wu, X.L.; Jones, T.A.; Jensen, K.B.; Chatterton, N.J.; Waldron, B.L.; Robins, J.G.; Bushman, B.S.; Palazzo, A.J. Comparative mapping of growth habit, plant height, and flowering QTLs in two interspecific families of *Leymus*. *Crop Sci.* **2006**, *46*, 2526–2539. [CrossRef]
32. Reynolds, T.D.; Fraley, L. Root profiles of some native and exotic plant-species in southeastern Idaho. *Environ. Exp. Bot.* **1989**, *29*, 241–248. [CrossRef]
33. Culumber, C.M.; Larson, S.R.; Jensen, K.B.; Jones, T.A. Genetic structure of Eurasian and North American *Leymus* (Triticeae) wildryes assessed by chloroplast DNA sequences and AFLP profiles. *Plant Syst. Evol.* **2011**, *294*, 207–225. [CrossRef]
34. Johnson, R.; Vance-Borland, K. Linking genetic variation in adaptive plant traits to climate in tetraploid and octoploid basin wildrye [*Leymus cinereus* (Scribn. & Merr.) a. Love] in the western U.S. *PLoS ONE* **2016**, *11*, e0148982.
35. Jones, T.A.; Parr, S.D.; Winslow, S.R.; Rosales, M.A. Notice of release of 'Continental' basin wildrye. *Nativ. Plants J.* **2009**, *10*, 57–61. [CrossRef]
36. USDA-NRCS. *Conservation Plant Release Brochure for 'Rio' Beardless Wild Rye (Leymus triticoides Buckley)*; United States Department of Agriculture, Natural Resources Conservation Service, California Plant Materials Center: Lockeford, CA, USA, 2014.
37. Suyama, H.; Benes, S.E.; Robinson, P.H.; Getachew, G.; Grattan, S.R.; Grieve, C.M. Biomass yield and nutritional quality of forage species under long-term irrigation with saline-sodic drainage water: Field evaluation. *Anim. Feed Sci. Technol.* **2007**, *135*, 329–345. [CrossRef]
38. Benes, S.E.; Adhikari, D.D.; Grattan, S.R.; Snyder, R.L. Evapotranspiration potential of forages irrigated with saline-sodic drainage water. *Agric. Water Manag.* **2012**, *105*, 1–7. [CrossRef]

39. Yun, L.; Larson, S.R.; Mott, I.W.; Jensen, K.B.; Staub, J.E. Genetic control of rhizomes and genomic localization of a major-effect growth habit QTL in perennial wildrye. *Mol. Genet. Genom.* **2014**, *289*, 383–397. [CrossRef] [PubMed]
40. Martinez-Reyna, J.M.; Vogel, K.P.; Caha, C.; Lee, D.J. Meiotic stability, chloroplast DNA polymorphisms, and morphological traits of upland × lowland switchgrass reciprocal hybrids. *Crop Sci.* **2001**, *41*, 1579–1583. [CrossRef]
41. Johnston, S.A.; Dennijs, T.P.M.; Peloquin, S.J.; Hanneman, R.E. The significance of genic balance to endosperm development in interspecific crosses. *Theor. Appl. Genet.* **1980**, *57*, 5–9. [CrossRef] [PubMed]
42. USDA-NRCS. *Notice of Release of 'Rio' Beardless Widlrye*; USDA-Natural Resources Conservation Service: Lockeford, CA, USA, 1991.
43. Brummer, E.C. Capturing heterosis in forage crop cultivar development. *Crop Sci.* **1999**, *39*, 943–954. [CrossRef]
44. Knowles, R.P. Comparison of cultivar hybrids and blends with pure cultivars in crested wheatgrass. *Can. J. Plant Sci.* **1979**, *59*, 1019–1023. [CrossRef]
45. Foster, C.A. Interpopulational and intervarietal hybridization in *Lolium perenne* breeding: Heterosis under non-competitive conditions. *J. Agric. Sci.* **1971**, *76*, 107–130. [CrossRef]
46. Foster, C.A. Interpopulational and intervarietal F1 hybrids in *Lolium perenne*: Performance in field sward conditions. *J. Agric. Sci.* **1973**, *80*, 463–477. [CrossRef]
47. Lemeziene, N.; Norkeviciene, E.; Liatukas, Z.; Dabkeviciene, G.; Ceceviciene, J.; Butkute, B. Switchgrass from North Dakota - an adaptable and promising energy crop for northern regions of Europe. *Acta Agric. Scand.* **2015**, *65*, 118–124.
48. Sage, R.F.; Peixoto, M.D.; Friesen, P.; Deen, B. C-4 bioenergy crops for cool climates, with special emphasis on perennial c-4 grasses. *J. Exp. Bot.* **2015**, *66*, 4195–4212. [CrossRef] [PubMed]
49. Cash, S.D.; Majerus, M.E.; Scheetz, J.C.; Holzworth, L.K.; Murphy, C.L.; Wichman, D.M.; Bowman, H.F.; Ditterline, R.L. Registration of 'Trailhead' basin wildrye. *Crop Sci.* **1998**, *38*, 278. [CrossRef]
50. Alderson, J.S.; Sharp, W.C.; Hanson, A.A.; U.S. Department of Agriculture. *Grass Varieties in the United States*; CRC Lewis Publishers: Boca Raton, FL, USA, 1995.
51. Wu, X.L.; Larson, S.R.; Hu, Z.M.; Palazzo, A.J.; Jones, T.A.; Wang, R.R.C.; Jensen, K.B.; Chatterton, N.J. Molecular genetic linkage maps for allotetraploid *Leymus* wildryes (Gramineae:Triticeae). *Genome* **2003**, *46*, 627–646. [CrossRef] [PubMed]
52. Jensen, K.B.; Zhang, Y.F.; Dewey, D.R. Mode of pollination of perennial species of the Triticeae in relation to genomically defined genera. *Can. J. Plant Sci.* **1990**, *70*, 215–225. [CrossRef]
53. PRISM Climate Group. Oregon State University. Available online: http://prism.oregonstate.edu (accessed on 23 December 2016).
54. Utah Climate Center, Utah State University. Available online: https://climate.usurf.usu.edu (accessed on 23 December 2016).
55. Vos, P.; Hogers, R.; Bleeker, M.; Reijans, M.; van de Lee, T.; Hornes, M.; Frijters, A.; Pot, J.; Peleman, J.; Kuiper, M.; et al. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* **1995**, *23*, 4407–4414. [CrossRef] [PubMed]
56. Falush, D.; Stephens, M.; Pritchard, J.K. Inference of population structure using multilocus genotype data: Dominant markers and null alleles. *Mol. Ecol. Notes* **2007**, *7*, 574–578. [CrossRef] [PubMed]
57. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **2000**, *155*, 945–959. [PubMed]
58. Leonard, A.C.; Franson, S.E.; Hertzberg, V.S.; Smith, M.K.; Toth, G.P. Hypothesis testing with the similarity index. *Mol. Ecol.* **1999**, *8*, 2105–2114. [CrossRef] [PubMed]
59. Pearson, C.H. An updated, automated commercial swather for harvesting forage plots. *Agron. J.* **2007**, *99*, 1382–1388. [CrossRef]

agronomy

MDPI

Article

Variation and Correlations among European and North American Orchardgrass Germplasm for Herbage Yield and Nutritive Value

Joseph G. Robins [1,*,†], B. Shaun Bushman [1,†], Ulf Feuerstein [2] and Greg Blaser [3]

1 USDA/ARS, Forage and Range Research, Logan, UT 84322, USA; shaun.bushman@ars.usda.gov
2 Deutsche Saatveredelung AG, Asendorf 27330, Germany; ulf.feuerstein@dsv-saaten.de
3 Brigham Young University—Idaho (Retired), Rexburg, ID 83440, USA; blaser.greg@gmail.com
* Correspondence: joseph.robins@ars.usda.gov; Tel.: +1-435-797-2905
† These authors contributed equally to this work.

Academic Editors: John W. Forster and Kevin F. Smith
Received: 28 September 2016; Accepted: 16 November 2016; Published: 2 December 2016

Abstract: Efforts to improve water-soluble carbohydrate (WSC) concentrations are common in perennial forage grass breeding. Perennial ryegrass (*Lolium perenne*) breeding has been very successful in developing new cultivars with high WSC and high agronomic performance. Breeding efforts are ongoing to improve the WSC of other perennial forage grasses, such as orchardgrass (*Dactylis glomerata*). The United States Department of Agriculture Forage and Range and Deutsche Saatveredelung orchardgrass breeding programs cooperated to characterize the expression and genotype by environment interaction (GEI) of water-soluble carbohydrates in a collection of orchardgrass populations from both breeding programs. Additionally, the effort characterized the relationship between water-soluble carbohydrates and other agronomic and nutritive value traits in these populations. Overall, the Deutsche Saatveredelung populations had higher herbage mass (15%), rust resistance (59%), and later maturity. The Forage and Range Research populations had higher water-soluble carbohydrates (4%), nutritive value, and earlier maturity. However, results were highly dependent on GEI. Differences were very pronounced at the French and German field locations, but less pronounced at the two US locations. Combining the germplasm from the Forage and Range Research and Deutsche Saatveredelung programs may be a way to develop an improved base germplasm source that could then be used separately in the EU and US for water-soluble carbohydrate and other trait improvement.

Keywords: cocksfoot; digestibility; genotype by environment; maturity; rust; water soluble carbohydrates

1. Introduction

Orchardgrass (*Dactylis glomerata*) is an auto-tetraploid, out-crossing forage grass, and is one of the main perennial grasses used for grazing and hay production in temperate climates [1]. Although *D. glomerata* is the sole species in the *Dactylis* genus, the species includes both Mediterranean and Continental ecogeographic subspecies that are diploid and tetraploid [2]. Orchardgrass is highly preferred by livestock, exhibits early season growth, and is one of the most compatible perennial forage grasses when sown with perennial legumes, e.g., alfalfa (*Medicago sativa* L.) [1,3,4]. It is less tolerant to abiotic stresses than tall fescue and, for winter injury, than timothy, but more tolerant than perennial ryegrass. The forage quality of orchardgrass is intermediate, less than perennial ryegrass (*Lolium perenne*), equal to timothy (*Phleum pratense*), but greater than tall fescue (*Lolium arundinaceum*). In the US orchardgrass is primarily utilized under rainfed conditions of the central and eastern portions, and under irrigated conditions in the semi-arid western portions [5].

Acute and chronic drought can result in substantial decreases of forage grass biomass production [6]. Winter damage caused by freezing includes delayed spring growth, uneven growth, and mortality. Available orchardgrass germplasm has substantial genetic variation for tolerance to drought and winter injury [7–9], and cultivars have been developed with drought tolerance in mind (e.g., cv. Paiute). However, cultivar performance under different or stressed conditions has been inconsistent [9], possibly due to a lack of uniformity in orchardgrass cultivars [10,11] or the unpredictability of appropriate environmental conditions in field settings [12]. Improving forage production and quality under abiotic stresses are objectives in orchardgrass breeding and genetics programs throughout the world (e.g., [13]).

In previous breeding efforts to improve perennial forage grass quality, it was noticed that water-soluble carbohydrate (WSC) accumulation in forage grasses was correlated with abiotic stress tolerance [14–19]. The WSC in crown tissues were found to stabilize lipids in cellular membranes [19], prevent membrane leakage, and maintain osmotic potential of the cells under cold and drought stress [17,18,20–22]. In addition to their role in abiotic stress tolerance, increased WSC in leaf and stem tissue can result in improved protein digestion, decreased nitrogen waste through feces and urine, and increased meat and milk productivity in feeding animals [23]. In orchardgrass, the overwhelming majority of all WSC are fructans [14,24], which bridge the gap between resource availability and demands [17]. Increasing WSC (i.e., fructans) is a tangible method of increasing both abiotic stress tolerance and forage quality in orchardgrass.

Although published reports on variation for orchardgrass WSC concentrations are limited, more information is coming forth in this arena. Robins et al. [25] identified substantial genetic variation for WSC within orchardgrass germplasm. Sanada et al. found significant differences in crown [13] and stubble WSC concentrations, and that WSC concentrations were related to winter hardiness and spring growth. Additionally they assessed European, Asian, and North American germplasm and found that European germplasm generally contained less WSC than other location sources [26]. Casler et al. [10] also detected differences in forage productivity and quality traits between North American and European orchardgrass varieties, although WSC was not included in that study. Other evaluations reported significant variation for herbage mass (HM) and forage quality under drought conditions, and found the WSC concentration to be correlated with forage productivity [27,28]. These reports indicate substantial variation for forage production and quality traits between different breeding programs, and suggested that increases in WSC can improve both traits. An understanding of the variation within a breeding program for WSC, and the relationship with forage productivity and quality, could result in great gains in this forage grass species.

In the study described herein, we examine the effect of differing environments in North America (US) and Europe (EU) on the expression of WSC, and the relationship between WSC and associated agronomic and nutritive value traits in populations of orchardgrass from the North American United States Department of Agriculture (USDA) Forage and Range (FRR) and European Deutsche Saatveredelung Aktiengesellschaft (DSV) breeding programs. The objective was to determine the utility of these breeding pools for the improvement of WSC and to determine how to capitalize on the genotype by environment interaction (GEI) effects underlying WSC, HM, and nutritive value expression in orchardgrass.

2. Results

2.1. Overall Model Effects

The overall statistical model identified differences ($p \leq 0.004$) among all main effects for each phenotype (Table 1). Year showed the largest effect (sums of squares) for WSC and acid detergent fiber (ADF); location for digestible dry matter (DDM), crude protein (CP), neutral detergent fiber (NDF), and neutral detergent fiber digestibility (NDFD); and population for maturity, rust rating (RR), and HM.

Table 1. Overall mean values for years, locations (HS—Hof Steimke; LR—Les Rosiers; LO—Logan; RE—Rexburg), and orchardgrass populations. Population values include the overall mean and range for each trait and population. Numbers followed by different letters in the same row (effect) differ statistically ($p < 0.05$).

	Year		Location				Entry		
	2014	**2015**	**HS**	**LR**	**LO**	**RE**	**Checks**	**DSV**	**FRR**
HM[1]	5.9 [a]	4.9 [b]	6.6 [a]	3.4 [c]	5.2 [b]	6.7 [a]	5.4 [b] (4.9–5.9)	5.8 [a] (5.4–6.2)	5.0 [c] (4.5–5.5)
DDM[2]	66.5 [b]	69.0 [a]	73.3 [b]	74.6 [a]	61.1 [d]	62.0 [c]	67.8 [b] (67.2–68.9)	67.3 [c] (66.7–68.1)	68.1 [a] (67.4–68.8)
CP[3]	11.3 [b]	14.7 [a]	8.1 [d]	14.4 [b]	14.0 [c]	15.5 [a]	12.9 [b] (12.1–13.6)	12.5 [c] (11.8–13.2)	13.5 [a] (12.7–14.4)
WSC[4]	7.6 [b]	8.4 [a]	1 [a]	8.7 [b]	6.4 [c]	6.8 [c]	8.3 [a] (7.7–1)	7.8 [b] (6.9–8.5)	8.1 [a] (7.2–9.2)
NDF[5]	61.5 [b]	56.2 [a]	64.6 [d]	58.3 [c]	57.1 [b]	55.5 [a]	58.8 [b] (56.7–6)	59.8 [c] (58.8–61.4)	58.0 [a] (57.0–59.3)
ADF[6]	35.6 [b]	31.5 [a]	34.6 [b]	29.5 [a]	35.7 [c]	34.6 [b]	33.5 [b] (32.4–34.5)	34.2 [c] (33.4–35.2)	33.0 [a] (32.2–33.8)
NDFD[7]	64.5 [b]	66.6 [a]	61.0 [c]	59.6 [d]	67.7 [b]	73.8 [a]	65.6[a] (64.6–66.4)	64.9 [b] (62.9–66.1)	66.1 [a] (63.8–67.9)
MAT[8]	3.0 [a]	2.8 [a]	-	2.6 [a]	2.9 [b]	3.1 [c]	2.7 [a] (0.8–3.9)	2.6 [a] (2.0–3.2)	3.1 [b] (2.8–3.4)
RR[9]	5.3 [b]	6.2 [a]	5.7 [b]	5.9 [a]	-	-	5.0 [b] (3.1–8.0)	7.7 [a] (6.5–8.4)	4.2 [c] (2.8–5.3)

[1] Herbage mass; [2] Digestible dry matter; [3] Crude protein; [4] Water soluble carbohydrates; [5] Neutral detergent fiber; [6] Acid detergent fiber; [7] Neutral detergent fiber digestibility; [8] Maturity; [9] Rust rating.

Differences among the locations during the study were large (Table 2). Annual maximum temperatures were higher at each location in 2015 compared to 2014, and growing degree days were higher in 2015 for each location but Hof Steimke. The two US locations experienced lower mean and minimum annual temperatures and annual precipitation compared to the European locations (Table 1). Therefore, the supplemental irrigation at these locations was used to compensate for the effect of the low precipitation. Although the annual maximum temperatures were similar among the four locations, there were lower winter and higher summer temperatures at the US locations. Interestingly, the growing degree days were also similar between Les Rosiers and Logan and between Hof Steimke and Rexburg.

Table 2. Mean weather characteristics at Hof Steimke, Germany; Les Rosiers, France; Logan, USA; and Rexburg, USA, during 2014 and 2015. Characteristics include latitude (Lat), longitude (Long), June photoperiod (June), soil type (Soil), annual precipitation excluding irrigation (Prec), mean annual temperature (Mean T), maximum annual temperature (Max T), minimum annual temperature (Min T), and growing degree days (GDD).

	Hof Steimke		**Les Rosiers**		**Logan**		**Rexburg**	
Lat	52.77° N		47.35° N		41.74° N		43.82° N	
Long	9.00° E		0.23° W		111.83° W		111.79° W	
June (h·day^{-1})	18–19		17–18		16–17		16–17	
Soil	Sandy, loam		Loamy, clay		Silty, clay loam		Silt loam	
Year	2014	2015	2014	2015	2014	2015	2014	2015
Prec (mm)	883	929	739	579	319	388	428	273
Mean T (°C)	11.2	10.5	13.7	13.5	8.9	10.0	7.2	8.3
Max T (°C)	32.7	37.5	35.0	37.2	36.7	37.8	33.3	35.6
Min T (°C)	−9.2	−4.8	−6.5	−6.0	−20.0	−22.2	−28.9	−26.7
GDD (50 °C)	1172	980	1628	1656	2314	2568	1828	2039

Rust resistance ratings were higher at Hof Steimke than Les Rosiers. Hof Steimke was characterized by relatively high WSC, HM, and NDF; intermediate DDM and ADF; and low CP. Les Rosiers was characterized by relatively high DDM; intermediate HM, NDF, CP, and WSC; and low ADF and NDFD. Logan exhibited relatively high ADF; intermediate levels of HM, NDF, NDFD, and CP; and low DDM and WSC. Rexburg exhibited relatively high HM, NDFD, and CP; intermediate DDM and ADF; and low NDF and WSC. Maturity ratings were similar at Les Rosiers and Logan, but earlier at Rexburg.

Overall, 13 populations (Muravka, Paykar and 11 FRR populations) had WSC values greater than 8.5% (Table S1). Twenty-three populations (Duero, Harvestar and 21 DSV populations) had HM greater than 5.7 kg·plot^{-1}. Twenty-four populations (Muravka, one DSV population, and 22 FFR populations)

had DDM greater than 68.0%. Twenty-five populations (Latar, Paiute, and 23 FRR populations) had CP greater than 13.5%. Twenty populations (Muravka, Paykar and 18 FRR populations) had NDF less than 58.1%. Seventeen populations (Muravka and 16 FFR populations) had ADF less than 33.0%. Twenty populations (Latar and 16 FRR populations) had NDFD greater than 66.2%. Seventeen populations (Duero and 16 DSV populations) had RR greater than 7.8. Twenty-five populations (Muravaka, Paiute, and 23 FRR populations) had maturity greater than 3.9 and were the earliest populations. Seventeen populations (Paykar and 16 DSV populations) had maturity less than 2.0.

Contrasts in the overall data showed that the check cultivars showed higher WSC and NDFD than the breeding program lines (Table 1). The DSV populations had higher HM, NDF, ADF, and RR; lower DDM, CP, and NDFD; and later maturity. The FFR populations had higher WSC, DDM, CP, NDFD; lower HM, NDF, ADF, and RR; and earlier maturity.

2.2. Principal Component Analysis and Correlations among Phenotype

Correlation estimates indicated a strong association among many of the measured phenotypes (Table 3). Only correlations > ±0.70 will be mentioned here. Herbage mass correlations were positive for NDF, ADF, and RR, but negative for DDM and CP. Digestible dry matter correlations were positive for WSC and NDFD, but negative for NDF, ADF, and RR. Crude protein was negatively correlated with NDF, ADF, and RR. Neutral detergent fiber was positively correlated with ADF and RR. Acid detergent fiber was positively correlated with RR. Maturity was not highly correlated with any phenotype, although correlations were highly driven by origin (EU vs. US).

Table 3. Pearson correlation estimates among orchardgrass populations among herbage mass (HM), dry matter digestibility (DDM), water soluble carbohydrates (WSC), neutral detergent fiber (NDF), acid detergent fiber (ADF), neutral detergent fiber digestibility (NDFD), maturity (MAT), rust resistance (RR), principal component 1 (PC1), and principal component 2 (PC2) evaluated across two years and four locations. All listed correlations significantly differ from zero ($p < 0.01$), unless noted NS (non-significant).

	DDM	**CP**	**WSC**	**NDF**	**ADF**	**NDFD**	**MAT**	**RR**
HM	−0.74	−0.74	−0.47	0.80	0.80	−0.51	−0.56	0.84
DDM		0.67	0.72	−0.92	−0.92	0.71	0.46	−0.73
CP			NS	−0.77	−0.83	0.54	0.41	−0.76
WSC				−0.66	−0.60	0.30	0.36	−0.45
NDF					0.96	−0.60	−0.51	0.83
ADF						−0.64	−0.51	0.82
NDFD							0.29	−0.53
MAT								−0.55

2.3. Interaction Effects between Population and Location

Significant interaction effects were identified for each phenotype. The year by location interaction was significant for all phenotypes but maturity. The year by population interaction was less consistent and differed only for maturity, RR, and HM. The location by population interaction differed for all phenotypes but maturity and ADF, although the evidence of interaction for CP was nominal ($p = 0.08$). The three-way year by location by population interaction was also inconsistent, differing for RR, HM, CP, and NDFD. The sums of squares associated with the interaction effects were generally smaller than population sums of squares, with exceptions of the year by location interaction for each of the nutritive value phenotypes; the location by population interaction for DDM, WSC, and NDFD; and the year by location by population interaction for NDFD. Due to the lack of differences for the year by population interaction, the inconsistency of the three-way interaction, and the perennial nature of orchardgrass [29], all further results are based on the location × population interaction analysis using AMMI analysis.

The Kendall (τ) statistic provided evidence for the nature (magnitude vs. rank changes) of the location × population interaction (Table 4). Population rankings between Hof Steimke and Les Rosiers were highly correlated ($\tau \leq 0.77$; $p < 0.0001$) for all phenotypes but WSC and NDFD. Hof Steimke rankings exhibited no to limited relationships with the Logan and Rexburg locations. Les Rosiers rankings also exhibited low to moderate relationships with the Logan and Rexburg locations, although the correlations were generally higher with the Rexburg rankings. The Logan and Rexburg rankings exhibited high correlation for DDM and WSC, moderate correlation for HM and NDFD, and low to no correlation for the remaining phenotypes. With the exception of the Hof Steimke and Les Rosiers rankings, the Kendall rankings provided strong evidence for changes in ranking leading to the underlying location by population interaction.

Table 4. Kendall concordance estimates of orchardgrass rankings for each phenotype across years and between locations for herbage mass (HM), dry matter digestibility (DDM), water soluble carbohydrates (WSC), neutral detergent fiber (NDF), acid detergent fiber (ADF), and neutral detergent fiber digestibility (NDFD). All listed correlations significantly differ from zero ($p < 0.05$), unless noted NS (non-significant).

	Les Rosiers	**Logan**	**Rexburg**
Hof Steimke			
HM	0.94	NS	0.35
DDM	0.95	NS	0.24
CP	0.77	0.61	0.44
WSC	0.47	NS	NS
NDF	0.88	0.54	0.69
ADF	0.94	0.37	0.72
NDFD	0.29	0.65	0.35
Les Rosiers			
HM		NS	0.42
DDM		0.17	0.30
CP		0.37	0.67
WSC		0.53	0.64
NDF		0.66	0.57
ADF		0.43	0.66
NDFD		NS	−0.37
Logan			
HM			0.62
DDM			0.88
CP			NS
WSC			0.89
NDF			0.22
ADF			0.09
NDFD			0.70

At Hof Steimke, Les Rosiers, and Rexburg there were differences ($p \leq 0.02$) among the populations for each phenotype (Table 5). At Logan there were pronounced population differences for maturity ($p = <2 \times 10^{-16}$), and HM ($p = 0.007$); less pronounced differences for DDM, NDF, and ADF ($p \leq 0.06$); and no differences for CP, WSC, and NDFD. The AMMI analysis identified the significance ($p \leq 0.0009$) of the first interaction principal component (IPCA) for each phenotype. The AMMI results separated the four locations into three groups for WSC and HM based on the high performance of a single population: (1) Hof Steimke and Les Rosiers; (2) Rexburg; and (3) Logan. All four locations grouped separately for DDM and CP. NDF resulted in two groups: (1) Hof Steimke, Les Rosiers, and Logan; and (2) Rexburg. ADF resulted in three groups: (1) Les Rosiers and Logan; (2) Hof Steimke; and (3) Rexburg. NDFD resulted in three groups: (1) Logan and Rexburg; (2) Hof Steimke; and (3) Les Rosiers. The two US locations grouped together less frequently than did the two EU locations, possibly

because of the lack of differences among the Logan populations for some traits. As demonstrated by the Kendall statistic, the performance of individual populations matched the grouping of locations for each phenotype, For example, nine populations showed high HM at both Hof Steimke and Les Rosiers.

Table 5. Population mean values corresponding to herbage mass (HM), dry matter digestibility (DDM), water soluble carbohydrates (WSC), neutral detergent fiber (NDF), acid detergent fiber (ADF), neutral detergent fiber digestibility (NDFD), maturity (MAT), and rust resistance (RR) for the orchardgrass population sources (DSV, FFR, and check) evaluated within each of the four locations and across the two production years. Numbers followed by different letters in the same column, and within the same location, differ statistically ($p < 0.05$).

	WSC	HM	DDM	CP	NDF	ADF	NDFD	MAT	RR
Hof Steimke									
Checks	10.2 [a]	5.6 [b]	75.9 [a]	11.3 [b]	60.3 [a]	31.3 [b]	64.1 [a,b]	-	5.7 [b]
DSV	8.9 [b]	6.0 [a]	75.3 [b]	11.3 [b]	61.4 [b]	31.8 [c]	63.8 [b]	-	7.6 [a]
FRR	10.3 [a]	4.9 [c]	76.3[a]	11.8 [a]	59.8 [a]	30.9 [a]	64.3 [a]	-	4.1 [c]
Les Rosiers									
Checks	8.9 [a]	3.3 [b]	74.9 [a]	14.4 [b]	57.9 [a]	29.2 [b]	59.6 [a]	3.0 [b]	5.8 [b]
DSV	8.4 [b]	4.0 [a]	74.2 [b]	13.4 [c]	59.2 [b]	30.3 [c]	59.7 [a]	2.1 [a]	7.9 [a]
FRR	8.8 [a]	2.8 [c]	74.9 [a]	15.3 [a]	57.6 [a]	28.8 [a]	59.5 [a]	4.3 [b]	4.3 [c]
Logan									
Checks	6.3 [a]	5.5 [a]	61.3 [a]	14.2 [a]	56.9 [a]	35.4 [a]	67.8 [a]	3.0 [b]	-
DSV	6.4 [a]	5.2 [b]	60.8 [b]	13.8 [b]	57.7 [b]	36.1 [b]	67.0 [b]	1.8 [a]	-
FRR	6.4 [a]	5.1 [b]	61.3 [a]	14.1 [a]	56.6 [a]	35.4 [a]	68.1 [a]	3.7 [c]	-
Rexburg									
Checks	6.8 [a]	6.6 [b]	62.0 [b]	15.4 [b]	55.6 [b]	34.6 [b]	73.9 [b]	3.1 [b]	-
DSV	6.8 [a]	7.0 [a]	61.6 [c]	15.2 [b]	56.5 [c]	35.1 [c]	72.5 [c]	2.1 [a]	-
FRR	6.7 [a]	6.5 [b]	62.5 [a]	16.0 [a]	54.4 [a]	34.0 [a]	75.1 [a]	3.8 [c]	-

3. Discussion

Previous evaluations of North American and European orchardgrass breeding programs [10,30] noted substantial differences between the two germplasm sources for HM and some forage quality traits. The current study focused on the characterization of the WSC concentration in the germplasm pools and the relationship between WSC and the other measured phenotypes. Additionally, this study focused on the GEI effects on orchardgrass from the DSV and FRR breeding programs when grown at sites in the EU and US. The DSV populations exhibited comparatively high HM and RR, but lower nutritive value, and late maturity. In contrast, the FRR populations exhibited higher nutritive value, including WSC, but lower HM and earlier maturity. Maturity, in particular, reflects the strong preference for much later material in the EU and the preference for seed production of early flowering cultivars in the US. Because rust infection was only rated in September at the EU locations, this study did not allow the further elucidation of this relationship by correlating the response at the individual harvests.

The success of breeding perennial ryegrass for increased WSC [31] has created a new objective for many forage breeding programs, including the DSV and FRR programs. Using near infrared spectroscopy approaches, WSC is easy to measure and characterize in targeted population improvement programs; however, its inheritance is strongly affected by location and GEI effects. High WSC perennial ryegrass cultivars may fail to express the phenotype in vastly differing environments, such as New Zealand [32] and the US [33]. The prevailing hypothesis for the GEI effect is that the higher summer temperatures limit the photosynthesis of the C_3 grasses and result in lower WSC concentrations in the forage [32]. This hypothesis is consistent with the results of the current analysis. While annual maximum temperatures did not differ substantially among the locations, the mean maximum temperatures and growing degree days during the growing season were higher at the US locations. Interestingly, the EU locations are at higher latitudes and receive more sunlight for

increased photosynthesis during the growing season, such that the EU locations resulted in a higher WSC concentration while the FRR populations exhibited a higher WSC than the DSV populations at those EU locations. However, there was no difference between the DSV and FRR populations for WSC at the US locations. These results are consistent with the report of Sanada et al. [26], which also found that European orchardgrass sources generally exhibited a lower WSC than orchardgrass from other regions. Further studies of the genetics and GEI of WSC will be required to determine appropriate selection targets for increased WSC in different regions and to determine limits of WSC expression in orchardgrass germplasm.

The GEI effects detected herein were also complicated by the relative RR of the two germplasm sources included in this evaluation. The relationship between the WSC and disease is not well defined, but Sanada et al. [26] found a negative relationship between rust infection and WSC. Thus, our results for WSC contradict this finding. The potential for increasing the WSC concentrations under US conditions is unclear. However, breeding for this trait is still in its infancy and substantial genetic variation exists in orchardgrass [25]. Thus, increases in WSC are likely, but overall expression levels comparable to EU conditions may not be attainable.

The higher HM of the DSV populations compared to the FRR populations, at both US and EU locations, is seemingly inconsistent with the higher HM of US cultivars at US locations in previous work [10]. At EU locations this difference may partially be due to disease pressure, where the effect of rust on the FRR populations was high and limited their productivity compared to the DSV populations. At the US locations rust was not a factor and the HM differences were not as striking, although the DSV populations as a whole had higher DM. Thus, the strong overall increased HM of the DSV populations was driven by their substantially higher HM at the EU locations.

Forage quality phenotypes included CP, cell wall components, and digestibility. At each individual location, the FRR populations had higher CP and DDM than DSV populations, but differences for NDF, ADF, NDFD were less or non-significant. In general, relationships between these phenotypes and the WSC were consistent with those identified previously in orchardgrass [25], including the negative relationship between HM and cell wall components (ADF and NDF) and the positive relationship between HM and digestibility (DDM and NDFD). The non-significant correlation between CP and WSC in this study was unexpected. Crude protein and WSC usually exhibit a negative correlation because photosynthate diverted to the production of WSC cannot be used for CP, and vice versa [23]. The non-significance in this evaluation is intriguing.

The genotype by environment (location by population) interaction was a key characteristic of this evaluation. The genotype by environment interaction is the rule rather than the exception in forage grass breeding [25,33]. However, a main objective of this study was the characterization of this interaction to allow future use of the GEI to maximize breeding goals in orchardgrass. The AMMI analysis suggested that a consistent relationship occurred between the two EU locations, but that the two US locations did not exhibit consistent phenotypic performance. Previously, the Logan and Rexburg locations exhibited substantial GEI for orchardgrass phenotypes, including WSC and HM [25], such that there were no specific populations that would perform uniformly well for the measured phenotypes across those two locations. However, the consistent performance of the DSV and FRR populations for phenotypes suggests that combining these populations through recurrent selection can result in germplasm improved for WSC, HM, and RR. These populations could then be specifically selected for improved performance in the EU and US, respectively.

4. Materials and Methods

4.1. Experimental Locations

The locations included in this evaluation were Hof Steimke, Lower Saxony, Germany; Les Rosiers-sur-Loire, Pays de la Loire, France; Logan, UT, USA; and Rexburg, ID, USA. Location descriptions, including weather data during the study, are included in Table 2.

4.2. Orchardgrass Populations

Eighty-four orchardgrass populations were evaluated, including 35 DSV orchardgrass breeding populations, 40 FRR orchardgrass breeding populations, and nine check cultivars from the EU and US (Table S1).

4.3. Experimental Design

The experimental design at Hof Steimke and Les Rosiers was a randomized complete block design with two complete blocks. Due to seed limitations only two blocks were included at each location. The experimental design at Logan and Rexburg was a double alpha-lattice with two complete blocks and 20 incomplete blocks. Plots were seeded in 2 m^2 plot sizes, at a rate of 17 $kg{\cdot}ha^{-1}$ during early fall 2013 at each location, but Hof Steimke, which was seeded in 2014. The EU locations employed no supplemental irrigation while the US locations applied supplemental irrigation (beginning after the first yearly harvest) to replace approximately 75% of the evapotranspiration rate each week. Following sowing and the establishment year, the fertilization schedule during the 2014 and 2015 production years were three split applications of 57 kg·N during the year.

4.4. Data Collection

At Logan and Rexburg there were three forage harvests in 2014 and four in 2015. At Les Rosiers there were four harvests in 2014 and five in 2015. At Hof Steimke no biomass or forage quality data was collected in 2014, but there were five harvests in 2015. Harvest intervals were roughly each five to six weeks at each location. Each spring, maturity ratings were made from 1–5, where a score of 1 corresponded to the earliest heading while a score of 5 corresponded to the latest heading. Following maturity ratings, each harvest removed all aboveground plant material to a stubble height of approximately 60 mm. Samples were collected from each plot to determine wet weights and for nutritive value analyses. The samples were then dried at 60 °C in forced air driers and re-weighed to determine moisture percentage. Thus plot values were adjusted to a dry matter basis to determine HM in $kg{\cdot}plot^{-1}$. Samples were then ground to pass a 1 mm screen for nutritive value analyses. The analyses used wet chemistry and near infrared spectroscopy according to established protocols [34] to determine values of WSC, DDM, CP, NDF, ADF, and NDFD. Nutritive values were not estimated from the first harvest to avoid the confounding effects of maturity differences. Additionally, nutritive value was only taken from two harvests from Les Rosiers in 2014. Herbage mass values were summed across the growing season, and the nutritive values were derived as a weighted average from each harvest based on the contribution of the corresponding harvest to the total yearly HM. Rust resistance was rated (1—highly susceptible to 9—highly resistant) only from the two EU locations during 2014 and 2015. There was no measurable rust at the US locations. Rust ratings did not attempt to distinguish between rust type, i.e., stem vs. leaf rust, but only provided an overall rating of plant health.

4.5. Data Analysis

The 'lme4' package [35] of the R software [36] was used to model the phenotypic data for the overall across years and locations and individual location models. The main effects of year, location, population, and their interactions were fixed effects. The main effects of block and incomplete block were random effects. The repeated effects of years were modeled using a compound symmetry structure. The lattice designs at the US locations were resolved to randomized complete blocks for the overall model. Contrasts between the population sources were used to determine differences in phenotypic values among the DSV, FFR, and check populations. The MATMODEL 3.0 software [37] was then used to characterize the underlying location by population effects for each genotype using the additive main effects and multiplicative interaction (AMMI) modeling. The Kendall concordance statistic was used to characterize the relative ranking of populations for each phenotype among

the locations. Simple correlations among cultivar means between different phenotypes in the same environment illustrated relationships among the phenotypes.

Supplementary Materials: Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/6/04/61/S1, Table S1: Overall across year (2014 and 2015) and location (Hof Steimke, Germany; Les Rosiers, France; Logan, UT, USA; and Rexburg, ID, USA) mean values for 84 orchardgrass populations corresponding to water soluble carbohydrates (WSC), dry matter yield (DMY), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), neutral detergent fiber digestibility (NDFD), rust resistance (RUST), and maturity (MAT).

Acknowledgments: Funding for this research came from internal budgets of the USDA/ARS and Deutsche Saatveredelung AG.

Author Contributions: J.G.R. conceived the research, conducted the US field experiments, analyzed the data, and wrote the manuscript. B.S.B. interpreted research results and wrote the manuscript. U.F. conceived the research and conducted the EU field experiments. G.B. conducted the Rexburg, ID, USA field experiments.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ADF	acid detergent fiber
CP	crude protein
DSV	Deutsche Saatveredelung AG
DDM	digestible dry matter
EU	European Union
FFR	Forage and Range Research
GEI	genotype by environment interaction
HM	herbage mass
NDF	neutral detergent fiber
NDFD	neutral detergent fiber digestibility
RR	rust resistance
US	United States
WSC	water soluble carbohydrates

References

1. Van Santen, E.; Sleper, D.A. Orchargrass. In *Agronomy Monograph 34, Cool-Season Forage Grasses*; Moser, L.E., Buxton, D.R., Casler, M.D., Eds.; ASA-CSSA-SSSA: Madison, WI, USA, 1996; pp. 503–534.
2. Stewart, A.V.; Ellison, N. The genus *Dactylis*. In *Wealth of Wild Species: Role in Plant Genome Elucidation and Improvement*; Kole, C., Ed.; Springer: New York, NY, USA, 2011; pp. 73–87.
3. Brummer, E.C.; Moore, K.J. Persistence of perennial cool-season grass and legume cultivars under continuous grazing by beef cattle. *Agron. J.* **2000**, *92*, 466–471. [CrossRef]
4. Chamblee, D.S.; Lovvorn, R.L. The effect of rate and method of seeding and the yield and botanical composition of alfalfa-orchardgrass and alfalfa-tall fescue. *Agron. J.* **1953**, *45*, 192–196. [CrossRef]
5. Moore, K.J. Compendium of common forages. In *Forages: An Introduction to Grassland Agriculture*, 6th ed.; Barnes, R.F., Nelson, C.J., Collins, M., Moore, K.J., Eds.; Iowa State University Press: Ames, IA, USA, 2003.
6. Norris, I.B.; Thomas, H. Recovery of ryegrass species from drought. *J. Agric. Sci.* **1982**, *98*, 623–628. [CrossRef]
7. Carlson, I.T.; Moll, R.H. Phenotypic and genotypic variation and covariation in quantitative characters in strains of orchardgrass. *Crop Sci.* **1962**, *2*, 281–286. [CrossRef]
8. Volaire, F. Drought survival, summer dormancy and dehydrin accumulation in contrasting cultivars of *Dactylis glomerata*. *Physiol. Plant.* **2002**, *116*, 42–51. [CrossRef] [PubMed]
9. Bushman, B.S.; Robins, J.G.; Jensen, K.B. Dry matter yield, heading date, and plant mortality of orchardgrass subspecies in a semiarid environment. *Crop Sci.* **2012**, *52*, 2745–2751. [CrossRef]
10. Casler, M.D.; Fales, S.L.; McElroy, A.R.; Hall, M.H.; Hoffman, L.D.; Leath, K.T. Genetic progress from 40 years of orchardgrass breeding in North America measured under hay management. *Crop Sci.* **2000**, *40*, 1019–1025. [CrossRef]

11. Xie, W.-G.; Bushman, B.S.; Ma, Y.-M.; West, M.S.; Robins, J.G.; Michaels, L.; Jensen, K.B.; Zhang, X.-Q.; Casler, M.D.; Stratton, S.D. Genetic diversity and variation in North American orchardgrass (*Dactylis glomerata* L.) cultivars and breeding lines. *Grassl. Sci.* **2014**, *60*, 185–193. [CrossRef]
12. Wilkins, P.W. Breeding perennial ryegrass for agriculture. *Euphytica* **1991**, *52*, 201–214. [CrossRef]
13. Sanada, Y.; Tamura, K.; Yamada, T. Relationship between water-soluble carbohydrates in fall and spring and vigor of spring regrowth in orchardgrass. *Crop Sci.* **2010**, *50*, 380–390. [CrossRef]
14. Smith, D.; Grotelueschen, R.D. Carbohydrates in grasses. I. Sugar and fructosan composition of the stem bases of several northern-adapted grasses at seed maturity. *Crop Sci.* **1966**, *6*, 263–266. [CrossRef]
15. Eagles, C.F. Variation in the soluble carbohydrate content of climatic races of *Dactylis glomerata* (cocksfoot) at different temperatures. *Ann. Bot.* **1967**, *31*, 645–651.
16. Hendry, G.A.F. Evolutionary origins and natural functions of fructans—A climatological, biogeographic and mechanistic appraisal. *New Phytol.* **1993**, *123*, 3–14. [CrossRef]
17. Valluru, R.; van den Ende, W. Plant fructans in stress environments: Emerging concepts and future prospects. *J. Exp. Bot.* **2008**, *59*, 2905–2916. [CrossRef] [PubMed]
18. Livingston, D.P., III; Hincha, D.K.; Heyer, A.G. Fructan and its relationship to abiotic stress tolerance in plants. *Cell. Mol. Life Sci.* **2009**, *66*, 2007–2023. [CrossRef] [PubMed]
19. Hincha, D.K.; Hagemann, M. Stabilization of model membranes during drying compatible solutes involved in the stress tolerance of plants and microorganisms. *Biochem. J.* **2004**, *383*, 277–283. [CrossRef] [PubMed]
20. Munns, R.; Weir, R. Contribution of sugars to osmotic adjustment in elongating and expanded zones of wheat leaves during moderate water deficits at light levels. *Aust. J. Plant Physiol.* **1981**, *8*, 93–105. [CrossRef]
21. Yamada, T.; Kuroda, K.; Jitsuyama, Y.; Takezawa, D.; Arakawa, K.; Fujikawa, S. Roles of the plasma membrane and the cell wall in the responses to plant cells freezing. *Planta* **2002**, *215*, 770–778. [CrossRef] [PubMed]
22. Volaire, F.; Lelièvre, F. How can resistant genotypes of *Dactylis glomerata* L. survive severe Mediterranean summer drought? In *Réhabilitation des Pâturages et des Parcours en Milieux Méditerranéens*; Cahiers Options Méditerranéennes; Ferchichi, A., Ed.; CIHEAM: Zaragoza, Spain, 2004; pp. 145–148.
23. Wilkins, P.W.; Humphreys, M.O. Progress in breeding perennial forage grasses for temperate agriculture. *J. Agric. Sci.* **2003**, *140*, 129–150. [CrossRef]
24. Chatterton, N.J.; Harrison, P.A.; Thornley, W.R.; Bennett, J.H. Structure of fructan oligomers in cheatgrass (*Bromus tectorum* L.). *New Phytol.* **1993**, *124*, 389–396. [CrossRef]
25. Robins, J.G.; Bushman, B.S.; Jensen, K.B.; Escribano, S.; Blaser, B. Genetic variation for dry matter yield, forage quality, and seed traits among the half-sib progeny of nine orchardgrass germplasm populations. *Crop Sci.* **2015**, *55*, 275–283. [CrossRef]
26. Sanada, Y.; Takai, T.; Yamada, T. Ecotypic variation of water-soluble carbohydrate concentration and winter hardiness in cocksfoot (*Dactylis glomerata* L.). *Euphytica* **2007**, *153*, 267–280. [CrossRef]
27. Volaire, F. Growth, carbohydrate reserves and drought survival strategies of contrasting *Dactylis glomerata* populations in a Mediterranean environment. *J. Appl. Ecol.* **1995**, *32*, 56–66. [CrossRef]
28. Rawnsley, R.P.; Donaghy, D.J.; Fulkerson, W.J.; Lane, P.A. Changes in the physiology and feed quality of cocksfoot (*Dactylis glomerata* L.) during regrowth. *Grass Forage Sci.* **2002**, *57*, 203–211. [CrossRef]
29. Robins, J.G.; Jensen, K.B. Identification of creeping foxtail germplasm with high dry matter yield and nutritive value. *Crop Sci.* **2011**, *51*, 728–735. [CrossRef]
30. Casler, M.D.; Fales, S.L.; Undersander, D.J.; McElroy, A.R. Genetic progress from 40 years of orchardgrass breeding in North America measured under management-intensive rotational grazing. *Can. J. Plant Sci.* **2001**, *81*, 713–721. [CrossRef]
31. Humphreys, M.O. Water-soluble carbohydrates in perennial ryegrass breeding. I. Genetic differences among cultivars and hybrid progeny grown as spaced plants. *Grass Forage Sci.* **1989**, *44*, 231–236. [CrossRef]
32. Parsons, A.J.; Rasmussen, S.; Xue, H.; Newman, J.A.; Anderson, C.B.; Cosgrove, G.P. Some 'high sugar grasses' don't like it hot. *Proc. New Zealand Grassl. Assoc.* **2004**, *66*, 265–271.
33. Robins, J.G.; Lovatt, J.A. Cultivar by environment effects of perennial ryegrass cultivars selected for high water soluble carbohydrates managed under differing precipitation levels. *Euphytica* **2015**, *208*, 571–581. [CrossRef]
34. Escribano, S.; Robins, J.G. Phenotypic performance of timothy accessions under irrigated and nonirrigated conditions. *Crop Sci.* **2014**, *54*, 1079–1086. [CrossRef]

35. Bates, D.; Maechler, M.; Bolker, B.; Walker, S. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **2015**, *67*, 1–48. [CrossRef]
36. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing: Vienna, Austria, 2014. Available online: http://www.R-project.org/ (accessed on 21 Novermber 2016).
37. Gauch, H.G. *MATMODEL Version 3.0: Open Source Software for AMMI and Related Analyses*; Crop and Soil Sciences, Cornell University: Ithaca, NY, USA, 2007.

agronomy

MDPI

Article

Development of a Molecular Breeding Strategy for the Integration of Transgenic Traits in Outcrossing Perennial Grasses

Pieter E. Badenhorst [1], Kevin F. Smith [2] and German Spangenberg [3,4,*]

1 Agriculture Victoria, Biosciences Research, Hamilton Centre, Private Bag 105, Hamilton 3300, Australia; pieter.badenhorst@ecodev.vic.gov.au
2 Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Private Bag 105, Hamilton 3300, Australia; kfsmith@unimelb.edu.au
3 Agriculture Victoria, Biosciences Research, AgriBio, Centre for AgriBioscience, Bundoora 3083, Australia
4 School of Applied Systems Biology, La Trobe University, AgriBio, Centre for AgriBioscience, Bundoora 3083, Australia
* Correspondence: german.spangenberg@ecodev.vic.gov.au; Tel.: +61-390-327-069

Academic Editor: Peter Langridge
Received: 29 September 2016; Accepted: 28 October 2016; Published: 2 November 2016

Abstract: Molecular breeding tools, such as genetic modification, provide forage plant breeders with the opportunity to incorporate high value traits into breeding programs which, in some cases, would not be available using any other methodology. Despite the potential impact of these traits, little work has been published that seeks to optimize the strategies for transgenic breeding or incorporate transgenic breeding with other modern genomics-assisted breeding strategies. As the number of new genomics assisted breeding tools become available it is also likely that multiple tools may be used within the one breeding program. In this paper we propose a strategy for breeding genetically-modified forages using perennial ryegrass as an example and demonstrate how this strategy may be linked with other technologies, such as genomic selection. Whilst the model used is perennial ryegrass the principles outlined are valid for those designing breeding strategies for other outcrossing forage species.

Keywords: forage; transgenic; breeding strategy

1. Introduction

"Roundup Ready" alfalfa was the first, and currently only, commercially available transgenic forage in the world [1], with a large range of transgenic traits being developed in a number of different forage species including white clover, subterranean clover, alfalfa, Italian ryegrass, perennial ryegrass, tall fescue, red fescue, creeping bentgrass, bahiagrass, and switchgrass. These traits include forage quality, tolerance to biotic and abiotic stress, and the manipulation of growth and development [1–5]. Genetic transformation of plants is only the beginning of the pathway towards the commercialization of a transgenic product and has not diminished the need for cultivar development through breeding [6].

Notwithstanding the technical and regulatory challenges that are sometimes associated with the development of forage cultivars using new technologies the fact remains that the targeting of high impact traits related to forage quality, productivity and persistence has the potential to greatly increase the profitability of grazing industries [7–9].

Transgenic breeding is an extension of conventional plant breeding technologies and, although it shares the same basic principles and guidelines with conventional plant breeding, it has its own challenges and breeding objectives [10]. These objectives include the selection of transgenic events that exhibit the targeted attribute across generations, whilst retaining all other non-target agronomic qualities [10,11]. Although the methodologies are in place to generate new transgenic events within

forage crops, they have not been assembled or optimised to be integrated into a transgenic breeding program for commercial development.

There is very limited literature related to breeding strategies for the development of transgenic forages from transformation to commercialisation [6,12], with no literature on the breeding strategies for transgenic *Lolium* grasses. Both Kalla et al. [2] and Woodfield and White [6] described the introgression of a transgene within a molecular breeding program in white clover, an outcrossing forage legume, and are focused on the incorporation of a novel trait into a known genetic background using repeated crossing to a transgenic donor plant to derive plants that are homozygous for the transgene. With minor adjustments, both of these breeding strategies could be applied to transgenic *Lolium* grasses. However, since these studies were published, progressive advances in molecular genetic and genomic technologies, and the ongoing expansion of phenomics capabilities, have allowed for designs of genome editing, marker assisted selection [4], genomic selection [13], and hybrid breeding strategies [14] in commercially-relevant breeding programs of temperate outcrossing grasses that could potentially change breeding strategies in forages [15–18]. These new methodologies can potentially increase the rate of genetic gain over generations, reduce time between generations and reduce the cost of phenotyping. The implementation of these technologies has the potential to address a number of short-falls of conventional breeding programs, when it comes to the integration of a transgene into a wider breeding population.

Some of these considerations and technologies will be discussed and an optimum strategy for the transgenic breeding of *Lolium* grasses is proposed. This transgenic breeding strategy will not replace the need for standard aspects of commercial breeding programs, such as the choice of adapted parental germplasm and multi-site screening for genotype × environment interactions, but rather allow the efficient introgression of transgenic technologies into such a program in a way that is also compatible with other molecular breeding tools.

The breeding strategy described aims to reduce the time-lag between transformation and commercialisation through the creation of market-ready transgenic events in perennial ryegrass. Elements of the strategy were validated during the development of transgenic perennial ryegrass with altered fructan biosynthesis [19]. However, the strategy proposed is not a description of what was done in that experiment, but rather how elements that were used there could be used more broadly in a breeding strategy, such as:

- screening agronomically superior populations for tissue culture responsive (TCR) genotypes to be used in genetic transformation;
- screening and selecting both T_0 events and recipient genotypes concurrently;
- ensuring agronomically fit, genetically-diverse, recipient genotypes were used in crossing,
- introducing endophytes in the T_1 generation via the recipient parent; and
- attempting to complete two cycles of crossing within one year.

This strategy allowed for the development of transgenic perennial ryegrass *Syn*0 germplasm for two transformation within five years of transformation. The strategy included four years of field evaluation on the primary T_0 parents and two years of field evaluation on the transgenic T_1 progeny. During these experiments, a number of areas, as listed below, were highlighted for consideration when attempting to create an optimized transgenic breeding strategy in *Lolium* grasses. These areas need to be considered when planning and developing transgenic *Lolium* for commercial purposes:

- selection methods of tissue culture responsive genotypes;
- development, evaluation, and selection of primary T_0 transgenic events in *Lolium*;
- integration of the gene technologies into the wider breeding population; and
- evaluation of progeny for trait stability and agronomic performance.

2. Selection Methods for Tissue Culture Responsive Genotypes

Callus production and regeneration protocols to produce fertile plants in *Lolium* are pre-requisites to *Lolium* transformation [20]. Thus, the development of an optimized tissue culture protocol is essential for successful genetic transformation [20]. However, not all agronomically elite cultivars/populations are amenable to genetic transformation, due to either poor callus induction or poor callus regeneration. Most genotypes selected for genetic transformation are, thus, selected based on their tissue culture responsiveness rather than agronomic performance. This can have a genetic drawback during initial cultivar development due to the presence of agronomically unfavourable alleles within the selected genotype used for genetic transformation [6,10]. Visarada [11] concluded that it is more realistic to use highly responsive genotypes for genetic transformation, and then use promising transgenic events in a backcrossing strategy with agronomically superior populations/cultivars. It will take at least five generations of backcrossing to regain roughly 97% of the backcrossed parents' genetic background [6]. In outcrossing species, such as *Lolium*, it is essential to backcross with a number of diverse recipient genotypes in each successive generation, to avoid inbreeding depression. This backcrossing strategy will maintain the transgene in a heterozygous state and so it will be present in only 50% of the progeny. Progeny will, thus, have to be screened for the transgene in each generation. Although achievable, this backcrossing strategy will add significant cost and an additional 2–5 years to the breeding process for commercialisation.

Genotypes selected for genetic transformation should, thus, not only be screened for TCR, but for agronomic performance under field conditions prior to transformation, as well. This could easily be implemented in a commercial breeding program, by screening selected superior genotypes from breeding populations for tissue culture responsiveness. This would allow for the identification of TCR genotypes that are agronomically superior well in advance of any transformation work, and would enable continues improvement of TCR genotypes through recurrent selection strategies. Screening for tissue culture responsiveness in a genomic selection reference population would enable the estimation of the marker effects that would allow for the development of genomic selection prediction equations. These prediction equations could then be used to estimate genomic estimated breeding value (GEBVs) for selection candidates for tissue culture responsiveness, as well as for important traits, such as biomass yield, nutritive quality, and persistence in a sward [16,17].

Another consideration in choosing the correct genotype for genetic transformation would be floral induction requirements. Floral induction marks the transition from a vegetative state to a reproductive state and requires a dual induction in most *Lolium* grasses [21–23]. The primary induction is brought on by short days and/or low temperatures (vernalisation), with an obligatory requirement of at least two weeks, followed with the secondary induction in response to a transition to longer days and moderately high temperatures [21,24,25]. The requirements for both primary and secondary induction can, however, vary greatly within perennial ryegrass germplasm [24,26]. The selection of agronomically fit TCR genotypes with short primary induction requirements will assist greatly with the crossing strategies in the initial cultivar development phase. Shorter obligatory PI requirements of 4–6 weeks, will allow for multiple crosses to be done in succession, allowing for a larger number of crosses to be completed within a year. In some of our research, the TCR genotype had a 12 week obligatory PI requirement. This long PI requirement restricted the number of crosses that could be produced within a year, and limited of generations that could be created to three generations every two years. As perennial ryegrass requires both primary and secondary flowering induction to occur, selection on shorter PI requirements alone should not alter heading dates. It would still be important to ensure that heading dates are in line with the breeding objectives. Crossing would have to be done in contained glasshouse facilities, which would most likely have space restrictions depending on the regulatory requirements of the jurisdiction in which the crosses are performed.

All of these considerations and technologies can assist with the selecting of an agronomically fit TCR genotype that would allow for the generation of two generations per year under glasshouse conditions, which could shorten breeding time requirements for commercialisation by 2–3 years.

3. Development, Evaluation and Selection of Primary T_0 Transgenic Events in *Lolium* Grasses

In the past two decades, enabling methodologies for the application of genetic transformation have been developed or improved for many important forage, turf, and bioenergy crops [1,5,27,28]. Genetic transformation technology allows for the introduction of novel genetic variation through the introgression of genes from related or unrelated species through transformation [1] and have been extensively reviewed [29–33]. The uncontrolled nature of both *Agrobacterium*-mediated transformation and biolistic transformation could lead to multiple insertion sites and complex integration patterns that could potentially lead to insertional mutagenesis [34]. This uncontrolled nature of transgene insertion could mean that each transformant has a different insertion site and potentially has a variable number of inserts of the transgene as well [10]. Somaclonal variation during tissue culture and insertional mutagenesis during transformation could also lead to significant phenotypic variation in primary T_0 transgenic events [10,11]. This could become a significant barrier in transgenic breeding but could be overcome by generating large numbers of primary T_0 transgenic events, to screen for T_0 events with low transgene copy number, that exhibit the desired trait without negatively affecting the agronomic performance of the plant [10]. Recent developments in genome editing techniques will also be able to address these concerns through the precise, targeted deletion, substitution, or addition of nucleotides within a specific site in the genome [15,35]. The advantage of these new techniques over *Agrobacterium*-mediated transformation and biolistic transformation is that the integration is site specific, which has the potential to reduce insertional site effects, complex integration patterns, and multiple copy insertions [35] although depending on the technology used there can be a large effort to define and characterize suitable introgression sites. It will also reduce the burden of identifying and characterising insertion sites for the deregulation of a selected transgenic event for commercial use [36]. There is currently no literature available that reports targeted genome editing in any forage species, but the use of genome editing has been reported in other plant species, including maize [37], rice [38], wheat [39], barley [40], and soybean [41].

For a transgenic event to be successful as a cultivar, it is expected that the trait will be stably inherited, consistently expressed, and with no significant negative impact on the agronomic performance [10,11]. In perennial ryegrass, the transgenic breeding strategy starts with the production, evaluation and selection of primary T_0 events for the targeted trait, followed by the introgression of the transgene into the larger population. The cost of running a field trial with transgenic events can be substantial due to regulatory requirements [36,42]. To reduce environmental risks that may arise from the release of transgenic material into the environment, restrictions may be placed on the number of T_0 events that can be evaluated, the length of the trial and the methods used for evaluation (e.g., no animal grazing) by the regulatory governing body. For these reasons, it is necessary to reduce the number of primary T_0 transgenic events to progress to field evaluation, by pre-screening the T_0 events in containment glasshouse facilities. Pre-screening can include gene expression analysis, estimation of transgene copy number, marker gene copy number, and phenotypic analysis of the target trait. For qualitative traits (e.g., disease resistance, herbicide tolerance, etc.), it is possible to pre-screen large numbers under containment conditions in the glasshouse and only take promising T_0 events for evaluation in targeted environments under field conditions. Although it is possible to pre-screen for quantitative traits (e.g., fructan biosynthesis) in the glasshouse as well, it is advisable to screen for the trait in targeted environments under field conditions, as promising events might not express the trait under glasshouse conditions. Due to the cost of deregulation of a transgenic event [43], it is most likely that only one primary T_0 event will be selected for deregulation. However, it is critical that between 4–20 of the most promising T_0 events be advanced in the breeding program, as transgenic events might be deemed unfit due to low T_0 fertility, limited seed production, and irregular trait heritability [10].

4. Introgression of the Transgene into the Wider Breeding Population

Genetic transformation and selection of elite T_0 transgenic events that exhibit the trait of interest is only the start of making a commercial transgenic product and has not diminished the need for cultivar

development through breeding [6]. The introgression of individual genes for cultivar development have been limited in outcrossing species, such as perennial ryegrass, as repeated backcrossing to a single parent is required [44] which, in turn, can lead to inbreeding depression. In this study, the method used to introgress the transgene within a breeding population was to use the clonal ramets of ryegrass to facilitate the simultaneous pair-crossing of a single transgenic event to a number of selected genotypes. Using multiple pair-crosses allowed for the introgression of the transgene into a range of genetically diverse genotypes of single/multiple breeding population(s), in the most cost-effective and controlled manner. To create a transgenic synthetic cultivar, while reducing the risk of inbreeding, around 4–20 recipient genotypes would need to be pair-crossed with each selected primary T_0 transgenic event [45]. This reduces the chance of a founder effect within the transgenic population and will reduce inbreeding depression in the following generations [46]. Kidwell et al. [47] have shown that fewer parents that are genetically diverse are more suited for cultivar development than highly related plants. Plant breeders have, however, until recently, been unable to distinguish genetically-diverse parents within breeding populations and had to rely on larger numbers of parents as a precaution against inbreeding [48,49]. New advances in molecular genetics have enabled breeders to determine the genetic distance between parents and, thus, select smaller subsets of parents, while maintaining genetic diversity and desired characteristics within the population [18]. Recipient genotypes can, thus, be selected based on genetic diversity between genotypes that will be used in crossing activities [47].

In recent years, genomic selection strategies suitable for use in *Lolium* have been proposed [13,16,17]. In a genomic selection breeding program, recipient parents could be selected based on GEBVs, as well as on phenotypic selection [17]. GEBVs could allow for the rapid identification of genetically-diverse parents that contain the desired genotypes for agronomic performance. Although the use of genomic selection is novel in plant species it is likely that for species where appropriate resources are placed into the development of genomic selection algorithms that the technology may be widely used. If genomic selection if combined with a transgenic breeding strategy it is import that both the transgenic genotype and the recipient genotypes should, however, be selected from the breeding population that is linked to the genomic selection training population, to ensure that the genomic predictions of the progenies are accurate [13,17].

The recipient parents could also be a vehicle to introduce endophyte strains into the breeding population. Endophyte/ryegrass symbiota have shown to have a competitive advantage over ryegrass without endophytes [50].

5. Evaluation of Progeny for Transgenic Trait Stability and Agronomic Performance

One of the characteristic features of biolistic transformation is the integration of both full length transgenes and possibly rearranged fragments of the transgenes, with variable copy numbers of both full-length transgenes and transgene fragments [51]. The multiple copies of the full transgenes and possibly rearranged fragments are most frequently inherited as a single locus [51] and is, thus, expected to exhibit a monogenic Mendelian segregation ratio of 1:1. However, studies have shown that between 10%–50% of transgenic events produced by either *Agrobacterium*-mediated transformation or biolistic transformation will show non-Mendelian inheritance of the transgene [10,51,52]. Yin et al. [52] have attributed the non-Mendelian segregation of the transgene to the nature of the recipient genome, the nature of the transgene and the interaction between them. The consequences of selecting a transgenic event that shows non-Mendelian inheritance for the genetic transgene is that an increased number of crosses should be produced after transformation [52]. The selection of a transgenic event that exhibits Mendelian segregation is, thus, preferable.

In this study, the method used to select for monogenic Mendelian inheritance in transgenic events was to pair-cross the primary T_0 event to a recipient genotype. This allowed for the calculation of segregation ratio within the T_1/F_1 progeny. All transgenic events that had a 1:1 segregation ratio in their T_1/F_1 progeny were progressed for further crossing. Using pair-crosses also allowed for cost

savings in molecular screening of progeny as the segregation ratio was likely to be 1:1. This would not have been the case if the transgenic events were placed in poly-crosses with multiple recipient genotypes in the initial integration step. A 1:1 segregation ratio was observed for three primary T_0 events. Using pair-crosses also allowed for full-sib or half-sib progeny testing, and for the selection of genetically-diverse parents to reduce the chances of inbreeding depression. To introgress the transgene into a diverse genetic background, the clonal propagation ability of ryegrass was used to allow for the simultaneous pair-crossing of a single transgenic event to a number of selected genotypes. Using multiple pair-crosses allowed for the introgression of the transgene into a range of genetically diverse genotypes of single/multiple breeding population/s. This crossing step was also used to integrate a novel fungal endophyte in the T_1/F_1 progeny, by only collecting seed from the recipient endophyte-containing maternal parents.

The introduction of a transgene into a recipient genome is complex and can potentially alter the transgene expression levels, depending on a number of factors, including the transgene integration site, transgene duplication, deletion, repeated sequence recombination, rearrangement, and gene interactions [10,52]. It is, thus, critical to screen the T_1 progeny for transgene expression and to evaluate the trait under field conditions. To reduce the number of T_1 progeny that requires screening, GEBVs can be used to select the T_1 progeny with the desired genotype first and then only screen those plants for transgene expression. In this study, transgenic T_1 progenies derived from three primary T_0 events were screened under field conditions, as GEBVs were not available.

A sub-selection of transgenic T_1 plants from each transgenic event can be used in a poly-cross to create T_2/F_2 progeny. The T_2/F_2 progeny should exhibit a 1:2:1 segregation ratio for the transgene as all T_1s used in the poly-cross would have been heterozygous for the transgene. T_2 progeny, homozygous for the transgene, can be used as *Syn*0 parents in a synthetic breeding program. Choices in regards to which event to deregulate can be made at this point. If the trait is deregulated, the progeny of the *Syn*0 parents could be used in a recurrent breeding nursery or genomic selection nursery, where sub-selections can be made for varietal development.

6. An Optimum Transgenic Breeding Strategy in *Lolium* Grasses that Is Compatible with Genomic Selection

An optimised transgenic breeding strategy in *Lolium* grasses will now be outlined. This strategy will address all of the abovementioned requirements, while making use of new technologies that have come to light in the last few years. This transgenic breeding strategy for *Lolium* grasses will allow for an efficient method to deploy transgenes into the wider perennial ryegrass breeding population for commercial cultivar development. This strategy is adapted from a genomic selection strategy described by Hayes et al. [16] (Figure 1) and reviewed by Simeão Resende et al. [17] and assumes that the genomic selection program for non-transgenic breeding material is well established, and that accurate GEBV's can be predicted on selection candidates for important traits, such as biomass yield, nutritive quality, and persistence in a sward [16,17]. With these basic genomic selection steps, a transgenic breeding strategy can be simplified to the following steps:

Year 1:

(i) Selection of TCR and agronomically superior genotypes for transformation—Figure 1, number 1.

Selection of superior genotypes from the genomic breeding nursery (Figure 1A) for transformation, based on genomic predictions of performance for a range of agronomic traits, as well as the TCR trait that is required both for callus induction and callus regeneration during transformation.

(ii) Creation of primary T_0 transgenic events in *Lolium* grasses through targeted genome editing—Figure 1, number 2.

Generation of primary T_0 events through targeted genome editing technology. Genome editing can accurately delete, substitute, or add nucleotides at a specific site within the genome [35]. This technology has distinct advantages over *Agrobacterium*-mediated transformation and biolistic transformation in that the transgene integration is site specific, which has the potential to reduce insertional mutagenesis, complex integration patterns, and multiple copy insertions [35]. It will also reduce the burden of identifying and characterising insertion sites for deregulation purposes, as the integration site is known and targeted for [35].

(iii) Application to Regulator for the limited release of transgenic *Lolium* grasses for evaluation.

Transgenic technology is governed by the Gene Technology Act 2000 and regulated by the Office of the Gene Technology Regulator in Australia. A licence by the Regulator is required for the intentional release of a transgenic event into the environment.

Year 2–3:

(i) Evaluation and selection of primary transgenic T_0 events in *Lolium* grasses—Figure 1, number 3.

Phase A: Pre-screening of T_0 events for transgene expression and qualitative traits under containment conditions, if possible.
Phase B: Establishment and evaluation of pre-screened T_0 events in a clonally-replicated, space-planted field trial for agronomic performance and performance of the targeted trait.
Phase C: Select 5–10 primary T_0 events for progression, based on "transgene traits" and transgene expression levels under field conditions. Data is based on phenotypic evaluation.

(ii) Selection of recipient genotypes to cross with the primary T_0 transgenic events—Figure 1, number 4.

Selection of genetically-diverse recipient genotypes from the genomic breeding nursery (Figure 1, box A) based on genomic predictions of diversity and performance for a range of traits and endophyte presence.

Year 4:

(i) Pair-crossing of recipient genotypes and primary T_0 transgenic events—Figure 1, number 5.

Four to eight genetically-diverse recipient genotypes (with endophyte) are to be pair-crossed to each of the selected T_0 events (5–10 events). Harvest seed from each recipient parent (endophyte present) as a half-sib family.

(ii) Selection of T_1 progeny for trait stability and agronomic performance.

Phase A: Screen all half-sib families for transgene presence. Transgenic events that exhibit a monogenic Mendelian segregation ratio of 1:1 for the transgene can be progressed. Using targeted genome editing, trait inheritance and trait expression should be more predictable in the progeny compared to alternative transformation methods.
Phase B: T_1 plants from selected events can be screened using genomic predictions for a range of traits/all traits. 4–6 T_1 plants that are genetically-diverse and show high transgene expression, can be selected for synthetic cultivar development through poly-crossing.

(iii) Poly-crossing of selected T_1 events—Figure 1, number 6.

Phase A: Poly-crosses are made for each selected transgenic event (3–5 events). All crosses are 4–6 parent synthetics. Seed should be harvested from each parent as a half-sib family. Molecular analysis for each half-sib family should indicate a segregation ratio of 1:2:1 for the transgene in the T_2/F_2 progeny.

Phase B: T_2 progeny homozygous for the gene of interest GOI can be used as Syn0 parents in a synthetic breeding program. The *Syn0* seed from the 3–5 transgenic events can progress to mini-sward evaluations.

Year 5–6:

(i) Mini-sward field evaluation of *Syn0* progeny for cultivar development—Figure 1, number 7.

All Phases: Mini swards are sown as *Syn0* half-sib populations. Each of the potential 4–6 parent synthetic populations, (i.e., the 4–6 mini-swards) will need to be observed together for assessment of flowering time uniformity. Phenotypic evaluation of the transgenic trait is measured on a population basis. Choices in regards to which event to deregulate can be made at this point. If the trait is deregulated, the progeny of the *Syn0* parents could be used in a recurrent breeding nursery or genomic selection nursery, where sub-selections can be made for varietal development.

The ability to do whole genome selection has been proven in the cattle industry and although the specific population structures and breeding strategies differ between species the general principles have been used to develop theoretical genomic selection strategies for ryegrass breeding as well [16,53,54]. These technologies have the power to maximise genetic gain whilst reducing the generational cycle time and have become practical within forage breeding [16,17], the result that the potential in commercial breeding programs is being evaluated [13,55]. All of these technologies have enabled a paradigm shift in the way that we evaluate *Lolium* grasses and will ensure a future for both forage development and the grazing industries.

