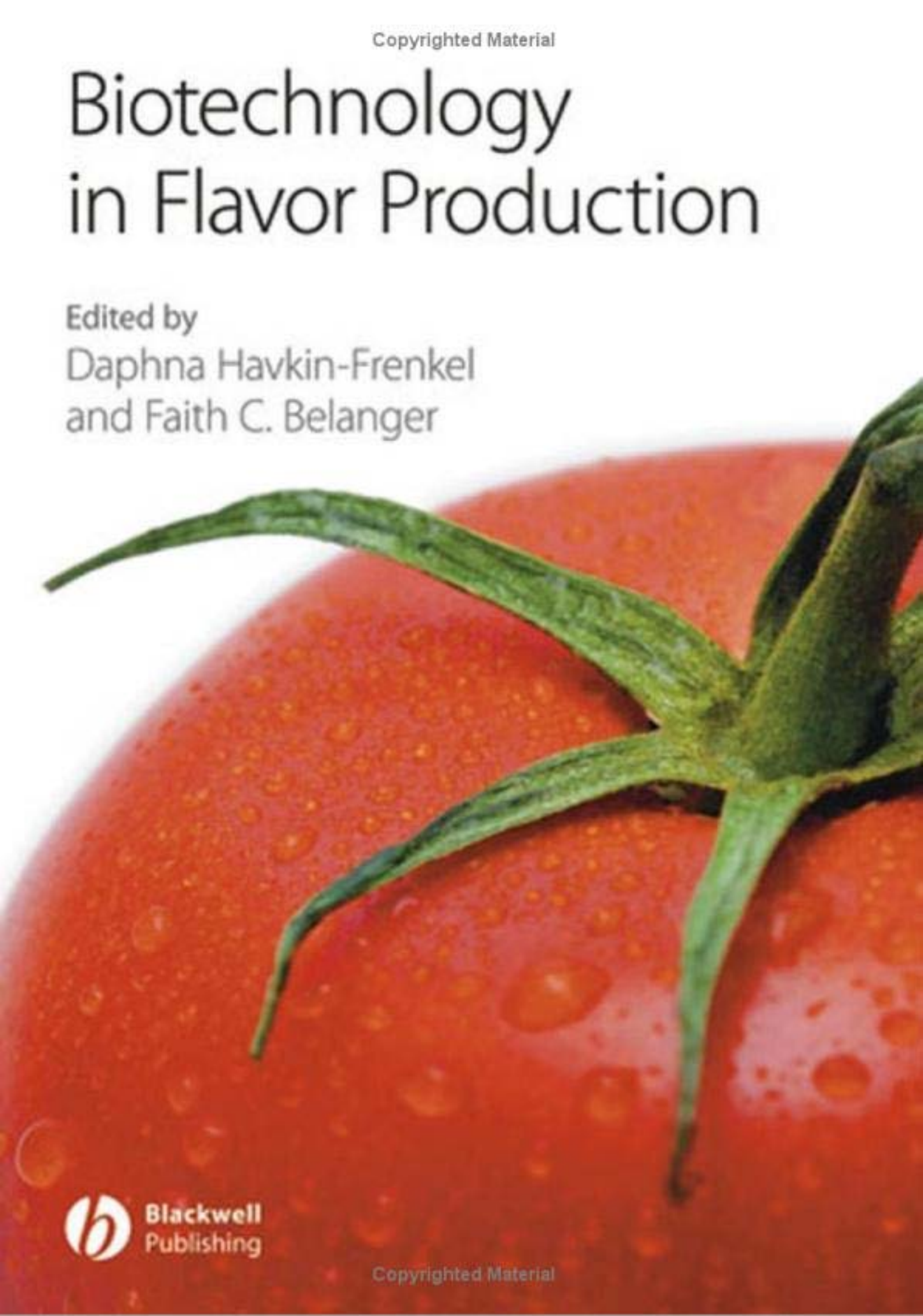
Biotechnology in Flavor Production

Edited by
Daphna Havkin-Frenkel
and Faith C. Belanger



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Biotechnology in Flavor Production: A Case-based Approach. Edited by Daphna Havkin-Frenkel, Faith C. Belanger.
 ISBN: 978-1-4051-5649-3

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Blackwell
Publishing

Blackwell Publishing editorial offices:
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Tel: +44 (0)1865 776868
Blackwell Publishing Professional, 2121 State Avenue, Ames, Iowa 50014-8300, USA
Tel: +1 515 292 0140
Blackwell Publishing Asia Pty Ltd, 550 Swanston Street, Carlton, Victoria 3053, Australia
Tel: +61 (0)3 8359 1011

First published 2008 by Blackwell Publishing Ltd

ISBN: 978-1-4051-5649-3

Library of Congress Cataloging-in-Publication Data
Biotechnology in flavor production/edited by Daphna Havkin-Frenkel and Faith C. Belanger.
p. cm.
Includes bibliographical references and index.
ISBN-13: 978-1-4051-5649-3 (hardback : alk. paper)
ISBN-10: 1-4051-5649-X (hardback : alk. paper) 1. Food — Biotechnology. 2. Flavor. I. Havkin-Frenkel, D. (Daphna), 1951– II. Belanger, Faith C.

TP248.65.F66B637 2008
664'.07 — dc22

2007032683

A catalogue record for this title is available from the British Library

Set in 10/13 pt TimesNRMT by Newgen Imaging Systems (P) Ltd, Chennai, India
Printed and bound in Singapore by C.O.S. Printers Pte Ltd

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Preface

Throughout history, human beings have sought ways to enhance the flavor of the foods they eat. In the 21st century, biotechnology is playing an important role in the flavor improvement of many types of foods.

This book covers several of the biotechnological approaches currently being applied to flavor enhancement. The contribution of microbial metabolism to flavor development in fermented beverages and dairy products has been exploited for thousands of years. The recent availability of whole genome sequences of the yeasts and bacteria involved in these processes is stimulating targeted approaches to flavor enhancement. The chapters by Swiegers *et al.* and by Weimer *et al.* discuss recent developments in the flavor modification of wine, beer, and dairy products through the manipulation of the microbial species involved. Biotechnological approaches to the production of specific flavor molecules in microbes and plant tissue cultures, and the challenges that have been encountered, are discussed in the chapters by Havkin-Frenkel and Belanger, and by Chin. Metabolic engineering of food crops for flavor enhancement is also a current area of research. The chapters by Davidovich-Rikanati *et al.* and by Di discuss metabolic engineering of tomato and potato for enhanced production of specific flavor compounds. Biotechnological approaches are also being applied to crop breeding through marker-assisted selection for important traits, including flavor. The chapters by Bradbury *et al.*, by Brown, and by Dudai and Belanger discuss the application of the biotechnological approach to breeding for enhanced flavor in rice, apple, and basil. The commercial application of metabolic engineering for flavor enhancement in foods or for extraction from microbes or tissue cultures is subject to governmental regulation, which is discussed in the chapter by Teske and Griffiths. The topics covered in this book will be of interest to those in the flavor industry as well as to academic researchers interested in flavors.

The authors of the chapters are experts in their fields and we would like to thank them all for an excellent job of summarizing the latest research developments regarding approaches to the flavor enhancement of foods.

Daphna Havkin-Frenkel
Faith C. Belanger

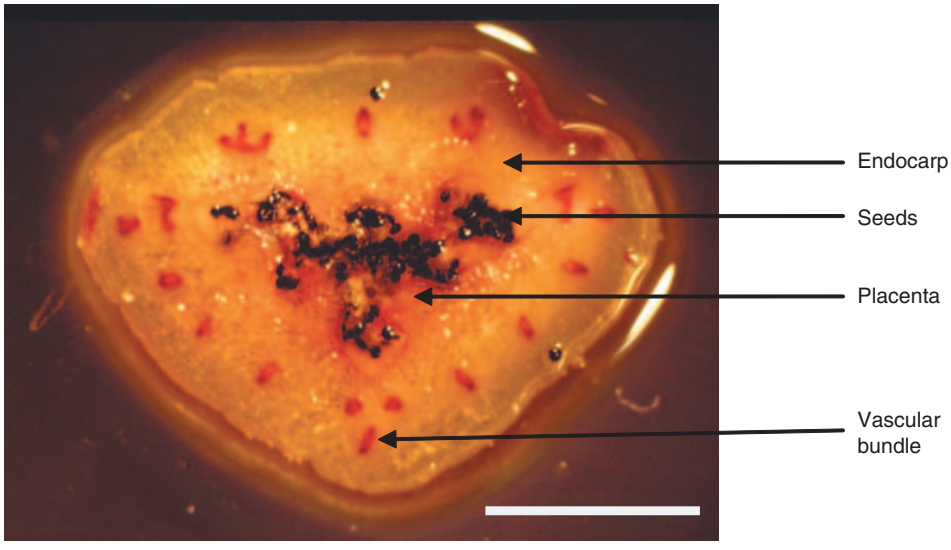

Plate 3.1 Cross-section of a mature green vanilla fruit after catechin-HCl staining. Vanillin is stained red in the placenta and neighboring endocarp cells, and also in the secreted matrix that surrounds the seeds in the fruit cavity. The fruit vascular bundles that are rich in lignin are also stained red. Scale bar = 5 mm. (From Joel *et al*. (2003); copyright material. Reprinted with permission of the publisher, LPP Ltd-Science, Israel.)

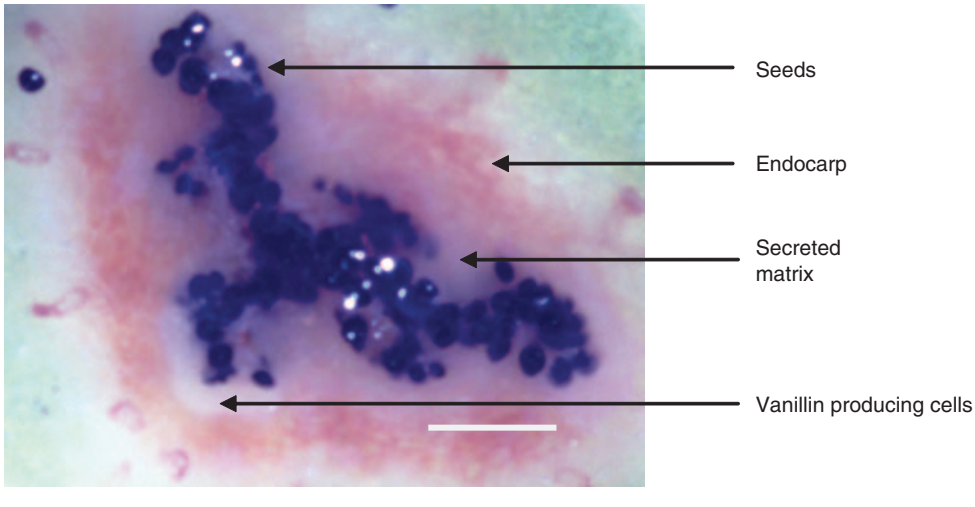

Plate 3.2 The central portion of the fruit, showing a dense catechin-HCl staining of the secreted matrix in the fruit cavity around the seeds, and a weaker staining in endocarp cells adjacent to the cavity. Scale bar = 2 mm. (From Joel *et al*. (2003); copyright material. Reprinted with permission of the publisher, LPP Ltd-Science, Israel.)

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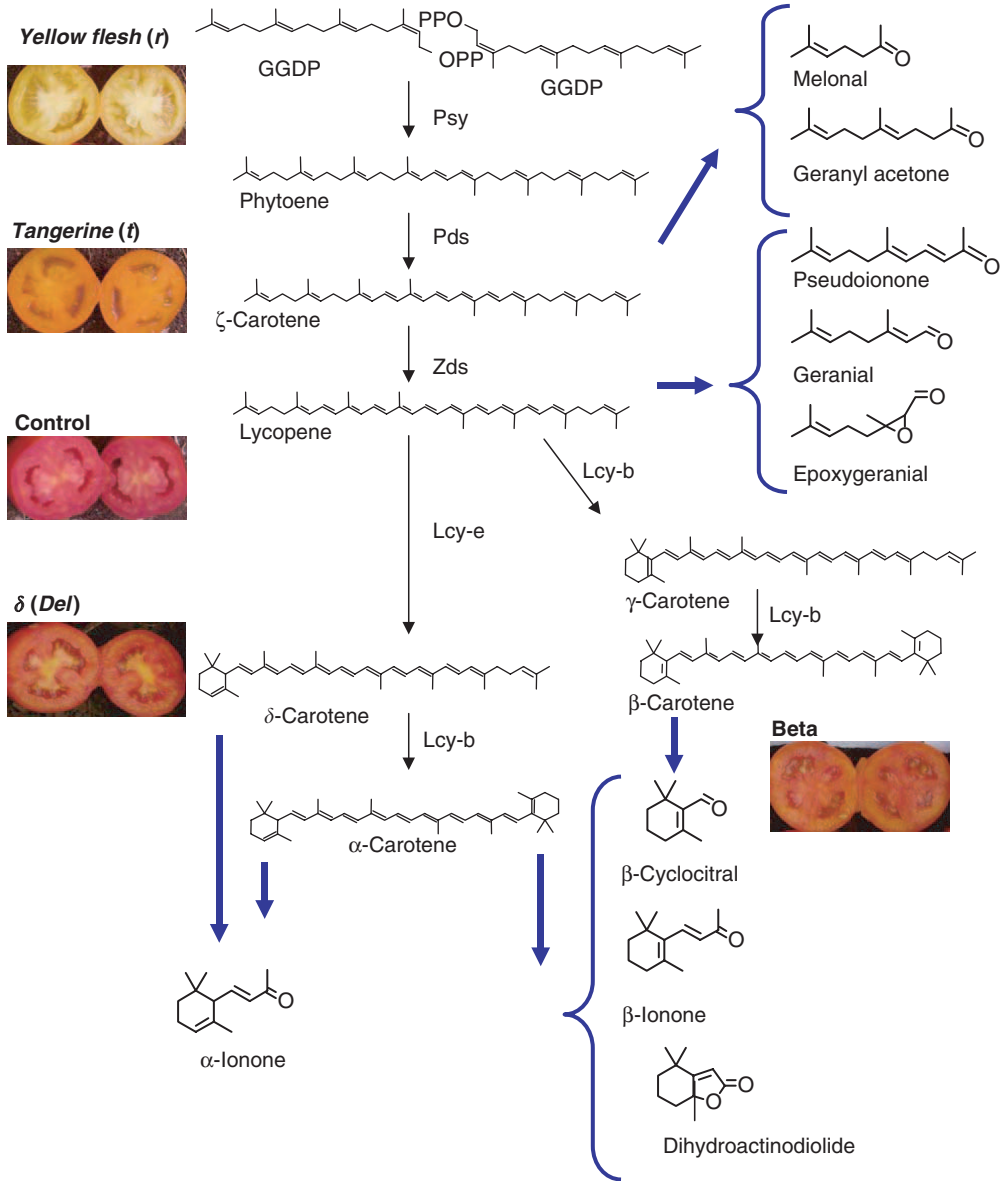

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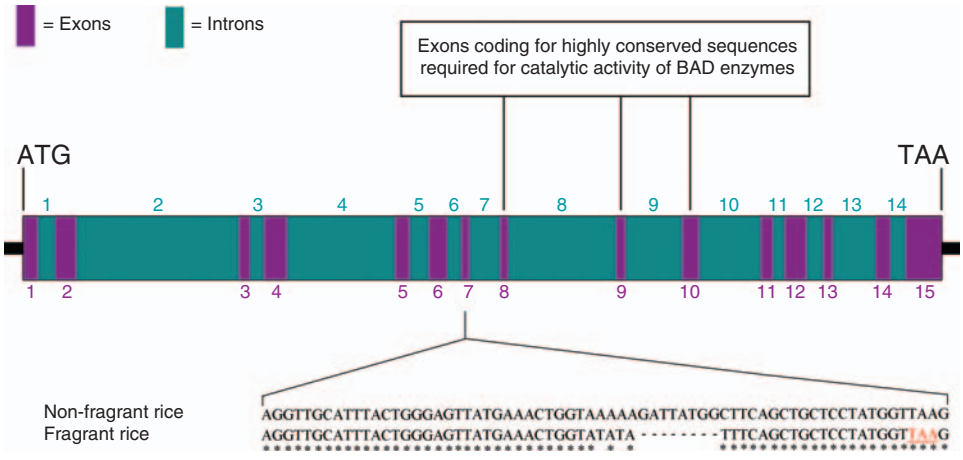

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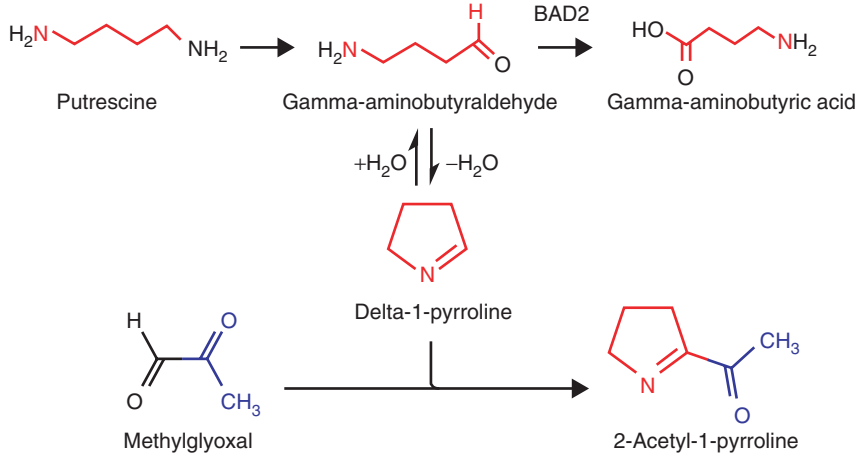

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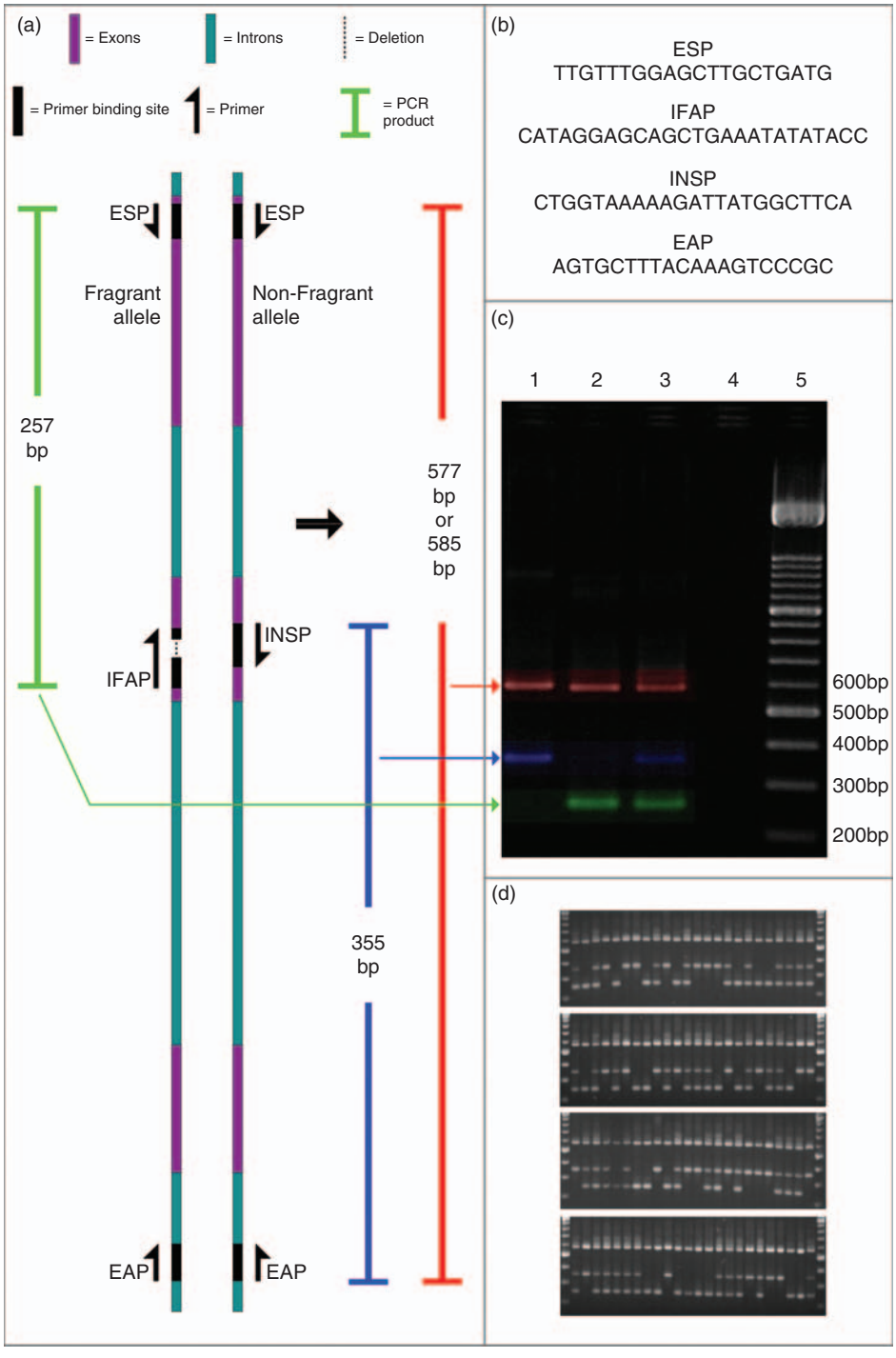

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Chapter 1
The development of yeast strains as tools for adjusting the flavor of fermented beverages to market specifications

Jan H. Swiegers, Sofie M.G. Saerens and Isak S. Pretorius

Introduction

It can be postulated that the very reason that humankind has prospered has been as a result of its dogged search for the 'good things in life'. At any time on any day, it is safe to say, someone is enjoying a glass of wine, beer or saké somewhere in the world. For those who imbibe in moderation, the pleasure is delivered in the aroma, flavor and mouth-feel of the beverage provided by a harmonious combination of flavor compounds and alcohol. What few of these appreciative drinkers realize, however, is that their enjoyment is made possible by a humble single-celled fungus. From the crudest home-brew to the most exquisite wine, the production of almost all alcoholic drinks depends on an extraordinary organism: the yeast *Saccharomyces*. In fact, it is remarkable that a single yeast is able to produce such a diverse variety of fermented beverages as those referred to in this chapter. It is even more remarkable that the same organism is able to release an equally diverse variety of flavors that enable us to enjoy – and therefore to seek out – these beverages.

Sometime in the distant past, *Saccharomyces* developed the ability to make and accumulate (and, under certain growth conditions, even consume!) alcohol while, at the same time, producing flavor-enhancing metabolites important to our sensory enjoyment of these beverages. A simple yeast with a unique set of capabilities, which has transformed human societies over the millennia, *Saccharomyces* has accompanied humankind's development to the extent that some anthropologists have even argued that the desire for alcoholic beverages was what persuaded us to become farmers and so led to the birth of civilization. Whether that is true or not, the ability of yeast to convert sugar-rich fruits and cereals into pleasurable alcoholic beverages such as wine, beer and saké has had an enormous influence on, and has been intertwined with, our history.

Wine

It is generally believed that human beings were making wine in Mesopotamia as early as 6000 BC (Robinson 2006). Evidence for winemaking has been found

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in Egypt and Phoenicia dating as far back as 5000 BC. The skills of cultivating grapes and making wine spread around the Mediterranean and, by 500 BC, wine was being produced in southern Europe and northern Africa. Later, vineyards were planted and wine was made in the Balkan States and in parts of northern Europe, eventually reaching as far as Great Britain. The first evidence for the spread of wine into the New World was in the years following 1530 when the Spanish conquistadors planted *Vitis vinifera* grapes in Mexico and South America. In 1655, Dutch settlers in South Africa planted vine cuttings from France in the Cape of Good Hope, and planting by the Spanish in California followed soon after. In Australia, two bunches of grapes were harvested in 1791 in the Governor's Garden (now Macquarie Street) in Sydney from vines brought from the Cape of Good Hope three years earlier. In New Zealand, missionaries were responsible for the planting of the first vines in 1819 at Kerikeri, in the far north of North Island.

Red wine is made from red (or 'black') grapes, and the color is extracted through the process of maceration, whereby the skins are left in contact with the juice during yeast fermentation and are regularly plunged or pumped over. On the other hand, white wines can be made from white or red grapes because the skins are separated from the juice during crushing. A white wine made from a very dark grape might appear pink and is known as a Rosé. Sparkling wines are those with carbon dioxide present, either from the yeast fermentation or added at a later stage. Champagne is made by fermenting twice: once in an open container to allow the carbon dioxide to escape and a second time in the bottle, where the gas is caught and remains in the wine. This traditional method of bottle fermentation is called *Méthode Champenoise*.

Beer

The history of beer brewing parallels that of wine: historians believe that beer was being brewed in Mesopotamia as early as 6000 BC (Protz 2004). It is likely that beer-like beverages were independently brewed by various cultures throughout the world because carbohydrate substrates are found in most foods. In Europe, beer was being produced as early as the seventh century AD, mostly in monasteries. By the fourteenth and fifteenth centuries, beer had achieved great popularity, partly because disease epidemics made drinking beer safer than drinking water. However, it is believed that beer did not take on the styles and flavors that we might recognize today until the seventeenth century (Nelson 2005).

Mashing barley is the first phase of brewing, when malted grains are crushed and soaked in warm water to create a malt extract. The starches are then converted to fermentable sugars over time. After mashing, water is filtered through the mash to dissolve the sugars, forming a dark, viscous liquid, which is called the wort. The wort is boiled and, after cooling and addition of hops, it is fermented by yeast.

Saké

Saké, or rice wine, is known to have been made since 3000 BC in China. It is thought that the refined technique for brewing saké was introduced to Japan in the fifth century AD. Saké is made by a process in which steamed rice is treated with a culture of *Aspergillus oryzae*, also called *koji*, which converts the starch into sugars. These are fermented by yeast to produce a beverage with about 15% alcohol (Rose 1983).

Wine, beer and saké yeasts

As is shown in the following sections, it is clear that, in '*consumerland*', not all members of the '*yeast tribe*' are considered '*equal citizens*'; the differences among them reflect their diversity in terms of robustness, fermentation performance and sensory attributes. Because, in the minds of the consumers, *product quality* is defined as '*sustainable customer satisfaction*', producers of fermented alcoholic beverages, all vying for the consumer dollar, seek to tailor their products to deliver satisfaction to specific markets. To do this, they have to select '*horses for courses*'; in other words, they want '*special yeasts for special treats*' (Pretorius 2000; Swiegers *et al*. 2005b). So, what makes *Saccharomyces* yeasts so special?

Unlike most other organisms, which resort to anaerobic respiration or fermentation only when oxygen is in short supply, *Saccharomyces* yeasts have the ability to convert sugars to ethanol, carbon dioxide and small quantities of sensorially important metabolites, even when oxygen is plentiful (Coghlan 2006). This physiological characteristic of the so-called Crabtree-positive yeasts is baffling. During aerobic respiration, the oxidative metabolic pathway generates 18 times more energy for cell growth and metabolic activities than can be delivered by the glycolytic pathway during fermentation (Pronk *et al*. 1996). In fact, high sugar concentrations repress respiration in these yeasts. Why would *Saccharomyces* sacrifice huge amounts of energy to ferment sugars in the presence of oxygen, and why would it produce alcohol instead of boosting cell growth? The explanation for this apparent anomaly is that ethanol is toxic to most microbes and, by churning out alcohol in the presence of oxygen, *Saccharomyces* gains a significant competitive advantage over other organisms in sugar-rich environments.

This unique capability of *Saccharomyces* can be attributed to the two versions of the alcohol dehydrogenase enzyme that it carries, that is, the enzymes encoded by *ADH1* and *ADH2*. The *ADH1*-encoded enzyme (Adh1p) converts the main breakdown product of sugar, acetaldehyde, into alcohol while the *ADH2*-encoded enzyme (Adh2p) catalyzes the reverse reaction. However, unlike Adh1p, Adh2p is produced only if the sugar levels drop; therefore, Adh2p is only available to turn alcohol back into acetaldehyde once *Saccharomyces* has scavenged the sugars away from rival microbes. In other words,

Saccharomyces, which has developed a high tolerance to ethanol, converts sugars into alcohol to 'defend its territory' in sugar-rich habitats. Put differently, by being able to sacrifice initial energy gains from aerobic respiration, *Saccharomyces* can rush into sugar-rich environments to rapidly turn sugars into a 'poison' that kills off any rivals, and then feasts on the 'poison' (Coghlan 2006). It is this ability to make, accumulate, tolerate and consume alcohol that was successfully harnessed by humankind to produce pleasurable fermented alcoholic beverages.

Over a period of several millennia, *Saccharomyces* yeasts have been selected for their robustness and the sensorial attributes they create in products such as wine, beer and saké. This has led to the generation of large collections of *Saccharomyces* yeast strains with which grape juice, beer wort and rice wort can be inoculated to deliver reliability of fermentation performance and diversity of predetermined flavors and product styles.

This section briefly outlines the yeast species and strains involved in the production of wine, beer and saké.

Wine yeasts

Saccharomyces cerevisiae is rarely isolated from vineyards and grape berries, but it is abundant in wine cellars, presses, tools and other environments that are high in sugars. Wine strains of *S. cerevisiae* are mostly homothallic, diploid/aneuploid and do not commonly sporulate under normal conditions. Unlike most laboratory strains, wine strains possess chromosomal-length polymorphisms and rearranged chromosomes with multiple translocations (Bidenne *et al*. 1992; Rachidi *et al*. 2000). *S. cerevisiae* is probably the most well-characterized eukaryotic organism known today, and this wealth of knowledge has boosted wine yeast research.

Owing to its low pH and high sugar content, which both exert selective pressure on the microorganisms present (Henschke 1997), grape must can support the growth of only a limited number of microbial species. Furthermore, sulfur dioxide (SO_2), added as an anti-oxidant and anti-microbial preservative, imposes additional selection, particularly against undesirable oxidative microbes. Once fermentation starts, oxygen and other nutrients are depleted and ethanol concentrations rise significantly – conditions that kill many microorganisms originally present (Henschke 1997). These selective pressures, which occur during the winemaking process, always favor yeasts with the most efficient fermentative catabolism and ethanol tolerance, particularly strains of *S. cerevisiae*. Therefore, *S. cerevisiae* is almost universally preferred for initiating alcoholic fermentation, and has earned itself the title of the *wine yeast*.

However, during the early stages of spontaneous wine fermentation, a number of non-*Saccharomyces* yeasts can have an influence. These include: *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora* and its asexual counterpart *Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*,

Saccharomycodes, *Schizosaccharomyces* and *Zygosaccharomyces* (Pretorius *et al*. 1999). Yeasts of the genera *Kloeckera*, *Hanseniaspora* and *Candida* predominate in the early stages, followed by several species of *Metschnikowia* and *Pichia* in the middle stages when the ethanol concentration rises to 3–4% (Fleet and Heard 1993). However, some species and strains of *Schizosaccharomyces*, *Zygosaccharomyces*, *Brettanomyces* and its sexual ('perfect') equivalent, *Dekkera*, are more resistant to high concentrations of ethanol and SO_2 and, if present under certain conditions, can adversely affect the sensory quality of wine. Nevertheless, the final stages in spontaneous fermentations are invariably being dominated by *S. cerevisiae* (Pretorius 2000).

A key question regarding commercial yeasts for inoculated wine ferments would be why there are so many strains of *S. cerevisiae* on offer. Why are there not only a couple of 'workhorse' strains that can turn grape sugars (glucose and fructose) into alcohol – one strain for white wine production and one for red winemaking? Although wine strains of *S. cerevisiae* are expected to perform a rapid, complete and efficient conversion of grape sugars to ethanol and carbon dioxide, they are also expected to produce sensorially important metabolites and to convert flavorless grape-derived constituents (e.g. glyco and cysteine conjugates) into flavor-enhancing compounds (Swiegers and Pretorius 2005, 2007; Swiegers *et al*. 2005a, 2007).

Beer yeasts

In brewing, top-fermenting (ale-brewing) yeasts form a diverse group of polyploid yeasts that are closely related to *S. cerevisiae* (Hansen and Kielland-Brandt 1997). Bottom-fermenting (lager-brewing) yeasts were first classified as *Saccharomyces carlsbergensis*, and were then later re-classified as *S. cerevisiae* before finally being re-classified as *Saccharomyces pastorianus* (Yarrow 1984; Vaughan-Martini and Martini 1987). Brewing strains are allo-tetraploid and exhibit poor sporulation and low spore viability, and they appear to contain sections of genomes that are probably derived from *S. cerevisiae* and *Saccharomyces monacencis* (Hansen and Kielland-Brandt 1997). However, other studies suggest that they are hybrids of *S. cerevisiae* and *Saccharomyces bayanus* (Vaughan-Martini and Kurtzman 1985; Yamagashi and Ogata 1999).

Saké yeasts

Although saké yeast is taxonomically classified as *S. cerevisiae*, there are many phenotypic and enological differences compared with wine and brewing strains (Kodama 1970). Saké yeast has particular; (i) vitamin requirements; (ii) sugar, acid and alcohol tolerance; and (iii) osmotic characters (Kodama and Yoshizawa 1977). Furthermore, saké yeast produces distinctive esters and other flavor compounds that are characteristic of rice wine (Asano *et al*. 1999; Arikawa *et al*. 2000; Akada *et al*. 2001).

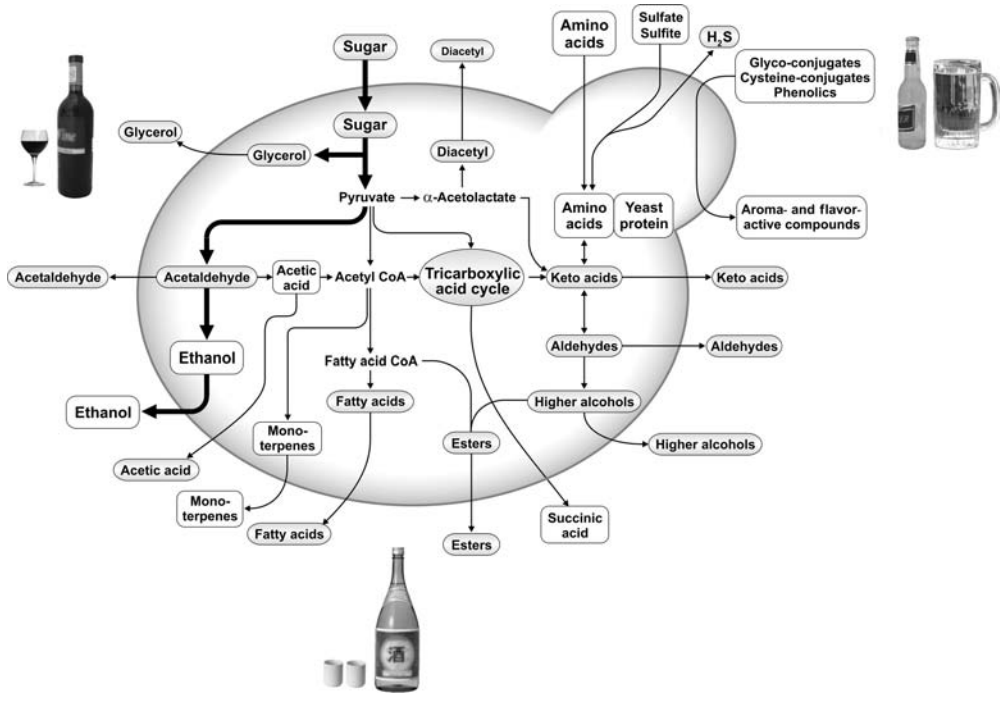

Fig. 1.1 The metabolic role of yeast in the development of flavor compounds in wine, beer and saké (adapted from Swiegers *et al*. 2005a).

Yeast, especially *S. cerevisiae*, clearly plays an important role in the development of flavor in wine beer and saké (Fig. 1.1). To put it simply, if a relatively flavorless sugar solution is fermented with *Saccharomyces*, the result will probably be a beverage with significantly more flavor. The following sections selectively discuss the various flavor compounds produced by the different wine, beer and saké yeasts, and the prospects for further genetic improvement of these starter strains.

Acids

Non-volatile acids

Non-volatile acids in grape juice play an important role in the physical, chemical and microbial stability of wines and have a significant impact on the taster's palate (Fowles 1992; Jackson 1994; Boulton *et al*. 1998). Non-volatile acids determine the acidity (including the pH) to a large extent and influence

(1) the survival and growth of all microorganisms
(2) the effectiveness of anti-oxidants, antimicrobial compounds and enzyme additions

(3) the solubility of proteins and tartrate salts
(4) the effectiveness of bentonite treatment
(5) the polymerization of the color pigments
(6) the oxidative and browning reactions and
(7) the freshness of some wine styles.

The most abundant non-volatile organic acids in grapes are tartaric acid and malic acid, accounting for 90% of the titratable acidity of grape juice. Tartaric acid is resistant to microbial activity and changes little during fermentation. Citric acid and lactic acid, which also contribute to the acidity of grape juice, are less abundant. Succinic acid is present only in trace amounts in grapes, but its concentration is significantly higher in wines because it is the main carboxylic acid produced during fermentation by wine yeast (Whiting 1976; Fowles 1992; Radler 1993; Boulton *et al.* 1998). Yeast can produce up to 2 g/L of succinic acid (succinate) in wine (Thoukis *et al.* 1965; Radler 1993; Coulter *et al.* 2004). There is a high degree of variation in the production of succinic acid by different strains of *S. cerevisiae*. *Saccharomyces uvarum* or *S. bayanus* strains tend to produce higher concentrations (Heerde and Radler 1978; Giudici *et al.* 1995; Eglinton *et al.* 2000). Succinic acid can affect wine quality because it has been described as having an 'unusual salty, bitter taste' (Whiting 1976). Succinic acid in wine is probably formed through the tri-carboxylic acid (TCA) cycle during anaerobic fermentation (Roustan and Sablayrolles 2002; Camarasa *et al.* 2003). Various factors affect succinic acid accumulation during fermentation, and these include yeast strain, fermentation temperature, aeration, must clarity and composition (including sugar concentration), nutrient content, pH, titratable acidity and SO_2 concentration (Coulter *et al.* 2004). It has been suggested that γ-amino butyric acid accounts for 'abnormal' concentrations of succinic acid in wine (Bach *et al.* 2004).

In beer, the most common non-volatile organic acids include pyruvate, citrate, malate, succinate, lactate and 2-oxoglutarate (Coote and Kirsop 1974; Klopper *et al.* 1986). The secretion of organic acids during wort fermentation varies according to the nature of the acid. Succinate, lactate and α-ketoglutarate accumulate during wort fermentation with succinate being the most abundant at the end of fermentation. α-Ketoglutarate and lactate are present in beer only in low concentrations. The quantities of citrate and malate in beer are largely determined by their concentrations in the wort, although their final levels might be slightly affected by the yeast strain. Citrate concentrations in beer vary between 6 and 322 mg/L. In pilsner beers and top-fermented beers, citrate concentrations of 107–186 and 173–211 mg/L are found, respectively. In acid beers, greater citrate variations occur, of between 6 and 146 mg/L. For malate in acid beers, concentrations from 6 to 136 mg/L are found (Coote and Kirsop 1974; Klopper *et al.* 1986).

Owing to their ability to bind SO_2 and to react with phenols (Rankine 1967; Rankine 1968a,b; Rankine and Pocock 1969), the keto acids, pyruvic and

α-ketoglutaric acid, can affect wine stability and quality. The keto acids are produced by wine yeast either during the early stages of fermentation through sugar metabolism, or from the amino acids alanine and glutamate. It has been shown that when nitrogen is adequate, α-ketoglutaric acid typically accumulates in wine to less than 50–100 mg/L, but when nitrogen is limited, several hundred mg/L can be produced by yeast (Rankine 1968a; Radler 1993). Apart from nitrogen, the type of yeast strain can also have an effect on the concentration of keto acids produced in wine (Rankine 1968a). Pyruvate is present in beer at concentrations of 1–127 mg/L. Top-fermented beers show greater variations in pyruvate content than do pilsner beers. During beer fermentation, pyruvate accumulation is followed by a period in which the pyruvate concentration remains approximately constant. Looking at the first phase of excretion, it seems likely that pyruvate is excreted in association with yeast growth. The concentration of pyruvate also depends on wort composition. The use of all-malt wort of higher specific gravity stimulates accumulation of pyruvate, as does the use of wort with increased soluble nitrogen content. When adjunct wort containing reduced concentrations of non-carbohydrate nutrients is used, pyruvate accumulation is diminished.

S. cerevisiae wine strains can degrade between 3% and 45% of malic acid during fermentation, whereas most strains of *Schizosaccharomyces pombe* and *Schizosaccharomyces malidevorans* can degrade it completely to ethanol and CO_2 (Rankine and Fornachon 1964; Radler 1993). One factor contributing to the inefficient use of malic acid by *S. cerevisiae* is its lack of an active malate transport system such as the one that occurs in *Sz. pombe*. Malate enters wine yeast by simple diffusion. The constitutive nicotinamide adenine dinucleotide (NAD)-dependent malic enzyme converts malate to pyruvate which, under anaerobic conditions, is converted to ethanol and carbon dioxide. Under aerobic conditions, malic acid is decarboxylated into water and carbon dioxide. However, the substrate specificity of the *S. cerevisiae* malic enzyme is about 15-fold lower than that of the *Sz. pombe* malic enzyme (Radler 1993; Ansanay *et al*. 1996) – another factor that is responsible for the inefficient metabolism of malate by wine yeast.

Several attempts have been made to genetically engineer wine yeast to perform alcoholic fermentation and malate degradation simultaneously (Dequin and Barre 1994; Volschenk *et al*. 1997; Ansanay *et al*. 1996). Malo-ethanolic wine yeast was developed by overexpressing the *mae1* malate permease gene and the *mae2* malic enzyme gene from *Sz. pombe* in *S. cerevisiae* (Volschenk *et al*. 1997). Recently, a genetically stable industrial wine strain of *S. cerevisiae* was constructed by integrating a linear cassette containing the *Sz. pombe* malate permease gene (*mae*1) and the *Oenococcus oeni* malolactic gene (*mle*A). This malolactic yeast strain, ML01, completely decarboxylated 5.5 g/L of malate in Chardonnay grape must during the alcoholic fermentation. Furthermore, genetic and phenotypic analysis indicated that the ML01 strain was similar to the parental industrial wine yeast (Husnik *et al*. 2007). The ML01 strain has been classified by the US Food and Drug Administration as

'Generally regarded as safe'. Recently, this strain was commercialized and used by some winemakers to produce commercial wine in North America.

In an attempt to reduce the acidity of highly acidic musts, a mutant of *Sz. malidevorans* was developed (through exposure to ultra-violet irradiation). The mutant consumed malic acid at a higher rate than did the wild-type and demonstrated reduced utilization of glucose in the presence of malic acid (Rodriguez and Thornton 1989). Therefore, a greater level of deacidification can be achieved by conversion of malic acid into ethanol and CO_2 under anaerobic conditions than is achieved by malolactic fermentation. Winemaking trials showed that the mutant could degrade 3.5–10 g/L of malic acid in juices over a 21–73-h period (Thornton and Rodriguez 1996).

Succinate production by yeast can have an important effect on the quality of saké (Tomizawa *et al*. 1960). To identify the origin of succinate biosynthesis in yeast, the fumarase-encoding gene, *FUM1*, was deleted in a saké yeast, and it was found that fumarate accumulated in the culture. Therefore, it was suggested that malate and succinate were produced from an oxidative pathway of the TCA cycle (Wu and Tzagoloff 1987). In support of this hypothesis, a saké yeast with a deleted aconitase-encoding gene, *ACO1*, was found to contain a two-fold higher concentration of malate and a two-fold lower concentration of succinate than was produced by the wild-type strain. Furthermore, deletion of the fumarate reductase gene, *OSM1*, produced saké containing a 15-fold higher concentration of succinate compared to the wild-type, whereas deletion of the α-ketoglutarate dehydrogenase-encoding gene, *KGD1*, and the *FUM1* fumarase gene resulted in lower succinate concentrations (Arikawa 1999).

Volatile acids

In wine, a group of volatile organic acids of short carbon-chain length is collectively known as volatile acidity (VA), and the volatile acid content of wine is, on average, between 500 and 1000 mg/L (Fowles 1992; Henschke and Jiranek 1993; Radler 1993). Acetic acid usually constitutes approximately 90% of the volatile acids in wine, while the rest of the volatile acids consist mostly of propionic acid and hexanoic acid, which are by-products of fatty acid metabolism by yeast and bacteria.

Acetic acid can have a significant impact on the quality of wine. At high concentrations, it imparts a vinegar-like character to wine, which can become objectionable at concentrations of 0.7–1.1 g/L, depending on the style of wine. The optimal concentration in wine is 0.2–0.7 g/L (Corison *et al*. 1979; Dubois 1983).

S. cerevisiae wine strains can produce varying amounts of acetic acid depending on the conditions and the type of strain, with concentrations varying from 100 mg/L to 2 g/L (Radler 1993). Strains of *S. bayanus* and *S. uvarum* usually produce less acetic acid than *S. cerevisiae* (Giudici *et al*. 1995; Eglinton *et al*. 2000).

In yeast, acetate is produced as an intermediate of the pyruvate dehydrogenase (PDH) pathway, which is responsible for the conversion of pyruvate into acetyl-CoA through a series of reactions catalyzed by pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase and acetyl-CoA synthase. The PDH pathway generates cytosolic acetyl-CoA, which is needed for anabolic processes such as lipid biosynthesis (Flikweert *et al*. 1996; Pronk *et al*. 1996). Acetate is formed by oxidizing the acetaldehyde produced from pyruvate during fermentation in a reaction catalyzed by acetaldehyde dehydrogenase. The mitochondrial isoforms are encoded by *ALD4* and *ALD5*, whereas the cytosolic acetaldehyde dehydrogenases are encoded by *ALD6*, *ALD2* and *ALD3* (Navarro-Avino *et al*. 1999). During anaerobic growth on glucose, in conditions resembling winemaking, it has been shown that Ald6p, Ald5p and Ald4p are the main enzymes responsible for acetate formation (Saint-Prix *et al*. 2004). In saké production, it has been shown that induction of *ALD2* and *ALD3* resulted in high acetic acid production in conditions of high glucose concentrations (Akamatsu *et al*. 2000). Other work has shown that the concentration of acetate produced by *S. cerevisiae* could be modulated by increasing or decreasing the expression of *ALD6* (Remize *et al*. 2000a). Deletion of both alleles of *ALD6* in a wine yeast led to a two-fold reduction in the amount of acetate produced during fermentation. However, as a consequence of the resulting redox imbalance, glycerol, succinate and 2,3-butanediol production is slightly increased (Remize *et al*. 2000a).

In beer, acetic acid can also have a negative effect when the concentrations are high (Mizuno *et al*. 2003). Excessive acetic acid production in beer has become a problem in high-gravity brewing, a process by which a wort of high sugar concentration (high gravity) is fermented, resulting in high concentrations of ethanol, before being diluted with carbonated water. Under these conditions, the yeast cells are stressed because of high sugar and ethanol concentrations and subsequently produce high amounts of acetic acid. Recently, a mutant brewing-yeast strain was isolated that produced low amounts of acetic acid and high amounts of ethanol (Mizuno *et al*. 2006). Transcript analysis indicated that the *ALD4*-encoded acetaldehyde dehydrogenase was expressed at significantly higher levels in the mutant than in the wild-type strain, confirming the importance of these enzymes in regulating acetic acid concentrations during fermentation. Beer brewed in pilot-scale high-gravity brewing using the mutant contained approximately half the amount of acetic acid and 1.1% more ethanol than beer brewed using the wild-type.

Alcohols

Ethanol

The fundamental physiological characteristic of wine and beer yeasts is their ability to degrade carbohydrates, usually six-carbon (C_6) molecules such as

glucose, to two-carbon (C_2) components, in particular ethanol, without completely oxidizing them to CO_2 (Piskur *et al*. 2006). *S. cerevisiae* is able to grow in high sugar (between 220 and 250 g/L) and at low pH (pH 3–4), and is able to survive in the presence of high ethanol concentrations. These attributes give the yeast a huge competitive advantage in grape must and wort, where it can ferment high concentrations of sugars such as glucose, fructose and maltose to ethanol and carbon dioxide.

Ethanol has a positive effect on the aroma sensations of wine and beer through its interaction with other compounds. In typical lager beers, ethanol significantly increases aldehyde retention, leading to a lower perception of the worty character (Perpete and Collin 2000). Ethanol also has an effect on the volatility of aroma compounds. The increase or decrease in the volatility of aromas can greatly influence the overall aroma of wine (Voilley and Lubbers 1998). Furthermore, ethanol leads to modifications in protein conformation that tend to reduce the number of binding sites of the aroma compounds.

Today, the reduction of ethanol in alcoholic beverages, especially beer and wine, is of considerable commercial interest. Consumer demand for reduced-ethanol beverages is increasing continuously because of increased awareness about health as well as stricter laws regarding drinking and driving (Pretorious, 2006). Current production methods of low-alcohol beer, that is manipulated fermentation or post-fermentation removal of ethanol, result either in a wort taste or in a loss of aroma components (Scott and Huxtable 1995; Zufall and Wackerbauer 2000). In an alcohol-free lager beer, produced by a cold-contact process, the absence of ethanol (<0.1%) strengthens the 'worty' off-flavors. In addition, the elimination of ethanol based on distillation or dialysis is expensive and labor-intensive.

The reduction of ethanol in wine can be achieved by various physical processes. These processes are sometimes used in combination and include reverse osmosis, adsorption, distillation, centrifugation, evaporation, extraction, freeze concentration, membrane filtration and partial fermentation. However, these methods involve expensive equipment and processing, and it is important to consider the impact of possible loss or modification of the aroma and flavor compounds during processing.

An alternative technique for producing wine or beer with a reduced ethanol content could be winemaking or brewing with a genetically modified (GM) yeast strain that produces less ethanol during complete fermentation of wort and must sugars, respectively. The reduction of ethanol production could be achieved by metabolic engineering of the carbon flux in yeast, resulting in increased formation of other fermentation products such as glycerol. However, only by-products that do not spoil the taste of wine or beer would be acceptable.

Recently, the ethanol content in beer was decreased by overexpressing the *GPD1* gene, which encodes glycerol-3-phosphate dehydrogenase (GPD), in an industrial lager brewing yeast (*S. cerevisiae* subsp. *carlsbergensis*) (Nevoigt *et al*. 2002). In fermentation experiments simulating brewing conditions, the

amount of glycerol produced by the strain overexpressing *GPD1* compared to the wild-type was increased 5.6 times and ethanol production was decreased by 18%. Only minor changes in the concentration of higher alcohols, esters and fatty acids could be observed in the final beer. However, the concentrations of several other by-products, particularly acetoin, diacetyl and acetaldehyde, were considerably increased. Hence, future developments in metabolic engineering will seek to combine the optimization of ethanol reduction and by-product formation, thus facilitating the direct use of modified brewing yeast to produce low-alcohol beer.