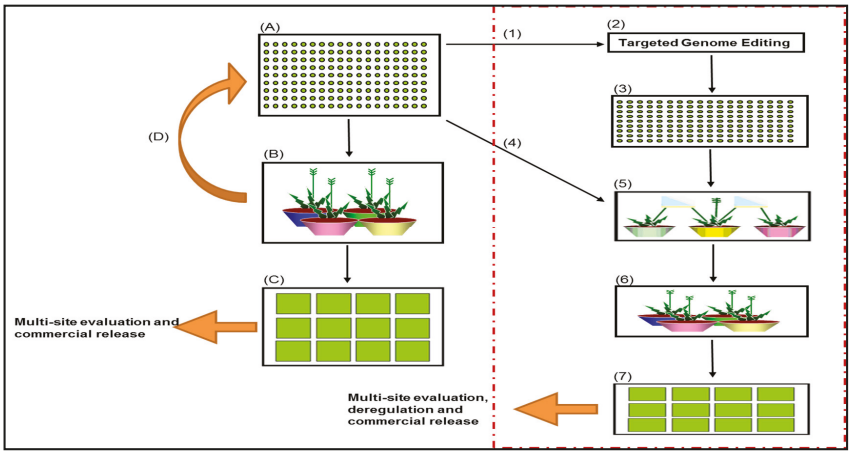


Figure 1. An optimum breeding strategy for transgenic *Lolium* grasses based on a genomic selection approach described by Hayes et al. Sections (**A**–**D**) are the phases of the genomic selection schema as proposed by Hayes et al. Sections 1–7 relate to the transgenic breeding strategy defined in this paper.

7. Conclusions

For novel forage traits to efficiently reach the marketplace, new breeding programs must evolve that optimize the rate of genetic gain, cost of implementation, and are compatible with current commercial breeding practices. Genetic simulation allows the modelling of multiple scenarios before they are implemented, as has recently been demonstrated for genomic selection of perennial ryegrass [13]. Similar challenges exist for transgenic breeding strategies with the added complexity of operating in a regulated environment. However, transgenic technologies offer the potential to incorporate novel

high impact traits into forage breeding programs. To reach this potential value, the technology needs to be incorporated into the wider perennial ryegrass breeding population for cultivar development. The challenges associated with using conventional breeding strategies have been discussed and an optimised transgenic breeding strategy in *Lolium* grasses was formulated, using a genomic selection strategy to deploy transgenes into the wider perennial ryegrass breeding population for commercial cultivar development. The strategy that is proposed is adapted from a genomic selection strategy described by Hayes et al. [16] and is compatible with modern breeding strategies, like F1 hybrid breeding and genomic selection, providing the opportunity for a fully-integrated molecular breeding strategy for perennial forage grasses.

Acknowledgments: Funding from the Molecular Plant Breeding Co-operative Research Centre is gratefully acknowledged.

Author Contributions: "P.B. K.S. and G.S. conceived and designed the experiments; P.B. performed the experiments; P.B. and G.S. analyzed and interpreted the data; P.B. and K.S. wrote the paper." All authors read and approved the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

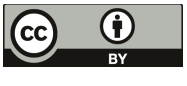
TCR	Tissue Culture Responsive
T_0	Primary Transformant
T_1	First Generation Transformant
GEBV	Genomic Estimated Breeding Value
GOI	Gene of Interest

References

1. Wang, Z.Y.; Brummer, E.C. Is genetic engineering ever going to take off in forage, turf and bioenergy crop breeding? *Ann. Bot.* **2012**, *110*, 1317–1325. [CrossRef] [PubMed]
2. OGTR. *The Biology of Lolium multiflorum Lam. (Italian ryegrass), Lolium perenne L. (perennial ryegrass) and Lolium arundinaceum (Schreb.) Darbysh (Tall Fescue)*; Office of the Gene Technology Regulator: Canberra, Australia, 2008.
3. Spangenberg, G.; Lidget, R.; Lidgett, A.; Sawbridge, T.; Ong, E.K.; John, U. Transgenesis and genomics in molecular breeding of forage plants. In Proceedings of the Australian Agronomy Conference, Hobart, Tasmania, 2001. Available online: http://www.regional.org.au/au/asa/2001 (accessed on 1 November 2016).
4. Smith, K.F.; Forster, J.W.; Spangenberg, G.C. Converting genomic discoveries into genetic solutions for diary pastures. *Aust. J. Exp. Agric.* **2007**, *47*, 1032–1038. [CrossRef]
5. Wang, Z.Y.; Ge, Y. *Agrobacterium*-mediated high efficiency transformation of tall fescue (*Festuca arundinacea*). *J. Plant Physiol.* **2005**, *162*, 103–114. [CrossRef] [PubMed]
6. Woodfield, D.R.; White, D.W.R. Breeding strategies for developing transgenic white clover cultivars. *Agron. Soc. N. Z. Spec. Publ.* **1996**, *11*, 125–130.
7. Smith, K.F.; Lewis, C.; Ludemann, C.I.; Malcolm, B.; Banks, R.G.; Jacobs, J.L.; Fennessy, P.F.; Spangenberg, G. Estimating the value of genetic gain in perennial pastures with emphasis on temperate species. *Crop Pasture Sci.* **2014**, *65*, 1238–1247. [CrossRef]
8. Ludemann, C.I.; Eckard, R.J.; Cullen, B.C.; Jacobs, J.L.; Malcolm, B.; Smith, K.F. Higher energy concentration traits in perennial ryegrass (*Lolium perenne* L.) may increase farm profits and improve energy conversion on dairy farms. *Agric. Syst.* **2015**, *137*, 89–100. [CrossRef]
9. Ludemann, C.I.; Smith, K.F. A comparison of methods to assess the likely on-farm value for meat production systems of pasture traits and genetic gain through plant breeding using phalaris (*Phalaris aquatica* L.) as an example. *Grass Forage Sci.* **2016**, *71*, 66–78. [CrossRef]
10. Zhong, G.Y. Genetic issues and pitfalls in transgenic plant breeding. *Euphytica* **2001**, *118*, 137–144. [CrossRef]
11. Visarada, K.; Meena, K.; Aruna, C.; Srujana, S.; Saikishore, N.; Seetharama, N. Transgenic breeding: Perspectives and prospects. *Crop Sci.* **2009**, *49*, 1555–1563. [CrossRef]

12. Kalla, R.; Chu, P.G.; Spangenberg, G. Molecular breeding of forage legumes for virus resistance. In *Molecular Breeding of Forages and Turf*; Kluwer Academin Publishers: Dordrecht, The Netherlands; Boston, MA, USA; London, UK, 2001; Volume 10, pp. 219–237.
13. Lin, Z.; Cogan, N.O.I.; Pembleton, L.W.; Spangenberg, G.C.; Forster, J.W.; Hayes, B.J.; Daetwyler, H.D. Genetic Gain and Inbreeding from Genomic Selection in a Simulated Commercial Breeding Program for Perennial Ryegrass. *Plant Genome* **2016**. [CrossRef]
14. Pembleton, L.W.; Shinozukam, H.; Wang, J.; Spangenberg, G.C.; Forster, J.W.; Cogan, N.O.I. Design of an F1 hybrid breeding strategy for ryegrass based on selection of self-incompatibility locus-specific alleles. *Front. Plant Sci.* **2015**, *6*, 764. [CrossRef] [PubMed]
15. Belhaj, K.; Chaparro-Garcia, A.; Kamoun, S.; Nekrasov, V. Plant genome editing made easy: Targeted mutagenesis in model and crop plants using the *CRISPR/Cas* system. *Plant Methods* **2013**, *9*, 39–49. [CrossRef] [PubMed]
16. Hayes, B.J.; Cogan, N.O.I.; Pembleton, L.W.; Goddard, M.E.; Wang, J.; Spangenberg, G.C.; Forster, J.W. Prospects for genomic selection in forage plant species. *Plant Breed.* **2013**, *132*, 133–143. [CrossRef]
17. Simeão Resende, R.M.; Casler, M.D.; Vilela de Resende, M.D. Genomic selection in forage breeding: Accuracy and methods. *Crop Sci.* **2014**, *54*, 143–156. [CrossRef]
18. Wang, J.; Pembleton, L.W.; Baillie, R.C.; Drayton, M.C.; Hand, M.L.; Bain, M.; Sawbridge, T.; Spangenberg, G.C.; Forster, J.W.; Cogan, N.O. Development and implementation of a multiplexed single nucleotide polymorphism genotyping tool for differentiation of ryegrass species and cultivars. *Mol. Breed.* **2014**, *33*, 435–451. [CrossRef]
19. Badenhorst, P.E.; Panter, S.; Palanisamy, R.; Georges, S.; Smith, K.F.; Mouradov, A.; Mason, J.; Spangenberg, G. Molecualr breeding of transgenic perennial ryegrass (*Lolium perenne* L.) with altered fructan biosynthesis through the expression of fructoyltransferases. *Mol. Breed. (submitted)* **2017**.
20. Bradley, D.E.; Bruneau, A.H.; Qu, R. Effects of cultivar, explant treatment, and medium suppliments on callus induction and plantlet regeneration in perennial ryegrass. *Int. Turfgrass Soc. Res. J.* **2001**, *9*, 152–155.
21. Heide, O.M. Control of flowering and reproduction in temperate grasses. *New Phytol.* **1994**, *128*, 347–362. [CrossRef]
22. Langer, R.H.M. *How Grasses Grow*; Edward Arnold: London, UK, 1972.
23. Sharman, B.C. Leaf and bud initiation in the gramineae. *Bot. Gaz.* **1945**, *106*, 269–289. [CrossRef]
24. Aamlid, T.S.; Heide, O.M.; Boelt, B. Primary and secondary induction requirements for flowering of contrasting European varieties of *Lolium perenne*. *Ann. Bot.* **2001**, *86*, 1087–1095. [CrossRef]
25. Meyer, W.A.; Watkins, E. Tall Fescue (*Festuca arundinacea*). In *Turfgrass Biology Genetics and Breeding*; Casler, M.D., Duncan, R.R., Eds.; John Wiley & Sons: Hobken, New Jersey, USA, 2003; pp. 107–127.
26. Cooper, J.P. Short-day and low-temperature induction in *Lolium*. *Ann. Bot.* **1960**, *24*, 232–247.
27. Spangenberg, G.S.; Wang, Z.Y.; Potrykus, I. *Biotechnology in Forage and Turf Grass Improvement*; Springer: Berlin, Germany, 1998.
28. Wang, Z.Y.; Scott, M.; Bell, J.; Hopkins, A.; Lehmann, D. Field performance of transgenic tall fescue (*Festuca arundinacea* Schreb.) plants and their progenies. *Theor. Appl. Genet.* **2003**, *107*, 406–412. [CrossRef] [PubMed]
29. Barampuram, S.; Zhang, Z.J. Recent advances in plant transformation. *Methods Mol. Biol.* **2011**, *701*, 1–35. [PubMed]
30. Birch, R.G. Plant transformation: Problems and strategies for practical application. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1997**, *48*, 297–326. [CrossRef] [PubMed]
31. Hooykaas, P.J.J. Plant transformation. In *Encyclopedia of Life Sciences*; John Wiley and Sons, Ltd.: Hoboken, NJ, USA, 2010. Available online: http://onlinelibrary.wiley.com/doi/10.1002/9780470015902.a0003070.pub2/full (accessed on 1 November 2016).
32. Newell, C.A. Plant transformation technology. *Mol. Biotechnol.* **2000**, *16*, 53–65. [CrossRef]
33. Rivera, A.L.; Gomez-Lim, M.; Fernandez, F.; Loske, A.M. Physical methods for genetic plant transformation. *Phys. Life Rev.* **2012**, *9*, 308–345. [CrossRef] [PubMed]
34. Wilson, A.K.; Latham, J.R.; Steinbrecher, R.A. Trnasformation-induced mutations in transgenic plants: Analysis and biosafety implications. *Biotechnol. Genet. Eng. Rev.* **2006**, *23*, 209–234. [CrossRef] [PubMed]
35. Urnov, F.D.; Rebar, E.J.; Holmes, M.C.; Zhang, H.S.; Gregory, P.D. Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.* **2010**, *11*, 636–646. [CrossRef] [PubMed]

36. Wozniak, C.A.; McHughen, A. *Regulation of Agricultural Biotechnology: The United States and Canada*; Springer: Dordrecht, The Netherlands; Heidelberg, Germany; New York, NY, USA; London, UK, 2013.
37. Shukla, V.K.; Doyon, Y.; Miller, J.C.; DeKelver, R.C.; Moehle, E.A.; Worden, S.E.; Mitchell, J.C.; Arnold, N.L.; Gopalan, S.; Meng, X.; et al. Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. *Nature* **2009**, *459*, 437–441. [CrossRef] [PubMed]
38. Li, T.; Liu, B.; Spalding, M.H.; Weeks, D.P.; Yang, B. High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat. Biotechnol.* **2012**, *30*, 390–392. [CrossRef] [PubMed]
39. Upadhyay, S.K.; Kumar, J.; Alok, A.; Tuli, R. RNA-guided genome editing for target gene mutations in wheat. *G3* **2013**, *3*, 2233–2238. [CrossRef] [PubMed]
40. Wendt, T.; Holm, P.B.; Starker, C.G.; Christian, M.; Voytas, D.F.; Brinch-Pedersen, H.; Holme, I.B. *TAL* effector nucleases induce mutations at a pre-selected location in the genome of primary barley transformants. *Plant Mol. Biol.* **2013**, *83*, 279–285. [CrossRef] [PubMed]
41. Curtin, S.J.; Anderson, J.E.; Starker, C.G.; Baltes, N.J.; Mani, D.; Voytas, D.F.; Stupar, R.M. Targeted mutagenesis for functional analysis of gene duplication in legumes. *Methods Mol. Biol.* **2013**, *1069*, 25–42. [PubMed]
42. Bradford, K.J.; Van Deynze, A.; Gutterson, N.; Parrott, W.; Strauss, S.H. Regulating transgenic crops sensibly: Lessons from plant breeding, biotechnology and genomics. *Nat. Biotechnol.* **2005**, *23*, 439–444. [CrossRef] [PubMed]
43. De Greef, W. GM Crops: The Cruching cost of Regulation. 2011. Available online: http://www.agbioworld.org/biotech-info/articles/biotech-art/crushingcost.html (accessed on 1 November 2016).
44. Wilkins, P.W.; Humphreys, M.O. Progress in breeding perennial forage grasses for temperate agriculture. *J. Agric. Sci.* **2003**, *140*, 129–150. [CrossRef]
45. Huyghe, C. *Sustainable Use of Genetic Diversity in Forage and Turf Breeding*; INRA—Poitou Charentes: Lusignan, France, 2010.
46. Ladizinsky, G. Founder effect in crop-plant evolution. *Econ. Bot.* **1985**, *39*, 191–200. [CrossRef]
47. Kidwell, K.K.; Hartweck, L.M.; Yandell, B.S.; Crump, P.M.; Brummer, J.E.; Moutray, J.; Osborn, T.C. Forage yields of alfalfa populations derived from parents selected on the basis of molecular marker dfiversity. *Crop Sci.* **1999**, *39*, 223–227. [CrossRef]
48. Fehr, W.R. *Principles of Cultivar Development*; MacMillan: New York, NY, USA, 1987.
49. Fejer, S.O. Genetic and environmental components of the productivity of perennial ryegrass (*Lolium perenne* L.). *N. Z. J. Agric. Res.* **1958**, *1*, 86–104.
50. Van Zijl de Jong, E.; Dobrowolski, M.P.; Bannan, N.R.; Stewart, A.; Smith, K.F.; Spangenberg, G.; Forster, J. Global genetic diversity of the perennial ryegrass fungal endophyte *Neotyphodium lolii*. *Crop Sci.* **2008**, *48*, 1487–1501. [CrossRef]
51. Pawlowski, W.P.; Somers, D.A. Transgene inheritance in plants genetically engineered by microprojectile bombardment. *Mol. Biotechnol.* **1996**, *6*, 17–30. [CrossRef] [PubMed]
52. Yin, Z.; Plader, W.; Malepszy, S. Transgene inheritance in plants. *J. Appl. Genet.* **2004**, *45*, 127–144. [PubMed]
53. Daetwyler, H.D.; Pong-Wong, R.; Villanueva, B.; Woolliams, J.A. The impact of genetic architechture on genome-wide evaluation methods. *Genetics* **2010**, *185*, 1021–1031. [CrossRef] [PubMed]
54. Hayes, B.J.; Bowman, P.J.; Chamberlain, A.J.; Goddard, M.E. Invited review: Genomic selection in dairy cattle: Progress and challenges. *J. Dairy Sci.* **2009**, *92*, 433–443. [CrossRef] [PubMed]
55. Fe, D.; Cericola, F.; Byrne, S.; Lenk, I.; Asraf, B.H.; Pedersen, M.G.; Roulund, N.; Asp, T.; Janss, L.; Jensen, C.S.; et al. Genomic dissection and prediction of heading date in perennial ryegrass. *BMC Genom.* **2015**, *16*, 921. [CrossRef] [PubMed]



agronomy

MDPI

Article

Quantitative Trait Loci (QTL) Identification in the Progeny of a Polycross

Laurence Pauly [1], Sandrine Flajoulot [1], Jérôme Garon [2], Bernadette Julier [3], Vincent Béguier [1] and Philippe Barre [3,*]

1 Jouffray-Drillaud La Litière, Saint Sauvant 86600, France; laurence.pauly@gmail.com (L.P.); sandrine.flajoulot@inra.fr (S.F.); vbeguier@jouffray-drillaud.fr (V.B.)
2 GIE GRASS La Litière, Saint Sauvant, 86600 France; jerome@garon-fils.fr
3 INRA, UR4, Unité de Recherche Pluridisciplinaire Prairies et Plantes Fourragères, RD150, Le Chêne, CS80006, Lusignan 86600, France; bernadette.julier@inra.fr
* Correspondence: philippe.barre@inra.fr; Tel.: +33-549-556-116

Academic Editors: John W. Forster and Kevin F. Smith
Received: 23 September 2016; Accepted: 20 October 2016; Published: 28 October 2016

Abstract: Connected multiparental crosses are valuable for detecting quantitative trait loci (QTL) with multiple alleles. The objective of this study was to show that the progeny of a polycross can be considered as connected mutiparental crosses and used for QTL identification. This is particularly relevant in outbreeding species showing strong inbreeding depression and for which synthetic varieties are created. A total of 191 genotypes from a polycross with six parents were phenotyped for plant height (PH) and plant growth rate (PGR) and genotyped with 82 codominant markers. Markers allowed the identification of the male parent for each sibling and so the 191 genotypes were divided into 15 full-sib families. The number of genotypes per full-sib family varied from 2 to 28. A consensus map of 491 cM was built and QTL were detected with MCQTL-software dedicated to QTL detection in connected mapping populations. Two major QTL for PH and PGR in spring were identified on linkage groups 3 and 4. These explained from 12% to 22% of phenotypic variance. The additive effects reached 12.4 mm for PH and 0.11 mm/C°d for PGR. This study shows that the progenies of polycrosses can be used to detect QTL.

Keywords: marker assisted selection; outbreeding species; forage species; connected populations; MCQTL

1. Introduction

Molecular markers are being used increasingly in plant breeding either to construct new genotypes having favourable alleles or to better estimate the breeding values of genotypes [1,2]. The first option implies the detection of the loci involved in the variation of quantitative traits (QTL). This detection is usually realised either in populations derived from bi-parental crosses between contrasting parents or in populations for which the lineage is unknown (association studies). The first strategy is interesting because linkage disequilibrium (LD) is long in the populations created, allowing the use of a limited number of markers to cover the genome. Nevertheless, this strategy can be limited by difficulties in creating the populations by the low number of alleles surveyed and by the accuracy of the location of the QTL. The second strategy (association studies) is interesting because the populations already exist and can be highly diverse. Nevertheless, it can be limited by short LD, forcing breeders to focus on just a few genes (candidate gene approach) or to use a very large number of markers [3]. In particular, LD is often very short in outbreeding species [3].

Synthetic varieties are produced in outbreeding species for which crosses cannot be controlled at large scale to produce hybrid varieties (e.g., in the majority of perennial forage and tree species).

Synthetic varieties are created by inter-crossing a number of selected plants (from four to several hundred parents (i.e., making a polycross)) and then multiplying up the resulting population over three or four generations (Syn 3–4), without selection, to obtain sufficient seed for commercialisation. The genetic diversity remaining in synthetic varieties avoids inbreeding depression and also allows better stability of performance in variable environments (spatial and temporal variations) [4]. When a low number of parents is used, these can be selected for their specific combining ability in addition to their general combining ability [5]. To apply marker assisted selection (MAS) in such species, QTL can be detected in bi-parental populations and then the favourable alleles must be introgressed into more diverse populations, such as elite varieties. This process is time consuming and the effect of the QTL can change in a new genetic background. Another way is to detect QTL directly in diverse populations such as in the progeny of a polycross or in a given generation of multiplication of a synthetic variety (Syn 2, 3 or 4). Linkage disequilibrium decreases with successive generations of multiplication [6]. Depending on the genetic architecture of selected traits, the knowledge of candidate genes and the budgetary allocation for genotyping (number of markers necessary to cover the genome depending on the LD) breeders can choose the best population for MAS. For example, a Syn 4 has been used to detect an association between a candidate gene and leaf elongation rate in perennial ryegrass [7].

Perennial ryegrass (*Lolium perenne* L.) is the most commonly sown forage and turf-grass species in temperate climates and it is considered to be a model for genomics in forage grasses [8]. Varieties are synthetics, due to biological constraints. In this species, LD decreases very rapidly ($r^2 < 0.2$ over less than 1 kb) in natural populations but also in synthetic varieties, except for in varieties produced from a very small number of parental plants [9,10]. Leaf length and leaf elongation rate are important traits affecting: (i) vegetative yield [11–15]; (ii) intake rate by dairy cows [16]; and (iii) plant survival under light competition conditions [17]. At the plant level, leaf length can be estimated by stretched plant height (PH) and leaf elongation rate by plant growth rate (PGR).

The objective of this study was to demonstrate that the progeny from a polycross can be used advantageously for the detection of QTL with interval mapping because (i) the variability within the progeny can be sufficient to avoid inbreeding depression in the following step of selection to produce a variety; (ii) LD is high, allowing the use of a moderate number of markers to cover the genome; and (iii) polycrosses are classically made in breeding programs for species in which synthetics are produced. Our approach was to identify QTL in the progeny of a polycross of perennial ryegrass including six parents, for PH and for PGR, the increase in plant height over a certain time after a defoliation event.

2. Results

2.1. Paternity Identification

A total of 82 codominant molecular markers allowed the identification of the male parent for the 191 genotypes (Table 1). The number of genotypes per full-sib family ranged from 2 to 28, clearly showing that the crosses within the polycross were not in panmixia. That is, Nemo A, Nemo B, Nemo D, and Nemo G tend to cross preferentially together, except for Nemo B and Nemo D. Also, Nemo C and Nemo H tend to cross preferentially together but there was a deficit of genotypes in all other crosses involving either Nemo C or Nemo H. In addition, few genotypes were obtained from Nemo B × Nemo D. This non-random mating could be at least partly due to differences in flowering date. Indeed, Nemo C and Nemo H showed heading dates three to four days earlier than the other potential parents (Table 1). Another reason for low mating between Nemo A × Nemo C, Nemo B × Nemo D, and Nemo D × Nemo H could have arisen from the spatial distance between parents in the polycross (Figure 1). These pairs were not nearby in any of the four repetitions of the polycross.

Table 1. Number of genotypes per family and heading dates of the parents.

	Nemo A	Nemo B	Nemo C	Nemo D	Nemo G	Total Number of Genotypes per HS Family	Heading Dates [1]
Nemo A						84	157
Nemo B	20					55	157
Nemo C	5	3				48	153
Nemo D	27	5	10			67	156
Nemo G	23	22	2	16		70	157
Nemo H	9	5	28	9	7	58	154

[1] Calendar day counting from 1 January; HS: half-sib.

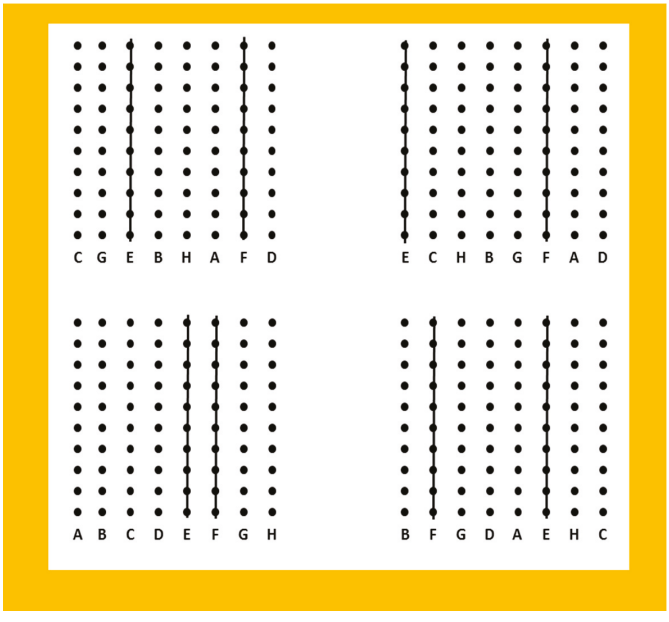


Figure 1. Design of the polycross including Nemo A–D, Nemo G, and Nemo H organised in four repetitions. Nemo E and Nemo F were discarded before crossing. In each repetition, each parent is represented by a line of 10 clones (dots). The polycross was planted in a field of rye (in yellow).

2.2. Phenotypic Analyses

The 191 genotypes were measured for PH in spring and autumn in a spaced-plant trial with four replicates. Six measurements of PH in April were used to estimate PGR (Figure 2). After the defoliation of all plants on 24 March, PH increased linearly up to a maximum reached after three to six weeks, depending on the plant. This means that at this date (24 March) all plants had re-started their growth after winter. The fast regrowth indicates all the plants were certainly induced for flowering [18].

For each trait, the distribution of the error term of the variance analysis was not significantly different from a normal distribution. The broad sense heritability was medium for PH and low for PGR (Table 2).

All traits showed large genetic variability (i.e., the variability of the mean per genotype (Table 3)). On average, PH reached 205 mm after six weeks of regrowth in spring and 203 mm after 11 weeks of regrowth in autumn.

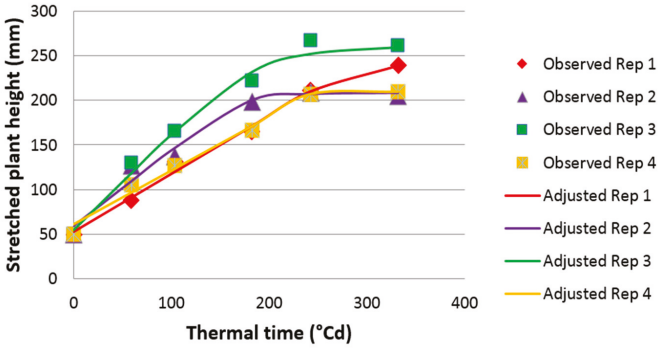


Figure 2. Example of plant regrowth in spring for the genotype 23 (four replicates). A non-equilateral hyperbolic function was fitted between stretched plant height (PH) and thermal time (mm/°Cd, base 0 °C).

Table 2. Genetic variance, error variance, and broad sense heritability ($H^2 = \sigma^2_g/(\sigma^2_g + \sigma^2_e)$) for stretched plant heights (PH in mm) in spring (0309 and 0409) and autumn (0909) and maximum plant growth rate (PGR in mm/°Cd, base 0 °C) in spring.

Traits	σ^2_g	σ^2_e	H^2
PH0309	414	382	0.52
PH0409_1	172	164	0.51
PH0409_2	451	451	0.50
PH0409_3	378	360	0.51
PH0409_4	512	552	0.48
PH0409_5	595	665	0.47
PGR	0.039	0.083	0.32
PH0909	426	394	0.52

Table 3. Distribution of the mean per genotype for stretched plant heights (PH in mm) and maximum plant growth rate (PGR in mm/ Cd, base 0 °C).

Traits	Average	Minimum	Maximum	Standard Deviation
PH0309	129	71	190	22
PH0409_1	114	66	147	15
PH0409_2	134	53	185	24
PH0409_3	137	96	237	22
PH0409_4	198	114	266	25
PH0409_5	205	123	282	28
PGR	0.95	0.48	1.66	0.25
PH0909	203	116	271	24

All PH measurements done in spring were highly inter-correlated and were also highly correlated with the PGR estimated in spring (Table 4). The high correlation between PH in March and in April indicates the majority of the plants had already started their regrowth in March, after winter. As expected from the estimation of PGR, PGR was very highly correlated with the first three PH measurements in April. The value of PGR was also highly correlated with the last two measurements in April, although slightly less than with the first three measurements. This indicates the rate of plant regrowth in spring had a greater influence on PH after six weeks of regrowth than the duration of the linear phase of regrowth. The PH in autumn was only poorly correlated with PH and PGR in spring.

Table 4. Pearson correlation coefficients for pair-wise combinations of mean data for each genotype for stretched plant heights (PH in mm) and maximum plant growth rate (PGR in mm/°Cd, base 0 °C).

Traits	PH0309	PH0409_1	PH0409_2	PH0409_3	PH0409_4	PH0409_5	PGR
PH0409_1	0.83 ***						
PH0409_2	0.89 ***	0.90 ***					
PH0409_3	0.83 ***	0.83 ***	0.88 ***				
PH0409_4	0.81 ***	0.83 ***	0.87 ***	0.88 ***			
PH0409_5	0.77 ***	0.82 ***	0.83 ***	0.87 ***	0.88 ***		
PGR	0.81 ***	0.86 ***	0.90 ***	0.78 ***	0.73 ***	0.68 ***	
PH0909	0.18 *	0.11 ns	0.15 *	0.13 ns	0.20 **	0.17 *	0.13 ns

*** significant at 0.001; ** significant at 0.01; * significant at 0.05; ns: not significant at 0.05.

2.3. Genetic Map

A consensus map was built based on the recombination information of the six parents included in the polycross (Figure 3). The map included seven linkage groups (LG) as expected in perennial ryegrass. The LG were numbered as in the map built by the International *Lolium* Genome Initiative [19]. It covered a distance of 491.4 cM with an average distance between two consecutive markers of 5.1 cM. The maximum distance between two markers was 36.9 cM on LG 2. LG 1 was surprisingly short, indicating a lack of markers on this LG.

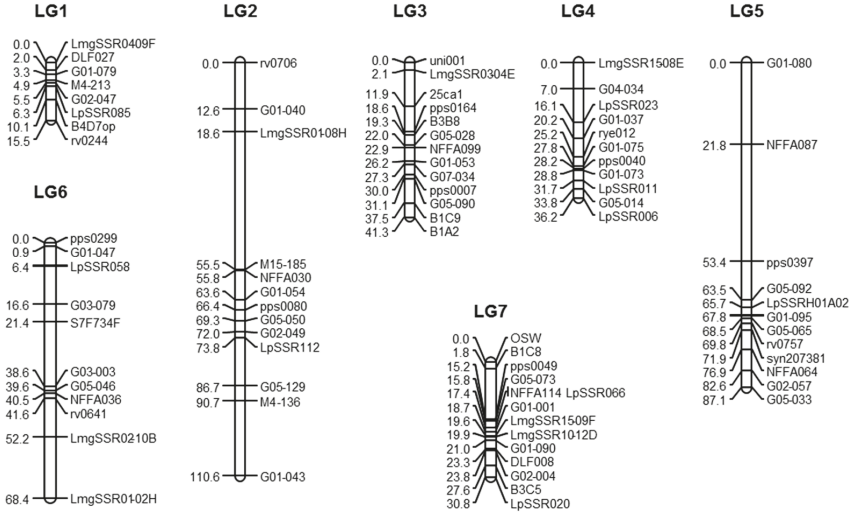


Figure 3. Consensus map of the six parents of the polycross. Seven linkage groups (LG) are presented.

2.4. QTL Identification

Two loci (LG 3 and 4) were identified with significant effects for PH and PGR in spring for all parents (Table 5). These explained between 12% and 22% of the variance of traits. The locus on LG 3 was detected only at the beginning of spring. No QTL was detected for PH in autumn. Significant effects were identified in all parents with Nemo C, showing the highest number of significant effects. The most favourable alleles were identified in Nemo C, Nemo D, and Nemo G on LG3 and Nemo B, Nemo C, and Nemo H on LG4.

Table 5. Quantitative traits (QTL) detected by the multipopulation connected analysis with additive effects. The software MCQTL with the Outbred module was used. Significant effects are indicated in bold.

							Additive Effects					
Traits	**LG**	**Markers**	**Map Location (cM)**	**Confidence Interval**	**r^2 (%)**	**Global r^2 (%)**	**Nemo A**	**Nemo B**	**Nemo C**	**Nemo D**	**Nemo G**	**Nemo H**
PH0309	3	pps0007	29.3	28.1–41.2	12.0	28.1	−1.8 [1]	4.5	5.2	4.6	**−9.5**	1.9
	4	LpSSR011	30.8	26.4–35.9	20.1		−3.8	**7.4**	**10.7**	−1.2	3.4	**10.8**
PH0409_1	3	pps0007	29.3	27.0–30.6	14.0	28.4	−1.7	0.9	**5.8**	**4.8**	**−5.5**	1.0
	4	G01-073	28.8	27.9–34.4	19.2		**−3.3**	**4.1**	**5.7**	0.1	**4.0**	**6.0**
PH0409_2	3	pps0007	29.3	27.2–30.6	15.6	27.7	−1.2	3.0	**8.8**	**8.3**	**−9.7**	3.1
	4	G01-075	27.8	26.2–32.6	17.3		**−4.6**	4.5	**9.6**	1.5	**5.6**	**10.6**
PH0409_3	4	pps0040	28.2	25.8–35.1	17.3		**−6.6**	4.3	**7.4**	1.2	**6.7**	**8.7**
PH0409_4	4	LpSSR011	31.7	26.2–35.0	16.1		**−5.3**	**11.8**	**7.8**	0.8	4.0	**9.1**
PH0409_5	4	LpSSR011	30.8	27.5–33.0	22.1		**−6.6**	**12.4**	**11.7**	−2.6	**8.4**	**11.6**
PGR	4	G05-014	33.8	19.2–36.2	12.3		−0.05	**0.08**	**0.11**	−0.04	0.02	**0.06**

[1] Within each parent, additive effects having the same sign are in phase.

3. Discussion

In this study, we successfully carried out genetic mapping and QTL detection on the progeny of a polycross, considered as a sum of connected populations. In the progeny, molecular markers allowed the identification of the male parent for each sibling. This approach has previously been used in red clover and alfalfa allowing increase of the selection gain [20]. As expected, we found the progeny of the polycross with six parents consisted of 15 full-sib families. However, the number of plants per full-sib family was highly variable with some families comprising fewer than five plants. This is a limitation for the estimation of a trait mean for very small families. To avoid this problem in future studies, it would be best to genotype a larger number of plants per half-sib family than the one needed for phenotyping and then to select seedlings based on their genotype so as to have equal numbers of plants per full-sib family. Another point that could be improved is the spatial design of the polycross, which should allow all crosses between the constituents of the polycross [21].

As expected in perennial ryegrass, we were able to build a genetic map with seven linkage groups, but the map was shorter than expected with only 491 cM, instead of between 700 and 800 cM [19,22–25]. The belonging of markers to LG was in agreement with previous studies [22,26–28]. The order of the markers on the LG was generally identical to the one in [27,28] except for LG3 and some punctual changes. For example, on LG2, the upper part of the LG, including G01_040 on the map in the present study, was at the opposite extremity in the map of [28]. The markers used in the present study cover the entire map of [28], but the size of the map was shrunk. For example, on LG1 the distance between LMgSSR04-09F and LpSSR085 was 33.5 cM in [28] and only 6.3 cM in this study. This could be due to the limited number of plants within each half-sib family and/or the number of markers in this study. In future, the number of markers used for this kind of study should be increased. This is now feasible at reasonable cost with marker platforms such as Genotyping by Sequencing (GBS) [29,30].

Values of QTL with strong effects were detected on LG 3 and 4 for PH and PGR in the spring. These regions have already been identified as QTL involved in traits related to leaf growth, such as leaf length, leaf elongation rate, and plant height [22,24,31–35]. In particular, the QTL on LG4 nearby LpSSR011 and G05_014 is located on the map of [22] nearby a QTL for leaf elongation rate, lamina length, and plant height. Moreover this QTL was near LpSSR082 which is close to the gene Gibberellic Acid Insensitive [9], which is involved in leaf elongation rate and leaf length [7]. The QTL on LG3 was not far from a QTL for leaf elongation rate in [32] (marker pps0164 in common) and in [34] (marker 25ca1 in common), but the very low number of common markers between studies does not allow a real accurate comparison. In the literature cited above, QTL were identified on all seven LG with the percentages of phenotypic variance explained by the QTL ranging from 5% to 43%. The fact that we detected only two strong QTL could arise from (i) the very small size of some full-sib families; (ii) a relatively low number of plants surveyed in comparison to the number of parents [36]; (iii) a partial genetic map; and (iv) the change of the effect of a QTL with genetic background. Nevertheless, the results obtained in this study are very encouraging for future work with larger populations and higher numbers of markers. It would improve the robustness of QTL to phenotype the plants on a multi-local trial, but due to the cost it could be a good option to keep the multi-local trials for advanced elite material tested on swards.

This study shows it was possible to detect QTL in the progeny of a polycross. This finding should be useful for breeding synthetic varieties because it allows maintenance of enough diversity to avoid inbreeding, while still selecting for the best alleles from different parents. Practically, from the progeny of a polycross, we propose to perform 1/ a selection based on molecular markers and on phenotypes (cycle 1) then 2/ a cycle of selection only on molecular markers (cycle 2) and 3/ a selection on seedlings based on molecular markers followed by a selection based on phenotypes on the rest of plants (cycle 3) (Figure 4). The selected plants could then be used to create a variety or to create new polycrosses for further cycles of selection. This breeding scheme could be done on several polycrosses at the same time and new polycrosses could be created with selected plants from different polycrosses. The selection on molecular markers should be performed for both increasing the frequencies of favourable alleles

and for maintaining diversity 1/ on the rest of the genome and if possible 2/ at the QTL locations by selecting different favourable alleles. In our case, genotypes bearing the favourable alleles from Nemo C, Nemo D, and Nemo G for the QTL on LG 3 and the favourable alleles from Nemo B, Nemo C, and Nemo H for the QTL on LG 4 were selected. In addition, we were careful to represent all six parents in the selected genotypes in order to avoid inbreeding depression. Moreover, the LD in the progeny allows the genome to be covered with a moderate number of markers. Connected populations have been used successfully for the detection of QTL in inbreeding [24,37] and outbreeding species [38], but it is the first time the progeny of a polycross has been used.

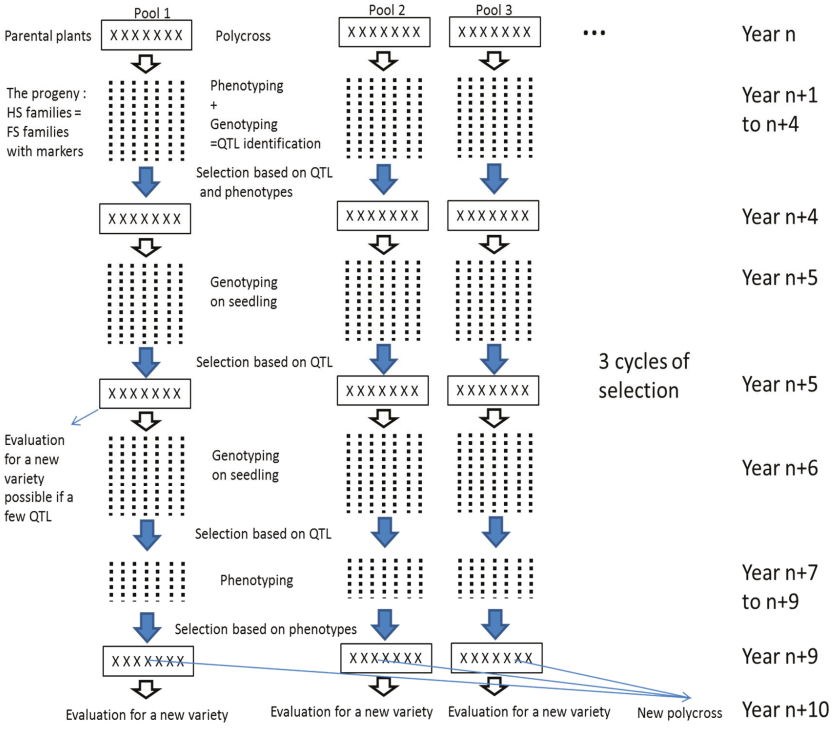


Figure 4. Breeding scheme including marker assisted selection (MAS) with QTL identification in the progenies of polycrosses.

4. Materials and Methods

The plants were obtained from Gie GRASS (a French plant breeding company) and come from their elite perennial ryegrass breeding programme. Plant material consisted of six half-sib (HS) families derived from a polycross including six parental plants: Nemo A–D, Nemo G, and Nemo H. The design of the polycross is presented in Figure 1. Four repetitions of lines (10 clones per line) of the parents were used to minimise any effects of spatial proximity. For each parent, all 40 clones were harvested and threshed together. A total of 42 genotypes per HS family were used. Four clones of each genotype were produced by splitting sets of three to five tillers from a mother plant grown in a spaced-plant nursery. Clones were planted in November 2008 in a spaced-plant trial in a randomised block design with four blocks, at Saint-Sauvant (France) (46°23′1″ N 0°05′ E). Non-surveyed plants of perennial ryegrass were planted around the trial to avoid border effects. The distance between plants was 75 cm in all directions.

Plants were cut at 5 cm height and fertilised with 50 U of nitrogen on 24 March 2009 and again on 16 July 2009. Stretched plant height (PH) was measured, on 18 March (PH0309), then every seven days from 1 April to 28 April (PH0409_1 to PH0409_5) and on 29 September after 11 weeks of regrowth (PH0909). The value of PH was measured using a modified HerboMETRE® (ARVALIS-Institut du Végétal, Paris, France) which made it possible to stretch the leaves and to record plant height from the ground to the top of the longest leaf.

For each genotype, 50 mg of fresh leaves were harvested and DNA was extracted as in [24] (i.e., CTAB (Cetyl Trimethyl Ammonium Bromide) followed by chloroform/octanol (24/1) purification). A total of 283 SSR (Simple Sequence Repeat) and STS (Sequence-Tagged Site) were tested for their polymorphism within the six parents of the polycross. A set of 82 markers evenly spread across the genome were selected to genotype the 252 genotypes (Table 6) as described in [24] (i.e., separation of PCR (Polymerase Chain Reaction) products on a 6.5% acrylamide gel with a LI-COR DNA sequencer 4200 and coding with SAGA Generation 2 software).

Table 6. Marker description: names, references, and linkage group (LG).

Marker Names	Reference	LG	Marker Names	Reference	LG
25ca1	[26]	3	LmgSSR01-08H	[39]	2
B1A2	[40]	3	LmgSSR02-10B		6
B1C8		7	LmgSSR03-04E		3
B1C9		3	LmgSSR04-09F		1
B3B8		3	LmgSSR10-12D		7
B3-C5		7	LmgSSR15-08E		4
B4D7op		1	LmgSSR15-09F		7
DLF008	[23]	7	LpSSR006	[23]	4
DLF027		1	LpSSR011		4
G01-001	[41]	7	LpSSR020		7
G01-037		4	LpSSR023		4
G01-040		2	LpSSR058		6
G01-043		2	LpSSR066		7
G01-047		6	LpSSR085		1
G01-053		3	LpSSR112		2
G01-054		2	LpSSRH01A02	[42]	5
G01-073		4	M15-185	[43]	2
G01-075		4	M4-136		2
G01-079		1	M4-213		1
G01-080		5	NFFA030	[44]	2
G01-090		7	NFFA036		6
G01-095		5	NFFA064		5
G02-004		7	NFFA087		5
G02-047		1	NFFA099		3
G02-049		2	NFFA114		7
G02-057		5	OSW	[45]	7
G03-003		6	pps0007	[25]	3
G03-079		6	pps0040		4
G04-034		4	pps0049		7
G05-014		4	pps0080		2
G05-028		3	pps0164		3
G05-033		5	pps0299		6
G05-046		6	pps0397		5
G05-050		2	rv0244	[26]	1
G05-065		5	rv0641		6
G05-073		7	rv0706		2
G05-090		3	rv0757		5
G05-092		5	rye012	[23]	4
G05-129		2	S7F7-3-4F	[39]	6
G07-034		3	syn20738-1	[22]	5
LmgSSR01-02H	[39]	6	uni001	[23]	3

To estimate the maximum plant growth rate (PGR) in spring 2009, a non-equilateral hyperbolic function was fitted between PH from 1 April to 28 April (five measurements) and thermal time (base 0 °C), using the NLIN procedure of SAS [46] as in [22] to estimate maximum leaf elongation rate.

For both PH and PGR, an analysis of variance was carried out using the GLM procedure in the SAS software package [46] with the following model: $Y_{ij} = \mu + G_i + B_j + E_{ij}$, where μ is the global mean, G_i is the effect of genotype i, B_j is the effect of replication (four replications), and E_{ij} is the error term. The Kolmogorov–Smirnov test in the UNIVARIATE procedure was used to test the normality of the error term E_{ij} in order to detect potential aberrant data. The adjusted mean for each genotype, used for QTL analyses, was computed with LSMEANS in the GLM procedure. The genotype and error variances were estimated with the VARCOMP procedure in the SAS software package. Broad-sense heritabilities (H^2) were calculated as $H^2 = \sigma^2_g/(\sigma^2_g + \sigma^2_e)$ [5], where σ^2_g is the genetic variance and σ^2_e is the error variance. Pearson correlation coefficients were calculated with the Statistica software [47] for pair-wise combinations of mean data for each genotype.

Molecular markers allowed the detection of 32 genotypes for which the male parent was not present in the polycross and 21 plants obtained by self-fertilisation. These plants were discarded in the analyses. Moreover, eight genotypes died in the field. Finally, 191 genotypes were used in the analyses.

For each of the six parents included in the polycross, one genetic map was built with the Joinmap software [48]. The Haldane distance was used. To increase the number of recombination events counted for each parent, the molecular data for Nemo A, Nemo B, and Nemo H from [24] were used to construct the maps of these genotypes and to determine the phase between markers belonging to the same linkage group. For each map, the linkage groups were defined with a Logarithm of Odds (LOD score) higher than 3. For each parent, the phases between markers belonging to the same linkage group were determined with Joinmap. The maps were then combined with the "combine maps" option of Joinmap. The linkage map was drawn using MapChart 2.1 [49].

The QTL were detected with the software MCQTL with the Outbred module [50] as in [24]. The multipopulation connected analysis was used [38] to detect QTL in the six parents within a single model, taking into account additive effects. For each parent, the additive effect was calculated as half the difference between the averages of the two allelic classes.

Acknowledgments: The authors thank the National Association for Research and Technology (ANRT) for funding. We thank the technicians of the experimental station of Jouffray-Drillaud and the GIE Grass for their help and advice on the trial. We thank the employees of the laboratory of biotechnologies of the INRA of Lusignan for helpful advice. We are grateful to Brigitte Mangin and Sylvain Jasson for their help on MCQTL.

Author Contributions: P.B., V.B., S.F. and B.J., conceived and designed the experiment; L.P., J.G., and S.F. carried out the experiments; L.P., S.F., and P.B. analysed the data; L.P., B.J., and P.B. wrote the paper.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Hill, W.G. Quantitative genetics in the genomic era. *Curr. Genom.* **2012**, *13*, 196–206. [CrossRef] [PubMed]
2. Pérez-de-Castro, A.M.; Vilanova, S.; Cañizares, J.; Pascual, L.; Blanca, J.M.; Diez, M.J.; Prohens, J.; Pico, B. Application of genomic tools in plant breeding. *Curr. Genom.* **2012**, *13*, 179–195. [CrossRef] [PubMed]
3. Rafalski, A. Application of single nucleotide polymorphisms in crop genetics. *Curr. Opin. Plant Biol.* **2002**, *54*, 94–100. [CrossRef]
4. Smith, S.E. Breeding synthetic cultivars. In *Encyclopedia of Plant and Crop Science*; Goodman, R.M., Ed.; Rutgers University: New Brunswick, NJ, USA, 2004; pp. 205–206.
5. Gallais, A. *Théorie De La Sélection en Amélioration Des Plantes*; Masson: Paris, France; Milan, Italy; Barcelon, Spain; Mexico, NM, USA, 1990; pp. 12–26.
6. Flint-Garcia, S.A.; Thornsberry, J.M.; Buckler, E.S. Structure of linkage disequilibrium in plants. *Annu. Rev. Plant Biol.* **2003**, *54*, 357–374. [CrossRef] [PubMed]

7. Auzanneau, J.; Huyghe, C.; Escobar-Gutierrez, A.J.; Julier, B.; Gastal, F.; Barre, P. Association study between the gibberellic acid insensitive gene and leaf length in a *Lolium perenne* L. Synthetic variety. *BMC Plant Biol.* **2011**, *11*, 183–196. [CrossRef] [PubMed]
8. Wilkins, P.; Humphreys, M. Progress in breeding perennial forage grasses for temperate agriculture. *J. Agric. Sci.* **2003**, *140*, 129–150. [CrossRef]
9. Auzanneau, J.; Huyghe, C.; Julier, B.; Barre, P. Linkage disequilibrium in synthetic varieties of perennial ryegrass. *Theor. Appl. Genet.* **2007**, *115*, 837–847. [CrossRef] [PubMed]
10. Brazauskas, G.; Lenk, I.; Pedersen, M.G.; Studer, B.; Lubberstedt, T. Genetic variation, population structure, and linkage disequilibrium in european elite germplasm of perennial ryegrass. *Plant Sci.* **2011**, *181*, 412–420. [CrossRef] [PubMed]
11. Rhodes, I. The relationship between productivity and some components of canopy structure in ryegrass (*Lolium* spp.). II. Yield, canopy structure and ligth interception. *J. Agric. Sci.* **1971**, *77*, 283–292. [CrossRef]
12. Rhodes, I. The relationship between productivity and some components of canopy structure in ryegrass (*Lolium* spp.) III. Spaced plant characters, their heritabilities and relationship to sward yield. *J. Agric. Sci.* **1973**, *80*, 171–176. [CrossRef]
13. Rhodes, I.; Mee, S. Changes in dry matter yield associated with selection for canopy characters in ryegrass. *Grass Forage Sci.* **1980**, *35*, 35–39. [CrossRef]
14. Horst, G.L.; Nelson, C.J.; Asay, K.H. Relationship of leaf elongation to forage yield of tall fescue genotypes. *Crop Sci.* **1978**, *18*, 715–719. [CrossRef]
15. Hazard, L.; Ghesquière, M. Productivity under contrasting cutting regimes of perennial ryegrass selected for short and long leaves. *Euphytica* **1997**, *95*, 295–299. [CrossRef]
16. Barre, P.; Emile, J.C.; Betin, M.; Surault, F.; Ghesquière, M.; Hazard, L. Morphological characteristics of perennial ryegrass leaves that influence short-term intake in dairy cows. *Agron. J.* **2006**, *98*, 978–985. [CrossRef]
17. Hazard, L.; Ghesquière, M. Evidence from the use of isozyme markers of competition in swards between short-leaved and long-leaved perennial ryegrass. *Grass Forage Sci.* **1995**, *50*, 241–248. [CrossRef]
18. Parsons, A.J.; Robson, M.J. Seasonal changes in the physiology of s24 perennial ryegrass (*Lolium perenne* L.). I. Response of leaf extension to temperature during the transition from vegetative to reproductive growth. *Ann. Bot.* **1980**, *46*, 435–444.
19. Jones, E.S.; Mahoney, N.L.; Hayward, M.D.; Armstead, I.P.; Jones, J.; Humphreys, M.O.; King, I.P.; Kishida, T.; Yamada, T.; Balfourier, F.; et al. An enhanced molecular marker based genetic map of perennial ryegrass (*Lolium perenne*) reveals comparative relationships with other poaceae genome. *Genome* **2002**, *45*, 282–295. [CrossRef] [PubMed]
20. Riday, H. Paternity testing: A non-linkage based marker-assisted selection scheme for outbred forage species. *Crop Sci.* **2011**, *51*, 631–641. [CrossRef]
21. Gillet, M. Un plan systématique de polycross pour les nombres de base pairs. *Ann. Amélior. Plantes* **1963**, *13*, 269–276.
22. Barre, P.; Moreau, L.; Mi, F.; Turner, L.; Gastal, F.; Julier, B.; Ghesquiere, M. Quantitative trait loci for leaf length in perennial ryegrass (*Lolium perenne* L.). *Grass Forage Sci.* **2009**, *64*, 310–321. [CrossRef]
23. Jensen, L.B.; Muylle, H.; Arens, P.; Andersen, C.H.; Holm, P.B.; Ghesquière, M.; Julier, B.; Lübberstedt, T.; Nielsen, K.K.; De Riek, J.; et al. Development and mapping of a public reference set of ssr markers in *Lolium perenne* L. *Mol. Ecol. Notes* **2005**, *5*, 951–957. [CrossRef]
24. Pauly, L.; Flajoulot, S.; Garon, J.; Julier, B.; Beguier, V.; Barre, P. Detection of favorable alleles for plant height and crown rust tolerance in three connected populations of perennial ryegrass (*Lolium perenne* L.). *Theor. Appl. Genet.* **2012**, *124*, 1139–1153. [CrossRef] [PubMed]
25. Faville, M.; Vecchies, A.; Schreiber, M.; Drayton, M.; Hughes, L.; Jones, E.; Guthridge, K.; Smith, K.; Sawbridge, T.; Spangenberg, G.; et al. Functionally associated molecular genetic marker map construction in perennial ryegrass (*Lolium perenne* L.). *Theor. Appl. Genet.* **2004**, *110*, 12–32. [CrossRef] [PubMed]
26. Gill, G.; Wilcox, P.; Whittaker, D.; Winz, R.; Bickerstaff, P.; Echt, C.; Kent, J.; Humphreys, M.; Elborough, K.; Gardner, R. A framework linkage map of perennial ryegrass based on ssr markers. *Genome* **2006**, *49*, 354–364. [CrossRef] [PubMed]

27. Studer, B.; Byrne, S.; Nielsen, R.O.; Panitz, F.; Bendixen, C.; Islam, M.S.; Pfeifer, M.; Lubberstedt, T.; Asp, T. A transcriptome map of perennial ryegrass (*Lolium perenne* L.). *BMC Genom.* **2012**, *13*, 140–153. [CrossRef] [PubMed]
28. Studer, B.; Kolliker, R.; Muylle, H.; Asp, T.; Frei, U.; Roldan-Ruiz, I.; Barre, P.; Tomaszewski, C.; Meally, H.; Barth, S.; et al. Est-derived ssr markers used as anchor loci for the construction of a consensus linkage map in ryegrass (*Lolium* spp.). *BMC Plant Biol.* **2010**, *10*, 177–187. [CrossRef] [PubMed]
29. Byrne, S.; Czaban, A.; Studer, B.; Panitz, F.; Bendixen, C.; Asp, T. Genome wide allele frequency fingerprints (gwaffs) of populations via genotyping by sequencing. *PLoS ONE* **2013**, *8*. [CrossRef] [PubMed]
30. Elshire, R.J.; Glaubitz, J.C.; Sun, Q.; Poland, J.A.; Kawamoto, K.; Buckler, E.S.; Mitchell, S.E. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS ONE* **2011**, *6*. [CrossRef] [PubMed]
31. Kobayashi, S.; Humphreys, M.O.; Tase, K.; Sanada, Y.; Yamada, T. Molecular marker dissection of ryegrass plant development and its response to growth environments and foliage cuts. *Crop Sci.* **2011**, *51*, 600–611. [CrossRef]
32. Sartie, A.M.; Matthew, C.; Easton, H.S.; Faville, M.J. Phenotypic and qtl analyses of herbage production-related traits in perennial ryegrass (*Lolium perenne* L.). *Euphytica* **2011**, *182*, 295–315. [CrossRef]
33. Yamada, T.; Jones, E.S.; Cogan, N.O.I.; Vecchies, A.C.; Nomura, T.; Hisano, H.; Shimamoto, Y.; Smith, K.F.; Hayward, M.D.; Forster, J.W. QTL analysis of morphological, developmental, and winter hardiness-associated traits in perennial ryegrass. *Crop Sci.* **2004**, *44*, 925–935. [CrossRef]
34. Turner, L.; Cairns, A.; Armstead, I.; Thomas, H.; Humphreys, M.W.; Humphreys, M.O. Does fructan have a functional role in physiological traits? Investigation by quantitative trait locus mapping. *New Phytol.* **2008**, *179*, 765–777. [CrossRef] [PubMed]
35. Studer, B.; Jensen, L.B.; Hentrup, S.; Brazauskas, G.; Kölliker, R.; Lübberstedt, T. Genetic characterisation of seed yield and fertility traits in perennial ryegrass (*Lolium perenne* L.). *Theor. Appl. Genet.* **2008**, *117*, 781–791. [CrossRef] [PubMed]
36. Muranty, H. Power of tests for quantitative trait loci detection using full-sib families in different schemes. *Heredity* **1996**, *76*, 156–165. [CrossRef]
37. Blanc, G.; Charcosset, A.; Mangin, B.; Gallais, A.; Moreau, L. Connected populations for detecting quantitative trait loci and testing for epistasis: An application in maize. *Theor. Appl. Genet.* **2006**, *113*, 206–224. [CrossRef] [PubMed]
38. Billotte, N.; Jourjon, M.F.; Marseillac, N.; Berger, A.; Flori, A.; Asmady, H.; Adon, B.; Singh, R.; Nouy, B.; Potier, F.; et al. QTL detection by multi-parent linkage mapping in oil palm (*Elaeis guineensis* Jacq.). *Theor. Appl. Genet.* **2010**, *120*, 1673–1687. [CrossRef] [PubMed]
39. Hirata, M.; Cai, H.W.; Inoue, M.; Yuyama, N.; Miura, Y.; Komatsu, T.; Takamizo, T.; Fujimori, M. Development of simple sequence repeat (SSR) markers and construction of an ssr-based linkage map in italian ryegrass (*Lolium multiflorum* Lam.). *Theor. Appl. Genet.* **2006**, *113*, 270–279. [CrossRef] [PubMed]
40. Lauvergeat, V.; Barre, P.; Bonnet, M.; Ghesquière, M. Sixty simple sequence repeat markers for use in the festuca—Lolium complex of grasses. *Mol. Ecol. Notes* **2005**, *5*, 401–405. [CrossRef]
41. Studer, B.; Asp, T.; Frei, U.; Hentrup, S.; Meally, H.; Guillard, A.; Barth, S.; Muylle, H.; Roldan-Ruiz, I.; Barre, P.; et al. Expressed sequence tag-derived microsatellite markers of perennial ryegrass (*Lolium perenne* L.). *Mol. Breed.* **2008**, *21*, 533–548. [CrossRef]
42. Jones, E.S.; Dupal, M.P.; Kölliker, R.; Drayton, M.C.; Forster, J.W. Development and characterisation of simple sequence repeat (SSR) markers for perennial ryegrass (*Lolium perenne* L.). *Theor. Appl. Genet.* **2001**, *102*, 405–415. [CrossRef]
43. Kubik, C.; Sawkins, M.; Meyer, W.A.; Gaut, B.S. Genetic diversity in seven perennial ryegrass (*Lolium perenne* L.) cultivars based on ssr markers. *Crop Sci.* **2001**, *41*, 1565–1572. [CrossRef]
44. Saha, M.C.; Rouf Mian, M.A.; Eujayl, I.; Zwonitzer, J.C.; Wang, L.; May, G.D. Tall fescue est-ssr markers with transferability across several grass species. *Theor. Appl. Genet.* **2004**, *109*, 783–791. [CrossRef] [PubMed]
45. Lem, P.; Lallemand, J. Grass consensus sts markers: An efficient approach for detecting polymorphism in *Lolium*. *Theor. Appl. Genet.* **2003**, *107*, 1113–1122. [CrossRef] [PubMed]
46. *SAS*, Version 9.3; Sas Institute Inc.: Cary, NC, USA, 2000.
47. StatSoft. Statistica Pour Windows. Available online: http://www.Statsoft.Fr (accessed on 20 September 2016).

48. Van Ooijen, J.W.; Voorrips, R.E. *Joinmap 3.0 Software for the Calculation of Genetic Linkage Maps*; Plant Research International: Wageningen, The Netherlands, 2001; pp. 1–51.
49. Voorrips, R.E. Mapchart: Software for the graphical presentation of linkage maps and qtls. *J. Hered.* **2002**, *93*, 77–78. [CrossRef] [PubMed]
50. Jourjon, M.F.; Jasson, S.; Marcel, J.; Ngom, B.; Mangin, B. Mcqtl: Multi-allelic qtl mapping in multi-cross design. *Bioinformatics* **2005**, *21*, 128–130. [CrossRef] [PubMed]

agronomy

MDPI

Article

Evidence for Heterosis in Italian Ryegrass (*Lolium multiflorum* Lam.) Based on Inbreeding Depression in F_2 Generation Offspring from Biparental Crosses

Junping Wang [1], Luke W. Pembleton [2], Noel O. I. Cogan [2,3] and John W. Forster [2,3,*]

1 Agriculture Victoria, Biosciences Research, Hamilton Centre, Hamilton, Victoria 3300, Australia; junping.wang@ecodev.vic.gov.au
2 Agriculture Victoria, Biosciences Research, AgriBio, Centre for AgriBioscience, Bundoora, Victoria 3083, Australia; luke.pembleton@ecodev.vic.gov.au (L.W.P.); noel.cogan@ecodev.vic.gov.au (N.O.I.C.)
3 School of Applied Systems Biology, La Trobe University, Bundoora, Victoria 3086, Australia
* Correspondence: john.forster@ecodev.vic.gov.au; Tel.: +61-03-90327054

Academic Editor: Peter Langridge
Received: 14 September 2016; Accepted: 25 October 2016; Published: 27 October 2016

Abstract: Italian ryegrass is one of the most important temperate forage grasses on a global basis. Improvement of both dry matter yield and quality of herbage have been major objectives of pasture grass breeding over the last century. F_1 and F_2 progeny sets derived from controlled pair-crosses between selected Italian ryegrass genotypes have been evaluated for yield and nutritive quality under field conditions. Linear regression of the performance of F_1 families under sward conditions on parental genotype means in a spaced plant trial was significant for quality characteristics, but not for herbage yield. This result suggests that phenotypic selection of individual plants from spaced plant nursery is feasible for improvement of nutritive quality traits, but not for yield. The presence of significant heterosis within F_1 populations was demonstrated by reduced herbage production in subsequent F_2 populations (generated by one cycle of full-sib mating), an up to 22.1% total herbage yield in fresh weight, and a 30.5% survival rate at the end of the second reproductive cycle. Potential optimal crosses for exploiting such heterosis are discussed, based on construction and the inter-mating of complementary parental pools, for the implementation of a novel F_1 hybrid production strategy.

Keywords: herbage quality; herbage yield; hybrid; heterosis; inbreeding

1. Introduction

Italian ryegrass (*L. multiflorum* Lam.) is one of the most important temperate forage grasses on a global basis, providing a high quality and cost-effective option for the supply of forage during the winter and spring seasons. Forage productivity traits such as yield, quality, and persistence have been the core targets of modern ryegrass breeding [1]. Improvements have been made in yield, digestibility, water soluble carbohydrate content for perennial, and Italian ryegrasses [2,3]. However, the magnitude of genetic gains made for dry matter production have been small relative to gains in cereal crops [4,5]. In some instances, no significant changes of forage production have been observed [6].

Despite the widespread use of hybrid cultivars (based on intercrossing of inbred lines) for crop species such as maize and rice, most varieties of forage crops are synthetic populations that do not express maximum heterosis for important agronomic traits such as yield [7]. Three types of methods for first filial generation (F_1) hybrid production have been proposed as a means to capture and exploit heterosis in forage grasses. These include varietal hybrid production [8–11], the use of cytoplasmic male

sterility (CMS) [12], and a restriction of allelic diversity at self-incompatibility loci by selfing [13,14] or by genotypic classification and selection [15]. The theoretical expectation from crossing of perennial ryegrass lines of divergent geographic origin is that 50% of progeny individuals would be intervarietal F_1 hybrids and the remainder would arise from intra-varietal mating, although several factors may produce bias in the proportions of hybrids and non-hybrids [8]. An empirical study of heterosis between 15 varieties of perennial ryegrass was conducted in spaced [9], simulated sward [10], and sown-sward [11] settings. Heterosis was highly specific to the test conditions, varying from up to 31% in spaced trial to only 3.6% in sown sward to the higher parent for herbage yield. Pure hybrids obtained by experimental pair-crossing outperformed the corresponding intervarietal hybrid by 8.4% in terms of yield [8].

Inbred lines were developed from several cycles of self-pollination in perennial ryegrass [14], and crosses between such lines identified a range of heterosis values, from negative to approximately. 20% of the mid-parental value. The author suggested that only the most vigorous individuals within inbred lines are able to survive under sward conditions, thus increasing the parental means and reducing the level of heterosis. Nonetheless, slightly lower levels of heterosis (up to 13% for yield) were observed in diallele crosses between eight distinct populations with distant geographic origins in Europe [16]. Considering the low levels of genetic gain that have been characteristic of ryegrass breeding programs based on individual or family-based phenotypic selection [2,3,5], access to even modest levels of heterosis would prove valuable for ryegrass improvement.

Recently, a F_1 hybrid breeding strategy for perennial ryegrass was developed, based on restriction of allelic diversity at the S and Z gametophytic self-incompatibility loci in complementary germplasm pools and crossing between pools (alleles) [15]. Since a similar self-incompatibility mechanism is active in Italian ryegrass, the same strategy would be applicable. From the perspective of seed production, Italian ryegrass may provide a larger potential market than perennial ryegrass due to more frequent sowing. If levels of heterosis in Italian ryegrass prove adequate, F_1 hybrid production would be an economically viable strategy. However, Italian ryegrass has a comparatively narrow genetic base compared to perennial ryegrass [17], and evidence for heterosis is relatively lacking. As a consequence, a crucial first step would be to identify heterotic combinations between Italian ryegrass germplasm sources.

In a previous study, the extent of trait variation and broad-sense heritability was reported for yield, nutritive values, and morphological traits evaluated in a spaced plant nursery trial containing 960 genotypes of Italian ryegrass [18]. The plants were also analysed for trait-marker associations using single nucleotide polymorphism (SNP) genetic marker data. In the present paper, the results of selection from those genotypes and the evaluation of the first and second generations derived from bi-parental crosses are described. The objective of this study was to estimate narrow-sense heritability as an indicator of feasibility for spaced plant selection for improvement of yield and quality, and to quantify the presence of heterosis in F_1 progeny sets and its subsequent erosion in succeeding generations.

2. Results

2.1. Field Evaluation of F_1 Progeny Sets

Changes in yield and quality parameters were observed across multiple harvests (Figure S1). Yield from the first harvest exhibited the broadest range and highest average value, representing the establishment phase of growth in the first three months after planting. Yield gradually declined following harvest-2 and havest-3, and recovered during harvest-4 in November, when plants were developing flowering tillers. Growth slowed again for harvest-5 and havest-6, and reached the lowest point during harvest-7, corresponding to the aftermath regrowth in summer. Yield from harvest-8 (conducted in July) represented autumn recovery following a dry summer and was slightly higher on average than the yields from harvest-6 and harvest-7. Herbage quality also altered according to harvest. Water-soluable carbohydrate (WSC) content was lowest for harvest-3 and harvest-8, while crude protein (CP) content was highest for these two harvests. Acid detergent fibre (ADF) and netural

detergent fibre (NDF) contents were high for harvest-4 and havest-5, while dry matter digestibility (DMD) was low for both harvests.

Simple linear regression of quality parameters for the F_1 progeny sets for each harvest and mean quality parameters across all harvests on those of the parental genotype means resulted in regression coefficients of 0.32 ($p < 0.001$), 0.21 ($p = 0.008$), 0.34 ($p < 0.001$) , 0.23 ($p = 0.002$), and 0.26 ($p = 0.029$) for mean WSC, CP, NDF, ADF, and DMD, respectively (Table 1).

Table 1. Regression of F_1 family performances on the parental means for quality parameters (b: regression coefficient; S.E.: standard error; WSC: water-soluble carbohydrate; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; DMD: dry matter digestibility).

	b	**S.E.**	***p*-Value**		**b**	**S.E.**	***p*-Value**
WSC-1	0.47	0.222	0.037	CP-1	0.42	0.184	0.026
WSC-2	0.31	0.110	0.006	CP-2	0.26	0.125	0.077
WSC-3	0.29	0.094	0.003	CP-3	0.28	0.111	0.012
WSC-4	0.33	0.095	<0.001	CP-4	0.29	0.103	0.006
WSC-5	0.33	0.102	0.002	CP-5	0.03	0.116	0.817
WSC-8	0.30	0.067	<0.001	CP-8	0.33	0.122	0.008
Mean WSC	0.32	0.078	<0.001	Mean CP	0.21	0.076	0.008
NDF-1	0.42	0.177	0.020	ADF-1	0.19	0.163	0.251
NDF-2	0.30	0.100	0.004	ADF-2	0.25	0.108	0.022
NDF-3	0.43	0.130	0.001	ADF-3	0.34	0.122	0.006
NDF-4	0.31	0.120	0.012	ADF-4	0.26	0.126	0.044
NDF-5	0.24	0.109	0.032	ADF-5	0.18	0.152	0.242
NDF-8	0.37	0.095	<0.001	ADF-8	0.21	0.128	0.110
Mean NDF	0.34	0.080	<0.001	Mean ADF	0.23	0.071	0.002
DMD-1	0.34	0.215	0.121				
DMD-2	0.14	0.148	0.338				
DMD-3	0.44	0.161	0.008				
DMD-4	0.15	0.241	0.547				
DMD-5	0.22	0.241	0.361				
DMD-8	0.38	0.185	0.042				
Mean DMD	0.26	0.118	0.029				

Total yield from F_1 swards was not significantly associated with the parental mean herbage yield from the spaced plant trial. Survival rate was highly correlated with fresh weight from harvest-8 (FW-8) ($r = 0.70$, $p < 0.001$), which was therefore taken as an indicator of persistence. Significant positive correlations were observed between parental mean plant height and F_1 plot total dry matter yield in 2012 ($r = 0.27$, $p = 0.0088$). However, parental plant height was also negatively correlated with F_1 plot persistence, as indicated by FW-8 ($r = -0.22$, $p = 0.03$). Genetic distance [17] between pairs of parental genotypes calculated on the basis of shared SNP markers was not correlated with the total herbage yield of the corresponding F_1 family ($r = 0.06$, $p = 0.57$).