Because the initial sugar concentration of grape must is important for producing wines with lower alcohol content, glucose oxidase (GOX) provides an approach for reducing the glucose content of must (Pickering *et al*. 1999a,b,c). Glucose is converted by GOX to D-glucone-δ-lactone and gluconic acid, rendering it unavailable for alcohol formation during fermentation. A reduction of almost 2% in ethanol concentration has been achieved by the expression of the *GOX1* gene of a food-grade fungus, *Aspergillus niger*, in yeast (Malherbe *et al*. 2003). Similarly, a significant decrease in alcohol concentration (up to 2%) and a concomitant increase in extracellularly accumulated glycerol have been achieved by overexpression of either the *GPD1*- or *GPD2*-encoded GPD isoenzymes of *S. cerevisiae* (Michnick *et al*. 1997; Remize *et al*. 1999).

Glycerol

Glycerol is quantitatively the most important fermentation product after ethanol and carbon dioxide. It is involved in osmotic cell regulation and, during alcoholic fermentation, the main role of glycerol formation is to equilibrate the intracellular redox balance by converting the excess NADH, generated during biomass formation, to NAD^+ (Taherzadeh *et al*. 2002). Its formation requires the reduction of the glycolytic intermediate, dihydroxyacetone phosphate, to glycerol-3-phosphate, a reaction catalyzed by GPD, followed by the dephosphorylation of glycerol-3-phosphate to glycerol by glycerol-3-phosphatase (GPP). Two isoenzymes of GPD encoded by the genes *GPD1* and *GPD2* have been characterized in *S. cerevisiae* (Albertyn *et al*. 1994; Ansell *et al*. 1997). The isoenzymes of GPP are encoded by *GPP1* and *GPP2* (Norbeck *et al*. 1996; Pahlman *et al*. 2001).

Glycerol is a key factor influencing the taste of wine, beer, saké and *shochu* (similar to saké). The usual glycerol concentration in wine ranges from 4 to 9 g/L (Ough *et al*. 1972; Radler and Schutz 1982). Red wines have been described as containing more glycerol than white wines, while flor sherries contain considerably less (Ough *et al*. 1972). In beer, glycerol concentration ranges from 1.3 to 2 g/L. Although it has no direct impact on aromatic characteristics, glycerol significantly contributes to wine quality by providing sweetness and fullness (Noble and Bursick 1984; Eustace and Thornton 1986). Despite a measurable effect on wine viscosity, this effect is perceived only for concentrations higher than 25 g/L (Noble and Bursick 1984). In contrast, a threshold

value for sweetness of 5.2 g/L has been determined in white wine (Noble and Bursick 1984). Owing to the favorable impact of glycerol on wine quality, the benefits of increasing glycerol production to improve the sensory characteristics of wines lacking in body have been emphasized (Butzke and Bisson 1996).

Glycerol production by yeast is influenced by many growth and environmental factors (Ough *et al*. 1972; Scanes *et al*. 1998). Numerous studies point to a significant influence of the strain, temperature, agitation and nitrogen composition on glycerol production, particularly under enological conditions. However, owing to the disparities in the conditions used (Remize *et al*. 2000b) the relative impact of these different factors remains difficult to assess. Therefore, genetic engineering of *S. cerevisiae* could be a better solution for optimizing glycerol production.

The strategy supporting most of the applied methods for enhancing glycerol production is, in a sense, 'indirect', because it aims to create conditions during which the NADH generation in metabolism is maximized. The consequent carbon flux redistribution is caused by the necessity for NAD^+ regeneration, giving increased glycerol production. A second strategy relies on direct interference with the carbon flux, for example by inhibiting the later part of glycolysis (Pretorius *et al.* 2006). This alternative strategy can be accomplished, for example, by overexpression of enzymes in the glycerol pathway, such as GPD and/or down-regulation of enzymes in the later part of glycolysis, such as alcohol dehydrogenase. In this case, the increased glycerol formation results in a need for increased NADH production, which has to be met by an increased production of oxidized products, for example carboxylic acids (Taherzadeh *et al*. 2002). It has been reported that glycerol formation could be enhanced by a factor of four from 0.069 to 0.275 mol/mol, by overexpression of *GPD1* in *S. cerevisiae* at the expense of ethanol production (Michnick *et al*. 1997).

A similar study was carried out in which overexpression of *GPD1* was analyzed in nine strains of *S. cerevisiae* (Remize *et al*. 1999). Glycerol production increased between 1.5- and 2.5-fold in the examined strains. The overexpression of *GPD2*, encoding the other isoform of GPD, is equally as effective as the overexpression of *GPD1* in increasing glycerol production (3.3-fold increase compared to the wild-type strain) and has similar effects on yeast metabolism. The biomass and ethanol yield decreased, whereas the yields of pyruvate, acetate, acetaldehyde, 2,3-butanediol, succinate and acetoin increased. The acetic acid concentrations increased to levels that were particularly unacceptable. This negative side effect was circumvented by deleting the *ALD6*-encoded acetaldehyde dehydrogenase activity, which is the main contributor to the oxidation of acetaldehyde during fermentation. For example, a laboratory strain of *S. cerevisiae* overexpressing *GPD2* and lacking the *ALD6* gene had the desired effect of producing more glycerol and less ethanol, without an increase in acetic acid (Eglinton *et al*. 2002; Remize *et al*. 1999; Remize *et al*. 2000a).

Recently, the effects of the overexpression of *GPD1* in three commercial wine yeast strains have been investigated (Cambon *et al*. 2006). Both alleles of

ALD6 were deleted to reduce the amount of acetic acid produced. The *GPD1 ald6Δ* wine strains produced 15–20% less alcohol compared to the wild-type without a major change in the concentration of acetate. However, these strains produced extremely high concentrations of acetoin, which had a negative impact on the aroma of the wine (Cambon *et al*. 2006).

Higher alcohols

In addition to ethanol, *S. cerevisiae* also produces a large number of long-chain and complex alcohols. These alcohols, called higher or fusel alcohols, are secondary yeast metabolites and can have both positive and negative impacts on the flavor and aroma of fermented beverages. Excessive concentrations of higher alcohols can result in a strong, pungent smell and taste, whereas optimal levels impart fruity characteristics in wine (Table 1.1) (Nykanen *et al*. 1977; Swiegers and Pretorius 2005; Lilly *et al*. 2006b). Concentrations of higher alcohols of less than 300 mg/L have been reported as contributing to the desirable complexity of wine, but above 400 mg/L the higher alcohols were found to have a negative influence on wine quality (Swiegers *et al*. 2005a).

Higher alcohols are comprised of two categories, aliphatic and aromatic, and they are extremely important in wine and distillates (Nykanen *et al*. 1977). The aliphatic alcohols include propanol, isoamyl alcohol, isobutanol and active amyl alcohol; the aromatic alcohols consist of 2-phenylethyl alcohol and tyrosol. In beer, the flavor of the aliphatic alcohols is distinctly alcoholic (like ethanol) and the aromatic alcohols have a rather sweet, alcoholic or bitter taste (Meilgaard 1975) (Table 1.2).

Many factors influence the final concentration of higher alcohols in alcoholic beverages. In wine, viticultural conditions and the use of different yeast strains during fermentation contribute considerably to variations in higher alcohol profiles and concentrations (Giudici *et al*. 1990). The amino acid concentration is another important factor influencing higher alcohol production (Schulthess and Ettlinger 1978). The total production of higher alcohols increases with increasing concentrations of the corresponding amino acids.

Table 1.1 Threshold values for higher alcohols produced by yeast, and their concentrations in wine (data from Swiegers *et al*. 2005a).

Compound	Concentration in wine (mg/L)	Threshold (mg/L)	Aroma descriptor
Propanol	9.0–68	500[a]	Alcohol
Butanol	0.5–8.5	150[b]	Fusel, spiritous
Isobutanol	9.0–174	40[b]	Fusel, spiritous
Isoamyl alcohol	6.0–490	30[b]	Harsh, nail polish
Hexanol	0.3–12.0	4[a]	Green, grass
2-Phenylethyl alcohol	4.0–197	10[b]	Floral, rose

[a]Wine, [b]10% ethanol.

Table 1.2 Threshold values for higher alcohols commonly found in beer (data from Meilgaard 1975).

Compound	Threshold value (mg/L)	Aroma descriptor
Propanol	800	Alcohol
Isobutanol	200	Fusel, spiritous
Isoamyl alcohol	70	Alcohol, banana, sweet, aromatic
Active amyl alcohol	65	Alcohol, banana, medicinal, solvent
2-Phenylethyl alcohol	125	Rose, sweet, perfumed
Tyrosol	200	Bitter, chemical

Ethanol concentration, fermentation temperature, the pH and composition of the grape must, aeration, level of solids, grape variety, maturity and skin contact time also affect the concentration of higher alcohols in the final product (Fleet and Heard 1993). In beer, many factors that stimulate yeast growth, such as oxygenation (Quain and Duffield 1985), addition of fatty acids and sterols (Taylor *et al*. 1979) or increased temperatures (Landaud *et al*. 2001), cause an increase in the fusel-alcohol concentration in the fermenting wort. Production of aromatic alcohols (especially 2-phenylethyl alcohol) appears to be particularly sensitive to temperature, whereas the synthesis of other higher alcohols is relatively unaffected by temperature.

Biosynthesis of higher alcohols involves the decarboxylation of keto acids to form aldehydes, followed by a reduction of the aldehydes to produce the alcohols. The branched-chain alcohols are synthesized from the α-keto acids during fermentation via the branched-chain amino acid metabolic pathway, by decarboxylation and by reduction (Dickinson *et al*. 1997). These α-keto acids are formed via two major pathways: the catabolic Erhlich pathway, involving degradation of amino acids to their corresponding alcohols (e.g. leucine to isoamyl alcohol, isoleucine to active amyl alcohol and valine to isobutanol) and anabolically via *de novo* synthesis of branched-chain amino acids from glucose (Hammond 1995). The uptake of branched-chain amino acids by *S. cerevisiae* is mediated by at least three transport proteins: the general *GAP1*-encoded amino acid permease, the *BAP2*-encoded branched-chain amino acid permease, and one or more unknown permeases (Didion *et al*. 1996).

The first step in the catabolism of branched-chain amino acids is deamination to form the corresponding α-keto acids, for example α-ketoisocaproic acid from leucine, α-ketoisovaleric acid from valine and α-keto-β-methylvaleric acid from isoleucine (Dickinson and Norte 1993). This reaction is catalyzed by mitochondrial and cytosolic branched-chain amino acid aminotransferases encoded by the *BAT1* and *BAT2* genes, respectively (Fig. 1.2) (Eden *et al*. 1996, 2001). A PDC then converts the resulting keto acid to the corresponding branched-chain aldehyde. In the leucine-degradation pathway, the major decarboxylase is encoded by *KID1* (Dickinson *et al*. 1997). In the valine-degradation pathway, any one of the three isozymes of PDC, encoded by *PDC1*, *PDC5* and *PDC6*, will decarboxylate α-ketoisovaleric acid

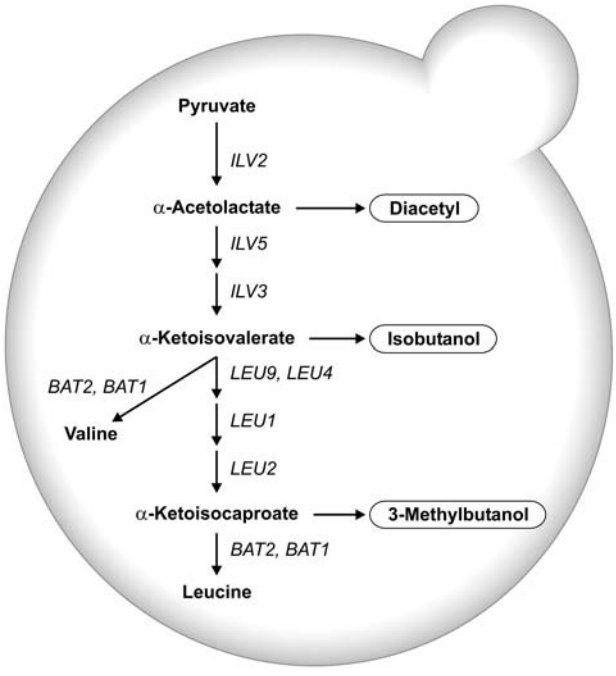

Fig. 1.2 Pathway of leucine and valine biosynthesis. *ILV2*: acetolactate synthase; *ILV5*: acetohydroxyacid reductoisomerase; *ILV3*: dihydroxy acid dehydratase; *BAT1*: branched-chain amino acid aminotransferase; *BAT2*: branched-chain amino acid transaminase; *LEU9*: α-isopropylmalate synthase; *LEU4*: α-isopropylmalate synthase; *LEU1*: isopropylmalate isomerase; *LEU2*: β-isopropylmalate dehydrogenase.

(Dickinson *et al*. 1998). In isoleucine catabolism, any one of the family of decarboxylases encoded by *PDC1*, *PDC5*, *PDC6*, *KID1* or *ARO10* is sufficient for the decarboxylation reaction (Dickinson *et al*. 2000). The final step of amino acid catabolism (conversion of an aldehyde to an alcohol) can be accomplished by any one of the ethanol dehydrogenases (encoded by *ADH1-5*) or by the *SFA1*-encoded formaldehyde dehydrogenase (Dickinson *et al*. 2003).

Pathways for the formation of the remaining alcohols, 2-phenylethyl alcohol and tyrosol, from their respective amino acids – phenylalanine and tryptophan – have been recently elucidated. It was demonstrated that phenylalanine and tryptophan are first deaminated to 3-phenylpyruvate and 3-indolepyruvate, respectively, and are then decarboxylated (Dickinson *et al*. 2003). This decarboxylation can be affected by any one of the enzymes encoded by *PDC1*, *PDC5*, *PDC6* or *ARO10* (Dickinson *et al*. 2003).

Recently, the effect of increased *BAT1*- and *BAT2*-encoded yeast branched-chain amino acid transaminase activity on the production of higher alcohols and on the flavor of wine and distillates has been investigated (Lilly *et al*. 2006b). The *BAT1* and *BAT2* genes were overexpressed in a widely used commercial wine yeast (VIN13) under the control of the constitutive phosphoglycerate

kinase I gene (*PGK1*) promoter and terminator sequences (Lilly *et al*. 2006b). The results indicated that the overexpression of *BAT1* increased the concentration of isoamyl alcohol, isoamyl acetate and, to a lesser extent, isobutanol and isobutyric acid. The overexpression of *BAT2* resulted in a substantial increase in the level of isobutanol, isobutyric acid and propionic acid production. Sensory analyses indicated that the wines and distillates produced with the strains in which the *BAT1* and *BAT2* genes were overexpressed had more fruity characteristics ('peach' and 'apricot' aromas) than the wines produced with the wild-type strains (Lilly *et al*. 2006b).

To determine whether the production of higher alcohols during wort fermentation is related to the assimilation of the corresponding amino acid, the *BAP2* gene was overexpressed under the glyceraldehyde 3-phosphate dehydrogenase promotor (*TDH3*) in a brewer's yeast (Kodama *et al*. 2001). Constitutive expression of the *BAP2* gene resulted in accelerated assimilation rates for leucine, valine and isoleucine. This caused increased production of isoamyl alcohol derived from leucine, while no increases of isobutyl alcohol derived from valine or of active amyl alcohol derived from isoleucine were observed. These results suggested that there were distinct, but interrelated, mechanisms for the production of each higher alcohol. Taken together, the results obtained with the overexpression of *BAT1*, *BAT2* and *BAP2* present interesting prospects for the development of wine or of brewing-yeast strains with optimized higher alcohol-producing capability that could assist winemakers and brewers in their endeavors to produce wine and beer with specific flavor properties.

Esters

Volatile esters are only trace compounds in fermented beverages such as beer, wine and saké, but they are extremely important for the flavor profile of these drinks (Engan 1972, 1974; Nykanen and Suomalainen 1983; Lambrechts and Pretorius 2000; Debourg 2000; Verstrepen *et al*. 2003a,b,c; Swiegers and Pretorius 2005). The most important flavor-active esters in wine are ethyl acetate ('solvent'-like aroma), isoamyl acetate ('fruity' and 'banana' aromas), ethyl caproate and ethyl caprylate ('sour apple' aroma) and phenyl ethyl acetate ('flowery', 'roses' and 'honey' aromas) (Table 1.3; Fig. 1.3).

In lager beers, only the concentration of isoamyl acetate reaches the threshold levels of detection (Table 1.4) (Dufour and Malcorps 1995). However, the presence of multiple esters can have a synergistic effect on the individual flavors, which means that they can affect beer flavor well below their individual threshold concentrations (Meilgaard 1975). Moreover, the fact that most esters are present in concentrations near their threshold values implies that minor changes in concentration might have dramatic effects on beer flavor (Hammond 1995).

Table 1.3 Threshold values for esters and their concentrations in wine (data from Swiegers *et al*. 2005a).

Compound	Chemical name	Concentration in wine (mg/L)	Threshold (mg/L)	Aroma descriptor
Ethyl acetate	Ethyl ethanoate	22.5–63.5	7.5[a]	Volatile acidity, nail polish, fruity
Isoamyl acetate	3-Methylbutyl acetate	0.1–3.4	0.03[a]	Banana, pear
2-Phenylethyl acetate	β-Phenylethyl acetate	0–18.5	0.25[a]	Flowery, rose, fruity
Isobutyl acetate	2-Methylpropyl acetate	0.01–1.6	1.6[b]	Banana, fruity
Hexyl acetate	Hexyl acetate	0–4.8	0.7[c]	Sweet, perfume
Ethyl butyrate	Ethyl butanoate	0.01–1.8	0.02[a]	Floral, fruity
Ethyl caproate	Ethyl hexanoate	0.03–3.4	0.05[a]	Green apple
Ethyl caprylate	Ethyl octanoate	0.05–3.8	0.02[a]	Sweet soap
Ethyl caprate	Ethyl decanoate	0–2.1	0.2[d]	Floral, soap

[a]10% Ethanol, [b]beer, [c]wine, [d]synthetic wine.

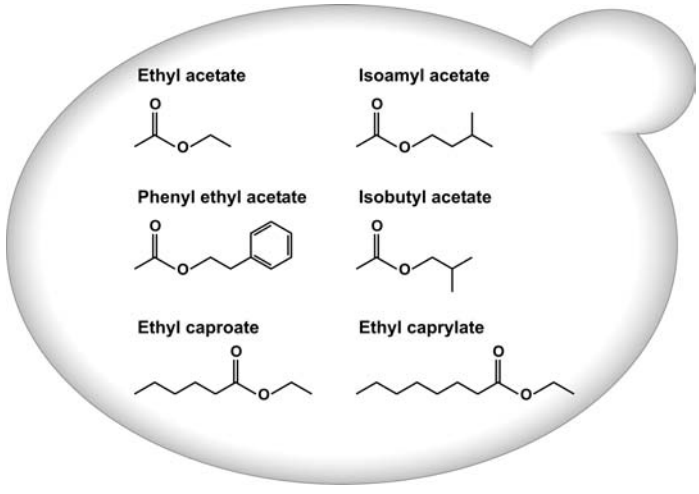

Fig. 1.3 Flavor-active esters produced by *Saccharomyces* yeast in wine, beer and saké.

In wine, fresh fruity aromas are derived mainly from mixtures of esters produced by the yeast during fermentation, but esters significant to specific grape cultivars have been identified. For example, Pinot Noir wines are known to exhibit distinct red fruity aromas that particularly evoke the odors of small stone fruit ('plum' and 'cherry'), 'strawberry', 'raspberry', 'black currant', 'blackberry', and often 'cherry stone' and 'cherry brandy' (Moio and Etievant 1995). Four volatile esters can influence the characteristic flavor quality exhibited by Pinot Noir wines. Ethyl anthranilate (ethyl 2-aminobenzoate) appears to be the most intense flavor compound among the four odorants in Pinot Noir wines. The second most intense flavor compound is ethyl cinnamate (ethyl 3-phenyl-2-propenoate), followed by ethyl 2,3-dihydrocinnamate (ethyl

Table 1.4 Threshold values, concentration range and average concentration of aroma-active esters in lager beer (data from Meilgaard 1975).

Component	Concentration range (mg/L)	Average (mg/L)	Threshold (mg/L)	Aroma description
Ethyl acetate	8–32	18.4	21–30	Fruity, solvent-like
Isoamyl acetate	0.3–3.8	1.72	0.6–1.2	Banana, pear
Ethyl caproate	0.05–0.3	0.14	0.17–0.21	Apple, aniseed
Ethyl caprylate	0.04–0.53	0.17	0.3–0.9	Apple
Phenyl ethyl acetate	0.10–0.73	0.54	3.8	Roses, honey

3-phenylpropanoate) and, finally, methyl anthranilate (methyl 2-aminobenzoate) (Moio and Etievant 1995).

The role of ester production in yeast metabolism is unclear, but several hypotheses have been proposed. One hypothesis is that esterification might be a detoxification mechanism for the medium-chain fatty acids (MCFAs) that are released during fatty acid synthesis under anaerobic conditions. It has been shown that fatty acids with chain lengths of C_8 to C_{14} are toxic to the yeast and exhibit strong antimicrobial activity, the effect of which is intensified if these fatty acids are unsaturated (Bardi *et al*. 1998). Upon release from the fatty acid synthesis complex, these toxic MCFAs rapidly dissociate and are thus unable to cross cellular membranes (Sumper 1974; Hundová and Fencl 1977; Taylor and Kirsop 1977; Wakil *et al*. 1983). Esterification allows partial diffusion of the fatty acid residues and could thus serve as a mechanism to remove these toxic intermediates. Another hypothesis is that ester formation could reduce the acetyl charge because it is essential for the yeast to maintain a balance between acetyl-CoA and CoA-SH (Thurston *et al*. 1982). In other words, yeast cells may produce esters in order to regenerate free CoA under conditions in which normal regeneration of acetyl-CoA through the TCA cycle or lipid synthesis is inadequate. Another possibility is that yeast cells may esterify free hydroxyl groups of certain membrane components to optimize membrane properties under stressful (e.g. anaerobic) conditions. This hypothesis agrees with the co-regulation of the *ATF1* alcohol acetyltransferase gene and the Δ9-desaturase-encoding gene, *OLE1*, by oxygen and unsaturated fatty acids (Fujii *et al*. 1997; Fujiwara *et al*. 1998, 1999). In addition, former studies have revealed that the enzymes encoded by *ATF1* and *EHT1* are located in the cellular lipid particles. This is also consistent with the possible role of ester production in fatty acid metabolism (Athenstaedt *et al*. 1999; Verstrepen *et al*. 2004).

Aroma-active esters are formed intracellularly by fermenting yeast cells. Being lipid-soluble, acetate esters rapidly diffuse through the cellular membrane into the fermenting medium. Unlike the situation with acetate esters, the proportion of the fatty acid ethyl esters transferred to the medium decreases with increasing chain length: 100% for ethyl caproate, 54–68% for

ethyl caprylate, and 8–17% for ethyl caprate. Longer-chain fatty acid ethyl esters all remain in the cell.

Volatile esters are the product of an enzyme-catalyzed condensation reaction between acyl-CoA and a higher alcohol (Nordström 1963, 1964). Several different enzymes are involved in the formation of esters in *S. cerevisiae*. The synthesis of acetate esters is catalyzed by the alcohol acetyltransferases I and II (AATase I and II; EC 2.3.1.84) which are encoded by the genes *ATF1* and *ATF2*. The *ATF1*- and *ATF2*-encoded enzymes catalyze the formation of acetate esters from two substrates: an alcohol and acetyl-CoA. It has been shown that during fermentation, acetate ester production rates are dependent on alcohol acetyltransferases activity (Malcorps *et al*. 1991). In addition, in beer, a clear correlation was found between the concentrations of ethyl acetate and isoamyl acetate, indicating that the same rate-limiting enzyme may synthesize both these esters (Alvarez *et al.* 1994). Purification of the enzymes that synthesize acetate esters has led to the identification of three distinct AATases: AATase I, its closely related homologue Lg-AATase I and AATase II. These AATases are encoded by *ATF1*, the *ATF1* homologue *Lg-ATF1* and *ATF2*, respectively (Fujii *et al.* 1994, 1996a,b; Malcorps and Dufour 1992; Nagasawa *et al*. 1998; Yoshimoto *et al*. 1998; Yoshioka *et al*. 1984). While *ATF1* and *ATF2* are present in both *S. cerevisiae* (ale) and *S. bayanus* (lager) strains, Lg-*ATF1* is found only in *S. bayanus* strains (Yoshimoto *et al*. 1998).

Although the yeast AATase was first considered to be a membrane-bound enzyme, the results of a hydrophobicity analysis indicated that the gene products of *ATF1* and *ATF2* did not have a membrane-spanning region. It has been reported that the *ATF1*-encoded enzyme is localized in lipid particles and that it is the target of the cAMP/PKA and FGM (Fermentable Growth Medium) nutrient signaling pathways (Verstrepen *et al*. 2003a,b).

Several studies have been carried out to determine the role of the *ATF1*- and *ATF2*-encoded alcohol acetyltransferases in acetate ester synthesis in *S. cerevisiae*. The roles of the known *S. cerevisiae* alcohol acetyltransferases in volatile ester production were investigated and compared by deleting or overexpressing *ATF1*, Lg-*ATF1* and *ATF2* in a laboratory yeast strain and a commercial brewing strain (Verstrepen *et al.* 2003b). The ester formation of the transformants was measured using headspace gas chromatography combined with mass spectrometry (GC-MS). Analysis of the fermentation products confirmed that the expression levels of *ATF1* and *ATF2* greatly affected the production of ethyl acetate and isoamyl acetate. GC-MS analysis revealed that the *ATF1*- and *ATF2*-encoded enzymes are also responsible for the formation of a broad range of less volatile esters, such as propyl acetate, isobutyl acetate, pentyl acetate, hexyl acetate, heptyl acetate, octyl acetate, and 2-phenylethyl acetate. With respect to the esters analyzed in this study (Verstrepen *et al*. 2003b), the *ATF2*-encoded enzyme appeared to play only a minor role compared to the *ATF1*-encoded enzyme. The *atf1Δ atf2Δ* double-deletion strain did not form any isoamyl acetate, showing that together the *ATF1*- and

ATF2-encoded alcohol acetyltransferases are responsible for the total cellular isoamyl alcohol acetyltransferase activity. However, the double-deletion strain still produced considerable amounts of certain other esters, such as ethyl acetate (50% of the wild-type strain), propyl acetate (50%) and isobutyl acetate (40%), which is evidence for the existence of additional, as yet unknown, ester synthases in the yeast proteome. Interestingly, overexpression of different alleles of *ATF1* and *ATF2* led to different ester-production rates, indicating that differences in the aroma profiles of yeast strains may be partially due to mutations in their *ATF* genes.

Also in wine yeasts, the *ATF1*- and *ATF2*-encoded alcohol acetyltransferases play an important role in acetate ester synthesis. It has been shown that, when *ATF1* and *ATF2* were overexpressed in the VIN13 wine yeast, the levels of ethyl acetate, isoamyl acetate, 2-phenylethyl acetate and ethyl caproate were increased in wine made with this GM yeast. The chemical changes had a pronounced effect on the 'solvent/chemical' and 'fruity/flowery' characteristics of the wines (Lilly *et al*. 2000, 2006a). The 'estery/synthetic' fruit flavor was overpowering in the wines fermented with the yeast in which *ATF1* was overexpressed, but was much more subtle in the strain that overexpressed *ATF2*.

In addition to these alcohol acetyltransferases, another enzyme, the *EHT1*-encoded ethanol hexanoyl transferase 1, has been described as being responsible for the production of ethyl hexanoate (Mason and Dufour 2000). However, in a recent study it has been demonstrated that a homologue of the *EHT1*-encoded ethanol hexanoyl transferase, namely an enzyme encoded by *EEB1*, is responsible for the majority of ethyl ester production in *S. cerevisiae* (Saerens *et al*. 2006). The levels of ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate produced during fermentation with an *eeb1Δ* deletion strain were reduced by 36%, 88%, 45% and 40%, respectively, in comparison with those produced by the wild type strain. Compared to the *eeb1Δ* strain, deletion of *EHT1* did not affect the production of ethyl butanoate and ethyl decanoate and resulted in only minor decreases in ethyl hexanoate formation (36%) and ethyl octanoate formation (20%). The double-deletion strain, *eht1Δ eeb1Δ*, produced similar levels of ethyl butanoate, ethyl hexanoate, and ethyl decanoate to the *eeb1Δ* single-deletion strain and a lower level of ethyl octanoate (82% reduction in comparison to the wild-type strain), indicating that *EHT1*-encoded ethanol hexanoyl transferase plays only a minor role in MCFA ethyl ester synthesis, while the *EEB1*-encoded enzyme is the most important enzyme for MCFA ethyl ester synthesis.

Overexpression of *EHT1* and *EEB1* did not enhance ethyl ester synthesis in a laboratory strain (Saerens *et al*. 2006). This could be due to the fact that both the *EHT1*- and *EEB1*-encoded enzymes, when purified as glutathione *S*-transferase-tagged proteins, exhibited both acyl-coenzymeA and ethanol *O*-acyltransferase activity *in vitro*, as well as esterase activity. However, in another investigation into the effect of the overexpression of an *EHT1* wine yeast allele, a marked increase in the concentrations of ethyl caproate, ethyl

caprylate and ethyl caprate was observed (Lilly *et al*. 2000, 2006a). An intense apple aroma was detected in the wines that were produced by the yeast in which *EHT1* was overexpressed. This result again suggested that differences in the aroma profiles of yeast strains might be partially due to mutations in the genes responsible for ester synthesis.

Furthermore, it has been shown that the balance between ester-synthesizing enzymes and esterases, which hydrolyze esters, might be important for the net rate of ester accumulation (Fukuda *et al.* 1998). Esterases represent a diverse group of hydrolases that catalyze the cleavage of esters and, in some cases, the formation of ester bonds. In ester breakdown, esterases catalyze the following reaction (from Peddie 1990):

$$RCOOR + H_2O \rightarrow ROH + RCOOH$$

Recently, the effect of the *IAH1*-encoded ester-degrading enzyme on the flavor profile of wine has been investigated (Lilly *et al*. 2006b). Overexpression of *IAH1* resulted in a significant decrease in ethyl acetate, isoamyl acetate, hexyl acetate and 2-phenylethyl acetate. The wines produced with the *IAH1* overexpression strain showed a significant decrease in ester concentrations when compared to the control wines. The concentration of isoamyl acetate decreased 11.4–15.6-fold and hexyl acetate was completely hydrolyzed. Ethyl acetate and 2-phenylethyl acetate concentrations decreased by 1.6–1.8- and 3.4–3.9-fold, respectively. All these investigations of enzymes involved in the synthesis and breakdown of flavor-active esters help to identify and develop non-GM and GM yeasts that could produce the desired amounts of esters, thereby assisting production of wines and beers with specific flavor profiles for specific market specifications.

The ratio of acetyl-CoA to CoA could play an important role in the production of acetate esters (Cordente *et al.* 2007). For this reason, the effect of overexpressing the mitochondrial carnitine acetyltransferase gene, *CAT2*, on the aroma profiles of fermentations has been investigated. The carnitine acetyltransferases catalyze the reversible reaction between carnitine and acetyl-CoA to form acetylcarnitine and CoA. In this investigation, overexpession of the wild-type *CAT2*-encoded mitochondrial carnitine acetyltransferase and a modified version, which was localized in the cytosol, resulted in a decrease in the concentration of acetate esters produced during fermentation. It is hypothesized that the overproduction of Cat2p favors the formation of acetylcarnitine and CoA and therefore limits the amount of precursor available for ester production (Cordente *et al.* 2007).

As in the case of wine and beer, esters are important flavor compounds in saké. Recently a saké yeast strain that overexpresses the *ATF1* gene was developed to improve the flavor profile of Japanese saké (Hirosawa *et al*. 2004). The *ATF1* gene was placed under the control of the constitutive yeast glyceraldehyde-3-phosphate dehydrogenase gene (*TDH3*) promoter and integrated into the genome of the saké yeast.

In another study, a non-GM, high ethyl caproate-producing mutant of a diploid saké yeast was developed (Inokoshi *et al*. 1994). Strains were mutagenized and mutants were selected for cerulenin resistance. Cerulenin is an inhibitor of fatty acid synthesis (Ichikawa *et al*. 1991). Analysis of the cerulenin-resistant saké mutant indicated a point mutation in the *FAS2* fatty acid synthase gene which resulted in higher production of ethyl caproate (Inokoshi *et al*. 1994).

Carbonyl compounds

Acetaldehyde

The most important aldehyde produced by yeast is acetaldehyde (Table 1.5). At low levels, it gives a pleasant, fruity aroma, but at high concentrations it possesses a pungent irritating odor (Miyake and Shibamoto 1993). Excess acetaldehyde produces a 'green', 'grassy' or 'apple-like' off-flavor in beer (Margalith 1981; Adams and Moss 2000), cider (Williams 1974) and wine (Henschke and Jiranek 1993). In beer, acetaldehyde is present at close to its flavor threshold of 15 mg/L (Engan 1981). It is one of the most important sensory carbonyl compounds formed during vinification and constitutes more than 90% of the total aldehyde content in wine (Nykanen 1986). Various levels of acetaldehyde are found in wine, with average values of approximately 80 mg/L for white wine, 30 mg/L for red wine and 300 mg/L for sherries (McCloskey and Mahaney 1981). While high levels of acetaldehyde are generally undesirable in table wines, high concentrations of this volatile compound are considered a unique feature of sherry-type wines (Sponholz 1993; Cortes *et al*. 1998).

Acetaldehyde is formed at the fastest rate during the primary phase of wort fermentation. The concentration of acetaldehyde in beer usually declines towards the end of fermentation and during maturation as a result of normal yeast activity. High concentrations normally reflect the absence of sufficient quantities of active yeast at this stage due either to premature flocculation or to a decline in yeast viability. In wine also, the accumulation of acetaldehyde

Table 1.5 Acetaldehyde concentrations in alcoholic beverages (data from Liu and Pilone 2000).

Beverage	Concentration (mg/L)
Red wine	4–212
White wine	11–493
Sweet wine	188–248
Sherry	90–500
Beer	5–12
Saké	15–60

occurs when the rate of carbon assimilation is at its maximum, and its levels drop towards the end of fermentation. Owing to the oxidation of ethanol, the activity of film yeast and aeration, the amount of acetaldehyde in wine can increase over time (Fleet and Heard 1993).

Because reduction of acetaldehyde to ethanol is dependent on zinc, a shortage of zinc ions in the wort can lead to excess acetaldehyde production. Other factors that affect acetaldehyde concentration are: the presence of oxygen late in the fermentation; the nature of insoluble material used to clarify the must; increasing fermentation temperature; and the use of high concentrations of SO_2 in grape must, which enhances its formation (Delfini and Costa 1993; Romano *et al*. 1994; Liu and Pilone 2000). The SO_2-induced production of acetaldehyde appears to be related to SO_2 resistance in yeast (Casalone *et al*. 1992). Acetaldehyde concentration can also vary considerably (from 0.5 to 286 mg/L) depending on the yeast strain (Liu and Pilone 2000).

The presence of acetaldehyde in white wines is an indication of wine oxidation. The process of converting ethanol to acetaldehyde in the presence of oxygen is also referred to as 'maderization', and this produces a slightly 'almond' flavor that resembles the fortified sweet wine, Madeira. It is usually facilitated by prolonged storage in barrels at high temperatures, and the resulting wine lacks freshness and has a musty taste known as *rancio* (Robinson 2006). Acetaldehyde in red wines can contribute to aroma complexity provided the concentration does not exceed 100 mg/L.

Acetaldehyde is one of the major metabolic intermediates in yeast fermentation because it is the last precursor formed before ethanol is produced. The end product of glycolysis, pyruvate, is converted to acetaldehyde by PDC enzymes encoded by three genes: *PDC1*, *PDC2* and *PDC3*. Acetaldehyde is then converted to ethanol by alcohol dehydrogenase enzymes, primarily the enzyme encoded by the *ADH1* gene (Pronk *et al*. 1996). This step is crucial for maintaining the cell's redox balance because it re-oxidizes NADH to NAD^+, which is required for glycolysis. While sugar is the primary substrate for acetaldehyde formation, metabolism of amino acids such as alanine also contributes to the formation of this compound (Henschke and Jiranek 1993; Boulton *et al*. 1998).

Acetaldehyde is extremely reactive and can react with various wine phenolic compounds to generate stable pigments in wine (Bakker and Timberlake 1997; Benabdeljalil *et al*. 2000; Hayasaka and Asenstorfer 2002; Eglinton *et al*. 2004). Acetaldehyde mediates anthocyanin–tannin and tannin–tannin condensation reactions. Model solutions containing malvidin-3-glucoside and (+)-catechin in the presence of acetaldehyde generally give rise to two major products corresponding to the dimeric structure of anthocyanin-ethyl-flavanol (Roggero *et al*. 1987; Bakker *et al*. 1993; Rivas-Gonzalo *et al*. 1995). Recently, it has been demonstrated that the acetaldehyde-mediated condensation reaction can also induce the polymerization of anthocyanins in the absence of flavanols, giving rise to dimers, trimers and tetramers composed of ethyl-linked anthocyanin units in different structural forms (cationic, hemiacetal

and quinoidal) (Atanasova *et al*. 2002). In another study, researchers investigated the mouth-feel attributes and bitterness perception of these compounds and concluded that these ethyl-bridged flavonols contributed particularly to the perception of bitterness and not to the astringency of a model wine (Vidal *et al.* 2003).

Diacetyl

Diacetyl is another important carbonyl compound in beer and wine, producing a 'butter' or 'butterscotch' aroma. At low concentrations in wine, it can be described as 'nutty' or 'toasty' but becomes objectionable at concentrations of between 1 and 4 mg/L (Sponholz 1993). Although yeasts biosynthesize some diacetyl (0.2–0.3 mg/L) in wine, most of it originates from the metabolic activities of lactic acid bacteria (Laurent *et al.* 1991; Bartowsky and Henschke 2004). However, diacetyl is a normal product of brewery fermentations, but is generally considered to be an undesirable contributor to the flavor of beer (Wainwright 1973). It is a characteristic flavor of some ale beers, but is undesirable in lagers and stouts. The rate of diacetyl formation in beer is affected by pH, temperature, oxygen and the presence of metal ions (Haukeli and Lie 1978). In particular, increasing temperature, decreasing pH (in the range 5.5–4.0) and the presence of copper and ferric ions all enhance the rate at which diacetyl is produced.

The final concentration of diacetyl in beer depends on three factors, namely synthesis and excretion of α-acetolactate, the immediate precursor of diacetyl, conversion of this precursor into diacetyl, and removal of diacetyl by yeast. The α-acetolactate is an intermediate in the pathway leading from pyruvate to the amino acids valine and leucine (Fig. 1.2). The α-acetolactate leaks from the cell and undergoes spontaneous oxidative decarboxylation to diacetyl. The yeast cell, however, is capable of reducing diacetyl, with the intermediate product being acetoin. The acetoin may be further reduced to 2,3-butanediol. Acetoin has a much higher flavor threshold (50 ppm), exhibits 'fruity', 'mouldy' and 'woody' flavors (Meilgaard 1975) and does not cause any off-flavors in the beer.

Because of the importance of diacetyl for beer flavor, a considerable amount of research has been concentrated on the pathway leading to the formation of α-acetolactate. The *ILV*-encoded enzyme forms α-acetolactate from pyruvate. This enzyme is subject to general amino acid control and very strong feedback inhibition by valine. There is potential for controlling the concentration of diacetyl by manipulation of this gene. This is of even greater importance in a brewing context because this pathway not only produces diacetyl, but is also the source of some higher alcohols. Diacetyl production is completely eliminated in mutants lacking *ILV2* but, because of their inability to synthesize valine and leucine, such yeasts ferment only poorly (Ryder and Masschelein 1983). By changing the upstream regulatory sequence of *ILV2*, it should be possible to reduce the level of this enzyme rather than to eliminate it completely (Petersen *et al.* 1983).

An alternative approach to reducing the concentration of diacetyl is to increase the flux through the pathways to amino acid synthesis. This has been achieved by transforming yeasts with multicopy plasmids containing copies of the *ILV3* and *ILV5* genes which code for the rate-limiting enzymes of the pathway. Yeasts transformed with multiple copies of the *ILV5* gene showed a five- to ten-fold increase in acetohydroxy acid reductoisomerase activity and a 60% decrease in diacetyl formation (Villaneuba *et al.* 1990; Goossens *et al.* 1991), whereas those transformed with the *ILV3* gene had no effect on diacetyl concentration despite a six-fold increase in dihydroxy acid dehydratase activity (Goossens *et al.* 1987). Since α-acetolactate is an intermediate in the biosynthetic pathway leading to the formation of valine and leucine, formation of this compound is linked to yeast growth and concentrations of nitrogenous compounds in wort. Because the amount of yeast growth during fermentation is inversely related to the amount of amino nitrogen in the wort, it is also possible to control diacetyl concentrations in beer by careful adjustment of the amino nitrogen concentrations in the wort.

Another strategy to overcome high diacetyl concentrations in beer involves the use of the enzyme α-acetolactate decarboxylase (Godtfredsen *et al*. 1982). This enzyme converts α-acetolactate directly into acetoin, and the chemical transformation of α-acetolactate into diacetyl occurs simultaneously. The use of α-acetolactate decarboxylase makes it possible to shorten primary fermentation in brewing until no further maturation is needed in regard to diacetyl (Linko *et al*. 1991; Aschengreen *et al*. 1992; Jepsen *et al*. 1993; Kabaktschieva 1994).

Once diacetyl is formed, it can be taken up and metabolized by yeast. Various enzymes are likely to be involved in the reduction of diacetyl by brewing yeast (Bamforth and Kanauchi 2004). Alcohol dehydrogenase is probably the main enzyme responsible for diacetyl reduction in both lager and ale strains. It has been reported that as much as 90–95% of the diacetyl reductase activity in the lager yeast may be accounted for by alcohol dehydrogenase (Bamforth and Kanauchi 2004). For the ale yeast, however, enzymes other than alcohol dehydrogenase appear to make a proportionately greater contribution to reductase activity, with perhaps no more than 60% of the diacetyl reductase activity being attributed to alcohol dehydrogenase.

Volatile phenols

Volatile phenols have a relatively low detection threshold and are, therefore, easily detected. In wine, they are formed by yeast from the hydroxycinnamic acid precursors in the grape must. Although volatile phenols can contribute positively to the aroma of some wines, they are better known for their contribution to off-flavors such as ‘Band-aid’, ‘barnyard’ or ‘stable’, which result from high concentrations of ethyl phenols (Dubois 1983). Ethyl phenols are the reduction products of vinyl phenols and originate from vinyl reductase

Table 1.6 Threshold values for volatile phenols and their concentrations in wine.

Compound	Concentration in wine (mg/L)	Aroma threshold (mg/L)	Aroma
4-Ethylphenol	0.012–6.5	0.14[a]/0.6[b]	Medicinal, barnyard
4-Ethyl guaiacol	0.001–0.44	0.033[a]/0.11[b]	Phenolic, sweet
4-Vinyl phenol	0.04–0.45	0.02[c]	Pharmaceutical
4-Vinyl guaiacol	0.0014–0.71	10[c]	Clove-like, phenolic

[a]10% ethanol, [b]red wine, [c]water.

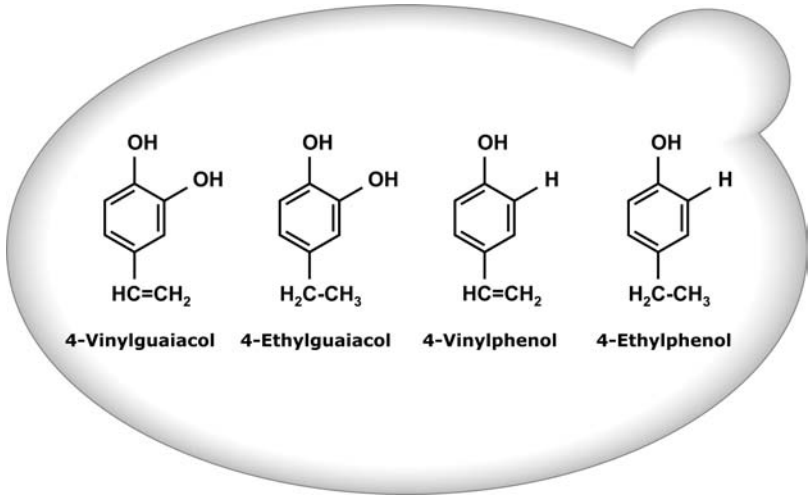

Fig. 1.4 Volatile phenols in wine produced by yeast (adapted from Swiegers and Pretorius 2005).

activity typically associated with *Brettanomyces* and *Dekkera* spp. High ethyl phenol concentrations are also a common problem in the brewing industry (Chatonnet *et al*. 1992; Licker *et al*. 1998). The prominent ethylphenols found in wine and beers are 4-ethyl guaiacol and 4-ethyl phenol. Vinyl phenols, especially 4-vinyl guaiacol and 4-vinyl phenol, produce a 'pharmaceutical' odor, particularly in white wines (Table 1.6; Fig. 1.4) (Ribéreau-Gayon *et al*. 2000).

In beer, most of the simple phenolic compounds originate from the raw materials used in the brewing process. Only some of them can be formed by yeast activity, namely 4-vinyl guaiacol and 4-vinyl phenol. The presence of these volatile vinyl phenols is considered undesirable when they are present in excessive concentrations in bottom-fermented pilsners. Hence the term 'phenolic off-flavor' (POF) is attributed to beers with a strong aroma described as 'pharmaceutical', 'medicinal', 'solvent', 'spicy', 'clove-like', 'smokey' or 'barbeque'. Despite being historically catalogued as an off-flavor, these compounds are known to be essential flavor contributors to the characteristic aroma of Belgian white beers (made with unmalted wheat), German rauch

beers and Weizen beers (made with malted wheat). Also, in many top-fermented blond and dark specialty beers, the phenolic flavor is essential for their overall flavor perception.

Volatile phenols are predominantly produced by yeast during fermentation, although trace amounts can be found in grape must (Baumes *et al*. 1988). The nonflavonoid hydroxycinnamic acids, such as *p*-coumaric acid and ferulic acid, are decarboxylated in a non-oxidative process by *S. cerevisiae* to form the volatile phenols 4-vinyl guaiacol and 4-vinyl phenol, respectively (Chatonnet *et al*. 1993). The *Brettanomyces* and *Dekkera* spp. yeasts are well known for their ability to form volatile phenols in wine and beer (Chatonnet *et al*. 1995; Du Toit and Pretorius 2000). These yeasts are associated with the more unpleasant odorous ethylphenols and are therefore regarded as spoilage organisms producing aromas described as 'Band-aid', 'medicinal', 'pharmaceutical', 'barnyard-like', 'horsey', 'sweaty', 'leathery', 'mouse urine', 'wet dog', 'smoky', 'spicy', 'cheesy', 'rancid' and 'metallic' (Chatonnet *et al*. 1995).

Another biological pathway by which volatile phenols are produced is through decarboxylation of phenolic acids, usually first into 4-vinyl derivatives that are then reduced to 4-ethyl derivatives through enzymes called phenolic acid decarboxylases (Cavin *et al*. 1993). A number of microorganisms have been found to contain the genes encoding phenolic acid decarboxylases, and these genes include *PAD1* (also known as *POF1*) from *S. cerevisiae*, *fdc* from *Bacillus pumilus*, *pdc* from *Lactobacillus plantarum*, *padc* from *Bacillus subtilis* and *PadA* from *Pediococcus pentosaceus* (Clausen *et al*. 1994; Zago *et al*. 1995; Cavin *et al*. 1997, 1998; Barthelmebs *et al*. 2000b). It has been shown that the production of volatile phenols by *S. uvarum* brewing strains is dependent on the presence of a functional allele of the *PAD1*/*POF1* phenylacrylic acid decarboxylase gene (Meaden and Taylor 1991; Hwang 1992; Clausen *et al*. 1994; Shinohara *et al*. 2000). The phenolic acid decarboxylases are not inhibited by other grape phenolics and they result in a high transformation of the vinyl phenol derivatives to the ethylphenol derivatives. *S. cerevisiae* does not use its phenolic acid decarboxylase as the sole defense against phenolic acid toxicity, which probably explains why phenolic acid decarboxylase activity is so low in most *S. cerevisiae* strains (Barthelmebs *et al*. 2000a,b).

Recently, wine yeasts with optimized phenolic acid decarboxylation activity were developed by overexpressing the *B. subtilis* phenolic acid decarboxylase gene (*padc*), the *L. plantarum p*-coumaric acid decarboxylase gene (*pdc*) and the *S. cerevisiae* phenylacrylic acid decarboxylase gene (*PAD1*/*POF1*) in a laboratory strain of *S. cerevisiae* (Smit *et al*. 2003). The overexpression of *padc* and *pdc* in *S. cerevisiae* showed high enzyme activity. However, this was not the case for the *PAD1*/*POF1*-encoded enzyme activity. Subsequently, the *padc* and *pdc* genes were also overexpressed in commercial yeasts, and these yeast strains were compared to both the original host strain and a mutant strain in which both alleles of *PAD1*/*POF1* were disrupted. Strains overexpressing *padc* and *pdc* gave almost a two-fold increase in volatile phenol

formation in a laboratory strain of *S. cerevisiae*. On the other hand, in wine made with commercial wine yeasts in which the *PAD1*/*POF1* gene was disrupted, no volatile phenols could be detected (Smit *et al*. 2003).

Sulfur compounds

Sulfides

Hydrogen sulfide (H_2S) is probably the best-known sulfur compound in wine and causes some of the most common problems associated with wineries (Henschke and Jiranek 1991; Rauhut 1993). It is a highly volatile sulfur compound that imparts a 'rotten egg' aroma and has a very low odour threshold of 50–80 μg/L (Table 1.7).

H_2S can be formed metabolically by wine yeast from either inorganic sulfur compounds (sulfate and sulfite) or from organic sulfur compounds (cysteine and glutathionine) (Henschke and Jiranek 1993; Rauhut 1993; Hallinan *et al*. 1999; Spiropoulos *et al*. 2000). In general, grape must is deficient in organic sulfur compounds and this can trigger synthesis of these sulfur compounds by yeast from inorganic sources, usually present in sufficient quantities in grape must (Henschke and Jiranek 1993; Park *et al*. 2000; Moreira *et al*. 2002). In *S. cerevisiae*, H_2S is the product of the *sulfate reduction sequence* (SRS) pathway (Fig. 1.5) (Yamagata 1989; Rauhut 1993). In the SRS pathway, H_2S is derived from the HS^- ion, a metabolic intermediate in the reduction of sulfate or sulfite, which is needed for the synthesis of organic sulfur compounds. If, during fermentation, these reactions proceed in the presence of a suitable nitrogen supply, the HS^- ion is sequestered by *O*-acetylserine and *O*-acetylhomoserine, which are derived from nitrogen metabolism, to form organic sulfur compounds such as methionine and cysteine (Henschke and Jiranek 1993; Park *et al*. 2000; Moreira *et al*. 2002). However, when nitrogen sources are insufficient or unsuitable, free H_2S can accumulate in the cell and diffuse into the fermenting must (Vos and Gray 1979; Henschke and Jiranek 1991; Giudici and Kunkee 1994; Jiranek *et al*. 1995, 1996).