2.2. Field Evaluation of F_2 Progeny Sets

Changes of yield and quality parameters across harvests in the sward trial of F_2 families (Figure S2) showed a similar trend to that observed for the F_1 sward trial. The first harvest showed the lowest ADF and NDF contents, and the highest DMD and WSC contents.

Residual maximum likelihood estimation (REML) analysis showed that different pair-wise combinations of parental genotypes produced significant effects on the yield and quality of offspring ($p < 0.001$). Identity of generation exerted a significant effect on herbage yield ($p < 0.001$), but not on quality. The total herbage yield of F_1 populations was significantly higher than that of F_2 populations (Figure 1A). The mean reduction of total herbage FW for F_2, as compared to F_1, populations was 3.96 kg, equivalent to 22.1% of the mean value across F_1 families. For the individual family-harvest

combinations, the corresponding value varied from negative in nature (such that higher yields were observed in the F_2 sets) to reductions of up to 56.1%. There was no significant difference between the yield measurements for F_2B and F_2W populations, which suggested that selection for vigor within-family at an intensity of 20 in 100 plants did not significantly further improve yield. Some F_1 families (1804 and 1836) were higher yielding than the best reference cultivars (Figure 1C). The total herbage yield of the best F_1 family (1836) was significantly higher than Hulk (14.4%) and Tabu (17.3%), and slightly higher than LM299 (4.3%) but not significant. It was also observed that the reduction of F_2 as compared to F_1 yield was larger during the first year (25.5%) than the second year (19.3%). F_1 populations also showed significantly higher survival rates than F_2 populations (Figure 1B). The absolute reduction of survival rate was 12.3% (from 40.3% for F_1 to 28.0% for F_2 families), and the proportion of inbreeding depression was 30.5%. This value varied across different families from 16.7% (1901) to 54.5% (1812). Survival of some F_1 families were superior to the reference cultivars (Figure 1D). Within-family selection slightly increased the survival rate from 28.0% (for F_2B) to 32.1% (for F_2W), but the difference was not significant.

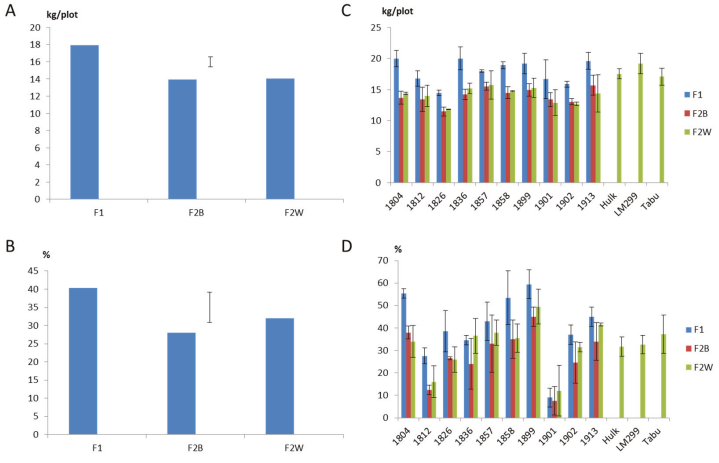


Figure 1. (**A**) Predict mean of different generations on total fresh herbage yield including LSD bar at $p = 0.05$. (**B**) Predict mean of different generations on survival rate including LSD bar at $p = 0.05$. (**C**) Total fresh herbage yield of different generation family combinations and reference cultivars. (**D**) Survival rate of different generation family combinations and reference cultivars.

3. Discussion

3.1. Effectiveness of Phenotypic Selection from Spaced Plant Nursery

The observed seasonal changes of herbage yield and quality are consistent with previous reports [19]. The herbage yield of hybrid progeny in a sward setting was not significantly associated with parental mean herbage yield in a spaced plant trial. Current practices of phenotypic recurrent selection also involve selection from a spaced plant nursery, followed by testing progenies in sward conditions [20]. This raises the issue of whether parental genotypic performances in a spaced plant trial may be trusted to be correlated with the performance of the progeny in a sward setting. The results of the present study suggest that selection of high-yielding individual Italian ryegrass genotypes from a spaced plant nursery cannot guarantee high-yielding progeny populations in a competitive sward condition. Spaced plant evaluation was also found to be unpredictive of sward yield in tall fescue [21]. Nonetheless, the observed correlation of progeny yield with mean parental plant height indicates that indirect selection for yield may be achievable by direct selection for plant height, which displays a

higher heritability value in Italian ryegrass. This correlation effect may be more relevant for Italian ryegrass than perennial ryegrass, as the former typically shows a more erect growth habit in which the vertical length of tillers will more accurately reflect total biomass. However, plant height was also found to be negatively correlated with persistence, such that any increases in yield would probably be achievable in the first growing season. Selection heavily and solely dependent on plant height would consequently not be ideal in the long term. Genotypic recurrent selection using full-sib or half-sib families [22] coupled with genomic selection [23] may hence be a necessary part of the breeding system if forage yield is the target.

The observed significant correlation between parental mean WSC content in a spaced plant trial and the WSC content of progeny under sward condition indicated that direct selection based on the former would be effective. The significant regression coefficient may arise because WSC content has never been the target of selection in the development histories of the particular varieties used in the present study. In such cases, there would be presumably a higher degree of genetic variability for the unselected character than for those traits (such as herbage yield) that have been subject to strong directional selection. This would ensure a broad range of WSC content in the regression analysis. In contrast, if the genetic variability was small and environmental effects were prominent, the regression analysis would probably not obtain significant results. Another possible explanation is that WSC content may be less sensitive to the inhibitory effects of plant neighbour interactions than a morphophysiological trait such as yield.

Based on these observations, phenotypic selection from spaced plant trials would be more effective for breeding of quality parameters than for yield. Since the quality parameters were affected by plant maturity, it would be important to measure at comparable stages of vegetative growth. In perennial grasses, selection is typically conducted on a single-plant basis and usually displays a realised heritability for digestibility of about 0.2 to 0.3 [24], in agreement with the results of the present study. Of the quality parameters, CP displayed lower heritability than for WSC content and NDF, consistent with reported sensitivity to environmental factors such as fertiliser applications [25].

3.2. Heterosis and Inbreeding Depression

In the present study, F_1 families were generated from pair crosses between two unique genotypes. As a consequence, mean values for a parental population in a field setting could not be obtained, except by vegetative propagation to produce multiple ramets from the selected genotype, which is logistically unfeasible, so the performance of F_1 families cannot be compared to those of parents. However, comparisons can be made between F_1 and F_2 progeny sets derived from a particular pair-wise genotypic combination in order to deduce the presence of heterosis in the F_1 generation and erosion by inbreeding depression in subsequent generations. High levels of inbreeding depression in parental lines has been shown to be associated with heterosis in hybrids of other plant species [26]. The magnitude of the observed inbreeding depression in the present study was on average 22.1% for total fresh herbage yield, with a range from negative values to 56.1% for different family-harvest combinations. In other studies of ryegrass species, yield in Italian ryegrass declined by 6% from Syn1 to Syn2, and remained constant from Syn2 and Syn3 [27], while that of perennial ryegrass declined by 11% from Syn1 to Syn3 of five-parent synthetic [28]. Dry matter yield in tall fescue declined by 6% from the Syn1 to Syn2 generations of four-parent synthetic [29]. In smooth bromegrass (*Bromus inermis* L.), the average level of heterosis for herbage yield in diallele crosses between seven parents was 14% with a range of −4 to 39%, and the level of inbreeding depression based comparison of selfed progenies with parents was 18%–33% [30]. The difference between species may be attributable to the number of genotypes used for crossing and different mechanisms of genetic control.

Of the 10 different families obtained in the present study, 8 were derived from pair-crosses of genotypes from different cultivars. If they were assumed to be unrelated, the inbreeding coefficient would be 0 and 0.25 for F_1 and F_2 progeny sets, respectively. For the other two families (1857 and 1826), the two parental genotypes were obtained from the same cultivar ("Tabu" for 1857 and "Warrior"

for 1826), and the two parents within each family, due to an origin from a single polycross, must by definition be to some extent related. "Tabu" was bred from a mass selection made from an 18-month old field trial of variety "Flanker" [31], while "Warrior" was bred from breeding pools obtained from various sources [32]. It is therefore likely that the two parental genotypes of family 1857 were more closely related than those of family 1826; consequently, the increase of inbreeding coefficient from the F_1 to F_2 generation for these two families will be less than 0.25, especially for 1857. A lesser degree of inbreeding depression would hence be expected in these two families. Results were consistent with this expectation, as the decline in total herbage yield for 1857 was 13.5%, the least among the 10 families. On average, heterosis increases as the genetic disparity of the parental stocks increases and interspecific crosses show greater heterosis than intraspecific crosses [33]. However, the extent of correlations between molecular marker-based genetic distance and heterosis are not conclusive. There are several prerequisites for positive correlations, such as high trait heritability and close marker linkage [34]. It is not a surprise that the present study revealed no correlation between yield in the F_1 generation and genetic distance between the parents, since yield showed low heritability and random low-density distribution of markers used for diversity analysis. Cultivars of the interspecific hybrid between perennial and Italian ryegrass species (*L. x boucheanum*), which combine advantages from each species, are available in the commercial marketplace. However, they are not F_1 hybrids, and any heterosis in the initial crosses would have been eroded through seed multiplication during the variety development process and so would not be expected to exploit the full potential of heterosis.

A 30.5% reduction in survival rate was seen in the F_2B generations as compared to F_1 generations across the various families, consistent with heterotic effects. Survival rate is a measure of persistence in forage grasses. It may be regarded as a fitness-related trait, which generally exhibit lower heritability values than morphological traits [35]. Nonetheless, heterosis for fitness-related traits is commonly observed [27]. Hybrid vigour for persistency in Italian ryegrass would be beneficial to farmers to allow the growth of more productive pastures for longer periods, with reducing re-sowing and establishment costs. Although inbreeding depression was observed for yield and persistence, no such effects were seen for quality parameters. Similarly, there is little evidence of heterosis for WSC content in perennial ryegrass [36] or for total soluble solid and crude protein content in sorghum [26]. Population hybrids (generated through controlled pair-crossing between varieties) of orchardgrass (*Dactylis glomerata* L.) exhibited higher mean phenotypic values than source cultivars for some trait-harvest combinations (such as increased CP, WSC, and decreased NDF); however, the magnitudes of difference were very small [37]. Based on these results, the feasibility of exploiting hybrids for improvement of quality traits in ryegrasses appears limited.

To date, very little information has been generated on the extent and prevalence of heterosis in Italian ryegrass. As the genetic base of Italian ryegrass is known to be narrow, it may be assumed that lower levels of differentiation between different germplasm pools are present, compared with the closely related species, perennial ryegrass. If so, a lesser extent of heterosis might be anticipated. However, the results of the present study suggest that the magnitude of heterosis in Italian ryegrass, for comparable traits, is no less than for perennial ryegrass. The significant heterosis observed in herbage yield and survival rate provided foundation data for the implementation of the F_1 hybrid breeding strategy in Italian ryegrass. However, controlled pair-crossing between specific genotypes is not a feasible approach for commercial seed production, especially as any advance for seed multiplication by reproduction will cause inbreeding depression. As a consequence, the development and maintenance of divergent and compatible parental population pools will be critical for F_1 hybrid breeding. Nonetheless, if an average of 20% yield advantages from F_1 hybrids could be achieved in commercial production, the gain would be enormous compared with the less than 1% of annual genetic gain in yield that has been achieved via conventional breeding programs.

4. Materials and Methods

4.1. Selection of Individual Plants as Parents

A total of 960 genotypes from 7 cultivars/breeding lines ("Accelerate", "AristocratII", "Hulk", "LM299", "LM414", "Tabu", and "Warrior") of Italian ryegrass were phenotypically assessed for herbage yield and quality in a spaced plant nursery trial with 4 replicates and genotyped with 384 SNP markers [18]. To conserve allelic diversity, an in-house R script was developed that selected a minimum core set of genotypes that contained all the alleles observed in the original population/nursery. This core set was made up of 29 individual plants, with representation from each of the cultivars/breeding lines. The core set was then expanded to 150 genotypes, weighted by phenotypic performance, i.e., herbage yield.

4.2. Generation of F_1 Full Sib Families and Field Test of Performance

The core set of 29 plants, along with 21 of the most elite genotypes (herbage yield) were propagated into two clonal ramets by a division of tillers. Each replicate of these 50 genotypes was then pair-crossed randomly with one of the remaining 100 selected genotypes. A total of 100 pair-crosses were conducted in glasshouses with pollen-proof bags. Seeds of each full-sib family were bulk-collected from each parent. Seeds were cleaned and germinated in a glasshouse, and 94 of the families obtained more than 100 seedlings each. Along with the original reference cultivars/breeding lines (2–4 entries each), the F_1 families were tested in a simulated sward setting during 2012–2013 with a total of 110 plots in a 10 (row) $\times$ 11 (column) layout. Each plot consisted of 100 plants in a 10 $\times$ 10 grid configuration. The distance between plots was 1 m, and the space between plants within a plot was 15 cm. The seedlings were transplanted into the field on the research farm of the Department of Economic Development, Transport, Jobs and Resources, Hamilton, Victoria, in May 2012. Apart from natural precipitation, irrigation was applied to the trial during the summer months (December, January, and February) in order to minimise plant death. Fertilizer "Grow Plus" (30 N, 10 P, 25 K, 10 S) was applied at a rate of 150 kg/ha on 18 April 2012 (before planting) and on 13 November 2012. A total of 8 harvests were conducted. The first 7 harvests were conducted every month from August 2012 onwards until February 2013 for production traits, and the last harvest was conducted in July 2013 for persistence. Fresh weight (FW) of each plot was recorded for every harvest, and herbage quality data was collected from 6 of the 8 harvests, apart from harvest-6 and harvest-7 which were performed in January and February 2013, respectively. When herbage quality data was collected, 200–300 g sub-samples from each plot were dried in a fan-forced oven at 60 °C, and dry matter yield was calculated. Herbage quality parameters including contents of WSC, CP, NDF, ADF, and DMD were assessed as described previously [38].

4.3. Generation of F_2 Families and Field Test of Performance

Based on the performance of the F_1 sward trial as described, 10 F_1 families were selected for high yield, high WSC content, and good persistence (Table 2). Within each of the 10 F_1 families, 20 plants were further selected based on visual assessment of vigour, and half of each selected plant was taken from the field and potted in a glasshouse in order to allow polycrossing (exclusive to a particular family) between extracted individuals (so providing the basis for between- and within-family selection, designated as F_2W). The 10 selected in-field plots were also covered with pollen proof cages to generate F_2 families based on inter-mating of members of the same F_1 family (so providing the basis for between-family selection, designated as F_2B). Seeds from these 20 polycrosses were harvested and cleaned, and used for in-field evaluation.

A simulated sward trial was conducted in 2014–2016 to test the performance of the F_2 families along with F_1 progeny sets and reference cultivars. The trial entries included 10 F_1 families, 10 F_2B families, 10 F_2W families, and the reference cultivars, "Tabu", "LM299" and "Hulk". The trial was basically designed as a split-plot design with family as whole plot and generation (F_1, F_2B, and F_2W)

as sub-plot, with 2 replicates. Reference cultivars were represented by 4 replicates in the trial. A total of 72 plots were organized in a 12 (row) × 6 (column) layout. Each of the plots contained 100 plants, and the space between plots and the distances between plants within a plot were the same as for the F_1 sward trial. Seeds from each entry were germinated in seedling trays in the glasshouse, and seedlings were transplanted to the field in May 2014. Irrigation was applied for the trial in the first summer months in order to keep plants alive. The trial was fertilized six times with nitrogen fertilizer and "Grow Plus" at the same rate as that of the F_1 trial at 2–3 month intervals, and a total of 12 harvests were conducted. The first 10 harvests were conducted every month starting from September 2014 to June 2015, and harvest-11 and harvest-12 were conducted in August and November 2015, respectively. Quality data were collected from harvests 1, 2, 7, 9, 10, and 11 following the same procedure as described in the F_1 trial. Plant survival rate was recorded on 4 March 2016.

Table 2. Selected 10 bi-parental F_1 families.

Family	Parent 1	Parent 2
1804	LM299-96	Tabu-37
1812	Hulk-35	LM299-85
1826	Warrior-77	Warrior-181
1836	LM299-48	Hulk-54
1857	Tabu-47	Tabu-60
1858	Hulk-81	Warrior-55
1899	LM299-20	LM414-55
1901	Tabu-58	Accelerate-89
1902	LM299-61	Warrior-26
1913	LM299-17	Accelerate-87

4.4. Statistical Analysis

Statistical analysis was carried out using GenStat [39]. Correlation coefficients between pairs of traits were calculated using the correlation command in GenStat. Narrow-sense heritability was estimated by simple linear regression of the F_1 progeny on parental mean for each trait. The narrow-sense heritability for the corresponding traits is equal to the regression coefficient [40]. Significance testing of family and generation differences was conducted using REML. The level of inbreeding depression from comparison of the F_1 and F_2 generations was calculated as the deviation of the mean performance from the mean of the previous generation.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/6/4/49/s1. Figure S1: Scatter plot of yield and quality across harvests from the F_1 field trial (FW: Fresh weight (g); WSC: water-soluble carbohydrate (% dry matter); CP: crude protein (% dry matter); NDF: neutral detergent fibre (% dry matter); ADF: acid detergent fibre (% dry matter); DMD: dry matter digestibility (%)), Figure S2: Scatter plot of yield and quality across harvests from the F_2 field trial (trait abbreviations and units see Figure S1).

Acknowledgments: The authors acknowledge financial support from the Victorian Department of Economic Development, Jobs, Transport and Resources, Geoffrey Gardiner Foundation, Meat and Livestock Australia and New Zealand Agriseeds, Christchurch, New Zealand, through the Dairy Futures Cooperative Research Centre.

Author Contributions: Noel O. I. Cogan and John W. Forster conceived the experiments. Junping Wang and Luke W. Pembleton designed and performed the experiments; Junping Wang analyzed the data; Junping Wang, Luke W. Pembleton, and John W. Forster prepared the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Stewart, A.; Hayes, R. Ryegrass breeding-balancing trait priorities. *Irish J. Agric. Food Res.* **2011**, *50*, 31–46.
2. Chaves, B.; de Vliegher, A.; van Waes, J.; Carlier, L.; Marynissen, B. Change in agronomic performance of *Lolium perenne* and *Lolium multiflorum* varieties in the past 40 years based on date from Belgian VCU trials. *Plant Breed.* **2009**, *128*, 680–690. [CrossRef]

3. Wilkins, P.W.; Lovatt, J.A. Gains in dry matter yield and herbage quality from breeding perennial ryegrass. *Irish J. Agriv. Food Res.* **2011**, *50*, 23–30.
4. Woodfield, D.R. Genetic improvement in New Zealand forage cultivars. In Proceedings of the New Zealand Grasslands Association, Hawkes Bay, New Zealand, 1999; Volume 61, pp. 3–7.
5. Gout, M.; Jones, S. Estimates of the annual sales of proprietary cultivars in Australia, and the value of pastures to the livestock and cropping industries. In Presentation to Pastures Australia, Adelaide, Australia, 23–24 February 2006.
6. Redfearn, D.D.; Venuto, B.C.; Pitman, W.D.; Blouin, D.C.; Alison, M.W. Multilocation annual ryegrass cultivar performance over a twelve-year period. *Crop Sci.* **2005**, *45*, 2388–2393. [CrossRef]
7. Brummer, E.C. Capturing heterosis in forage crop cultivar development. *Crop Sci.* **1999**, *39*, 943–954. [CrossRef]
8. Foster, C.A. A study of the theoretical expectation of F_1 hybridity resulting from bulk interpopulation hybridization in herbage grasses. *J. Agric. Sci.* **1971**, *76*, 295–300. [CrossRef]
9. Foster, C.A. Interpopulational and intervarietal hybridization in *Lolium perenne* breeding: Heterosis under non-competitive conditions. *J. Agric. Sci.* **1971**, *76*, 107–130. [CrossRef]
10. Foster, C.A. Interpopulational and intervarietal hybridization in *Lolium perenne*: Heterosis under simulated-sward conditions. *J. Agric. Sci.* **1971**, *76*, 401–409. [CrossRef]
11. Foster, C.A. Interpopulational and intervarietal F_1 hybrids in *Lolium perenne*: Performance in field sward conditions. *J. Agric. Sci.* **1973**, *80*, 463–477. [CrossRef]
12. Islam, M.S.; Studer, B.; Møller, I.M.; Asp, T. Genetics and biology of cytoplasmic male sterility and its applications in forage and turf grass breeding. *Plant Breed.* **2014**, *133*, 299–312. [CrossRef]
13. England, F. The use of incompatibility for the production of F_1 hybrids in forage grasses. *Heredity* **1974**, *32*, 183–188. [CrossRef]
14. Posselt, U.K. Hybrid production in *Lolium perenne* based on incompatibility. *Euphytica* **1993**, *71*, 29–33. [CrossRef]
15. Pembleton, L.W.; Shinozuka, H.; Wang, J.; Spangenberg, G.C.; Forster, J.W.; Cogan, N.O.I. Design of an F_1 hybrid breeding strategy for ryegrasses based on selection of self-incompatibility locus-specific alleles. *Front. Plant Sci.* **2015**, *6*, 764. [CrossRef] [PubMed]
16. Posselt, U.K. Identification of heterotic patterns in perennial ryegrass. In *Sustainable Use of Genetic Diversity in Forage and Turf Breeding*; Huyghe, C., Ed.; Springer: Dordrecht, The Netherlands, 2010; pp. 569–572.
17. Wang, J.; Pembleton, L.W.; Baillie, R.C.; Drayton, M.C.; Hand, M.L.; Bain, M.; Sawbridge, T.I.; Spangenberg, G.C.; Forster, J.W.; Cogan, N.O.I. Development and implementation of a multiplexed single nucleotide polymorphism genotyping tool for differentiation of ryegrass species and cultivars. *Mol. Breed.* **2014**, *33*, 435–451. [CrossRef]
18. Wang, J.; Cogan, N.O.I.; Pembleton, L.W.; Forster, J.W. Variance, inter-trait correlation, heritability and trait-marker association of herbage yield, nutritive values, and morphological characteristics in Italian ryegrass (*Lolium multiflorum* Lam.). *Crop Pasture Sci.* **2015**, *66*, 973–984.
19. Radojevic, I.; Simpson, R.J.; St. John, J.A.; Humphreys, M.O. Chemical composition and *in vitro* digestibility of lines of *Lolium perenne* selected for high concentrations of water-soluble carbohydrate. *Aust. J. Agric. Res.* **1994**, *45*, 901–912. [CrossRef]
20. Vogel, K.P.; Pederson, J.F. Breeding systems for cross pollinated perennial grasses. In *Plant Breeding Reviews*; Janick, J., Ed.; John Wiley & Sons, Inc.: Oxford, UK, 1993; Volume 11, pp. 251–274.
21. Waldron, B.L.; Robins, J.G.; Peel, M.D.; Jensen, K.B. Predicted efficiency of spaced-plant selection to indirectly improve tall fescue sward yield and quality. *Crop Sci.* **2008**, *48*, 443–449. [CrossRef]
22. Conaghan, P.; Casler, M.D. A theoretical and practical analysis of optimum breeding system for perennial ryegrass. *Irish J. Agric. Food Res.* **2011**, *50*, 47–63.
23. Hayes, B.; Cogan, N.O.I.; Pembleton, L.; Goddard, M.; Wang, J.; Spangenberg, G.C.; Forster, J.W. Prospects for genomic selection in forage plant species. *Plant Breeding* **2013**, *132*, 133–143. [CrossRef]
24. Casler, M.D.; Vogel, K.P. Accomplishments and impact from breeding for increased forage nutritional value. *Crop Sci.* **1999**, *39*, 12–20. [CrossRef]
25. McGrath, D. A note on the influence of nitrogen application and time of cutting on water soluble carbohydrate production by Italian ryegrass. *Irish J. Agric. Food Res.* **1992**, *31*, 189–192.

26. Bhatt, A. Studies on heterosis and inbreeding depression in forage sorghum [*Sorghum bicolor* (L.) Moench]. *Agric. Sci. Digest* **2008**, *28*, 258–261.
27. Becker, H.C. Breeding synthetic varieties of crop plants. *Plant Genet. Breed. Rev.* **1988**, *1*, 31–54.
28. Hayward, M.D.; Abdultah, I.B. Selection and stability of synthetic varieties of *Lolium perenne* 1. The selected character and its expression over generations of multiplication. *Theor. Appl. Genet.* **1985**, *70*, 48–51. [CrossRef] [PubMed]
29. Piano, E.; Annicchiarico, P.; Romani, M.; Pecetti, L. Effect of the number of parents and their combining ability on the performance of synthetic varieties in tall fescue. *Aust. J. Agric. Res.* **2007**, *58*, 1100–1105. [CrossRef]
30. Casler, M.D.; Diaby, M.; Stendal, C. Heterosis and inbreeding depression for forage yield and fiber concentration in smooth bromegrass. *Crop Sci.* **2005**, *45*, 44–50. [CrossRef]
31. 'Tabu' in *Plant Varieties J.* **2002**, *15*, 60.
32. 'Warrior' in *Plant Varieties J.* **2003**, *20*, 162.
33. East, E.M. Heterosis. *Genetics* **1936**, *21*, 375–397. [PubMed]
34. Bernardo, R. Relationship between single-cross performance and molecular marker heterozygosity. *Theor. Appl. Genet.* **1992**, *83*, 628–634. [CrossRef] [PubMed]
35. Visscher, P.M.; Hill, W.G.; Wray, N.R. Heritability in the genomics era—Concepts and misconceptions. *Nat. Rev. Genet.* **2008**, *9*, 255–266. [CrossRef] [PubMed]
36. Wilkins, P.W.; Lovatt, J.A. Chromosome doubling and top-crossing as a means of exploiting heterosis in perennial ryegrass. In Proceedings of Breeding and Seed Production for Conventional and Organic Agriculture, Proceeding of the 26th EUCAPRIA Fodder Crops and Amenity Grasses Section and the 16th Medicago spp. Group Joint Meeting, Perugia, Italy, 3–7 September 2006; pp. 52–55.
37. Robins, J.; Bushman, B.; Escribano, S.; Jensen, K. Heterosis for protein, digestibility, fiber, and water soluble carbohydrates in nine sources of orchardgrass germplasm. *Euphytica* **2015**, *204*, 503–511. [CrossRef]
38. Pembleton, L.W.; Wang, J.; Cogan, N.O.I.; Pryce, J.E.; Ye, G.; Bandaranayake, C.K.; Hand, M.L.; Baillie, R.C.; Drayton, M.C.; Lawless, K.; et al. Candidate gene-based association genetics analysis of herbage quality traits in perennial ryegrass (*Lolium perenne* L.). *Crop Pasture Sci.* **2013**, *64*, 244–253. [CrossRef]
39. Payne, R.W.; Murray, D.A.; Harding, S.A.; Baird, D.B.; Soutar, D.M. *GenStat for Windows Introduction*; VSN International: Hemel Hempstead, UK, 2009.
40. Falconer, D.S.; Mackay, T.F.C. *Introduction to Quantitative Genetics*, 4th ed.; Pearson Education Limited: Harlow, UK, 1996; pp. 160–181.

agronomy

MDPI

Review

Opportunities for Napier Grass (*Pennisetum purpureum*) Improvement Using Molecular Genetics

Alemayehu Teressa Negawo [1], Abel Teshome [1], Alok Kumar [1,2], Jean Hanson [1] and Chris S. Jones [1,*]

[1] Feed and Forage Development, International Livestock Research Institute, Box 5689, Addis Ababa, Ethiopia; a.teresssa@cgiar.org (A.T.N.); a.teshome@cgiar.org (A.T.); alok.igib@gmail.com (A.K.); j.hanson@cgiar.org (J.H.)

[2] School of Plant Science, College of Agriculture and Environmental Science, Haramaya University, Box 138, Dire Dawa, Ethiopia

* Correspondence: c.s.jones@cgiar.org; Tel.: +25-111-617-2352

Academic Editors: John W. Forster and Kevin F. Smith
Received: 28 February 2017; Accepted: 4 April 2017; Published: 13 April 2017

Abstract: Napier grass (*Pennisetum purpureum* Schumach.) is a fast-growing perennial grass native to Sub-Saharan Africa that is widely grown across the tropical and subtropical regions of the world. It is a multipurpose forage crop, primarily used to feed cattle in cut and carry feeding systems. Characterization and diversity studies on a small collection of Napier grasses have identified a moderate level of genetic variation and highlighted the availability of some good agronomic traits, particularly high biomass production, as a forage crop. However, very little information exists on precise phenotyping, genotyping and the application of molecular technologies to Napier grass improvement using modern genomic tools which have been applied in advancing the selection and breeding of important food crops. In this review paper, existing information on genetic resources, molecular diversity, yield and nutritional quality of Napier grass will be discussed. Recent findings on characterizing disease resistance and abiotic stress (drought) tolerance will also be highlighted. Finally, opportunities and future prospects for better conservation and use arising from the application of modern genomic tools in Napier grass phenotyping and genotyping will be discussed.

Keywords: Napier grass; elephant grass; Uganda grass; *Pennisetum purpureum*; diversity analysis; characterization; phenotype; genotype

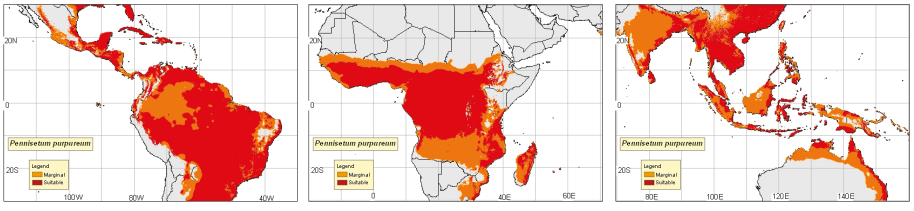
1. Introduction

Sustainable livestock production is highly dependent on the availability of quality feed and forage resources. Napier grass, also known as elephant or Uganda grass, is one of the most important tropical forage crops. It is widely used in cut and carry feeding systems [1–3] and is of growing importance in other agricultural systems. Napier grass possesses many desirable characteristics, including high yield per unit area, tolerance to intermittent drought and high water use efficiency [2], making it a forage of choice. It has the ability to withstand repeated cutting and will rapidly regenerate, producing palatable leafy shoots [4]. Consequently, enhancing the knowledge-based use and conservation of the available Napier grass resources promises to substantially benefit livestock value chains.

1.1. Origin, Propagation and Distribution

Napier grass is a monocotyledonous flowering plant belonging to the family Poaceae (the family of grasses) and the genus *Pennisetum* [5,6]. *Pennisetum* is a highly diverse genus consisting of a heterogeneous group of approximately 140 species [7–9] with different basic chromosome numbers of 5, 7, 8 or 9, a range of ploidy levels from diploid to octoploid, both sexual and apomictic reproductive

behaviours and life cycles of an annual, biennial or perennial nature [10]. Napier grass is a perennial C4 grass species [11,12] native to Sub-Saharan Africa from where it is believed to have been distributed to other tropical and subtropical regions around the world. It has been reported to be adapted to grow across a wide range of soil conditions and agro-ecologies, from sea level to 2100 m, and it can withstand minor dry spells, although it grows best in areas where the annual rainfall is between 750 and 2500 mm [6]. Given its wide agro-ecological adaptation, Napier grass has been naturalized in areas of Central and South America, tropical parts of Asia, Australia, the Middle East and the Pacific islands [6,13]. As a result, today it is widely grown in tropical and subtropical regions of the world, for use predominantly as animal fodder (Figure 1). Napier grass can be more commonly distributed by vegetative cuttings and tillers [6], since the grass cannot produce many seeds and those that are produced are normally very small, light, of poor quality and the spikelets are prone to shattering [6,14]. Consequently, the seeds are considered inappropriate for propagation as they produce weak seedlings and, as Napier grass is an open pollinated crop, the seedlings are also highly heterozygous [6,14]. Therefore, propagation by stem cuttings is currently the dominant practice for the distribution of Napier grass propagules [6,15].



Caribbean, Central and Southern America regions

Africa

Melanesia, Southeast Asia and surrounding regions

Figure 1. Regional distribution of Napier grass around the world (reproduced from [13]).

1.2. Economic Importance

A range of grass species are used as fodder crops by farmers in Africa, Asia and other tropical/subtropical regions of the world. Napier grass is one of the most important fodder crops, particularly in Eastern and Central African smallholder farming communities [1,2]. It is mainly used to feed livestock in cut and carry feeding systems [3,16,17]. It is a multipurpose forage crop that can be grazed directly, or made into silage or hay [18] and there have also been reports of using Napier grass as fish food, for example for feeding grass carp and tilapia in Nepal [19,20] and Bangladesh [21]. A recent report from Nigeria also indicated that young shoots of Napier grass were used as a cooked vegetable [22]. These varied uses provide an indication of the diversity of roles that Napier grass could contribute to the reduction of poverty and nutritional insecurity.

In addition to its value as a forage crop, Napier grass can also be used to make fences, as a windbreak, to demarcate boundaries among neighbouring farmers, and the dried material can be used as a fuel source [18]. In crop land management systems, it is used as a mulch to control weed infestation and soil erosion [2] and as a trap plant in the push–pull strategy, a pest management practice which uses repellent intercrop 'push' plants and attractant trap 'pull' plants [23] for insect pest control in Africa, particularly for the maize stem borer [24,25]. Plants are also used to scavenge pollutants, such as heavy metals, and Napier grass has been used in phytoremediation strategies, for example for the cleanup of cadmium-affected soil, reducing the concentration of cadmium to a depth of 15 cm in soil [17].

With growing global interest in reducing fossil fuel consumption and concerns about the impacts of climate change, the search for alternative biofuel sources has led to the promotion of large biomass plants as second- or next-generation biofuel crops. Napier grass, with its perennial nature

and fast growing characteristics, has been reported to produce a dry matter (DM) yield of up to 78 tons/ha/annum (35–41 tons/ha average) [26]. Rengsirikul et al. [27] estimated a maximum ethanol production of 350–460 L/ton DM from Napier grass varieties grown in Thailand, and an estimated ethanol yield of 329 L/ton DM. Lima et al. [28] demonstrated that this potential was 6% and 15% higher than for the tropical forages *Brachiaria brizantha* and *Panicum maximum*, respectively, around 15% higher than *Eucalyptus* bark and 17% higher than for sugarcane. Consequently, the potential exists for the use of Napier grass for phytoremediation purposes, after which the large harvest could go into processing plants for biofuel production.

1.3. Genetic Resources, Molecular Diversity and Breeding

Napier grass is considered to be a socio-economically important tropical grass species and is therefore available across most of the tropical and subtropical regions of the world. As reviewed in Sanghu et al. [5], a number of genebanks (for example: the International Center for Tropical Agriculture (CIAT); the Commonwealth Scientific and Industrial Research Organisation (CSIRO); the International Livestock Research Institute (ILRI); and the National Bureau of Plant Genetic Resources (NBPGR)) are involved in conserving a substantial amount of tropical and sub-tropical forage genetic resources. Through early exploration, Napier grass germplasm has been collected from various geographical regions and is conserved by these different institutions [5,29,30]. Consequently, over 300 accessions of Napier grass are currently being maintained in different genebanks (Table 1). Genetic resources form an essential component of agriculture and livestock production value chains where in-depth knowledge of the existing resources is required. Accurate passport, characterization and evaluation data, together with an overall understanding of the diversity of the genetic resources, are considered the primary reasons for the conservation and use of available genetic resources [5]. For example, a broad array of Napier grass accessions are currently being maintained by the ILRI forage genebank in the field at Debre Zeit and Zwai, Ethiopia with considerable diversity in growth and form (Figure 2). However, germplasm available from genebanks has so far been largely underutilized [5].

Napier grass is a cross-pollinating allotetraploid species with a chromosome number of 2n = 4x = 28 (genome A'A'BB) [11,31,32]. Although there is no clear information on the genetic origin of allotetraploidy in Napier grass, the A'A' genome has been reported to be homologous to the AA genome of pearl millet (*Pennisetum glaucum* (L.)) and the A' chromosomes are larger than the B chromosomes, which contribute genes controlling the perennial growth habit [31]. To date, Napier grass 'improvement' has mainly been based on the evaluation and selection of existing accessions for traits of interest. For example, accessions were screened for resistance to diseases, and Napier grass head smut- and stunt-resistant lines were identified from the existing collections [33,34]. Plant breeding and selection in Napier grass has primarily been aimed at improving different agronomic traits such as disease resistance, yield, nutritional quality, growth habit (dwarfing), palatability and abiotic stress tolerance [6,11,35]. Napier grass is cross-compatible with the closely related species pearl millet (*Pennisetum glaucum*) (2n = 2x = 14, genome AA) [6,15]; the resultant hybrids are triploid and sterile [6] and can only be propagated by vegetative means which, although labour intensive, ensure a true-to-type variety [15]. A number of agronomically important traits, nutritional quality and palatability for example, have been introgressed into the genome of Napier grass from pearl millet through conventional plant breeding [29] and hybrids have become a crucial part of the forage crop value chain in Africa, Asia and South America [6,36].

Table 1. Napier grass distribution and accessions in various genebanks around the world.

Native to *:	Number of Accessions at **:						
	ILRI	**ICRISAT**	**CIAT**	**EMBRAPA [1]**	**USDA GRIN [2]**	**RBG [3]**	**Total *****
Tanzania	6	9					15
Uganda					1		1
Ethiopia	1				12 [c]		13
Malawi	1				2	1	4
Mozambique	2						2
Zimbabwe	11	5			8		24
Côte d'Ivoire					1		1
Nigeria	1				1	3	5
Cameroon		8	1				9
Sub-total	22	22	1	0	25	4	74
Collected from							
Australia					4		4
Brazil	8			39	7		54
Burkina Faso						1	1
Burundi	1						1
Central African R.		7				2	9
China					1		1
Colombia	1			5			6
Costa Rica	1			1	1		3
Cuba	2			4			6
DRC (Zaire)					4		4
Ecuador						1	1
India	2	8		3	2		15
Mexico					2		2
Namibia	1						1
Panama				1			1
South Africa					12		12
Sudan		2					2
Swaziland	6				3		9
USA	16 [a]			1	6		23
Unknown	14 [b]		2	29	44		90
Sub-total	52	17	2	83	86	4	244
Total	74	39	3	83	111	8	318

[a] Breeding lines; [b] Includes 2 cultivars 'Mott' (=PI517947) and 'Kizizi'. ILRI14983 may = PI667853; [c] 11 duplicates of ILRI accessions, not actually from Ethiopia, and none of the 12 are available; * Native distribution taken from [13]; ** Number of accessions from the forage registry, except for Brazil, United States Department of Agriculture (USDA) Germplasm Resources Information Network (GRIN), ICRISAT and RBG (Genesys [37]); *** Some of the accessions listed here are in fact duplicated between the collections (for example 20 of the ILRI collection are part of the Brazilian collection); [1] Brazilian Agricultural Research Corporation (EMBRAPA, *Empresa Brasileira de Pesquisa Agropecuária*); [2] Most USDA accessions not available; [3] Millennium Seedbank, Royal Botanic Gardens (RBG), Wakehurst Place, UK. ILRI: International Livestock Research Institute; CIAT: International Center for Tropical Agriculture.

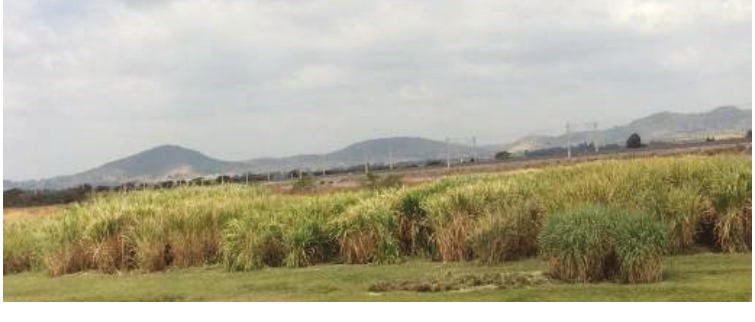


Figure 2. Partial view of the Napier grass field genebank in Debre Zeit (Ethiopia).

Since the early 1990s there have been a number of reports characterizing the genetic diversity of Napier grass. Tcacenco and Lance [38] evaluated the usefulness of morphological characteristics for the characterization of Napier grass and were able to differentiate nine accessions based on leaf, stem and inflorescence characteristics. Van De Wouw et al. [39] also studied a collection of Napier grass using morphological and agronomic characters where the collection was clustered into six groups. Smith and co-workers [40] were the first group to develop and use molecular restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNA (RAPD), markers in Napier grass studies and they were able to link quantitative trait loci to several plant traits. A diversity analysis, based on RAPD molecular markers, revealed a moderate level of diversity with clear differentiation of Napier grass accessions from pearl millet and its hybrids, and the accessions were clustered into groups according to their geographical origin [4,41]. However, the difficulty of differentiating some of the accessions based on their RAPD profile was also acknowledged [4]. Bhandari and co-workers [42] were able to differentiate 64 accessions of Napier grass based on polymorphisms in isozymes and total proteins and reported the availability of a wide range of genetic diversity. They suggested that the markers could be used to efficiently complement the morphological traits for diversity assessment and varietal identification of Napier grass accessions.

Harris et al. [11] were able to study the genetic relationship among 89 nursery accessions using amplified fragment length polymorphism (AFLP) markers and the results revealed a moderate to high degree of genetic relatedness among the accessions. In addition, clustering of the accessions into five groups in line with geographical origin was observed, which was a similar result to that observed using RAPD markers [11]. However, in another study using AFLP markers, Napier grass accessions of different geographical background obtained from research centres in Botswana, Mozambique, Ghana, South Africa, and Ethiopia (ILRI forage genebank collection) came together into different groups, with no clear evidence of clustering according to geographical origin [43]. Recent studies using AFLP markers [44] provided an indication that there was little to moderate within population diversity and a clustering of two groups in the Napier grass collection held at the ILRI forage genebank together with some additional accessions collected from Kenya, Tanzania and Uganda. Interestingly, these results also did not reveal the clustering of different accessions according to their geographical origin, which was demonstrated by the previous morphological, agronomic and RAPD marker studies. Other types of molecular markers, such as inter-sequential simple repeat (ISSR) markers [41,45] and sequence-related amplified polymorphisms (SRAPs) [46] have also been used for the characterization and identification of Napier grass clones.

Finally, a number of microsatellite, or simple sequence repeat (SSR), markers from pearl millet genetic studies have been demonstrated to be transferable to Napier grass [47–51]. The transferrable markers were successfully used in diversity analyses and clone identification of Napier grass accessions [12,52]. Expressed sequence tag (EST)-based SSR markers have been successfully used to identify pearl millet-Napier grass hybrids (the majority of hybrid Napier grass varieties under cultivation have been developed using pearl millet as maternal parents and Napier grass as paternal parents) [53]. Napier grass collections from Kenya and Uganda [54], the United States Department of Agriculture-Agricultural Research Service (USDA-ARS, Tifton nursery) [12] and the ILRI forage genebank (unpublished data) have also been characterized using SSR markers. Results from the SSR analyses demonstrated the availability of a broad array of genetic diversity in Napier grass germplasm while some duplicates were also identified in the collections. Kawube and his colleagues [54] also reported the allelic uniqueness of Napier grass from Uganda when compared with some of the accessions from the ILRI forage genebank. This array of outcomes highlights the need for the integration of modern molecular tools (for example, genotyping by sequencing) for the establishment and management of core collections in order to better capture the available genetic diversity.

2. Current Status

Due to the fact that most smallholder livestock producers predominantly own small and fragmented pieces of land, grasses such as Napier grass offer a best-fit alternative to other feed options, as these are high yielding forages which require a minimum amount of inputs and acreage. Napier grass possesses a number of attributes including: high biomass yield [55,56]; rapid re-growth potential and ease of propagation [57]; attributes that help with the control of soil erosion [58]; resistance to a broad spectrum of pests and diseases [59]; and suitability for cellulosic biofuel production [60]. The growth rate and biomass production of Napier grass surpasses other tropical grasses including Johnson grass (*Sorghum halepense*), switchgrass (*Panicum virgatum*), maize (*Zea mays*) and sugarcane (*Saccharum officinarum*) [61]. In addition, by following best management practices (regular cutting between 60 to 90 days and keeping soil moisture level at an optimum level) and applying fertilizer when required, harvesting of Napier grass can be maintained for decades [39].

The aforementioned qualities of Napier grass make it an attractive option for livestock production systems. However, the adoption and utilization of Napier as an alternative forage crop has not been totally successful due to the limited amount of research and attention given to this crop [62]. At present, only a handful of molecular characterization studies have been reported and its genome is yet to be sequenced. Therefore, in order to increase the utility of Napier grass and advance its breeding initiatives, genotyping by sequencing (GBS) of the Napier grass collection held at ILRI is currently underway. This characterization will be of great importance to assess the available diversity within ILRI collections. Furthermore, GBS characterization will also help develop sufficient SNP markers for marker-assisted breeding of Napier grass.

2.1. Yield and Morphology

Napier grass cultivars have been reported to yield around 60 tonnes dry matter/ha/year, with some studies indicating significantly higher yields [26,27]. The yield of Napier grass mainly depends on the type of cultivar used which in turn is influenced by both the environment and management practices employed. Nevertheless, there are two major categories of Napier grass cultivars based on their morphology, the normal or tall (up to 4–7 m) varieties (for example 'Australiano', 'Bana' and 'French Cameroon') and the dwarf or semi-dwarf (<2 m) varieties (for example 'Mott') [27]. The normal varieties have been reported to produce up to twice as much yield as the dwarf ones [27,63]. However, dwarf varieties also have a number of positive attributes, including enhanced overwintering capacity in the border areas between subtropics and temperate zones, better nutritive value, and ease of management and harvesting [64,65]. Therefore, different cultivars of Napier grass can be adopted by farmers depending on their situation and ultimate use of the crop.

The performance and yield of Napier grass is heavily influenced by agro-ecology, climatic conditions, management practices and other edaphic factors [27,66]. According to Kebede et al. [67], the most significant factor affecting DM production of Napier grass is the environment, followed by genotype by environment interactions and then the genotype. However, the genotype is still important and the DM yield of Napier grass has been demonstrated to be superior to other tropical forages including Guinea grass (*Megathysus maximus*) and Rhodes grass (*Chloris gayana*) [68]. Table 2 provides a summary of the dry matter yield, and other important forage quality attributes, obtained in different studies conducted on Napier grass.

Table 2. Yield and nutritional qualities of Napier grass accessions across different studies.

Country	DM (t/ha/year)	CP (%)	NDF (%)	ADF (%)	No. of Accessions Evaluated	Ref.
Bangladesh	14.9–16.5	10.3–11.4	NA	29.9–45.9	4	[69]
Brazil	14.9–78	NA	NA	NA	85	[26]
Ethiopia	4.6–20.5	7.5–15.7	52–64.6	28.8–36.6	9	[70]
Kenya	12.1–19	NA	NA	NA	8	[71]
Malaysia	43.7–65.9	10–12	60–70	35–40	9	[64]
Mexico	NA	9.2–9.9	65.2–69.7	42.2–44.7	3	[72]
Thailand	27.1–58.4	NA	NA	NA	8	[27]
USA	25.3–28.2	12.42–15.68	62.7–66.8	37.2–39.6	2	[57]
Zimbabwe	90.2	5.35	56.8	39.2	2	[73]

DM: dry matter production; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; NA: 'Not available' is given when that particular component was not measured in that study.

2.2. Nutritional Qualities

Significant variation in dry matter production (DM), crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and other nutritional qualities have been reported across different studies and accessions of Napier grass (Table 2). Nutritional quality is strongly influenced by management practices and age at harvest but, on average, Napier grass is considered to contain 9% CP, 20% DM, 70% NDF, 50% ADF, 9% ash and 6% lignin in samples taken from 10–15 week old plants [74,75]. Apart from genetics, the nutritional qualities of forages are influenced by many factors including the climate [76], soil nutrition [77], season and grazing pressure [78], management [65,79,80] and fertilizer application [81]. Consequently, great care should be taken to determine the optimum time when planning to harvest or graze Napier grass in order to maximize both yield and nutritional qualities [82].

An important aspect for most forages is that cutting treatments and interval can have a significant impact on both yield and nutritional qualities [80,83]. For example, the CP content of Napier grass has been demonstrated to decrease significantly from 28.2% at a 40-day cutting interval to 8.8% at an 80-day interval [56,84]. In addition, the CP content of Napier grass has been shown to be negatively impacted over recurrent cuttings, although the possibility exists to increase both DM and protein content through increased fertilizer application [85]. On the other hand, DM production has been shown to increase significantly over consecutive cuttings from the first to the third [69]. Although cultivar and environment specific, Wanghchuck et al. [56] recommended a 60-day cutting interval in the optimum growing season to maintain a high yield without compromising nutrient composition too much.

Forages, when harvested during the early stages of their development, are considered to possess relatively higher crude protein content [76,86]. On the other hand, plant structural components (NDF, ADF and lignin) increase during later harvests, resulting in decreased dry matter digestibility [76,86]. Lignin, an integral component of the plant secondary cell wall [87], is the primary factor limiting organic matter digestibility and nutrient availability in forages [88,89] by interfering (as a physical barrier) with microbial enzymatic activity [88,90]. Despite many desirable characteristics, Napier grass is generally considered to be of inferior nutritional quality depending on management (particularly in terms of metabolisable energy, digestion kinetics and percentage crude protein) and palatability when compared to other forage crops [16,64,84]. Napier grass is reported to possess around 50% NDF, which is higher than the recommended NDF content for forage grasses [64]. Feeding experiments using Friesian cows revealed a loss of body weight and reduced milk yield when solely fed Napier grass varieties [71,74]. Lactating cows that were producing 10 kg/day milk yield, when fed on Rhodes grass (*Chlolris gayana*), produced only 6–8 kg/day, which was accompanied by a loss of body weight, when fed solely on Napier grass [74]. Therefore, when using Napier grass as a main forage supplement, it is recommended that a portion of the ration needs to be substituted with high energy/protein feed to prevent reduced rumen microbial activity and depressed digestibility [74]. On the other hand, dwarf and semi-dwarf Napier grass varieties have been shown to contain a higher CP content and

lower ADF and hence are considered to be more suited as a forage for dairy farming applications [64]. In general, in areas where supplementary feed is scarce, intercropping Napier grass with legumes is considered to be a better option and such an approach could be the best alternative for smallholders who cannot supplement their milking cows with additional protein sources. In addition, using an increased amount of fertilizers can enhance organic matter content of Napier grass and subsequently enhance its nutritive quality as a feed [80].

2.3. Water Use Efficiency

A number of traits, including high dry matter production, ease of establishment and regeneration, persistence, and enhanced water use efficiency make Napier grass the primary forage of choice in the regions of Eastern and Central Africa where smallholder dairy farmers and pastoralists suffer from sporadic droughts and possess limited irrigation infrastructure [71]. Grasses which possess a C4 photosynthetic pathway are considered to have a competitive edge over C3 grass species when grown in tropical and subtropical regions [91]. Napier grass is a C4 grass species that also has the capacity to reduce shoot dry matter and maximize carbon assimilation during times of water stress, making it a desirable forage crop in areas subjected to intermittent droughts [92]. Napier grass undergoes changes in its morphology including leaf rolling, reduced stomatal conductance and enhanced water use efficiency when subjected to water stress conditions [93]. Since Napier grass is a perennial crop, it is expected to face rainfall fluctuations which would induce water stress at some point during the year and cultivars have been reported to lose as much as 20% of their yield potential when grown under water-deficient conditions in comparison to a control environment [93]. Hence, the development of cultivars that can withstand and produce during short periods of drought is considered to be useful for areas without irrigation, particularly as the effects of climate change are expected to impact on a growing number of regions.

Successful forage cultivation is influenced by the ability to minimize the trade-off between DM production and yield potential when grown under stress conditions such as drought. Biomass yield loss in Napier grass has been demonstrated to be less severe than in Guinea grass when grown under water stress conditions [93]. However, due to the fact that Napier grass has so far received little attention in terms of research investment, its advancement through breeding is considered to be minimal and lags behind other forages [62]. In addition, the lack of available genomic tools for Napier grass has hampered breeding initiatives. If an appropriate genomic toolbox is established and physiological responses to water stress are well understood, cultivars that can cope with intermittent drought should be able to be developed in the foreseeable future. The water use efficiency of a range of accessions from the ILRI forage genebank are currently being evaluated in irrigated and non-irrigated blocks, which will help further our understanding of their drought response mechanisms and provide the basis for the development of more drought tolerant Napier grass cultivars.

2.4. Pests and Diseases

Napier grass has been shown to be affected by many insects and other pests, bacteria, viruses, fungi and phytoplasmas, although most of them do not produce severe disease symptoms [3]. There have been numerous records of insect infestation on Napier grass. Farrell et al. [3] listed seventy-two different insects and mites that infect the species although for most of them, Napier grass acts as a reservoir in which the insect can survive between the growing seasons of other crops. To date, there has only been a single report of a bacterial infection (*Xanthomonas albilineans*), the causal agent of scald disease in sugarcane, in Napier grass and in this case the disease symptoms were unclear [94]. Potyviruses are considered to be the emerging problem for Napier grass but at the moment the two most significant threats to its productivity are the diseases smut and stunt which are caused by a fungus and a phytoplasma, respectively.

2.4.1. Viral Diseases

There have only been nine reported cases of viral infection of Napier grass worldwide. These viruses belong to the genera *Mastrevirus*, *Potyvirus* and *Sobemovirus* [3]. A geminivirus was the first virus reported to naturally infect Napier grass, described in Zimbabwe [94]. Subsequently, sugarcane mosaic virus [95], sugarcane chlorotic streak [96], maize mosaic (stripe disease) [97], die-back virus [98], maize streak geminivirus [99], elephant grass mosaic virus [100], a member of the potyviruses [101] and Johnson grass mosaic virus [3,95] have been reported. However, none of these were reported to produce serious disease symptoms or any significant productivity loss. The only report of symptoms was by Mih and Hanson [101], who reported that infection by one of the potyvirus isolates (Is16840), among three potyviruses isolated from the ILRI field genebank at Debre Zeit in Ethiopia in 1994–1996, produced severe mosaic and stunting symptoms in Napier grass that would cause productivity losses. Apart from this, an unclassified insect-borne virus was suggested to cause stunt disease in Napier grass in Uganda [102], that may also cause productivity loss.

2.4.2. Fungal Diseases

There have been as many as seventy-one different fungi reported to infect Napier grass [3]. Among them, only three diseases, namely eyespot, snow mould and head smut, have been addressed by researchers, mainly because the other fungi do not appear to have a significant effect on plant growth and productivity. The eyespot disease, caused by the fungus *Helminthosporium* spp., was first reported in the Caribbean in 1938 [96]. Although Burton [97] later reported that a Napier grass variety, 'Merkeron', was resistant to this disease (as the fungus did not cause a severe disease outbreak) there has been no significant effort to further any studies on this disease. The snow mould disease, also known as white mould disease, caused by the fungus *Beniowskia sphaeroidea*, was first reported to affect Napier grass in Kenya [98,99] and was later discovered in Malawi, Tanzania, Mauritius, Uganda and Zimbabwe [100,101]. The disease symptoms only appear during heavy rains and there is limited damage, restricted to during that season; it also does not appear to affect the vigour of the plants and livestock feeding on the diseased leaves do not appear to suffer any adverse effects. However, efforts have been made to introduce resistance to this disease and a resistant variety, 'Clone 13', was developed by conventional plant breeding in French Cameroon in the early 1970s [103].

The fungus responsible for head smut disease caused a severe disease outbreak with huge productivity losses of Napier grass in Kenya [18,95,102]. The causal agent of this disease is a fungus from the genus *Ustilago*, which was initially named as the species "*kamerunensis*" based on the place 'Cameroon' from where it was first isolated [104] and then later described as *Ustilago kamerunensis*" by Sydow and Sydow in 1911 [105]. The fungus appears to be slowly spreading from West Africa to the eastern parts of Africa as the disease was first reported in Cameroon [104], and subsequently in Uganda [106], Congo [107], Rwanda, Tanzania and then Kenya [108]. However, head smut occurs only in Africa as it has not been reported elsewhere outside the continent so far [95,109]. The mode of transmission of this disease is either by wind-borne spores or infected planting materials [110]. The spores of the smut-causing fungus are very light and have been reported to be able to spread by wind over large distances [111]. However, during a survey of the smut-infected districts of Kenya it was found that the disease spreads mainly by infected planting materials, as the farmers were completely unaware of this disease and the possible symptoms [110]. Although the smut disease of Napier grass has been recorded in many African countries, Kenya was the first country to be threatened by a potential epidemic. This could be because the strain identified in central Kenya causes the greatest yield losses when compared with the strains reported in other African countries [95,109,110]. The disease is widely spread across the central regions of Kenya and has been reported to cause 25–46% loss of biomass production [18,95,102]. The infected plants have thinner and shorter stems, a reduced numbers of leaves, and suffer from slower re-growth after cutting. The continual spread of the disease to other parts of the country, including the Rift valley and lower eastern region has also been recorded [1], which raises concerns about the possible future spread of this disease. Fungicide treatment is not currently an

option to control the disease, especially for vegetatively propagated cuttings. Following diagnosis of infected planting material, destroying these materials by burning is currently the only option to control this disease. Efforts to select and breed resistant accessions or varieties have led to the identification of two resistant varieties, namely 'Kakamega 1' and 'Kakamega 2' [95,112] and the Muguga South branch of the Kenya Agricultural and Livestock Research Organization (KALRO) is promoting and distributing planting materials of these varieties to farmers in the affected regions in order to minimize the effect of this disease in the country. Co-evolutionary modification of some African accessions, particularly from Southern Africa, has also resulted in the development of resistance to the disease over time [113,114] and further focus is currently being placed on developing resistant plant material to manage the disease in the future. Many Napier grass clones have been collected from various sites across the world and are currently under trial in an attempt to discover whether they exhibit any selection bias related to their geographic origin which may have developed due to a co-evolutionary cycle of selection [109,112]. However, this co-evolutionary process in the induction of resistance may also lead to the selection of more virulent strains of the pathogen *U. kamerunensis*, in case of widespread use of selected resistant accessions [114]. Therefore, it is considered advisable to adopt a strategy which promotes the planting of varieties of mixed origin and resistance levels that could slow down the likely natural selection of the pathogen into a more virulent strain [33].

2.4.3. Phytoplasma (Stunt) Disease

Napier grass stunt disease is by far the most devastating disease of Napier grass as the infected plant material shows severe stunting symptoms, resulting in eventual death of the plant [115–117]. The disease was first observed in western Kenya in 1997 and has been reported to spread quickly, causing serious economic losses [118]. It has been demonstrated that the disease is associated with the 16SrXI phytoplasma (*Candidatus* (*Ca*.) Phytoplasma oryzae) group [118,119]. Subsequently, the disease has been reported to occur in Ethiopia [120], Uganda [116] and Tanzania and Rwanda [2]. The phytoplasma responsible for the stunt disease in Uganda was discovered to be similar to the Kenyan strain; however, in Ethiopia the strain was found to be a member of the 16SrIII group, '*Ca*. Phytoplasma pruni' or X-disease [116,121], which caused symptoms similar to those observed for stunt disease in Uganda and Kenya but without the severe stunting [117]. The disease symptoms include yellowing of foliar material, smaller leaves, a proliferation of tillers, yellow to purple streaking and shortening of internodes to the extent that clumps appear severely stunted, resulting in a low biomass yield and eventual death of the plant, although this only occurs after cutting or grazing the grass [118]. However, the level of expression of the symptoms in phytoplasma-infected plants partly depends on the virulence of the strain, strain interference and phytoplasma concentration [122] and the abundance of insect vectors and phytoplasma-infested host plants [123]. The primary mode of transmission of the disease is by vegetative propagation of infected planting material or by phloem-feeding insects belonging to the families Cicadellidae (leafhoppers), Delphacidae (planthoppers) and some psyllids (Psylloidea) [124,125]. Obura et al. [126] identified *Maiestas banda* Kramer (Hemiptera:Cicadellidae) as a vector for Napier grass stunt disease phytoplasma in Kenya and *Leptodelphax dymas* and *Exitianus* spp. have been recorded in Ethiopia [127]. However, so far no vector has been identified in Uganda [126]. The vector–phytoplasma–host plant three-way interaction plays an important role in determining the spread of the disease [128]. There is the possible involvement of other phytoplasma susceptible food crops and grasses which could act as a reservoir, providing a source of inoculum for the spread of the disease [127] which would present a challenge to the development and implementation of management strategies for the disease. Two stunt-resistant varieties, 'Ouma 2' and 'South Africa', were selected by the International Centre of Insect Physiology and Ecology (icipe) in collaboration with KALRO and Rothamsted Research (UK) [129]. However, despite the efforts made to date to develop resistant varieties by national research organizations at various locations in western Kenya, many selected accessions have ultimately been found to be susceptible [130]. Consequently, the only guaranteed way to control the disease is through removal of the infected plants [131–133].

3. Future Prospects

Through years of effort, a number of cultivars have been selected and are currently in production in different regions of the world [6]. In addition, active breeding programs have been established to generate and capture greater diversity for both animal production and biofuel applications [134]. However, despite the efforts made so far, the production and use of Napier grass remains constrained by many factors. Nutritional quality, palatability, and propagation by seed or vegetative organs are currently the main limitations, and the diseases Napier grass stunt and head smut are significantly challenging its production in some regions of Africa. Moreover, enhancing the crop's water use efficiency is another key area of research which will allow for production and use in areas with annual rainfall below its optimal range (<750 mm), and maintenance of current areas under the threat of climate change. In this review, we have compiled an extensive amount of evaluation and characterisation data which has been derived from various collections over the past few decades and demonstrated that significant diversity exists in these traits of interest which have the potential to be captured [39,44,54]. Consequently, by integrating modern molecular approaches into improvement strategies, some of the constraints in Napier grass production and use could now be efficiently addressed [44].

Opportunities to help capture the genetic diversity in crops for plant breeding and crop improvement have recently been revolutionized by the integration of advances in molecular genetics and genomics, plant biotechnology and next-generation sequencing. These advances have already been widely applied to crop improvement and offer the opportunity for new approaches to enhance quality and performance traits of feeds and forages at a relatively low cost. However orphan crops, which include tropical forages in general and Napier grass in particular, have not yet substantially benefited from these advances in molecular genetics and the associated modern tools that are available. There remain few reports on characterizing the genetic diversity of Napier grass through the application of molecular markers, and genetic maps and genome sequence information is largely lacking. As a result, there is little molecular information on Napier grass, which has implications for the knowledge-based use and conservation of available genetic resources for sustainable development. This, for example, has limited the ability to locate genomic regions controlling traits of interest and gene discovery. Consequently, the potential to use Napier grass as a 'climate-smart' forage crop, with traits such as enhanced water use efficiency, disease resistance and temperature tolerance stacked in new varieties which perform well in the face of climate change, has not been fully realised. Breeding efforts are also limited in Napier grass [6], which could be due to poor quality and limited seed production. The current distribution of planting materials to farmers is considered bulky, expensive [6] and carries the potential risk of disease distribution (for example, stunt disease) to new areas. In other vegetatively propagated crops such as potato, cassava and sugarcane, the use of diseased planting materials has been demonstrated to be the main source of inoculum for disease-causing agents [135]. Therefore, improving the seed production ability (both in terms of quantity and quality) of Napier grass potentially conveys a multitude of benefits including using seeds for distribution to farmers, creating genetic variation and new hybrid varieties through crossing and reducing the risk of disease spread related to distribution of vegetative propagules.

With respect to advances in nutritional quality of Napier grass, a number of opportunities exist to leverage the knowledge and advances seen in other fodder crops to the improvement of Napier grass and the benefit of livestock productivity. The plant cell wall provides the major source of dietary fibre and the nutritional availability of forage fibre to livestock is highly dependent on its composition and structure [136]. The plant cell wall is a complex biological structure, mainly composed of cellulose, hemicellulose, protein and lignin, which varies greatly depending on developmental stage, tissue type and plant species [137]. The bioavailability of cellulose, the major structural polymer of plants and the most abundant organic polymer on Earth [138] as an energy source is restricted by the β-glucosidic linkages, making it insoluble in water in its native form [139] and the lignin complex [140]. Lignin affects the digestion of cell-wall polysaccharides by interfering (as a physical barrier) with microbial enzymatic activity [88,90] and therefore, developing low-lignin Napier grass lines could substantially

improve its digestibility and nutritional quality for enhanced livestock productivity. For example, it has been reported that a 1% increase in in vitro dry matter digestibility of forages leads to a 3.2% increase in daily weight gains of beef cattle [141]. Thus, the selection and/or development of low lignin varieties is another area of research where modern genomic tools could contribute substantially to improved feed quality in Napier grass. For example, the brown midrib mutants could offer an opportunity for selection in Napier grass. These mutants, which contain mutations in the lignin biosynthesis pathway and offer improved forage digestibility for livestock, have been selected in maize (*Zea mays*), sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum*) and a similar approach could be employed on Napier grass to improve its nutritional deficiency as a forage crop [142]. Transgenic approaches have also been used to enhance the nutritional quality of forages; for example, low-lignin alfalfa lines with enhanced digestibility have been developed [143] and similar technology can also be an option for Napier grass improvement. However, it is not only the reduction but the modification of lignin structure that can be important and the incorporation of *p*-coumaric acid instead of ferulic acid can improve cell wall digestibility in grasses by reducing cross linking [144].

In addition to enhancing nutritional quality, the improvement of other attributes of Napier grass, such as resistance to pests and diseases, also requires attention in order for this species to realise its full potential. Despite evidence demonstrating that insect vectors are responsible for transmitting diseases such as stunt and viruses, information on the impact of insects on feed yield and quality in Napier grass remains largely ambiguous. By employing modern molecular technologies and tapping into the genetic diversity available, we can develop a better understanding of the potential impacts and identify accessions which are tolerant to certain pests and diseases which could be used to introgress plant-derived resistance mechanisms into modern varieties. Two accessions, 'Kakamega 1' and 'Kakamega 2', have been identified and developed as varieties which provide resistance to the smut disease [95,112]. However, the mechanism of resistance is not fully understood and molecular approaches can play a role to augment and transfer the resistance genes in to commercial varieties. In addition, more severe strains of the pathogen may develop in the future through co-evolutionary mechanisms. Consequently, a more proactive effort is required, directed towards the discovery and development of new varieties with alternative resistance mechanisms to help address this threat in the future. The same applies for stunt disease, caused by the phytoplasmas, which results in severe productivity loss of Napier grass. To date there has been limited success achieved in the development of resistant varieties to combat this disease [133]. Therefore, the primary approach towards this disease would be screening the primary and secondary gene pool of Napier grass held in global collections that could lead to the identification of disease resistance genes with different modes of action against the phytoplasma.

The development of disease resistance in plants by introducing a gene, or a part thereof, from the pathogen is another approach which could be applied. For example, many viral diseases have been reported to infect Napier grass [3] which could have implications for both yield and quality, and the transgenic expression of viral coat proteins, replicases or other sequences from the virus genome could potentially be used to introduce resistance into the grass. Similarly, antifungal genes such as chitinases and glucanases could be introduced into the genome in order to confer resistance to fungal diseases such as the head smut disease reported to substantially affect household feed supply in Eastern and Central Africa. The use of antimicrobial genes to engineer the plant to produce antimicrobial proteins could also be considered to strengthen resistance mechanisms. Also, engineering with genes producing antibodies against a protein crucial for pathogenesis could result in a level of immunity or resistance to the pathogen. Alternatively, genetic modification or the recently developed technique of gene editing could be used in Napier grass to combat economically important insect pests. Accordingly, transgenic lines with resistance to different groups of insect pests can be generated using genes from various origins (Bt *cry* genes, the insect chitinase gene, RNA interference (RNAi) technology, plant-derived genes for proteinase inhibitors, and α-amylase inhibitors and lectins for example).

Good agricultural practices and management of diseases are currently the only option to protect against the spread of Napier grass diseases. The development and deployment of management practices to guard against any disease in a particular geographical area is guided by quantitative information on the existing levels of disease risk, definitive identification of the pathogen and a clear understanding of the factors that correlate strongly with disease/pathogen risk within a defined host population [145]. Although most of the viruses infecting Napier grass do not appear to cause any severe disease symptoms and productivity losses, there have been some reports on the effects of the potyvirus (Is 16840) identified in Ethiopia [146] and an insect-borne virus identified in Uganda [147] which need to be investigated more thoroughly, especially in terms of potential productivity loss, as they were reported to cause stunting in infected plants. There is limited information available on farmers' knowledge and understanding of Napier grass diseases, in particular, and forages in general, which need to be addressed through extension packages. Similarly, in order to manage head smut disease of Napier grass, there is a need to educate farmers on the identification of disease symptoms and implementation of management strategies. Although burning of infected material is a good option to destroy the source of infection, the development of visible symptoms can take time, which allows the disease to spread further. Therefore, efficient diagnostic tools (serological or molecular) could offer a valuable asset for the early detection and diagnosis of the disease and to monitor its spread for improved management and containment. A number of studies have been undertaken towards the identification of the pathogen, possible vectors and disease severity for an outbreak of Napier grass stunt disease in Kenya [118,126,148]. However, further studies will be required to elucidate factors involved in the plant–host–vector three-way interaction related to the spread of stunt disease in Ethiopia and Uganda. There is also a need for further research to confirm whether *Exitianus* sp., *L. dymas*, or both species, act as a vector(s) of stunt disease. Further, for the disease outbreak in Uganda, there is a lack of information regarding the possible vectors involved in the transmission of the disease. Molecular studies would provide more information about the identity of the causal agent, vectors involved in disease transmission and the factors supporting the spread of the disease, which may help in the development of an effective management tool to control/minimize its spread. Seasonal monitoring of the insect vector populations could also provide information on the spread of the disease, and should facilitate the prediction of future Napier grass stunt disease outbreaks.

In a similar manner to the approaches reported for other crops, improvements in tolerance to abiotic stresses such as drought, salinity, soil pH and extreme temperatures in Napier grass could be achieved by employing a range of modern molecular tools. Despite the successful selection of a few accessions with resistance to the diseases head smut and stunt, the introgression of stress resistance into advanced breeding lines is yet to be effectively tackled in Napier grass. Moreover, genomic regions controlling desirable characteristics such as the dwarf growth habit, smooth (hairless) leaf, water use efficiency, etc. remain to be elucidated in Napier grass. Genetically Napier grass has two different sets of genomes: A'A' and BB. The homologous nature of the A'A' genome with the AA genome of pearl millet and the contribution of the B genome to perennial growth habit offers many other opportunities for future genomic studies in Napier grass.