It appears that the ability of a strain to produce H_2S is at least partly genetic because its production by different wine strains varies under the same conditions (Thornton and Bunker 1989; Henschke and Jiranek 1993; Jiranek

Table 1.7 Threshold values for hydrogen sulfide and mercaptans, and their concentrations in wine.

Compound	Aroma descriptor	Aroma threshold (μg/L)	Concentration in wine (μg/L)
Hydrogen sulfide	Rotten egg	10–80	Trace to >80
Ethanethiol	Onion, rubber, natural gas	1.1	1.9–18.7
Dimethyl sulfide	Asparagus, corn, molasses	25	1.4–61.9

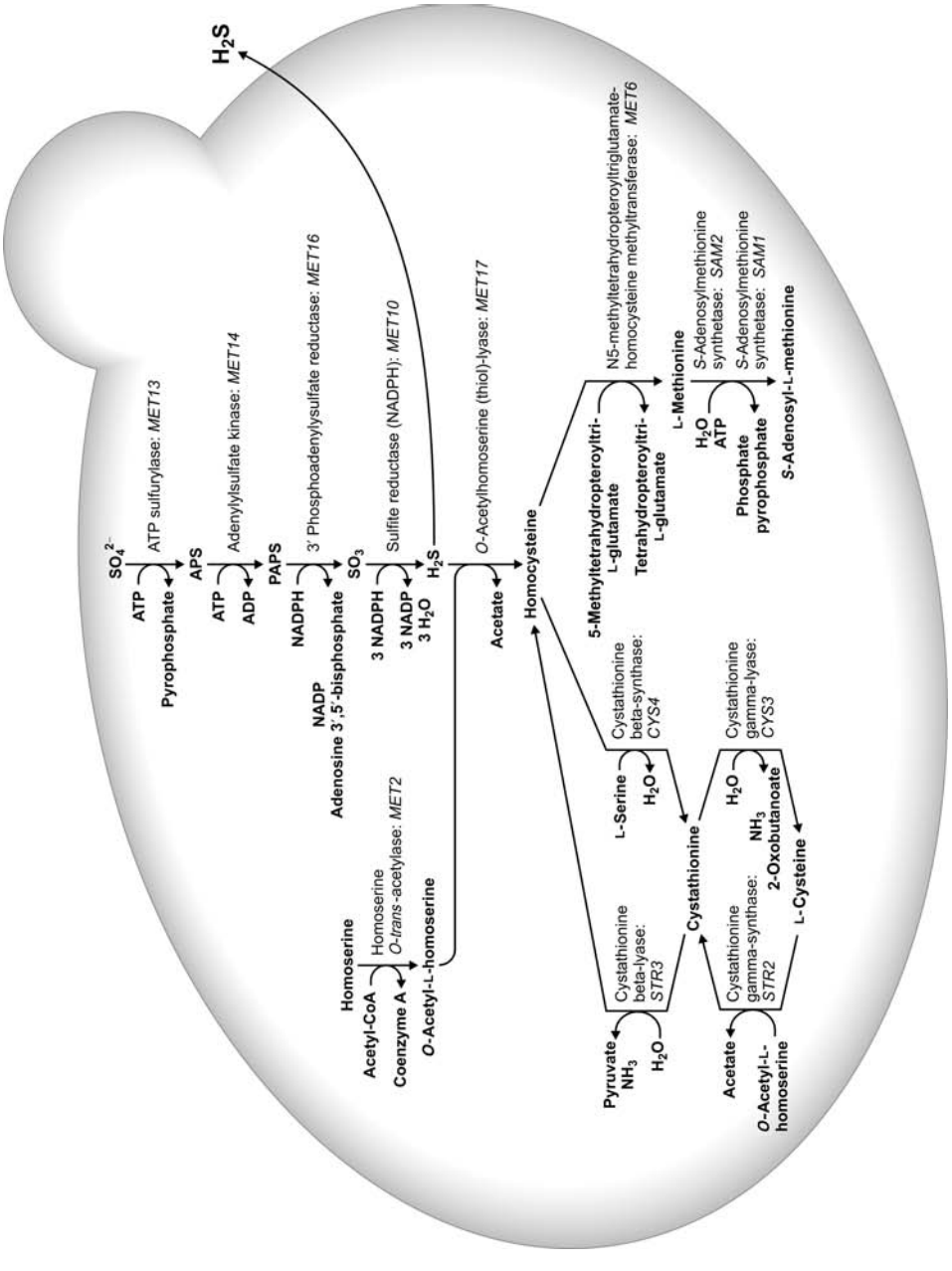

Fig. 1.5 Sulfur amino acid metabolism in *S. cerevisiae* and its role in the production of hydrogen sulfide.

et al. 1995; Spiropoulos *et al*. 2000). The first step of the SRS metabolic pathway involves the transport of sulfate from the medium into the yeast cell by sulfate permease. Sulfate is then reduced to sulfide through a series of steps using the enzymes ATP-sulfurylase (using two ATP molecules) and sulfite reductase. The next step leads to the sequestering of the sulfide: *O*-acetylserine (from the amino acid serine) combines with sulfide to form cysteine, and *O*-acetylhomoserine (from the amino acid aspartate) combines with sulfide to form homocysteine, which can then be converted to methionine (Thornton and Bunker 1989; Yamagata 1989; Henschke and Jiranek 1993; Rauhut 1993; Jiranek *et al*. 1995; Spiropoulos *et al*. 2000).

Because the concentrations of cysteine and methionine in grape juices are usually not sufficient to meet the metabolic needs of growing cells, the SRS metabolic pathway is activated to meet this demand (Henschke and Jiranek 1993). When enough nitrogen is present in the medium, sufficient precursors for these amino acids (*O*-acetylserine and *O*-acetylhomoserine) will be available to sequester the sulfide. If nitrogen is limited, insufficient precursors will be present; the SRS pathway will be activated and sulfide will accumulate. This surplus sulfide is then liberated from the cell as hydrogen sulfide (Henschke and Jiranek 1993; Rauhut 1993; Jiranek *et al*. 1995; Spiropoulos *et al*. 2000). Significant amounts of H_2S are sometimes produced when sulfite, which diffuses into the cell, is present in the fermentation medium. Therefore, in conditions of nitrogen depletion, high and continuous production of H_2S is observed in the presence of sulfite (Jiranek *et al*. 1995; Hallinan *et al*. 1999).

Several attempts have been made to modulate H_2S production by industrial wine yeast (Spiropoulos and Bisson 2000; Donalies and Stahl 2002; Sutherland *et al*. 2003). The overexpression of the *MET17* gene, which encodes *O*-acetylserine and *O*-acetylhomoserine sulfhydrylase, in a strain of *S. cerevisiae,* resulted in greatly reduced H_2S formation in a wine ferment. However, this was not the case with another *S. cerevisiae* strain in which *MET17* was overexpressed (Spiropoulos and Bisson 2000). The strategy of overexpressing *MET17* was also investigated in brewing yeast, and it resulted in a ten-fold decrease in H_2S production in beer produced on a pilot scale (Omura *et al*. 1995). Therefore, it appears that the success of this strategy is dependent on the strain.

In another attempt, the overexpression of two genes, *MET14* (encoding an adenosylphosphosulfate kinase) and *SSU1* (encoding a sulfite transporter), has been shown to increase the formation of sulfite (Donalies and Stahl 2002). Therefore, it has been postulated that the deletion of the *MET14* gene or the *MRX1* gene, the latter encoding a methionine sulfoxide reductase, might be the most effective way to prevent wine yeast from producing H_2S in fermentations (Pretorius 2000, 2003, 2004; Pretorius and Høj 2005).

Another approach to preventing H_2S formation was carried out through modifying the activity of the sulfite reductase enzyme by engineering one of the enzyme subunits (Sutherland *et al*. 2003). Sulfite reductase is a heterotetramer, consisting of two α- and two β-subunits, which are encoded by the

MET10 and *MET5* genes, respectively (Sutherland *et al*. 2003). The enzyme, a hemoflavoprotein, binds three cofactors: flavin adenine dinucleotide, flavin mononucleotide and siroheme. Mutations were introduced into the *MET10* gene such that the α-subunit could no longer bind cofactors but could still form a heterotetramer protein complex with the β-subunit. In this way, overexpression of the mutant *met10* gene would produce a nonfunctional subunit, which could reduce the proportion of functional sulfite reductase in the cell, and hence reduce sulfide formation. However, more research needs to be undertaken to confirm whether this strategy would prevent the formation of H_2S in wine ferments.

In beer, low levels of H_2S can also be produced by *S. cerevisiae* through the reduction of sulfate during methionine synthesis. Even small amounts of H_2S can be detrimental to the flavor of beer, and it is therefore important for the brewer to try to inhibit the formation of this compound. One strategy to achieve this is to develop brewing yeasts with reduced H_2S formation. Increased expression of *CYS4*, encoding the cystathionine β-synthase in brewing yeast, has been shown to suppress the formation of H_2S in beer produced on a laboratory scale, without affecting other fermentation characteristics (Tezuka *et al*. 1992). Another approach that has been investigated is to partially or fully eliminate *MET10*, which encodes a putative sulfite reductase subunit that transforms sulfite (SO_3^{2-}) into H_2S. The results showed a substantial reduction in H_2S and, not surprisingly, in the accumulation of sulfite. No H_2S was produced, and the beer that was made showed increased flavor stability (Hansen and Kielland-Brandt 1996).

Mercaptans

H_2S is a reactive compound and, when it reacts with ethanol or acetaldehyde, it forms ethanethiol, which displays an aroma like 'onion' (Rauhut 1993). How dimethyl sulfide (DMS) is formed in wine is not clear. This compound displays 'asparagus', 'corn' and 'molasses' characters (Table 1.7). DMS is found in wine well above its sensory threshold of 25 μg/L (white wine) and 60 μg/L (red wine). DMS might be formed by yeast via cleavage of *S*-methyl-L-methionine to homoserine and DMS. In beer production, heat decomposition during malting of *S*-methylmethionine produces dimethyl sulfoxide (DMSO), which can be reduced to DMS, presumably during storage (Rauhut 1993). DMS formation during fermentation has also been linked to cysteine, cystine or glutathione metabolism in yeast (Rauhut 1993; Ribéreau-Gayon *et al*. 2000).

In some lager beers, DMS is produced during fermentation by reduction of DMSO. In *S. cerevisiae*, the *MXR1* gene has been shown to encode a methionine sulfoxide reductase, which could possibly lead to the production of DMS (Hansen 1999). To investigate this possibility, a *mxr1* disruption mutant was constructed, and this yeast was unable to reduce DMSO under laboratory conditions. This investigation paves the way for producing brewing and wine strains that do not produce DMS.

Table 1.8 Threshold values for volatile thiols and their concentrations in wine (data from Swiegers *et al*. 2005).

Compound	Aroma descriptor	Threshold (μg/L)	Concentration in wine (μg/L)
4MMP	Cat urine, box tree/ blackcurrant, broom	3 ng/L	0–30 ng/L
3MH	Passion-fruit, grapefruit	60 ng/L	50–5000 ng/L
3MHA	Riesling-type note, passion-fruit, box tree	4 ng/L	1–100 ng/L

Thiols

The volatile thiols 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) are important aroma compounds in wine (Table 1.8). These volatile thiols are extremely potent, having low perception thresholds: 0.8 ng/L (4MMP), 60 ng/L (3MH) and 4 ng/L (3MHA). In Sauvignon Blanc wines, these compounds are of particular importance to varietal character because they impart 'box tree' (4MMP), 'passionfruit', 'grapefruit', 'gooseberry' and 'guava' aromas (3MH and 3MHA) (Tominaga *et al*. 1995, 1998a,b; Dubourdieu *et al*. 2006). However, 4MMP, 3MH and 3MHA have also been identified in varying concentrations in wines made from Colombard, Riesling, Semillon, Merlot and Cabernet Sauvignon grapes and could, therefore, have an impact on the aroma and quality of these wines (Tominaga *et al*. 1995, 1998a,b; Murat *et al*. 2001b).

Little is known about the occurrence of volatile thiols in beer. However, researchers used a specific extraction method with *p*-hydroxymercuribenzoic acid to reveal the presence of more than ten polyfunctional thiols in fresh lager beers (Vermeulen *et al*. 2006). All of these were absent from wort, suggesting a key role for the H_2S produced by the brewing yeast during fermentation. Three thiols, 3-methyl-2-buten-1-thiol, 2-mercapto-3-methylbutanol and 3-mercapto-3-methylbutanol, appear to be created from hop allylic alcohols with the first of these thiols being the most powerful thiol in beer (Vermeulen *et al*. 2006). It is possible that the thiols 2-mercaptoethanol and 3-mercaptopropanol, and their corresponding acetates, might be derived from Ehrlich degradation of sulfur amino acids, while 2-methyl-3-furanthiol could be produced through Maillard reactions.

It has been proposed that the cysteine desulfhydrase enzyme, which is required for the production of cysteine in *S. cerevisiae*, catalyzes the formation of furfurylthiol from furfural (Tominaga *et al*. 2000). The HS^- anion is also produced by this enzyme, resulting in the formation of hydrogen sulfide. The formation of H_2S enhances the formation of furfurylthiol from furfural. This has been confirmed by demonstrating that ferments with an added nitrogen source (thus inhibiting H_2S formation) do not produce as much furfurylthiol. Thus, production of furfurylthiol is linked to the production of the HS^- anion, which is not produced when ammonium sulfate is added to a ferment in sufficient quantities (Tominaga *et al*. 2000).

The volatile thiols 4MMP, 3MH and 3MHA are almost non-existent in grape juice and develop only during fermentation. However, it has been shown that 4MMP and 3MH do exist in grapes in the form of non-volatile, cysteine-bound conjugates and that wine yeast is responsible for the cleaving of the thiol from the precursor (Darriet *et al*. 1995). Experiments that showed a cell-free enzyme extract of *Eubacterium limosum* (which contains carbon–sulfur lyase enzymes) could release 4MMP from its precursor *S*-4-(4-methylpentan-2-one)-L-cysteine (Cys-4MMP) indicated that a similar mechanism of release through yeast carbon–sulfur lyases possibly occuring during wine fermentation (Tominaga *et al*. 1995). This hypothesis has been tested by investigating the ability of yeast to release 4MMP from Cys-4MMP when genes encoding putative yeast carbon–sulfur lyases were deleted in a laboratory strain of *S. cerevisiae* (Howell *et al*. 2005). Four genes, identified as encoding putative carbon–sulfur lyase enzymes, influenced the release of the volatile thiol 4MMP, indicating that the mechanism of release probably involves multiple genes. The identified genes were also deleted in a homozygous derivative of the commercial wine yeast, VL3, and the results showed that deletion of the four putative carbon–sulfur lyase genes led to a decrease in the amount of 4MMP released (Howell *et al*. 2005). The latter study, however, did not show that overexpression of these genes resulted in an increase of 4MMP release.

Recently, a heterologous carbon–sulfur lyase gene was overexpressed in a wine yeast (Swiegers *et al*. 2007). The yeast strain overexpressing carbon–sulfur lyase released up to ten times more 4MMP and 3MH from their chemically synthesized precursors than the wild-type yeast strain. Sauvignon Blanc wine produced by the modified wine yeast had an overpowering passionfruit aroma, not detected in the control wine. Therefore, it is possible to engineer wine yeast to unlock grape-derived thiol precursors and thereby enhance the aroma of wine (Swiegers *et al*. 2007).

Previously, it has been shown that the amount of 4MMP released in wine ferments depends on which yeast strain is used to conduct the fermentation (Dubourdieu *et al*. 2006). Therefore, the genetic and physiological nature of the yeast strain determines its ability to release volatile thiols. It has been shown that two commercially available *S. cerevisiae* wine strains, VL3 and EG8, release more thiols than strains VL1 and 522d. Furthermore, *S. bayanus* strains release more 4MMP than do *S. cerevisiae* strains VL3 and EG8. Wines made with *S. bayanus*/*S. cerevisiae* hybrid strains have been shown to contain more of the volatile thiols (Murat *et al*. 2001a). These findings have been confirmed by showing that different commercial wine strains have varying abilities to release 4MMP from the Cys-4MMP precursor in model ferments (Howell *et al*. 2004). Commercial wine yeast strains that release even more thiols than VL3 have been identified (Howell *et al*. 2004). In a follow-up study, Sauvignon Blanc produced by seven different commercial wine yeast strains (including VL3) showed unique volatile thiol profiles, with the VIN7 strain producing the highest concentration of 4MMP and 3MHA and the VIN13 strain producing the highest concentration of 3MH (Swiegers *et al*. 2006a).

It has been shown that, during fermentation, 3MHA is formed from 3MH by the action of alcohol acetyltransferase, encoded by the *ATF1* gene (Swiegers *et al*. 2006b). Overexpression of the *ATF1* gene in the VIN13 yeast strain resulted in a significant increase in the amount of 3MHA produced. On the other hand, overexpression of the gene *IAH1*, which encodes an ester-degrading enzyme, resulted in a reduction in the concentration of 3MHA. The ability of different commercial wine yeasts to convert 3MH into 3MHA during fermentation has also been investigated. Large variations in 3MHA concentrations were observed and, in most cases, this did not correspond with the ability of the yeasts to release 4MMP (Swiegers *et al*. 2006b). Therefore, it is clear that yeast strain selection is of extreme importance in modulating volatile thiol concentrations in wine.

It has been shown that when the thiol precursor *S*-3-(hexan-1-ol)-L-cysteine (Cys-3MH) decreases in concentration, 3MH increases during fermentation. However, only a small fraction (1.6%) of the cysteine-bound precursor originally present is released as 3MH (Dubourdieu *et al*. 2006). Furthermore, in Cabernet Sauvignon and Merlot musts, it has been shown that the amount of 3MH released is proportional to the Cys-3MH concentration present at the start of fermentation. Therefore, the higher the concentration of the cysteine-conjugated thiol precursors in the must, the higher the volatile thiol concentration in the resulting wine (Murat *et al*. 2001b). However, in the latter study, only 3.2% of the precursor originally present in the must was released as volatile thiols during fermentation.

Recently, the impact of fermentation temperature on the production and retention of volatile thiols has been determined in a model medium and in grape juice. It was shown that the concentrations of 4MMP, 3MH and 3MHA were higher when the alcoholic fermentation was conducted at 20°C compared to 13°C, irrespective of the yeast strain used (Masneuf-Pomarède *et al*. 2006). In contrast, Swiegers *et al*. (2006a) have shown that in model ferments more 4MMP was released and more 3MH was converted to 3MHA at lower temperatures (18°C) compared to higher temperatures (23°C and 28°C) at the end of fermentation. However, more volatile thiols were present in the warmer ferments at the start of fermentation (Swiegers *et al*. 2006a)

Monoterpenoids

Monoterpenoids are a class of compounds produced by higher plants, algae, fungi and even some yeasts and are derived from a common precursor, isopentyl pyrophosphate. Monoterpenoids are compounds with strong sensory qualities, and different isomers of a given terpenoid can have varying aromas. For example, geraniol has a 'rose'-like, 'citrus' aroma, whereas the *cis*-isomer, nerol, has a fresh, 'green' odor (Table 1.9). In plants, monoterpenoids and sesquiterpenoids are produced via the 1-deoxy-D-xylulose-5-phosphate pathway. Two plant species that produce significant amounts of

Table 1.9 Threshold values for monoterpenes and their concentrations in wine.

Compound	Concentration in wine (μg/L)	Threshold (mg/L)	Aroma descriptor
Linalool	0.0017–.010	0.0015[c]/0.025[b]	Rose
Geraniol	0.001–0.044	5[c]/30[a]	Rose-like
Citronellol	0.015–0.042	8[c]/100*	Citronella

[a]10% ethanol, [b]synthetic wine, [c]water.

monoterpenoids are *V. vinifera* (grapes) and *Humulus lupulus* (hops) (King and Dickinson 2000). The aromatic grape varieties, such as Muscat, Riesling and Gewürztraminer, contain large amounts of the monoterpenes, geraniol and nerol (Simpson 1979). However, some fungal (*Penicillium*) and yeast species are also able to produce monoterpenoids (Larsen and Frisvad 1994). Yeast species that produce terpenoids include *Kluyveromyces lactis*, *Torulaspora delbrueckii* (formerly *Saccharomyces fermentati*) and *Ambrosiozyma monospora* (Drawert and Barton 1978; Fagan *et al*. 1981; Klingenberg and Sprecher 1985). Although *S. cerevisiae* is known to produce only trace amounts of monoterpenoids, a recent study investigating naturally isolated wine strains showed that several strains were capable of significant production of monoterpenoids (Carrau *et al*. 2005). Therefore, *Saccharomyces* yeasts could contribute to the floral aroma of wine by *de novo* synthesis of monoterpenes.

Apart from the free fraction of volatile terpenoids, naturally non-odorous and non-volatile precursors exist in grapes and can significantly contribute to the aroma of wine if released. The aglycon moiety of the glucoside precursor can be linked to β-D-glucose or to the disaccharides 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranose, 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose and 6-*O*-β-D-apiofuranosyl-β-D-glucopyranose (Günata *et al*. 1985; Voirin *et al*. 1990). Terpenols such as linalool, nerol, geraniol, α-terpineol, citronellol and, in some cases, linalool oxides and terpene diols and triols can act as aglycon precursors (Günata *et al*. 1985; Park and Noble 1993). Usually, bound glycosides are more abundant than the free terpenoids in grapes, and the ratios of bound to free terpenoids can also vary amongst different grape cultivars. Alexandria Muscat grapes, for example, have a ratio of 5:1, whereas some non-Muscat varieties have a ratio of 1:1 (Williams *et al*. 1984).

During the process of winemaking, these bound terpenoids can be released by the action of glycosidase enzymes, which are produced by the grapes, yeast and bacteria (Fig. 1.6). Enzymatic hydrolysis of bound terpenoids involves two steps:

(1) An α-L-rhamnosidase and an α-L-arabinofuranosidase or a β-D-apiofuranosidase (depending on the structure of the aglycon moiety) cleave the 1,6-glycosidic linkage.

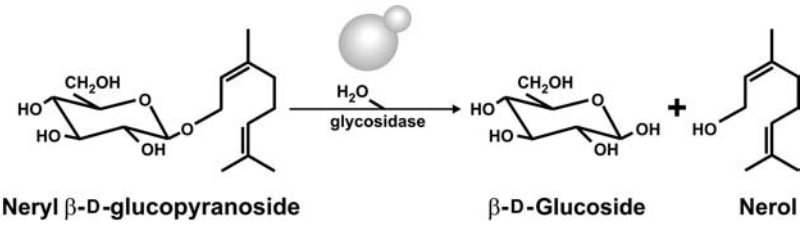

Fig. 1.6 The hydrolysis of neryl β-D-glucoside to nerol and glucose through β-glycosidase enzymes produced by yeast (adapted from Swiegers and Pretorius 2005).

(2) The monoterpenols are liberated from the monoterpenyl β-D-glucosides by the action of a β-glucosidase (Günata *et al.* 1988, 1990).

The origin of the enzyme and the structure of the aglycon determine the efficiency of the hydrolysis of monoterpenyl β-D-glucosides by β-glucosidases. Endogenous grape β-glucosidases, resulting from the maturation of grapes, cleave off monoterpenyl β-D-glucosides. Grape β-glucosidases, however, exhibit almost no activity towards grape terpenyl-glycosides in must and wine, probably because they are inhibited by glucose and exhibit poor stability in low pH and high ethanol concentrations in wine (Bayonove *et al.* 1984; Aryan *et al.* 1987). Although some strains of *S. cerevisiae* possess β-glucosidase activity, their activity towards glycoside precursors appears to be very low (Günata *et al.* 1986; Delcroix *et al.* 1994; Hernández *et al.* 2003). However, non-*Saccharomyces* yeasts such as *Brettanomyces*/*Dekkera*, *Candida*, *Debaryomyces*, *Hanseniaspora* and *Pichia* have been shown to have strong β-glucosidase activity (Vasserot *et al.* 1989; Rosi *et al.* 1994; McMahon 1999; Fernández *et al.* 2000; Garcia *et al.* 2002).

In order to enhance wine flavor, the addition of functional exogenous β-glucosidases to the fermentation is the most effective way to improve the hydrolysis of the glycoconjugated aroma compounds (Aryan *et al.* 1987; Shoseyov *et al.* 1990; Vasserot *et al.* 1993). These glycosidases need to have: (1) a high affinity for grape-derived terpenoid aglycons; (2) optimal activity at wine pH (pH 2.5–3.8); (3) resistance to glucose inhibition and (4) high tolerance to ethanol (Riou *et al.* 1998).

Efforts have been made to express heterologous glucosidase enzymes in wine yeast. A yeast expressing the β-1,4-glucanase gene from *Trichoderma longibratum* has been constructed, and wine made with this yeast was shown to be more intense in aroma than the wild-type (Villanueva *et al.* 2000). In other attempts to develop flavor-enhancing yeast, the *BGL1* and *BGL2* β-glucosidase genes of *Saccharomycopsis fibuligera*, the *ABF2* α-L-arabinofuranosidase gene of *Aspergillus niger* and a glucanase-encoding gene cassette consisting of several glucanase genes (*BEG1*, *END1* and *EXG1*) were expressed in wine yeast (Pretorius 2000, 2003, 2004; van Rensburg and Pretorius 2000; Pretorius and Bauer 2002; de Barros Lopes *et al.* 2006). In general, wines

produced by the modified yeast displayed more aroma characteristics than wines made with the control strains.

Conclusion

Yeast is no longer considered a 'one-size-fits-all' input into production processes or a 'workhorse' responsible for little more than alcohol production; rather, yeast is now considered a critical and profound driver of the composition of fermented alcoholic beverages.

The versatile yeast *S. cerevisiae* and related species are powerful 'flavor factories'. Significant progress has been made in elucidating the flavor biochemical pathways and the genes involved in these pathways. This information has been used to genetically improve yeast starter strains with enhanced flavor-producing capabilities, and has proved that significant improvements can be made to the flavor of fermented beverages. However, there still remain many unanswered questions, and continued research in this field will further our understanding and ultimately lead to the development of yeast strains that can predictably produce alcoholic beverages with specific metabolic profiles. Such predictability would allow producers to 'tailor-make' their fermented beverages to suit certain consumer preferences and/or add value to otherwise low-value products. Armed with the ability to 'shape' the flavors of their fermented beverages, producers could secure the highest value for their products in multiple markets, each characterized by inherent style preferences. Accordingly, the choice of yeast is a critical factor in the search for value-adding within a rapidly globalizing marketplace.

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Chapter 2
Biotechnology of flavor production in dairy products

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Introduction

The methods involved in the production of fermented dairy products follow generally similar steps (Fig. 2.1). The steps that involve biotechnology are mostly limited to the addition of a recombinant coagulant or of a modified bacterium as a starter culture. In both cases, the primary motivation is to reduce production costs and to improve flavor production. Flavor production in dairy products is the result of microbial metabolism which, in turn, is determined by the entire set of genes related to the culture's metabolic capability. The interaction between processing conditions and the microbial cell has a profound influence on the exact set of metabolites produced during production of fermented dairy products. The abiotic conditions that have an impact on the culture's metabolism include temperature, osmotic status, nutrient availability, and the interaction between these parameters to induce stress responses that shape the pool of metabolic products important in flavor.

Use of genetic modification in lactic acid bacteria (LAB) has a long and successful history in the production of fermented dairy products. Single-gene effects for milk fermentation are important for the primary traits – sugar use (acid production), bacteriophage resistance, and protein degradation – that lead to a successful fermentation. Genome sequences and functional genomic tools are now being used to reveal additional interactions and to identify specific genes that are important for flavor production. It is becoming increasingly clear that complex, multi-gene/protein interactions are needed to optimize

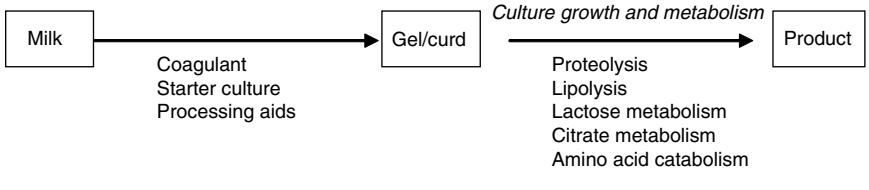

Fig. 2.1 Representation of fermented dairy products production. In some cases, coagulants are not added. Processing aids may include sodium chloride, calcium chloride, or citrate, among others.

Biotechnology in Flavor Production: A Case-based Approach. Edited by Daphna Havkin-Frenkel, Faith C. Belanger.
 ISBN: 978-1-4051-5649-3

the fermentation of milk into a consistently high-quality product. For example, lactose utilization and proteolysis require multiple genes organized into an operon to control functions ranging from lactate transport to generation of the final peptide products.

LAB genome sequencing is revolutionizing dairy-fermentation biotechnology, because it provides a platform to improve the system-wide understanding of metabolism and dairy fermentation, thereby optimizing the fermentation process and achieving a new, previously lacking, level of control. The historical focus on the genetics of sugar metabolism, proteolysis, and resistance to bacteriophage infection via single genes or a handful of genes can now be expanded to include the entire set of interactions, including the regulation of gene expression, stress response, and flavor production. With the availability of many genome sequences for the bacteria associated with dairy fermentation, the biotechnological questions open to investigation are substantially larger today, as compared to only 5 years ago. Metabolic shifts between substrates, amino acid metabolism, and nucleic acid metabolism are now possible based on the genome sequences.

Functional genomics is generating tools to explore the entire set of gene, protein, and metabolite changes that occur during the fermentation process, in order to provide a new picture of the response of the culture to processing. This new ability allows researchers to frame questions that are more specific and to provide additional answers to hitherto undefined mechanisms responsible for flavor-compound production during dairy fermentations. Integration of genetics, biochemistry, computer science, and statistics to intertwine genome, proteome, and metabolome information so that it is useful to the dairy industry remains a challenge. This chapter focuses on the functional genomics of LAB as a system for the optimization of flavor production, with specific phenotypic examples relevant to important flavors in fermented dairy products.

Biochemistry of dairy fermentations

Bacteria added to milk for the production of fermented dairy products produce flavor compounds via the metabolism of sugar, milk fat, and protein (Fig. 2.1). The most challenging dairy product to understand regarding flavor formation is cheese because of the extensive number of flavorful end-products, the variety of environmental conditions used in production, and the numerous types of starter cultures that are added to create an enormous variety of cheeses. The aging process provides time for the metabolism of substrates into new products that are the specific flavor constituents of each variety. Production of flavor in fresh cheeses and short-shelf-life products is largely associated with added enzymes to form the curd and to ferment lactose. Therefore, this chapter will focus on the fermentation process from the perspective of the bacterial metabolism of cheese-making, since addition of the starter culture is the most likely step for biotechnological application.

Table 2.1 Flavor compounds formed during cheese ripening (adapted from Urbach 1993).

Product	Associated flavor compounds	Impact compounds
Yogurt	Lactic acid	Acetylaldehyde
Cheddar	Lactic acid, acetic acid, amino acids, sulfur compounds, ammonia	Sulfur compounds and fatty acids in low quantities
Gouda cheese	Amino acids, fatty acids	
Swiss-type cheeses	Lactic acid, propionic acid, acetic acid, amino acids (proline), sulfur compounds, alkyl pyrazines	Propionic acid
Italian cheeses	Volatile fatty acids, amino acids, alcohol, ketones	Fatty acids
Blue-veined cheeses	Volatile fatty acids, ketones, amino acids, lactones, aromatic hydrocarbons, methyl ketones, secondary alcohols	Ketones
Tilsit cheese	Methanethiol, methyl thio-acetate, methyl thio-propionate, hydrogen sulfide	Sulfur compounds and acids

The unique flavor of fermented dairy products is completely dependent on the set of compounds produced through bacterial metabolism (Table 2.1) (Reiter *et al*. 1967). The generation of flavor is a complex web of metabolic processes between milk enzymes, added enzymes, the native milk microflora, and the starter culture, and this is often categorized into four metabolic stages:

(1) sugar (lactose) metabolism to lactic acid
(2) lipolysis of milk fat to liberate straight-chain fatty acids
(3) proteolysis of casein to produce peptides and amino acids
(4) protein and amino acid metabolism.

Lactose is metabolized to lactic acid during the first step of milk fermentations. In cheese, the lactose is utilized almost completely within 1 week of cheese manufacture (Fox *et al*. 1993). By 30 days, lactose is completely exhausted from the cheese matrix, and is not available for the generation of glycolytic products by bacteria. In some cheeses, lactate is utilized by mold and yeast to produce a number of compounds, ultimately generating ammonia, which is the primary compound that changes the texture of products that are aged for a number of months or years. In the absence of lactose, LAB may utilize lactate to produce acetate, ethanol, and carbon dioxide (Fox *et al*. 2000). However, it is becoming increasingly clear that these intermediates are used as intersecting metabolites in the metabolism of other substrates (i.e. protein, fat, and nucleic acids) (Fig. 2.2). As the sugar is depleted, LAB metabolize caseins to peptides and amino acids, which generate energy (via adenosine triphosphate [ATP]) and the metabolic precursors of volatile sulfur compounds (VSCs), aldehydes, ketones, and fatty acids (Urbach 1993) to produce energy and new flavor compounds not found during growth in the laboratory (Ganesan *et al*. 2006, 2007).

While lipolysis is the mechanism involved in generating the lipolytic flavor of Italian cheeses and flavor defects in milk and other dairy products (Law 1984; Seitz 1990), VSCs, alcohols, aldehydes, and fatty acids involved in flavor arise due to amino acid metabolism in Italian and other cheeses. The lipolytic

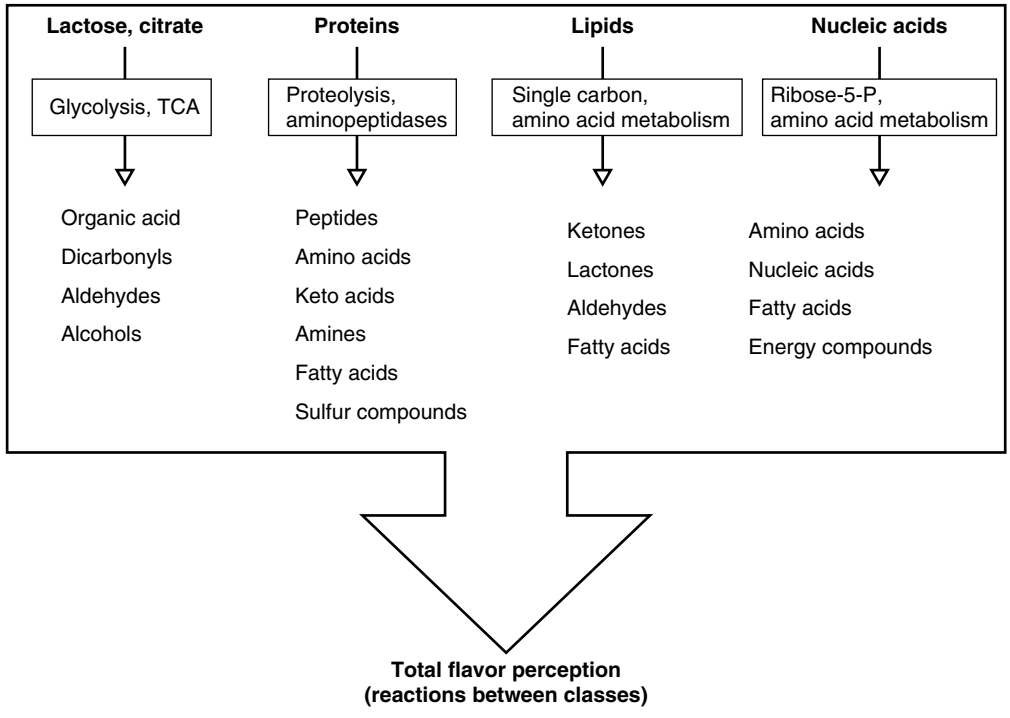

Fig. 2.2 General scheme for flavor production in fermented dairy products.

process is highlighted by the limited lipolytic capability of the starter culture and non-starter bacteria in fermented dairy products (Medina *et al*. 2004). Fatty acids play a role in cheese flavor, and are produced by LAB via glycolysis, lipolysis, and proteolysis via amino acid metabolism to branched chain fatty acids (Ganesan *et al*. 2006).

Protein metabolism and amino acid metabolism dominate the later phase of ripening (Christensen *et al*. 1999; Tammam *et al*. 2000). The catabolic mechanisms that are active during flavor generation depend on the bacterial genera and their physiological state and the specific metabolic processes that are in place (Ganesan *et al*. 2004a,b; Ganesan and Weimer 2004; Stuart *et al*. 1999). Sugar starvation and low pH activate the metabolic mechanisms in lactococci that prevail during cheese ripening for amino acid metabolism. VSCs, together with other classes of compounds, commonly play a major role in overall flavor perception from a number of different organisms related to cheese production (Ferchichi *et al*. 1985; Hemme *et al*. 1982; Weimer *et al*. 1999).

Nucleotide metabolism in LAB is largely restricted to understanding salvage pathways for survival during growth (Kilstrup *et al*. 2005). The connection with flavor production is only beginning to be explored as common intermediates are used for amino acid and vitamin metabolism. However, bioinformatic evaluations of these pathways infer that nucleotide metabolism may also play a role via ribose-5-phosphate metabolism, thus making a link to

sugars as well. The exact nature of the interconnections between these substrates remains to be elucidated with respect to the exact impact on flavor production and biotechnological applications for dairy fermentations. Nucleotides intersent with common intermediates for sugar and amino acid metabolism. For example, uridine-diphosphate is linked to sugar metabolism (sucrose and galactose) and amino acids (via tetrahydrofolate), cytidine-triphosphate is linked to sugar metabolism (sucrose and galactose) and amino acids (His), and uridine-triphosphate is linked to sugar metabolism (sucrose, galactose, lactate) and amino acids (His) (http://www.biocyc.org/META/server.html).

A long history of compound discovery has evolved over the past 50 years (Urbach 1993). The existing challenge is to define the genes and metabolic routes that produce the vast array of compounds found in fermented dairy products. A few of the metabolic routes for producing flavor compounds are defined, but the precise control and interconversion mechanisms are not elucidated. The use of genome-sequence data, gene expression arrays, and metabolomics holds great promise to define the exact mechanisms behind the biotechnological applications for dairy fermentations.

Biotechnology and flavor

Single-gene manipulation is highly developed in LAB. This was promoted by the extensive plasmid biology undertaken in lactococci with the realization that the important milk-fermentation traits are plasmid-borne (Mills *et al.* 2006). Based on this extensive body of work, numerous expression vectors, recombination tools, and genes were developed for use with LAB; this is especially true for lactococci. With the availability of the genome sequence of lactococci, it is now possible to understand the genome structure and to control, relative to growth, survival, stress response, and flavor production with specific integration sites.

A scientist's ability to collect, process, and understand the rapidly accumulating genomic data is a significant challenge. This is especially true for dairy fermentations because the organisms behave differently in the laboratory compared to the food-processing setting. The opportunity to use genomics for dairy fermentations is ever growing. At the time of writing, the number of public microbial genomes was more than 925, with the total number of genomes (including eukaryotic) exceeding 2000. The challenge has shifted from obtaining additional genome sequences to the more difficult task of using and understanding the impact of specific genes on specific cellular processes. However, for dairy scientists, this requires the combination of metabolomics, gene expression, and bioinformatics with the target of improving the production of flavors. These studies are important in providing answers to the flavor industry as to how the microbes produce these compounds in the vat. Undoubtedly, detailed understanding of the regulatory mechanisms associated with gene expression of flavor-producing pathways will ultimately promote consistent

production of specific flavors from the varying milk substrate. To this end, a number of LAB genomes were sequenced and released into public databases (see NCBI; http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

Dairy fermentations use a very large number of strains in the vat to avoid bacteriophage infection and to stabilize acid production. The increasing sequencing capacity led the Lactic Acid Bacteria Genome Consortium (Weimer and Mills 2002) to sequence 11 genomes of bacteria involved in food fermentation and probiotic applications (Markarova *et al*. 2006) with the goal of defining the strain differences to optimize food fermentations. The first LAB genome reported was that of *Lactococcus lactis* subsp. *lactis* IL1403 (Bolotin *et al*. 2001), with the most recent being *L. lactis* subsp. *cremoris* MG1363 (Wegmann *et al*. 2007). A number of LAB genomes related to dairy fermentations are available (reviewed by Weimer 2007).

Comparative analyses of bacterial genomes hold great promise to provide new practical information because their genomes are very dynamic (Hughes 2000). Gene duplication, translocation, inversion, deletion, and horizontal transfer are often found to facilitate genome rearrangement, which is known for lactococci (Campo *et al*. 2002; Davidson *et al*. 1995). Presumably, such rearrangements mediate rapid strain evolution, allowing adaptation of the strain to selective pressures (Guédon *et al*. 2000; Hughes 2000). Comparing sequenced genomes is an excellent approach to understanding genome plasticity and how it has an impact on the metabolism of organisms used for flavor production. A key biotechnological advance allowed with multiple genome sequences is specific comparisons of important industrial traits (Table 2.2). For example, streptococcal bacteriophage elements are broadly distributed between LAB, and even *Brevibacterium linens* and *Saccharomyces cerevisiae* contain these elements. However, among LAB, transport of sulfate via a symport process is confined to lactococci. Additional examples of the differences that can have an impact on the expected growth and flavor production abilities of LAB for dairy fermentations are found in Table 2.2.

Extensive comparisons between specific genes at the sequence level are required on a large scale for each metabolic pathway of interest to define the exact structural differences that will lead to genetic regulatory differences between strains. A number of comparisons for LAB have found genome reduction to be the common theme. Bolotin *et al*. (2004) compared the genome sequences of two *Streptococcus thermophilus* strains and found extensive genome reduction during its evolution (reviewed by Hols *et al*. 2005). Similar conclusions were made for *Lactobacillus bulgaricus* (van de Guchte *et al*. 2006). Markarova *et al*. (2006) reported the largest genome comparison to date for any set of organisms. They found that all LAB evolved via genome reduction from a common *Bacillus* ancestor. Despite these comparisons, the presence of unknown genes leaves functional holes that can be filled by examining the specific domains present in those genes to estimate their cellular function (Ganesan *et al*. 2006). This approach will provide additional information about each gene in the genome and will complete the task of annotation,

Table 2.2 Genomic comparison of lactic acid bacteria for functional characteristics important to cheese flavor development.

Metabolic role	Genome features	*Lactococcus lactis* subsp. *cremoris* SK11	*Lactococcus lactis* subsp. *lactis* IL1403	*Streptococcus thermophilus* LMD-9	*Streptococcus thermophilus* CNRZ1066 (IG-40)	*Pediococcus pentosaceus* ATCC25745	*Leuconostoc mesenteroides* ATCC8293	*Lactobacillus plantarum* WCFS1	*Lactobacillus casei* ATCC334	*Brevibacterium linens* ATCC9174	*Saccharomyces cerevisiae*
Phage		131	149	53	48	111	67	176	114	58	30
	Lactococcal	10	8	2	2	7	4	10	6	0	0
	Listerial	88	88	24	21	72	28	118	62	27	17
	Streptococcal	99	117	27	24	82	39	141	78	24	0
	Enterobacteria-phage	11	8	12	10	12	14	15	17	19	19
	Phage regulatory proteins	12	13	2	2	7	4	11	8	2	0
	Prophage proteins	0	7	0	0	0	0	0	0	0	0
	Phage structural proteins	7	15	1	1	9	4	10	4	0	0
Membrane transport		239	241	233	230	198	243	372	371	391	86
	Complex exo-polysaccharide transport	2	0	0	1	0	1	0	2	0	0
	Aromatic compound transport	0	0	0	0	0	0	0	0	2	0
	Unknown substrate transporters	51	47	50	50	43	59	80	108	120	22
	Drug export	31	30	28	23	18	21	49	37	29	4
	Coenzyme membrane transport	4	6	8	4	7	12	8	8	29	3
	Purine membrane transport	2	2	3	4	5	4	5	2	4	10
	Pyrimidine membrane transport	1	1	1	1	2	2	2	1	5	5
	Ion membrane transport	49	57	44	37	29	38	60	52	86	19

	Carbohydrate membrane transport	39	39	21	29	51	37	91	74	39	9
	Osmoprotectant uptake	6	6	5	8	7	5	7	10	18	1
	Membrane transport of nitrogen compounds	7	7	3	4	3	2	7	3	3	8
	Amino acid membrane transport	38	38	70	63	32	55	60	64	80	7
	Gln/Asn uptake	2	3	11	14	7	16	7	14	4	0
	Amino acid uptake, unknown specificity, proton symport	5	5	3	3	10	8	12	13	8	1
	Amino acid efflux	4	4	2	2	4	5	12	10	6	2
	Ser/Thr uptake	0	0	7	6	0	5	6	6	10	0
	Phe/Tyr/Trp His uptake	0	0	3	3	4	4	5	2	0	2
	Cys/Met uptake	1	1	3	5	0	7	5	2	0	2
	Ala/Gly/ Pro uptake	0	0	6	6	0	5	5	5	20	1
	Glu/Asp uptake	2	2	0	0	2	0	2	0	10	0
	Polyamine transport	3	5	7	4	1	4	4	4	3	0
	Lys/Arg/Orn uptake	8	8	9	11	9	12	10	8	5	0
	Leu/Ile/Val uptake	1	3	6	6	0	6	8	7	10	0
	Peptide uptake	18	13	30	22	7	9	12	19	32	0
Sulfur compound membrane transport		2	2	0	0	0	3	0	3	4	4
	Aryl-sulfonates uptake ABC transporter	0	0	0	0	0	0	0	0	0	0

(Continued)

Table 2.2 Continued

Metabolic role	Genome features	*Lactococcus lactis* subsp. *cremoris* SK11	*Lactococcus lactis* subsp. *lactis* IL1403	*Streptococcus thermophilus* LMD-9	*Streptococcus thermophilus* CNRZ1066 (IG-40)	*Pediococcus pentosaceus* ATCC25745	*Leuconostoc mesenteroides* ATCC8293	*Lactobacillus plantarum* WCFS1	*Lactobacillus casei* ATCC334	*Brevibacterium linens* ATCC9174	*Saccharomyces cerevisiae*
	Sulfate uptake proton symport	2	2	0	0	0	0	0	0	4	4
	Alkane-sulfonates uptake ABC transporter	0	0	0	0	0	3	0	3	0	0
	Taurine uptake ABC transporter	0	0	0	0	0	0	0	0	0	0
	Thiosulfate uptake sulfate ABC transporter	0	0	0	0	0	0	0	0	0	0
	Membrane transport of phosphorus-containing compounds	12	11	6	7	5	6	13	14	10	0
Sulfur metabolism		8	11	13	12	11	9	15	14	19	19
	Cytosolic reactions of sulfur metabolism	2	2	4	3	3	4	6	4	11	17
	Extracellular and periplasmic reactions of sulfur metabolism	0	0	0	0	0	0	0	0	1	0
	Fe–S cluster assembly	6	9	9	9	8	5	9	10	7	2

Nitrogen metabolism		16	19	14	14	10	11	18	18	19	10
	Cytosolic reactions of nitrogen metabolism	16	19	14	14	10	11	18	18	19	10
	Urea degradation	0	0	3	3	0	0	0	0	0	2
	Nitrogen fixation	11	13	9	9	7	8	12	12	9	2
	Ammonia assimilation	3	3	2	2	1	3	2	6	9	6
	Bicarbonate, cyanate-carbamoyl phosphate anabolism (cytosol)	2	3	0	0	2	0	0	0	0	0
	Cyanate (periplasm), H^+ (periplasm) – CO_2, NH_3 catabolism (plasma membrane, cytosol)	0	0	0	0	0	0	0	0	0	0
	Assimilatory ferredoxin nitrite reductase	0	0	0	0	0	0	0	0	1	0
	Coenzyme and cofactor metabolism	70	69	51	52	44	67	77	54	125	124
Aromatic compound degradation		6	7	7	8	5	9	14	6	61	11
	Aromatic compound degradation via catechol	0	0	0	0	2	1	3	0	18	2

(Continued)

Table 2.2 Continued

Metabolic role	Genome features	*Lactococcus lactis* subsp. *cremoris* SK11	*Lactococcus lactis* subsp. *lactis* IL1403	*Streptococcus thermophilus* LMD-9	*Streptococcus thermophilus* CNRZ1066 (IG-40)	*Pediococcus pentosaceus* ATCC25745	*Leuconostoc mesenteroides* ATCC8293	*Lactobacillus plantarum* WCFS1	*Lactobacillus casei* ATCC334	*Brevibacterium linens* ATCC9174	*Saccharomyces cerevisiae*
	3-Hydroxy-phenylacetate and 4-hydroxy-phenylacetate degradation	0	0	0	0	1	3	2	1	9	1
	3-Hydroxy-benzoate and 4-hydroxy-benzoate degradation	0	0	0	0	1	0	2	0	3	0
	Aromatic compound degradation via proto-catechuate	6	6	2	2	2	4	6	4	17	8
	3-Phenyl-propionate and hydroxy-phenyl-propionate degradation	0	1	4	5	0	1	2	1	1	0
	Phenyl-acetate degradation	0	0	1	1	0	0	0	0	25	2
Lipid metabolism		52	53	31	31	38	39	60	49	105	73
	Lipid biosynthesis	6	7	8	8	8	8	7	8	10	26
	Poly-hydroxy-alkanoate metabolism	2	2	1	1	0	0	0	1	18	3
	Fatty acid modification	1	2	1	1	1	1	2	1	1	4
	Bacterial metabolism of steroids	2	1	0	0	1	0	5	1	0	0

	Carotenoid biosynthesis	2	2	0	0	1	3	5	2	7	2
	Lipid degradation	3	5	1	1	3	5	5	7	6	11
	Isoprenoid biosynthesis	24	24	11	11	12	14	23	22	27	19
	Fatty acid degradation	1	1	1	1	0	0	0	0	28	9
	Fatty acid biosynthesis	16	14	12	12	15	12	18	12	27	9
Amino acid metabolism		151	150	143	144	75	148	158	125	292	209
	Peptide metabolism	2	2	2	2	2	5	3	1	5	6
	Polyamine metabolism	1	3	0	0	3	1	0	1	15	3
	Amino acid catabolism	46	45	40	47	28	38	47	51	151	93
	Degradation of taurine and ethanolamine	1	2	6	8	0	2	1	2	4	5
	Degradation of aromatic amino acids	4	5	5	6	2	9	7	5	26	20
	Degradation of Glu, Gln, Lys, Pro, and Arg	14	12	9	11	7	10	11	11	54	28
	Degradation of Cys, Ser, and Ala	14	13	14	14	10	9	14	14	13	13
	Degradation of branched-chain amino acids	6	6	10	12	4	6	7	14	39	20
	Degradation of Met and Thr	8	8	6	8	4	6	5	6	33	15
	Degradation of His, Asp, and Asn	9	8	5	9	5	7	10	6	27	18

(Continued)

Table 2.2 Continued

Metabolic role	Genome features	*Lactococcus lactis* subsp. *cremoris* SK11	*Lactococcus lactis* subsp. *lactis* IL1403	*Streptococcus thermophilus* LMD-9	*Streptococcus thermophilus* CNRZ1066 (IG-40)	*Pediococcus pentosaceus* ATCC25745	*Leuconostoc mesenteroides* ATCC8293	*Lactobacillus plantarum* WCFS1	*Lactobacillus casei* ATCC334	*Brevibacterium linens* ATCC9174	*Saccharomyces cerevisiae*
Nucleotide metabolism		60	64	60	62	64	67	69	64	74	78
	Purine biosynthesis	23	24	27	27	30	30	31	31	29	34
	Purine salvage pathways	11	11	13	11	10	16	14	11	15	20
	Purine degradation	2	2	2	2	2	4	3	1	12	7
	Pyrimidine biosynthesis	23	27	25	24	27	24	26	24	24	27
	Pyrimidine salvage pathways	17	19	16	19	14	15	16	18	19	15
	Pyrimidine degradation	0	0	0	0	1	0	0	0	1	2
	Sum of genes in these features	1534	1612	1262	1257	1218	1347	2060	1767	2478	1241
	Total genes	2155	1973	1808	1763	1567	1703	2554	2313	2919	1595
	Ratio involved in these traits	71.2	81.7	69.8	71.3	77.7	79.1	80.7	76.4	84.9	77.8

ABC, ATP-binding cassette.