It is expected that research in Napier grass characterization, phenotyping, genotyping and breeding will be aided by the application of modern tools in the near future. This will facilitate clone identification, the establishment, management and exploitation of core collections, generation of sequence information, development of genetic maps and identification of high throughput marker systems such as SSRs and single-nucleotide polymorphisms (SNPs) for the localization of genomic region(s) and discovery of genes controlling traits of interest in Napier grass. Once linkages between traits of interest and known genetic markers are well established, marker-assisted selection/breeding could facilitate the selection of new clones and/or varieties with improved agronomic traits. In general, modern molecular genetics should be quickly integrated into the current conservation, use and improvement strategies to address nutritional quality and palatability concerns, and biotic and abiotic stresses in Napier grass.

Acknowledgments: We acknowledge Michael Bolton for his analysis of the distribution of Napier grass accessions in genebanks and the generation of Table 1. We also acknowledge Michael Bolton for critical review of the manuscript. The authors were supported by the CGIAR Research Programs for Livestock and Fish and for Managing and Sustaining Crop Collections during the writing of this paper. ILRI fully supports the publication of open access articles.

Author Contributions: All authors contributed to writing the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ADF	Acid Detergent Fibre
AFLP	Amplified Fragment Length Polymorphism
Bt	Bacillus thuringiensis
CIAT	International Center for Tropical Agriculture
CP	Crude Protein
CSIRO	Commonwealth Scientific and Industrial Research Organization
DM	dry Matter
DRC	Democratic Republic of Congo
EST	Expressed Sequence Tag
GBS	Genotyping by Sequencing
GRIN	Germplasm Resources Information Network
ha	hectare
icipe	International Centre of Insect Physiology and Ecology
ILRI	International Livestock Research Institute
ISSR	Inter-Sequential Simple Repeat
KALRO	Kenya Agricultural and Livestock Research Organization
L	Litre
NBPGR	National Bureau of Plant Genetic Resources, Delhi, India
NDF	Neutral Detergent Fibre
RNAi	RNA Interference
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RBG	Royal Botanic Gardens
SNPs	single-nucleotide polymorphisms
SRAPs	Sequence-Related Amplified Polymorphisms
SSR	Simple Sequence Repeat
USDA	United States Department of Agriculture

References

1. Lukuyu, B.A.; Gachuiri, C.K.; Lukuyu, M.N.; Lusweti, C.; Mwendia, S. *Feeding Dairy Cattle in East Africa*; East Africa Dairy Development Project: Nairobi, Kenya, 2012.
2. Kabirizi, J.; Muyekho, F.; Mulaa, M.; Msangi, R.; Pallangyo, B.; Kawube, G.; Zziwa, E.; Mugerwa, S.; Ajanga, S.; Lukwago, G.; et al. *Napier Grass Feed Resource: Production, Constraints and Implications For Smallholder Farmers in Eastern and Central Africa*; The Eastern African Agricultural Productivity Project: Naivasha, Kenya, 2015.
3. Farrell, G.; Simons, S.; Hillocks, R. Pests, diseases and weeds of Napier grass, *Pennisetum purpureum*: A review. *Int. J. Pest Manag.* **2002**, *48*, 39–48. [CrossRef]
4. Lowe, A.J.; Thorpe, W.; Teale, A.; Hanson, J. Characterisation of germplasm accessions of Napier grass (*Pennisetum purpureum* and *P. purpureum*× *P. glaucum* hybrids) and comparison with farm clones using RAPD. *Genet. Resour. Crop Evol.* **2003**, *50*, 121–132. [CrossRef]

5. Sandhu, J.S.; Kumar, D.; Yadav, V.K.; Singh, T.; Sah, R.P.; Radhakrishna, A. Recent trends in breeding of tropical grass and forage species. In Proceedings of the 23rd International Grassland Congress, New Delhi, India, 20–24 November 2015; Vijay, D., Srivastava, M.K., Gupta, C.K., Malaviya, D.R., Roy, M.M., Mahanta, S.K., Singh, J.B., Maity, A., Ghosh, P.K., Eds.; Range Management Society of India: Jhansi, India, 2015; pp. 337–348.
6. Singh, B.P.; Singh, H.P.; Obeng, E. Elephant grass. In *Biofuel Crops: Production, Physiology and Genetics*; Singh, B.P., Ed.; CAB International: Fort Valley State University, Fort Valley, GA, USA, 2013; pp. 271–291.
7. Brunken, J.N. A systematic study of *Pennisetum* sect. *Pennisetum* (*Gramineae*). *Am. J. Bot.* **1977**, *64*, 161–176. [CrossRef]
8. Donadío, S.; Giussani, L.M.; Kellogg, E.A.; Zuolaga, F.O.; Morrone, O. A preliminary molecular phylogeny of *Pennisetum* and *Cenchrus* (Poaceae-Paniceae) based on the trnL-F, rpl16 chloroplast markers. *Taxon* **2009**, *58*, 392–404.
9. Dos Reis, G.B.; Mesquita, A.T.; Torres, G.A.; Andrade-Vieira, L.F.; Vander Pereira, A.; Davide, L.C. Genomic homeology between *Pennisetum purpureum* and *Pennisetum glaucum* (Poaceae). *Comp. Cytogenet.* **2014**, *8*, 199. [PubMed]
10. Martel, E.; De Nay, D.; Siljak-Yakoviev, S.; Brown, S.; Sarr, A. Genome size variation and basic chromosome number in Pearl millet and fourteen related *Pennisetum* species. *J. Hered.* **1997**, *88*, 139–143. [CrossRef]
11. Harris, K.; Anderson, W.; Malik, R. Genetic relationships among Napier grass (*Pennisetum purpureum* Schum.) nursery accessions using AFLP markers. *Plant Genet. Resour.* **2010**, *8*, 63–70. [CrossRef]
12. Kandel, R.; Singh, H.P.; Singh, B.P.; Harris-Shultz, K.R.; Anderson, W.F. Assessment of genetic diversity in Napier Grass (*Pennisetum purpureum* Schum.) using microsatellite, single-nucleotide polymorphism and insertion-deletion markers from Pearl Millet (*Pennisetum glaucum* (L.) R. Br.). *Plant Mol. Biol. Rep.* **2016**, *34*, 265–272. [CrossRef]
13. Cook, B.G.; Pengelly, B.C.; Brown, S.D.; Donnelly, J.L.; Eagles, D.A.; Franco, M.A.; Hanson, J.; Mullen, B.F.; Partridge, I.J.; Peters, M.; et al. *Tropical Forages: An Interactive Selection Tool*; [CD-ROM], CSIRO, DPI&F(Qld), CIAT and ILRI: Brisbane, Australia, 2005.
14. Diz, D.A. Breeding Procedures and Seed Production Management in *Pearl millet* × *Elephant grass* Hexaploids Hybrids. Ph.D. Thesis, University of Florida, Gainesville, FL, USA, 1994.
15. Cheng, Y. Forage breeding in Taiwan-Review. *Asian Australas. J. Anim.Sci.* **1991**, *4*, 203–209. [CrossRef]
16. Heuzé, V.; Tran, G.; Giger-Reverdin, S.; Lebas, F. Elephant grass (*Pennisetum purpureum*). Available online: http://www.feedipedia.org/node/395 (accessed on 21 September 2016).
17. Ishii, Y.; Hamano, K.; Kang, D.J.; Kannika, R.; Idota, S.; Fukuyama, K.; Nishiwaki, A. C4-Napier grass cultivation for cadmium phytoremediation activity and organic livestock farming in Kyushu, Japan. *J. Agric. Sci. Technol.* **2013**, *3*, 321.
18. Orodho, A.B. *The Role and Importance of Napier Grass in the Smallholder Dairy Industry in Kenya*; Food and Agriculture Organization: Rome, Italy, 2006; p. 2011.
19. Pandit, N.P.; Shrestha, M.K.; Yi, Y.; Diana, J.S.; Rampur, C. Polyculture of grass carp and Nile tilapia with Napier grass as the sole nutrient input in the subtropical climate of Nepal. In Proceedings of the 6th International Symposium on Tilapia in Aquaculture, Manila, Philippines, 12–16 September 2004; pp. 12–16.
20. Shrestha, M.; Yadav, C. Feeding of Napier (*Pennisetum purpureum*) to grass carp in polyculture: A sustainable fish culture practice for small farmers. *Asian Fish. Sci.* **1998**, *11*, 287–294.
21. Shaha, D.C.; Kundu, S.R.; Hasan, M.N. Production of organic grass carp (*Ctenopharyngodon idella*) and GIFT tilapia (*Oreochromis niloticus*) using Napier grass, *Pennisetum purpureum*. *J. Fish.* **2015**, *3*, 233–238. [CrossRef]
22. Akah, N.; Onweluzo, J. Evaluation of water-soluble vitamins and optimum cooking time of fresh edible portions of Elephant Grass (*Pennisetum purpureum* L. Schumach) shoot. *Niger. Food J.* **2014**, *32*, 120–127. [CrossRef]
23. Khan, Z.; Chiliswa, P.; Ampong-Nyarko, K.; Smart, L.; Polaszek, A.; Wandera, J.; Mulaa, M. Utilisation of wild gramineous plants for management of cereal stemborers in Africa. *Int. J. Trop. Insect Sci.* **1997**, *17*, 143–150. [CrossRef]
24. Khan, Z.R.; Midega, C.A.; Hutter, N.J.; Wilkins, R.M.; Wadhams, L.J. Assessment of the potential of Napier grass (*Pennisetum purpureum*) varieties as trap plants for management of *Chilo partellus*. *Entomol. Exp. Appl.* **2006**, *119*, 15–22. [CrossRef]

25. Khan, Z.R.; Midega, C.A.; Wadhams, L.J.; Pickett, J.A.; Mumuni, A. Evaluation of Napier grass (*Pennisetum purpureum*) varieties for use as trap plants for the management of African stemborer (*Busseola fusca*) in a push–pull strategy. *Entomol. Exp. Appl.* **2007**, *124*, 201–211. [CrossRef]
26. Oliveira, M.L.F.; Daher, R.F.; Gravina, G.D.A.; da Silva, V.B.; Viana, A.P.; Rodrigues, E.V.; Shimoya, A.; Junior, A.T.D.A.; Menezes, B.R.D.S.; Rocha, A.D.S. Pre-breeding of Elephant grass for energy purposes and biomass analysis in Campos dos Goytacazes-RJ, Brazil. *Afr. J. Agric. Res.* **2014**, *9*, 2743–2758.
27. Rengsirikul, K.; Ishii, Y.; Kangvansaichol, K.; Sripichitt, P.; Punsuvon, V.; Vaithanomsat, P.; Nakamanee, G.; Tudsri, S. Biomass yield, chemical composition and potential ethanol yields of eight cultivars of Napier grass (*Pennisetum purpureum* Schumach.) harvested 3-monthly in central Thailand. *J. Sustain. Bioenergy Syst.* **2013**, *3*, 107. [CrossRef]
28. Lima, M.A.; Gomez, L.D.; Steele-King, C.G.; Simister, R.; Bernardinelli, O.D.; Carvalho, M.A.; Rezende, C.A.; Labate, C.A.; McQueen-Mason, S.J.; Polikarpov, I. Evaluating the composition and processing potential of novel sources of Brazilian biomass for sustainable biorenewables production. *Biotechnol. Biofuels* **2014**, *7*, 1. [CrossRef] [PubMed]
29. Hanna, W.W.; Monson, W.G. Yield, quality, and breeding of *Pearl millet* × *Napier grass* interspecific hybrids. *Agron. J.* **1980**, *72*, 358–360. [CrossRef]
30. Pitman, W.D.; Sotomayor-Rios, A. *Tropical Forage Plants: Development and Use*; CRC Press: Boca Raton, FL, USA, 2000.
31. Anderson, W.F.; Casler, M.D.; Baldwin, B.S. Improvement of perennial forage species as feedstock for bioenergy. In *Genetic Improvement of Bioenergy Crops*; Vermerris, W., Ed.; Springer Science + Business Media LLC: New York, NY, USA, 2008; pp. 347–376.
32. Paiva, E.A.; Bustamante, F.O.; Barbosa, S.; Pereira, A.V.; Davide, L.C. Meiotic behavior in early and recent duplicated hexaploid hybrids of Napier grass (*Pennisetum purpureum*) and Pearl millet (*Pennisetum glaucum*). *Caryologia* **2012**, *65*, 114–120. [CrossRef]
33. Omayio, D.O.; Ajanga, S.I.; Muoma, J.V.; Muyekho, F.N.; Yamame, M.K.; Kariuki, I.M.S. Using Napier grass accessions' origins, neighbour joining groups and their response to *Ustilago kamerunensis* to predict a probable co-evolutionary scenario. *Int. J. Recent Sci. Res.* **2015**, *6*, 2639–2645.
34. Kawube, G.; Alicai, T.; Otim, M.; Mukwaya, A.; Kabirizi, J.; Talwana, H. Resistance of Napier grass clones to Napier grass stunt Disease. *Afr. Crop Sci. J.* **2014**, *22*, 229–236.
35. Hanna, W.W.; Monson, W.G. Registration of dwarf Tift N75 Napier grass germplasm. *Crop Sci.* **1988**, *28*, 870–871. [CrossRef]
36. Premaratne, S.; Premalal, G.G.C. Hybrid Napier (*Pennisetum perpureum* × *Pennisetum americarnum*) var. CO-3: A resourceful fodder grass for dairy development in Sri Lanka. *J. Agric. Sci.* **2006**, *2*. [CrossRef]
37. Genesys. Available online: https://www.genesys-pgr.org/welcome (acessed on 30 November 2016).
38. Tcacenco, F.A.; Lance, G.N. Selection of morphological traits for characterisation of Elephant grass accessions. *Trop. Grassl.* **1992**, *26*, 145–155.
39. Van De Wouw, M.; Hanson, J.; Leuthi, S. Morphological and argonomic characterisation of a collection of Napier grass (*Pennisetum purpureum*) and *P. purpureum* × *P. glaucum*. *Trop. Grassl.* **1999**, *33*, 150–158.
40. Smith, R.L.; Schweder, M.; Chowdhury, M.; Seib, J.; Schank, S. Development and application of RFLP and RAPD DNA markers in genetic improvement of *Pennisetum* for biomass and forage production. *Biomass Bioenergy* **1993**, *5*, 51–62. [CrossRef]
41. Babu, C.; Sundaramoorthi, J.; Vijayakumar, G.; Ram, S.G. Analysis of genetic diversity in Napier grass (*Pennisetum purpureum* Schum) as detected by RAPD and ISSR markers. *J. Plant Biochem. Biotechnol.* **2009**, *18*, 181–187. [CrossRef]
42. Bhandari, A.P.; Sukanya, D.; Ramesh, C. Application of isozyme data in fingerprinting Napier grass (*Pennisetum purpureum* Schum.) for germplasm management. *Genet. Resour. Crop Evol.* **2006**, *53*, 253–264. [CrossRef]
43. Struwig, M.; Mienie, C.; Van Den Berg, J.; Mucina, L.; Buys, M. AFLPs are incompatible with RAPD and morphological data in *Pennisetum purpureum* (Napier grass). *Biochem. Syst. Ecol.* **2009**, *37*, 645–652. [CrossRef]
44. Wanjala, B.W.; Obonyo, M.; Wachira, F.N.; Muchugi, A.; Mulaa, M.; Harvey, J.; Skilton, R.A.; Proud, J.; Hanson, J. Genetic diversity in Napier grass (*Pennisetum purpureum*) cultivars: Implications for breeding and conservation. *AoB Plants* **2013**, *5*, plt022. [CrossRef] [PubMed]

45. De Lima, R.; Daher, R.; Goncalves, L.; Rossi, D.; do Amaral Júnior, A.; Pereira, M.; Lédo, F. RAPD and ISSR markers in the evaluation of genetic divergence among accessions of Elephant grass. *Genet. Mol. Res.* **2011**, *10*, 1304–1313. [CrossRef] [PubMed]
46. Xie, X.-M.; Zhou, F.; Zhang, X.-Q.; Zhang, J.-M. Genetic variability and relationship between MT-1 Elephant grass and closely related cultivars assessed by SRAP markers. *J. Genet.* **2009**, *88*, 281–290. [CrossRef] [PubMed]
47. Allouis, S.; Qi, X.; Lindup, S.; Gale, M.; Devos, K. Construction of a BAC library of pearl millet, *Pennisetum glaucum*. *Theor. Appl. Genet.* **2001**, *102*, 1200–1205. [CrossRef]
48. Budak, H.; Pedraza, F.; Cregan, P.; Baenziger, P.; Dweikat, I. Development and utilization of SSRs to estimate the degree of genetic relationships in a collection of pearl millet germplasm. *Crop Sci.* **2003**, *43*, 2284–2290. [CrossRef]
49. Mariac, C.; Luong, V.; Kapran, I.; Mamadou, A.; Sagnard, F.; Deu, M.; Chantereau, J.; Gerard, B.; Ndjeunga, J.; Bezançon, G. Diversity of wild and cultivated pearl millet accessions (*Pennisetum glaucum* (L.) R. Br.) in Niger assessed by microsatellite markers. *Theor. Appl. Genet.* **2006**, *114*, 49–58. [CrossRef] [PubMed]
50. Qi, X.; Lindup, S.; Pittaway, T.; Allouis, S.; Gale, M.; Devos, K. Development of simple sequence repeat markers from bacterial artificial chromosomes without subcloning. *Biotechniques* **2001**, *31*, 358–362.
51. Senthilvel, S.; Jayashree, B.; Mahalakshmi, V.; Kumar, P.S.; Nakka, S.; Nepolean, T.; Hash, C. Development and mapping of simple sequence repeat markers for pearl millet from data mining of expressed sequence tags. *BMC Plant Biol.* **2008**, *8*, 119. [CrossRef] [PubMed]
52. Sousa Azevedo, A.L.; Costa, P.P.; Machado, J.C.; Machado, M.A.; Pereira, A.V.; José da Silva Lédo, F. Cross species amplification of microsatellite markers in and genetic diversity of Napier grass accessions. *Crop Sci.* **2012**, *52*, 1776–1785. [CrossRef]
53. Dowling, C.D.; Burson, B.L.; Foster, J.L.; Tarpley, L.; Jessup, R.W. Confirmation of Pearl millet-Napier grass hybrids using EST-derived simple sequence repeat (SSR) markers. *Am. J. Plant Sci.* **2013**, *4*, 1004–1012. [CrossRef]
54. Kawube, G.; Alicai, T.; Wanjala, B.; Njahira, M.; Awalla, J.; Skilton, R. Genetic diversity in Napier Grass (*Pennisetum purpureum*) assessed by SSR Markers. *J. Agric. Sci.* **2015**, *7*, 147. [CrossRef]
55. Morais, R.F.D.; Souza, B.J.D.; Leite, J.M.; Soares, L.H.D.B.; Alves, B.J.R.; Boddey, R.M.; Urquiaga, S. Elephant grass genotypes for bioenergy production by direct biomass combustion. *Pesqui. Agropecu. Bras.* **2009**, *44*, 133–140. [CrossRef]
56. Wangchuk, K.; Rai, K.; Nirola, H.; Dendup, C.; Mongar, D. Forage growth, yield and quality responses of Napier hybrid grass cultivars to three cutting intervals in the Himalayan foothills. *Trop. Grassl. Forrajes Trop.* **2015**, *3*, 142–150. [CrossRef]
57. Lee, C.N.; Fukumoto, G.K.; Thorne, M.S.; Stevenson, M.H.; Nakahata, M.; Ogoshi, R.M. *Bana Grass (Pennisetum purpureum): A Possible Forage for Ruminants in Hawai'i*; University of Hawai'i: Honolulu, HI, USA, 2016.
58. Magcale-Macandog, D.; Predo, C.; Menz, K.; Predo, A. Napier grass strips and livestock: A bioeconomic analysis. *Agrofor. Syst.* **1998**, *40*, 41–58. [CrossRef]
59. Van den Berg, J.; Van Hamburg, H. Trap cropping with Napier grass, *Pennisetum purpureum* (Schumach), decreases damage by maize stem borers. *Int. J. Pest Manag.* **2015**, *61*, 73–79. [CrossRef]
60. Tsai, W.-T.; Tsai, Y.-L. Thermochemical characterization of Napier grass as an energy source and its environmental and economic benefit analysis. *Energy Sources Part B Econ. Plan. Policy* **2016**, *11*, 130–136.
61. Ra, K.; Shiotsu, F.; Abe, J.; Morita, S. Biomass yield and nitrogen use efficiency of cellulosic energy crops for ethanol production. *Biomass Bioenergy* **2012**, *37*, 330–334. [CrossRef]
62. Mwendia, S.; Yunusa, I.; Whalley, R.; Sindel, B.; Kenney, D.; Kariuki, I. Use of plant water relations to assess forage quality and growth for two cultivars of Napier grass (*Pennisetum purpureum*) subjected to different levels of soil water supply and temperature regimes. *Crop Pasture Sci.* **2014**, *64*, 1008–1019.
63. Williams, M.J.; Hanna, W.W. Performance and nutritive quality of dwarf and semi-dwarf Elephant grass genotypes in the south-eastern USA. *Trop. Grassl.* **1995**, *29*, 122–127.
64. Halim, R.A.; Shampazuraini, S.; Idris, A.B. Yield and nutritive quality of nine Napier grass varieties in Malaysia. *Malays. J. Anim. Sci.* **2013**, *16*, 37–44.

65. Mukhtar, M.; Ishii, Y.; Tudsri, S.; Idota, S.; Sonoda, T. Dry matter productivity and overwintering ability of the dwarf and normal Napier grasses as affected by the planting density and cutting frequency. *Plant Prod. Sci.* **2003**, *6*, 65–73. [CrossRef]
66. Utamy, R.F.; Ishii, Y.; Idota, S.; Harada, N.; Fukuyama, K. Adaptability of dwarf Napier grass under cut and carry and grazing sysytems for smallholder beef farmers in southern Kyhushu, Japan. *J. Warm Reg. Soc. Anim. Jpn.* **2011**, *54*, 87–98.
67. Kebede, G.; Feyissa, F.; Assefa, G.; Alemayehu, M.; Mengistu, A.; Kehaliew, A.; Melese, K.; Mengistu, S.; Tadesse, E.; Wolde, S. Chemical composition and in vitro organic matter digestibility of Napier Grass (*Pennisetum purpureum* (L.) Schumach) accessions in the mid and highland areas of Ethiopia. *Int. J. Livest. Res.* **2016**, *6*, 41–59. [CrossRef]
68. Relwani, L.L.; Nakat, R.V.; Kandale, D.Y. Intercropping of four leuceana cultivars with three grasses. *Leuceana Res. Rep.* **1982**, *3*, 41.
69. Amin, R.; Ali, N.R.S.M.Y.; Abul, H.M.; Khatun, M. Study on cutting intervals on biomass yield, nutritive value and their oxalate content of different high yielding Napier (*P. purpureum*) cultivars. *Asian Australas. J. Biosci. Biotechnol.* **2016**, *1*, 100–107.
70. Zewdu, T. Variation in growth, yield, chemical composition and in vitro dry matter digestibility of Napier grass accessions (*Pennisetum purpureum*). *Trop. Sci.* **2005**, *45*, 67–73. [CrossRef]
71. Nyambati, E.M.; Muyekho, F.N.; Onginjo, E.; Lusweti, C.M. Production, characterization and nutritional quality of Napier grass (*Pennisetum purpureum* (Schum.)) cultivars in Western Kenya. *Afr. J. Plant Sci.* **2010**, *4*, 496–502.
72. Ortega-Gómez, R.; Castillo-Gallegos, E.; Jarillo-Rodríguez, J.; Escobar-Hernández, R.; Ocaña-Zavaleta, E.; de la Mora, B.V. Nutritive quality of ten grasses during the rainy season in a hot-humid climate and ultisol soil. *Trop. Subtrop. Agroecosyst.* **2011**, *13*, 481–491.
73. Tavirimirwa, B.; Manzungu, E.; Ncube, S. The evaluation of dry season nutritive value of dominant and improved grasses in fallows in Chivi district, Zimbabwe. *Online J. Anim. Feed Res.* **2012**, *2*, 470–474.
74. Gwayumba, W.; Christensen, D.; McKinnon, J.; Yu, P. Dry matter intake, digestibility and milk yield by Friesian cows fed two Napier grass varieties. *Asian Australas. J. Anim. Sci.* **2002**, *15*, 516–521. [CrossRef]
75. Islam, M.; Saha, C.; Sarker, N.; Jalil, M.; Hasanuzzaman, M. Effect of variety on proportion of botanical fractions and nutritive value of different Napier grass (*Pennisetum purpureum*) and relationship between botanical fractions and nutritive Value. *Asian Australas. J. Anim. Sci.* **2003**, *16*, 837–842. [CrossRef]
76. Keba, H.T.; Madakadze, I.C.; Angassa, A.; Hassen, A. Nutritive value of grasses in semi-arid rangelands of Ethiopia: Local experience based herbage preference evaluation versus laboratory analysis. *Asian Australas. J. Anim. Sci.* **2013**, *26*, 366. [CrossRef] [PubMed]
77. Tessema, Z.K.; De Boer, W.F.; Baars, R.M.T.; Prins, H.H.T. Changes in soil nutrients, vegetation structure and herbaceous biomass in response to grazing in a semi-arid savanna of Ethiopia. *J. Arid Environ.* **2011**, *75*, 662–670. [CrossRef]
78. Henkin, Z.; Ungar, E.D.; Dvash, L.; Perevolotsky, A.; Yehuda, Y.; Sternberg, M.; Voet, H.; Landau, S.Y. Effects of cattle grazing on herbage quality in a herbaceous Mediterranean rangeland. *Grass Forage Sci.* **2011**, *66*, 516–525. [CrossRef]
79. Van der Westhuizen, H.; Snyman, H.; Fouché, H. A degradation gradient for the assessment of rangeland condition of a semi-arid sourveld in southern Africa. *Afr. J. Range Forage Sci.* **2005**, *22*, 47–58. [CrossRef]
80. Okwori, A.I.; Magani, I.E. Influence of nitrogen sources and cutting interval on the digestibility of four grass species in the southern guinea savanna of Nigeria. *Agric. Biol. J. N. Am.* **2010**, *1*, 526–533.
81. Hasyim, H.; Ishii, Y.; Ahmad, W.; Sachiko, I. Quality herbage production of dwarf Napier grass with Italian Ryegrass cropping under digested effluent application in Southern Kyushu, Japan. *Am. J. Agric. Biol. Sci.* **2015**, *11*, 35–44. [CrossRef]
82. Na, C.I.; Fedenko, J.R.; Sollenberger, L.E.; Erickson, J.E. Harvest management affects biomass composition responses of C4 perennial bioenergy grasses in the humid subtropical USA. *GCB Bioenergy* **2016**. [CrossRef]
83. Butt, N.M.; Donart, G.B.; Southwara, M.G.; Pieper, R.D. Effect of defoliation on plant growth of Napier grass. *Trop. Sci. Lond.* **1993**, *33*, 111.
84. Bayble, T.; Melaku, S.; Prasad, N. Effects of cutting dates on nutritive value of Napier (*Pennisetum purpureum*) grass planted sole and in association with Desmodium (*Desmodium intortum*) or Lablab (*Lablab purpureus*). *Livest. Res. Rural Dev.* **2007**, *19*, 120–136.

85. Carvalho, C.A.B.D.; Menezes, J.B.D.O.X.D.; Cóser, A.C. Effect of fertilizer and cutting frequency on yield and nutritive value of Elephant grass. *Cienc. Agrotecnol.* **2000**, *24*, 233–241.
86. Mirza, S.N.; Muhammad, N.; Qamar, I.A. Effect of growth stages on the yield and quality of forage grasses. *Pak. J. Agric. Res.* **2002**, *17*, 145–147.
87. Boudet, A.M. Towards an understanding of the supramolecular organization of the lignified wall. *Plant Cell Wall* **2003**, *8*, 155–182.
88. Jung, H.-J.G. Forage digestibility: The intersection of cell wall lignification and plant tissue anatomy. In Proceedings of the 23rd Annual Florida Ruminant Nutrition Symposium, Gainesville, FL, USA, 31 January–1 February 2012; University of Florida: Gainesville, FL, USA, 2012; pp. 162–174.
89. Moore, K.J.; Jung, H.-J.G. Lignin and fiber digestion. *J. Range Manag.* **2001**, *54*, 420–430. [CrossRef]
90. Agbor, V.B.; Cicek, N.; Sparling, R.; Berlin, A.; Levin, D.B. Biomass pretreatment: Fundamentals toward application. *Biotechnol. Adv.* **2011**, *29*, 675–685. [CrossRef] [PubMed]
91. Taylor, S.; Ripley, B.; Woodward, F.; Osborne, C. Drought limitation of photosynthesis differs between C3 and C4 grass species in a comparative experiment. *Plant Cell Environ.* **2011**, *34*, 65–75. [CrossRef] [PubMed]
92. Cardoso, J.A.; Pineda, M.; de la Cruz Jiménez, J.; Vergara, M.F.; Rao, I.M. Contrasting strategies to cope with drought conditions by two tropical forage C4 grasses. *AoB Plants* **2015**, *7*, plv107. [CrossRef] [PubMed]
93. Purbajanti, E.; Anwar, S.; Wydiati, F.K. Drought stress effect on morphology characters, water use efficiency, growth and yield of guinea and napier grasses. *Int. Res. J. Plant Sci.* **2012**, *3*, 47.
94. Rott, P.; Chatenet, M.; Granier, M.; Baudin, P. L'échaudure des feuilles de canne à sucre provoquée par *Xanthomonas albilineans* (Ashby) Dowson. II: Diagnostic et spectres d'hôtes de l'agent pathogène en Afrique tropicale. *L'Agron. Trop.* **1988**, *43*, 244–251.
95. Farrell, G. Towards the Management of *Ustilago kamerunensis* H Sydow and Sydow, a Smut Pathogen of Napier Grass (*Pennisetum purpureum* Schum.) in Kenya. Ph.D. Thesis, University of Greenwich, London, UK, 1998.
96. Paterson, D.D. Further experiments with cultivated tropical fodder crops. *Emp. J. Exp. Agric.* **1938**, *6*, 323–340.
97. Burton, G.W. Registration of 'Merkeron' Napier grass. *Crop Sci.* **1989**, *29*, 1327. [CrossRef]
98. Maher, C. Elephant grass (*Pennisetum purpureum*) as a cattle fodder in Kenya. *East Afr. Agric. J.* **1936**, *1*, 340–342. [CrossRef]
99. Nattrass, R.M. Notes on plant diseases. *East Afr. Agric. J.* **1941**, *7*, 56.
100. Lenné, J.M. *A World List of Fungal Diseases of Tropical Pasture Species*; CIAT: Wallingford, UK, 1990.
101. Mtisi, E.; de Milliano, W. False mildew on Pearl millet and other hosts in Zimbabwe. *East Afr. Agric. For. J.* **1993**, *59*, 145–153.
102. Farrell, G.; Simons, S.; Hillocks, R. A novel technique for measuring biomass loss in a diseased tussock grass. *Trop. Grassl.* **2000**, *34*, 118–124.
103. Boonman, G. *East Africa's Grasses and Fodders: Their Ecology and Husbandry*; Springer Science + Business Media, B.V.: Dordrecht, The Netherlands, 1993.
104. Ledermann, C. Herbarium Record 162103, Systematic Botany and Mycology Laboratory, USDA, Maryland, USA. 1998. Available online: http://nt.ars-grin.gov/fungaldatabases/fungushost (accessed on 15 September 2016).
105. Sydow, H.; Sydow, P. Fungi Africani novi. Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie/begr. *Von A. Engler* **1911**, *45*, 259–265.
106. Snowden, J.D. Herbarium Record 162104, Systematic Botany and Mycology Laboratory, USDA, Maryland, USA. 1998. Available online: http://nt.ars-grin.gov/fungaldatabases/fungushost (accessed on 15 September 2016).
107. Watson, A.J. *Foreign Bacterial and Fungus Diseases of Food, Forage, and Fiber Crops: An Annotated List*; Agricutural Research service, United States Department of Agrculture: Washington, DC, USA, 1971.
108. Kung'u, J.N.; Waller, J.M. Occurrence of smut of Napier grass caused by *Ustilago kamerunensis* H Sydow and Sydow in Kenya. *Int. J. Pest Manag.* **2001**. [CrossRef]
109. Association of Strengthening Agricultural Research in East and Central Africa (ASARECA). *Workshop on Mitigating the Impact of Napier Grass Smut and Stunt Diseases for the Smallholder Dairy Sector-Sharing Results: Final Report, June 1–3, 2010*; ILRI: Addis Ababa Ethiopia, Ethiopia, 2010.
110. Farrell, G.; Simons, S.; Hillocks, R. *Ustilago kamerunensis* on Napier grass in Kenya. *Int. J. Pest Manag.* **2002**, *48*, 25–28. [CrossRef]

111. Simmonds, N.W. Some speculative calculations on the dispersal of sugarcane smut disease. *Sugar Cane* **1994**, *1*, 2–5.
112. Mwendia, S.; Wanyoike, M.; Wahome, R.; Mwangi, D. Effect of napier head smut disease on Napier yields and the disease coping strategies in farming systems in central Kenya. *Livest. Res. Rural Dev.* **2007**, *19*. Available online: http://www.lrrd.org/lrrd19/8/mwen19109.htm (accessed on 11 November 2016).
113. Friedman, A.R.; Baker, B.J. The evolution of resistance genes in multi-protein plant resistance systems. *Curr. Opin. Genet. Dev.* **2007**, *17*, 493–499. [CrossRef] [PubMed]
114. Rausher, M.D. Co-evolution and plant resistance to natural enemies. *Nature* **2001**, *411*, 857–864. [CrossRef] [PubMed]
115. Alicai, T.; Kabirizi, J.; Byenkya, S.; Kayiwa, S.; Ebong, C. *Assessment of the Magnitude and Farmers' Management Practices of the Elephant Grass Stunting Disorder in Masaka District*; Namulonge Agricultural and Animal Production Research Institute: Kampala, Uganda, 2004.
116. Nielsen, S.L.; Ebong, C.; Kabirizi, J.; Nicolaisen, M. First report of a 16SrXI group phytoplasma (*Candidatus phytoplasma* oryzae) associated with Napier grass disease in Uganda. *Plant Pathol.* **2007**, *56*, 1039. [CrossRef]
117. Rosete, Y.A.; Jones, P. Phytoplasma diseases of the Gramineae. In *Phytoplasmas: Genomes, Plant Hosts and Vectors*; Weintraub, P.G., Jones, P., Eds.; CAB International: Wallingford, UK, 2010; pp. 170–187.
118. Jones, P.; Devonshire, B.; Holman, T.; Ajanga, S. Napier grass stunt: A new disease associated with a 16SrXI group phytoplasma in Kenya. *Plant Pathol.* **2004**, *53*, 519. [CrossRef]
119. Jones, P.; Arocha, T.; Zerfy, J.; Proud, J.; Abebe, G.; Hanson, J. A stunting syndrome of Napier grass in Ethiopia is associated with a 16SrIII Group phytoplasma. *New Dis. Rep.* **2006**, *10*, 2006–2019. [CrossRef]
120. Jones, P.; Arocha, Y.; Zerfy, T.; Proud, J.; Abebe, G.; Hanson, J. A stunting syndrome of Napier grass in Ethiopia is associated with a 16SrIII group phytoplasma. *Plant Pathol.* **2007**, *56*, 345. [CrossRef]
121. Asudi, G.O.; Van den Berg, J.; Midega, C.A.; Schneider, B.; Seemüller, E.; Pickett, J.A.; Khan, Z.R. Detection, identification, and significance of Phytoplasmas in Wild Grasses in East Africa. *Plant Dis.* **2016**, *100*, 108–115. [CrossRef]
122. Marcone, C. *Movement of Phytoplasmas and the Development of Diseases in the Plant*; CAB International: Wallingford, UK, 2010.
123. Sharon, R.; Soroker, V.; Wesley, S.D.; Zahavi, T.; Harari, A.; Weintraub, P.G. Vitex agnus-castus is a preferred host plant for *Hyalesthes obsoletus*. *J. Chem. Ecol.* **2005**, *31*, 1051–1063. [CrossRef] [PubMed]
124. Lee, I.-M.; Davis, R.E.; Gundersen-Rindal, D.E. Phytoplasma: Phytopathogenic Mollicutes. *Annu. Rev. Microbiol.* **2000**, *54*, 221–255. [CrossRef] [PubMed]
125. Weintraub, P.G.; Beanland, L. Insect vectors of phytoplasmas. *Annu. Rev. Entomol.* **2006**, *51*, 91–111. [CrossRef] [PubMed]
126. Obura, E.; Midega, C.A.; Masiga, D.; Pickett, J.A.; Hassan, M.; Koji, S.; Khan, Z.R. *Recilia banda* Kramer (Hemiptera: Cicadellidae), a vector of Napier stunt phytoplasma in Kenya. *Naturwissenschaften* **2009**, *96*, 1169–1176. [CrossRef] [PubMed]
127. Arocha, Y.; Zerfy, T.; Abebe, G.; Proud, J.; Hanson, J.; Wilson, M.; Jones, P.; Lucas, J. Identification of potential vectors and alternative plant hosts for the phytoplasma associated with Napier grass stunt disease in Ethiopia. *J. Phytopathol.* **2009**, *157*, 126–132. [CrossRef]
128. Lee, I.-M.; Martini, M.; Bottner, K.; Dane, R.; Black, M.; Troxclair, N. Ecological implications from a molecular analysis of phytoplasmas involved in an aster yellows epidemic in various crops in Texas. *Phytopathology* **2003**, *93*, 1368–1377. [CrossRef] [PubMed]
129. International Centre of Insect Physiology and Ecology (ICIPE). *Solving Napier Stunt Disease to Save the Smallholder Dairy Sector in East Africa—A Success Story*; ICIPE: Nairobi, Kenya, 2014; Available online: http://www.push-pull.net/napier_stunt_brochure.pdf (accessed on 25 July 2016).
130. Mulaa, M.; Awalla, B.; Hanson, J.; Proud, J.; Cherunya, A.; Wanyama, J.; Lusweti, C.; Muyekho, F. Stunting disease incidence and impact on Napier grass (*Pennisetum purpureum* Schumach) in western Kenya. In *12th Biennial Kenya Agricultural Research Institute (KARI) Conference: Transforming Agriculture for Improved Livelihoods through Agricultural Product Value Chains*; Wasilwa, L.A., Ed.; Kenya Agricultural Research Institute: Nairobi, Kenya, 2010; pp. 936–943.
131. Asudi, G.O.; van den Berg, J.; Midega, C.A.; Pittchar, J.; Pickett, J.A.; Khan, Z.R. Napier grass stunt disease in East Africa: Farmers' perspectives on disease management. *Crop Prot.* **2015**, *71*, 116–124. [CrossRef]

132. Kabirizi, J.; Nielsen, S.; Nicolaisen, M.; Byenkya, S.; Alacai, T. Napier stunt disease in Uganda: Farmers' perceptions and impact on fodder production. In Proceedings of the 8th African Crop Science Society Conference Proceedings, El-Minia, Egypt, 27–31 Octber 2007; Ahmed, K.Z., Ed.; Volume 8, pp. 895–897.
133. Khan, Z.R.; Midega, C.A.O.; Nyang'au, I.M.; Murage, A.; Pittchar, J.; Agutu, L.O.; Amudavi, D.M.; Pickett, J.A. Farmers' knowledge and perceptions of the stunting disease of Napier grass in Western Kenya. *Plant Pathol.* **2014**, *63*, 1426–1435. [CrossRef]
134. Faleiro, F.G.; Kannan, B.; Altpeter, F. Regeneration of fertile, hexaploid, interspecific hybrids of Elephant grass and pearl millet following treatment of embryogenic calli with antimitotic agents. *Plant Cell Tissue Organ Cult. (PCTOC)* **2016**, *124*, 57–67. [CrossRef]
135. Sastry, K.S.; Zitter, T.A. Management of virus and viroid diseases of crops in the tropics. In *Plant Virus and Viroid Diseases in the Tropics, volume 2: Epidemiology and management*; Springer Netherlands: Dordrecht, The Netherlands, 2014; pp. 149–480.
136. Buxton, D.R.; Redfearn, D.D. Plant limitations to fiber digestion and utilization. *J. Nutr.* **1997**, *127*, 814S–818S. [PubMed]
137. Jung, H.; Allen, M. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. *J. Anima. Sci.* **1995**, *73*, 2774–2790. [CrossRef]
138. Berg, J.M.; Tymoczko, J.L.; Stryer, L. *Biochemistry*, 5th ed.; W H Freeman: New York, NY, USA, 2002.
139. Bhatt, N.; Gupta, P.; Naithani, S. Preparation of cellulose sulfate from α-cellulose isolated from Lantana camara by the direct esterification method. *J. Appl. Polym. Sci.* **2008**, *108*, 2895–2901. [CrossRef]
140. Klemm, D.; Heublein, B.; Fink, H.P.; Bohn, A. Cellulose: Fascinating biopolymer and sustainable raw material. *Angew. Chem. Int. Ed.* **2005**, *44*, 3358–3393. [CrossRef] [PubMed]
141. Casler, M.D.; Vogel, K.P. Accomplishments and impact from breeding for increased forage nutritional value. *Crop Sci.* **1999**, *39*, 12–20. [CrossRef]
142. Sattler, S.E.; Funnell-Harris, D.L.; Pedersen, J.F. Brown midrib mutations and their importance to the utilization of Maize, Sorghum, and Pearl millet lignocellulosic tissues. *Plant Sci.* **2010**, *178*, 229–238. [CrossRef]
143. Guo, D.; Chen, F.; Wheeler, J.; Winder, J.; Selman, S.; Peterson, M.; Dixon, R.A. Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin O-methyltransferases. *Transgenic Res.* **2001**, *10*, 457–464. [CrossRef] [PubMed]
144. Hatfield, R.D.; Rancour, D.M.; Marita, J.M. Grass Cell Walls: A story of cross-Linking. *Front. Plant Sci.* **2017**, *7*, 2056. [CrossRef] [PubMed]
145. Thébaud, G.; Yvon, M.; Alary, R.; Sauvion, N.; Labonne, G. Efficient transmission of '*Candidatus phytoplasma* Prunorum' is delayed by eight months due to a long latency in its host-alternating vector. *Phytopathology* **2009**, *99*, 265–273. [CrossRef] [PubMed]
146. Mih, A.M.; Hanson, J. Identification of potyviruses infecting forage grasses in Ethiopia. *J. Cameroon Acad. Sci.* **2004**, *4*, 205–210.
147. Tiley, G.E.D. *Elephant Grass. Kawanda Technical Communication No.23*; Kawanda Research Station: Kawanda, Unganda, 1969.
148. Mudavadi, P.O.; Otieno, K.; Wanambacha, J.W.; Odenya, J.O.; Odendo, M.; Njaro, O.K. *Smallholder Dairy Production and Marketing in Western Kenya: A Review of Literature*; ILRI: Nairobi, Kenya, 2001.

agronomy

MDPI

Review

Optimal Regulation of the Balance between Productivity and Overwintering of Perennial Grasses in a Warmer Climate

Åshild Ergon

Department of Plant Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway; ashild.ergon@nmbu.no

Academic Editors: John W. Forster and Kevin F. Smith
Received: 2 January 2017; Accepted: 21 February 2017; Published: 23 February 2017

Abstract: Seasonal growth patterns of perennial plants are linked to patterns of acclimation and de-acclimation to seasonal stresses. The timing of cold acclimation (development of freezing resistance) and leaf growth cessation in autumn, and the timing of de-acclimation and leaf regrowth in spring, is regulated by seasonal cues in the environment, mainly temperature and light factors. Warming will lead to new combinations of these cues in autumn and spring. Extended thermal growing seasons offer a possibility for obtaining increased yields of perennial grasses at high latitudes. Increased productivity in the autumn may not be possible in all high latitude regions due to the need for light during cold acclimation and the need for accumulating a carbohydrate storage prior to winter. There is more potential for increased yields in spring due to the availability of light, but higher probability of freezing events in earlier springs would necessitate a delay of de-acclimation, or an ability to rapidly re-acclimate. In order to optimize the balance between productivity and overwintering in the future, the regulation of growth and acclimation processes may have to be modified. Here, the current knowledge on the coordinated regulation of growth and freezing resistance in perennial grasses is reviewed.

Keywords: CBF; climate change; cold acclimation; de-acclimation; freezing; growth; light; photoperiod; seasonality; stress; temperature; winter survival

1. Introduction

Changes in atmospheric CO_2 concentration, temperature, and precipitation patterns are expected to affect plant productivity in a complex manner due to a set of mechanisms and interactions at different scales from leaf to agro-ecosystem [1]. In regions or periods where water availability is sufficient, elevated atmospheric CO_2 concentrations and higher temperatures can potentially increase growth rates of many plant species, including C_3 grasses and forage legumes, and thus increase grassland productivity [2]. In addition to this direct effect of temperature and CO_2 concentration, cool regions with non-productive winters are likely to have longer thermal growing seasons (often defined as the part of the year when the daily mean temperature exceeds 5 °C), with earlier springs and later autumns. For example, in Finland, where the annual mean temperature has most likely increased by at least 2 °C during the last 150 years [3], the thermal growing season was predicted to become one to three months longer by the end of the century as compared to the period 1971–2000 [4]. Such extended growing seasons are expected to contribute to the increase in annual grassland yields in temperate climates [5–9]. Although the prediction models used so far account for drought limitations on growth, they do not account for possible effects of plant survival during seasonal stresses. However, a recently developed model, which incorporates both the cold acclimation process in autumn and winter survival, will improve predictions for high latitudes in this respect [10].

Perennial grasses have the potential to utilize the light energy over a larger part of the year than most annual crops, but should survive and produce biomass for several years. The annual recurrent periods of winter stresses or summer droughts in some regions have led to the evolution of seasonal acclimation and de-acclimation processes regulating the level of resistance to seasonal stresses [11–13]. These processes, which are largely regulated by temperature and photoperiod, correlate with changes in growth, development, and dormancy status [13–15], and latitudinal clines in growth responses to temperature and photoperiod have been described [16]. Acclimation and de-acclimation are associated with cessation and resumption of leaf growth, respectively, suggesting a classical growth–stress survival trade-off [17] in the adaptation to seasonal stresses [15]. It is important to note, though, that cessation of leaf growth does not always mean cessation of biomass accumulation, but rather a shift in allocation of photosynthates from leaf blades to newly formed tillers, roots, and storage tissues [18]. Although some perennial grasses have been shown to possess summer endodormancy of shoot meristems [12,14], the existence of winter endodormancy has not been demonstrated to my knowledge, although perennial grasses are obviously ecodormant during cold winter periods. Instead, the shoot meristems that are formed during the latter part of summer and/or during autumn (variations between species) are more or less unresponsive to long day-induction of reproductive development. They gradually become responsive during weeks of low temperature (vernalization), a process which occurs faster in short than in long photoperiods [19].

Winter survival of perennial grasses can be measured directly at the individual plant level, but is frequently measured at sward level as the relative recovery in spring, a measure which also encompasses rate of tiller survival, and earliness and rate of regrowth. Winter survival is an extremely complex trait being the result of both an acclimation process and responses to numerous types of stresses that plants encounter and must endure during winter and early spring [20]. The term "cold acclimation" refers to the development of resistance to freezing stress. Temperature is a major environmental factor controlling cold acclimation and cold de-acclimation in perennial grasses, although light factors are also of importance. Low temperature induces not only freezing resistance, but also resistance to other winter stresses, such as ice encasement/anoxia [21], and fungal pathogens (snow molds) [22,23], which, depending on climatic conditions, may have a much stronger influence on winter survival than freezing [20].

Temperatures are increasing in most regions of the world [24,25], but the annual variation in photoperiod will remain the same. We will therefore have new seasonal combinations of temperature and photoperiod in the future, particularly at high latitudes, where photoperiod changes dramatically during the course of a year. This may lead to mismatches between annual cycles of growth, development, and stress resistance. In order to utilize the new seasonal patterns to maximize the production of biomass from perennial grasses, while maintaining sufficient survival through stressful parts of the year, we need species and varieties with temperature and photoperiod responses conferring an annual growth pattern that optimizes the balance between growth and survival. This review aims to describe the physiological and genetic factors that determine the balance between the productivity and overwintering of perennial grasses in the longer thermal growing seasons expected at high latitudes in the future.

2. Can We Increase Autumn Productivity at High Latitudes?

Autumn-extended thermal growing seasons, combined with higher atmospheric CO_2 concentrations, have the potential to increase the productivity of perennial grasses in the autumn [6–9]. However, since the annual variation in temperature lags behind the annual variation in photoperiod, there is less light in autumn than at comparable temperatures in spring (Figure 1). Therefore, light availability may limit the utilization of the extended growing season in autumn [26,27]. Insufficient light not only limits growth rates, but can also prevent proper acclimation to several types of winter stress [28–33]. Light factors during the cold acclimation period can affect the freezing tolerance of herbaceous overwintering plants in several ways [34,35]. Firstly, a certain irradiance combined with low temperature increases photosystem II (PSII) excitation pressure, which is a signal leading to the development of freezing resistance [36–40].

Secondly, short photoperiod and low red to far red light (R:FR) ratio can interact with low temperature to stimulate the development of freezing resistance [41–44]. Thirdly, irradiance is the energy source for the accumulation of carbohydrates with a functional role in freezing resistance [40]. In addition, irradiance is the energy source for the accumulation of carbohydrate reserves needed for maintenance and stress responses during winter, as well as for early regrowth in spring.

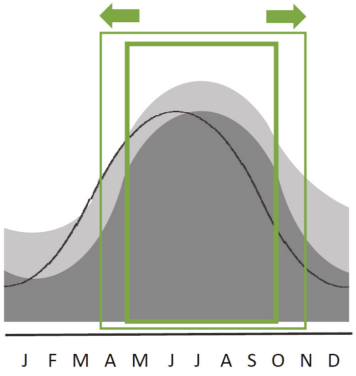


Figure 1. The thermal growing season and annual variation in photoperiod and temperature at a northern latitude. With higher temperatures in the future (light grey area), the thermal growing season will extend in both ends (outer green box) as compared to today (dark grey area and inner green box). The annual changes in temperature (grey area) lags behind the annual changes in photoperiod (black line). With higher temperatures in the future, but the same photoperiod variation as today, we will have new combinations of temperature and photoperiod.

In order to obtain high productivity over several years, a high rate of winter survival and vigorous spring regrowth is necessary, and therefore increased autumn productivity through an extended growing season can only be sustainably achieved if there is enough light during the delayed cold acclimation period. The required levels of acclimation and of stored carbohydrate reserves depend on the severity of the prevailing stresses and on the magnitude of the net photosynthetic deficit that might accumulate during winter, both which can vary greatly from year to year, and which do not necessarily diminish with climate change [20]. There are, in theory, two contrasting options for genetic adaptation to autumn-extended thermal growing seasons at high latitudes: either utilize the extended growing period and cold acclimate later in the autumn, but at the same temperature as today, or cease leaf growth and cold acclimate at the same photoperiod as today, but at higher temperatures (Figure 2). Which option is the best one with regard to optimization of long-term yield would depend on the amount of light needed for sufficient cold acclimation and for sufficient accumulation of carbohydrates. The relative importance of temperature and light factors in cold acclimation and cessation of leaf growth in perennial grasses is not very well characterized, neither are the interactions between temperature and light. It has been suggested that plants adapted to the extreme north rely more on photoperiod than temperature for timing of cold acclimation and cessation of leaf growth than other plants, and that such photoperiodic control will be of increasing importance in southern Scandinavia in the future [32]. The genetic association between cold acclimation and cessation of leaf growth is also not well characterized. The negative correlation between accumulation of carbohydrate reserves and production of harvestable biomass (i.e., assimilate partitioning) is difficult to get around. The association between leaf growth and cold acclimation may have a regulatory cause (discussed below) rather than a physiological cause, and thus it may be possible to break this association through breeding, as has been suggested for alfalfa (*Medicago sativa* L.) [45–47].

When considering the possibilities for adaptation to autumn-extended thermal growing seasons, it would be relevant to know: (1) to what extent do perennial grasses depend on PSII excitation pressure for induction of cold acclimation and how much light is required for this? (2) how much carbohydrate reserves are needed, and how much light is required after cessation of leaf growth, if any, to build this storage? and (3) how are the different aspects of growth, allocation of photosynthates, and cold acclimation regulated by environmental and genetic factors?

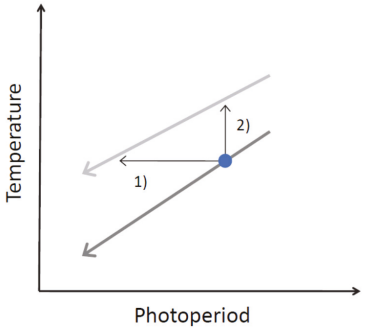


Figure 2. What is the optimal timing of cold acclimation and cessation of leaf growth in autumn? The grey arrows indicate the change in photoperiod and temperature during current (dark grey) and future (light grey) autumns. The timing of cold acclimation and cessation of leaf growth (blue dot) in the future climate could occur (1) at the same temperature as today, but shorter photoperiods (horizontal black arrow), or (2) at the same photoperiod as today, but warmer temperatures (vertical black arrow). Option (1) would allow higher biomass production, but cold acclimation and accumulation of sufficient carbohydrate reserves might be compromised by limited light energy.

2.1. The Role of Light in Signalling Mechanisms Inducing Cold Acclimation

Low temperature limits the rates of enzymatic reactions of photosynthesis more than the rates of electron transfer reactions in the light harvesting systems. As a result, low temperature in light creates an energy imbalance in the photosystems, leading to a change in the redox state of photosynthetic electron-transport components and a high excitation pressure of PSII [36–38]. The altered photosystem redox state functions as an irradiance-dependent cold sensor. As a result of the PSII over-excitation, reactive oxygen species (ROS) are generated, and these may act in signaling pathways leading to the expression of genes involved in freezing resistance [48,49] as well as a compact growth habit in overwintering herbaceous plants [36,37,50–53]. This mechanism of sensing cold might become less efficient if the cold acclimation period is shifted to a later time in autumn in the future, particularly at high latitudes, were irradiance levels are rapidly declining during that time of year. There are, however, also other mechanisms by which plants may sense low temperature and initiate development of freezing resistance, such as changes in membrane rigidity, temperature-dependent histone-DNA interactions, and conformational changes of RNA and protein structure [48,54]. These mechanisms may increase in importance if the cold acclimation period is postponed to a later time of year in the future.

Overwintering plant species have different strategies to handle the potentially damaging excess energy associated with elevated PSII excitation pressure [37–39]. In winter wheat and winter rye the photosynthetic capacity is upregulated during cold acclimation (photochemical quenching, q_p), ensuring utilization of the energy available from the light harvesting complexes. Compared to winter cereals, spring cereals exhibit less photosynthetic acclimation, rely more on dissipation of excess energy by release of heat (non-photochemical quenching, NPQ) and less on q_p, and are more sensitive to both photoinhibition and freezing [55–57]. In the perennial grasses meadow fescue (*Schedonorus pratensis* Huds., syn. *Festuca*

pratensis Huds.), perennial ryegrass (*Lolium perenne* L.), and timothy (*Phleum pratense* L.), NPQ appears to be a more important mechanism of photosynthetic acclimation to cold than q_p [58–60]. In the less freezing resistant Italian ryegrass (*L. multiflorum* L.), q_p increased after cold acclimation and NPQ decreased, while a part of meadow fescue chromosome 4 introgressed into Italian ryegrass was associated with higher cold-induced NPQ and freezing resistance [59]. However, it appears that both NPQ and q_p take place and that the relative importance of these two mechanisms vary among genotypes. For example, Kosmala et al. [61] found higher amounts of some proteins involved in photosynthetic carbon metabolism in a freezing resistant genotype of meadow fescue than a less tolerant genotype, suggesting a role of q_p. Moreover, the relative magnitudes of NPQ and q_p may be related to the carbohydrate status of the plant, with higher q_p in plants with less stored carbohydrates. Selection of photochemical quenching rather than non-photochemical quenching as a mode of photoacclimation to cold appears to be favorable as some of the accumulating photosynthates could either support survival during a long winter or be converted into forage production the following spring.

2.2. The Role of Photosynthates in Winter Survival

In temperate perennial grasses, simple carbohydrates accumulate during cold acclimation and most of these are converted into fructans, which accumulate mainly in the basal part of the shoot [18,62,63]. In regions with a long winter, a storage of organic reserves, particularly carbohydrates, are necessary for maintenance respiration, stress responses, and early spring regrowth. In addition, carbohydrates have specific roles as osmolytes and protectants of cellular components [13,40], and winter survival ability is often associated with a higher concentration of both simple sugars and fructans in the basal parts of the shoot attained during cold acclimation [64–67]. As described above, winter cereals maintain CO_2-fixation rates at low temperatures due to photosynthetic acclimation, a mechanism, which combined with restrictions on leaf growth, ensures that a storage of carbohydrates is accumulating. Elevated atmospheric CO_2 concentrations may affect cold acclimation and winter survival in several ways. For example, higher CO_2 concentrations could inhibit the generation of a PSII excitation pressure signal or enhance the accumulation of carbohydrate reserves. The few studies of perennial grasses have contrasting results regarding the impact of elevated CO_2 on freezing resistance [30,68,69].

The amount of carbohydrate reserves that have to be stored in order to ensure winter survival depends on several factors. A general assessment is that a larger carbohydrate storage will be required in areas were photosynthesis is limited for a long period due to low irradiance, thick snow cover, freezing temperatures, or dying leaf blade tissues, meaning that the plants must draw on stored reserves. Obtaining as high annual yields as possible while maintaining tiller survival is a fine-tuned balance between the allocation of autumn photosynthates into leaf growth versus storage. The potential for utilizing light energy in longer growing seasons (beyond the autumn equinox) for increased autumn yield, rather than for storage, may therefore be highest at lower latitudes and diminish as we move towards higher latitudes with darker autumns and longer winters. Interestingly, during a relatively mild winter without snow cover (approximate average temperature 2 °C) at 61° latitude in western Norway, timothy and perennial ryegrass both accumulated carbohydrates in the shoot base during winter, and more so in the most winter hardy species/cultivars [62,70]. Although this could be due to reallocation within the plant, it is known that photosynthesis can operate at very low temperatures around or even below 0 °C [71,72]. This result indicates that at this latitude, there may be possibilities for maintaining or even accumulating a carbohydrate storage during such mild winters. It is, however, a prerequisite that the leaf tissue survives, and it is likely that there will be strong effects of the timing of the last defoliation, as the amount of new leaf area developed prior to the cessation of leaf growth is critical. Predictions of ideotypes for different regions in a future climate [73] could aid breeding efforts to achieve the optimum balance between allocation of photosynthates to leaf growth versus storage. The severity of winter conditions vary greatly from year to year, and plants need to be designed to be able to survive winters that are harsher than the average winter. In any case, when it comes to

photosynthates, these are possibly better invested in rapid regrowth in spring, when light conditions are very good, than in autumn growth.

2.3. Regulation of Leaf Growth versus Cold Acclimation

In herbaceous overwintering plants, leaf growth inhibition in response to low temperature is not simply a result of lower metabolic rates, but an actively regulated process, which is coordinated with changes in carbon metabolism [74]. Concomitantly with the development of freezing resistance at low temperatures, winter rye, winter wheat, *Arabidopsis thaliana*, and *Brassica napus* develop a dwarf phenotype with shorter, thicker leaves that have a distinct anatomy and high concentrations of proteins and carbohydrates [75]. In addition, these species upregulate their photosynthetic capacity to compensate for the slower rates of enzymatic reactions at low temperature, and accumulate carbohydrates in storage organs. Unlike winter cereals, spring cereals do not develop a dwarf phenotype during cold acclimation, do not upregulate the photosynthetic capacity to the same level, and are not able to attain the same level of freezing resistance [57,75].

In experiments with several perennial forage grasses exposed to different photoperiods, but the same total amount of photosynthetic active radiation (PAR), it was shown that long photoperiods stimulate increased specific leaf area (SLA, leaf area per unit of leaf dry weight) and increased dry matter production, and conversely, that short photoperiods result in lower SLA and productivity [76–78]. Short photoperiod also stimulates tiller formation in perennial forage grasses [79,80], a process which likely improves the potential for spring productivity. Interestingly, at short photoperiods, low irradiance levels, and relatively high autumn temperatures (12 °C), a stimulation of leaf elongation was observed in perennial ryegrass and timothy [33], indicating that when the temperature is not low enough, the shade avoidance syndrome [81] can override the photoperiod response of leaf elongation. This could possibly become a problem during mild, rainy autumns at high latitudes in the future.

C-REPEAT BINDING FACTORs (CBFs) appear to be central in the coordinated regulation of leaf growth and freezing tolerance in response to low temperature [34,74] (Figure 3). *CBF* transcription factors upregulate whole sets of genes encoding proteins with direct functions in freezing resistance [82]. Temperate grasses have a large number of *CBF* genes, which are differentially expressed in response to various environmental signals [82–88]. Exactly which of the *CBF* genes has the largest influence on freezing resistance appears to vary between species, genetic background, and/or environment [89–92]. One of the three *CBF*s in *A. thaliana* is upregulated by PSII excitation pressure, redox state of the plastoquinon pool, and ROS signaling [49]. However, barley mutants with an impaired chloroplast development had normal cold-induced expression of at least some *CBFs*, indicating that their expression does not depend solely on a signal generated in the chloroplasts [93]. Indeed, Marozsán-Tóth et al. [85] showed that some *CBFs* in barley were regulated through Ca^{2+} signaling. Short photoperiods and low R:FR ratios can interact with temperature to induce freezing resistance in *A. thaliana*, and this effect is mediated by the circadian clock through its control of *CBF* expression [42,43]. In wheat and barley, low R:FR ratios upregulate expression of *CBF14* as well as freezing resistance [44], and in meadow fescue *CBF6* expression is affected by interactions between temperature, light quality, and irradiance [94]. In *A. thaliana*, *CBFs* can down-regulate leaf growth by down-regulating the content of gibberellic acid (GA), thereby allowing the accumulation of DELLA proteins which inhibits growth, and in addition increases freezing resistance by a reduction of ROS [95,96]. *CBFs* can also enhance photosynthetic capacity in *B. napus* [56,97,98], and may therefore play a role in photosynthetic quenching and maintenance of biomass accumulation at low temperatures. All this points to the *CBF* family of transcription factors as integrators of many different adaptive responses to autumn conditions leading to winter survival.

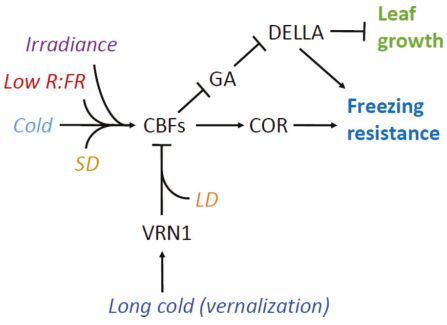


Figure 3. Putative model showing the central role of CBF transcription factors in coordinating freezing resistance and leaf growth in response to temperature and light. Cold induces expression of *CBFs*, which are important in the development of freezing resistance through the induction of the CBF regulon of cold-induced genes (here collectively indicated with *COR*), and also play a role in down-regulation leaf growth via gibberellic acid (GA). Short days (SD), irradiance, and low red to far red light-ratio (R:FR), can take part in the induction of *CBFs*. Prolonged cold leads to a gradual increase in *VRN1* transcripts. VRN1 can down-regulate *CBFs* and freezing resistance, particularly in long days (LD). In temperate grasses there are around 20 *CBF* genes which are likely to have partly differentiated roles.

3. Can We Increase Spring Productivity at High Latitudes?

Unlike the situation in autumn, it is not light availability, but temperature, which is currently limiting biomass production in spring at high latitudes (Figure 1). Thus, there is a potential to utilize an earlier thermal growing season in the future. However, with the growing season starting earlier in the year, and possibly before the spring equinox, the day-night temperature amplitude and the probability of night frost or longer freezing periods increases. For example, the probability of spring frost damage was predicted to increase in the western part of the Nordic area in 2040–2065 as compared to 1960–1990 [5].

In general, exposure to higher temperatures in spring results in stimulation of growth and at the same time, loss of freezing resistance (de-acclimation) [20]. There are several reports describing de-acclimation and re-acclimation responses in perennial grass species [99–105], and some of them also report a negative association between freezing resistance and leaf growth during the de-acclimation period. In cereals, it has been shown that both freezing resistance and expression of cold-induced genes are down-regulated in shoot base tissue when the vernalization requirement is saturated, but before any development of the apex is visible in the microscope [106–108]. There is an interaction between vernalization and photoperiod on de-acclimation. In cultivars with a long day requirement for flowering, the negative effect of vernalization on freezing resistance is stronger when plants are vernalized under long days than under short days, whereas vernalization- and photoperiod-insensitive cultivars are not able to develop much freezing resistance at all [109–112]. Also, plants vernalized and de-acclimated under long days are often found to be less able to re-acclimate [113,114]. Vernalization not only enhances de-acclimation, as well as competency to flower in response to long photoperiod in a large number of perennial grass species [19], but also increases the rate of leaf expansion, specific leaf area, and photosynthetic rate of perennial ryegrass leaves developed after transfer to 15 °C, particularly under long photoperiods [115].

In order to utilize more of the spring light for increased productivity, it would be necessary to grow plants that are capable of maintaining freezing tolerance during early spring growth, and/or able to rapidly re-acclimate upon demand. In this context, it would be desirable to have a better understanding of (1) how do temperature, vernalization, and photoperiod together control leaf growth and freezing resistance in spring? and (2) which mechanisms govern rapid re-acclimation after de-acclimation, and to which extent do these function in growing plants?

3.1. Regulation of Leaf Growth versus Freezing Resistance in Spring

VRN1, an inducer of the transition to generative development in cereals and other temperate grass species, is gradually up-regulated during vernalization [116,117]. Several studies indicate that there is a negative association between expression level of *VRN1* and the expression of cold-induced genes and freezing resistance. Using near-isogenic lines of wheat and barley, and a *T. monococcum* deletion mutant, it was shown that, under 16 h photoperiod, the *VRN1* locus controls expression of *VRN1*, *COR14B*, and other cold induced-genes [106–108,112,118]. High expression levels of *VRN1* were associated with the down-regulation of cold-induced genes and freezing resistance. From these studies, however, it is not entirely clear whether it is *VRN1* itself, or very closely linked genes, that is responsible. However, using a transgenic approach combined with chromatin immunoprecipitation sequencing and RNA sequencing, Deng et al. [119] showed that in barley grown at 16 h photoperiod, VRN1 binds to the promoter of several *CBF* genes. After short-term cold exposure, when the expression level of *VRN1* is still very low, Oliver et al. [120] found similar kinetics in the initial transcription of *VRN1* and *COR14B* upon cold exposure (24 h) in barley. Under short photoperiods, high *COR14B* expression or positive correlation between expression of *VRN1* and *COR14B* remained after long-term cold treatment in *T. monococcum* [112], barley [121], and meadow fescue [105]. *COR14B* is induced by *CBF*s, and barley *VRN1* also has a putative *CBF* binding site in its promoter [122]. Oliver et al. [107] therefore suggested that *VRN1* and *COR14B* may be regulated by similar mechanisms in early cold acclimation, possibly through the action of CBF transcription factors. Several studies show that *CBF6* and *COR14B* are down-regulated in cereals and meadow fescue by prolonged cold, but only under long photoperiods [105]. Taken together, these results suggest that *VRN1* and the *CBF* regulon are co-regulated during cold acclimation of temperate grasses and as long as photoperiods are short, but that VRN1 down-regulates *CBF*s when photoperiods become long (Figure 3). This is a possible explanation for the interaction between vernalization and photoperiod during de-acclimation in temperate grasses. Also, given that CBFs can inhibit leaf growth via GA and DELLA proteins [34], the regulatory effect of VRN1 on *CBF* expression may explain the effects of vernalization on leaf growth and photosynthetic activity observed by Stapleton and Jones [115], and also its interaction with photoperiod.

3.2. Is Re-Acclimation in Spring Different from Cold Acclimation in Autumn?

Under controlled conditions, the re-acclimation of temperate grasses differs somewhat from the initial cold acclimation. For example, carbohydrates did not accumulate to the same extent during re-acclimation as during initial cold acclimation in winter wheat [114], and several cold induced genes upregulated by cold acclimation in meadow fescue were not upregulated during re-acclimation [105]. The mechanisms behind these differences are not known, but are likely to be related to a coordinated regulation of growth and freezing tolerance as described in Section 2.3. In the field, re-acclimation may also be inhibited if plants are exhausted from carbohydrate reserves or devoid of functional leaf area. Re-acclimation at a time when spring growth has been initiated may be provided by other mechanisms than those employed during initial cold acclimation in the autumn. For example, while expression of *COR14B* could explain some of the variation in freezing resistance in de-acclimated meadow fescue, *CR7* was, unlike *COR14B*, significantly upregulated by re-acclimation and could explain some of the variation in freezing resistance after re-acclimation [105].

4. Conclusions

The expected prolonged growing season in future autumns can probably be utilized for higher autumn productivity in some areas, but in areas were photosynthesis is prohibited during long winters, such as at high latitudes, areas with a deep long-lasting snow cover, or with severe stresses killing leaf blades, this may not be possible due to the need for storage carbohydrates. There is a larger potential for utilizing the earlier springs to increase productivity in such areas, but it will be necessary with

varieties that maintain some level of freezing resistance and re-acclimation ability also during early spring regrowth.

The many *CBF* genes in perennial grasses, which are differentially regulated and probably have different functions, could possibly be utilized in developing varieties that combine some autumn productivity with cold acclimation. Similarly, a possibility of at least partly breaking the association between growth, de-acclimation, and loss of re-acclimation ability in spring may lay in playing with alleles of the various *CBF* genes and their differential functions. The interaction between temperature and light factors, particularly photoperiod, on *CBF* regulation is critical in this respect. An improved understanding of the specific functions of the various *CBF* genes—how they are regulated and which parts of the *CBF* regulon they control, as well as an overview of allelic variation—could aid in the development of perennial grass varieties with an optimal balance between growth and perennial persistence under future climates.

Conflicts of Interest: The author declares no conflict of interest.