Amino acid abbreviations: alanine, Ala, A; arginine, Arg, R; asparagine, Asn, N; aspartic acid, Asp, D; cysteine, Cys, C; glutamic acid, Glu, E; glutamine, Gln, Q; glycine, Gly, G; Histidine, His, H; isoleucie, Ile, I; leucine, Leu, L; lysine, Lys, K; methionine, Met, M; phenylalanine, Phe, F; proline, Pro, P; serine, Ser, S; theonine, Thr, T; tryptophan, Trp, W; tyrosine, Tyr, Y; valine, Val, V.

which is an on-going process for each genome. A direct method to compare genomes is to conduct a genomic hybridization using a gene expression array. This approach provides direct biological evaluation of gene content and diversity (reviewed by Weimer 2007).

The next logical step in comparative genomics is to conduct comparisons of metabolic capabilities that are predicted by the genome sequence using *in silico* tools. These comparisons are possible using a number of bioinformatic tools, such as KEGG and Pathway Tools, which are designed to predict the metabolic potential from the gene content (Kanehisa 2002; Karp *et al*. 2005). MetaCyc (Caspi *et al*. 2006) provides a generalized inventory of metabolic pathways which is dynamic, as opposed to KEGG which provides a static view of metabolism in a generalized fashion (Kaneshisa 2002). BioCyc is a web-based database of more than 160 organisms which allows complete customization of metabolic pathways for specific organisms (Karp *et al*. 2005). This provides users with very extensive predictive abilities to study the most important pathways for flavor production and their interconnections. Pathway Tools databases are available for *L. lactis* IL1403 (http://www.biocyc.org; Notebaart *et al*. 2006), *L. lactis* subsp. *cremoris* SK11 (http://www.biosystems.usu.edu), and *Lactobacillus plantarum* WCSF1 (http://www.lacplantcyc.nl). Additional databases are being constructed for each of the LAB in the public domain (http://www.biosystems.usu.edu). These databases are available to query for specific compounds of interest, and they provide a method to link function to genome structure with a genome viewer.

Additional tools for metabolite interconnections are being developed to predict the complex web of metabolism. This approach provides a new level of insight into the coupling between genetic, metabolic, and expression events that lead to phenotypic traits. Weimer's group created a tool as a plug-in to Pathway Tools, and this plug-in allows pathway interconnections to be displayed for any queries of genes, intermediates, or proteins. For example, a query with glutamic acid resulted in more than 150 interconnections in *L. lactis* subsp. *cremoris* SK11. Complexity of metabolism for glutamic acid is expected, but the extent of the pervasive connections for this amino acid in lactococcal metabolism was unexpected. This approach highlights the potential metabolic routes that will have an impact on the flavor of dairy fermentations for common substrates found in milk.

Predictions of metabolism can be verified with gene expression and metabolite analysis. However, the use of DNA arrays requires immense data-handling capacity for analysis and visualization. A single DNA array with an entire genome, even a small one, requires approximately 4500 individual spots of the DNA probes. A single experiment carried out with microarrays using two treatments (e.g. a single variable) and three replications results in 27,000 discrete data points for analysis. Statistical analysis is done using individual pair-wise comparisons, with a correction for the large number of multiple comparisons, to define the expression differences. Subsequently, these significant genes are associated with a biological function to determine the metabolic difference between the test conditions (Ganesan *et al*. 2006, 2007).

Flavor production from bacteria

Biotechnology of dairy fermentations for flavor production has been very focused on the proteolytic system of lactococci and, to some degree, lactobacilli. This focus is largely due to the role of lactococci in acid production during the production stages and the adventitious growth of lactobacilli during aging. Due to these roles, specific lactococci and lactobacilli are used as flavor-adjunct bacteria and are added to milk during the production stage. The critical nature of the starter culture is borne out by the lack of flavor development during ripening without added starter cultures (Law 1984). In the initial stages of fermentation (Fig. 2.1), LAB catabolize lactose to lactic acid. Lactose is reduced to undetectable levels by 30 days (Crow *et al*. 1993). Proteinases and aminopeptidases from the starter culture subsequently act on the residual peptides to continue peptide hydrolysis and amino acid production (Christensen *et al*. 1999; Law *et al*. 1992). It is estimated that approximately 25% of the proteins in cheese are hydrolyzed to peptides and amino acids in ripening (Chapman and Sharpe 1990) and, if this is not achieved, it contributes directly to bitterness (Broadbent *et al*. 1998). Amino acids are subsequently metabolized to important positive flavor compounds (Dias and Weimer 1999; Harper and Wang 1980).

The role of different genera in Cheddar cheese flavor production continues to be elusive, and the cumulative interpretations from numerous studies add to the controversy. Products that require long ripening times tend to develop non-starter lactobacilli that also contribute to flavor and that have been adopted for addition as starter cultures in hard cheeses (Fox *et al*. 1993). However, lactococci have a unique causative role in flavor development and, consequently, remain at the heart of the starter cultures for many fermented dairy products. In recent experiments, the metabolic state of the starter was investigated, and it was found that non-culturable cells develop new metabolic capabilities important for flavor production in response to lactose starvation (Ganesan *et al.* 2006, 2007).

Each fermented product has a specific group of flavor compounds that are responsible for its flavor (Table 2.1; Fig. 2.2). Compounds contribute specific flavor attributes based on their physico-chemical properties (Urbach 1993). Multiple lists of flavor compounds are available in the literature (Fox *et al*. 1993; Urbach 1993). For example, fatty acids and VSCs correlate with flavor development during ripening. VSCs are one of the major classes of flavor compounds that correlate with good Cheddar cheese flavor (Dias and Weimer 1999; Manning *et al*. 1976; Weimer *et al*. 1999), yet knowledge of the exact genes and proteins needed for this production is lacking. Fatty acids exist both alone and in combinations with VSCs as thioesters. While microbial mechanisms of thioester production exist, they are yet to be characterized for their contribution to cheese flavor (Cuer *et al*. 1979a,b). In cheese, the actual sources of these fatty acids – particularly branched-chain fatty acids – are branched-chain amino acids and their metabolic products.

Amino acids are essential for bacterial growth, especially LAB. Casein yields flavor compounds by the conversion of amino acids to volatile end-products

(Hemme *et al*. 1982). Consequently, amino acid transport is an important event for metabolism and is a key point of control for biotechnological focus. Amino acids are transported in lactococci via three different mechanisms. Alanine, serine, lysine, and the branched-chain amino acids leucine, isoleucine, and valine, are transported by a proton-motive force-driven mechanism. Arginine is transported inside the cell by an ornithine antiporter system. Similar antiporter systems also exist for histidine–histamine, tyrosine–tyramine, and aspartic acid–alanine couples (Poolman 1993). Glutamate, glutamine, aspartate, and asparagine are transported by a phosphate-bond-driven transport system (Driessen *et al*. 1989), which is likely to be ATP-driven. Hence, transport of most amino acids will require either a gradient (i.e. proton or positive ion) or energy. Permeases also play a role for specific compounds, such as amino acids and sugars, and are just beginning to be understood using gene expression arrays during laboratory and cheese fermentations (Ganesan *et al*. 2006, 2007).

Several mechanisms for the production of flavor compounds from amino acids are detailed in the literature, and these include both enzymatic and non-enzymatic degradation of amino acids in cheese. The importance of amino acid metabolism lies in the cellular necessity for surviving LAB to acquire energy in the form of ATP together with carbon, sulfur and nitrogen for physiological maintenance. Glutamate, valine, methionine, leucine, isoleucine, and histidine are essential for LAB to produce flavor compounds that are new to the fermented product, rather than being produced by a simple hydrolysis of existing substrates in the milk.

A few amino acids are key to the survival and production of flavor compounds in fermented dairy products. These include arginine, glutamic acid, branched-chain amino acids, and sulfur-containing amino acids. An extensive review of amino acid metabolism is available (Weimer 2007). The implication for these amino acids in biotechnology extends to flavor production and cellular survival. For example, arginine and branched-chain amino acids extend LAB metabolism and survival during carbohydrate starvation (Chou *et al*. 2001; Ganesan and Weimer 2004; Ganesan *et al*. 2006).

Comparative genomics of flavor production

The availability of LAB genome sequences allows a considerable amount of bioinformatic analysis using various software tools to examine gene location (also called genome context), orientation, operon structure, and alignment with other genomes. Broad comparisons of LAB genomes provide a key set of information on which to focus biotechnological development (Table 2.2). Using these sequences, comparisons of very closely related subspecies and strains can be carried out in order to determine the exact genetic differences that result in the phenotypic variances between the strains, and this in turn allows the metabolic impact to be assessed and verified with experimentation.

Owing to the lack of plasmids, the obvious differences between the strains that have been sequenced are lactose metabolism and proteolysis. MG1363

and IL1403 are laboratory strains that lack plasmids, and therefore lack all plasmid-associated traits for the use of lactose and for the entire proteolytic cascade. SK11 is a commercial strain that has five plasmids, and which therefore has these traits as well as many others for phage resistance. All three sequenced strains of lactococci, as well as other LAB, encode various parts of the tri-carboxylic acid (TCA) cycle. The TCA cycle provides key intermediates that link to other metabolic traits which lead, in turn, to flavor-compound production, including α-keto glutarate, succinate, fumarate, and malate (Fig. 2.3). TCA intermediate compounds bridge the connection between sugar, protein, lipids, and nucleotide metabolism. Gene dosage plays a role in sugar-utilization differences in lactococci. For example, SK11 has two copies of glutamate decarboxylase, while MG1363 and IL1403 have a single

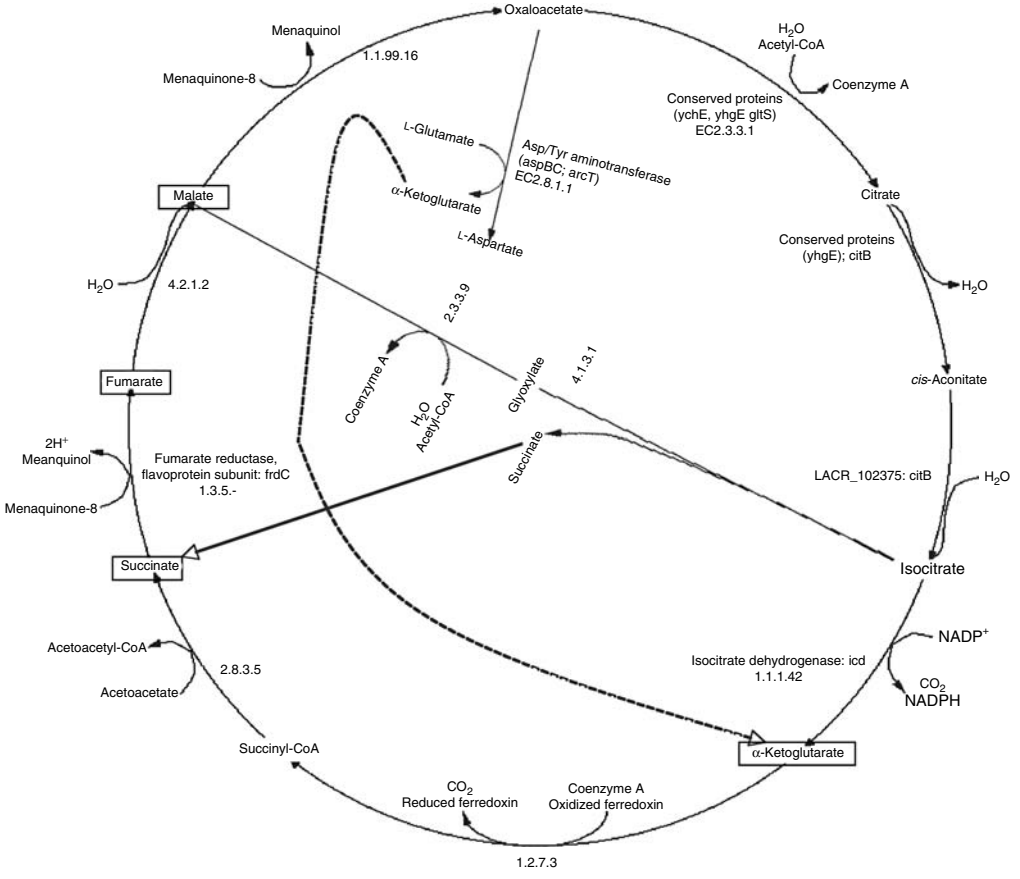

Fig. 2.3 Genetic reconstruction of the TCA cycle in *Lactococcus lactis* subsp. *cremoris* SK11. The glyoxylate shunt (solid line across the circle) and the keto glutarate bridge (dashed line) are shown. Boxes indicate specific compounds that interconnect with other pathways of importance for dairy fermentations. This was reconstructed using Cremacyc, a Pathway Tools database built using the SK11 genome, which predicts metabolic ability based on the genome sequence (http://www.biocyc.org/META/server.html). Gene symbols and EC numbers are shown between the reactions.

copy of this gene. This is also true for fumarate reductase which requires NADH–SK11 has a single copy, while the other two lactococcal strains lack this gene.

Amino acid metabolism in bacteria provides ATP generation (via substrate-level phosphorylation), pH regulation, and redox maintenance (Harwood and Canale-Parola 1981; Zhang *et al.* 1999). Keto acids, largely produced from amino acid metabolism, are subsequently metabolized or interact with a broad range of molecules to yield a series of compounds that includes fatty acids, alcohols, and aldehydes among others (Fig. 2.4). An increasing number of unique LAB pathways that involve keto acids as intermediates for flavor-compound formation are being discovered using genomics. A central link between sugar and amino acid metabolism is α-ketoglutarate in the TCA cycle and amino acid interconversion, as well as other major pathways that were not previously considered as an interconnecting system for flavor production (Fig. 2.4). Use of pathway webbing with the *L. lactis* subsp. *cremoris* SK11 pathway-reconstruction database (http://www.biosystems.usu.edu) indicates that keto acids play a central role in flavor production. The central question remains as to how and which specific genes between the connections are important during processing and storage.

Other connections with the TCA cycle that were not appreciated prior to genome sequencing and metabolic reconstruction also exist. For example, the involvement of succinate in flavor is well documented (Díaz-Muñiz *et al.* 2006), but the exact genes and metabolic routes were not known at the time of this work. Figure 2.5 demonstrates the numerous interactions between succinate (i.e. the TCA cycle) and other processes that are critical to the survival of the cell. This expanded view of the interactions between these intermediates provides new biotechnological targets for modification and flavor improvment. A critical interaction for flavor is sulfur compounds. The production

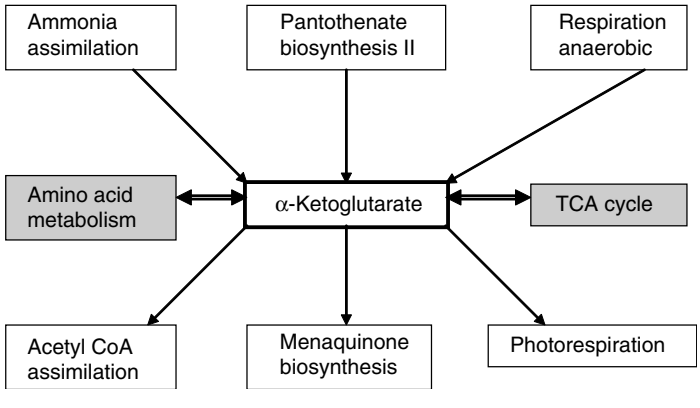

Fig. 2.4 Metabolic interconnections for the production and consumption of α-keto glutarate (and keto acids generally) in *L. lactis* subsp. *cremoris* SK11. This was predicted using Cremacyc, a Pathway Tools database built using the SK11 genome (http://www.biocyc.org/META/server.html) using pathway webbing.

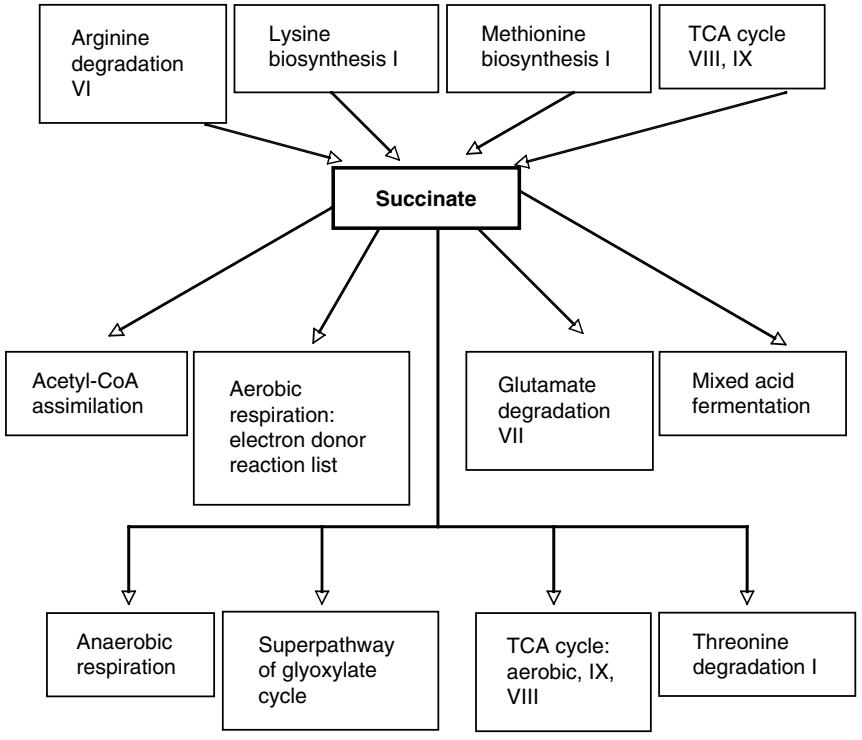

Fig. 2.5 Metabolic interconnections for the production and consumption of succinate in *L. lactis* subsp. *cremoris* SK11. This was predicted using Cremacyc, a Pathway Tools database built using the SK11 genome (http://www.biocyc.org/META/server.html) using pathway webbing.

VSCs from methionine and other sulfur-containing compounds is well known, but the specific pathways have been only partially defined (Seefeldt *et al.* 2000). Use of pathway webbing indicates that succinate and methionine metabolism are interconnected (Fig. 2.6), providing a new insight that remains to be explored. However, keto acid involvement is likely to be critical.

The metabolism of amino acids in LAB typically requires an amino-acceptor keto acid to form another amino acid (Yvon *et al.* 1997). Keto glutarate has been the choice to promote aminotransferase action by providing it as the primary acceptor (Yvon *et al.* 1999). While this work ignored auto-degradation of keto glutarate to flavor-related fatty acids (Ganesan *et al.* 2004a), metabolic engineering was carried out to convert the glutamate produced from this reaction back to ketoglutarate by glutamate dehydrogenase (Tanous *et al.* 2002).

Ganesan *et al.* (2004a, 2006, 2007) showed that keto acids are also converted to fatty acids, although they are not the same as the products from their precursor amino acids. Several keto acids are present in intracellular fractions of long-term starved lactococci (Ganesan 2006), suggesting that amino-acceptors are not limiting for fatty acid production during starvation and non-culturability. The lack of an aminotransferase caused lactococci to

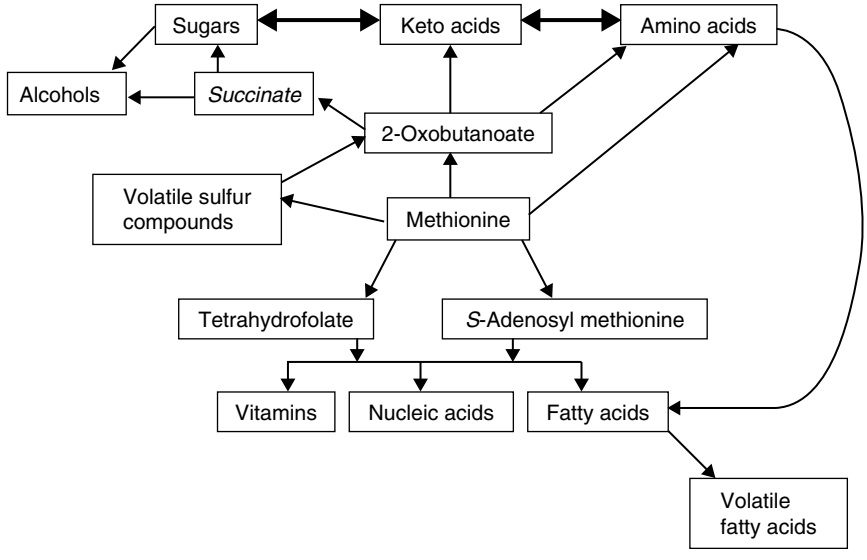

Fig. 2.6 Metabolic interconnections for the production and consumption of methionine in *L. lactis* subsp. *cremoris* SK11. This was predicted using Cremacyc, a Pathway Tools database built using the SK11 genome (http://www.biocyc.org/META/server.html) using pathway webbing.

produce different end-products from precursor amino acids and the products of keto acids (Ganesan and Weimer 2004), suggesting that different aminotransferases are involved in the regulation of keto acid metabolism. The ability of long-term starved lactococci to produce fatty acids without any external supplementation with keto acids (Ganesan *et al*. 2006, 2007) also brings into question the importance of a single keto acid as the primary amino-acceptor for amino acid metabolism. However, the extent of interconnections between keto acids and other metabolic processes is clearly demonstrated in Fig. 2.4, which implies that keto acid metabolism is very important for flavor production. The combination of Figs 2.4, 2.5, and 2.6 highlight the intersections between protein metabolism (keto acids), sugar metabolism (via succinate in the TCA), and sulfur metabolism (succinate and keto acids). Verification of these intersections is needed, which can be done using gene expression analysis.

Expression and metabolite analysis

Gene expression arrays are the immediate next step for the functional use of the genome sequence. Essentially, expression arrays monitor how the cell communicates and regulates biological functions. To make an expression array, small segments of DNA (i.e. probes that are homologous to the genome) are anchored to a glass slide or membrane. Subsequently, RNA is collected during the experiment and is reverse transcribed into cDNA that is labeled with a fluorescent tracer molecule. This is hybridized to the array, and a fluorescent signal

is obtained on small individual spots that contain the homologous probe. Analysis of the fluorescent signals indicates the transcripts (and by extrapolation the proteins) that were present or absent due to the specific cellular treatment.

Visualization of these data provides an unprecedented view of the complex web of genetic networks that can affect flavor-compound development, growth, and response to the stress conditions of cheese-making and aging. Xie *et al.* (2004) described the first gene expression array using small oligonucleotide probes for use with LAB in a functional setting related to cheese conditions. This study determined the effect of stress on lactococci to identify the common and unique genes regulated by acid, salt, and heat stress. It also demonstrated that gene expression arrays provide useful and reliable information about gene expression, as every known stress-linked gene was verified by the study in a single experiment – in addition to finding new stress-related networks that were previously unreported. Use of this array in cheese during ripening provided evidence that the arginine catabolic pathway is active and is stimulated by a variety of nutrients and co-factors throughout ripening to 45 days (Fig. 2.1).

Use of gene expression arrays provides a set of genes that are regulated during a specific condition and at a specific time. To be truly useful for flavor production, this information must be translated into biologically meaningful information. This is a significant challenge, especially for cheese flavor compounds, and is being addressed using high-throughput biochemistry (e.g. metabolomics) and computer science (e.g. bioinformatics).

Gene expression data often need additional or supporting evidence to provide assurance that the metabolism extrapolated from the gene expression pattern is reliable. Ganesan *et al.* (2006) used NMR, gene expression analysis, and metabolic network maps to delineate the exact metabolic pathway used by lactococci to convert branched-chain amino acids to branched-chain fatty acids. The study demonstrated the usefulness of gene expression for flavor production, but it also found that the aminotransferase genes thought to catalyze the initial step of this transformation were not expressed in these conditions, but rather another two of the possible nine genes were induced for this purpose, which changed the view of the specific genes involved in flavor formation.

Gene expression arrays are also useful for measuring the impact of metabolic end-products from flavor formation. For example, Pieterse *et al.* (2005) used microarray analysis to determine the impact of lactic acid exposure on *Lactobacillus plantarum*. Array analysis also provides new tools to unravel the changes in metabolism that are due to nutrient availability. In this case, depletion of lactose in cheese during ripening is easy to measure, but determining the metabolic impact is very difficult. Stuart *et al.* (1999) determined that lactococci lose the ability to produce colonies after carbohydrate exhaustion, which is accompanied by the release of methionine and serine into the medium – just as is observed during cheese ripening. Ganesan *et al.* (2006) extended this investigation using gene expression studies to confirm Stuart's work and further demonstrated that the cells continue to transcribe RNA

even without the ability to form colonies. This observation was used to demonstrate branched-chain fatty acid production from amino acids using gene expression arrays. Ganesan and colleagues also demonstrated that this cellular state can last for atleast 5 years without induction of the genes needed for cellular lysis. They proposed that ~0.001% of the starter culture lysed during this state. Hence, the culture appears to die because it cannot form colonies, yet the cells are intact and continue to metabolize peptides and amino acids to end-products that affect flavor. The full impact of this metabolic state during cheese ripening remains to be clarified.