References

1. Hatfield, J.L.; Prueger, J.H. Agroecology: Implications for plant response to climate change. In *Crop Adaptation to Climate Change*; Yadav, S.S., Redden, R.J., Hatfield, J.L., Lotze-Campen, H., Hall, A.E., Eds.; Wiley-Blackwell: Chichester, UK, 2011; pp. 27–43.
2. Soussana, J.-F.; Lüscher, A. Temperate grasslands and global atmospheric change: A review. *Grass For. Sci.* **2007**, *62*, 127–134. [CrossRef]
3. Mikkonen, S.; Laine, M.; Mäkelä, H.M.; Gregow, H.; Tuomenvirta, H.; Lahtinen, M.; Laaksonen, A. Trends in the average temperature in Finland, 1847–2013. *Stoch. Environ. Res. Risk Assess.* **2015**, *29*, 1521–1529. [CrossRef]
4. Ruosteenoja, K.; Räisänen, J.; Pirinen, P. Projected changes in thermal seasons and the growing season in Finland. *Int. J. Climatol.* **2011**, *31*, 1473–1487. [CrossRef]
5. Höglind, M.; Thorsen, S.M.; Semenov, M.A. Assessing uncertainties in impact of climate change on grass production in Northern Europe using ensembles of global climate models. *Agric. For. Meteorol.* **2013**, *170*, 103–113. [CrossRef]
6. Jing, Q.; Bélanger, G.; Qian, B.; Baron, V. Timothy yield and nutritive value under climate change in Canada. *Agron. J.* **2013**, *105*, 1683–1694. [CrossRef]
7. Jing, Q.; Bélanger, G.; Qian, B.; Baron, V. Timothy yield and nutritive value with a three-harvest system under the projected future climate in Canada. *Can. J. Plant Sci.* **2014**, *94*, 213–222. [CrossRef]
8. Thivierge, M.-N.; Jégo, G.; Bélanger, G.; Bertrand, A.; Tremblay, G.F.; Rotz, C.A. Predicted yield and nutritive value of an alfalfa–timothy mixture under climate change and elevated atmospheric carbon dioxide. *Agron. J.* **2016**, *108*, 1–19. [CrossRef]
9. Graux, A.-I.; Bellocchi, G.; Lardy, R.; Soussana, J.-F. Ensemble modelling of climate change risks and opportunities for managed grasslands in France. *Agric. For. Meteorol.* **2013**, *170*, 114–131. [CrossRef]
10. Höglind, M.; van Oijen, M.; Cameron, D.; Persson, T. Process-based simulation of growth and overwintering of grassland using the BASGRA model. *Ecol. Model.* **2016**, *335*, 1–15. [CrossRef]
11. Laude, H.M. The nature of summer dormancy in perennial grasses. *Bot. Gaz.* **1953**, *114*, 282–292. [CrossRef]
12. Volaire, F.; Norton, M. Summer dormancy in perennial temperate grasses. *Ann. Bot.* **2006**, *98*, 927–933. [CrossRef] [PubMed]
13. Preston, J.C.; Sandve, S.R. Adaptation to seasonality and the winter freeze. *Front. Plant Sci.* **2013**, *4*, 167. [CrossRef] [PubMed]
14. Norton, M.R.; Volaire, F.; Lelièvre, F.; Fukai, S. Identification and measurement of summer dormancy in temperate perennial grasses. *Crop Sci.* **2009**, *49*, 2347–2352. [CrossRef]
15. Gillespie, M.; Volaire, F.A. Are winter and summer dormancy symmetrical seasonal adaptive strategies? The case of temperate herbaceous perennials. *Ann. Bot.* **2017**, *119*, 311–323. [CrossRef] [PubMed]
16. Cooper, J.P. Climatic Variation in Forage Grasses. I. Leaf Development in Climatic Races of *Lolium* and *Dactylis*. *J. Appl. Ecol.* **1964**, *1*, 45–61. [CrossRef]
17. Sibly, R.M.; Calow, P. A life-cycle theory of responses to stress. *Biol. J. Linn. Soc.* **1989**, *37*, 101–116. [CrossRef]

18. Hisano, H.; Kanazawa, A.; Yoshida, M.; Humphreys, M.O.; Iizuka, M.; Kitamura, K.; Yamada, T. Coordinated expression of functionally diverse fructosyltransferase genes is associated with fructan accumulation in response to low temperature in perennial ryegrass. *New Phytol.* **2008**, *178*, 766–780. [CrossRef] [PubMed]
19. Heide, O.M. Control of flowering and reproduction in temperate grasses. *New Phytol.* **1994**, *128*, 347–362. [CrossRef]
20. Rapacz, M.; Ergon, Å.; Höglind, M.; Jørgensen, M.; Jurczyk, B.; Østrem, L.; Rognli, O.A.; Tronsmo, A.M. Overwintering of herbaceous plants in a changing climate. Still more questions than answers. *Plant Sci.* **2014**, *225*, 34–44. [CrossRef] [PubMed]
21. Andrews, C.J.; Gudleifsson, B.E. A comparison of cold hardiness and ice encasement tolerance of timothy grass and winter wheat. *Can. J. Plant Sci.* **1983**, *63*, 429–435. [CrossRef]
22. Årsvoll, K. Effects of hardening, plant age, and development in *Phleum pratense* and *Festuca pratensis* on resistance to snow mould fungi. *Sci. Rep. Agric. Univ. Nor.* **1977**, *56*, 1–14.
23. Tronsmo, A.M. Predisposing effects of low temperature on resistance to winter stress factors in grasses. *Acta Agric. Scand.* **1983**, *34*, 210–220. [CrossRef]
24. Stocker, T.F.; Qin, D.; Plattner, G.-K.; Alexander, L.V.; Allen, S.K.; Bindoff, N.L.; Bréon, F.-M.; Church, J.A.; Cubasch, U.; Emori, S.; et al. Technical summary. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*; Stocker, T.F., Qin, D., Plattner, K.-G., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M., Eds.; Cambridge University Press: Cambridge, UK; New York, NY, USA, 2013.
25. Kirtman, B.; Power, S.B.; Adedoyin, J.A.; Boer, G.J.; Bojariu, R.; Camilloni, I.; Doblas-Reyes, F.J.; Fiore, A.M.; Kimoto, M.; Meehl, G.A.; et al. Near-term climate change: Projections and predictability. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*; Stocker, T.F., Qin, D., Plattner, K.-G., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M., Eds.; Cambridge University Press: Cambridge, UK; New York, NY, USA, 2013.
26. Uleberg, E.; Hanssen-Bauer, I.; van Oort, B.; Dalmannsdóttir, S. Impact of climate change on agriculture in Northern Norway and potential strategies for adaptation. *Clim. Chang.* **2014**, *122*, 27–39. [CrossRef]
27. Virkajärvi, P.; Rinne, M.; Mononen, J.; Niskanen, O.; Järvenranta, K.; Sairanen, A. Dairy production systems in Finland. *Grassl. Sci. Eur.* **2015**, *20*, 51–66.
28. Lawrence, T.; Cooper, J.P.; Breese, E.L. Cold tolerance and winter hardiness in *Lolium perenne*: II. Influence of light and temperature during growth and hardening. *J. Agric. Sci.* **1973**, *80*, 341–348. [CrossRef]
29. Nakajima, T.; Abe, J. Environmental factors affecting expression of resistance to pink snow mold caused by *Microdochium nivale* in winter wheat. *Can. J. Bot.* **1996**, *74*, 1783–1788. [CrossRef]
30. Harrison, J.; Tonkinson, C.; Eagles, C.; Foyer, C. Acclimation to freezing temperatures in perennial ryegrass *(Lolium perenne)*. *Acta Phys. Plant.* **1997**, *19*, 505–515. [CrossRef]
31. Hanslin, H.M.; Höglind, M. Differences in winter-hardening between phenotypes of *Lolium perenne* with contrasting water-soluble carbohydrate concentrations. *Grass For. Sci.* **2009**, *64*, 187–195. [CrossRef]
32. Østrem, L.; Rapacz, M.; Larsen, A.; Dalmannsdóttir, S.; Jørgensen, M. Influences of growth cessation and photoacclimation on winter survival of non-native *Lolium–Festuca* grasses in high-latitude regions. *Environ. Exp. Bot.* **2015**, *111*, 21–31. [CrossRef]
33. Dalmannsdottir, S.; Jørgensen, M.; Rapacz, M.; Østrem, L.; Larsen, A.; Rognli, O.A. Cold acclimation in warmer extended autumns impairs freezing tolerance of perennial ryegrass (*Lolium perenne* L.) and timothy (*Phleum pratense* L.). *Physiol. Plant.* **2017**, in press. [CrossRef] [PubMed]
34. Kurepin, L.V.; Dahal, K.P.; Savitch, L.V.; Singh, J.; Bode, R.; Ivanov, A.G.; Hurry, V.; Hüner, N.P. Role of CBFs as integrators of chloroplast redox, phytochrome and plant hormone signaling during cold acclimation. *Int. J. Mol. Sci.* **2013**, *14*, 12729–12763. [CrossRef] [PubMed]
35. Kovi, M.R.; Ergon, Å.; Rognli, O.A. Freezing tolerance recisited-effects of variable temperatures on gene regulation in temperate grasses and legumes. *Curr. Opin. Plant Biol.* **2016**, *33*, 140–146. [CrossRef] [PubMed]
36. Gray, G.R.; Chauvin, L.P.; Sarhan, F.; Hüner, N.P.A. Cold acclimation and freezing tolerance (a complex interaction of light and temperature). *Plant Physiol.* **1997**, *114*, 467–474. [CrossRef] [PubMed]
37. Hüner, N.P.A.; Öquist, G.; Sarhan, F. Energy balance and acclimation to light and cold. *Trends Plant Sci.* **1998**, *3*, 224–230. [CrossRef]

38. Ensminger, I.; Busch, F.; Hüner, N.P.A. Photostasis and cold acclimation: Sensing low temperature through photosynthesis. *Physiol. Plant.* **2006**, *126*, 28–44. [CrossRef]
39. Wilson, K.E.; Ivanov, A.G.; Öquist, G.; Grodzinski, B.; Sarhan, F.; Hüner, N.P.A. Energy balance, organellar redox status, and acclimation to environmental stress. *Can. J. Bot.* **2006**, *84*, 1355–1370. [CrossRef]
40. Sandve, S.R.; Kosmala, A.; Rudi, H.; Fjellheim, S.; Rapacz, M.; Yamada, T.; Rognli, O.A. Molecular mechanisms underlying frost tolerance in perennial grasses adapted to cold climates. *Plant Sci.* **2011**, *80*, 69–77. [CrossRef] [PubMed]
41. Franklin, K.A.; Whitlam, G.C. Light-quality regulation of freezing tolerance in *Arabidopsis thaliana*. *Nat. Genet.* **2007**, *39*, 1410–1413. [CrossRef] [PubMed]
42. Kinmonth-Schultz, H.A.; Golembeski, G.S.; Imaizumi, T. Circadian clock-regulated physiological outputs: Dynamic responses in nature. *Semin. Cell Dev. Biol.* **2013**, *24*, 407–413. [CrossRef] [PubMed]
43. Franklin, K.A.; Toledo-Ortiz, G.; Pyott, D.E.; Halliday, K.J. Interaction of light and temperature signalling. *J. Exp. Bot.* **2014**, *65*, 2859–2871. [CrossRef] [PubMed]
44. Novák, A.; Boldizsár, Á.; Ádám, É.; Kozma-Bognár, L.; Majláth, I.; Båga, M.; Tóth, B.; Chibbar, R.; Galiba, G. Light-quality and temperature-dependent *CBF14* gene expression modulates freezing tolerance in cereals. *J. Exp. Bot.* **2016**, *67*, 1285–1295. [CrossRef] [PubMed]
45. Castonguay, Y.; Laberge, S.; Brummer, E.C.; Volenec, J.J. Alfalfa winter hardiness: A research retrospective and integrated perspective. *Adv. Agron.* **2006**, *90*, 203–265.
46. Brummer, E.C.; Shah, M.M.; Luth, L. Reexamining the relationship between fall dormancy and winter hardiness in alfalfa. *Crop Sci.* **2000**, *40*, 971–977. [CrossRef]
47. Li, X.; Alarcon-Zuniga, B.; Kang, J.; Tahir, M.H.N.; Jiang, Q.; Wei, Y.; Reyno, R.; Robins, J.G.; Brummer, E.C. Mapping fall dormancy and winter injury in tetraploid alfalfa. *Crop Sci.* **2005**, *55*, 1995–2011. [CrossRef]
48. Winfield, M.O.; Lu, C.; Wilson, I.D.; Coghill, J.A.; Edwards, K.J. Plant responses to cold: Transcriptome analysis of wheat. *Plant Biotechnol. J.* **2010**, *8*, 749–771. [CrossRef] [PubMed]
49. Bode, R.; Ivanov, A.G.; Hüner, N.P.A. Global transcriptome analyses provide evidence that chloroplast redox state contributes to intracellular as well as long-distance signalling in response to stress and acclimation in Arabidopsis. *Photosynth. Res.* **2016**, *128*, 287–312. [CrossRef] [PubMed]
50. Ndong, C.; Danyluk, J.; Hüner, N.P.A.; Sarhan, F. Survey of gene expression in winter rye during changes in growth temperature, irradiance or excitation pressure. *Plant Mol. Biol.* **2001**, *45*, 691–703. [CrossRef] [PubMed]
51. Dal Bosco, C.; Busconi, M.; Govoni, C.; Baldi, P.; Stanca, A.M.; Crosatti, C.; Bassi, R.; Cattivelli, L. *cor* Gene expression in barley mutants affected in chloroplast development and photosynthetic electron transport. *Plant Physiol.* **2003**, *131*, 793–802. [CrossRef] [PubMed]
52. Crosatti, C.; Rizza, F.; Badeck, F.W.; Mazzucotelli, E.; Cattivelli, L. Harden the chloroplast to protect the plant. *Physiol. Plant.* **2013**, *147*, 55–63. [CrossRef] [PubMed]
53. Rapacz, M. The effects of day and night temperatures during early growth of winter rape seedlings on their morphology and cold acclimation responses. *Acta Physiol. Plant.* **1998**, *20*, 67–72. [CrossRef]
54. McClung, C.R.; Davies, S.J. Ambient Thermometers in Plants: From Physiological Outputs towards Mechanisms of Thermal Sensing. *Curr. Biol.* **2010**, *20*, R1086–R1092. [CrossRef] [PubMed]
55. Pocock, T.H.; Hurry, V.; Savitch, L.V.; Huner, N.P.A. Susceptibility to low-temperature photoinhibition and the acquisition of freezing tolerance in winter and spring wheat: The role of growth temperature and irradiance. *Physiol. Plant.* **2001**, *113*, 499–506. [CrossRef]
56. Dahlal, K.; Kane, K.; Gadapati, W.; Webb, E.; Savitch, L.V.; Singh, J.; Sharma, P.; Sarhan, F.; Longstaffe, F.J.; Grodzinski, B.; et al. The effects of phenotypic plasticity on photosynthetic performance in winter rye, winter wheat and *Brassica napus*. *Physiol. Plant.* **2012**, *144*, 169–188. [CrossRef] [PubMed]
57. Dahlal, K.; Kane, K.; Sarhan, F.; Grodzinski, B.; Hüner, N.P.A. Cold acclimation inhibits CO_2-dependent stimulation of photosynthesis in spring wheat and spring rye. *Botany* **2012**, *90*, 433–444. [CrossRef]
58. Rapacz, M.; Gasior, D.; Zwierzykowski, Z.; Lesniewska-Bocianowska, A.; Humphreys, M.W.; Gay, A.P. Changes in cold tolerance and the mechanisms of acclimation of photosystem II to cold hardening generated by anther culture of *Festuca pratensis* × *Lolium multiflorum* cultivars. *New Phytol.* **2004**, *162*, 105–114. [CrossRef]

59. Humphreys, M.W.; Gasior, D.; Lesniewska-Bocianowska, A.; Zwierzykowski, Z.; Rapacz, M. Androgenesis as a means of dissecting complex genetic and physiological controls: Selecting useful gene combinations for breeding freezing tolerant grasses. *Euphytica* **2007**, *158*, 337–345. [CrossRef]
60. Dalmannsdóttir, S.; Rapacz, M.; Jørgensen, M.; Østrem, L.; Larsen, A.; Rødven, R.; Rognli, O.A. Temperature before cold acclimation affects cold tolerance and photoacclimation in timothy (*Phleum pratense* L.), perennial ryegrass (*Lolium perenne* L.) and red clover (*Trifolium pratense* L.). *J. Agron. Crop Sci.* **2016**, *202*, 320–330. [CrossRef]
61. Kosmala, A.; Bocian, A.; Rapacz, M.; Jurczyk, B.; Zwierzykowski, Z. Identification of leaf proteins differentially accumulated during cold acclimation between *Festuca pratensis* plants with distinct levels of frost tolerance. *J. Exp. Bot.* **2009**, *60*, 3595–3609. [CrossRef] [PubMed]
62. Pollock, C.J.; Jones, T. Seasonal patterns of fructan metabolism in forage grasses. *New Phytol.* **1979**, *83*, 9–15. [CrossRef]
63. Dionne, J.; Castonguay, Y.; Nadeau, P.; Desjardins, Y. Freezing tolerance and carbohydrate changes during cold acclimation of green-type annual bluegrass (*Poa annua* L.) ecotypes. *Crop Sci.* **2001**, *41*, 443–451. [CrossRef]
64. Østrem, L.; Rapacz, M.; Jørgensen, M.; Höglind, M. Effect of developmental stage on carbohydrate accumulation patterns during winter of timothy and perennial ryegrass. *Acta Agric. Scand. Sect. B Soil Plant Sci.* **2011**, *61*, 153–163. [CrossRef]
65. Yoshida, M.; Abe, J.; Moiyama, M.; Kuwabara, T. Carbohydrate levels among winter wheat cultivars varying in freezing tolerance and snow mold resistance during autumn and winter. *Physiol. Plant.* **1998**, *103*, 8–16. [CrossRef]
66. Sanada, Y.; Takai, T.; Yamada, T. Ecotypic variation of water-soluble carbohydrate concentration and winter hardiness in cocksfoot (*Dactylis glomerata* L.). *Euphytica* **2007**, *153*, 267–280. [CrossRef]
67. Abeynayake, S.W.; Byrne, S.; Nagy, I.; Jonavičien, K.; Etzerodt, T.P.; Boelt, B.; Asp, T. Changes in *Lolium perenne* transcriptome during cold acclimation in two genotypes adapted to different climatic conditions. *BMC Plant Biol.* **2015**, *15*, 250. [CrossRef] [PubMed]
68. Obrist, D.; Arnone , J.A., III; Körner, C. In situ effects of elevated atmospheric CO_2 on leaf freezing resistance and carbohydrates in a native temperate grassland. *Ann. Bot.* **2001**, *87*, 839–844. [CrossRef]
69. Jurczyk, B.; Rapacz, M.; Krepski, T. Short-term growth of meadow fescue with atmospheric CO_2 enrichment decreases freezing tolerance, modifies photosynthetic apparatus performance and changes the expression of some genes during cold acclimation. *Acta Physiol. Plant.* **2013**, *35*, 1543–1554. [CrossRef]
70. Østrem, L.; Rapacz, M.; Jørgensen, M.; Höglind, M. Impact of frost and plant age on compensatory growth in timothy and perennial ryegrass during winter. *Grass For. Sci.* **2010**, *65*, 15–22. [CrossRef]
71. Eagles, C.F. Apparent photosynthesis and respiration in populations of *Lolium perenne* from contrasting climatic regions. *Nature* **1967**, *215*, 100–101. [CrossRef]
72. Skinner, H. Winter carbon dioxide fluxes in humid-temperate pastures. *Agric. For. Meteorol.* **2007**, *144*, 32–43. [CrossRef]
73. Van Oijen, M.; Höglind, M. Toward a Bayesian procedure for using process-based models in plant breeding, with application to ideotype design. *Euphytica* **2016**, *207*, 627–643. [CrossRef]
74. Wingler, A. Comparison of signaling interactions determining annual and perennial plant growth in response to low temperature. *Front. Plant Sci.* **2014**, *5*, 794. [CrossRef] [PubMed]
75. Hüner, N.P.A.; Dahal, K.; Bode, R.; Kurepin, L.V.; Ivanov, A.G. Photosynthetic acclimation, vernalization, crop productivity and "the grand design of photosynthesis". *J. Plant Physiol.* **2016**, *203*, 29–43. [CrossRef] [PubMed]
76. Hay, R.K.M. The influence of photoperiod on the dry matter production of grasses and cereals. *New Phytol.* **1990**, *116*, 233–254. [CrossRef]
77. Solhaug, K.A. Long day stimulation of dry matter production in *Poa alpina* along a longitudinal gradient in Norway. *Holarct. Ecol.* **1991**, *14*, 161–168.
78. Wu, Z.; Skjelvåg, A.O.; Baadshaug, O.H. Quantification of photoperiodic effects on growth of *Phleum pratense*. *Ann. Bot.* **2004**, *94*, 535–543. [CrossRef] [PubMed]
79. Ryle, G.J.A. Effects of photoperiod in the glasshouse on the growth of leaves and tillers in three perennial grasses. *Ann. Appl. Biol.* **1966**, *57*, 257–268. [CrossRef]

80. Aamlid, T.S. Effects of temperature and photoperiod on growth and development of tillers and rhizomes in *Poa pratensis* L. ecotypes. *Ann. Bot.* **1992**, *69*, 289–296. [CrossRef]
81. Kebrom, T.H.; Brutnell, T.P. The molecular analysis of the shade avoidance syndrome in the grasses has begun. *J. Exp. Bot.* **2007**, *58*, 3079–3089. [CrossRef] [PubMed]
82. Galiba, G.; Vágújfalvi, A.; Li, C.; Soltesz, A.; Dubcovsky, J. Regulatory genes involved in the determination of frost tolerance in temperate cereals. *Plant Sci.* **2009**, *176*, 12–19. [CrossRef]
83. Skinner, J.S.; von Zitzewitz, J.; Szücs, P.; Marquez-Cedillo, L.; Filichkin, T.; Amundsen, K.; Stockinger, E.J.; Thomashow, M.F.; Chen, T.H.H.; Hayes, P.M. Structural, functional, and phylogenetic characterization of a large *CBF* gene family in barley. *Plant Mol. Biol.* **2005**, *59*, 533–551. [CrossRef] [PubMed]
84. Badawi, M.; Danyluk, J. The *CBF* gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal *CBF*s. *Mol. Genet. Genom.* **2007**, *277*, 533–554. [CrossRef] [PubMed]
85. Marozsán-Tóth, Z.; Vashegyi, I.; Galiba, G.; Tóth, B. The cold response of *CBF* genes in barley is regulated by distinct signaling mechanisms. *J. Plant Physiol.* **2015**, *181*, 42–49. [CrossRef] [PubMed]
86. Alm, V.; Busso, C.S.; Ergon, Å.; Rudi, H.; Larsen, A.; Humphreys, M.W.; Rognli, O.A. QTL analyses and comparative genetic mapping of frost tolerance, winter survival and drought tolerance in meadow fescue (*Festuca pratensis* Huds.). *Theor. Appl. Genet.* **2011**, *123*, 369–382. [CrossRef] [PubMed]
87. Xiong, Y.; Fei, S.-Z.; Arora, R.; Brummer, E.C.; Barker, R.E.; Jung, G.; Warnke, S.E. Identification of quantitative trait loci controlling winter hardiness in an annual—Perennial ryegrass interspecific hybrid population. *Mol. Breed.* **2007**, *19*, 125–136. [CrossRef]
88. Ryu, J.Y.; Hong, S.-Y.; Jo, S.-H.; Woo, J.-C.; Lee, S.; Park, C.-M. Molecular and functional characterization of cold-responsive C-repeat binding factors from *Brachypodium distachyon*. *BMC Plant Biol.* **2015**, *14*, 15. [CrossRef] [PubMed]
89. Fricano, A.; Rizza, F.; Faccioli, P.; Pagani, D.; Pavan, P.; Stella, A.; Rossini, L.; PiVanelli, P.; Cattivelli, L. Genetic variants of *Hvcbf14* are statistically associated with frost tolerance in a European germplasm collection of *Hordeum vulgare*. *Theor. Appl. Genet.* **2009**, *119*, 1335–1348. [CrossRef] [PubMed]
90. Zhu, J.; Pearce, S.; Burke, A.; See, D.R.; Skinner, D.Z.; Dubcovsky, J.; Garland-Campbell, K. Copy number and haplotype variation at the *VRN-A1* and central *FR-A2* loci are associated with frost tolerance in hexaploid wheat. *Theor. Appl. Genet.* **2014**, *127*, 1183–1197. [CrossRef] [PubMed]
91. Sieber, A.-N.; Longin, C.F.H.; Leiser, W.L.; Würschum, T. Copy number variation of *CBF-A14* at the *Fr-A2* locus determines frost tolerance in winter durum wheat. *Theor. Appl. Genet.* **2016**, *129*, 1087–1097. [CrossRef] [PubMed]
92. Frankia, E.; Morcia, C.; Pasquariello, M.; Mazzamurro, V.; Milc, J.A.; Rizza, F.; Terzi, V.; Pecchioni, N. Copy number variation at the *HvCBF4–HvCBF2* genomic segment is a major component of frost resistance in barley. *Plant Mol. Biol.* **2016**, *92*, 161–175. [CrossRef] [PubMed]
93. Svensson, J.T.; Crosatti, C.; Campoli, C.; Bassi, R.; Stanca, A.M.; Close, T.J.; Cattivelli, L. Transcriptome analysis of cold acclimation in barley *Albina* and *Xantha* mutants. *Plant Physiol.* **2006**, *141*, 257–270. [CrossRef] [PubMed]
94. Jurczyk, B.; Rapacz, M.; Budzisz, K.; Barcik, W.; Sasal, M. The effects of cold, light and time of day during low-temperature shift on the expression of *CBF6*, *FpCor14b* and *LOS2* in *Festuca pratensis*. *Plant Sci.* **2012**, *183*, 143–148. [CrossRef] [PubMed]
95. Archard, P.; Gong, F.; Cheminant, S.; Alioua, M.; Hedden, P.; Genschik, P. The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. *Plant Cell* **2008**, *20*, 2117–2129. [CrossRef] [PubMed]
96. Achard, P.; Renou, J.P.; Berthome, R.; Harberd, N.P.; Genschik, P. Plant DELLAs restrain growth and promote survival of adversity by reducing the levels of reactive oxygen species. *Curr. Biol.* **2008**, *18*, 656–660. [CrossRef] [PubMed]
97. Savitch, L.V.; Allard, G.; Seki, M.; Robert, L.S.; Tinker, N.A.; Hüner, N.P.A.; Shinozaki, K.; Singh, J. The effect of over-expression of two *Brassica* CBF/DREB1-liketranscription factors on photosynthetic capacity and freezing tolerance in *Brassica napus*. *Plant Cell Physiol.* **2005**, *46*, 1525–1539. [CrossRef] [PubMed]
98. Dahal, K.; Gadapati, W.; Savitch, L.; Singh, J.; Hüner, N.P.A. Cold acclimation and *BnCBF17*-over-expression enhance photosynthetic performance and energy conversion efficiency during long-term growth of *Brassica napus* under elevated CO_2 conditions. *Planta* **2012**, *236*, 1639–1652. [CrossRef] [PubMed]

99. Tronsmo, A.M. Effects of dehardening on resistance to freezing and to infection by T*yphula ishikariensis* in *Phleum pratense*. *Acta Agric. Scand.* **1985**, *35*, 113–116. [CrossRef]
100. Gay, A.P.; Eagles, C.F. Quantitative analysis of cold hardening and dehardening in *Lolium*. *Ann. Bot.* **1991**, *67*, 339–345.
101. Tompkins, D.K.; Ross, J.B.; Moroz, D.L. Dehardening of annual bluegrass and creeping bentgrass during late winter and early spring. *Agron. J.* **2000**, *92*, 5–9. [CrossRef]
102. Jørgensen, M.; Østrem, L.; Höglind, M. De-hardening in contrasting cultivars of timothy and perennial ryegrass during winter and spring. *Grass For. Sci.* **2010**, *65*, 38–48. [CrossRef]
103. Espevig, T.; Höglind, M.; Aamlid, T.S. Dehardening resistance of six turfgrasses used on golf greens. *Environ. Exp. Bot.* **2014**, *106*, 082–188. [CrossRef]
104. Hoffmann, L.; DaCosta, M.; Ebdon, J.S. Examination of cold deacclimation sensitivity of annual bluegrass and creeping bentgrass. *Crop Sci.* **2014**, *54*, 413–420. [CrossRef]
105. Ergon, Å.; Melby, T.I.; Höglind, M.; Rognli, O.A. Vernalization requirement and the chromosomal *VRN1*-region can affect freezing tolerance and expression of cold-regulated genes in *Festuca pratensis*. *Front. Plant Sci.* **2016**, *7*, 207. [CrossRef] [PubMed]
106. Fowler, D.B.; Chauvin, L.P.; Limin, A.E.; Sarhan, F. The regulatory role of vernalization in the expression of low-temperature-induced genes in wheat and rye. *Theor. Appl. Genet.* **1996**, *93*, 554–559. [CrossRef] [PubMed]
107. Limin, A.E.; Fowler, D.B. Low-temperature tolerance and genetic potential in wheat (*Triticum aestivum* L.): response to photoperiod, vernalization, and plant development. *Planta* **2006**, *224*, 360–366. [CrossRef] [PubMed]
108. Laudencia-Chingcuanco, D.; Ganeshan, S.; You, F.; Fowler, B.; Chibbar, R.; Anderson, O. Genome-wide gene expression analysis supports a developmental model of low temperature tolerance gene regulation in wheat (*Triticum aestivum* L.). *BMC Genom.* **2011**, *12*, 99. [CrossRef] [PubMed]
109. Mahfoozi, S.; Limin, A.E.; Fowler, D.B. Influence of vernalization and photoperiod responses on cold hardiness in winter cereals. *Crop Sci.* **2001**, *41*, 1006–1011. [CrossRef]
110. Mahfoozi, S.; Limin, A.E.; Ahakpaz, F.; Roustaii, M.; Ketata, H.; Fowler, D.B. Regulation of low-temperature tolerance in barley under field conditions in northwest Iran. *Can. J. Plant Sci.* **2005**, *85*, 587–592. [CrossRef]
111. Mahfoozi, S.; Limin, A.E.; Ahakpaz, F.; Fowler, D.B. Phenological development and expression of freezing resistance in spring and winter wheat under field conditions in northwest Iran. *Field Crop Res.* **2006**, *97*, 182–187. [CrossRef]
112. Dhillon, T.; Pearce, S.P.; Stockinger, E.J.; Distelfeld, A.; Li, C.; Knox, A.K.; Vashegyi, I.; Vágújfalvi, A.; Galiba, G.; Dubcovsky, J. Regulation of freezing tolerance and flowering in temperate cereals: The *VRN-1* connection. *Plant Physiol.* **2010**, *153*, 1846–1858. [CrossRef] [PubMed]
113. Mahfoozi, S.; Limin, A.E.; Fowler, D.B. Developmental regulation of low-temperature tolerance in winter wheat. *Ann. Bot.* **2001**, *87*, 751–757. [CrossRef]
114. Trischuk, G.; Schilling, B.S.; Low, N.H.; Gray, G.R.; Gusta, L.V. Cold acclimation, de-acclimation and re-acclimation of spring canola, winter canola and winter wheat: the role of carbohydrates, cold-induced stress proteins and vernalization. *Environ. Exp. Bot.* **2014**, *106*, 156–163. [CrossRef]
115. Stapleton, J.; Jones, M.B. Effects of vernalization on the subsequent rates of leaf extension and photosynthesis of perennial ryegrass (*Lolium perenne* L.). *Grass For. Sci.* **1987**, *42*, 27–31. [CrossRef]
116. Trevaskis, B. The central role of the *VERNALIZATION 1* gene in the vernalization response of cereals. *Funct. Plant Biol.* **2010**, *37*, 479–487. [CrossRef]
117. Fjellheim, S.; Boden, S.; Trevaskis, B. The role of seasonal flowering responses in adaptation of grasses to temperate climates. *Front. Plant Sci.* **2014**, *5*, 431. [CrossRef] [PubMed]
118. Cuesta-Marcos, A.; Muñoz-Amatriaín, M.; Filichkin, T.; Karsai, I.; Trevaskis, B.; Yasuda, S.; Hayes, P.; Sato, K. The relationships between development and low temperature tolerance in barley near isogenic lines differing for flowering behavior. *Plant Cell Physiol.* **2015**, *56*, 2312–2324. [CrossRef] [PubMed]
119. Deng, W.; Casao, M.C.; Wang, P.; Sato, K.; Hayes, P.M.; Finnegan, E.J.; Trevaskis, B. Direct links between vernalization response and other key traits of cereal crops. *Nat. Commun.* **2015**, *6*, 5882. [CrossRef] [PubMed]
120. Oliver, S.N.; Deng, W.; Casao, M.C.; Trevaskis, B. Low temperatures induce rapid changes in chromatin state and transcript levels of the cereal *VERNALIZATION1* gene. *J. Exp. Bot.* **2013**, *64*, 2413–2422. [CrossRef] [PubMed]

121. Greenup, A.G.; Sasani, S.; Oliver, S.N.; Walford, S.A.; Millar, A.A.; Trevaskis, B. Transcriptome analysis of the vernalization response in barley (*Hordeum vulgare*) seedlings. *PLoS ONE* **2011**, *6*, e17900. [CrossRef] [PubMed]
122. Alonso-Peral, M.M.; Oliver, S.N.; Casao, M.C.; Greenup, A.A.; Trevaskis, B. The promoter of the cereal *VERNALIZATION1* gene is sufficient for transcriptional induction by prolonged cold. *PLoS ONE* **2011**, *6*, e29456. [CrossRef] [PubMed]

agronomy

MDPI

Review

Molecular Regulation of Flowering Time in Grasses

Fiorella D. B. Nuñez [1] and Toshihiko Yamada [2,*]

[1] Graduate School of Environmental Science, Hokkaido University, Kita 10 Nishi 5, Sapporo, Hokkaido 060-0810, Japan; fiorepkm@gmail.com
[2] Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan
* Correspondence: yamada@fsc.hokudai.ac.jp; Tel.: +81-11-706-3644

Academic Editors: John W. Forster and Kevin F. Smith
Received: 29 November 2016; Accepted: 13 February 2017; Published: 20 February 2017

Abstract: Flowering time is a key target trait for extending the vegetative phase to increase biomass in bioenergy crops such as perennial C_4 grasses. Molecular genetic studies allow the identification of genes involved in the control of flowering in different species. Some regulatory factors of the *Arabidopsis* pathway are conserved in other plant species such as grasses. However, differences in the function of particular genes confer specific responses to flowering. One of the major pathways is photoperiod regulation, based on the interaction of the circadian clock and environmental light signals. Depending on their requirements for day-length plants can be classified as long-day (LD), short-day (SD), and day-neutral. The *CONSTANS* (*CO*) and *Heading Date 1* (*Hd1*), orthologos genes, are central regulators in the flowering of *Arabidopsis* and rice, LD and SD plants, respectively. Additionally, *Early heading date 1* (*Ehd1*) induces the expression of *Heading date 3a* (*Hd3a*), conferring SD promotion and controls *Rice Flowering Locus T 1* (*RFT1*) in LD conditions, independently of *Hd1*. Nevertheless, the mechanisms promoting flowering in perennial bioenergy crops are poorly understood. Recent progress on the regulatory network of important gramineous crops and components involved in flowering control will be discussed.

Keywords: flowering; *Arabidopsis*; grasses; photoperiod; circadian clock

1. Introduction

Global climate change and energy security issues have promoted interest in the production and increased availability of alternative energy sources. Lignocellulosic biomass is a promising feedstock source for biorefineries producing biofuel, which can mitigate greenhouse gas emissions [1–3] and reduce dependency on fossil oil [4,5]. Perennial C_4 bioenergy crops such as switchgrass (*Panicum virgatum* L.) and *Miscanthus* spp. provide good targets as non-edible plant species [6–8] having advantages with regard to land utilization and the avoidance of conflict with food security [9–11] to provide efficient production systems at low cost.

One of the most important traits in the plant life cycle is the timing of flowering, the floral transition between the vegetative and reproductive phases of plant development [12,13]. Consideration of flowering time is an important strategy in the cultivation of grain crops in northern latitudes. Early flowering is useful in regions where growing seasons are short to enhance grain yield stability by avoiding drought or adverse temperatures [14–16]. Flowering time is also a major determinant of biomass yield in perennial C_4 bioenergy crops, because delayed flowering time allows an extended period of vegetative growth and produces more biomass. Thus, earlier flowering will produce lower yields than late flowering in terms of feedstock production [17]. However, biomass potential also depends on environmental conditions. In switchgrass, lowland ecotypes that originate from southern areas flower later in high latitude areas, but the yield advantages of these southern switchgrasses are often not realized at northern latitudes due to high winter mortality [18].

Natural variation in flowering time is related to latitude in several plant species. Migration of plants into different latitudes often require the adoption of different signals to induce flowering and promote adaptive responses to diverse growing seasons [19]. Factors such as photoperiod and temperature that vary over large geographical scales are involved [20]. Plants possess an internal biological clock providing circadian rhythms that respond to fluctuations in day-length and thus anticipate upcoming seasonal changes [21] to regulate flowering. Depending on their requirements for day-length (light period in a 24-h cycle) to promote flowering, plants can be classified as long-day (LD) plants when photoperiod exceeds a critical day-length, short-day (SD) plants when photoperiod is shorter than a critical day-length and day-neutral plants when flowering occurs irrespective of day-length [22–24]. Winter annuals (e.g., wheat (*Triticum aestivum* L.)), biennials (e.g., sugar beet (*Beta vulgaris* L.)) and numerous perennials (e.g., orchardgrass (*Dactylis glomerata* L.)) are obligatory LD plants. These plants, however, flower only after vernalization during a cold period [23]. The molecular basis of flowering time regulation has been extensively studied using classical Quantitative Trait Loci (QTL) approaches in model plant species such as Arabidopsis thaliana [25–27], a LD plant, and rice (*Oryza sativa* L.), a SD plant [28]. Grasses have multiple pathways to control flowering time but only some of them are conserved in *Arabidopsis thaliana* (L.) Heynh. [29]. These studies have been crucial in establishing the multiple pathways that control flowering, of which the photoperiod pathway is of major importance [30]. The use of model species has played a major role in understanding the molecular mechanisms involved in flowering time to help in the genetic improvement of crop development. Nevertheless, little is known about the mechanisms promoting flowering in perennial C_4 bioenergy crops. In this review, we discuss recent progress concerning the regulatory network and components involved with flowering control in different species including C_4 grasses such as sorghum (*Sorghum bicolor* (L.) Moench), switchgrass and *Miscanthus* spp. To understand how plants initiate flowering is a crucial step in developing selection criteria in breeding programs of grasses used as bioenergy crops.

2. Conservation and Divergence in Flowering Pathways

2.1. Arabidopsis and Rice

In the last decades, studies on the model plant *Arabidopsis* have revealed that molecular mechanisms discovered in that species are evolutionally conserved in other species [15]. Genetic approaches in *Arabidopsis* have identified three genes that control flowering: *GIGANTEA (GI)—CONSTANS (CO)—FLOWERING LOCUS (FT)* [15,28]. Loss of function mutations in each gene for flowering control delay flowering under LD conditions but no effect is produced under SD [31]. *GI* is a key regulator of the photoperiodic pathway and in the evening promotes *CO* transcription under LD conditions [32]. The most extensively gene studied in *Arabidopsis* flowering is *CO* that confers LD responses. *CO* encodes a B-box zinc finger transcription factor and CCT domain genes that promote flowering under LD conditions and activate the expression of *FT* [33–35], a major component of the florigen that induces flower differentiation [29]. Its inactivation causes flowering delay, while its over-expression induces early flowering. *CO* and *FT* are expressed in the phloem and act there to promote flowering [31]. This signalling pathway is conserved in rice: *OsGI-Hd1-Hd3a* [28], mediated by LD responses. *OsGIGANTEA (OsGI)* acts an activator of *Heading date1 (Hd1)*, an ortholog of *CO*, and controls flowering time by modulating rhythmic flowering under SD [36]. *Hd1* encodes a zinc finger type transcriptional activator with the conserved CCT *(CO*, *CO-like*, *TIMING OF CAB EXPRESSION1* (*TOC1*)) domain [15,37]. In contrast to *Arabidopsis CO*, *Hd1* promotes *Heading date 3a (Hd3a)* expression in SD but expression is modified in LD conditions [38,39] where *Hd1* function is converted into a repressor. Rice involves at least two flowering pathways that control the expression of the florigens: *Hd1* that is conserved in rice and *Arabidopsis*, and *Early heading date 1*(*Ehd1*), without an ortholog in *Arabidopsis* [39,40] (Figure 1). Moreover, *Grain Number, Plant Height, and Heading Date7* (*Ghd7*) is unique in grasses [16]. *Ehd1* is a B-type response regulator that induces the expression of *Hd3a* in rice, conferring SD promotion of

flowering in the absence of a functional allele of *Hd1*. It also controls *Rice Flowering Locus T (RFT)* gene in LD conditions independently of *Hd1* [40]. In LD conditions *Hd1* acts as a flowering repressor inhibiting *Hd3a* expression but promotes its expression and subsequent flowering in *SD* [41]. *Ghd7* is a small protein with a CCT-domain that represses *Ehd1* expression and downstream *Hd3a/RFT1* expression in LD conditions to delay flowering [42–45]. Recent studies demonstrated that the interaction between *Ghd7* and *Hd1* can play a critical role in repressing *Ehd1*. Under SD conditions *Hd1* activates the expression of *Ehd1* at night but not in the day while under LD conditions *Hd1* represses its expression in the morning. Indeed, *Hd1* repressor activity requires a proper *Ghd7* function under LD conditions to repress *Ehd1* in the morning. In contrast, *Ghd7* can repress the expression of *Ehd1*, *Hd3a* and *RFT1* by itself under all photoperiod conditions [15].

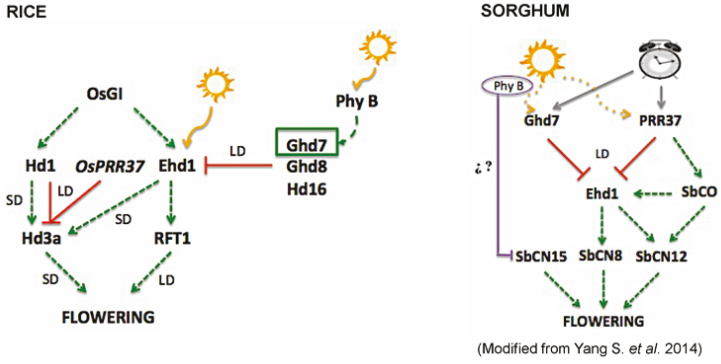


Figure 1. A simplified model of flowering time under short-day (SD) and long-day (LD) conditions in rice and sorghum. A dashed green arrow indicates transcriptional activation and a solid red line indicates transcriptional repression.

2.2. C_4 Grasses

Previous studies identified two floral activators in sorghum, a SD plant: *SbEhd1* and *SbCO*. *SbCO* is a homolog of the floral activator *CO* in *Arabidopsis* and an ortholog of *Hd1* in rice. It promotes early flowering in both LD and SD conditions, and increases the expression of *SbEhd1*, *SbCN8*, *SbCN12* and *SbCN15* [16]. Genetic analyses and expression studies in sorghum reveal that *SbCO* shares a conserved CCT-domain with *TOC1*, *PSEUDORESPONSE REGULATOR PROTEIN 37* (*PRR37*), *Ghd7* and *HEME ACTIVATOR PROTEINS (HAP)*. *SbCO* also increases expression of *SbEhd1*, a promoter of *Hd3a* in rice. In comparison with rice, *Ehd1* regulates positively the expression of *RFT1* to promote flowering; however, no ortholog of *RFT1* is present in the sorghum genome. *SbPRR37* (Ma_1) and *Ghd7* (Ma_6) inhibit flowering, reducing the expression of *SbEhd1* and *SbCN8/12* (florigens) under LD conditions, but not in SD (Figure 1). The ability of *SbPRR37* to inhibit their expression could be due to inhibition of *SbEhd1* or *SbCO*, activators of *SbCN8* and *SbCN12* expression [14,16]. The *PhyB* regulation of *SbCN15* expression may modify flowering time in a photoperiod-insensitive manner [46] (Figure 1). In switchgrass, the flowering time regulatory network is similarly to maize (*Zea mays* L.) and is regulated by both photoperiod-dependent and autonomous pathways. Some conserved flowering genes such as *FT-like gene* (*ZCN8* in maize) and *INDETERMINATE 1 (ID1)* have also been identified in the maize genome. The study of genes involved in flowering of switchgrass is relatively new. Hence, the functions of *FT-like* gene in switchgrass germplasm have not been clarified yet but may contribute to delayed flowering time as in maize [18]. Thus, the switchgrass *FT* homolog may have similar functions to the maize *FT* gene and is down-regulated by the expression of *LONG VEGETATIVE PHASE ONE (AtLOV1)* in switchgrass [18]. Overexpression of *AtLOV1* causes delayed flowering time in switchgrass but does not enhance cold tolerance as in *Arabidopsis* [18]. Sorghum is closely related

to *Miscanthus* spp., a promising candidate C_4 bioenergy crop in temperate climates. The *CO/Hd1* sequence in *Miscanthus sinensis* Andersson was identified as *MsiHd1* with two types of diverged loci, *MsiHd1a* and *MsiHd1b*. The *MsiHd1* gene encodes two conserved B-box zinc finger domains and a CCT domain. Two to five different alleles of *MsiHd1* were found in *Miscanthus* accessions from mainland Asia and from Japan, suggesting that *MsiHd1* consists of at least three loci in the *Miscanthus* genome with small differences in the number of functional alleles [38]. From preliminary data we identified at least three alleles suggesting that *MsiEhd1* has two loci in the *Miscanthus* genome, *MsiEhd1*a and *MsiEhd1b* in comparison to sorghum, rice and maize, which have only one gene. We also detected two loci in *MsiGhd7*. The current diploid *M. sinensis* evolved from genome duplication of its progenitor that was very close to a sorghum ancestor [46]. Gene duplication is a key mechanism in evolution because it can provide genes with new functions.

3. QTLs Analysis

To understand the complex genetic network of flowering in perennial ryegrass (*Lolium perenne* L.), a C_3 forage grass, a number of genes have been identified through QTL mapping, using different plant material and genetic maps and by sequence homology with *Arabidopsis*, rice and maize [47]. The genomic and phenotypic variations associated with perennial ryegrass *LpFT3*, an ortholog of *FT*, were assessed in a diverse collection of nine European germplasm populations, identifying a total of 7 haplotypes. The results indicated a significant association between allelic variation in the *LpFT3* gene and flowering time. Haplotype C was associated with early flowering and the A and B haplotypes with late flowering. The variations were identified in the predicted sequence and in non-coding regions, mainly within the 5′ region of the coding sequence which is strongly conserved [48]. Comparative analysis established close proximity between genetic markers related to the *DGL1*, *Ph1* and *OsPIPK1* ortholoci and the corresponding perennial ryegrass QTLs. This suggests that *DGL1* and *Ph1* ortholoci may provide candidate genes for the herbage yield-related QTLs on linkage group 3 (LG3). The physical location of the *OsPIPK1* gene (a heading date locus) was located at the 28.2 Mb position of rice chromosome 3, close to the predicted *CDO795* ortholocus (23.1 Mb). Further studies have suggested that the *CDO795*-linked heading date QTL was equivalent to a rice heading date QTL, *dth3.3* (Gramene QTL Acc. ID AQFE011). As a consequence, the perennial ryegrass *OsPIPK1* ortholocus may be related to the heading date QTLs on LG4 [49]. In addition, the major QTL in the F_2/WSC and ILGI perennial ryegrass populations was identified on LG7, which is associated with the position of the genes *Hd3a* and *Hd1*, two heading date genes of rice on chromosome 6. However, analysis of the ILGI population grown in Japan identified a QTL on LG4, but not the QTL reported on LG7 [50,51]. This result emphasizes the importance of adaptation in plants to the broad range of agro-environmental conditions in which they grow. In sorghum, three significant QTL associated with flowering time, *PHYB (Ma3), PHYC (Ma5)* [52] and *SbGHD7 (Ma6)* [14,52] were identified, through analysis of flowering variation in LD using an F_2 population, which explained *~50% of the* phenotypic variance for flowering time [52]. Recessive *ma3R* alleles from 58 M populations associated with *Ma3* QTL produced early flowering time phenotypes; however, dominant alleles of Sb*Ghd7 (Ma6)* and Sb*PRR37* act in an additive manner to delay floral initiation for ~175 days until day-lengths decrease below 12.3 h [14,52]. Sorghum accessions exhibit significant variations in flowering time in response to day-length. One QTL controlling photoperiod sensitivity was detected on chromosome 1 under SD, and one QTL controlling photoperiod insensitivity expression was detected on chromosome 4 under 12 h and natural photoperiod conditions, from the SSR markers *Xtxp61* and *Xtxp51* respectively [53]. Under LD, *a cross between tropical and temperate sorghums* (Sorghum *propinquum* (Kunth) Hitchc. × *S. bicolor* (L.) Moench), revealed one QTL *FlrAvgD1* located in chromosome 6 in a 10 kb interval, which accounted for 85.7% of the variation in flowering time. This interval contains a single annotated gene, *Sb06g012260*, which is a member of the *FT* family of transcription factors. *Sb06g012260* is unique to panicoids and suppresses flowering, although it is quite distant evolutionarily from other *FT* family members that are floral suppressors [54]. In *M. sinensis* five putative flowering QTLs were detected using the Multiple

QTL model (MQM) approach for plants grown in the years 2000 and 2001 [55]. Only QTL F12 was detected in both years on LG1, F11 and F13 were only detected in the first year while F14 and F15 were detected in the second year. So these QTLs may depend on interactions between genotype and environment. A genome-wide association study may be identified association with gene network in flowering time.

4. Circadian Clock and Photoperiod Response

The circadian clock plays an important role in seasonal flowering time regulation of angiosperms; photoperiodic time measurement is based on the interaction between the endogenous circadian clock and environmental light signals in *Arabidopsis* [56,57]. The plant circadian system consists of biochemical timing mechanisms that temporarily modulate the function of several signalling pathways to measures changes in day-length and promote suitable timing of flowering to maximize reproductive success [2,3,6,7,9,10,13–15,21,24,26,28,29,31,36,38,41,42,46,48,49,56–66]. The photoperiod response on flowering time varies among grasses. Barley (*Hordeum vulgare* L.) and wheat are LD plants, while rice and sorghum are SD plants [52]. Flowering is regulated through the *CO* and *FT* genes [66]. *GI* plays an important role in regulating the circadian clock and flowering, promoting *CO* gene expression and light response. The rice ortholog of *GI, OsGI,* is a positive regulator of *Hd1* expression under both SDs and LDs [24]. Mutation in *OsGI* reduced photoperiod sensitivity in rice [36] and affected the expression of *LATE ELONGATED HYPOCOTYL (LHY)* and several *PSEUDO RESPONSE REGULATOR (PRR)* genes. However, *PRR37* expression was not affected in the *osgi* mutant, suggesting independent control of heading date by these factors [66]. *Hd1* is predominantly regulated by the circadian clock through *OsGIGANTEA (OsGI)* [64] and possesses two contrasting functions in the regulation of the rice ortholog of *Arabidopsis FT* gene, *Hd3a*. The bi-functionally mechanism of *Hd1* involves the action of the red-light photoreceptor *phytochrome B (phyB)*, a primary cause of long-day suppression of flowering in rice [39,60]. Over-expression of *Hd1* causes a delay in flowering under SD conditions and a single extension of day-length decreases *Hd3a* expression consistently with the duration of daylight [44,59]. The repression of flowering by *Hd1* under LD conditions is enhanced by the kinase activity of *Heading date 6 (Hd6),* a gene encoding the α subunit of protein kinase CK2 (CK2α) [58]. *Hd6* is a QTL involved in photoperiod response in rice. To induce delayed flowering under LD conditions, *Hd6* requires the presence of functional *Hd1* alleles and plays a critical role in *Hd1* activity. Despite this, *Hd6* regulation is not mediated by changes in the circadian clock [37,65]. The rice genome contains two important genes for photoperiodic regulation: *Ehd1* and *Ghd7*, specific to grass species such as rice, maize and sorghum but absent in the *Arabidopsis* genome [44]. The expression of *Hd3a* is also regulated by *Ehd1* conferring SD promotion of flowering and controlling *FT-like* gene expression independently of the *Hd1/CO* photoperiodic flowering pathway [30,40] (Figure 1). *Hd1* and *Ehd1* expression are controlled by the circadian clock, although *Ehd1* is also regulated by both blue and red light. In sorghum, *SbCO* expression is not altered significantly in response to day-length. However, *Ghd7*, a floral repressor regulated by the circadian clock and light, represses the expression of *SbEhd1* and *SbCN8* [14]. *Ehd1* expression in rice is strongly repressed by *Ghd7* in LD conditions but in SD conditions *Ghd7* rarely affects flowering time [36].

5. Photoreceptors Involved in Flowering Time

Plants use the phytochrome system to regulate time of flowering and adjust growth based on the duration of dark and light periods (photoperiodism), while the spectrum of the light also affects flowering. Plants use many photoreceptors to detect the intensity and quality of light, including PHYTOCHROMES (PHY), which absorb the red and far-red region of the visible spectrum, and the CRYPTOCHROMES (CRY) [63]. *Arabidopsis* contains five PHYs (A-E), where accumulation of *CO* in LD is due to stabilization mediated by *phytochrome A* (*PHYA*), *cryptochromes* (*CRY1/2*) and *SUPPRESSOR OF PHYA-105 (SPA1)* [61]. However, it has been shown that PHYB signals delay flowering by destabilizing CO protein during the morning and have an inhibitory effect on *FT*

expression [60]. The phytochromes PHYA, PHYB, CRY1 and CRY2 are directly clock regulated under specific light conditions. For example, over-expression of the photoreceptor PHYA under SD conditions promotes flowering, but *phyA* mutants delay flowering in LD conditions. In contrast to *Arabidopsis*, rice and sorghum encodes three phytochromes (PHYA, PHYB and PHYC) [12,52], where *phyA* mutants of rice do not produce significant alterations in flowering time [52,63]. This is despite the high similarity between the *PHYA* locus in *Arabidopsis* rice, sorghum and maize [62] suggesting that a similar response would be expected. However, PHYA mutations in combination with PHYB or PHYC cause early flowering in rice [61]. In addition, studies have shown that PHYC plays an essential role in the acceleration of wheat flowering under LD photoperiods. Moreover, it is stable and functionally active even in the absences of other phytochromes, compared with rice and *Arabidopsis* [67]. Blue and far-red lights promote flowering in *Arabidopsis* and rice, acting through the action of PHYA, CRY1 and CRY2 photoreceptors in *Arabidopsis*, while red light delays flowering [12,33,43,67]. On the other hand, PHYB modulates the expression of genes in response to red light and is the main component of the shade-avoidance mechanism in *Arabidopsis*. *PhyB* mutants revealed that PHYB inhibits flowering under both LD and SD photoperiods, but an over-expression of PHYB in LDs results in early flowering [22]. PHYB is responsible for delayed flowering and *Hd3a* suppression in the presence of a night-break (NB) treatment and activates the *Hd1* expression in rice. The NB treatment is a short exposure to light in the middle of night and was widely used to understand the role of the circadian clock and light on flowering. In recent studies, *photoperiodic sensitivity 5* (*SE5*) and PHYB also suppress *Ehd1* expression, by suppressing *Oryza sativa CO-like4 (OsCOL4)* [39]. Rice mutants deficient in PHYB have reduced sensitivity to red light and are early flowering [43,60]. In contrast, *phyB* null mutations in wheat are connected with delayed flowering [24]. In sorghum, under LD, PHYB (Ma_3) is required for elevated expression of *SbPRR37* and *SbGHD7* during the evening to inhibit flowering (Figure 1). This response results in repression of *SbEHD1*, *SbCN12*, *SbCN8* and floral initiation. *Ghd7* represses *Ehd1* expression in response to the red light signal in the morning mediated by phytochromes. In SD conditions, PHYB may have a limited effect on the expression of these genes as peak *SbPRR37* and *SbGHD7* expression is highest in the morning and lowest during the evening compared with expression in LD. The inactivation of PHYB results in early flowering in LD [43,52].

6. Conclusions and Perspectives

Genetic analysis in *Arabidopsis* has allowed the identification of different pathways that promote flowering in response to environmental conditions and developmental regulation. The primary mechanism of the photoperiod pathway in plants is evolutionary conserved for flowering signalling. *CO* is the central regulator in promoting flowering and exhibits complex regulation. In addition day-length and the circadian clock control critical aspects of flowering. The effect of *GI* on flowering is associated with promoting expression of genes related to the circadian pathway. *Arabidopsis* is considered as a model plant to understand flower development while some grasses have defined their own responses and adaptation strategies. For example, *Ehd1* and *Ghd7* genes are unique in grasses in relation to the promotion and repression of flowering time respectively. Due to the lack of nucleotide information in *Miscanthus*, few genetic resources have been developed to clarify the relationship of the *Miscanthus* genome to its close relatives, sorghum and sugarcane (*Saccharum* spp.). Comparisons between the *Sorghum* genome and the genus *Miscanthus* reveal that whole genome duplication occurred in *Miscanthus* after its divergence from a common ancestor shared with sorghum. The base chromosome number of *Miscanthus* is approximately twice that of sorghum with nominally diploid and tetraploid species [46,68]. Analysis of natural variation in flowering in different ecotypes of grasses with economic value, such as *Miscanthus* spp. and switchgrass, is necessary to clarify the molecular network of flowering time control in these species. Through breeding programs, favorable alleles of QTLs then can then be efficiently introduced into elite cultivars to generate new varieties with high biomass productivity and beneficial adaptations to environmental changes.

Acknowledgments: We gratefully acknowledge Mervyn O. Humphreys, for critical reading of the manuscript. F.D.B.N. acknowledgments the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) scholarship for Master Course study at the Hokkaido University, Japan.

Author Contributions: F.D.B.N. written paper; T.Y. edited paper, mentored MS student (F.D.B.N.)

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Clifton-Brown, J.C.; Stampfl, P.F.; Jones, M.B. *Miscanthus* biomass production for energy in Europe and its potential contribution to decreasing fossil fuel carbon emission. *Glob. Chang. Biol.* **2004**, *10*, 509–518. [CrossRef]
2. Lewandowski, I.; Kicherer, A.; Vonier, P. CO_2-balance for the cultivation and combustion of *Miscanthus*. *Biomass Bioenerg.* **1995**, *8*, 81–90. [CrossRef]
3. Lewandowski, I.; Kicherer, A. Combustion quality of biomass: Practical relevance and experiments to modify the biomass quality of *Miscanthus* × *giganteus*. *Eur. J. Agr.* **1997**, *6*, 163–177. [CrossRef]
4. Fargione, J.; Hill, J.; Tilman, D.; Polasky, S.; Hawthorne, P. Land clearing and the biofuel carbon debt. *Science* **2008**, *319*, 1235–1238. [CrossRef] [PubMed]
5. Timothy, S.; Ralph, H.; Houghton, R.A.; Fengxia, D.; Amani, E.; Jacinto, F.; Simla, T.; Dermot, H.; Tun-Hsiang, Y. Use of US croplands for biofuels increases greenhouse gases through emissions from land-use change. *Science* **2008**, *319*, 1238–1240.
6. Oliver, R.J.; Finch, J.W.; Taylor, G. Second generation bioenergy crops and climate change: A review of the effects of elevated atmospheric CO_2 and drought on water use and the applications for yield. *GCB Bioenerg.* **2009**, *1*, 97–114. [CrossRef]
7. Somerville, C. Biofuels. *Curr. Biol.* **2007**, *17*, 115–119. [CrossRef] [PubMed]
8. Yuan, J.S.; Tiller, K.H.; Al-Ahmad, H.; Stewart, N.R.; Stewart, C.N. Plants to power: Bioenergy to fuel the future. *Trends Plant Sci.* **2008**, *13*, 421–429. [CrossRef] [PubMed]
9. Heaton, E.A.; Frank, G.; Dohleman, F.G.; Long, S.P. Meeting biofuel goals with less land: The potential of *Miscanthus*. *Glob. Chang. Biol.* **2008**, *14*, 2000–2014. [CrossRef]
10. Henry, R.J. Evaluation of plant biomass resources available for replacement of fossil oil. *Plant Biotechnol. J.* **2010**, *8*, 288–293. [CrossRef] [PubMed]
11. Tilman, D.; Socolow, R.; Foley, J.A.; Hill, J.; Larson, E.; Lynd, L.; Pacala, S.; Reilly, J.; Searchinger, T.; Somerville, C.; et al. Beneficial biofuels-the food, energy, and environment trilemma. *Science* **2009**, *325*, 270–271. [CrossRef] [PubMed]
12. Galvão, V.C.; Schmid, M. Regulation of flowering by endogenous signals. In *Advance in Botanical Research*; Fornara, F., Ed.; Elsevier: Amsterdam, The Netherlands, 2014; Volume 72, pp. 63–102.
13. Joshi, C.P.; DiFazio, S.P.; Chittaranjan, K. *Genetics, Genomics and Breeding of Poplar*; CRC Press: Boca Raton, FL, USA, 2011.
14. Murphy, R.L.; Morishige, D.T.; Brady, J.A.; Rooney, W.L.; Yang, S.; Klein, P.E.; Mullet, J.E. *Ghd7* (*Ma6*) represses sorghum flowering in long days: *Ghd7* alleles enhance biomass accumulation and grain production. *Plant Genome* **2014**, *7*, 1–10. [CrossRef]
15. Nemoto, Y.; Nonoue, Y.; Yano, M.; Izawa, T. *Hd1*, a *CONSTANS* ortholog in rice, functions as an *Ehd1* repressor through interaction with monocot-specific CCT-domain protein Ghd7. *Plant J.* **2016**, *86*, 221–233. [CrossRef] [PubMed]
16. Yang, S.; Weers, B.D.; Morishige, D.T.; Mullet, J.E. *CONSTANS* is a photoperiod regulated activator of flowering in sorghum. *BMC Plant Biol.* **2014**, *14*, 148. [CrossRef] [PubMed]
17. Grabowski, P.P.; Evans, J.; Daum, C.; Deshpande, S.; Barry, K.W.; Kennedy, M.; Casler, M.D. Genome-wide associations with flowering time in switchgrass using exome-capture sequencing data. *New Phytol.* **2017**, *213*, 154–169. [CrossRef] [PubMed]
18. Xu, B.; Sathitsuksanoh, N.; Tang, Y.; Udvardi, M.K.; Zhang, J.; Shen, Z.; Balota, M.; Harich, K.; Zhang, P.; Zhao, B. Overexpression of *AtLOV1* in switchgrass alters plant architecture, lignin content, and flowering time. *PLoS ONE* **2012**, *7*, e47399. [CrossRef] [PubMed]
19. Colasanti, J.; Coneva, V. Mechanisms of floral induction in grasses: Something borrowed, something new. *Plant Physiol.* **2009**, *149*, 56–62. [CrossRef] [PubMed]

20. Brachi, B.; Faure, N.; Horton, M.; Flahauw, E.; Vazquez, A.; Nordborg, M.; Roux, F. Linkage and association mapping of *Arabidopsis thaliana* flowering time in nature. *PLoS Genet.* **2010**, *6*, e1000940. [CrossRef] [PubMed]
21. Hayama, R.; Coupland, G. Shedding light on the circadian clock and the photoperiodic control of flowering. *Curr. Opin. Plant Biol.* **2003**, *6*, 13–19. [CrossRef]
22. Blázquez, M.A.; Piñeiro, M.; Valverde, F. Bases moleculares de la floración. Prensa científica. Temas 61. *Investig. Cienc.* **2011**, *416*, 29–36.
23. Gardner, F.P.; Pearce, B.; Mitchell, R. *Physiology of Crop Plants*, 1st ed.; Iowa State University Press: Ames, IA, USA, 1985.
24. Pearce, S.; Kippes, N.; Chen, A.; Debernardi, J.M.; Dubcovsky, J. RNA-seq studies using wheat *PHYTOCHROME B* and *PHYTOCHROME C* mutants reveal shared and specific functions in the regulation of flowering and shade-avoidance pathways. *BMC Plant Biol.* **2016**, *16*, 141. [CrossRef] [PubMed]
25. Coupland, G. Regulation of flowering time: Arabidopsis as a model system to study genes that promote or delay flowering. *Philos. Trans. R Soc. Lond. B Biol. Sci.* **1995**, *350*, 27–34. [CrossRef] [PubMed]
26. Simpson, G.G.; Dean, C. Arabidopsis, the Rosetta stone of flowering time? *Science* **2002**, *296*, 285–289. [CrossRef] [PubMed]
27. Valentin, F.L.; Van Mourik, S.; Posé, D.; Kim, M.C.; Schmid, M.; Van Ham, R.; Busscher, M.; Sanchez-Perez, G.F.; Molenaar, J.; Angenent, G.C.; et al. A quantitative and dynamic model of the Arabidopsis flowering time gene regulatory network. *PLoS ONE* **2015**, *10*, e0116973.
28. Peña, N. Caracterización de la Variación Natural de *Hd3a* y *RFT1* en Cultivares de Tipo Japónica de *Oryza sativa*. Máster's Thesis in Molecular and Cellular Biotechnology of Plants, The Technical University of Valencia, Valencia, Spain, 2013.
29. Mauro-Herrera, M.; Wang, X.; Barbier, H.; Brutnell, T.P.; Devos, K.M.; Doust, A.N. Genetic control and comparative genomic analysis of flowering time in *Setaria* (Poaceae). *G3 (Bethesda)* **2013**, *3*, 283–295. [CrossRef] [PubMed]
30. Wada, M.; Shimazaki, K.; Lino, M. *Light Sensing in Plants*; Springer Science & Business Media: New York, NY, USA, 2005; pp. 333–346.
31. Mizoguchi, T.; Wright, L.; Fujiwara, S.; Cremer, F.; Lee, K.; Onouchi, H.; Mouradov, A.; Fowler, S.; Kamada, H.; Putterill, J.; et al. Distinct roles of *GIGANTEA* in promoting flowering and regulating circadian rhythms in *Arabidopsis*. *Plant Cell* **2005**, *17*, 2255–2270. [CrossRef] [PubMed]
32. David, K.M.; Armbruster, U.; Tama, N.; Putterill, J. *Arabidopsis* GIGANTEA protein is post-transcriptionally regulated by light and dark. *FEBS Lett.* **2006**, *580*, 1193–1197. [CrossRef] [PubMed]
33. Max Planck Institute for Plant Breeding Research. Control of Flowering Time. Available online: http://www.mpipz.mpg.de/coupland (accessed on 8 September 2016).
34. Valverde, F. *CONSTANS* and the evolutionary origin of photoperiodic timing of flowering. *J. Exp. Bot.* **2011**, *62*, 2453–2463. [CrossRef] [PubMed]
35. Wang, C.C.; Chang, P.; Ng, K.; Chang, C.; Sheu, P.; Tsai, J. A model comparison study of the flowering time regulatory network in *Arabidopsis*. *BMC Syst. Biol.* **2014**, *8*, 15. [CrossRef] [PubMed]
36. Lee, Y.S.; An, G. *OsGI* controls flowering time by modulating rhythmic flowering time regulators preferentially under short day in rice. *J. Plant Biol.* **2015**, *58*, 137–145. [CrossRef]
37. Takahashi, Y.; Shomura, A.; Sasaki, T.; Yano, M. Hd6, a rice quantitative trait locus involved in photoperiod sensitivity, encodes the subunit of protein kinase CK2. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 7922–7927. [CrossRef]
38. Nagano, H.; Clark, L.V.; Zhao, H.; Peng, J.; Yoo, J.H.; Heo, K.; Yu, C.Y.; Anzoua, K.G.; Matsuo, T.; Sacks, E.; et al. Contrasting allelic distribution of *CO/Hd1* homologues in *Miscanthus sinensis* from the East Asian mainland and the Japanese archipelago. *J. Exp. Bot.* **2015**, *66*, 4227–4237. [CrossRef] [PubMed]
39. Sun, C.; Chen, D.; Fang, J.; Wang, P.; Deng, X.; Chu, C. Understanding the genetic and epigenetic architecture in complex network of rice flowering pathways. *Protein Cell* **2014**, *5*, 889–898. [CrossRef] [PubMed]
40. Doi, K.; Izawa, T.; Fuse, T.; Yamanouchi, U.; Kubo, T.; Shimatani, Z.; Yano, M.; Yoshimura, A. *Ehd1*, a B-type response regulator in rice, confers short-day promotion of flowering and controls *FT-like* gene expression independently of *Hd1*. *Genes Dev.* **2004**, *18*, 926–936. [CrossRef] [PubMed]
41. Song, Y.H.; Ito, S.; Imaizumi, T. Similarities in the circadian clock and photoperiodism in plants. *Curr. Opin. Plant Biol.* **2010**, *13*, 594–603. [CrossRef] [PubMed]

42. Itoh, H.; Nonoue, Y.; Yano, M.; Izawa, T. A pair of floral regulators sets critical day length for *Hd3a* florigen expression in rice. *Nat. Genet.* **2010**, *42*, 635–638. [CrossRef] [PubMed]
43. Tsuji, H.; Taoka, K.; Shimamoto, K. Regulation of flowering in rice: Two florigen genes, a complex gene network, and natural variation. *Curr. Opin. Plant Biol.* **2011**, *14*, 45–52. [CrossRef] [PubMed]
44. Tsuji, H.; Taoka, K. Florigen signaling. In *Signaling Pathways in Plants*; The Enzymes, Machida, Y., Lin, C., Tamanoi, F., Eds.; Elsevier: Amsterdam, The Netherlands, 2015; Volume 35, pp. 130–135.
45. Xue, W.; Xing, Y.; Weng, X.; Zhao, Y.; Tang, W.; Wang, L.; Zhou, H.; Yu, S.; Xu, C.; Li, X.; et al. Natural variation in *Ghd7* is an important regulator of heading date and yield potential in rice. *Nat. Genet.* **2008**, *40*, 761–767. [CrossRef] [PubMed]
46. Ma, X.F.; Jensen, E.; Alexandrov, N.; Troukhan, M.; Zhang, L.; Thomas-Jones, S.; Farrar, K.; Clifton-Brown, J.; Donnison, I.; Swaller, T.; et al. High resolution genetic mapping by genome sequencing reveals genome duplication and tetraploid genetic structure of the diploid *Miscanthus sinensis*. *PLoS ONE* **2012**, *7*, e33821. [CrossRef] [PubMed]
47. Fe, D.; Cericola, F.; Byrne, S.; Lenk, I.; Asraf, B.H.; Pedersen, M.G.; Roulund, N.; Asp, T.; Janss, L.; Jensen, C.S.; et al. Genomic dissection and prediction of heading date in perennial ryegrass. *BMC Genom.* **2015**, *16*, 921. [CrossRef] [PubMed]
48. Skøt, L.; Sanderson, R.; Thomas, A.; Skøt, K.; Thorogood, D.; Latypova, G.; Asp, T.; Armstead, I. Allelic variation in the perennial ryegrass *FLOWERING LOCUS T* gene is associated with changes in flowering time across a range of populations. *Plant Physiol.* **2011**, 1013–1022. [CrossRef] [PubMed]
49. Shinozuka, H.; Cogan, N.O.; Spangenberg, G.C.; Forster, J.W. Quantitative Trait Locus (QTL) meta-analysis and comparative genomics for candidate gene prediction in perennial ryegrass (*Lolium perenne* L.). *BMC Genet.* **2012**, *8*, 101. [CrossRef] [PubMed]
50. Armstead, I.P.; Turner, L.B.; Marshall, A.H.; Humphreys, M.O.; King, I.P.; Thorogood, D. Identifying genetic components controlling fertility in the outcrossing grass species perennial ryegrass (*Lolium perenne*) by quantitative trait loci analysis and comparative genetics. *New Phytol.* **2008**, *178*, 559–571. [CrossRef] [PubMed]
51. Yamada, T.; Jones, E.S.; Cogan, N.O.I.; Vecchies, A.C.; Nomura, T.; Hisano, H.; Shimamoto, Y.; Smith, K.F.; Hayward, M.D.; Forster, J.W. QTL analysis of morphological, developmental, and winter hardiness associated traits in perennial ryegrass. *Crop Sci.* **2004**, *44*, 925–935. [CrossRef]
52. Yang, S.; Murphy, R.L.; Morishige, D.T.; Klein, P.E.; Rooney, W.L.; Mullet, J.E. Sorghum phytochrome B inhibits flowering in long days by activating expression of *SbPRR37* and *SbGHD7*, repressors of *SbEHD1*, *SbCN8* and *SbCN12*. *PLoS ONE* **2014**, *9*, e105352. [CrossRef] [PubMed]
53. El Mannai, Y.; Shehzad, T.; Okuno, K. Variation in flowering time in sorghum core collection and mapping of QTLs controlling flowering time by association analysis. *Genet. Resour. Crop Evol.* **2011**, *58*, 983–989.
54. Cuevas, H.E.; Zhou, C.; Tang, H.; Khadke, P.P.; Das, S.; Lin, Y.; Ge, Z.; Clemente, T.; Upadhyaya, H.D.; Hash, T.C.; et al. The evolution of photoperiod-insensitive flowering in sorghum, a genomic model for panicoid grasses. *Mol. Biol. Evol.* **2016**. [CrossRef]
55. Atienza, S.G.; Ramirez, M.C.; Martin, A. Mapping QTLs controlling flowering date in *Miscanthus sinensis* Anderss. *Cereal Res. Commun.* **2003**, *31*, 265–271.
56. Hajdu, A.; Ádám, É.; Sheerin, D.J.; Dobos, O.; Bernula, P.; Hiltbrunner, A.; Kozma-Bognar, L.; Nagy, F. High-level expression and phosphorylation of phytochrome B modulates flowering time in *Arabidopsis*. *Plant J.* **2015**, *83*, 794–805. [CrossRef] [PubMed]
57. Izawa, T.; Mihara, M.; Suzuki, Y.; Gupta, M.; Itoh, H.; Nagano, A.J.; Motoyama, R.; Sawada, Y.; Yano, M.; Hirai, M.Y.; et al. *Os-GIGANTEA* confers robust diurnal rhythms on the global transcriptome of rice in the field. *Plant Cell* **2011**, *23*, 1741–1755. [CrossRef] [PubMed]
58. Hori, K.; Nonoue, Y.; Ono, N.; Shibaya, T.; Ebana, K.; Matsubara, K.; Ogiso-Tanaka, E.; Tanabata, T.; Sugimoto, K.; Taguchi-Shiobara, F.; et al. Genetic architecture of variation in heading date among Asian rice accessions. *BMC Plant Biol.* **2015**, *15*, 115. [CrossRef] [PubMed]
59. Ishikawa, R.; Aoki, M.; Kurotani, K.; Yokoi, S.; Shinomura, T.; Takano, M.; Shimamoto, K. Phytochrome B regulates *Heading date 1* (*Hd1*)-mediated expression of rice florigen *Hd3a* and critical day length in rice. *Mol. Genet. Genom.* **2011**, *285*, 461–470. [CrossRef] [PubMed]

60. Ishikawa, R.; Tamaki, S.; Yokoi, S.; Inagaki, N.; Shinomura, T.; Takano, M.; Shimamoto, K. Suppression of the floral activator *Hd3a* is the principal cause of the night break effect in rice. *Plant Cell* **2015**, *17*, 3326–3336. [CrossRef] [PubMed]
61. Jarillo, J.A.; Del Olmo, I.; Gómez-Zambrano, A.; Lázaro, A.; López-González, L.; Miguel, E.; Narro-Diego, L.; Sáez, D.; Piñeiro, M. Photoperiodic control of flowering time. *Span. J. Agric. Res.* **2008**, *6*, 221–244. [CrossRef]
62. Morishige, D.T.; Childs, K.L.; Moore, L.D.; Mullet, J.E. Targeted analysis of orthologous *phytochrome A* regions of the sorghum, maize, and rice genomes using gene-island sequencing. *Plant Physiol.* **2002**, *130*, 1614–1625. [CrossRef] [PubMed]
63. Mouradov, A.; Cremer, F.; Coupland, G. Control of flowering time: Interacting pathways as a basis for diversity. *Plant Cell* **2002**, S111–S130.
64. Naranjo, L.; Talon, M.; Domingo, C. Diversity of floral regulatory genes of japonica rice. *BMC Genom.* **2014**, *15*, 101. [CrossRef] [PubMed]
65. Ogiso, E.; Takahashi, Y.; Sasaki, T.; Yano, M.; Izawa, T. The role of casein kinase II in flowering time regulation has diversified during evolution. *Plant Physiol.* **2010**, *152*, 808–820. [CrossRef] [PubMed]
66. Shrestha, R.; Gómez-Ariza, J.; Brambilla, V.; Fornara, F. Molecular control of seasonal flowering in rice, *arabidopsis* and temperate cereals. *Ann. Bot.* **2014**, *114*, 1445–1458. [CrossRef] [PubMed]
67. Chen, A.; Li, C.; Hu, W.; Lau, M.Y.; Lin, H.; Rockwell, N.C.; Martin, S.S.; Jernstedt, J.A.; Lagarias, J.C.; Dubcovsky, J. PHYTOCHROME C plays a major role in the acceleration of wheat flowering under long-day photoperiod. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 10037–10044. [CrossRef] [PubMed]
68. Swaminathan, K.; Chae, W.B.; Mitros, T.; Varala, K.; Xie, L.; Barling, A.; Glowacka, K.; Hall, M.; Jezowski, S.; Ming, R.; et al. A framework genetic map for *Miscanthus sinensis* from RNAseq-based markers shows recent tetraploidy. *BMC Genom.* **2012**, *13*, 142. [CrossRef] [PubMed]

agronomy

MDPI

Review

Diurnal Leaf Starch Content: An Orphan Trait in Forage Legumes

Michael E. Ruckle [1], Michael A. Meier [1], Lea Frey [1], Simona Eicke [2], Roland Kölliker [1,3], Samuel C. Zeeman [2] and Bruno Studer [1,*]

[1] Molecular Plant Breeding, Institute of Agricultural Sciences, ETH Zurich, 8092 Zurich, Switzerland; mruckle@ethz.ch (M.E.R.); michael.meier@huskers.unl.edu (M.A.M.); lea.frey@usys.ethz.ch (L.F.); roland.koelliker@usys.ethz.ch (R.K.)

[2] Plant Biochemistry, Institute of Agricultural Sciences, ETH Zurich, 8092 Zurich, Switzerland; eickes@ethz.ch (S.E.); szeeman@ethz.ch (S.C.Z.)

[3] Molecular Ecology, Agroscope, 8046 Zurich, Switzerland

* Correspondence: bruno.studer@usys.ethz.ch; Tel.: +41-44-632-0157

Academic Editors: John W. Forster and Kevin F. Smith
Received: 30 November 2016; Accepted: 6 February 2017; Published: 20 February 2017

Abstract: Forage legumes have a relatively high biomass yield and crude protein content, but their grazed and harvested biomass lacks the high-energy carbohydrates required to meet the productivity potential of modern livestock breeds. Because of their low carbohydrate content, forage legume diets are typically supplemented with starch rich cereal grains or maize (*Zea mays*), leading to the disruption of local nutrient cycles. Although plant leaves were first reported to accumulate starch in a diurnal pattern over a century ago, leaf starch content has yet to be exploited as an agronomic trait in forage crops. Forage legumes such as red clover (*Trifolium pratense*) have the genetic potential to accumulate up to one third of their leaf dry mass as starch, but this starch is typically degraded at night to support nighttime growth and respiration. Even when diurnal accumulation is considered with regard to the time the crop is harvested, only limited gains are realized due to environmental effects and post-harvest losses from respiration. Here we present original data for starch metabolism in red clover and place it in the broader context of other forage legumes such as, white clover (*T. repens*), and alfalfa (*Medicago sativa*). We review the application of recent advances in molecular breeding, plant biology, and crop phenotyping, to forage legumes to improve and exploit a potentially valuable trait for sustainable ruminant livestock production.

Keywords: forage legumes; non-soluble carbohydrates; starch; water soluble carbohydrates

1. Introduction

In many areas of the world, ruminant-based livestock production has for centuries been a key component of sustainable agriculture and cultural traditions. These traditionally pasture and grassland-based agroecosystems maintain carbon balances, nutrient cycles, biodiversity, and water quality. However, in the past decades a growing global population with more purchasing power has placed a greater demand on livestock production. To meet demand, traditional forage-based production has ever-increasingly been intensified and replaced by confined feeding operations (CFOs) [1]. One of the primary reasons for this transition is that the high-energy feeds, which are required for maximum animal productivity, are difficult or too costly to distribute to livestock that graze on pasture lands. Because of the reduced efficiency of grassland-based livestock production, these traditional systems are not economically competitive with CFOs. The supplementation of local perennial forage with externally grown maize (*Zea mays*) and cereals has led to the disruption of the local nutrient, carbon, and water cycles that are maintained by the buffering capacity of perennial grassland agriculture.

Society is increasingly becoming aware of this environmental impact, and the challenges modern livestock production places on global water pollution, land use, and greenhouse gas emissions [1,2].

Although grassland-based forages can supply energy, protein, and fiber into animal diets, consistent nutritional quality required for optimal animal productivity is difficult to obtain. In the humid cool temperate to sub-tropic climates of Europe and Eastern North America, clovers and ryegrasses are major components of pastures and meadows. While perennial ryegrass (*Lolium perenne*) is valued for its high soluble sugar content, low lignin content, and high digestibility, red (*Trifolium pratense*) and white clover (*T. repens*) are valued for their high protein content. White clover is generally grown in pastures, while red clover in cut grasslands. Ryegrasses and clover are typically grown in mixed swards, because of their complementary nutritive and yield traits. Alfalfa (*Medicago sativa*) is the principle forage legume grown in dryer climates such as Western North America and the Mediterranean. It is typically grown in cut grasslands in monoculture for its high protein content and high digestibility. These legumes produce forage normally containing between 15% and 30% protein by dry weight (DW), depending on conditions during harvest [3,4].

The primary advantage of CFOs is the supplementation of readily transportable, starch rich grains such as barley (*Secale cereale*), wheat (*Triticum aestivum*), and maize into the animal's diet. Depending on animal species, age, and production type, the optimal total non-structural carbohydrate (NSC) concentration in the diet typically ranges between 25% and 60% [5,6]. Simple or soluble carbohydrates such as glucose and sucrose are readily broken down in the rumen, while NSCs with a more complex structure, such as starch, are more resistant to degradation. Due to their high digestibility, mono- and disaccharides improve animal performance by maintaining efficient rumen fermentation and the production of bacterial protein. Unlike simple sugars, up to 40% of ingested starch can pass through the rumen and eventually into the small intestine, because of its complex structure [6,7]. There, starch is broken down by glucosidases and amylases into glucose, which facilitates improved protein and energy uptake [6]. These combined effects on ruminal and intestinal digestion are the primary reasons why dietary soluble and insoluble carbohydrates are important for reaching the animal's productivity potential.

Although classically characterized as low in energy content, forage legumes are one of the most attractive crops for sustainable agriculture, because of their high cropping versatility, high digestibility, high leaf-to-stem ratio, high biomass yield potential, and high protein content [8]. Through their mutualistic relationship with soil rhizobia and their control of nodule number, forage legumes fix and furnish atmospheric nitrogen to companion species, buffer excess nitrogen to maintain soil quality with reduced input requirements, and subsequently reduce freshwater nitrate pollution [2,9]. Therefore, forage legumes are essential components of well managed pastures and grasslands that can coexist with native ecosystems more readily than modern farming systems based on arable crops [10]. As a major carbon sink, well managed perennial pastures and grasslands could play a major role in reducing global warming. Based on the modeling of global carbon balances, it was estimated that shifting annual crops toward managed perennial grasslands is part of a strategy to reverse current greenhouse gas levels using "climate smart soils" [11].

Despite their potential to contribute to sustainable agriculture, dedicated forage crops such as perennial grasses and legumes have not seen the same investment in research as maize or soy (*Glycine max*). Consequently, the genetic and genomic resources for breeding more productive forage crops have lagged behind high input crops. In the last two decades, forage crops have only seen yield improvements of 8%–10%, whereas in the same time period, yield improvements of maize and soy were in the range of 50%–60% [12,13]. This review aims to present the potential of integrating modern genetic tools and resources, the current knowledge of plant biology, and contemporary breeding strategies to address the low energy content in forage legumes. Although this review addresses concepts that in principle can be applied to all forage legumes, original research from red clover is presented as a model.

2. Energy Content in Forage Crops

As a quality trait in forages, NSCs are differentiated from structural carbohydrates, which are major components of digestible fiber. NSCs are further divided into water soluble carbohydrates (WSCs) and starch. In forage legumes, WSCs are primarily glucose, fructose, and sucrose, but osmolytes such as pinitol can make up a significant fraction of the WSCs during stress [14–16]. Although NSCs have received little to modest attention in forage legumes, perennial ryegrass varieties with high sugar content, such as "AberMagic" [17,18], have been readily accepted by the pasture livestock community and have been a commercial success. Unlike forage legumes, perennial ryegrass accumulates fructans in the vacuole. These high fructan varieties can accumulate up to 30% more WSCs than traditional varieties [19]. Although reported to increase animal productivity by up to 10%–15% in comparison to traditional varieties [20,21], high sugar grass diets only marginally increased animal productivity in comparison to grain supplementation, because the energy from the soluble sugars is rapidly converted to bacterial protein in the rumen [22]. Therefore, further increases in forage WSC concentration will not replace starch rich grain supplementation, because unlike starch, sugars do not contribute to the synergistic action in the animal's small intestine required for maximum production.

Starch is an important form of assimilated carbohydrates in forage legume herbage, which diurnally accumulates in the leaf and nocturnally mobilizes to support growth. Reported starch concentration in forage legumes is inconsistent, but typically ranges from 0.5% to 10% of DW [23–25]. This 20-fold disparity is in part due to varying sampling strategies, weather conditions, the time of day the samples were harvested, postharvest treatment, and genotypic variation. The accuracy and standardization of quantification protocols also limits direct comparison of reported starch values. Moreover, genetic variation is often not addressed, as a majority of reports focus on bulked field samples and not on single genotypes. Recently, conventional breeding programs in alfalfa successfully selected for increased NSC concentration, and synthetics with a 10%–20% higher starch concentration were developed [26,27]. However, due to the low harvestable starch concentration of less than 10% the full benefit to animals' diets cannot be realized and the trait has so far been elusive for forage legume breeders.

3. Physiology, Biochemistry, and Genetics of Starch Metabolism in Model Plants

Plant carbohydrates and their metabolism have received considerable research attention because of their high energy density, economic value, and dietary importance [28]. Leaf starch synthesis, structure, and degradation is best understood in the model plant arabidopsis (*Arabidopsis thaliana*), where ample knowledge on starch physiology, mutants, genes, and quantitation methods has been developed for over two decades. In leaves, starch and most of the protein is located in the chloroplast. The majority of leaf based protein content is chloroplast derived (from Rubisco and photosynthetic enzyme complexes) [29]. As a direct product of photosynthesis, starch accumulates in the chloroplast and it occupies a substantial proportion of the chloroplast volume at the end of the day (ED) (Figure 1). Starch can accumulate to significant levels, and up to 25% of the leaves' dry biomass can be starch [30]. Ultimately, this starch is typically mobilized during the night to facilitate growth and cellular respiration, when, at the end of the night (EN), the diel (or daily) cycle repeats.

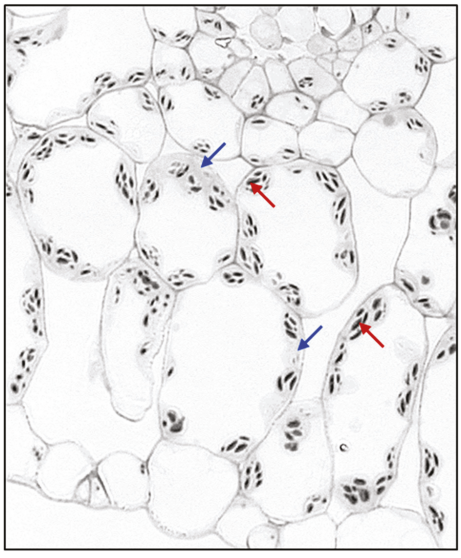


Figure 1. Light micrograph of starch granules in chloroplasts of arabidopsis (*Arabidopsis thaliana*) mesophyll cells harvested at the end of the day. Protein dense chloroplasts (the light-greyregions) and starch granules (the black regions) are indicated with a blue and a red arrows, respectively. Details are described in Supplemental Methods.

Leaf starch directly accepts and stores fixed carbon from photosynthesis and the Calvin cycle, and its diurnal accumulation and depletion ultimately depends on the balance of starch synthesis and degradation. The enzymes required for starch synthesis and degradation are well understood in leaves, cereal endosperms, roots, and tubers in several plant species [28,31]. Although there are similarities between different starch forms, the regulation, structure, and utilization are distinct. Much of the detailed knowledge of leaf starch metabolism was derived from mutant studies in arabidopsis. As illustrated in Figure 2 in detail, starch metabolism consists of four basic processes; synthesis, remodeling, degradation, and sugar export. Mutants in starch synthesis accumulate less starch, mutants in starch remodeling loose starch structure, degradation mutants accumulate starch, and export mutants accumulate soluble sugars during granule degradation [28,32,33]. Degradation mutants are particularly interesting for developing a high starch trait, because mutants in the enzymes that solubilize the granule surface of starch or hydrolyze glucan chains, such as GWD1, SEX4, and BAM1/3/4, can accumulate up to 8-fold more starch than wild type arabidopsis [30,34,35].

Detailed knowledge of genes, enzymes, and processes involved in starch metabolism are key for developing modern breeding strategies. Analysis of QTL for primary leaf metabolism traits in arabidopsis have shown that a portion of the genetic variation causing the metabolite differences corresponds to genetic variants of known biosynthetic enzymes [36,37]. In the model forage legume birdsfoot trefoil (*Lotus japonicus*), this knowledge was used to guide a Targeted Induced Lesions In Genomes (TILLING) approach, which identified both *starchless* and *starch excess* mutants [38].

Remobilization of leaf starch serves to support both nocturnal respiration and facilitate continued leaf and root growth in the dark. Growth throughout the combined diurnal and nocturnal, or diel cycle is determined by carbohydrate availability, carbohydrate deposition, construction of the cell wall, and cell wall expansion. Since quantitation of leaf growth only directly measures cell wall expansion, diel growth patterns do not always reflect carbon availability [31]. For example in wild type arabidopsis, leaf relative growth rates (RGR) increase during the late night until dawn, when a burst of cell wall expansion is observed, presumably following carbohydrate deposition in the late

day and early night [39]. Alternatively in a *starchless* mutant, RGR increase in late afternoon when soluble sugars accumulate and carbohydrate availability is high, and decrease at night and dawn [39]. In soy, RGR correlates more directly with carbohydrate concentration, which peaks at ED, and linearly decreases to the lowest level at dawn [40]. Taken together utilization of leaf starch provides flexibility to plant growth strategies during the diel cycle, but how starch concentration and this added flexibility relates to overall biomass potential is still not well understood.

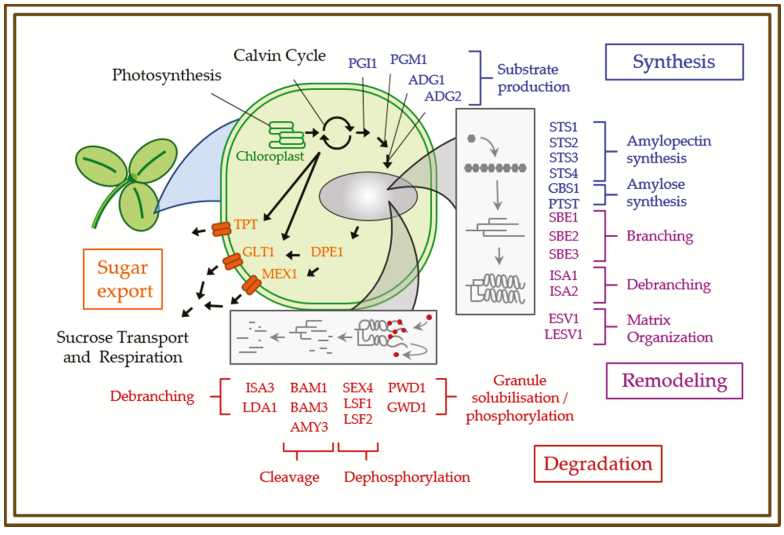


Figure 2. The synthesis and degradation of the complex semi-crystalline structure of starch is orchestrated by a series of enzymes that are involved in four basic processes. Synthesis: fructose-6-P from the Calvin cycle is isomerized (PGI1; PGM2) before it is activated with a UDP moiety (ADG1; ADG2). Soluble and granule bound starch synthases polymerize UDP-Glucose to produce amylose and amylopectin (STS1-4; GBS). Remodeling: the crystalline helical structure of starch requires the amylopectin to be remodeled, which is carried out by branching enzymes (SBE1-3) and debranching isoamylase (ISA1-2). Degradation: To release the soluble sugars from the starch granule, first the amorphization of the crystalline structure must be carried through glucan phosphorylation, which is done by glucan dikinases (GWD1; PWD1). Once amorphous, phosphatases remove the phosphate from the glucan chain (SEX4, LSF1-2), allowing hydrolyzing enzymes such as the β-amylases, (BAM1-3), α-amylases (AMY3), isoamylase (ISA3), and limit dextranase (LDA1) to cleave the glucan polymer. Export: Prior to export, oligomaltosaccharides are disproportioned to glucose and maltose (DPE1). These soluble sugars are exported out of the chloroplast by the maltose exporter (MEX1) and glucose transporter (GLT1). Enzyme nomenclature is based on arabidopsis for the enzymes that are conserved in higher plants.

In arabidopsis, a broad analysis of metabolites in natural accessions found that genetic variation in starch concentration inversely correlates with biomass. Because of this inverse correlation, starch concentration is hypothesized to be a major integrator of plant growth through a regulatory network that balances carbon availability with growth [41]. This hypothesis is supported by the reduced growth of both *starch excess* and *starchless* mutants, which has been observed in various species [31,38]. The reduction in growth of the *starchless* mutants is thought to come from nighttime starvation. In *starch excess* mutants, the unused sequestered carbon delays the exponential increase in biomass, which is observed during the development of wild type plants [31]. Because plant metabolism is highly plastic, one hypothesis would be that weak mutations that only moderately disrupt starch metabolism

would be tolerated without a growth penalty. In birdsfoot trefoil and maize, disruption of the starch synthesis and degradation, respectively, led to altered starch concentration, but without an observed biomass penalty, suggesting that the reliance of growth on starch is complex and likely varies among species [38,42]. Although starch is a central component of plant metabolism, the potential to develop a high starch trait could be achieved by exploiting this innate flexibility between starch metabolism and growth.

4. The Potential of Leaf Starch Content as a Trait in Forage Legumes

We adapted the knowledge from model species to assess the genetic potential of starch production in forage legumes. A controlled growth regime, optimized sampling strategy, and accurate quantification method was used to determine leaf starch concentration in single red clover genotypes. Leaf starch accumulation was estimated with a visual screen based on iodine staining of 32 plants derived from a genetically diverse population grown under simulated partially sunny conditions. To accurately quantify leaf starch, eight genotypes were selected based on their iodine stained phenotype and vegetatively propagated into biological replicates. These replicates were harvested at ED and EN (Figure 3). In these eight genotypes, the observed variation in leaf starch concentration at ED ranged between 6% and 35% by DW, and the variation in leaf starch concentration at EN ranged from 1% to 15% by DW. Moreover, two basic patterns of accumulation were observed in this population. The majority of the genotypes degraded almost all of their starch at night and their ED starch concentration was dependent on diurnal accumulation. These genotypes were designated diurnal dependent starch accumulation and two examples are presented, *TpDST05* and *TpDST20*. Two of the eight genotypes degraded less than 50% of their starch at night, and their ED starch concentration was more independent from diurnal accumulation and more dependent on starch retention at night (Figure 3). These genotypes were designated diurnal independent starch and two examples are presented, *TpDIST31* and *TpDIST27*. Although the diel usage of starch is well studied in model species, little is described about the genetic variation in diurnal starch accumulation and nocturnal mobilization. These diurnal dependent and independent starch concentration patterns illustrate the genetic potential of red clover to not only accumulate significant levels of starch during the day, but also the genetic potential of some genotypes to maintain high starch levels during the night.

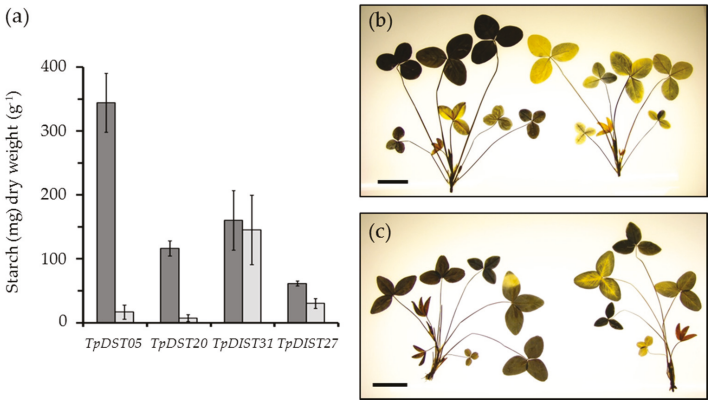


Figure 3. Diel patterns of leaf starch accumulation in selected red clover genotypes. (**a**) The starch concentration in four genotypes harvested at the end of the day (ED; dark-gray bars), and at the end of the night (EN; light-gray bars). Plants were grown at a fluence rate of 250–350 μmol·m^2·s^{-1} in a 14:10 light/dark and 14–16 °C/19–23 °C diel cycle. Error bars indicate 95% confidence intervals (N = 8); (**b**,**c**) Iodine staining of ED (left) and EN (right) plants of genotypes *TpDST05* (b) and *TpDIST31* (c). Details are described in Supplementary Methods.

The reduced nocturnal starch degradation observed in the genotypes with weak or no diurnal response is consistent with reduced mobilization, respiration, or growth [31]. Respiration in plants is temperature dependent, as reduced respiration rates are observed at lower temperatures [43]. Although in forage legumes little is known about the direct effect of night-time temperature on starch concentration, in other legume crops such as soy and white clover, studies have shown that lower temperatures lead to reduced degradation of starch at night. The temperature at which starch turnover is reduced differs between species, as reduced starch turnover occurs at 17 °C in soy, and at 7 °C in white clover [44,45].

One of the major challenges with assessing starch concentration is its dependency on the environment. Light is arguably the greatest determinant of starch concentration; During intense light, such as direct sunlight, starch accumulates to a greater degree, but under low light conditions, less starch is produced [46]. To determine the effect of light on starch concentration in red clover, four of the genotypes from above were re-grown in simulated cloudy conditions and compared to plants grown in simulated partially sunny conditions (Figure 4). The total leaf starch concentration in the plants grown in the cloudy conditions was between 0.5% and 1.0% by DW at ED and undetectable at EN, while it reached 16% and 4% at ED and EN, respectively under partially sunny conditions (Figure 4a). Taken together, the potential of red clover to accumulate starch is strongly dependent on light intensity.

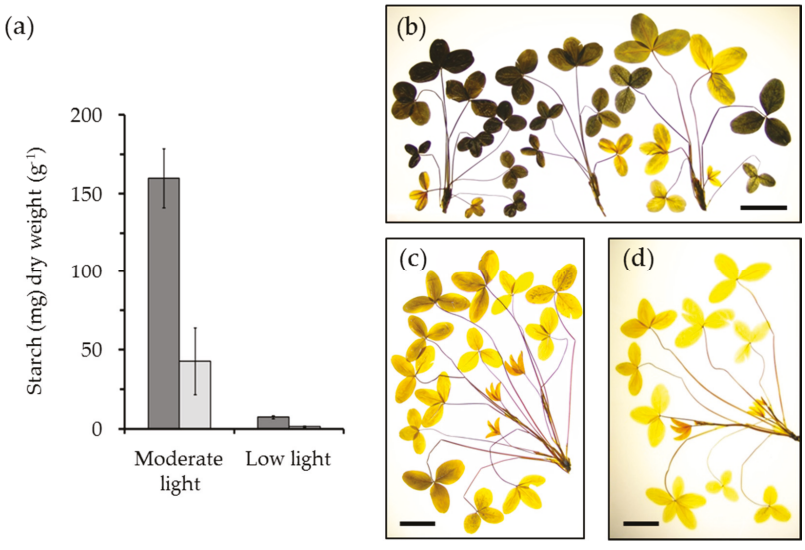


Figure 4. Starch concentration in red clover is dependent on the light intensity. (**a**) The starch concentration in *TpDST34* harvested at the ED (dark-gray bars), and at the EN (light-gray bars). Plants of *TpDST34* were grown under a moderate fluence rate of 250–350 μmol ·m^2· s^{-1} or a low fluence rate of 50–150 μmol·m^2·s^{-1}. Other growth conditions were the same as described in Figure 3. Error bars indicate 95% confidence intervals (N = 8); (**b**–**d**) Iodine staining of moderate light grown ED (left 2 plants) and EN (right) *TpDIST34*, and low light ED (c) and EN (d) *TpDIST34*. Details are described in Supplementary Methods.

Another major challenge with utilizing leaf starch content as a trait in forage legumes is the influence of harvest time and postharvest loss. As a diurnal trait, the peak starch concentration is observed at ED. This time is typically less convenient for farmers to cut forage fields, and the late cutting time also reduces the critical drying time in the sun. Although less feasible, studies have

demonstrated that hay produced from alfalfa fields harvested later in the evening contain 20% more NSCs than equivalent fields cut in the morning [47]. This higher NSC content was primarily attributed to higher starch concentration. The higher NSC content from the evening cutting resulted in higher digestibility, and when evening cut forage was fed to dairy cows, they produced up to 5% more milk compared to the morning cut [47,48]. Breeding for traits, such as diurnally independent starch turnover, as seen in *TpDIST27* and *TpDIST31* genotypes, would increase the harvestable starch content.

Unfortunately, maximizing starch concentration in forage legumes through breeding and adjustment of cutting time is only marginally useful, due to immense postharvest losses. After cutting, plants are unable to carry out transpiration and the production of carbohydrates from photosynthesis is not possible. Moreover, cut forage immediately begins to respire the produced energy. In forage crops, the yield loss due to postharvest respiration can be 10%–15% of the DW [49]. This yield loss is primarily due to a loss in total NSC concentration, as sugars and starch are readily mobilized after cutting to facilitate respiration [49]. Therefore, most of the energy potential of forage legumes is lost during harvest. Although the development of better varieties that accumulate more starch will be important, breeding for reduced postharvest respiration and loss will be crucial for successfully developing high starch content in red clover. Such strategies would include specifically targeting and selecting genotypes with reduced postharvest respiration rates, and reduced postharvest starch mobilization.

Because of starch's physiological relationship to growth, improvement of a leaf starch trait could appear counterproductive in forage crop species, since it could come at the cost of reduced biomass yield. To test the relationship between biomass and starch concentration in red clover, 128 genotypes were analyzed for ED starch concentration and biomass. Phenotypic variation in starch concentration ranged from 10% to above 40%, and a 10-fold difference in biomass was observed (Figure 5). Little to no correlation between starch concentration and biomass in this population was observed (Pearson $r = 0.1223$, $p = 0.169$) (Figure 5). Although under controlled conditions, phenotypic variation in starch concentration is less than the genetic variation observed between genotypes (Figure 3), the lack of phenotypic correlation between biomass and starch concentration does not imply a genetic correlation to distinguish these as independent traits. This observation, along with observations on starch mutants in birdsfoot trefoil [38], raises the hypothesis that forage legumes may have more flexibility in the regulatory and metabolic networks that link starch with growth.

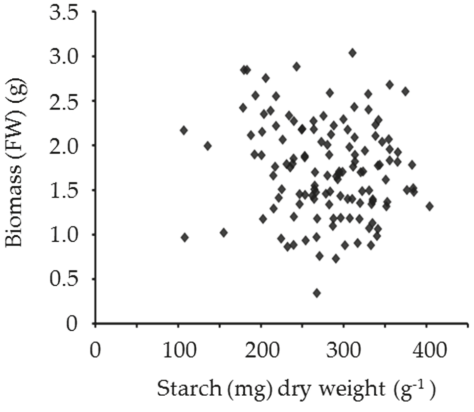


Figure 5. Biomass yield does not correlate with starch concentration in 128 red clover genotypes. The starch concentration and biomass were measured in 50 day old red clover plants grown under controlled growth conditions with a fluence rate of 200 μmol·m^2·s^{-1} in a 14:10 light/dark, 13–14 °C/18–19 °C diel cycle and harvested at the end of the day. Details are described in Supplementary Methods.

Although, diurnal accumulation of starch in the leaves facilitates diel growth, forage legumes also accumulate long term storage of starch in heterotrophic tissues, such as roots and stolons. This starch is important for the remobilization of carbon during stress or during specific stages of development to support growth [16]. In forage legumes, remobilization of starch in the roots and stolons is critical for regrowth after cutting, which is a key determinant of overall biomass potential [50]. Although there is overlap between the genes, and enzymes that control starch metabolism in leaves and heterotrophic tissue, their regulation is independent, leading to distinct traits, and allowing for the positive selection for leaf starch concentration, without impacting forage regrowth after cutting [28].

Based on the analysis of single genotypes in red clover, it is likely that forage legumes share a similar genetic potential to produce different amounts of leaf starch. Although a leaf starch concentration of over 30% by DW was observed in some red clover genotypes, to be a practical trait, the dependence on the environment and harvest loss must be feasibly addressed.

5. The Potential of Modern Genomic and Phenomic Tools to Develop Leaf Starch as a Trait in Forage Legumes

In the past decade, the scientific resource gap between forage crops, model species, and high value crops has narrowed due to an ever-increasing plant biology knowledge-base and better genomic tools. Therefore, many benefits of functional genomics and molecular breeding techniques are now accessible to forage crop breeders. Alfalfa, red clover, and white clover have described genetic linkage maps and partial genome sequences based on assembled contigs and scaffolds [51–55]. Contig mapping and gene annotation of these genomes has been aided by cDNA sequencing, and genomic synteny mapping using well defined legume genomes, such as *M. truncatula* [53,55–57]. Given these genomic tools, modern breeding methods such as genotyping by sequencing (GBS), genome-wide association study (GWAS), marker assisted selection (MAS), and TILLING can be integrated into traditional trait selection pipelines [58]. Combined with progress made in the field of starch metabolism, these methods have the potential to address the issues that have made starch content so far an elusive trait.

For complex traits such as leaf starch content, MAS can play an important role to overcome the limitations of conventional selection [59]. A basic prerequisite for MAS is the identification of genomic regions linked to the trait of interest. Under controlled conditions, quantitative trait loci (QTL) for leaf starch concentration have been identified in arabidopsis [36]. In the field, GWAS are challenging for complex traits, because the identified QTL are dependent on environmental context, but strategies have been developed to identify QTL for diel traits that are influenced by light and temperature, similar to starch [60]. Using ecophysiological modeling, QTL were identified for diel leaf elongation rates in maize [61]. Such ecophysiological modeling could be useful to apply the appropriate context to starch concentration QTL identified in the field.

An alternative approach would be to influence starch metabolism through mutagenesis. Starch degradation mutants would have less variation in genetic background, time of day, and environment [62]. Starch degradation mutants identified through TILLING in birds-foot trefoil maintained high levels of leaf starch concentration throughout the diel cycle [38]. TILLING has been adapted to breeding programs in many crop species, but it remains challenging to identify induced mutations in populations that are genetically heterogeneous, such as forage legumes [63,64]. Because transformation methods exist for alfalfa and red clover, CRISPR/Cas9 mediated mutagenesis has the potential to replicate well described alleles in starch degradation from other species and overcome challenges that TILLING has in heterogeneous genomes [65,66]. Such approaches have been used to specifically target branched amino acid metabolism to confer herbicide resistance in maize [67].

In addition to targeted genetic approaches, a rapid, cost-effective, high-throughput, phenotyping method is needed to allow for developing starch content into a selectable trait. Such a method would have to be field based and allow for assessing the trait throughout the season to compensation for seasonal variation. Due to the lack of uniformity of starch concentration between leaves (Figure 3b,c), sample homogenization is a major bottleneck for accurate inferred or biochemical analysis. Therefore,

technologies such as hyperspectral imaging offer the greatest promise for efficiently and accurately measuring starch in the field as has been shown for WSC in wheat [68].

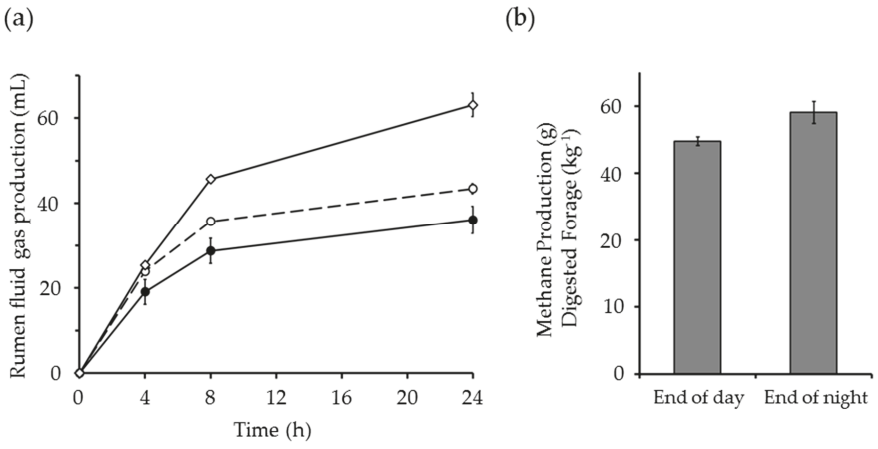


Figure 6. Diurnal starch accumulation increases the digestibility of red clover. The same tissue that was analyzed in Figure 3a for genotype *TpDST20* was analyzed by the Hohenheim gas test. (**a**) Gas production from rumen fluid digestion of *TpDST20* samples harvested at the ED (empty circles), or the EN (filled circles). Gas production from the Hohenheim concentrate standard is presented as a reference (empty diamonds); (**b**) Methane production from ED and EN samples from Figure 6a after 24 h of digestion. Error bars represent 95% confidence intervals (N = 5). Details are described in Supplementary Methods.

6. The Potential to Reduce Concentrate Feeding

Forage legumes such as red clover have the potential to accumulate one third of their dry matter biomass as starch, but due to environmental, harvest, and post-harvest limitations, a 10%–15% final starch proportion in post-harvest biomass is optimistic. An amount of 10%–15% starch is 2- to 10-fold greater than the starch concentration reported in bulk field samples of red clover [23,24,69]. This concentration of starch in an animal's diet is sufficient to promote ruminant microbial activity, better protein absorption, and ultimately better productivity with less nitrate and methane emissions [13,70]. Although leaf starch has been reported to be completely fermented during in vitro rumen assays, much of the research regarding the role of starch in the small intestine has focused on grain supplements [6,23]. An amount of 10%–15% dietary starch is adequate to support the ruminant microbial activity required for better protein absorption, and for the high protein content of forage legumes to be fully utilized [3,4]. Higher protein absorption rates facilitated by 10%–15% grain starch supplementation can lead to 25% more daily animal productivity, 30% reduced methane emissions and 20% reduced nitrate emissions [71–73].

To determine whether red clover leaf starch improves rumen fermentation, we tested the in vitro digestibility of one particular red clover genotype which accumulates around 12% of its DW as starch at ED and little to no starch at EN under standard growing conditions (Figure 3a). The ED and EN samples were assessed for digestibility in comparison to a high energy concentrate using the Hohenheim Gas Test [74]. Based on the gas produced after 24 h of rumen fluid digestion, the starch containing ED samples had 20% more digestible organic material than the EN samples (Figure 6a). Because the rumen degrades compounds at different rates, the major class of compounds leading to the digestibility gain can be estimated. As observed in the concentrate control, soluble sugars and proteins are rapidly digested in the first 4 h, starch is degraded for up to 8 h, and structural carbohydrates such as cellulose are degraded beyond 8 h [74]. Therefore, the increased digestibility observed between 4 h and 8 h, is

consistent with higher starch concentration of the ED sample compared to the EN sample (Figure 6a). The slower rumen digestion of leaf starch should allow it to move to the small intestine, where it is hypothesized to have more impact on animal productivity [6]. Moreover, because the ED sample is more efficiently digested than the EN sample, the ED sample produced almost 15% less methane per unit of digested of organic material than the EN sample (Figure 6b). Since in vivo rumen digestibility is a strong measure of the productivity potential of a feed, diurnal leaf starch has the potential to increase productivity and reduce grain starch supplementation.

7. Conclusions

The goal of this review was to illustrate the potential that forage legumes have to produce starch, and to dissect the major factors that currently limit the development of a high starch trait. For decades, traditional breeding strategies to increase NSC content, decrease post-harvest dry matter loss, and increase digestibility have resulted in minimal gains in alfalfa starch content [26]. Arguably, these minimal gains are due to the complexity of the trait. The lack of success in selecting a high starch trait has led it to receiving much less attention than other forage legume traits in recent years. Today, the merging of novel phenotyping platforms, molecular breeding tools, knowledge of plant biology, and genetic resources with traditional breeding allows for a greater level of creativity to develop novel traits that have never seen selection, or were considered too challenging to develop. Theoretically, the tools and knowledge are available to develop high starch content in forage legumes, but there is still significant investment required to merge basic biology with modern breeding.

Investment into developing a high starch trait in forage legumes is particularly imperative to address negative aspects of modern agriculture. Traditionally, the driving force behind the development of a high NSC trait was the economic benefit it offered to farmers. These economic incentives have increased with increased awareness of sustainable livestock production by the public. Because a high-starch content in forage legumes would have the potential to reduce grain supplementation in the livestock's diet, the demand for high-input grain crops, such as maize and soy would be reduced. Therefore, livestock production based on sustainable pastures and grasslands would be more economically competitive. Because of the potential value and impact that a high starch trait could have on agriculture, the concerns about the feasibility of this orphan trait should be re-evaluated.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/7/1/16/s1. Detailed growth conditions and experimental methods are supplied as supplemental Materials and Methods.

Acknowledgments: We would like to thank Beat Boller of Agroscope (Reckenholz, CH) for the seeds of the red clover accession, TP1245. We thank Gavin M. George, Angela Schwarm, and Shaopu Wang for helpful discussions and technical advice. We thank Michael Kreuzer for the use of technical equipment and beneficial advice. We thank Diana Zwahlen, Brigitta Herzog, and Andrea Ruckle for their expert care of the plant material. This research was supported by the COOP—Research Fellowship Program through the ETH—World Food System Center and the Swiss Federal Office for Agriculture (FOAG).

Author Contributions: Michael E. Ruckle, Samuel C. Zeeman and Bruno Studer conceived the design of the experiments. Michael E. Ruckle, Michael Meier, Lea Frey and Simona Eicke performed the experiments and analyzed the data. Michael E. Ruckle, Roland Kölliker and Bruno Studer prepared the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Steinfeld, H.; Gerber, P.; Wassenaar, T.; Castel, V.; Rosales, M.; De Haan, C. *Livestock's Long Shadow*; FAO Rome: Rome, Italy, 2006.
2. Robertson, G.P.; Vitousek, P.M. Nitrogen in agriculture: Balancing the cost of an essential resource. *Annu. Rev. Environ. Resour.* **2009**, *34*, 97–125. [CrossRef]
3. Broderick, G.; Albrecht, K.; Owens, V.; Smith, R. Genetic variation in red clover for rumen protein degradability. *Anim. Feed Sci. Technol.* **2004**, *113*, 157–167. [CrossRef]
4. Frame, J.; Charlton, J.; Laidlaw, A.S. *Temperate Forage Legumes*; Cab International: Wallingford, UK, 1998.

5. Nkrumah, J.; Okine, E.; Mathison, G.; Schmid, K.; Li, C.; Basarab, J.; Price, M.; Wang, Z.; Moore, S. Relationships of feedlot feed efficiency, performance, and feeding behavior with metabolic rate, methane production, and energy partitioning in beef cattle. *J. Anim. Sci.* **2006**, *84*, 145–153. [CrossRef] [PubMed]
6. Nocek, J.E.; Tamminga, S. Site of digestion of starch in the gastrointestinal tract of dairy cows and its effect on milk yield and composition. *J. Dairy Sci.* **1991**, *74*, 3598–3629. [CrossRef]
7. Reynolds, C. Production and metabolic effects of site of starch digestion in dairy cattle. *Anim. Feed Sci. Technol.* **2006**, *130*, 78–94. [CrossRef]
8. Taylor, N.; Quesenberry, K. *Red Clover Science. Current Plant Sciences and Biology in Agriculture*; Kluwer Academic Publisher: Dordrecht, The Netherlands, 1996; Volume 28, pp. 141–160.
9. Graham, P.H.; Vance, C.P. Legumes: Importance and constraints to greater use. *Plant Physiol.* **2003**, *131*, 872–877. [CrossRef] [PubMed]
10. Gibon, A. Managing grassland for production, the environment and the landscape. Challenges at the farm and the landscape level. *Livest. Prod. Sci.* **2005**, *96*, 11–31. [CrossRef]
11. Paustian, K.; Lehmann, J.; Ogle, S.; Reay, D.; Robertson, G.P.; Smith, P. Climate-smart soils. *Nature* **2016**, *532*, 49–57. [CrossRef] [PubMed]
12. Fischer, R.; Edmeades, G.O. Breeding and cereal yield progress. *Crop Sci.* **2010**, *50*, S-85–S-98. [CrossRef]
13. Kingston-Smith, A.; Marshall, A.; Moorby, J. Breeding for genetic improvement of forage plants in relation to increasing animal production with reduced environmental footprint. *Animal* **2013**, *7*, 79–88. [CrossRef] [PubMed]
14. Bertrand, A.; Dhont, C.; Bipfubusa, M.; Chalifour, F.-P.; Drouin, P.; Beauchamp, C.J. Improving salt stress responses of the symbiosis in alfalfa using salt-tolerant cultivar and rhizobial strain. *Appl. Soil Ecol.* **2015**, *87*, 108–117. [CrossRef]
15. Brito, A.; Tremblay, G.; Bertrand, A.; Castonguay, Y.; Bélanger, G.; Michaud, R.; Lafrenière, C.; Martineau, R.; Berthiaume, R. Alfalfa baleage with increased concentration of nonstructural carbohydrates supplemented with a corn-based concentrate did not improve production and nitrogen utilization in early lactation dairy cows. *J. Dairy Sci.* **2014**, *97*, 6970–6990. [CrossRef] [PubMed]
16. Turner, L.; Pollock, C. Changes in stolon carbohydrates during the winter in four varieties of white clover (trifolium repensl.) with contrasting hardiness. *Ann. Bot.* **1998**, *81*, 97–107. [CrossRef]
17. Humphreys, M. Water-soluble carbohydrates in perennial ryegrass breeding. *Grass Forage Sci.* **1989**, *44*, 237–244. [CrossRef]
18. Turner, L.; Cairns, A.; Armstead, I.; Ashton, J.; Skøt, K.; Whittaker, D.; Humphreys, M. Dissecting the regulation of fructan metabolism in perennial ryegrass (*Lolium perenne*) with quantitative trait locus mapping. *New Phytol.* **2006**, *169*, 45–58. [CrossRef] [PubMed]
19. Turner, L.B.; Humphreys, M.O.; Cairns, A.J.; Pollock, C.J. Comparison of growth and carbohydrate accumulation in seedlings of two varieties of *Lolium perenne*. *J. Plant Physiol.* **2001**, *158*, 891–897. [CrossRef]
20. Moorby, J.; Evans, R.; Scollan, N.; MacRae, J.; Theodorou, M. Increased concentration of water-soluble carbohydrate in perennial ryegrass (*Lolium perenne* L.). Evaluation in dairy cows in early lactation. *Grass Forage Sci.* **2006**, *61*, 52–59. [CrossRef]
21. Proctor, L.; Craig, H.; Mclean, N.; Fennessy, P.; Kerslake, J.; Behrent, M.; Chuah, J.; Campbell, A. The effect of grazing high-sugar ryegrass on lamb performance. *Proc. N. Z. Soc. Anim. Prod.* **2015**, *75*, 235–238.
22. Staerfl, S.; Amelchanka, S.; Kälber, T.; Soliva, C.; Kreuzer, M.; Zeitz, J. Effect of feeding dried high-sugar ryegrass ('abermagic') on methane and urinary nitrogen emissions of primiparous cows. *Livest. Sci.* **2012**, *150*, 293–301. [CrossRef]
23. Åman, P.; Nordkvist, E. Chemical composition and in-vitro degradability of major chemical constituents of red clover harvested at different stages of maturity. *J. Sci. Food Agric.* **1983**, *34*, 1185–1189. [CrossRef]
24. Mackenzie, D.; Wylam, C.B. Analytical studies on the carbohydrates of grasses and clovers. Viii.—changes in carbohydrate composition during the growth of perennial rye-grass. *J. Sci. Food Agric.* **1957**, *8*, 38–45. [CrossRef]
25. Owens, V.; Albrecht, K.; Muck, R. Protein degradation and fermentation characteristics of unwilted red clover and alfalfa silage harvested at various times during the day. *Grass Forage Sci.* **2002**, *57*, 329–341. [CrossRef]
26. Chouinard-Michaud, C.; Michaud, R.; Castonguay, Y.; Bertrand, A.; Bélanger, G.; Tremblay, G.F.; Berthiaume, R.; Allard, G. Increasing alfalfa non structural carbohydrates through genetic selection and cutting management. In *Ruminants Physiology Digestion, Metabolisms and Effects of Nutrition on Reproduction and Welfare*; Wagennigen Academic Publishers: Wagennigen, The Netherlands, 2009; pp. 138–139.

27. Claessens, A.; Castonguay, Y.; Bertrand, A.; Bélanger, G.; Tremblay, G. Breeding for improved nonstructural carbohydrates in alfalfa. In *Breeding in a World of Scarcity*; Springer: Cham, CH, Switzerland, 2016; pp. 231–235.
28. Zeeman, S.C.; Kossmann, J.; Smith, A.M. Starch: Its metabolism, evolution, and biotechnological modification in plants. *Annu. Rev. Plant Biol.* **2010**, *61*, 209–234. [CrossRef] [PubMed]
29. Jones, B.; Hatfield, R.; Muck, R. Characterization of proteolysis in alfalfa and red clover. *Crop Sci.* **1995**, *35*, 537–541. [CrossRef]
30. Kötting, O.; Santelia, D.; Edner, C.; Eicke, S.; Marthaler, T.; Gentry, M.S.; Comparot-Moss, S.; Chen, J.; Smith, A.M.; Steup, M. Starch-excess4 is a laforin-like phosphoglucan phosphatase required for starch degradation in *Arabidopsis thaliana*. *Plant Cell* **2009**, *21*, 334–346. [CrossRef] [PubMed]
31. Stitt, M.; Zeeman, S.C. Starch turnover: Pathways, regulation and role in growth. *Curr. Opin. Plant Biol.* **2012**, *15*, 282–292. [CrossRef] [PubMed]
32. Feike, D.; Seung, D.; Graf, A.; Bischof, S.; Ellick, T.; Coiro, M.; Soyk, S.; Eicke, S.; Mettler-Altmann, T.; Lu, K.-J. The starch granule-associated protein early starvation1 (esv1) is required for the control of starch degradation in *Arabidopsis thaliana* leaves. *Plant Cell* **2016**. [CrossRef]
33. Seung, D.; Soyk, S.; Coiro, M.; Maier, B.A.; Eicke, S.; Zeeman, S.C. Protein targeting to starch is required for localising granule-bound starch synthase to starch granules and for normal amylose synthesis in arabidopsis. *PLoS Biol.* **2015**, *13*, e1002080. [CrossRef] [PubMed]
34. Stettler, M.; Eicke, S.; Mettler, T.; Messerli, G.; Hörtensteiner, S.; Zeeman, S.C. Blocking the metabolism of starch breakdown products in arabidopsis leaves triggers chloroplast degradation. *Mol. Plant* **2009**, *2*, 1233–1246. [CrossRef] [PubMed]
35. Yu, T.-S.; Kofler, H.; Häusler, R.E.; Hille, D.; Flügge, U.-I.; Zeeman, S.C.; Smith, A.M.; Kossmann, J.; Lloyd, J.; Ritte, G. The arabidopsis sex1 mutant is defective in the r1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. *Plant Cell* **2001**, *13*, 1907–1918. [CrossRef] [PubMed]
36. Calenge, F.; Saliba-Colombani, V.; Mahieu, S.; Loudet, O.; Daniel-Vedele, F.; Krapp, A. Natural variation for carbohydrate content in arabidopsis. Interaction with complex traits dissected by quantitative genetics. *Plant Physiol.* **2006**, *141*, 1630–1643. [CrossRef] [PubMed]
37. Gibon, Y.; Blaesing, O.E.; Hannemann, J.; Carillo, P.; Höhne, M.; Hendriks, J.H.; Palacios, N.; Cross, J.; Selbig, J.; Stitt, M. A robot-based platform to measure multiple enzyme activities in arabidopsis using a set of cycling assays: Comparison of changes of enzyme activities and transcript levels during diurnal cycles and in prolonged darkness. *Plant Cell* **2004**, *16*, 3304–3325. [CrossRef] [PubMed]
38. Vriet, C.; Welham, T.; Brachmann, A.; Pike, M.; Pike, J.; Perry, J.; Parniske, M.; Sato, S.; Tabata, S.; Smith, A.M. A suite of lotus japonicus starch mutants reveals both conserved and novel features of starch metabolism. *Plant Physiol.* **2010**, *154*, 643–655. [CrossRef] [PubMed]
39. Wiese, A.; Christ, M.; Virnich, O.; Schurr, U.; Walter, A. Spatio-temporal leaf growth patterns of *Arabidopsis thaliana* and evidence for sugar control of the diel leaf growth cycle. *New Phytol.* **2007**, *174*, 752–761. [CrossRef]
40. Mielewczik, M.; Friedli, M.; Kirchgessner, N.; Walter, A. Diel leaf growth of soybean: A novel method to analyze two-dimensional leaf expansion in high temporal resolution based on a marker tracking approach (martrack leaf). *Plant Methods* **2013**, *9*, 30. [CrossRef] [PubMed]
41. Sulpice, R.; Pyl, E.-T.; Ishihara, H.; Trenkamp, S.; Steinfath, M.; Witucka-Wall, H.; Gibon, Y.; Usadel, B.; Poree, F.; Piques, M.C. Starch as a major integrator in the regulation of plant growth. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 10348–10353. [CrossRef] [PubMed]
42. Weise, S.E.; Aung, K.; Jarou, Z.J.; Mehrshahi, P.; Li, Z.; Hardy, A.C.; Carr, D.J.; Sharkey, T.D. Engineering starch accumulation by manipulation of phosphate metabolism of starch. *Plant Biotechnol. J.* **2012**, *10*, 545–554. [CrossRef] [PubMed]
43. Atkin, O.K.; Tjoelker, M.G. Thermal acclimation and the dynamic response of plant respiration to temperature. *Trends Plant Sci.* **2003**, *8*, 343–351. [CrossRef]
44. Boller, B.C.; Nösberger, J. Effects of temperature and photoperiod on stolon characteristics, dry matter partitioning, and nonstructural carbohydrate concentration of two white clover ecotypes. *Crop Sci.* **1983**, *23*, 1057–1062. [CrossRef]
45. Warrington, I.; Peet, M.; Patterson, D.; Bunce, J.; Haslemore, R.; Hellmers, H. Growth and physiological responses of soybean under various thermoperiods. *Funct. Plant Biol.* **1977**, *4*, 371–380. [CrossRef]

46. Weston, E.; Thorogood, K.; Vinti, G.; López-Juez, E. Light quantity controls leaf-cell and chloroplast development in *Arabidopsis thaliana* wild type and blue-light-perception mutants. *Planta* **2000**, *211*, 807–815. [CrossRef] [PubMed]
47. Brito, A.; Tremblay, G.; Lapierre, H.; Bertrand, A.; Castonguay, Y.; Bélanger, G.; Michaud, R.; Benchaar, C.; Ouellet, D.; Berthiaume, R. Alfalfa cut at sundown and harvested as baleage increases bacterial protein synthesis in late-lactation dairy cows. *J. Dairy Sci.* **2009**, *92*, 1092–1107. [CrossRef] [PubMed]
48. Brito, A.; Tremblay, G.; Bertrand, A.; Castonguay, Y.; Bélanger, G.; Michaud, R.; Lapierre, H.; Benchaar, C.; Petit, H.; Ouellet, D. Alfalfa cut at sundown and harvested as baleage improves milk yield of late-lactation dairy cows. *J. Dairy Sci.* **2008**, *91*, 3968–3982. [CrossRef] [PubMed]
49. Lowell, M.E. Post-harvest physiological changes in forage plants. In *Post-Harvest Physiology and Preservation of Forages*; Crop Science Society of America: Madison, WI, USA, 1995; pp. 1–19.
50. Li, R.; Volenec, J.; Joern, B.; Cunningham, S. Seasonal changes in nonstructural carbohydrates, protein, and macronutrients in roots of alfalfa, red clover, sweetclover, and birdsfoot trefoil. *Crop Sci.* **1996**, *36*, 617–623. [CrossRef]
51. De Vega, J.J.; Ayling, S.; Hegarty, M.; Kudrna, D.; Goicoechea, J.L.; Ergon, Å.; Rognli, O.A.; Jones, C.; Swain, M.; Geurts, R. Red clover (*Trifolium pratense* L.) draft genome provides a platform for trait improvement. *Sci. Rep.* **2015**, *5*, 17394. [CrossRef] [PubMed]
52. Ištvánek, J.; Jaroš, M.; Křenek, A.; Řepková, J. Genome assembly and annotation for red clover (trifolium pratense; fabaceae). *Am. J. Bot.* **2014**, *101*, 327–337. [CrossRef] [PubMed]
53. Griffiths, A.G.; Barrett, B.A.; Simon, D.; Khan, A.K.; Bickerstaff, P.; Anderson, C.B.; Franzmayr, B.K.; Hancock, K.R.; Jones, C.S. An integrated genetic linkage map for white clover (*Trifolium repens* L.) with alignment to medicago. *BMC Genom.* **2013**, *14*, 388. [CrossRef] [PubMed]
54. Annicchiarico, P.; Nazzicari, N.; Brummer, E. Alfalfa genomic selection: Challenges, strategies, transnational cooperation. In *Breeding in a World of Scarcity*; Springer: Cham, CH, Switzerland, 2016; pp. 145–149.
55. Li, X.; Wei, Y.; Acharya, A.; Jiang, Q.; Kang, J.; Brummer, E.C. A saturated genetic linkage map of autotetraploid alfalfa (*Medicago sativa* L.) developed using genotyping-by-sequencing is highly syntenous with the medicago truncatula genome. *G3: Genes Genomes Genet.* **2014**, *4*, 1971–1979. [CrossRef] [PubMed]
56. O'Rourke, J.A.; Fu, F.; Bucciarelli, B.; Yang, S.S.; Samac, D.A.; Lamb, J.F.; Monteros, M.J.; Graham, M.A.; Gronwald, J.W.; Krom, N. The *Medicago sativa* gene index 1.2: A web-accessible gene expression atlas for investigating expression differences between *Medicago sativa* subspecies. *BMC Genom.* **2015**, *16*, 502. [CrossRef] [PubMed]
57. Yates, S.A.; Swain, M.T.; Hegarty, M.J.; Chernukin, I.; Lowe, M.; Allison, G.G.; Ruttink, T.; Abberton, M.T.; Jenkins, G.; Skøt, L. De novo assembly of red clover transcriptome based on rna-seq data provides insight into drought response, gene discovery and marker identification. *BMC Genom.* **2014**, *15*, 453. [CrossRef] [PubMed]
58. Manzanares, C.; Yates, S.; Ruckle, M.; Nay, M.; Studer, B. Tilling in forage grasses for gene discovery and breeding improvement. *New Biotechnol.* **2016**, *33*, 594–603. [CrossRef] [PubMed]
59. Cooper, M.; van Eeuwijk, F.A.; Hammer, G.L.; Podlich, D.W.; Messina, C. Modeling qtl for complex traits: Detection and context for plant breeding. *Curr. Opin. Plant Biol.* **2009**, *12*, 231–240. [CrossRef]
60. Hammer, G.; Messina, C.; van Oosterom, E.; Chapman, S.; Singh, V.; Borrell, A.; Jordan, D.; Cooper, M. Molecular breeding for complex adaptive traits: How integrating crop ecophysiology and modelling can enhance efficiency. In *Crop Systems Biology*; Springer: Cham, CH, Switzerland, 2016; pp. 147–162.
61. Reymond, M.; Muller, B.; Leonardi, A.; Charcosset, A.; Tardieu, F. Combining quantitative trait loci analysis and an ecophysiological model to analyze the genetic variability of the responses of maize leaf growth to temperature and water deficit. *Plant Physiol.* **2003**, *131*, 664–675. [CrossRef] [PubMed]
62. Lloyd, J.R.; Kossmann, J.; Ritte, G. Leaf starch degradation comes out of the shadows. *Trends Plant Sci.* **2005**, *10*, 130–137. [CrossRef] [PubMed]
63. Shu, Q.; Forster, B.P.; Nakagawa, H.; Nakagawa, H. *Plant Mutation Breeding and Biotechnology*; CABI: Wallingford, UK, 2012.
64. Tsai, H.; Howell, T.; Nitcher, R.; Missirian, V.; Watson, B.; Ngo, K.J.; Lieberman, M.; Fass, J.; Uauy, C.; Tran, R.K. Discovery of rare mutations in populations: Tilling by sequencing. *Plant Physiol.* **2011**, *156*, 1257–1268. [CrossRef] [PubMed]
65. Sullivan, M.L.; Quesenberry, K.H. Clover, red (*Trifolium pratense*). *Agrobact. Protoc.* **2015**, *1*, 237–254.

66. Fu, C.; Hernandez, T.; Zhou, C.; Wang, Z.-Y. Alfalfa (*Medicago sativa* L.). *Agrobact. Protoc.* **2015**, *1*, 213–221.
67. Svitashev, S.; Young, J.K.; Schwartz, C.; Gao, H.; Falco, S.C.; Cigan, A.M. Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using cas9 and guide rna. *Plant Physiol.* **2015**, *169*, 931–945. [CrossRef] [PubMed]
68. Dreccer, M.F.; Barnes, L.R.; Meder, R. Quantitative dynamics of stem water soluble carbohydrates in wheat can be monitored in the field using hyperspectral reflectance. *Field Crops Res.* **2014**, *159*, 70–80. [CrossRef]
69. Bowley, S.; Taylor, N.; Dougherty, C. Physiology and morphology of red clover. *Adv. Agron.* **1984**, *37*, 317–347.
70. Huntington, G.B. Starch utilization by ruminants: From basics to the bunk. *J. Anim. Sci.* **1997**, *75*, 852–867. [CrossRef]
71. Benchaar, C.; Pomar, C.; Chiquette, J. Evaluation of dietary strategies to reduce methane production in ruminants: A modelling approach. *Can. J. Anim. Sci.* **2001**, *81*, 563–574. [CrossRef]
72. Reis, R.; Combs, D. Effects of increasing levels of grain supplementation on rumen environment and lactation performance of dairy cows grazing grass-legume pasture. *J. Dairy Sci.* **2000**, *83*, 2888–2898. [CrossRef]
73. Van Dorland, H.; Wettstein, H.-R.; Leuenberger, H.; Kreuzer, M. Effect of supplementation of fresh and ensiled clovers to ryegrass on nitrogen loss and methane emission of dairy cows. *Livest. Sci.* **2007**, *111*, 57–69. [CrossRef]
74. Menke, K.H.; Steingass, H. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim. Res. Dev.* **1988**, *28*, 7–55.

agronomy

MDPI

Review

The Current Status, Problems, and Prospects of Alfalfa (*Medicago sativa* L.) Breeding in China

Shangli Shi [1,*], Lili Nan [1] and Kevin F. Smith [2]

[1] Ministry of Education Key Laboratory of Ecosystems, College of Pratacultural Science, Gansu Agricultural University, Lanzhou 730070, Gansu, China; nanll@gsau.edu.cn

[2] Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Private Bag 105 Hamilton, Victoria 3300, Australia; kfsmith@unimelb.edu.au

* Correspondence: shishl@gsau.edu.cn; Tel.: +139-1905-1530

Academic Editor: John W. Forster
Received: 23 September 2016; Accepted: 3 November 2016; Published: 1 January 2017

Abstract: This paper reviews the current status, methodology, achievements, and prospects of alfalfa (*Medicago sativa* L.) breeding in China. There are 77 cultivars that have been registered in the country, these include 36 cultivars bred through breeding programs, 17 introduced from overseas, 5 domesticated from wild ecotypes, and 19 through regional collection/breeding programs. Cultivars have been selected for cold resistance, disease resistance, salt tolerance, grazing tolerance, high yield, and early maturity. Most of these cultivars have been developed through conventional breeding techniques, such as selective and cross breeding, and some are now being evaluated that have been developed through the application of transgenic technology. The major problems for alfalfa breeding in China include low success rate, slow progress to breed resistant varieties, lack of breeding materials and their systematic collection, storage and evaluation, lack of advanced breeding techniques, and low adoption rate of new cultivars. There are gaps in alfalfa breeding between China and the developed world. Improvement of alfalfa breeding techniques, development of cultivars with adaptations to different regions within China, and the protection and utilization of alfalfa germplasm resources have been identified as major strategies to improve the efficiency of alfalfa breeding in China.

Keywords: China; alfalfa; varieties; breeding; selection; *Medicago sativa* L.

1. Introduction

China has abundant genetic resources of *Medicago* spp. with 12 species *M. sativa* subsp. *sativa* L., *M. ruthenica* L. Ledeb., *M. pubescens* Sirj., *M. archiducis-nicolai* Sirjaev., *M. platycarpos* L. Trautv., *M. sativa* subsp. *falcata*, *M. sativa* subsp. *x varia*, *M. polymorpha* L., *M. minima* L. Grnfb, *M. lupulina* L., and *M. orbicularis* L. Bart., which are mainly distributed in northern and southwest China [1].

M. sativa L. is a perennial species of the genus, originating in Asia Minor, Transcaucasia, Iran and Turkmenistan highlands. The common name "alfalfa" refers to the main species *M. sativa* subsp. *sativa* but may also describe *M. sativa* subsp. *falcata* and *M. sativa* subsp. *x varia* (a hybrid between subsp. *sativa* and subsp. *falcata*) which is closely related to *M. sativa* in morphology. Among the *Medicago* species, *M. sativa* has the largest cultivation area in the world at present because of its numerous superior traits such as cold resistance, salt tolerance, wide adaptability, high yield, good herbage quality, resistance to frequent cutting, good persistence, soil amelioration, and economic benefit. It is therefore regarded as "the queen of forages". As the concept of "Pasture-based livestock industries" gained prominence in China in recent years, and with the application of new policies such as subsidy for grassland ecological protection, revitalization of alfalfa for dairying, and conversion of crops to forest and grassland, *M. sativa* has become the most widely used species in integrated farming systems, grazing, and ecological conservation in China. It is therefore imperative to develop new alfalfa varieties

to meet production targets under local conditions, which presents both opportunities and challenges for alfalfa breeding. Although there have been previous reviews of alfalfa breeding in China [2–4] these have not been widely available to an international audience, this review seeks to provide an updated review of the status of alfalfa breeding in China and a comment on the future needs for research and development.

2. Alfalfa Breeding in China

Forage breeding in China commenced about half a century later than the developed world. Until 1949, only a few scientists collected and evaluated wild forage germplasm for agronomic adaptation. Modern herbage breeding programs commenced in the 1950s and have been developed extensively since the 1980s. The National Committee for the Examination and Approval of Pasture Variety (NCEAPV) was established officially in 1986, and has promoted the selection and development of new pasture varieties, the collection and domestication of local ecotypes, and the introduction of foreign forage germplasm.

As of 2015, a total of 77 Alfalfa varieties were registered by NCEAPV, including 36 domestic varieties, 17 introduced varieties, 5 domesticated varieties, and 19 local varieties [5,6]. These do not include varieties registered by regional authorities (provinces and autonomous regions).

Varieties developed in China can be divided into eight categories based on their main characteristics (Figure 1), which have been described briefly below:

1. *High yield*: such as cv. Gannong 3 and 4;
2. *Disease resistance*: such as cv. Zhonglan 1, which has high resistance to downy mildew (*Peronospera trifoliorum*), and moderate resistance to brown leaf spot and rust disease, with resulting increases of herbage production of ca. 30% compared with cv. Longdong in the presence of these diseases. The resistance to downy mildew and brown leaf spot of cv. Xinmu 4 is better than cv. Xinjiang Daye;
3. *Cold resistance*: such as cvv. Longmu 801, Longmu 803, Longmu 806, Longmu 808, Caoyuan 1, Caoyuan 2, Caoyuan 3, Tumu 1, Xinmu 1, Xinmu 3, and Chicao 1. Most of these have been developed for planting in high latitude and high altitude areas in North China;
4. *Salt tolerant*: such as cvv. Zhongmu 1, Zhongmu 3, and Zhongmu 5; these have more than 10% higher yields compared with control cultivars when planted in saline-alkali soil;
5. *Grazing tolerance*: creeping-rooted types alfalfa such as cvv. Gannong 2, Zhongmu 2, and Gongnong 3. This suitability to grazing is conferred through strong root systems and greater ground cover. These cultivars are also highly suitable for conservation of water and soil, wind-breaks and sand/dune stability, soil reinforcement, and slope protection which represent other important areas of use for alfalfa in the pastoral regions of China;
6. *Early maturity*: such as cv. Xinmu 2, which matures earlier (about three to five days) than cv. Xinjiang Daye, and has high rates of regrowth following grazing;
7. *Lower fiber concentrations*: such as cv. Gannong 7 which has Neutral Detergent Fiber(NDF) and Acid Detergent Fiber (ADF) concentrations 2 percentage points lower than that in other alfalfa, and crude protein 1 percentage point higher than other cultivars, and hence improved palatability;
8. *Insect resistance*: such as cv. Gannong 5, with high levels of resistance to aphids with yields increased 14.22% [7] compared with the control cv. Golden Empress, cv. Caoyuan 4 is a variety developed for regions with serious thrip infestations.

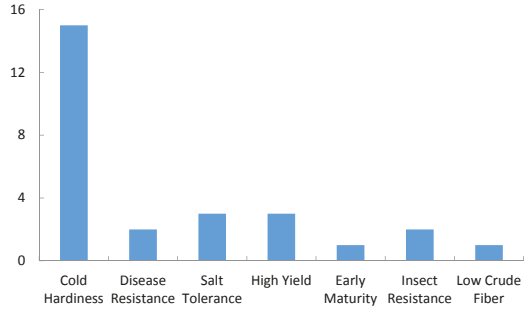


Figure 1. The number and reported general attributes of alfalfa varieties bred in China.

The introduction of new alfalfa cultivars from overseas has been increasing with the rapid development of the alfalfa industry in China. The number of alfalfa varieties imported to China has increased dramatically to a current level of approximately 400. There are presently 17 varieties registered by the NCEAPV, including cvv. AmeriGraze 401+Z, Derby, Sanditi, and WL525HQ. (tab:agronomy-07-00001-t001). This has been attributed to the improved production and forage quality that can be directly utilized for animal production. These importations also contribute to the pool of the alfalfa germplasm resources for alfalfa breeding in China.

Table 1. Introduced *Medicago sativa* varieties registered by the National Committee for the Examination and Approval of Pasture Varieties.

Num.	Cultivar	Country	Main Characteristics
1	AmeriGraze 401+Z	USA	Cold tolerance, regeneration
2	Derby	France	Lodging resistance, cold tolerance, high yield
3	Eureka	Australia	Heat tolerance
4	Golden Empress	USA	Cold tolerance, drought tolerance, regeneration
5	Crown	USA	Cold tolerance, drought tolerance, regeneration
6	Sanditi	France	Lodging resistance, cold resistance, yield
7	Sitel	France	Lodging resistance, yield
8	Victor	Canada	Drought tolerance, insect resistance, heat tolerance
9	Victoria	Canada	Moderate cold tolerance, heat tolerance
10	WL232HQ	USA	Cold tolerance, regeneration
11	WL323ML	USA	Cold tolerance, yield
12	Rambler	Canada	Cold tolerance, drought tolerance, grazing tolerance
13	Caribou	Canada	Cold tolerance
14	WL525HQ	USA	Heat tolerance, yield
15	Wisdom	USA	Heat tolerance
16	WL343HQ	USA	Resistance to diseases and insect pests, cold tolerance, regeneration, frequent cutting tolerance
17	Qiuliu	Russia	Cold tolerance, drought tolerance, saline-alkali soil tolerance

Local varieties are mainly distributed in Xinjiang, Inner Mongolia, Gansu, Shaanxi, Shanxi, Hebei, Shandong, Heilongjiang, Jiangsu, and Yunnan provinces. They have resistance to environmental stresses, for example, cv. Zhaodong and cv. Aohan are resistant to cold.

Alfalfa has been cultivated for more than 2000 years in China; a nation with vastly diverse climate, soil, and other ecological conditions across different regions. Under the long-term influence of natural conditions and farming conditions in different parts of the country, the formation of the local varieties has primarily been achieved by natural selection (Local varieties, tab:agronomy-07-00001-t002). These local varieties play a significant role in production, are extremely valuable germplasm resources, and have strong resistance, wide adaptability, and yield stability.

Table 2. Local alfalfa varieties registered by the National Committee for the Examination and Approval of Herbage Varieties.

Num.	Cultivar	Region	Main Characteristics
1	Beijiang	Xinjiang	Drought tolerance, cold tolerance, poor regeneration
2	Xinjiang Daye	Xinjiang	Large leaves, good regeneration
3	Hexi	Gansu	Late maturing, poor regeneration
4	Longdong	Gansu	Drought tolerance, poor regeneration
5	Longzhong		Drought tolerance
6	Tianshui	Gansu	
7	Guanzhong	Shanxi	Early regrowth, early maturing
8	Shanbei	Shanxi	Late maturing, drought tolerance
9	Jinnan	Shanxi	Good regeneration, early maturing
10	Pianguan	Shanxi	Late maturing
11	Aohan	Inner Mongolia	Drought tolerance, cold tolerance, suitable for dryland cultivation
12	Junggar	Inner Mongolia	Drought tolerance, suitable for dryland cultivation
13	Cangzhou	Hebei	Comparative tolerance to saline-alkali soils
14	Weixi	Hebei	Drought tolerance
15	Baoding	Hebei	Good regeneration
16	Zhaodong	Heilongjiang	Cold tolerance
17	Huiyin	Jiangsu	Heat tolerance, early maturing
18	Chuxiong	Yunnan	Annual or biennial, high nitrogen-fixing ability, cold tolerance, drought tolerance
19	Wudi	Shandong	Highly salt tolerant

The five wild varieties registered for cultivation are cvv. Aletai, Longdong, Hulunbeier, Deqin, and Qingshui. Among them, cv. Qingshui is the first rhizomatous alfalfa registered in China, and plays an important role in production for some regions. The width of the crown is 25–30 cm with well-developed underground horizontal roots, which enables the variety to produce abundant shoots (25–46 shoots/plant) and to have a strong regenerative ability following grazing.

3. Breeding Methods

Alfalfa is a perennial predominantly cross-pollinated plant; self-fertility is affected by many factors. Morphological features and physiological characteristics prevent alfalfa from self-pollination which results in low selfing rates. Hence, alfalfa is classified as a cross-pollinated species, and its natural cross-pollination rate is between 25% and 75%. As an autotetraploid, it has its specific requirements in breeding methods and selection of offspring that are different to diploid plant species; these factors have also contributed to the delay in utilizing some modern breeding technologies in alfalfa compared to major food crops. Currently selective breeding, cross breeding, male sterile line breeding, space breeding, and biotechnology-assisted breeding have been adopted for alfalfa breeding in China, among which selective breeding and cross breeding are the most widely applied.

3.1. Selective Breeding

Selective breeding is the selection of improved varieties and genotypes with advantageous characteristics to develop new varieties with specific breeding objectives. It is an important method to improve both existing cultivars and breed new varieties from germplasm collections or other sources. The methods of selective breeding include individual selection, mass selection, improved mass selection, group selection, and recurrent selection etc. [8]. There are a large number of new varieties developed and registered in China using this method, including cvv. Zhongmu 1, Zhongmu 2, Zhongmu 3, Gongnong 1, Gongnong 2, Gongnong 3, Xinmu 1, Xinmu 2, Xinmu 3, and Gannong 2. Selective breeding is practical, effective, manageable, and is one of the most commonly used methods in alfalfa breeding in China.

3.2. Cross Breeding

Cross breeding uses cross-pollination between different species or sub-species to breed varieties by selection from the hybrid progeny. It can recombine the parents' genes to form a variety of different types and provide abundant material for selection. Genetic recombination allows the accumulation of desirable alleles from parents that differ for major traits. Choosing the correct parents and making suitable combinations are critical for cross breeding. Among alfalfa varieties that have been bred using cross breeding and registered in China are cv. Gannong 3 and Tumu 2. *M. sativa* subsp *x. varia* cvv. Caoyuan 1, Caoyuan 2, Tumu 1, and Gannong 1 were developed through crossing subsp. *sativa* and subsp. *falcata.* Intergeneric reciprocal hybridization combined with radiation breeding techniques were used to develop *Melilotoides ruthenicus* Sojak hybrids cvv. Longmu 801 and Longmu 803.

3.3. Male Sterile Line Breeding

Male sterile line breeding is where the male organs in flowers do not function to produce pollen whereas the female organs function normally. No seeds of male sterile lines are produced by self-pollination but seeds can be produced when pollinated with other non-male sterile lines.

The first alfalfa male sterile line was found in Canada in 1958. Since then researchers in the USA, Russia, Hungary, Bulgaria, France, and Japan used male sterile lines in their breeding programs. Petkov discovered that the maintainer line (line to help maintain the male sterile lines) for sterile line breeding in 1979. Other researchers conducted hybridization experiments with the sterile line and obtained excellent hybrids, which showed obvious heterosis. Herbage yield increased by 30% compared with control species, with improved resistance to stress. From the 1980s, researchers have studied a range of aspects related to the basic biology and agronomic suitability of male sterility in alfalfa breeding including pollen sterility and fertility, regarding the characterization of male sterile lines, and investigated the sterility mechanism of alfalfa pollen. More recently, Hybrid force-620 that was bred with male sterile line technique by Dairyland company in the US has been widely sown by dairy farmers due to its high yield and improved quality [9].

In China, Yongfu Wu [10] from Inner Mongolia University selected and bred six male sterile lines from *M. varia* Martin. cv. Caoyuan 1; scientists from the Institute of Animal Science, Chinese Academy of Agricultural Sciences developed three male sterile lines from *M. sativa* cv. Atlantic Ocean; and scientists from the Institute of Grassland Science, Jilin Academy of Agricultural Sciences pioneered the male sterile variety test in alfalfa and obtained F1 seeds under open pollination [2]. However, there has been no practical use of male sterile line technology in the development of new cultivars of *M. sativa* in China.

3.4. Biotechnology Assisted Breeding

Biotechnology assisted breeding is the use of genomic techniques such as molecular markers and transgenesis to incorporate new traits or increase the rate of genetic gain [11–14]. Research on biotechnology assisted breeding for alfalfa in China began in late 1970s. We used tissue culture and somatic cell hybridization initially, and then moved to the development and application of transgenesis and molecular markers. At present, studies are focused on the transformation and expression of genes related to stress resistance and tolerance. Although there are few varieties developed using this technology to date, the methods adopted in China are briefly summarized below.

3.5. Transgenic Technology

Different genetic transformation methods significantly affect the efficiency of exogenous genes transfer into *M. sativa*, which is the basis for the acquisition and development of transgenic plants. There are three main methods in genetic transformation of *M. sativa*, germplasm line transformation, direct transformation, and indirect transformation. Germplasm line transformation method uses a plant's own pollen, ovary, and other germ cells to introduce exogenous genes, such as via the

ovary injection and pollen tube method. Direct transformation methods use chemical or physical methods to introduce exogenous DNA into plant cells and obtain transgenic plants. Biolistic, ultrasonic, polyethelene glycol (PEG)-mediated, and microinjection are all examples of direct transformation methods. Indirect transformation methods use carriers to introduce DNA into the plant cell, and Agrobacterium-mediated transformation is the major example of this method.

In genetically modified breeding for alfalfa salt tolerance [15], Li et al. overexpressed the Alfinl transcription factor in alfalfa via Agrobacterium-mediated transformation, which enhanced the expression of endogenous gene MsPRP2 and improved the salt tolerance of transgenic alfalfa plants. Liang et al. [14] studied several factors that influence the efficiency of Agrobacterium-mediated genetic transformation in alfalfa and obtained transgenic plants expressing the betaine aldehyde dehydrogenase (BADH) gene. Yan et al. [16] showed that the BADH gene can enhance the salt tolerance of alfalfa. Wang et al. [17] used the vacuum infiltration-aided Agrobacterium-mediated method to transfer salt tolerance gene *AtNHXl* into Alfalfa and obtained salt tolerant somatic embryos. Pan et al. [18] used Agrobacterium EHA105-mediated cotyledon dissemination method to transfer *Cg-NHXl* gene into cv. Xinjiang Daye. Zhao et al. [19], transferred the *Atriplex dimorphostegia NHX* salt tolerance gene into aseptic seedling leaves and cotyledons of cv. Xinmu No.1 and cv. Xinjiang Daye, and obtained salt tolerant plants. Li et al. [20] studied genetic transformation of *M. sativa* and transferred *DsNRT2* (a Nitrate Transporter) that was cloned from *Dunaliella salina* into cv. Zhongmu No.1 and 7 with positive seedlings obtained. Liu et al. [21] transferred the *HAL1* gene that was cloned from yeast into the embryogenic callus of cv. Longmu 803 with the Agrobacterium-mediated method and obtained 11 transgenic plants. Expression of HAL1 gene improved the salt tolerance characteristics of alfalfa. Sui [22] studied a vacuolar Na^+/H^+*antiporter* gene (*ScNHXI*) from *Suaeda corniculata*, which was introduced into *M. sativa* and improved salt tolerance of transgenic alfalfa. Sheng [23] successfully introduced the *DREB2A* gene into a range of alfalfa genotypes cvv. Zhaodong, Aoha, Gongnong 1, and Gongnong 2, primarily from Heilongjiang province with the aim of improving drought tolerance characteristics. Wang et al. [24] transferred the *LEA3* gene from barley into cv. Zhongmu 1 through biolistic transformation and obtained an increased survival rate of transgenic plants under high salt stress. The salt tolerance of the transgenic plants was greater than that of the control plants, which shows that *LEA3* gene has potential for application in developing drought resistance and salt tolerance for alfalfa breeding. The improvement of salt tolerance was also the aim of Bao et al. [25] where the transgenic co-expression of *tonoplast Cation*/H^+ antiporter and H^+*-pyrophosphatase* genes from the xerophytic plant *Zygophyllum xanthoxylum* increased the growth and salt tolerance of genetically modified alfalfa plants under field conditions. The use of transgenesis to introduce genes into alfalfa to confer stress tolerance outside of the range that is presented within sexually compatible species offers the potential to greatly increase the adaptation of cultivated alfalfa in stressed and degraded environments in China.

The recent advent of transcriptomics and next generation sequencing technologies offers the potential to identify genes involved in stress tolerance on a broader scale than previous technologies. Deep sequencing of the transcriptome of alfalfa was used recently to identify 5605 differentially expressed genes in the crown buds of cv. Zhaodong when grown under naturally occurring freezing stress in northern China [26]. These studies may identify further candidates for deployment using transgenic or genome editing technologies.

Due to various constraints, the further development of transgenic alfalfa in China has not been widely carried out. Although progress and achievements have been made, most are still at the laboratory experiment phase. Currently there are no transgenic alfalfa cultivars developed and adopted in practice.

3.6. Molecular Marker Technology

In stress resistance breeding, Yang et al. [27] used *M. sativa* L. cv. Zhongmu No. 1 and salt sensitive alfalfa as materials to screen from and build up the basis for research on salt tolerance genes for molecular marker development in alfalfa, using the Random Amplified Polymorphism

(RAPD) technique. In disease resistance breeding, Gui et al. [28] used RAPD technique and Bulked Segregation Analysis to study molecular markers linked to resistance genes against brown spot disease in five *Medicago* species, and selected eight random primers which are able to indicate polymorphism between resistant and susceptible materials in more than three species simultaneously. Wei [29] used RAPD, Simple Sequence Repeat (SSR), and Inter Simple Sequence Repeat (ISSR) molecular marker and field experiments to study DNA fingerprints and genetic diversity of 84 alfalfa lines. Xu and Jia [30] regenerated somatic hybrid plants between alfalfa and sainfoin by protoplast fusion and culture, and extracted DNA from leaf material from regenerants of hybrid tissue; they also analyzed the recombination of the genetic materials by RAPD and Southern hybridization techniques. Yu et al. [31] combined SSR and Expressed Sequence Tag derived SSR (EST-SSR) molecular marker techniques to construct molecular marker profiles of alfalfa germplasm. Su et al. [32] established a tetraploid F2 mapping family in a hybrid between subsp. *sativa* and susbsp. *falacata*, and constructed a genetic map of this population. Fu and Pauls [33] used 20 imported alfalfa accessions and demonstrated that RAPD markers could determine the relationship between alfalfa varieties. SSR marker technologies have been used to characterize and contrast the genetic diversity within and between cultivars and landraces of Chinese origin with those from other geographic regions, thereby providing insights into the nature of population structure and variation that could be used in a genomics assisted breeding program [34] Again, there have been no bred varieties of alfalfa registered in China using these technologies to date.

3.7. Space Breeding

Space breeding, also called space technology breeding or spaceflight breeding, takes advantage of space environment which has been known to induce physiological changes in plants and heritable mutations, although the mechanisms through which these mutations occur are not fully understood [35,36]. Space breeding has been performed in China since 1987 with recoverable spacecraft allowing the recovery of plant material. Space breeding has resulted in new cultivars of rice, wheat, and soybean, and although the efforts have focused on major food crops approximately 1% of the seed accessions in China's space breeding program have been pastures [35]. Ren [37] studied alfalfa seed carried by recoverable satellite using Fourier Transform (FT)-Raman spectroscopy, which showed that the content of DNA and Ca^{2+} increased and that of sugar and lipid decreased compared with the control seed on the ground. Zhang [38] analyzed leaf microstructure of four alfalfa varieties grown from seeds carried by Shenzhou-3 spacecraft in comparison with control plants, and found that leaf thickness and palisade tissue thickness of the varieties are much greater than that of the control plants, with their leaf protuberance degree being much less than the control plants. In addition, the cell structure tightness of the four alfalfa varieties was different from the control plants. These mutations may have influenced its resistance performance, and could be used for further resistance breeding. Zhang [39] studied the space mutation of cv. Longmu 803 and cv. Zhaodong with their seeds carried by recoverable satellite. The results indicated that the types of alfalfa chromosome aberration produced by space mutation were mainly the formation of micronuclei. Visible mutations occurred in mitotic cell chromosomes due to space mutation effects.

Space breeding technology has been widely used in the breeding of other crops; however, there is no alfalfa variety registered on a national scale. Nevertheless, there are several varieties registered in provinces or autonomous regions of China.

4. Problems and Challenges

Although China has made considerable achievements in alfalfa breeding, there is room for improvement compared with other countries, and the overall level of alfalfa breeding is still low and cannot meet the requirements of development in grassland agriculture, grassland animal husbandry, and ecological reconstruction. The main problems and challenges are discussed below.

4.1. Slow Breeding Cycle

The number of varieties bred in China is relatively low, and the progress of resistance breeding is slow. Up to date, only 36 bred Alfalfa varieties have been registered by the National Committee for the Examination and Approval of Herbage Variety in China, compared with 192 alfalfa varieties registered in the USA in 2015. The breeding target is more focused on yield in China, and overwintering rates has also become one of the priority breeding targets due to northern China being one of the major regions of *M. sativa* plantation. Hence, the target characteristics of bred varieties include high yield and high overwintering rates, and to a lesser degree, cold tolerance and fall dormancy. In disease and insect resistance, the main focus is on pathogen identification and field investigation of disease conditions [40,41], disease resistance identification methods, and the influence of environment on alfalfa resistance performance. There are few varieties that have been specifically bred for disease resistance, insect resistance, and drought tolerance, and almost no varieties for herbicide resistance.

4.2. Breeding Methods

At present, alfalfa varieties registered in China are mainly bred with conventional breeding methods. These methods are simple and widely adopted, and take advantage of excellent germplasm resources in different ecological regions to develop new varieties suitable for regional conditions. However, these methods normally take a long time to develop cultivars that are used on farms. The application of genetic engineering and other modern biotechnology in alfalfa breeding has been put in place in China for only 20 years, and research is currently focused on target gene cloning, construction of expression vectors, and related laboratory experiments with the commencement of some field trials.

4.3. Low Adoption Rate of Bred Varieties

The supply chain for seed production, distribution, and marketing of seed is less well developed in China than some other countries where specialized breeding companies are often involved and responsible for this process. In China, however, alfalfa breeding resources are mainly centered in universities and research institutes. As a result, the breeding targets are not well placed to meet the market demands, and breeding research is disconnected with business development and without effective involvement from industries, thereby leading to poor adoption of new varieties [42]. Largely due to the slow process of seed production of new varieties, most enterprises choose to purchase imported varieties, further reducing the adoption rates of newly bred varieties in China.

5. Opportunities and Further Research

In recent years, the collection of germplasm resources, innovation of breeding theory and technology, and molecular biology assisted breeding have played an important role in accelerating alfalfa breeding in China. These experiences confirmed that we could learn from the experience of developed countries and take integrated approaches in enhancing alfalfa breeding in China.

5.1. Strengthening the Research and Utilization of Alfalfa Germplasm Resources

Many developed countries give great emphasis on the collection, preservation, research, and utilization of germplasm resources. China covers a large territory with very diverse ecological environments due to the influence of latitude, elevation, and topography. This makes the country rich and unique in alfalfa germplasm resources, including conventional local and wild types, improved varieties, and new breeding materials such as alfalfa mutants, all of which form the basis of alfalfa breeding. In addition to the development of breeding methods and the adoption of new technology, a critical step for the success of modern breeding programs is to widely collect and make use of the right germplasm resources. Due to poor awareness in germplasm protection, overgrazing, and excessive exploitation, some excellent forage germplasm resources have been lost. Lack of desirable germplasm

resources for screening and breeding has seriously affected the process and results for developing new varieties. Therefore, it is imperative to learn from this experience, conduct further research and identification of existing germplasm in a systematic way, screen materials with desirable characteristics, and conduct research in an innovative manner [43].

5.2. Improving Alfalfa Breeding Theory

In the developed world, great efforts have been made to develop the basic theory of breeding. Forage breeding in China is not only behind developed countries, but also behind crop breeding domestically. This is largely due to lack of advanced forage breeding theory and technology. In order to make a breakthrough in alfalfa breeding, we need to strengthen research on forage breeding theory and techniques; otherwise, slow breeding cycle/process will be unavoidable.

5.3. Enhancing the Exploration of New Techniques in Alfalfa Breeding

Conventional breeding methods are basic methods to breed new variety all over the world and will continue to play an important role in alfalfa breeding in China in the near future. With the rapid development of science and technology, molecular techniques and transgenic technology have been widely used in alfalfa breeding worldwide. Transgenic technology has been used to improve the resistance of alfalfa to cold, drought, salinity, alkalinity, disease, herbicides, and insect pests. Molecular technology has been widely used in assisted alfalfa breeding and germplasm introgression research, genetic linkage map, germplasm identification, and genetic diversity studies. The combination of conventional breeding techniques with modern breeding techniques can help to break through some of the constraints and speed up the breeding cycle, which is critical for improving alfalfa breeding technology in China.

5.4. Conservation of Distinctive and Special-Purpose Alfalfa Materials

Alfalfa breeding in China has the following characteristics: broad cultivation area, complex ecological conditions, and a wide range of uses in production. According to local conditions and uses, specific alfalfa varieties can be selected and cultivated. For example, in germplasm collection and application in western China, research should focus on germplasms that are resistant/tolerant to drought, cold, and saline soils of sandy texture. In these regions, new varieties that are able to prevent wind erosion and fix sandy soils, and to conserve water and soil resources could be the major breeding targets for alfalfa. On the other hand, improved animal production through high forage production and better forage quality could also be a high priority target. Therefore, selection and breeding of special-purpose varieties that fit both the environment/soil constraints and production requirements for various feeding systems, such as hay and silage, and different growing seasons are necessary to address regional production and sustainability issues [2]. Strategies must be worked out on a regional basis to achieve specific needs.

Acknowledgments: The authors would like to thank Zhongnan Nie for his assistance during the development of this manuscript.

Author Contributions: S.S., L.L. and K.S. wrote and edited this review.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Geng, H.Z. *Alfalfa of China*; China Agriculture Press: Beijing, China, 1995. (In Chinese)
2. Shi, S.L.; Nan, L.L.; Guo, Q.E. Achievements and prospect of alfalfa breeding in China. *J. Plant Genet. Res.* **2010**, *11*, 46–51. (In Chinese)
3. Yang, Q.C.; Sun, Y. The History, Current situation and development of alfalfa breeding in China. *Chin. J. Grassl.* **2011**, *33*, 95–101. (In Chinese)

4. Yang, Q.C.; Kang, J.M.; Zhang, T.J.; Liu, F.Q.; Long, R.C.; Sun, Y. Distribution, breeding and utilization of alfalfa germplasm resources. *Chin. Sci. Bull.* **2016**, *61*, 261–270.
5. Chinese Forage Cultivar Registration Board (CHCRB). *Licensed Cultivars of Herbage Crops in China*; China Agricultural University Press: Beijing, China, 1999. (In Chinese)
6. Chinese Herbage Cultivar Registration Board (CHCRB). *Licensed Cultivars of Herbage Crops in China (1999–2006)*; China Agriculture Press: Beijing, China, 2008. (In Chinese)
7. Wu, D.G. Resistance of Alfalfa (*Medicago sativa* L.) to Aphid and Regional Adaptability Experiment of New Alfalfa Line HA-3. Master's Thesis, Gansu Agricultural University, Lanzhou, China, 2007. (In Chinese)
8. Gui, Z.; Gao, J.M. Advances in research of alfalfa breeding in China. *J. Tianjin Agric. Coll.* **2003**, *10*, 37–41. (In Chinese)
9. Cai, L.Y.; Shi, F.L.; Zhang, F.S.; Gao, C.P. Research progresses in alfalfa heterosis. *Chin. J. Grassl.* **2010**, *32*, 92–97. (In Chinese)
10. Wu, Y.F. Researching on breeding Alfalfa Male Sterile Line. *Chin. J. Grassl.* **1980**, 37–39. (In Chinese)
11. Smith, K.F.; Forster, J.W.; Spangenberg, G.C. Converting genomic discoveries into genetic solutions for diary pastures. *Anim. Prod. Sci.* **2007**, *47*, 1032–1038. [CrossRef]
12. Kolliker, R.; Rosellini, D.; Wang, Z.-Y. Development and application of technological and molecular genetic tools. In *Fodder Crops and Amenity Grasses. Vol. 5 in the Series: Handbook of Plant Breeding*; Boller, B., Posselt, U., Veronesi, F., Eds.; Springer: New York, NY, USA; pp. 89–113.
13. Li, X.; Brummer, E.C. Applied genetics and genomics in alfalfa breeding. *Agronomy* **2012**, *2*, 1–44. [CrossRef]
14. Li, H.W.; Ma, D.M.; Xu, X. Advance and prospects on salt-tolerant transgenic alfalfa. *North. Hortic.* **2012**, *19*, 184–188. (In Chinese)
15. Liang, H.-M.; Xia, Y.; Sun, Z.-X.; Wang, T.-M.; Liu, D.-X.; Wang, G.-L.; Huang, J.; Chen, S.-Y. Establishment of genetic transformation system of *Medicago sativa* mediated by *Agrobacterium tumefaciens*. *J. Agric. Biotechnol.* **2015**, *13*, 152–156. (In Chinese)
16. Yan, L.P.; Xia, Y.; Mao, X.H.; Liu, C.; Liang, H. Breeding and Salt Resistance Evaluation of *BADH* Transgenic Alfalfa Cultivar Shanmu 2. *Chin. Bull. Bot.* **2011**, *46*, 293–301. (In Chinese)
17. Wang, Q.L.; Wang, S.M.; Zhang, J.L.; Bao, A.K.; Chen, T.X.; Lou, J.Q.; Lu, N. Transformation Studies of *Medicago sativa* Mediated by *Agrobacterium tumefaciens* with *AtNHX1* Gene. *Pratacult. Sci.* **2005**, *23*, 55–59. (In Chinese)
18. Pan, J.L. Plant Regeneration and Molecule Detection for the Transgenic Xinjiang Big Leaf Alfalfa of Cg-*NHX1* Gene from Halophyte Chenopodium glaucum. Ph.D. Thesis, Xinjiang Agricultural University, Urumchi, China, 2007. (In Chinese)
19. Zhao, H.J.; Zhang, B.; Li, P.Y.; Chen, A.P. Influencing Factors on Alfalfa Transformation with *Atriplex dimorphostegia NHX* Gene Mediated by *Agrobacterium tumefaciens*. *Acta Agrestia Sin.* **2007**, *15*, 418–422. (In Chinese)
20. Li, S.L.; Yang, G.; Yi, X.L.; Ma, G.; Ma, X.Z.; Li, Y.; Cao, Y. Transformation of Alfalfa (*Medicago sativa* L.) by Agrobacterium-mediated Process with *DsNRT2* Gene from *Dunaliella salina*. *J. Sichuan Univ.* **2008**, *45*, 409–412. (In Chinese)
21. Liu, Y.Z.; Wei, Z.Y.; Xing, S.C.; Tan, H.; Gao, M.; Dong, Y.S. Transgenic Alfalfa with *HAL1* Gene and Its Salt Tolerance. *J. Jilin Agric. Sci.* **2008**, *33*, 21–24. (In Chinese)
22. Sui, X. Cloning of Vacuolar Na^+/H^+ *Antiporter* Gene from *Suaeda corniculata (ScNHX1)* and Transformation Studies of *ScNHX1* in Medicago sativa. Master's Thesis, Jilin Agricultural University, Changchun, China, 2007. (In Chinese)
23. Sheng, H. Construction of Plant Expressive Vectors of *DREB2A, SCMRP* Gene and Genetic Transformation into Alfalfa. Master's Thesis, Northeast Agricultural University, Harbin, China, 2006. (In Chinese)
24. Wang, Y.; Zhu, B.C.; Sun, Y. Transformation of Barley *LEA3* Gene into Alfalfa (*Medicago sativa* L.). *J. Nucl. Agric. Sci.* **2007**, *21*, 249–252. (In Chinese)
25. Bao, A.K.; Du, B.Q.; Touil, L.; Kang, P.; Wang, Q.L.; Wang, S.W. Co-expression of tonoplast Cation/H^+ antiporter and H^+-pyrophosphatase from xerophyte *Zygophyllum xanthoxylum* improves alfalfa plant growth under salinity, drought and field conditions. *Plant Biotech. J.* **2016**, *14*, 964–975. [CrossRef] [PubMed]
26. Song, L.; Jiang, L.; Chen, Y.; Shu, Y.; Bai, Y.; Guo, C. Deep-sequencing transcriptome analysis of field-grown *Medicago sativa* L. crown buds acclimated to freezing stress. *Funct. Integr. Genom.* **2016**, *5*, 495–511. [CrossRef] [PubMed]

27. Yang, Q.C.; Han, J.G. A Study on Using RAPD in Alfalfa Salt Tolerant Breeding. *Acta Agrestia Sin.* **2003**, *11*, 27–32. (In Chinese)
28. Gui, Z.; Yuan, Q.H. Studies on RAPD Polymorphic in Alfalfa Resistant to Brown Blot. *Acta Agrestia Sin.* **2002**, *10*, 274–278. (In Chinese)
29. Wei, Z.W. DNA Fingerprint of *Medicago sativa* Variety Genomes Using SSR, ISSR and RAPD. *Acta Pratacult. Sin.* **2004**, *13*, 62–67. (In Chinese)
30. Xu, Z.Q.; Jia, J.F. Molecular Identification of Intergeneric Somatic Hybrid Plants between Alfalfa and Sainfoin. *Chin. J. Biotechnol.* **2000**, *16*, 173–178. (In Chinese)
31. Yu, L.Q.; Liu, R.X.; Su, D.; Yun, J.F. Genetic Diversities of Fall Dormancy Alfalfa Based on SSR and EST-SSR. *Acta Agrestia Sin.* **2009**, *31*, 52–58. (In Chinese)
32. Su, D.; Yu, L.Q.; Zhou, Y.L.; Sun, J.J.; Wang, F.G.; Wu, R.N. Establishment of a Tetraploid Alfalfa Family and Analysis of Its Genetic Variability. *Acta Agrestia Sin.* **2011**, *19*, 657–662. (In Chinese)
33. Fu, J.H.; Pauls, K.P. Identification of Alfalfa Populations by Using RAPD Molecular Marker Method. *Crops* **1995**, *6*, 28. (In Chinese)
34. Qiang, H.; Chen, Z.; Zhang, Z.; Wang, X.; Gao, H.; Wang, Z. Molecular diversity and population structure of a worldwide collection of cultivated tetraploid alfalfa (*Medicago sativa* subsp. *sativa* L.) germplasm as revealed by microsatellite markers. *PLoS ONE* **2015**, *10*, e0124592.
35. Chengzhi, L. Agronomy in space—China's crop breeding program. *Space Policy* **2011**, *27*, 157–164. [CrossRef]
36. Li, Y.; Liu, M.; Cheng, Z.; Sun, Y. Space environment induced mutations prefer to occur at polymorphic sites of rice genomes. *Adv. Space Res.* **2007**, *40*, 523–527. [CrossRef]
37. Ren, W.B.; Han, J.G.; Zhang, Y.W. A Study of the effect of space mutagenesis of grass seeds. *Pratacult. Sci.* **2006**, *23*, 72–76. (In Chinese)
38. Zhang, W.J. Effects of Four Alfalfa Cultivars on Mutagenic of Spaceflight. Master's Thesis, Gansu Agricultural University, Lanzhou, China, 2010. (In Chinese)
39. Zhang, Y.X.; Liu, J.L.; Han, W.B.; Tang, F.; Hao, R.; Shang, C.; Du, Y.; Li, J.; Wang, C. Biological effect of aerospace environment of alfalfa. *J. Nucl. Agric. Sci.* **2009**, *23*, 266–269. (In Chinese)
40. Chen, Y.J.; Liu, X.M.; Cui, G.W.; Xi, Q.X. Research progress in root rot of alfalfa. *Grassl. China* **2000**, *22*, 51–56. (In Chinese)
41. Chen, S.K.; Yao, G.J. Resistance to common leafspot in different alfalfa varieties. *Pratacult. Sci.* **1994**, *11*, 61–62. (In Chinese)
42. Yang, Q.C.; Sun, J.; Han, G.D. Studies on RAPD Polymorphic in salt-tolerant alfalfa and salt-sensitive alfalfa. *Acta Agrestia Sin.* **2001**, *9*, 83–86. (In Chinese)
43. Wang, Y.H.; Gao, H.W.; Wang, Z.; Li, Y.; Liu, G.B. Study and evaluation of drought resistance capacity of *Medicago glutinosa* germplasm resources at seedling stage. *J. Plant Genet. Resour.* **2009**, *10*, 443–447. (In Chinese)

agronomy

MDPI

Review

Haploid and Doubled Haploid Techniques in Perennial Ryegrass (*Lolium perenne* L.) to Advance Research and Breeding

Rachel F. Begheyn [1], Thomas Lübberstedt [2] and Bruno Studer [1,*]

[1] Molecular Plant Breeding, Institute of Agricultural Sciences, ETH Zurich, 8092 Zurich, Switzerland; rachel.begheyn@usys.ethz.ch

[2] Department of Agronomy, Iowa State University, 100 Osborne Drive, Ames, IA 50011, USA; thomasl@iastate.edu

* Correspondence: bruno.studer@usys.ethz.ch; Tel.: +41-446-320-157

Academic Editors: John W. Forster and Kevin F. Smith
Received: 28 September 2016; Accepted: 10 November 2016; Published: 28 November 2016

Abstract: The importance of haploid and doubled haploid (DH) techniques for basic and applied research, as well as to improve the speed of genetic gain when applied in breeding programs, cannot be overstated. They have become routine tools in several major crop species, such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), and barley (*Hordeum vulgare* L.). DH techniques in perennial ryegrass (*Lolium perenne* L.), an important forage species, have advanced to a sufficiently successful and promising stage to merit an exploration of what their further developments may bring. The exploitation of both in vitro and in vivo haploid and DH methods to (1) purge deleterious alleles from germplasm intended for breeding; (2) develop mapping populations for genetic and genomic studies; (3) simplify haplotype mapping; (4) fix transgenes and mutations for functional gene validation and molecular breeding; and (5) hybrid cultivar development are discussed. Even with the comparatively modest budgets of those active in forage crop improvement, haploid and DH techniques can be developed into powerful tools to achieve the acceleration of the speed of genetic gain needed to meet future agricultural demands.

Keywords: anther culture; doubled haploid (DH); forage crops; microspore culture; perennial ryegrass (*Lolium perenne* L.); plant breeding

1. Introduction

Biomass, produced by agriculture, is humanity's main source of food, feed, and functional materials, such as fibre for cloth, construction wood, and industrial starches. In the latter half of the previous century, our agricultural systems generated higher yields than ever before during the Green Revolution. Presently, driven by predictions of global population growth, changing environmental conditions and the claim to arable land made by the biofuel sector, it is crucial that the yields increase sharply once again [1]. A doubling of the speed of yield growth is necessary in order to be able to feed the world's population in 2050 [2]. Since the Green Revolution, however, the agricultural paradigm has changed. More output with less input, in terms of agrochemicals, fertilizers, and water, on the existing cultivated land area is the current mandate [3]. Sustainability issues, such as biodiversity conservation, maintaining ecological services, and safeguarding soil fertility should be addressed by modern agriculture in order to achieve both global food security and environmental sustainability [4].

Aside from the undesirable option of expanding the agricultural area, production increases can be achieved in two ways. Firstly, by optimizing management practices, for example through precision agriculture or increasing water and nutrient supply to marginal lands, the gap between attainable

and actual yields may be decreased [3,5]. The second and generally considered most sustainable way to increase outputs is via genetic crop improvements by plant breeding, raising potential yields [6,7]. Perhaps even more importantly, plant breeding enables the integration of novel traits, which is essential in achieving yield stability in the changing climatic conditions we are facing [8]. There has, therefore, been a shift in emphasis towards breeding for crop characteristics, such as nutrient and water use efficiency, tolerance to drought or salt stress, and the ability to produce high and stable yields under sub-optimal conditions [9]. The challenge for contemporary plant breeding is to not only integrate new traits into our crops, but to accelerate the genetic gain of its breeding programs at the same time, in order to achieve a doubling in speed of yield increase.

The potential impact of haploid and doubled haploid (DH) techniques on improving the speed of genetic gain when applied in breeding programs, as well as their importance and diverse applicability in basic and applied research, cannot be overstated and has been the subject of numerous reviews [10–14]. DH techniques have been, and are being, used to accelerate the breeding programs of a range of crops, most notably maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.) [15,16]. A plethora of in vivo and in vitro protocols exist to accommodate the widely varying degrees of response between species to DH induction [11]. Even so, a number of scientifically (*Arabidopsis thaliana*) and economically important species, such as tomato (*Solanum lycopersicum* L.), cotton (*Gossypium* spp.), grape (*Vitis* spp.), trees, and medicinals are still considered recalcitrant [14].

This review aims to illustrate the importance of allocating time and resources towards further developing the efficiency and efficacy of haploid and DH techniques for forage crop breeding. In forage-based agriculture, to which close to 70% of the world's agricultural land is devoted (FAOstat, 2013), the challenges described above are no less pressing [17]. This extensive area is not only at the basis of global meat and milk production, but also plays a major role in ecosystem processes, such as nutrient cycling and carbon sequestration, as well as being a reservoir for the preservation of biodiversity [18]. Technologies, such as DH induction, can play a key role in accelerating breeding of forages, which we will illustrate using the economically important crop perennial ryegrass (*Lolium perenne* L.) as an example.

2. Haploids and Doubled Haploids: Their Production and Use in Breeding and Research

Haploids are defined as plants with a single chromosome set (n) and DHs as 100% homozygous individuals stemming from chromosome doubled haploids ($2n$). The preferred method for the production of haploid or DH plants differs per species and depends on protocol availability, as well as efficiency in terms of investments and yields. Immature microspores, which are abundant in most flowering plants, can be induced to develop into embryos and, subsequently, into plants in vitro (androgenesis) [19,20]. Isolated microspore culture (IMC), although technically more challenging, is preferred over anther culture (AC) because of its higher efficiency [19] and has been routine in barley, tobacco (*Nicotiana tabacum* L.) and rapeseed (*Brassica napus* L.) breeding for some time [14]. Response to in vitro DH induction is highly genotype-dependent and colchicine may be needed for chromosome doubling [11]. Additionally, factors such as donor plant growing conditions, stress pre-treatment, medium composition and culture conditions all influence the embryo induction rates, number of regenerated plants and, especially in *Poaceae* species, the ratio between green and albino regenerants [11,21–23]. After roughly 50 years of research into DH induction methods it must be concluded that there probably is no single 'master switch' to stimulate the formation of embryos from any species of microspore [20]. Successful protocols, therefore, differ significantly between, and even within, species or are not yet available at all [24–26].

Haploid seed production can be induced in vivo by using irradiated or heat-treated non-functional pollen, pollen of distantly related species followed by uniparental genome elimination (wide hybridization), or pollen from haploid inducer genotypes [13]. DH wheat (*Triticum aestivum* L.) plants, for example, can be efficiently produced via wide crosses with maize, embryo rescue, and chemical chromosome duplication [27]. In hybrid maize breeding, haploid inducer lines are routinely used

to obtain an average of 10% haploid kernels on the seed parent. The resulting haploid seedlings are treated with colchicine to obtain DHs [16]. Ovule culture (gynogenesis) is mainly used in species recalcitrant to androgenesis, since its efficiency is much lower due to the smaller number of ovules available per flower. The value of the DH can make gynogenesis an economical option, however, for example in sugar beet (*Beta vulgaris* L.), onion (*Allium cepa* L.), and some tree species [28].

Haploid and DH technologies have found wide application, especially in the field of plant breeding, for those crops where protocols are sufficiently effective [29]. Major reductions in the time needed for cultivar development have been realized, since the availability of DHs eliminates the need for the 5–7 generations of selfing traditionally required to produce inbred lines [14]. In combination with marker assisted selection (MAS), DH induction has significantly increased the efficiency of backcross breeding [30]. By applying DH induction to one of the early backcross generations, genotypes carrying the trait to be introgressed, as well as having the highest possible proportion of the elite genome, can be selected quickly. DHs have been released directly as cultivars in barley [31], rice (*Oryza sativa* L.) [32], rapeseed [33], wheat [27], and other crops, or used as parents of F_1 hybrids of vegetables and maize [16], in order to benefit from hybrid vigour (heterosis) [34,35]. In ornamental breeding, haploid plants have commercial value of their own because of their smaller size compared to diploids [36].

Furthermore, DH populations have been invaluable for QTL discovery, especially in cereals, since their immortality enables robust phenotyping data to be gathered in different locations and over several years [37]. In outcrossers, which suffer from inbreeding depression, using at least one DH parent to create mapping populations has been effective [10]. Genome sequencing studies have used haploids or DHs to reduce the complexity of assembly, for example in peach (*Prunus persica*), citrus (*Citrus* spp.), coffee (*Coffea* spp.), apple (*Malus pumila*), and pear (*Pyrus* spp.) [13]. Microspores of tobacco, rapeseed, wheat, and barley are exploited in transformation and mutagenesis programs, in order to fix mutations and transgenes in a single step through subsequent DH induction [38–42]. For example in Brassicas, microspore mutation studies have enabled modifications of disease resistance, cold tolerance, and fatty acid composition [43]. Additionally, in vitro microspore culture systems have allowed for detailed study of embryogenesis, early cell fate decisions, embryogenesis, and totipotency [44–46]. All of these applications of haploid and DH techniques, as well as many others not mentioned here, could confer the same benefits to perennial ryegrass breeding and research as they have done, and currently do, in species for which effective and efficient DH induction protocols are available [14].

3. Perennial Ryegrass

3.1. Perennial Ryegrass Breeding

Perennial ryegrass, the economically most significant forage grass worldwide, is popular for its good yields, high digestibility for animals, and excellent grazing tolerance. Natural populations are diploid ($2n = 2x = 14$) but both diploid and artificially-created tetraploid cultivars are available. Compared to diploids, tetraploid plants are larger, have a higher nutritive value for animals and better abiotic and biotic stress tolerances, but reduced sward density and lower persistence [47–49]. In contrast to the thousands of years of breeding effort in annual grasses, such as wheat, barley, and rice, perennial grass breeding is not even a century old [50]. Nevertheless, important improvements in yield potential, persistency and disease resistance, as well as feeding value—through increased water-soluble carbohydrate content, for example—have been achieved [51,52]. The allogamous nature of this species, due to its highly effective gametophytic self-incompatibility system [53], has, until now, restricted breeding to the population level, resulting in marked genetic diversity and heterozygosity within cultivars [54]. Modern perennial ryegrass varieties are synthetic populations, selected from the progeny of a polycross between elite genotypes, obtained by recurrent selection, with a good agronomical performance [50]. As a consequence, genes governing key agricultural traits are rarely completely fixed and cultivar characterization for variety registration purposes is complicated [55].

Compared to cereal grain yield increases, perennial ryegrass yield gains have been described as low [52]. Commonly cited causes for this relatively slow progress, calculated as an increase in dry matter yield of just 3.2% per decade [51], are (1) the longer breeding cycle of perennial forage crops; (2) the absence of a harvest index trait to facilitate partitioning of dry matter into the marketed product; (3) a lack of commercial exploitation of heterosis; (4) a focus on breeding for other traits than yield, such as resistance to crown rust (*Puccinia coronata*), reduced aftermath heading and early spring growth [56]. In effect, the modest yield increases in perennial ryegrass are most likely due to constraints stemming from life history traits and the techniques available to forage breeders rather than physiological limitations or lack of genetic variation [51]. If genetic gains are to be improved, it is, therefore, imperative to expand the arsenal of breeding tools, as well as to find ways of working with, or around, the characteristic life cycle of perennial ryegrass. As has been shown in other crops, DH techniques have excellent credentials to help address both of these challenges [14].

3.2. Doubled Haploids in Perennial Ryegrass

Perennial ryegrass DH production through AC (Figure 1) was first attempted in the late 1970s. Initially, only embryos and albino plants were obtained, until the first green regenerants were reported in 1984 [57]. During the following decades, contemporary protocols for barley [58] and wheat [59] AC were adapted for use in perennial ryegrass by optimizing the pre-culture temperature stress, in vitro carbohydrate source, growth regulator additions, and culture conditions, such as light and temperature [58,60–62]. The total number of regenerated plants was thus increased, although the percentage of albinos remained high and genotypes capable of producing green plants by androgenesis were described as being rare exceptions [59,63]. For example, only 71 out of 229 genotypes, derived from 15 cultivars, produced regenerants after DH induction, and only one genotype produced green plants [64]. Only one study recounts green plant regeneration via IMC in perennial ryegrass, while in vitro gynogenesis or in vivo haploids have never been reported [65].

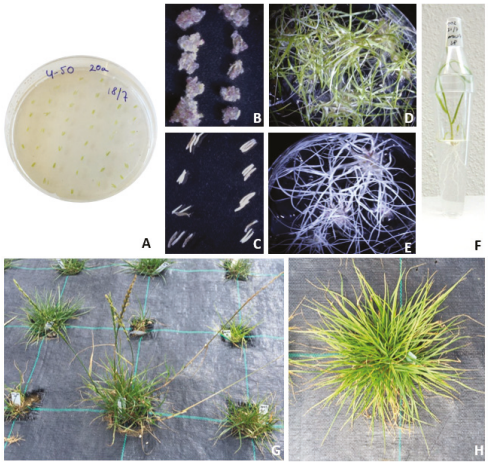


Figure 1. Different stages of perennial ryegrass (*Lolium perenne* L.) anther culture (AC) and doubled haploid (DH) production. (**A**) Fresh anthers on DH induction medium; (**B**) anthers of a responsive donor genotype producing many embryo-like structures (ELS) on DH induction medium, six weeks post AC; (**C**) anthers of an unresponsive donor genotype, six weeks post AC; (**D**) green and albino putative DH plantlets growing from ELS cultured on DH regeneration medium, four weeks post subculture; (**E**) albino plantlets growing from ELS, four weeks post subculture; (**F**) putative DH plantlet on regeneration medium; (**G**) flowering DH plant in the field; and (**H**) a vigorous vegetative DH plant in the field (photographs by Begheyn, R. F.).

As in other crops, the ability to (1) form embryos or embryo-like structures (ELS); (2) regenerate plants; and (3) the ratio of green and albino regenerants, is under genetic control in perennial ryegrass [59,61,63,66]. Anther donor genotype was found to determine 73% of the variation in embryo yield [59] and 80% of the variation in green plant production [63]. Evidence of transgressive segregation was reported when a 60% higher green plant yield, up to 59 green plants per 100 anthers, was observed in the progeny of crosses between three genotypes responsive to AC [66,67]. Based on these results, Halberg et al. proposed the so-called 'inducer approach': introducing the ability to produce high numbers of green plants into recalcitrant germplasm by crosses with genotypes exhibiting that ability (the inducers; not to be confused with in vivo haploid inducers) [66]. Evidence for the efficacy of the inducer approach was reported by Madsen et al., who achieved a 7.3-fold increase in green plant yield in crosses between inducers and regular breeding germplasm [63]. The inducer approach was also effective in darnel ryegrass (*L. temulentum* L.) where, using an adapted perennial ryegrass AC protocol, a higher responsiveness was achieved through intercrossing good responders [68].

Androgenic capacity is recessively inherited in perennial ryegrass and probably controlled by a relatively small number of genes with a large effect [63], similar to what is supposed to be the case in barley [69]. The ability to develop ELS seems to be controlled by additive gene effects, while total plant regeneration capacity and green plant percentage seem to be controlled by dominance [67]. There may be different genes with epistatic interactions affecting green and albino plant production (as was found in wheat, [70]). However, since it has been hypothesised that in vitro conditions cause the mutations that lead to plastid development defects, albinism rates are likely to be influenced more by environment than by genetics [67,71]. To date, no QTL studies on androgenetic capacity in perennial ryegrass have been published. In the cereals, a limited number of reports exist, typically finding between 1 and 8 QTL for each component trait, explaining 3%–65% of the variation [72]. For example, two QTL, explaining 53% of the variation in green plant regeneration, were recently found in wheat [73]. In a triticale (×*Triticosecale*) QTL study on androgenic responsiveness, 28 QTL on five chromosomes were found, explaining 5%–22% of the phenotypic variation [74].

Relatively high spontaneous chromosome doubling rates, between 50% and 80%, are common in perennial ryegrass AC [59,61,66,75]. Colchicine, a toxic chromosome doubling agent, is, therefore, usually not applied, making the whole procedure safe for human health [76]. Isozyme pattern characterization studies have confirmed that nearly all diploid regenerants are, in fact, homozygous. Andersen et al., for example, reported that only three out of 913 anther-derived plants were heterozygous [65]. Non-diploid regenerants are mostly haploids, but also include a small percentage of homozygous polyploids. Apart from ploidy level, confirmation of homozygosity and reports of obvious signs of inbreeding depression, there is a marked lack of information on the performance of the DH plants. One field study compared the biomass and seed yields of DH lines and their parents, finding reductions of 80 and over 90%, respectively [77]. A different field trial evaluated seed set in 75 DH lines selected for their vigorous growth and found a 70% lower performance compared to their parents [65]. Nevertheless, one DH clone produced 5.08 g seeds/plant compared to the 1.44 g of its parent, suggesting that it is possible to identify DH plants with both vigorous growth and excellent fertility. Self-fertility of DH plants was investigated in a study by Madsen et al. and their seed set was found to be very low at 0–0.4 seeds per spike [78].

4. Future Applications of Haploid and Doubled Haploid Techniques in Perennial Ryegrass

Strategies to accelerate perennial ryegrass breeding in order to increase yield gains have been, and are being, discussed [56,79–82], but the role that DH techniques could play in this context has hardly ever been considered. DH techniques have the potential to help unlock and increase the genetic variation available for selection, as well, as facilitate the development of more rapid and efficient selection and breeding procedures [83]. An effective perennial ryegrass AC protocol is available; however, transitioning to IMC would certainly increase efficiency and yield even further. Major leaps in efficiency are likely to be achievable for both methods, since adaptations which increase the yields

of already highly successful protocols continue to be published regularly [11,84,85]. Only this year for example, a two- to four-fold increase in the number of embryos and regenerated plants in both barley and wheat was reported, achieved through the addition of dimethyl sulfoxide (DMSO) to the pre-treatment medium [86].

In vivo haploid induction has never been reported in perennial ryegrass even though, due to low genotypic specificity and relatively simple technical demands, it could prove highly practical. Additionally, segregation distortion due to in vitro androgenesis, resulting in higher allele frequencies of the donor most amenable to tissue culture, can thus be avoided [29,87–91]. Recently, two DH inducer lines of annual ryegrass (*L. perenne* L. subsp. *multiflorum* (Lam.) Husnot [syn. *L. multiflorum* Lam.]) have been registered which, when crossed with tall fescue (*L. arundinaceum* (Schreb.) Darbysh. [syn. *Festuca arundinacea* Schreb.]), produce F_1 hybrids that can yield low percentages of both tall fescue and annual ryegrass DHs [92]. Selection could be performed on the vigorous F_1 hybrids prior to inflorescence harvest and the subsequent recovery of 1–5 DHs per plant [93]. Perhaps this technique could be applied to perennial ryegrass as well. Alternatively, it might be worthwhile to investigate the existence of perennial ryegrass haploid inducing genes, such as CENH3 discovered in Arabidopsis [94], which seems to also affect centromere disruption and genome elimination in maize [95], barley, and sugar beet [96].

Dwivedi et al. have recently published a comprehensive review of the myriad applications of DHs in plant breeding and research [14], many of which may be applicable in perennial ryegrass as well. However, it is important to realize that the costs associated with the implementation of the DH applications which have been proposed, or indeed are already applied elsewhere, may be prohibitive in a forage crop. The significantly lower economic value of perennial ryegrass compared to crops, such as maize, barley, and rice, limits the available resources for using and further developing DH techniques. Nevertheless, there definitely are exciting possibilities for exploiting DHs within the budgetary reach of perennial ryegrass researchers and breeders, some of which will be highlighted in this section.

4.1. Purging Deleterious Alleles

Recessive deleterious mutations are thought to play a major role in inbreeding depression, which is observed when allogamous species, such as perennial ryegrass, are selfed [97]. Such recessive alleles are masked in heterozygous genotypes, but are carried at one or more loci in a majority of gametes. To purge deleterious alleles from germplasm intended for breeding, DH induction has been proposed as the most effective method. By fixing maize landraces as DH lines, the genetic basis of elite maize germplasm could be broadened [98]. In one field study, for example, genetically highly diverse and distant lines with a grain yield similar to elite lines could be selected [99]. The introgression of interesting traits from landraces without incurring yield impairments is thus enabled by using a DH selection phase. Introgression breeding is commonly practiced in the *Lolium-Festuca* complex as well, for example, to improve winter hardiness, crown rust resistance, and drought tolerance [52]. A recent study revealed extensive genetic variation in European ecotypes of perennial ryegrass, much of which has not yet been exploited in modern cultivars [54]. Germplasm intended for population and synthetics breeding can benefit from the inclusion of a DH step to reduce deleterious genetic load. Given the status of DH techniques in perennial ryegrass, purging deleterious alleles from natural populations for introgression breeding is not feasible using current in vitro methods. The development of an in vivo haploid induction system would be exceedingly useful for work with natural populations of the *Lolium-Festuca* complex.

4.2. Doubled Haploids for Genetics and Genomics

To accelerate perennial ryegrass breeding, increased knowledge on the genetics underlying traits of interest allows for a more informed and, thus, effective selection process to exploit available genetic variation. DH technologies can significantly reduce the time and costs required for genomics and

genetics studies of perennial ryegrass, as it has done in other crops [13,14]. Since the existing DH induction procedure is not yet successful in all genotypes, it may be sensible to restrict some of the methods proposed in the following section to a few 'model genotypes' at this time. These genotypes should be selected for their ability to produce large numbers of green, chromosome-doubled, vigorous and fertile DHs. Similar to the highly androgenetic barley cultivar 'Igri' [39,89,100–102], such model genotypes may yield a wealth of information which can then be used in perennial ryegrass breeding.

4.2.1. Transformation and Mutation

An efficient transformation system is an important tool for functional gene validation and molecular breeding. Protocols to transform (embryogenic) calli using *Agrobacterium*, particle bombardment, and electroporation have been published and successfully used in perennial ryegrass (reviewed in [103]), although problematically low regeneration efficiencies are often reported. Additionally, it is challenging to perform the selfings needed in order to fix a transgene for further evaluation in this SI species. In vitro or in vivo microspore transformation, either by *Agrobacterium*, particle bombardment or cell-penetrating peptides (CPPs), and subsequent DH induction and regeneration can overcome both these issues [104]. *Agrobacterium*-mediated transformation was successfully used in concert with IMC in barley and resulted in single-copy [39] and even transcription activator-like effector nuclease (TALEN)-mediated gene knock-out DH transformants [102]. Similar successes were reported in other crops, such as wheat [38] and rapeseed [33]. Linear DNA, enzymes, and proteins could be delivered into triticale [105] and wheat [106] microspores by CPPs. These types of peptides can thus be used for both transformation and transgene-free genome editing, if for example proteins and guide RNAs of the CRISPR/Cas system are introduced [107].

Mutation techniques combine well with DH technology, because, as with transgene approaches, homozygous and stable integration of (recessive) mutations does not require additional generations of selfing [108]. Additionally, DHs or their microspores are excellent targets for mutagenesis since the absence of background variation in their genomes allows for easy identification of mutants. Both seeds derived from DHs, as well as haploid microspores, have been mutagenized in a range of crop species, such as wheat, barley, and rice [109]. A number of microspore mutagenesis studies have been performed to target oil quality characteristics in several *Brassica* species and significant diversity could be induced for these traits [43]. The detection of false-positives can be prevented by using DH starting material for TILLING experiments, a technique that has recently received attention to advance forage grass breeding [79]. In barley, seeds of a DH line were used for a TILLING experiment [110], however, DH derived microspores would be a better target in perennial ryegrass in order to circumvent the need for selfing.

Combining mutagenesis and transformation studies with DH techniques significantly reduces the time and costs required to obtain modified homozygotes for genotype-phenotype validation, as well as generate and fixate genetic variation [42]. Markedly smaller populations are required to obtain genotypes with multiple homozygous transgene inserts or mutations when DH induction is used instead of self-fertilization [111]. Considerable reductions in space can be gained if the selection of regenerants can be done during the in vitro stage. Small tissue samples for DNA extraction and further analysis may be taken from in vitro cultures, for example, to detect genotypes with modifications to the gene of interest in reverse genetics screens. Alternatively, a selection agent, such as NaCl or a pathogen-derived substance, can be added to the culture medium after mutagenesis, in order to obtain DH mutants with tolerances to certain stresses [10].

4.2.2. Population Development for Mapping

Chromosome maps as well as a vast number of mapped genetic markers have been established through DH techniques in a range of species, such as rapeseed [112], wheat [113], and barley [114]. Indeed, the genetic map of the International *Lolium* Genome Initiative (ILGI) was constructed from a cross between a DH and a heterozygous perennial ryegrass genotype [115,116]. Segregating DH

populations provide excellent opportunities to find marker-trait associations through linkage mapping and have been extensively used in many crops [117]. Tuvesson et al. describe how a DH mapping population for marker-trait associations can be created and maintained in rye, a crop which, like perennial ryegrass, suffers from inbreeding depression [30]. Two distinct DHs are used to produce an F_1 population, individuals of which are then subjected to DH induction. Both the parental DHs and the F_1-derived DHs are crossed to a tester in order to keep them alive. *Lolium-Festuca* hybrids exhibit high levels of recombination within their gametes and thus offer unique opportunities to determine genome organization, elucidate genetic control of key agricultural traits and map markers [118]. Such introgression mapping combines well with DH induction and this approach has already been successfully used to obtain and select useful gene combinations for freezing-tolerance [119].

Inducing a large number of DHs from a single, highly heterogeneous genotype could circumvent the construction of a designed population altogether and allow for direct haplotype mapping (Figure 2). Every single microspore-derived plant is a unique product of recombination between the chromosomal pairs of the donor and an analysis of the inheritance of markers and genes is, therefore, possible in such a population. Single pollen grain PCR-based sequencing methods for recombination studies have been described in barley, maize, sorghum, and other crops [120–122]. However, a major advantage of DH induction over these approaches is that it allows for phenotyping, in addition to genotyping. Additionally, a sufficiently high number of DHs can be regenerated from the microspores of a single plant to allow for fine-mapping or even map-based cloning approaches, without being dependent on the seed set of specific crosses.

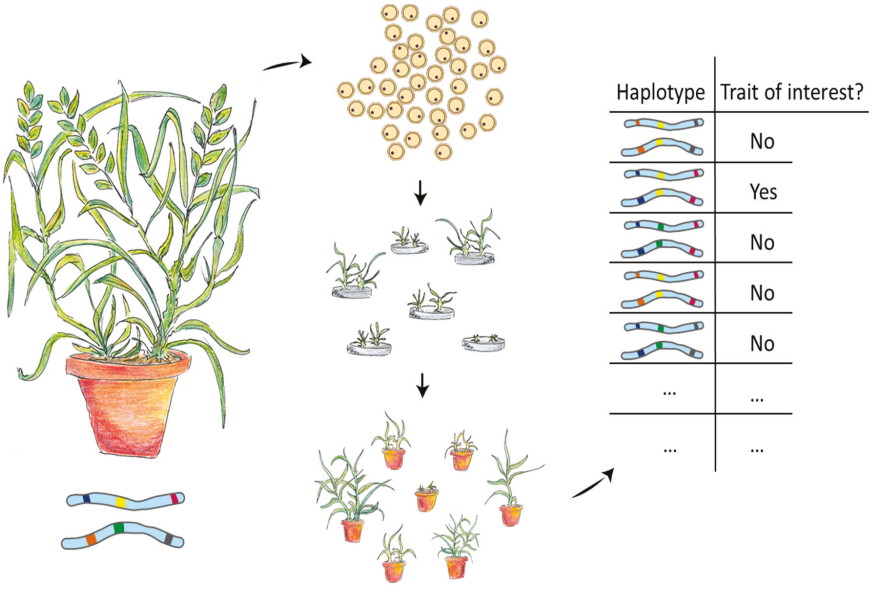


Figure 2. Schematic representation of a direct haplotype mapping strategy by DH induction of microspores derived from a single, heterozygous genotype exhibiting a trait of interest. Through recombination during meiosis in the donor plant, the microspore population represents a large diversity of possible haplotypes. Both donor and microspore-derived DH plants are genotyped and phenotyped so that statistical methods can be used to infer the haplotype associated with the trait of interest (in this case the dark blue, yellow, and red alleles).

4.3. Doubled Haploids for Hybrid Breeding

Hybrid breeding has made a significant contribution to the acceleration of yield gains of many important crops through the exploitation of the phenomenon of heterosis [123]. In perennial ryegrass, DH induction may well be the most practical method for the development of homozygous lines for hybrid breeding (Table 1). Currently, most breeding germplasm is recalcitrant to DH induction, necessitating the introgression of androgenic capacity [63,66]. Using modern mapping approaches, it should be feasible to obtain markers associated with the few major genes expected to control ELS formation and green plant regeneration [63,67], in order to accelerate their introgression into elite material [72].

Table 1. Comparison of three methods to obtain inbred or 100% homozygous lines in perennial ryegrass.

	Repeated Self-Fertilization	**In Vitro Doubled Haploid Induction**	**In Vivo Doubled Haploid Induction**
Method available	yes	yes [1]	no
Genotype specificity	low	high	unknown
Efficiency	low	high [2]	unknown
Required skill	low	moderate	low [3]
Space required	high	low	high
Lab requirements	none	high	low
Generations required	5–6 [4]	1	1
Diploid regenerants	100%	50%–80%	unknown [5]
Obstacles	self-incompatibility inbreeding depression	albinism inbreeding depression	inbreeding depression
Side effects of procedure [6]	allows selection every generation	gametoclonal variation somatoclonal variation ploidy level variation segregation distortion	part of inducer genome could integrate

[1] Anther culture, possibly isolated microspore culture; [2] Up to several hundreds of plants per 100 cultured anthers using current anther culture techniques; [3] High if embryo rescue is required; [4] Some residual heterozygosity (theoretically 1.6% after six generations); [5] If the chosen method generates haploids, colchicine will be needed to double the chromosomes; [6] Can be positive or negative depending on end-use.

Even though inbreeding depression is severe in perennial ryegrass DHs, reports of vigorous and fertile plants do exist [65]. In the early days of maize [124] and rye hybrid breeding [125], both allogamous cereals suffered from inbreeding depression as well, and selection among inbred lines was successfully used to improve their vigour and fertility to economically practical levels. Similar to the purging of deleterious alleles described above, DH induction may, thus, be used as a selection tool against inbreeding depression (Niels Roulund, personal communication; [97]). Additionally, negative effects from inbreeding depression may be averted if diploid or tetraploid single-cross hybrids are used as parents to produce double-cross hybrids. Indeed, tetraploid cultivars are of special interest in the context of hybrid breeding, because polyploids often exhibit progressive heterosis the larger the genetic diversity between their component genomes is [35,126]. A single-cross hybrid between two homozygous autotetraploids (AABB) will display heterosis, but a double-cross hybrid from two distinct single-cross hybrids (ABCD) is nearly always more heterotic [127]. Conversely, tetraploid *Lolium-Festuca* hybrids have been converted into diploids by AC to reduce vigour and plant size for turf applications [128]. DH techniques can, thus, allow breeders to manipulate ploidy level and homozygosity in order to maximize the exploitation of heterosis in future perennial ryegrass cultivars.

Hybrid seed production requires efficient multiplication of inbred lines, as well as an effective method to control pollination. Elucidation of the SI system and the development of markers for its components are now within reach [129–131] and should enable maintenance and multiplication of DH lines through seed. Interestingly, repeated selfing of DHs has been proposed as a method to cause the

breakdown of SI, since rare mutations in SI genes in pollen grains can, thus, be selected [78]. Schemes to produce F_1 seed of perennial ryegrass based on population hybridization [50,126], cytoplasmic male sterility (CMS) [82,132], and SI [82,133,134] have been proposed (see also [135]), although opinions differ on which method is the most practically and economically feasible.

5. Concluding Remarks

DH techniques in perennial ryegrass have advanced to a sufficiently successful and promising stage to warrant a tentative glance at what future developments in this field may bring to both breeding and research [12]. Some exceedingly useful applications require the realization of relatively small improvements to existing in vitro protocols which could be derived from successful work in barley, rye, and wheat [86]. For example, highly androgenic genotypes may be used as models in DH or microspore transformation and mutagenization studies, or for direct haplotype mapping [39]. Homozygous line production for hybrid breeding, however, will require either improved in vitro protocols that are effective in recalcitrant genotypes or the introgression of androgenic capacity into breeding germplasm [63,66]. Additionally, investigations into and improvement of the agronomic performance of perennial ryegrass DHs, as well as an efficient seed production system, are required for an economically feasible production of hybrid cultivars. An alternative strategy to significantly reduce genotype specific responses to in vitro DH production, would be the development of in vivo haploid or DH inducers [92]. These would also allow the purging of deleterious alleles from natural populations, thus enabling a broadening of the genetic variation available for breeding without incurring high levels of performance impairment [99].

All of the haploid and DH techniques discussed here should reduce the time, space, and investment required to perform effective perennial ryegrass research and breeding. Since this species can be regarded as a model for other grasses, any progress made should be beneficial to them as well. Even with the comparatively modest budgets of those active in forage crop improvement, haploid and DH techniques can be developed into powerful tools to achieve the acceleration of the speed of genetic gain needed to meet future agricultural demands.

Acknowledgments: This work was supported by ETH Research Grant ETH-34 14-1 and the Swiss National Science Foundation (SNSF Professorship grant No.: PP00P2 138988). RFB and BS wish to express their gratitude to Niels Roulund and Kirsten Vangsgaard for their support in their work on perennial ryegrass DH induction. RFB gratefully acknowledges Léonor Bonnafous for her help with Figure 2.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AC	anther culture
DH	doubled haploid
CENH3	centromere-specific histone 3 variant
CRISPR	clustered regularly interspaced short palindrome repeats
DMSO	dimethyl sulfoxide
ELS	embryo-like structures
IMC	isolated microspore culture
MAS	marker-assisted selection
QTL	quantitative trait locus/loci
SI	self-incompatible/self-incompatibility
TALEN	transcription activator-like effector nuclease
TILLING	targeting induced local lesions in genomes

References

1. Tilman, D.; Balzer, C.; Hill, J.; Befort, B.L. Global food demand and the sustainable intensification of agriculture. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 20260–20264. [CrossRef] [PubMed]
2. Fischer, R.A.; Byerlee, D.; Edmeades, G.O. *Crop Yields and Global Food Security: Will Yield Increase Continue to Feed the World?* ACIAR Monograph No. 158; Australian Centre for International Agricultural Research: Canberra, Australia, 2014.
3. Foley, J.A.; Ramankutty, N.; Brauman, K.A.; Cassidy, E.S.; Gerber, J.S.; Johnston, M.; Mueller, N.D.; O'Connell, C.; Ray, D.K.; West, P.C.; et al. Solutions for a cultivated planet. *Nature* **2011**, *478*, 337–342. [CrossRef] [PubMed]
4. Brummer, C.E.; Barber, W.T.; Collier, S.M.; Cox, T.S.; Johnson, R.; Murray, S.C.; Olsen, R.T.; Pratt, R.C.; Thro, A.M. Plant breeding for harmony between agriculture and the environment. *Front. Ecol. Environ.* **2011**, *9*, 561–568. [CrossRef]
5. Fischer, R.A.; Edmeades, G.O. Breeding and cereal yield progress. *Crop Sci.* **2010**, *50*, S85–S98. [CrossRef]
6. Stamp, P.; Visser, R. The twenty-first century, the century of plant breeding. *Euphytica* **2012**, *186*, 585–591. [CrossRef]
7. Fischer, R.A. Definitions and determination of crop yield, yield gaps, and of rates of change. *Field Crops Res.* **2015**, *182*, 9–18. [CrossRef]
8. Tester, M.; Langridge, P. Breeding technologies to increase crop production in a changing world. *Science* **2010**, *327*, 818–822. [CrossRef] [PubMed]
9. Dawson, I.K.; Russell, J.; Powell, W.; Steffenson, B.; Thomas, W.T.B.; Waugh, R. Barley: A translational model for adaptation to climate change. *New Phytol.* **2015**, *206*, 913–931. [CrossRef] [PubMed]
10. Germanà, M.A. Gametic embryogenesis and haploid technology as valuable support to plant breeding. *Plant Cell Rep.* **2011**, *30*, 839–857. [CrossRef] [PubMed]
11. Seguí-Simarro, J.M. Androgenesis revisited. *Bot. Rev.* **2010**, *76*, 377–404. [CrossRef]
12. Forster, B.P.; Heberle-Bors, E.; Kasha, K.J.; Touraev, A. The resurgence of haploids in higher plants. *Trends Plant Sci.* **2007**, *12*, 368–375. [CrossRef] [PubMed]
13. Dunwell, J.M. Haploids in flowering plants: Origins and exploitation. *Plant Biotechnol. J.* **2010**, *8*, 377–424. [CrossRef] [PubMed]
14. Dwivedi, S.L.; Britt, A.B.; Tripathi, L.; Sharma, S.; Upadhyaya, H.D.; Ortiz, R. Haploids: Constraints and opportunities in plant breeding. *Biotechnol. Adv.* **2015**, *33*, 812–829. [CrossRef] [PubMed]
15. Seguí-Simarro, J.M. Editorial: Doubled haploidy in model and recalcitrant species. *Front. Plant Sci.* **2015**, *6*, 1–2. [CrossRef] [PubMed]
16. Geiger, H.H.; Gordillo, G.A. Doubled haploids in hybrid maize breeding. *Maydica* **2009**, *54*, 485–499.
17. Smith, K.F.; Spangenberg, G. Forage breeding for changing environments and production systems: An overview. *Crop Pasture Sci.* **2014**, *65*. [CrossRef]
18. Reheul, D.; De Cauwer, B.; Cougnon, M. The role of forage crops in multifunctional agriculture. In *Fodder Crops and Amenity Grasses*; Boller, B., Posselt, U., Veronesi, F., Eds.; Springer: New York, NY, USA, 2010; pp. 457–476.
19. Ferrie, A.M.R.; Caswell, K.L. Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. *Plant Cell Tissue Organ Cult.* **2011**, *104*, 301–309. [CrossRef]
20. Seguí-Simarro, J.M.; Nuez, F. How microspores transform into haploid embryos: Changes associated with embryogenesis induction and microspore-derived embryogenesis. *Physiol. Plant.* **2008**, *134*, 1–12. [CrossRef] [PubMed]
21. Shariatpanahi, M.E.; Bal, U.; Heberle-Bors, E.; Touraev, A. Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis. *Physiol. Plant.* **2006**, *127*, 519–534. [CrossRef]
22. Islam, S.M.S.; Tuteja, N. Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species. *Plant Sci.* **2012**, *182*, 134–144. [CrossRef] [PubMed]
23. Kumari, M.; Clarke, H.J.; Small, I.; Siddique, K.H.M. Albinism in plants: A major bottleneck in wide hybridization, androgenesis and doubled haploid culture. *CRC. Crit. Rev. Plant Sci.* **2009**, *28*, 393–409. [CrossRef]

24. Maluszynski, M.; Kasha, K.J.; Forster, B.P.; Szarejko, I. *Doubled Haploid Production in Crop Plants: A manual*; Maluszynski, M., Kasha, K.J., Forster, B.P., Szarejko, I., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2003.
25. Touraev, A.; Forster, B.P.; Mohan Jain, S. *Advances in Haploid Production in Higher Plants*; Touraev, A., Forster, B.P., Jain, S.M., Eds.; Springer: Dordrecht, The Netherlands, 2009.
26. Germanà, M.A.; Lambardi, M. *In Vitro Embryogenesis in Higher Plants*, 1st ed.; Germanà, M.A., Lambardi, M., Eds.; Humana Press: New York, NY, USA, 2016.
27. Niu, Z.; Jiang, A.; Abu Hammad, W.; Oladzadabbasabadi, A.; Xu, S.S.; Mergoum, M.; Elias, E.M. Review of doubled haploid production in durum and common wheat through wheat × maize hybridization. *Plant Breed.* **2014**, *133*, 313–320. [CrossRef]
28. Chen, J.-F.; Cui, L.; Malik, A.A.; Mbira, K.G. In vitro haploid and dihaploid production via unfertilized ovule culture. *Plant Cell Tissue Organ Cult.* **2010**, *104*, 311–319. [CrossRef]
29. Pink, D.; Bailey, L.; McClement, S.; Hand, P.; Mathas, E.; Buchanan-Wollaston, V.; Astley, D.; King, G.; Teakle, G. Double haploids, markers and QTL analysis in vegetable brassicas. *Euphytica* **2008**, *164*, 509–514. [CrossRef]
30. Tuvesson, S.; Dayteg, C.; Hagberg, P.; Manninen, O.; Tanhuanpää, P.; Tenhola-Roininen, T.; Kiviharju, E.; Weyen, J.; Förster, J.; Schondelmaier, J.; et al. Molecular markers and doubled haploids in European plant breeding programmes. *Euphytica* **2006**, *158*, 305–312. [CrossRef]
31. Devaux, P.; Kasha, K.J. Overview of barley doubled haploid production. In *Advances in Haploid Production in Higher Plants*; Touraev, A., Forster, B.P., Jain, S.M., Eds.; Springer: Dordrecht, The Netherlands, 2009; pp. 47–64.
32. Mishra, R.; Jwala, G.; Ao, N.R. In Vitro androgenesis in rice: Advantages, constraints and future prospects. *Rice Sci.* **2016**, *23*, 57–68. [CrossRef]
33. Ferrie, A.M.R.; Möllers, C. Haploids and doubled haploids in *Brassica* spp. for genetic and genomic research. *Plant Cell Tissue Organ Cult.* **2010**, *104*, 375–386. [CrossRef]
34. Lippman, Z.B.; Zamir, D. Heterosis: Revisiting the magic. *Trends Genet.* **2006**, *23*, 60–66. [CrossRef] [PubMed]
35. Birchler, J.A.; Yao, H.; Chudalayandi, S.; Vaiman, D.; Veitia, R.A. Heterosis. *Plant Cell* **2010**, *22*, 2105–2112. [CrossRef] [PubMed]
36. Ferrie, A.M.R. Doubled haploidy as a tool in ornamental breeding. *Acta Hortic.* **2012**, *953*, 167–171. [CrossRef]
37. Obsa, B.T.; Eglinton, J.; Coventry, S.; March, T.; Langridge, P.; Fleury, D. Genetic analysis of developmental and adaptive traits in three doubled haploid populations of barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.* **2016**, *129*, 1139–1151. [CrossRef] [PubMed]
38. Brew-Appiah, R.A.T.; Ankrah, N.; Liu, W.; Konzak, C.F.; Von Wettstein, D.; Rustgi, S. Generation of doubled haploid transgenic wheat lines by microspore transformation. *PLoS ONE* **2013**, *8*, e80155. [CrossRef] [PubMed]
39. Kumlehn, J.; Serazetdinova, L.; Hensel, G.; Becker, D.; Loerz, H. Genetic transformation of barley (*Hordeum vulgare* L.) via infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*. *Plant Biotechnol. J.* **2006**, *4*, 251–261. [CrossRef] [PubMed]
40. Kapusi, E.; Hensel, G.; Coronado, M.J.; Broeders, S.; Marthe, C.; Otto, I.; Kumlehn, J. The elimination of a selectable marker gene in the doubled haploid progeny of co-transformed barley plants. *Plant Mol. Biol.* **2013**, *81*, 149–160. [CrossRef] [PubMed]
41. Huang, S.; Liu, Z. A new method for generation and screening of Chinese cabbage mutants using isolated microspore culturing and EMS mutagenesis. *Euphytica* **2016**, *207*, 23–33. [CrossRef]
42. Shen, Y.; Pan, G.; Lübberstedt, T. Haploid strategies for functional validation of plant genes. *Trends Biotechnol.* **2015**, *33*, 611–620. [CrossRef] [PubMed]
43. Ferrie, A.M.R.; Caswell, K.L. Applications of doubled haploidy for improving industrial oilseeds. In *Industrial Oil Crops*; McKeon, T.A., Hayes, D.G., Hildebrand, D.F., Weselake, R.J., Eds.; Elsevier Inc.: London, UK, 2016; pp. 359–378.
44. Soriano, M.; Li, H.; Boutilier, K. Microspore embryogenesis: Establishment of embryo identity and pattern in culture. *Plant Reprod.* **2013**, *26*, 181–196. [CrossRef] [PubMed]
45. Daghma, D.E.S.; Hensel, G.; Rutten, T.; Melzer, M.; Kumlehn, J. Cellular dynamics during early barley pollen embryogenesis revealed by time-lapse imaging. *Front. Plant Sci.* **2014**, *5*, 1–14. [CrossRef] [PubMed]

46. Seifert, F.; Bössow, S.; Kumlehn, J.; Gnad, H.; Scholten, S. Analysis of wheat microspore embryogenesis induction by transcriptome and small RNA sequencing using the highly responsive cultivar "Svilena". *BMC Plant Biol.* **2016**, *16*, 97. [CrossRef] [PubMed]
47. Smith, K.F.; Simpson, R.J.; Culvenor, R.A.; Humphreys, M.O.; Prud'Homme, M.P.; Oram, R.N. The effects of ploidy and a phenotype conferring a high water-soluble carbohydrate concentration on carbohydrate accumulation, nutritive value and morphology of perennial ryegrass (*Lolium perenne* L.). *J. Agric. Sci.* **2001**, *136*, 65–74. [CrossRef]
48. Smith, K.F.; McFarlane, N.M.; Croft, V.M.; Trigg, P.J.; Kearney, G.A. The effects of ploidy and seed mass on the emergence and early vigour of perennial ryegrass (*Lolium perenne* L.) cultivars. *Aust. J. Exp. Agric.* **2003**, *43*, 481–486. [CrossRef]
49. Nair, R. Developing tetraploid perennial ryegrass (*Lolium perenne* L.) populations. *N. Z. J. Agric. Res.* **2004**, *47*, 45–49. [CrossRef]
50. Wilkins, P.W. Breeding perennial ryegrass for agriculture. *Euphytica* **1991**, *52*, 201–214. [CrossRef]
51. Sampoux, J.P.; Baudouin, P.; Bayle, B.; Béguier, V.; Bourdon, P.; Chosson, J.F.; Deneufbourg, F.; Galbrun, C.; Ghesquière, M.; Noël, D.; et al. Breeding perennial grasses for forage usage: An experimental assessment of trait changes in diploid perennial ryegrass (*Lolium perenne* L.) cultivars released in the last four decades. *Field Crops Res.* **2011**, *123*, 117–129. [CrossRef]
52. Humphreys, M.O. Genetic improvement of forage crops—Past, present and future. *J. Agric. Sci.* **2005**, *143*, 441–448. [CrossRef]
53. Cornish, M.A.; Hayward, M.D.; Lawrence, M.J. Self-incompatibility in ryegrass. I. Genetic control in diploid *Lolium perenne* L. *Heredity* **1979**, *43*, 95–106. [CrossRef]
54. Blackmore, T.; Thorogood, D.; Skøt, L.; Mcmahon, R.; Powell, W.; Hegarty, M. Germplasm dynamics: The role of ecotypic diversity in shaping the patterns of genetic variation in *Lolium perenne*. *Sci. Rep.* **2016**, *6*, 22603. [CrossRef] [PubMed]
55. Wang, J.; Pembleton, L.W.; Baillie, R.C.; Drayton, M.C.; Hand, M.L.; Bain, M.; Sawbridge, T.I.; Spangenberg, G.C.; Forster, J.W.; Cogan, N.O.I. Development and implementation of a multiplexed single nucleotide polymorphism genotyping tool for differentiation of ryegrass species and cultivars. *Mol. Breed.* **2014**, *33*, 435–451. [CrossRef]
56. Casler, M.D.; Brummer, E.C. Theoretical expected genetic gains for among-and-within-family selection methods in perennial forage crops. *Crop Sci.* **2008**, *48*, 890–902. [CrossRef]
57. Stanis, V.A.; Butenko, R.G. Developing viable haploid plants in anther culture of ryegrass. *Dokl. Biol. Sci.* **1984**, *275*, 249–251.
58. Boppenmeier, J.; Zuchner, S.; Foroughi-Wehr, B. Haploid production from barley yellow dwarf virus resistant clones of *Lolium*. *Plant Breed.* **1989**, *103*, 216–220. [CrossRef]
59. Olesen, A.; Andersen, S.B.; Due, I.K. Anther culture response in perennial ryegrass (*Lolium perenne* L.). *Plant Breed.* **1988**, *101*, 60–65. [CrossRef]
60. Opsahl-Ferstad, H.-G.; Bjornstad, A.; Rognli, O.A. Influence of medium and cold pretreatment on androgenetic response in *Lolium perenne* L. *Plant Cell Rep.* **1994**, *13*, 594–600. [CrossRef] [PubMed]
61. Bante, I.; Sonke, T.; Tandler, R.F.; van den Bruel, A.M.R.; Meyer, E.M. Anther culture of Lolium perenne and Lolium multiflorum. In *The Impact of Biotechnology in Agriculture*; Sangwan, R., Sangwan-Norreel, B.S., Eds.; Springer: Dordrecht, The Netherlands, 1990; pp. 105–127.
62. Creemers-Molenaar, J.; Beerepoot, L.J. In Vitro Culture and Micropropagation of Ryegrass (*Lolium* spp.). In *High-Tech and Micropropagation III*; Bajaj, Y.P.S., Ed.; Springer: Berlin/Heidelberg, Germany, 1992; Volume 19, pp. 549–575.
63. Madsen, S.; Olesen, A.; Dennis, B.; Andersen, S.B. Inheritance of anther-culture response in perennial ryegrass (*Lolium perenne* L.). *Plant Breed.* **1995**, *114*, 165–168. [CrossRef]
64. Hussain, S.W.; Richardson, K.; Faville, M.; Woodfield, D. Production of haploids and double haploids in annual (*Lolium multiflorum*) and perennial (*L. perenne*) ryegrass. In Proceedings of the 13th Australasian Plant Breeding Conference, Christchurch, New Zealand, 18–21 April 2006; Mercer, C.F., Ed.; pp. 531–536.
65. Andersen, S.B.; Madsen, S.; Roulund, N.; Halberg, N.; Olesen, A. Haploidy in ryegrass. In *In Vritro Haploid Production in Higher Plants*; Jain, S.M., Sopory, S.K., Veilleux, R.E., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1997; Volume 4, pp. 133–147.

66. Halberg, N.; Olesen, A.; Tuvesson, I.K.D.; Andersen, S.B. Genotypes of perennial ryegrass (*Lolium perenne* L.) with high anther-culture response through hybridization. *Plant Breed.* **1990**, *105*, 89–94. [CrossRef]
67. Opsahl-Ferstad, H.G.; Bjørnstad, N.; Rognli, O.A. Genetic control of androgenetic response in *Lolium perenne* L. *Theor. Appl. Genet.* **1994**, *89*, 133–138. [CrossRef] [PubMed]
68. Wang, Z.-Y.; Ge, Y.; Mian, R.; Baker, J. Development of highly tissue culture responsive lines of *Lolium temulentum* by anther culture. *Plant Sci.* **2005**, *168*, 203–211. [CrossRef]
69. Chen, X.-W.; Cistué, L.; Muñoz-Amatriaín, M.; Sanz, M.; Romagosa, I.; Castillo, A.-M.; Vallés, M.-P. Genetic markers for doubled haploid response in barley. *Euphytica* **2006**, *158*, 287–294. [CrossRef]
70. Agache, S.; Bachelier, B.; De Buyser, J.; Henry, Y.; Snape, J. Genetic analysis of anther culture response in wheat using aneuploid, chromosome substitution and translocation lines. *Theor. Appl. Genet.* **1989**, *77*, 7–11. [CrossRef] [PubMed]
71. Torp, A.M.; Andersen, S.B. Albinism in microspore culture. In *Advances in Haploid Production in Higher Plants*; Touraev, A., Forster, B., Jain, S.M., Eds.; Springer: New York, NY, USA, 2009; pp. 155–160.
72. Bolibok, H.; Rakoczy-Trojanowska, M. Genetic mapping of QTLs for tissue-culture response in plants. *Euphytica* **2006**, *149*, 73–83. [CrossRef]
73. Nielsen, N.H.; Andersen, S.U.; Stougaard, J.; Jensen, A.; Backes, G.; Jahoor, A. Chromosomal regions associated with the in vitro culture response of wheat (*Triticum aestivum* L.) microspores. *Plant Breed.* **2015**, *134*, 255–263. [CrossRef]
74. Krzewska, M.; Czyczyło-Mysza, I.; Dubas, E.; Gołębiowska-Pikania, G.; Golemiec, E.; Stojałowski, S.; Chrupek, M.; Zur, I. Quantitative trait loci associated with androgenic responsiveness in triticale (×Triticosecale Wittm.) anther culture. *Plant Cell Rep.* **2012**, *31*, 2099–2108. [CrossRef] [PubMed]
75. Begheyn, R.F.; Vangsgaard, K.; Roulund, N. Efficient doubled haploid production in perennial ryegrass (*Lolium perenne* L.). In *Breeding in a World of Scarcity, Proceedings of the 2015 Meeting of the Section "Forage Crops and Amenity Grasses" of Eucarpia, Ghent, Belgium, 14 June 2016*; Springer: Basel, Switzerland; pp. 151–155.
76. Melchinger, A.E.; Molenaar, W.S.; Mirdita, V.; Schipprack, W. Colchicine alternatives for chromosome doubling in maize haploids for doubled-haploid production. *Crop Sci.* **2016**, *56*, 559–569. [CrossRef]
77. Opsahl-Ferstad, H.G. Androgenetic Response in Grasses. I. Anther Culture in Perennial Ryegrass (*Lolium perenne* L.). II. Molecular Studies of Embryogenesis in Barley (*Hordeum vulgare* L.). Ph.D. Thesis, Agricultural University of Norway, Ås, Norway, 1993.
78. Madsen, S.; Olesen, A.; Andersen, S.B. Self-fertile doubled haploid plants of perennial ryegrass (*Lolium perenne* L.). *Plant Breed.* **1993**, *110*, 323–327. [CrossRef]
79. Manzanares, C.; Yates, S.; Ruckle, M.; Nay, M.; Studer, B. TILLING in forage grasses for gene discovery and breeding improvement. *New Biotechnol.* **2016**, *33*, 594–603. [CrossRef] [PubMed]
80. Conaghan, P.; Casler, M.D. A theoretical and practical analysis of the optimum breeding system for perennial ryegrass. *Irish J. Agric. Sci.* **2011**, *50*, 47–63.
81. Wang, Z.Y.; Brummer, E.C. Is genetic engineering ever going to take off in forage, turf and bioenergy crop breeding? *Ann. Bot.* **2012**, *110*, 1317–1325. [CrossRef] [PubMed]
82. Arias Aguirre, A.; Studer, B.; Frei, U.; Lübberstedt, T. Prospects for hybrid breeding in bioenergy grasses. *BioEnergy Res.* **2011**, *5*, 10–19. [CrossRef]
83. Langridge, P.; Fleury, D. Making the most of "omics" for crop breeding. *Trends Biotechnol.* **2011**, *29*, 33–40. [CrossRef] [PubMed]
84. Sinha, R.K.; Eudes, F. Dimethyl tyrosine conjugated peptide prevents oxidative damage and death of triticale and wheat microspores. *Plant Cell Tissue Organ Cult.* **2015**, *122*, 227–237. [CrossRef]
85. Sriskandarajah, S.; Sameri, M.; Lerceteau-Köhler, E.; Westerbergh, A. Increased recovery of green doubled haploid plants from barley anther culture. *Crop Sci.* **2015**, *55*, 2806–2812. [CrossRef]
86. Echavarri, B.; Cistue, L. Enhancement in androgenesis efficiency in barley (*Hordeum vulgare* L.) and bread wheat (*Triticum aestivum* L.) by the addition of dimethyl sulfoxide to the mannitol pretreatment medium. *Plant Cell Tissue Organ Cult.* **2016**, *125*, 11–22. [CrossRef]
87. Devaux, P.; Zivy, M. Protein markers for anther culturability in barley. *Theor. Appl. Genet.* **1994**, *88*, 701–706. [CrossRef] [PubMed]
88. Zhang, L.; Zhang, L.; Luo, J.; Chen, W.; Hao, M.; Liu, B.; Yan, Z.; Zhang, B.; Zhang, H.; Zheng, Y.; et al. Synthesizing double haploid hexaploid wheat populations based on a spontaneous alloploidization process. *J. Genet. Genom.* **2011**, *38*, 89–94. [CrossRef] [PubMed]

89. Muñoz-Amatriaín, M.; Castillo, A.M.; Chen, X.W.; Cistué, L.; Vallés, M.P. Identification and validation of QTLs for green plant percentage in barley (*Hordeum vulgare* L.) anther culture. *Mol. Breed.* **2008**, *22*, 119–129. [CrossRef]
90. Bélanger, S.; Esteves, P.; Clermont, I.; Jean, M.; Belzile, F. Genotyping-by-sequencing on pooled samples and its use in measuring segregation bias during the course of androgenesis in barley. *Plant Genome* **2016**, *9*, 1–13. [CrossRef]
91. Hayward, M.D.; Olesen, A.; Due, I.K.; Jenkins, R.; Morris, P. Segregation of isozyme marker loci amongst androgenetic plants of *Lolium perenne* L. *Plant Breed.* **1990**, *104*, 68–71. [CrossRef]
92. Kindiger, B. Generation of paternal dihaploids in tall fescue. *Grassl. Sci.* **2016**, *62*, 1–5. [CrossRef]
93. Kindiger, B. Sampling the genetic diversity of tall fescue utilizing gamete selection. In *Genetic Diversity in Plants*; Caliskan, M., Ed.; InTech: Rijeka, Croatia, 2012; pp. 271–284.
94. Ravi, M.; Chan, S.W. Centromere-mediated generation of haploid plants. In *Plant Centromere Biology*; Jiang, J., Birchler, J.A., Eds.; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2013; pp. 169–181.
95. Kelliher, T.; Starr, D.; Wang, W.; Mccuiston, J.; Zhong, H.; Nuccio, M.L.; Martin, B. Maternal haploids are preferentially induced by CENH3-tailswap transgenic complementation in maize. *Front. Plant Sci.* **2016**, *7*, 1–11. [CrossRef] [PubMed]
96. Karimi-Ashtiyani, R.; Ishii, T.; Niessen, M.; Stein, N.; Heckmann, S.; Gurushidze, M.; Banaei-Moghaddam, A.M.; Fuchs, J.; Schubert, V.; Koch, K.; et al. Point mutation impairs centromeric CENH3 loading and induces haploid plants. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 11211–11216. [CrossRef] [PubMed]
97. Charlesworth, D.; Willis, J.H. The genetics of inbreeding depression. *Nat. Rev. Genet.* **2009**, *10*, 783–796. [CrossRef] [PubMed]
98. Wilde, K.; Burger, H.; Prigge, V.; Presterl, T.; Schmidt, W.; Ouzunova, M.; Geiger, H.H. Testcross performance of doubled-haploid lines developed from European flint maize landraces. *Plant Breed.* **2010**, *129*, 181–185. [CrossRef]
99. Strigens, A.; Schipprack, W.; Reif, J.C.; Melchinger, A.E. Unlocking the genetic diversity of maize landraces with doubled haploids opens new avenues for breeding. *PLoS ONE* **2013**, *8*, e57234. [CrossRef] [PubMed]
100. Jacquard, C.; Nolin, F.; Hécart, C.; Grauda, D.; Rashal, I.; Dhondt-Cordelier, S.; Sangwan, R.S.; Devaux, P.; Mazeyrat-Gourbeyre, F.; Clément, C. Microspore embryogenesis and programmed cell death in barley: Effects of copper on albinism in recalcitrant cultivars. *Plant Cell Rep.* **2009**, *28*, 1329–1339. [CrossRef] [PubMed]
101. Larsen, E.T.; Tuvesson, I.K.; Andersen, S.B. Nuclear genes affecting percentage of green plants in barley (*Hordeum vulgare* L.) anther culture. *Theor. Appl. Genet.* **1991**, *82*, 417–420. [CrossRef] [PubMed]
102. Gurushidze, M.; Hensel, G.; Hiekel, S.; Schedel, S.; Valkov, V.; Kumlehn, J. True-breeding targeted gene knock-out in barley using designer TALE-nuclease in haploid cells. *PLoS ONE* **2014**, *9*, 1–9. [CrossRef] [PubMed]
103. Giri, C.C.; Praveena, M. In vitro regeneration, somatic hybridization and genetic transformation studies: An appraisal on biotechnological interventions in grasses. *Plant Cell Tissue Organ Cult.* **2014**, *120*, 843–860. [CrossRef]
104. Eudes, F.; Shim, Y.S.; Jiang, F. Engineering the haploid genome of microspores. *Biocatal. Agric. Biotechnol.* **2014**, *3*, 20–23. [CrossRef]
105. Chugh, A.; Amundsen, E.; Eudes, F. Translocation of cell-penetrating peptides and delivery of their cargoes in triticale microspores. *Plant Cell Rep.* **2009**, *28*, 801–810. [CrossRef] [PubMed]
106. Bilichak, A.; Luu, J.; Eudes, F. Intracellular delivery of fluorescent protein into viable wheat microspores using cationic peptides. *Front. Plant Sci.* **2015**, *6*, 666. [CrossRef] [PubMed]
107. Kumar, V.; Jain, M. The CRISPR-Cas system for plant genome editing: Advances and opportunities. *J. Exp. Bot.* **2015**, *66*, 47–57. [CrossRef] [PubMed]
108. Maluszynski, M.; Ahloowalia, B.S.; Sigurbjörnsson, B. Application of in vivo and in vitro mutation techniques for crop improvement. *Euphytica* **1995**, *85*, 303–315. [CrossRef]
109. Szarejko, I.; Forster, B.P. Doubled haploidy and induced mutation. *Euphytica* **2007**, *158*, 359–370. [CrossRef]
110. Kurowska, M.; Labocha-Pawłowska, A.; Gnizda, D.; Maluszynski, M.; Szarejko, I. Molecular analysis of point mutations in a barley genome exposed to MNU and Gamma rays. *Mutat. Res.* **2012**, *738–739*, 52–70. [CrossRef] [PubMed]

111. Lübberstedt, T.; Frei, U.K. Application of doubled haploids for target gene fixation in backcross programmes of maize. *Plant Breed.* **2012**, *131*, 449–452. [CrossRef]
112. Delourme, R.; Falentin, C.; Fomeju, B.F.; Boillot, M.; Lassalle, G.; André, I.; Duarte, J.; Gauthier, V.; Lucante, N.; Marty, A.; et al. High-density SNP-based genetic map development and linkage disequilibrium assessment in *Brassica napus* L. *BMC Genom.* **2013**, *14*, 120. [CrossRef] [PubMed]
113. Cabral, A.L.; Jordan, M.C.; McCartney, C.A.; You, F.M.; Humphreys, D.G.; MacLachlan, R.; Pozniak, C.J. Identification of candidate genes, regions and markers for pre-harvest sprouting resistance in wheat (*Triticum aestivum* L.). *BMC Plant Biol.* **2014**, *14*, 340. [CrossRef] [PubMed]
114. Sannemann, W.; Huang, B.E.; Mathew, B.; Léon, J. Multi-parent advanced generation inter-cross in barley: High-resolution quantitative trait locus mapping for flowering time as a proof of concept. *Mol. Breed.* **2015**, *35*, 86. [CrossRef]
115. Bert, P.F.; Charmet, G.; Sourdille, P.; Hayward, M.D.; Balfourier, F. A high-density molecular map for ryegrass (*Lolium perenne*) using AFLP markers. *Theor. Appl. Genet.* **1999**, *99*, 445–452. [CrossRef] [PubMed]
116. Jones, E.S.; Mahoney, N.L.; Hayward, M.D.; Armstead, I.P.; Jones, J.G.; Humphreys, M.O.; King, I.P.; Kishida, T.; Yamada, T.; Balfourier, F.; et al. An enhanced molecular marker based genetic map of perennial ryegrass (*Lolium perenne*) reveals comparative relationships with other Poaceae genomes. *Genome* **2002**, *45*, 282–295. [CrossRef] [PubMed]
117. Guo, B.; Wang, D.; Guo, Z.; Beavis, W.D. Family-based association mapping in crop species. *Theor. Appl. Genet.* **2013**, *126*, 1419–1430. [CrossRef] [PubMed]
118. King, J.; Armstead, I.P.; Donnison, I.S.; Harper, J.A.; Roberts, L.A.; Thomas, H.; Ougham, H.; Thomas, A.; Huang, L.; King, I.P. Introgression mapping in the grasses. *Chromosom. Res.* **2007**, *15*, 105–113. [CrossRef] [PubMed]
119. Humphreys, M.W.; Gasior, D.; Lesniewska-Bocianowska, A.; Zwierzykowski, Z.; Rapacz, M. Androgenesis as a means of dissecting complex genetic and physiological controls: Selecting useful gene combinations for breeding freezing tolerant grasses. *Euphytica* **2007**, *158*, 337–345. [CrossRef]
120. Petersen, G.; Johansen, B.; Seberg, O. PCR and sequencing from a single pollen grain. *Plant Mol. Biol.* **1996**, *31*, 189–191. [CrossRef] [PubMed]
121. Dreissig, S.; Fuchs, J.; Cápal, P.; Kettles, N.; Byrne, E.; Houben, A. Measuring meiotic crossovers via multi-locus genotyping of single pollen grains in barley. *PLoS ONE* **2015**, *10*, 1–10. [CrossRef] [PubMed]
122. Chen, P.H.; Pan, Y.B.; Chen, R.K. High-throughput procedure for single pollen grain collection and polymerase chain reaction in plants. *J. Integr. Plant Biol.* **2008**, *50*, 375–383. [CrossRef] [PubMed]
123. Goff, S.A. Tansley review—A unifying theory for general multigenic heterosis: Energy efficiency, protein metabolism, and implications for molecular breeding. *New Phytol.* **2011**, *189*, 923–937. [CrossRef] [PubMed]
124. Hallauer, A.R.; Carena, M.J.; Filho, J.B.M. Inbreeding. In *Quantitative Genetics in Maize Breeding*; Hallauer, A.R., Carena, M.J., Filho, J.B.M., Eds.; Springer: New York, NY, USA, 2010; pp. 425–475.
125. Geiger, H.H.; Miedaner, T. Rye breeding. In *Cereals*; Carena, M.J., Ed.; Springer: New York, NY, USA, 2009; pp. 157–181.
126. Brummer, E.C. Capturing heterosis in forage crop cultivar development. *Crop Sci.* **1999**, *39*, 943–954. [CrossRef]
127. Riddle, N.C.; Birchler, J.A. Comparative analysis of inbred and hybrid maize at the diploid and tetraploid levels. *Theor. Appl. Genet.* **2008**, *116*, 563–576. [CrossRef] [PubMed]
128. Kopecky, D.; Lukaszewski, A.J.; Gibeault, V. Reduction of ploidy level by androgenesis in intergenic *Lolium—Festuca* hybrids for turf grass breeding. *Crop Sci.* **2005**, *45*, 274–281.
129. Yang, B.; Thorogood, D.; Armstead, I.P.; Franklin, F.C.H.; Barth, S. Identification of genes expressed during the self-incompatibility response in perennial ryegrass (*Lolium perenne* L.). *Plant Mol. Biol.* **2009**, *70*, 709–723. [CrossRef] [PubMed]
130. Klaas, M.; Yang, B.; Bosch, M.; Thorogood, D.; Manzanares, C.; Armstead, I.P.; Franklin, F.C.H.; Barth, S. Progress towards elucidating the mechanisms of self-incompatibility in the grasses: Further insights from studies in *Lolium*. *Ann. Bot.* **2011**, *108*, 677–685. [CrossRef] [PubMed]
131. Manzanares, C.; Barth, S.; Thorogood, D.; Byrne, S.L.; Yates, S.; Czaban, A.; Asp, T.; Yang, B.; Studer, B. A gene encoding a DUF247 domain protein cosegregates with the S self-incompatibility locus in perennial ryegrass. *Mol. Biol. Evol.* **2016**, *33*, 870–884. [CrossRef] [PubMed]

132. Islam, M.S.; Studer, B.; Møller, I.M.; Asp, T. Genetics and biology of cytoplasmic male sterility and its applications in forage and turf grass breeding. *Plant Breed.* **2014**, *133*, 299–312. [CrossRef]
133. Pembleton, L.W.; Shinozuka, H.; Wang, J.; Spangenberg, G.C.; Forster, J.W.; Cogan, N.O.I. Design of an F1 hybrid breeding strategy for ryegrasses based on selection of self-incompatibility locus-specific alleles. *Front. Plant Sci.* **2015**, *6*, 764. [CrossRef] [PubMed]
134. Do Canto, J.; Studer, B.; Lubberstedt, T. Overcoming self-incompatibility in grasses: A pathway to hybrid breeding. *Theor. Appl. Genet.* **2016**, *129*, 1815–1829. [CrossRef] [PubMed]
135. Posselt, U.K. Heterosis in grasses. *Czech J. Genet. Plant Breed.* **2003**, *39*, 48–53.

agronomy

MDPI

Review

Considerations for Managing Agricultural Co-Existence between Transgenic and Non-Transgenic Cultivars of Outcrossing Perennial Forage Plants in Dairy Pastures

Kevin F. Smith [1,*] and German Spangenberg [2]

[1] Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Private Bag 105, Hamilton 3300, Australia

[2] Agriculture Victoria, Biosciences Research & School of Applied Systems Biology, La Trobe University, AgriBio, the Centre for AgriBioscience, 5 Ring Rd, Bundoora 3083, Australia; german.spangenberg@ecodev.vic.gov.au

* Correspondence: kfsmith@unimelb.edu.au; Tel.: +61-355-730-951

Academic Editors: John W. Forster and Peter Langridge
Received: 28 September 2016; Accepted: 10 November 2016; Published: 25 November 2016

Abstract: Many of the major forage species used in agriculture are outcrossing and rely on the exchange of pollen between individuals for reproduction; this includes the major species used for dairy production in grazing systems: perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). Cultivars of these species have been co-existing since contrasting cultivars were developed using plant breeding, but the consequences and need for strategies to manage co-existence have been made more prominent with the advent of genetic modification. Recent technological developments have seen the experimental evaluation of genetically modified (GM) white clover and perennial ryegrass, although there is no current commercial growing of GM cultivars of these species. Co-existence frameworks already exist for two major cross-pollinated grain crops (canola and maize) in Europe, and for alfalfa (*Medicago sativa* L.) in the US, so many of the principles that the industry has developed for co-existence in these crops such as detection techniques, segregation, and agronomic management provide lessons and guidelines for outcrossing forage species, that are discussed in this paper.

Keywords: pasture; GMO; co-existence

1. Introduction

Many of the major forage species used in agriculture are outcrossing and rely on the exchange of pollen between individuals for reproduction. This includes the major species used for dairy production in grazing systems: perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). Cultivars of these species have been co-existing since contrasting cultivars were developed using plant breeding, but the consequences and the need for strategies to manage co-existence have been made more prominent with the advent of genetic modification.

Genetically modified (GM) crops have been grown commercially for more than 20 years, with more than 170 million hectares sown across 28 countries in 2015 [1]. The majority (70%–90%) of these GM crops are used for animal feed [2] with up to 95% of the 9 billion animals grown for food production each year in the USA consuming diets containing GM ingredients [2]. Until recently this consumption was entirely based on the use of grains (soy, maize) or crop residues (cottonseed meal and canola meal). However, the recent release of Roundup Ready alfalfa (*Medicago sativa*) and research

in other forage crops such as perennial ryegrass and white clover [3,4] suggests that it is timely to consider the co-existence of GM and non-GM pastures.

Co-existence frameworks already exist for two major cross-pollinated grain crops (canola and maize) and alfalfa for seed and hay production, so many of the principles that the industry has developed for co-existence in these crops such as detection techniques, segregation, and agronomic management provide lessons and guidelines for outcrossing forage species.

Some of the principles that underpin a co-existence framework include,

- The ability to detect the transgene or its products in relevant commodities.
- A knowledge of the mechanism and extent of pollen (gene) flow and seed dispersal in the species.
- The strategic use of management interventions to "separate" GM and non-GM crops and prevent gene flow between them.
- The equivalence or otherwise in the agronomic or nutritional aspects of the GM and non-GM crops.
- The segregation of products during marketing and supply.

An example of these co-existence frameworks for grain crops are those developed within the European Union (EU) [5] which allow "the ability of farmers to make a practical choice between conventional, organic, and GM crop production" considering issues such as the segregation of GM and non-GM crops and the cost of this segregation. The principles of such a framework have been adopted by several European nations for the commercial cultivation of GM maize [6]. The technical and social aspects of the co-existence of GM and non-GM maize crops have been reviewed for Spain [7] and the EU [6]. The isolation distances for GM maize proposed by European member states vary from state to state and whether the GM crop is neighbouring a conventional or organic crop with distances varying from 25 to 800 m [8]. A recent review found that the large and fixed isolation distances proposed by some countries were not consistent with either the co-existence principles outlined by the European Commission and were excessive based on scientific evidence [6]; they recommend that isolation distances within the range of 10–50 m would in most instances be sufficient to keep GM inputs from cross-pollination below the legal tolerance level of 0.9%. Therefore, despite inconsistency in the application of the guidelines for co-existence, the principles are well established in grain crops such as maize.

In contrast to grain crops where maximising seed set (through gene flow) is usually the goal, the perennial forage supply chain has two distinct phases,

1. Seed production where high pollination is required
2. Pasture production where most management seeks to minimise reproductive development and seed set—particularly in dairy grazing systems.

In contrast to grain crops where the adoption of GM crops has been widespread, in some countries a range of technical and economic constraints [4] has meant there is only one perennial outcrossing forage crop; glyphosate resistant alfalfa (*Medicago sativa* L.) that was finally approved for fully deregulated, commercial release in the United States (US) in January 2011. The National Alfalfa and Forage Alliance in the US has developed a set of guidelines for the co-existence of alfalfa seed crops [9], and the technical aspects of co-existence and market assurance for alfalfa hay and forage production in an era of biotech crops have been summarized by Putnam et al. [10].

In this paper, we will summarise the literature on aspects of functional equivalence and co-existence in perennial outcrossing forage species with a particular emphasis on those used in dairy production systems drawing on examples using both GM and non-GM plants.

2. Detection of Transgenes in Forages and Related Agricultural Products

Fundamental to the process of monitoring transgenic crops and agricultural commodities is the ability to detect the transgene or its products. Although DNA fragments from high copy number

endogenous plant genes such as rubsico have been detected in the blood and digesta [11,12], transgenic DNA (tDNA) has been shown to be broken down in the rumen and duodenum of cattle [9], and a number of studies have shown that tDNA was not detected in the milk of cows fed diets containing GM feeds [11,13–19]. These data from milking trials are consistent with those from wide ranging reviews of animal production trials that have focused on meat producing animals [2,20] with the conclusion that there are no detectable or reliably quantifiable traces of GM feed components in eggs, meat, or milk [2,20].

Therefore, efforts in GM detection for perennial forages should not focus on milk or meat but rather on other agricultural products such as pollen, seed, and herbage. While the need to detect transgenes in these products themselves is obvious as pollen is the vehicle for gene transfer, seed is traded for sowing new pastures and herbage is the diet of grazing ruminants.

There is also the need to monitor the pollen of perennial forage species that are pollinated by honeybees, as this pollen may find its way into honey. Honey containing traces of pollen from genetically modified plants is currently subject to marketing and labelling regulations in the European Union [21] following the commercial expansion of genetically modified field crops. Studies of the amount of canola pollen occurring in honey from hives foraging non-GM crops in Australia and GM crops in Canada found canola pollen levels on the order of 0.2% and thus well below the 1% threshold for labelling in Australia [22]. White clover pollen is also present in honey produced by bees foraging white clover flowers [23]. The exact cut-off levels for detection are likely to vary from jurisdiction to jurisdiction as well as whether pollen is seen as a constituent or an ingredient of honey [21]. Regardless of these trade and regulatory discussions, the likely presence of genetically modified pollen in honey following commercial release of GM white clover will most likely create a situation analogous to that in canola (e.g., [22]) where commercial honey supplies will need to be monitored.

An example to demonstrate the ability to detect tDNA in a range of products from an insect pollinated perennial forage species comes from research associated with the development and evaluation of white clover with transgenic virus coat protein mediated resistance to Alfalfa Mosaic Virus (AMV) [24]. During the production of genetically modified white clover seed, white clover plants were pollinated by honey bees under containment conditions and PCR-based techniques were developed to detect the AMV coat protein gene and the *neomycin phosphotransferase 2* (*npt2*) selectable marker gene in genetically modified white clover pollen, whether this pollen was collected fresh from honey bees that have been foraging white clover or from honey [25]. Similarly, the AMV coat protein gene was able to be detected in seed, fresh leaves (as would be fed at grazing), air dried leaves (as would be fed as hay) when the leaves were either pure white clover, or in a mixture with perennial ryegrass (simulating the mixed sward systems where white clover is most commonly grown) [26].

These results demonstrate that it is possible to develop molecular diagnostics for pollen, herbage, or seeds from forage plants. The issues of the cost of real-time PCR based systems, which are the standard reference method for transgene detection, when used for routine field-based applications have been addressed in grain crops already through efficient sampling strategies [27–29] and through the development of other diagnostic tools such as semi-quantitative enzyme-linked immunosorbent assay (ELISA) (e.g., [29]) which have also been used to detect the presence of the *CP4 EPSPS* gene in GM bentgrass [30] and alfalfa [10], and through the use of plasmid DNA for the calibrated detection of specific transgenic events (e.g., [31,32]. More recently novel DNA amplification techniques such as recombinase polymerase amplification (RPA) for the rapid point-of-use screening of transgenic soybean seeds [33] have been developed. The ultimate choice of molecular diagnostics for forage samples will depend on the cost and target detection limits, but the experience from major food crops and initial data from forage samples demonstrates that it will be possible to develop these tools for samples from dairy pastures.

Whilst there is no evidence to suggest that tDNA will find its way into the muscles or milk of lactating cows following digestion, the development of methodologies to detect the presence or absence of the *cp4epsps* transgene from soybean meal and the *cry1a*[*b*] transgene from GM corn grain in

the rumen fluid, duodenal digesta, milk, blood, and faeces of lactating cows when fed these diets or their near-isogenic comparators [11] demonstrates that it is possible to monitor the digestion of tDNA in the digestive tract of ruminants.

3. Composition and Performance of GM Feeds and Forages

Although the entry of GM forages into the marketplace is relatively new, the use of GM grain as animal feed has occurred for more than 20 years. Both the experimental studies and the trends following the commercial feeding of GM feed to over 100 billion animals were reviewed by Van Eenennaamm and Young [2] with the conclusion that no study had revealed any difference in the nutritional composition of animals fed GM or non-GM diets nor where there any negative trends in commercial animal health or productivity [2]. Of specific interest to this paper are the results of a 2 year feeding study on the feeding of GM corn (whole-crop silage; kernels and cobs) of GM corn modified with the Bt-MON810 event and an isogenic comparator to dairy cows. This long term study concluded that there were no consistent effects on milk composition or cow body condition and hence the GM corn and its isogenic comparator could be said to have nutritional equivalence, and the milk produced had no functional reason to be classified differently [18,19].

Given the small number of genetically modified perennial forages that have progressed to feed trials, it is not surprising that there are few data sets describing the agronomic or nutritional equivalence of GM cultivars and the non-GM cultivars that the transgenic event has been crossed in to. However, in each of the cases that have been published to date, the data shows no evidence that the performance of the GM cultivars is different to equivalent conventional cultivars other than for the trait controlled by the transgene.

For instance, in a feeding experiment with Holstein cows diets were prepared that were nutritionally similar and contained approximately 40% (by dry matter) of lucerne hay that was either "Roundup Ready" (containing the *cp4epsps* protein) or three conventional cultivars that had been selected to have similar nutritive characteristics to the hay derived from the GM cultivar [17]. In this experiment there were no differences in daily milk yield, fat corrected milk yield, milk fat, milk lactose, non-fat milk solids, nor dry matter intake of cows consuming GM or non-GM diets [17].

In white clover in Australia (where licence conditions prevented the feeding of GM clover herbage to animals), a proximate analysis of both nutritional and anti-nutritional characteristics of the virus coat protein mediated AMV resistant GM white clover was performed [24]. In this study there was no difference in the nutritional characteristics (crude protein, in vitro dry matter digestibility, neutral detergent fibre, and water soluble carbohydrates) and anti-nutritional characteristics (cyanogenic glucosides, phytoestrogens, and saponins) were compared for two conventional white clover cultivars (Mink, Grasslands Sustain) and their GM AMV resistant derivatives when grown under field or glasshouse conditions [34].

A further study on Zoysia grass (*Zoysia japonica* Steud), genetically modified to be resistant to the herbicide glufosinate [35], was shown to be no different to non-GM plants for a range of morphological traits related to turfgrass agronomy and also the allergenicity of the pollen to humans as assessed through skin prick tests [36].

4. Gene Flow in Out-Crossing Perennial Forage Species

The major biosafety concern with cross-pollinated perennial forages is the gene flow from GM to non-GM crops [4]. However, in the case of commodities where the GM and non-GM crops have substantive equivalence with respect to all traits rather than the GM trait, and that the GM trait has been deregulated, the issue is not really one of biosafety per se but rather one of compliance with regulatory guidelines for co-existence. The setting of these thresholds is a matter for commerce and industry; the following discussion describes the aspects of gene flow in perennial forage species during both seed and forage productions and how these data can be effectively used during the design and implementation of a co-existence strategy for forage species.

Perennial forage species may be either wind or insect pollinated. We have shown gene flow in field grown wind pollinated, perennial ryegrass [37], and insect pollinated, white clover [38] follows a leptokurtic distribution with a rapid decline in effective pollen flow such that greater than 95% of gene flow occurs within a relatively short distance of the pollen source (30–50 m) under field conditions. However, a small amount of pollen moves a long distance from the pollen source. These data are consistent with that observed internationally and for major outcrossing grain crops such as maize and canola. These principles have been used to develop isolation distances used during seed production [39].

As the pattern of pollen flow in both wind and insect pollinated species is leptokurtic, it is possible for small amounts of pollen to travel a large distance. If this pollen finds a suitable recipient population it is possible for novel traits (including GM) to establish themselves a long way from the pollen source [30,40,41]. The extent to which these novel traits will establish themselves in the new populations will depend on the reproductive fitness of the plants containing the new trait, the ability of the species to establish new plants through seedling recruitment, and also adaptation of the background genetics of the pollen donor to the new environment.

4.1. Isolation, Separation, and Segregation of Seed Crops

As there is no reason to believe that the pollen of genetically modified forages generally behave differently to that from non GM crops during seed production, the existing seed production guidelines that are used internationally to isolate and segregate cultivars are likely to apply (e.g., [39,41]). It is also worth noting the development of a range of marker tools for the determination of varietal purity based on plant [42,43] or endophyte [44] DNA are now available to assist with varietal identification and seed production QA, along with the previously mentioned methods to detect transgenes to assess not only the presence or absence of GM seeds but also the background in which these events occur. It is also possible for industry to develop protocols to further minimize gene flow between GM crops and those being grown for markets sensitive to GM such as the "Grower Opportunity Zones" or GOZ as defined by the National Alfalfa and Forage Alliance in the USA [9].

4.2. Transport of Seed and Hay Crops

Another aspect to consider is the spillage of seed during the transport of seed and hay crops. A recent study in the US has shown that in a survey of 4190 sites on roadside verges in 2011/2012, 185 contained feral alfalfa (lucerne) populations of which 38 tested positive for the presence of the *CP4 EPSPS* transgene [45]. These authors concluded that the distribution of feral alfalfa populations was not random and tended to be clustered in seed and hay production areas where transport of seed was likely, and that efforts to minimise seed spillage during transit and eradicating feral alfalfa along roadsides would be effective strategies to minimize the flow of transgenes. They also used spatial analysis to suggest that these feral populations started independently to provide further evidence that these populations were the likely result of seed spillage or some other mechanism of seed transfer.

4.3. Isolation, Separation, and Segregation of Forage Crops

In order for gene flow to occur not only must pollen find a synchronously flowering plant, pollination must occur, a fertile and mature seed must form, and this seed must join the seed bank, germinate, and establish itself in an established pasture.

4.3.1. Seedbank and Recruitment of Perennial Ryegrass into Existing Pastures

Perennial ryegrass seeds are not persistent in the soil, forming a transient type 1 seed bank [46] due to low seed dormancy and the ability to germinate across a range of environmental conditions. For instance, only 14% of the perennial ryegrass seed bank remained 14 months after release and all had gone after 2 years [47]. Little is known about the seedling recruitment of perennial ryegrass

into established dairy pastures in Australia. However, a recent study in New Zealand showed no germination of sown grasses in contrast to weedy annual and perennial grasses [48].

Seedling recruitment of sown perennial grasses into established pastures in Australia is generally poor [49] and is considered to be a cause of the poor persistence of these pastures under grazing. However, under conditions where grazing management encourages the development of mature heads it is possible to see seedling recruitment of perennial ryegrass, particularly in marginal conditions (e.g., [50–52]). There are attempts to manage perennial ryegrass pastures to facilitate seedling recruitment in meat production systems—for instance, the following from an EverGraze guide to encourage seedling recruitment of perennial ryegrass under grazing in Australia,

- Allow pasture to increase to 3000 kg/ha by the end of November
- Remove stock from mid-November to mid-January
- Graze the dry standing feed down to 1000 kg/ha before the autumn break

It can be seen that this management is not consistent with modern management of dairy pastures, where either grazing management or fodder conservation would be used in November to handle this Spring flush rather than allowing it to go to head and have the seed ripen over a 2 month period.

4.3.2. Management Practices That Could Be Used to Further Minimise Any Gene Flow between Adjacent Forage Crops

A review of co-existence strategies for maize grain crops in the EU found that a reliance merely on isolation distances often led to legislation of isolation distances that were not based on scientific principles [6], and that management and biological issues such as

- pollen barriers
- flowering coincidence
- crop rotation
- regional strategies
- biological confinement

should all be considered when developing co-existence frameworks.

Along with these general guidelines a number of specific interventions have been proposed to facilitate the co-existence of GM and non-GM alfalfa hay crops [9,10]. These are also applicable to perennial forages grown for dairy grazing and include,

- Selecting seed that is certified for purity and quality
- Preventing transfer during harvest through cleaning machinery
- Testing to confirm non-GM status, if required

The following section of this review will address how these issues may be considered for a grazed dairy system.

Given the paucity of data on seedling recruitment in dairy pastures, it is not possible to state that the following interventions would reduce the amount of gene flow from X to Y, nor is it possible to state whether the isolation distances used in seed production could be reduced by Z. Therefore the following section describes some general principles and practices that could be used to reduce gene flow but does not seek to quantify their relative efficiency. It is also important to note that most gene flow occurs from plants that are near to each other so if the large pollen source is a paddock adjacent to a well-managed dairy pasture and this paddock is laxly grazed and allowed to set seed, then most of the pollination will occur from plants within that paddock rather than by pollen from the well managed neighbour. Regardless of the absolute amount, the relative amount of pollen shed by a paddock or plot is also based on the amount of "edge" of that paddock relative to its overall size.

So within a large square paddock most of the pollen that is shed falls within the paddock boundary (this is why seed production isolation distances do not increase as paddock size increases; in fact, the opposite is true [39]).

4.3.3. Management of the "Donor Paddock" and "Recipient Paddock"

4.3.3.1. Sow One Large Paddock as Opposed to Multiple Small Ones

This minimises the proportional amount of area for pollen shed and also in isolation areas if these are used.

4.3.3.2. Utilise Management to Avoid Flowering and Seed Set

This is consistent with modern dairy pasture management and includes both grazing and the option for fodder conservation and silage, in order to remove flower heads before anthesis and/or seed set.

4.3.3.3. Consider the Use of a Boundary Crop Sown to a Non-GM Cultivar Around the "Donor" Paddock or Farm

This area can be managed in exactly the same way as the GM pasture but its physical presence will minimise the potential for gene flow.

4.3.3.4. Consider the Use of "Reproductive" Barriers to Gene Flow Such as Flowering Time and Ploidy

Modern perennial ryegrass cultivars exist as either diploids or induced tetraploids and these two classes are effectively reproductively isolated from each other outside of the laboratory. This reproductive isolation was actually used to allow the gene flow work of Cunliffe et al. [37] to occur in a region with endemic presence of diploid ryegrass pasture. Examples of tetraploid cultivars are Bealey and Banquet, and diploid cultivars include Tolosa, AberDart, and Avalon.

There is also a wide range of flowering times in perennial ryegrass used commercially today. For instance, in Australia there is approximately a 50 day range in flowering date from early maturing types such as Barberia through to late heading types such as Shogun. However, there is a range even within maturity types. A full list of categories of all cultivars may be found in publications such as the Australian Seed Federation Pasture database [53].

Obviously the most extreme reproductive isolation would come from sowing an early maturing diploid adjacent to a late maturing tetraploid, but increased isolation (and hence reduction in gene flow) would also occur with less extreme contrasts.

4.3.3.5. Consider the Use of Shelter Belts between Farm Boundaries

As well as the physical effect of increasing the distance between neighbouring pastures, shelter belts also decrease wind flow [54] and are therefore likely to decrease gene flow.

5. Conclusions

This paper discusses issues related to the design and implementation of a framework for agricultural co-existence of GM and non-GM perennial pastures with a particular emphasis on high intensity commercial grazing systems such as dairy where it is likely that these perennial pastures will be sown. Therefore, it focuses on issues related to approved transgenic events for which there will already have been an assessment of the likely environmental impact of the GM product. For instance, in Australia this falls under the responsibility of the Office of the Gene Technology Regulator (OGTR) where the likely impact of the combination of the transgenic event and the recipient species to human health and the environment are assessed prior to approval to release.

As with the cross-pollinated grain crops, maize and canola, it would be possible to develop a co-existence framework for seed production in forage plants using existing principles that are

used for conventional forage seed production. There is likely to be less gene flow between adjacent grazed and established pastures under intensive grazing than between neighbouring grain or seed production paddocks. There are also a range of management interventions (on top of distance between neighbours) to further reduce gene flow. Therefore, it is concluded that it would be possible for industry to develop a co-existence framework for GM perennial pastures including perennial ryegrass for both seed production and grazing.

Acknowledgments: The authors acknowledge financial support from the Dairy Futures Co-operative Research Centre.

Author Contributions: German Spangenberg and Kevin F. Smith wrote and edited this review.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. James, C. *20th Anniversary (1996 to 2015) of the Global Commercialization of Biotech Crops and Biotech Crop Highlights in 2015. ISAAA Brief No. 51*; ISAAA: Ithaca, NY, USA, 2015.
2. Van Eeenennaam, A.L.; Young, A.E. Prevalence and impacts of genetically engineered feedstuffs on livestock populations. *J. Anim. Sci.* **2014**, *92*, 4255–4278. [CrossRef] [PubMed]
3. Smith, K.F.; Forster, J.W.; Spangenberg, G.C. Converting genomic discoveries into genetic solutions for diary pastures. *Aust. J. Exp. Agric.* **2007**, *47*, 1032–1038. [CrossRef]
4. Wang, Z.Y.; Brummer, E.C. Is genetic engineering ever going to take off in forage, turf and bioenergy crop breeding. *Ann. Bot.* **2012**, *110*, 1317–1325. [CrossRef] [PubMed]
5. European Commission. Commission recommendation of 23 July 2003 on guidelines for the development of national strategies and best practices to ensure the coexistence of genetically modified crops with conventional and organic farming. *Off. J. Eur. Comm.* **2003**, *L189*, 36–47.
6. Devos, Y.; Demont, M.; Dillen, K.; Reheul, D.; Kaiser, M.; Sanvido, O. Coexistence of genetically modified (GM) and non-GM crops in the European Union. A review. *Agron. Sustain. Dev.* **2009**, *29*, 11–30. [CrossRef]
7. Binimelis, R. Coexistence of plants and coexistence of farmers: Is individual choice possible? *J. Agric. Environ. Ethics* **2008**, *21*, 437–457. [CrossRef]
8. European Commission. Report on the Implementation of National Measures on the Co-Existence of Genetically Modified Crops with Conventional and Organic Farming. European Commission. 2006. Available online: http://ec.europa.eu/agriculture/coexistence/sec313_en.pdf (accessed on 24 November 2016).
9. National Alfalfa and Forage Alliance Co-Existence Documents. 2016. Available online: https://www.alfalfa.org/CSCoexistenceDocs.html (accessed on 2 November 2016).
10. Putnam, D.H.; Woodward, T.; Reisen, P.; Orloff, S. Coexistence and market assurance for production of non-genetically engineered alfalfa hay and forage in a biotech era. *Crop Forage Turfgrass Manag.* **2016**, *2*. [CrossRef]
11. Phipps, R.H.; Deaville, E.R.; Maddison, B.C. Detection of transgenic and endogenous plant DNA in rumen fluid, duodenal digesta, milk, blood and feces of lactating dairy cows. *J. Dairy Sci.* **2003**, *86*, 4070–4078. [CrossRef]
12. Bertheau, Y.; Helbling, J.C.; Fortabat, M.N.; Makhzami, S.; Sotinel, I.; Audeon, C.; Nignol, A.C.; Koblinsky, A.; Petit, L.; Fach, P.; et al. Persistence of plant DNA sequences in the blood of dairy cows fed with genetically modified (Bt176) and conventional corn silage. *J. Agric. Food Chem.* **2009**, *57*, 509–516. [CrossRef] [PubMed]
13. Klotz, A.; Einspanier, R. Nachweis von "novel-feed" in tier? Beeintrchtigung des verbrauchers von fleisch oder milch ist nicht zu erwarten. *Mais* **1998**, *3*, 109–111.
14. Einspanier, R.A.; Klotz, A.; Kraft, J.; Aulrich, K.; Poser, R.; Schwaegle, F.; Jahreis, G.; Flachowsky, G. The fate of forage plant DNA in farm animals: A collaborative case study investigating cattle and chicken fed recombinant plant material. *Eur. Food Res. Technol.* **2001**, *212*, 129–134. [CrossRef]
15. Phipps, R.H.; Beever, D.E.; Humphries, D.J. Detection of transgenic DNA in milk from cows receiving herbicide tolerant (CP4EPSPS) soybean meal. *Livest. Prod. Sci.* **2002**, *73*, 269–273. [CrossRef]
16. Poms, R.E.; Hochsteiner, W.; Luger, K.; Glossl, J.; Foissy, H. Model studies on the detectability of genetically modified feeds in milk. *J. Food Prot.* **2003**, *66*, 304–310. [PubMed]
17. Combs, D.K.; Hartnell, G.F. Alfalfa containing the glyphosate-tolerant trait has no effect on feed intake, milk composition, or milk production of dairy cattle. *J. Dairy Sci.* **2008**, *91*, 673–678. [CrossRef] [PubMed]

18. Guertler, P.; Paul, V.; Steinke, K.; Weidemann, S.; Preissinger, W.; Albrecht, C.; Spiekers, H.; Schwarz, F.J.; Meyer, H.H.D. Long-term feeding of genetically modified corn (MON810)—Fate of cry 1 Ab DNA and recombinant protein during the metabolism of the dairy cow. *Livest. Sci.* **2010**, *131*, 250–259. [CrossRef]
19. Steinke, K.; Guertler, P.; Paul, V.; Weidemann, S.; Ettle, T.; Albrecht, C.; Meyer, H.H.; Spiekers, H.; Schwarz, F.J. Effects of long-term feeding of genetically modified corn (event MON810) on the performance of lactating dairy cows. *J. Anim. Physiol. Anim. Nutr. (Berl.)* **2010**, *94*, E185–E193. [CrossRef] [PubMed]
20. Einspanier, R. The fate of transgenic DNA and newly expressed proteins. In *Animal Nutrition with Transgenic Plants*; CABI Biotechnology Series; Flachowsky, G., Ed.; CABI: Oxfordshire, UK, 2013; pp. 112–127.
21. Birkman, L.; van den Bosse, E.; Carrero, I.; Vogt, A.; Weistra, K.; Oortwijn, W. Clarifying the Status of Pollen in Honey. Substitute Impact Assessment of EC Directive Amending Council Honey Directive 2001/110/EC. 2013. Available online: http://www.europarl.europa.eu/committees/en.studiesdownload.html?languageDocument=EN&file=96720 (accessed on 24 November 2016).
22. Hornitzky, M.; Ghalayini, A. Honey produced from genetically modified canola (*Brassica napus*) nectar will not need to be labelled as a GM food under current Australian guidelines. *Aust. J. Exp. Agric.* **2006**, *46*, 1101–1104. [CrossRef]
23. Moar, N.T. Pollen analysis of New Zealand honey. *N. Z. J. Agric. Res.* **1985**, *28*, 39–70. [CrossRef]
24. Panter, S.; Chu, P.G.; Ludlow, E.; Garrett, R.; Kalla, R.; Jahufer, M.Z.Z.; de Lucas Arbiza, A.; Mouradov, A.; Smith, K.F.; Spangenberg, G. Molecular breeding of transgenic white clover (*Trifolium repens* L.) with field resistance to alfalfa mosaic virus through the expression of the AMV coat protein. *Transgenic Res.* **2012**, *21*, 619–632. [CrossRef] [PubMed]
25. Panter, S.; Mouradov, A.; Smith, K.F.; Spangenberg, G. Development and validation of protocols for product stewardship in transgenic white clover (*Trifolium repens* L.): Detection of the AMV CP and npt2 transgenes in pollen, honey and honeybees. *Crop Pasture Sci.* **2015**, *66*, 474–480. [CrossRef]
26. Panter, S.; Mouradov, A.; Smith, K.F.; Spangenberg, G. Development and validation of protocols for product stewardship in transgenic white clover (*Trifolium repens* L.): Detection of the *AMV CP* and *npt2* transgenes in seed, herbage and hay. *Crop Pasture Sci.* **2015**, *66*, 1039–1048. [CrossRef]
27. Remund, K.M.; Dixon, D.A.; Wright, D.L.; Holden, L.R. Statistical considerations in seed purity testing for transgenic traits. *Seed Sci. Res.* **2001**, *11*, 101–119.
28. Koblinksy, A.; Bertheau, Y. Minimum cost acceptance sampling plans for grain control, with application to GMO detection. *Chemom. Intell. Lab. Syst.* **2005**, *75*, 189–200.
29. Elmslie, K.R.; Whaites, L.; Griffiths, K.R.; Murby, E.J. Sampling plan and test protocol for the semiquantitative detection of genetically modified canola (*Brassica napus*) seed in bulk canola seed. *J. Agric. Food Chem.* **2007**, *55*, 4414–4421. [CrossRef] [PubMed]
30. Watrud, L.S.; Lee, E.H.; Fairbrother, A.; Burdick, C.; Reichman, J.R.; Bollman, M.; Storm, M.; King, G.; Van de Water, P.K. Evidence for landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with CP4 EPSPS as a marker. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14533–14538. [CrossRef] [PubMed]
31. Zhang, H.; Yang, L.; Guo, J.; Li, X.; Jiang, L.; Zhang, D. Development of one novel multiple-target plasmid for duplex quantitative PCR analysis of Roundup Ready soybean. *J. Agric. Food Chem.* **2008**, *56*, 5514–5520. [CrossRef] [PubMed]
32. Chaouachi, M.; Hafsa, A.B.; Nabi, N.; Zellema, M.S.; Said, K. A new dual plasmid calibrator for the quantification of the construct specific GM canola Oxy-235 with duplex real-time PCR. *Food Chem.* **2014**, *145*, 49–56. [CrossRef] [PubMed]
33. Chandu, D.; Paul, S.; Parker, M.; Dudin, Y.; King-Sitzes, J.; Perez, T.; Mittanck, D.W.; Shah, M.; Glenn, K.C.; Piepenburg, O. Development of rapid point-of-use DNA test for the screening of Genuity Roundup Ready 2 Yield soybean in seed samples. *Biomed. Res. Int.* **2016**. [CrossRef] [PubMed]
34. De Lucas, A.; Panter, S.; Mouradov, A.; Rochfort, S.; Smith, K.F.; Spangenberg, G. Assessment of Nutritional Characteristics of Virus-Resistant Transgenic White Clover (*Trifolium repens L.*) Grown under Field and Glasshouse Conditions. *Mol. Breed.* **2015**, *35*, 147. [CrossRef]
35. Toyama, K.; Bae, C.H.; Kang, J.G.; Lim, Y.P.; Adachi, T.; Riu, K.Z.; Song, P.S.; Lee, H.Y. Production of herbicide-tolerant Zoysiagrass by Agrobacterium-mediated transformation. *Mol. Cells* **2003**, *16*, 19–27. [PubMed]

36. Bae, T.W.; Vanjildorj, E.; Song, S.Y.; Nishiguchi, S.; Yang, S.S.; Song, I.J.; Chandrasekhar, T.; Kang, T.W.; Kim, J.I.; Koh, Y.J.; et al. Environmental risk assessment of genetically engineered herbicide-tolerant *Zoysia japonica*. *J. Environ. Qual.* **2008**, *37*, 207–218. [CrossRef] [PubMed]
37. Cunliffe, K.V.; Vecchies, A.C.; Jones, E.S.; Kearney, G.A.; Forster, J.W.; Spangenberg, G.C.; Smith, K.F. Assessment of gene flow using tetraploid genotypes of perennial ryegrass (*Lolium perenne* L.). *Aust. J. Agric. Res.* **2004**, *55*, 389–396. [CrossRef]
38. De Lucas, J.A.; Forster, J.W.; Smith, K.F.; Spangenberg, G.C. Assessment of gene flow in white clover (*Trifolium repens* L.) under field and conditions in Australia using phenotypic characters and genetic markers. *Crop Pasture Sci.* **2011**, *63*, 155–163. [CrossRef]
39. Smith, P.; Baxter, L. South Australian Seed Certification Scheme—Procedures and Standards Manual. Seed Services, Primary Industries & Resources South Australia, Plant Research Centre, Hartley Grove, Urrbrae, SA 5064. 2002. Available online: http://www.ruralsolutions.sa.gov.au/__data/assets/pdf_file/0005/43349/seeds_manual.pdf (accessed on 24 November 2016).
40. Rognli, O.A.; Nilsson, N.O.; Nurminiemi, M. Effects of distance and pollen competition on gene flow in the wind-pollinated grass *Festuca pratensis* Huds. *Heredity* **2000**, *85*, 550–560. [CrossRef] [PubMed]
41. Anon Oregon Seed Certification Handbook. 2014. Available online: http://seedcert.oregonstate.edu/sites/default/files/publications/handbook.pdf (accessed on 24 November 2016).
42. Wang, J.; Dobrowolski, M.P.; Cogan, N.O.I.; Forster, J.W.; Smith, K.F. Assignment of individual genotypes to specific forage cultivars of perennial ryegrass based on SSR markers. *Crop Sci.* **2009**, *49*, 49–58. [CrossRef]
43. Wang, J.; Pembleton, L.W.; Baillie, R.C.; Drayton, M.C.; Hand, M.L.; Bain, M.; Sawbridge, T.I.; Spangenberg, G.C.; Forster, J.W.; Cogan, N.O.I. Development and implementation of a multiplexed single nucleotide polymorphism genotyping tool for differentiation of ryegrass species and cultivars. *Mol. Breed.* **2014**, *33*, 435–451. [CrossRef]
44. Van Zijll de Jong, E.; Dobrowolski, M.P.; Sandford, A.; Smith, K.F.; Willocks, M.J.; Spangenberg, G.C.; Forster, J.W. Detection and characterisation of novel fungal endophyte variation in cultivars of perennial ryegrass (*Lolium perenne* L.). *Aust. J. Agric. Res.* **2008**, *59*, 214–221. [CrossRef]
45. Greene, S.L.; Kesoju, S.R.; Martin, R.C.; Kramer, M. Occurrence of transgenic feral alfalfa (*Medicago sativa* subsp. *sativa* L.) in alfalfa seed production areas in the United States. *PLoS ONE* **2015**, *10*, e0143296. [CrossRef] [PubMed]
46. Thompson, K.; Grime, J.P. Seasonal Variation in the Seed Banks of Herbaceous Species in Ten Contrasting Habitats. *J. Ecol.* **1979**, *67*, 893–921. [CrossRef]
47. Lodge, G.M. Seed dormancy, germination, seedling emergence, and survival of some temperate perennial pasture grasses in northern New South Wales. *Aust. J. Agric. Res.* **2004**, *55*, 345–355. [CrossRef]
48. Tozer, K.N.; Barker, G.M.; Cameron, C.A.; James, T.K. Relationship between seedbank and above-ground botanical composition during spring. *N. Z. Plant Prot.* **2010**, *63*, 90–95.
49. Sanderson, M.A.; Goslee, S.C.; Klement, K.D.; Soder, K.J. Soil seed bank composition in pastures of diverse mixtures of temperate forages. *Agron. J.* **2007**, *99*, 1514–1520. [CrossRef]
50. Hume, D.E.; Barker, D.J. Natural reseeding of five grass species in summer dry hill country. *Proc. N. Z. Grassl. Assoc.* **1991**, *53*, 97–104.
51. Waller, R.A.; Sale, P.W.G.; Saul, G.R.; Quigley, P.E.; Kearney, G.A. Tactical versus continuous stocking for persistence of perennial ryegrass (*Lolium perenne* L.) in pastures grazed by sheep in south-western Victoria. *Aust. J. Exp. Agric.* **1999**, *39*, 265–274. [CrossRef]
52. Edwards, G.R.; Hay, M.J.M.; Brock, J.L. Seedling recruitment dynamics of forage and weed species under continuous and rotational sheep grazing in a temperate New Zealand pasture. *Grass Forage Sci.* **2005**, *60*, 186–199. [CrossRef]
53. Australian Seeds Federation. Database of Pasture Cultivars and Varieties. Available online: http://www.asf.asn.au/seed_database_mobile.php (accessed on 24 November 16).
54. Bird, P.R.; Jackson, T.T.; Kearney, G.A.; Williams, K.W. Effect of two tree windbreaks on adjacent pastures in south-western Victoria Australia. *Aust. J. Exp. Agric.* **2002**, *42*, 809–830. [CrossRef]

MDPI AG
St. Alban-Anlage 66
4052 Basel, Switzerland
Tel. +41 61 683 77 34
Fax +41 61 302 89 18
http://www.mdpi.com

Agronomy Editorial Office
E-mail: agronomy@mdpi.com
http://www.mdpi.com/journal/agronomy

MDPI AG
St. Alban-Anlage 66
4052 Basel
Switzerland

Tel: +41 61 683 77 34
Fax: +41 61 302 89 18

www.mdpi.com

ISBN 978-3-03842-543-4