Non-culturable lactococci

The current dogma states that lactococci are alive only in the early steps of cheese-making and produce acid (Arora *et al*. 1995; Crow *et al*. 1993). A number of studies suggest that once acid production is over, starter cultures are simply a delivery mechanism for enzymes that act on the substrates from milk after lysis to produce flavor (Buist *et al*. 1998; Ostlie *et al*. 1995; Wilkinson *et al*. 1994). The ability of lactococci to survive under stress conditions (Rallu *et al*. 1996) and continue protein turnover, RNA synthesis (Thomas and Batt 1969), and degradation also indicates their ability to actively metabolize proteins and amino acids. Hence, owing to the lack of a sugar source and the presence of amino acids, they shift towards a non-lactic, nitrogenous metabolism in their non-culturable state. However, increasing evidence suggests that a minor proportion of the lactococcal population may die, although a larger population continues to exist in a non-culturable state (Díaz-Muñiz *et al*. 2006; Ganesan *et al*. 2006, 2007; Stuart *et al*. 1999). Recent evidence demonstrates that, owing to repression of the cytoskeleton genes (*fts*) that are required to initiate and carryout replication, the cells lose the ability to grow on media. The non-culturable population consists of cells with intact cell walls, and these remain capable of amino acid and peptide transport as well as the metabolism – including fatty acids – of new compounds not observed during the logarithmic phase growth (Ganesan *et al*. 2004a,b, 2006, 2007; Ganesan and Weimer 2004).

Summary

Compounds related to flavor perception and to the presence of flavor in cheese have been a topic of research since the 1940s. Despite great efforts, the mechanisms of flavor production employed by the microbes used in cheese production have been elusive. With the rapid increase in genome-sequencing efforts, the genetic and metabolic causes of flavor-compound production are becoming increasingly clear. The genome sequences are also providing surprises by revealing novel metabolic pathways that were not previously associated or thought possible by LAB. This is also true of genes for phage resistance. The use

of high-throughput tools such as gene expression analysis and metabolomics will provide new answers to age-old questions in flavor production and LAB metabolism – for example, questions concerning the induction of new compounds, such as branched-chain fatty acids during metabolic states that previously were not known to exist in lactococci. Further advances in genomics and bioinformatics will enhance our ability to link genes to the industrial processes that are needed for consistent and flavorful cheese production.

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Chapter 3
Biotechnological production of vanillin

Daphna Havkin-Frenkel and Faith C. Belanger

Introduction

Vanilla is the most popular and widely used flavor by both the dollar and the tonnage basis. Vanilla extract is obtained by aqueous ethanolic extraction of cured vanilla beans, the pod-like fruit of vanilla, a tropical climbing orchid. Vanilla beans are harvested green and are cured under high-heat and high-humidity conditions to release vanillin and other flavor compounds from their glucoside precursors (Ranadive 1994; Dignum *et al*. 2001; Havkin-Frenkel *et al*. 2005). Vanilla extract contains more than 250 compounds (Hartman *et al*. 1992), and chief among them is vanillin (3-methoxy-4-hydroxybenzaldehyde), the most abundant component in the vanilla flavor. In addition to being an important flavor molecule, vanillin is valued also for other properties, including anti-oxidant, antimicrobial, and anti-inflammatory properties. Vanillin is also considered as having aphrodisiac properties and is used in folklore medicine for soothing the stomach and as a relaxant.

Vanilla beans are produced in tropical regions, and the supply, cost and quality of the beans are subject to fluctuations, primarily because of severe weather episodes. Most of the vanilla beans produced are used in the US market. The US annual consumption of vanilla beans is around 1200–1400 tons with a market value of approximately US$100 million. Around 40% of the beans imported into the USA are used in ice creams. Approximately 300–400 tons of vanilla beans are used in the rest of the world. In the past few years, the vanilla market has been volatile and the rate of consumption has been fluctuating.

The demand for vanilla flavor cannot be met by vanilla extract, but this deficiency is not only because of the price of vanilla beans. Many flavors require a large amount of vanillin, but this high demand for vanillin cannot be met by using vanilla extract alone, since most vanilla beans on the market contain less than 2% vanillin on a dry-weight basis. In addition, vanilla extract is dark in color, and this can affect the appearance of the food. Only a few applications require the use of pure vanillin. Pure vanillin is one of the most important aromatic flavor compounds used in foods, beverages, perfumes, and pharmaceuticals, and synthetic vanillin is produced on a scale of more than 10 000 tons per year.

By the US Food and Drug Administration (FDA) definition, vanillin can be labeled as 'natural vanillin' only when it is derived from vanilla beans. The European regulations are different and allow vanillin from natural sources, and obtained by natural processes, to be labeled as 'natural vanillin'. Currently, the price of natural vanillin, obtained through extraction of vanilla beans, is

Biotechnology in Flavor Production: A Case-based Approach. Edited by Daphna Havkin-Frenkel, Faith C. Belanger.
 ISBN: 978-1-4051-5649-3

around US$1000–2000/kg of vanillin. Many studies suggest that vanillin might be produced through biotechnological methods at a relatively low cost (Barghini *et al*. 2007, Dignum *et al*. 2001, Overhage *et al*. 2003, Priefert *et al*. 2001). Results of the past 20 years indicate, however, that vanillin produced through biotechnological methods would have a high market cost at around US$800/kg of vanillin. Vanillin is also produced by hydrolysis of eugenol using physical means, providing around 50 tons annually worldwide at an average price of US$50–60/kg of vanillin. Synthetic vanillin is produced from guaiacol and lignin. Approximately 13 000 tons of synthetic vanillin are produced annually worldwide (Desmurs *et al*. 2004) at a price of US$16–20/kg of vanillin. Ten to fifteen percent of the synthetic vanillin is obtained from lignin, and the remainder from guaiacol.

Vanillin was first isolated in 1858 by Gobley (Gobley 1858), who evaporated a vanilla extract to dryness, and recrystallized the vanillin. Vanillin was synthesized in 1874 by Tiemann and Haarmann from coniferin found in pine tree tissues. Two years later, vanillin was synthesized by Reimer from guaiacol (Reimer 1876). In the late nineteenth century, synthesis of vanillin was also achieved by hydrolysis of eugenol, the main component of clove oil. Presently vanillin is derived by a similar bydrolytic process from lignin, a waste product of the pulp industry. Another source of vanillin is from synthesis with guaiacol, a petrochemical constituent, as the starting material (Esposito *et al*. 1997).

Vanillin, the major flavor constituent of vanilla flavor, accumulates in vanilla beans as glucovanillin. Synthesis of glucovanillin ensues when the vanilla pod has reached its maximum size, about 3–4 months after pollination, and is carried out in the interior of the vanilla pod in specialized cells (Joel *et al*. 2003). Accumulation of glucovanillin continues at a rapid rate for an additional 3–4 months and then tapers off gradually for an additional few months. At the end of the vanilla bean development, glucovanillin is found in the central cavity of the pod in significant amounts (Havkin-Frenkel *et al*. 1999). Mature beans are then harvested and subjected to curing, a process aimed at releasing vanillin in a free form from glucovanillin, as well as other compounds that make up the prized vanilla flavor (Adedeji *et al*. 1993).

In commercial practice, however, curing usually yields 2.5–4.5% vanillin or less, on a dry-weight basis of cured beans (Bala 2003; Ranadive 2003), corresponding roughly to 1.75–2.1% of vanillin in cured beans containing 30% moisture. An additional portion of vanillin, generated during the curing process, might be lost when cured beans are extracted for the preparation of vanilla extract (Clark 1990). Yet another fraction of vanillin, added to foods or to other materials as vanilla extract or pure vanillin, appears to fade away with time. Our studies on the physics and chemistry of vanillin (Frenkel and Havkin-Frenkel 2006) suggest that escape tendencies of vanillin from cured vanilla beans are governed by the hydration state of the compound, as well as by acid–base conditions. Water structuring in the bean microenvironment governs the chemical reactivity of the release of vanillin, mostly Schiff base formation with amines. By controlling these conditions, we found that the content of vanillin may reach around 8–10%, on a dry-weight basis, using an appropriate

curing protocol (Frenkel and Havkin-Frenkel 2006). Thus, an understanding of the physical and chemical processes leading to losses in vanillin may provide practical tools for preventing losses in vanillin in cured vanilla beans or during vanillin accumulation in reaction media employed in biotechnology.

Biotechnology-based approaches for the production of vanillin are based on bioconversion of lignin, phenolic stilbenes, isoeugenol, eugenol, ferulic acid, curcumin, or aromatic amino acids, and on *de novo* biosynthesis, using microorganisms, such as fungi or bacteria as well as plant cells, or enzymes.

The present chapter is concerned with biotechnological studies on the production of vanillin. We will analyze the current state of the art, indicating some problems such as accessibility of vanillin precursors, yield, and the need for controlling the biosynthetic pathways. These issues are constraints to the economic production of vanillin via the biotechnology route and, in addition, biotechnologically produced vanillin is not well defined from the standpoint of regulation.

Biosynthesis of vanillin

Natural occurrence of vanillin

Vanillin is found in trace amounts in many plants. Many essential oils, such as clove, cinnamon, and mace contain vanillin (Clark 1990). Table 3.1 lists plant species that produce detectable amounts of vanillin. Large amounts of vanillin are only found, however, in plants from the genus *Vanilla*. There are around 130 species of *Vanilla*, but only two species, *Vanilla planifolia* and *Vanilla tahitensis*, are allowed to be used in food. Most other *Vanilla* species have not been characterized with regard to vanillin content.

Site of vanillin production in vanilla beans

In an early observation on vanilla pod structure, Swamy (1947) posited that vanillin is secreted 'in tissues around the seeds'. Jones and Vicente (1949) also

Table 3.1 Natural occurrence of vanillin in plants.

Species	Tissue	Percentage of dry weight*
Unicorn plant (*Proboscidae cuisianica*)	Roots, pod	0.01
Potato (*Solanum tuberosum*)	Tuber skin	0.01
Clove (*Syzygium aromaticum*)	Dry flower buds	Trace
Sian benoin	Vascular tissue exudates	Trace
Narcissus (*Triandrus narcissi, Tazetta arsissi*)	Roots, basal plate	0.01–0.60
Hyacinth (*Hyacinthus orientalis*)	Roots, basal plate	0.20–0.50
Vanilla planifolia	Pod (cured)	1.00–8.00
Vanilla tahitensis	Pod (cured)	0.50–2.00
Vanilla pompona	Pod (cured)	0.01–2.00

*The values for vanillin content represent studies carried out by a large number of research groups.

pointed out that most of the vanillin and other compounds involved in vanilla flavors were found in the middle part of the bean around the seeds. In a comprehensive study on vanilla fruit (pod) development, we found that vanillin is specifically present in the non-photosynthetic white parenchyma cells of the endocarp in the pod interior (Joel *et al.* 2003). Separation of these 'white' inner-fruit portions from the outer 'green' fruit exocarp revealed that the former contains 95% of the total vanillin found in the vanilla pod. We also used catechin-HCl, which binds to various phenolic compounds including vanillin, as a staining agent for localizing accumulated vanillin in the developing fruit. We found, accordingly, that both the placenta and the adjacent endocarp parenchymatic cells were red-stained (Plate 3.1), indicating the presence of vanillin and intermediates in the vanillin biosynthetic pathway. Catechin-HCl also stained the densely packed secreted matrix that accumulates in the fruit cavity (Plate 3.2), clearly showing a descending staining gradient, from endocarp in the fruit cavity outwards.

The use of this method also revealed that vanillin accumulation begins after 3 to 4 months of fruit development. However, no staining was observed in the longitudinal strips of brilliant whitish secretory tissue located in the gaps between the placentas along the central fruit cavity (Plates 3.1 and 3.2). Because vanillin is sparsely water-soluble, particularly in acidic plant vacuoles (Frenkel and Havkin-Frenkel 2006), glycosylation of vanillin to glucovanillin is a likely mechanism for increasing the hydrophilicity of the compound, thus aiding in the trafficking and storage of the compound in aqueous extracellular regions. Additional observations indicate that vanillin accumulates predominantly in the intercellular space, while little or no vanillin is found in the cell interior. This observation might be important in understanding some of the problems found in microbial systems that can be transformed to produce vanillin, as discussed below.

We believe that the special cells in the pod interior are dedicated to vanillin biosynthesis. We therefore used these cells to construct a cDNA library and are currently characterizing putative genes in the vanillin biosynthetic pathway.

Vanillin biosynthetic pathway in V. planifolia

There is general agreement in the literature that vanillin is a product of the shikimic acid pathway. In this pathway, phenylalanine or tyrosine undergo deamination to a C_6–C_3 phenylpropanoid, which then serves as a precursor for the biosynthesis of vanillin. A general view on the metabolic origin of vanillin is outlined in Figs 3.1 and 3.2. Although it is generally agreed that vanillin originates from a phenylpropanoid C_6–C_3 compound, there are two major views as to how a phenylpropanoid precursor is converted to vanillin. One school of thought, proposed by Zenk (1965) suggested that the aromatic ring on C_6–C_3 compounds (*trans*-cinnamic, *p*-coumaric acids) undergoes

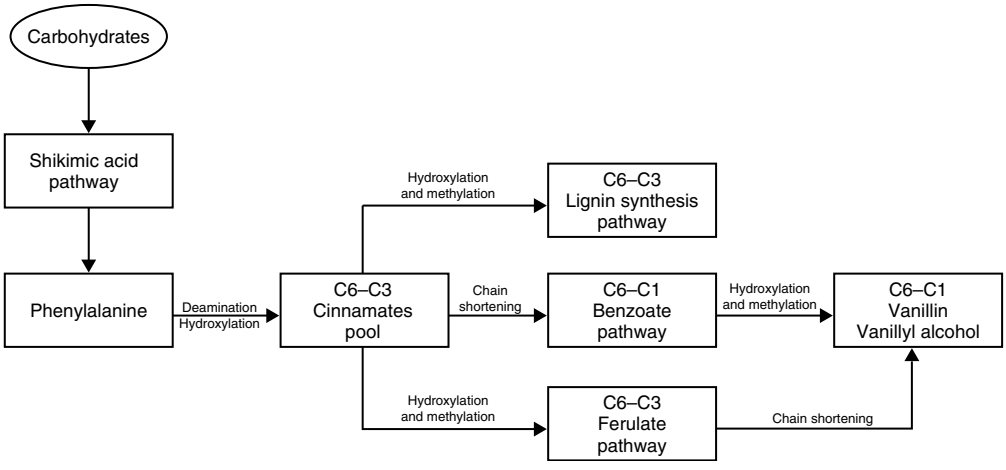

Fig. 3.1 Possible biosynthetic route to vanillin in *Vanilla planifolia*, showing the ferulate and benzoate pathways.

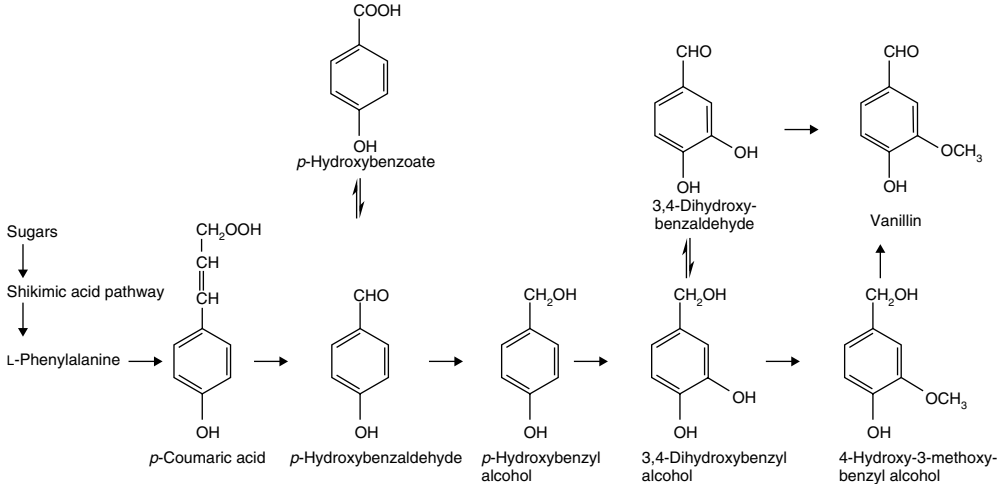

Fig. 3.2 Proposed vanillin biosynthetic pathway in *Vanilla planifolia* (Havkin-Frenkel *et al*. 1996).

hydroxylation and methylation giving rise to ferulic acid. The later then undergoes chain shortening to vanillin. This scheme is termed the 'ferulate pathway'. Another view argues that chain shortening of a phenylpropanoid is the first metabolic event, followed by hydroxylation and methylation of the aromatic ring to yield vanillin. This is termed the 'benzoate pathway' (Podstolski *et al*. 2002). It is also possible that an early intermediate in the shikimic acid pathway gives rise directly to the benzoate pool, bypassing the production of phenylpropanoids and their degradation to benzoate-pathway intermediates (Wildermuth *et al*. 2001).

Production of vanillin by biotechnology

Introduction

Higher plants, notably *Vanilla* species, synthesize vanillin through committed pathways. Several plant species can produce vanillin in trace amounts or at significant levels (Table 3.1), but it is not clear whether they share a common biosynthetic route with vanillin production in *V. planifolia*. The only extensive information regarding vanillin biosynthesis is from *V. planifolia*, but there are opposing views as to exactly how the process is carried out. Vanillin production in microorganisms is principally through the degradation of metabolic precursors. Skilled scientists can also construct an artificial pathway for the synthesis of vanillin, from glucose for example (Frost *et al*. 1998).

The principal methods for the production of natural vanillin consist of cell or tissue culture for the biosynthesis of vanillin as well as the use of cell or tissue culture for bioconversion of natural precursors to vanillin. Other methods consist of releasing vanillin from lignin by enzymatic degradation as well as the use of microbial or fungal cultures for the bioconversion of natural precursors to vanillin. Cell-free systems, using enzyme technology, may be used for bioconversion of precursors, provided there is no need for expensive co-factors. Use of microorganisms for breakage of ferulic acid or eugenol has been studied extensively in many microbial and fungal systems. Some appropriate examples will be cited below in detail.

Use of microorganisms

Degradation of lignin to vanillin

Enzymatic degradation of lignin to useful compounds, including vanillin, has been the topic of many studies for the past 100 years. Lignin, a cell wall constituent in plants, harbors vanillin subunits in its polymeric structure and is an abundant by-product of the paper industry (Janshekar and Fiechter 1983). Chemical breakage of lignin may yield around 4% vanillin. Companies utilize enzyme-catalyzed oxidative degradation of lignin to obtain vanillin. The processes are carried out by extracellular enzymes from the white rot fungus *Phanerochaete crysosporium* (Tien 1987), composed of heme-containing peroxidases (including lignin peroxidase) and manganese-dependent peroxidase as well as laccase, a copper-containing phenol oxidase. The lignin-degrading system does not readily attack native lignin but can degrade lignin fragmentation products obtained by harsh chemical processing with sulfuric acid, for instance. The activity of lignin-degrading enzymes also depends on supplementation with co-factors including veratryl alcohol (3,4-dimethoxybenzoyl alcohol), which is the redox mediator for lignin peroxidase. Mn^{2+} ions mediate the activity of the manganese-dependent peroxidase, when properly chelated with the fungal organic acid metabolites (e.g. oxalate). The process yields

around 1% vanillin as well as a vast array of other by-products. However, the starting substrate, degradable lignin fragments obtained by harsh chemical treatments, may not be regarded as a natural material and so the resulting vanillin is considered synthetic.

Bioconversion of ferulic acid and eugenol to vanillin

Biosynthesis of vanillin by microbial and fungal fermentation is based on bioconversion or degradation but not on *de novo* synthesis in the manner found in plants (Barghini *et al*. 2007; Overhage *et al*. 2003; Priefert *et al*. 2001). There is plentiful literature on the subject. Although there are many reports on successful laboratory-scale production of vanillin, there are only a few companies producing vanillin on a commercial scale (US$500–1000/kg of vanillin). Other companies abandoned this route for vanillin production because of high production costs. Synthetic vanillin commands a price of US$16–20/kg of vanillin. Most of the flavors made in the USA are labeled NAA (natural and artificial). For these flavors, there is no need to buy biotechnologically produced vanillin. For flavors that are labeled 'natural', companies use vanillin produced by physical means, which carries a price of US$40–60/kg of vanillin. Neither source of vanillin may be denoted as 'natural vanillin', which can come only from vanilla beans. This is the authors' view, although there is some controversy and a continued debate on this issue. Our purpose, in this chapter, is to highlight the issues that constrain the production of vanillin, including the use of appropriate precursors and the use of appropriate fermentation systems.

Several C_6–C_3 source compounds, mainly eugenol and ferulic acid, are currently being explored or are in use in conjunction with fermentation technologies for the biotechnological production of vanillin (Benz and Muheim 1996; Priefer *et al*. 2001; Lesage-Meessen *et al*. 2002; Walton *et al*. 2003; Desmurs *et al*. 2004; Mathew and Abraham 2006). This chapter will deal with only some representative examples of microorganisms producing vanillin. More information on this subject can be found in the following references (Labuda *et al*. 1994; Frost 1998; Lesage-Meessen *et al*. 1999; Oddou *et al*. 1999; Li and Rosazza 2000; Muheim *et al*. 2001; Priefert *et al*. 2001; Mathew and Abraham 2006).

Eugenol, a major aromatic constituent in clove oil, is converted by a *Pseudomonas* strain to ferulic acid through successive steps involving formation of coniferyl alcohol, coniferyl aldehyde, and finally ferulic acid (De Jong *et al*. 1992; Fraaije *et al*. 1995; Furukawa *et al*. 1998; Priefert *et al*. 1999; Van den Heuvel *et al*. 2001). Eugenol is inexpensive, comprising 80% of clove oil and is readily accessible. However, eugenol is not water-soluble and, in addition, must be isomerized to isoeugenol. Furthermore, eugenol is toxic to microorganisms, even at low concentrations, thus putting in doubt the usefulness of the compound for biotechnological production of vanillin. These problems might be overcome with the use of organisms that are resistant to eugenol toxicity and that can grow and function in non-aqueous media. Chemical modifications of eugenol to increase solubility will render the product non-natural.

Ferulic acid is found in numerous cereal crops (Clifford 1999), where the compound is esterified to arabinose moieties in plant cell walls and may be cross-linked to diferulate or other polymeric forms of the compound. Cross-linking may be to another wall-bound ferulic acid or to other cinnamic acid derivatives. The ferulic acid content of cell wall material, on a dry-weight basis, is around 0.4–0.7% of the cell wall material of wheat, 3% in maize bran, 1.2% in rice endosperm, and 0.5–1.0% in sugar beet (Walton *et al*. 2000). Ferulic acid may be released from the cell wall matrix by hydrolyzing the ester bond with the use of strong alkali, but biotechnological production of natural vanillin relies on enzymatic cleavage of the wall material using cinnamoyl esterase in combination with cell wall hydrolyzing enzymes (Williamson *et al*. 1998).

Accordingly, wall hydrolyzing enzymes, in combination with cinnamoyl esterase from *Aspergillus niger*, were observed to release a high proportion of ferulate from cereal bran (Faulds and Williamson 1995) or sugar beet pulp (Kroon and Williamson 1996). Cinnamoyl esterase from *Pseudomonas* (Ferreira *et al*. 1993) released both monomeric and dimeric forms of ferulic acid from cereal bran and spent barley grain (Bartolomé *et al*. 1997). Yields of released ferulic acid may approach up to 1% of the content present in the parent materials (Ferreira *et al*. 1993; Faulds and Williamson 1995; Kroon and Williamson 1996; Bartolomé *et al*. 1997; Couteau and Mathaly 1997). On the other hand, diferulate found in various plant materials (Parr *et al.* 1996; Saulnier and Thibault 1999) is released together with free ferulic acid. Levels of diferulates released from cell walls or from other plant tissue preparations, comprise around 21% of the free-form ferulic acid in maize sheath (Santiago *et al*. 2006), 14% of the ferulate content in wheat flour (Vansteenkiste *et al*. 2004), and 24% in maize bran (Lapierrea *et al*. 2001). Hence, the presence of these compounds, released together with ferulic acid, interferes with the final product. Enzymes for cleaving the diferulate to the monomeric form of ferulic acid are not known, although degradation of diferulates might be carried out with lignin-metabolizing enzymes from microorganisms, as outlined above.

In microorganisms, there are two main known routes from ferulic acid to vanillin. The first one is a CoA-dependent non-oxidative chain-shortening mode of action. This well-characterized enzyme system is part of the hydroxycinnamate-degradation process in *Pseudomonas* (Walton *et al*. 2000) as outlined in Fig. 3.3. The initial reaction is ligation of ferulic acid to CoA, catalyzed by 4-hydroxycinnamate: CoA ligase. An enzyme termed 4-hydroxycinnammoyl-CoA hydratase/lyase (HCHL) next catalyses the hydration and cleavage of feruloyl-CoA to vanillin and acetyl-CoA (Gasson *et al*. 1998). Accompanying this pathway to the production of vanillin is the formation of vanillic acid, protochatechuic acid, and products of ring cleavage. This chain of events is characteristic of hydroxycinnamate utilization by microorganisms, where ingested compounds are degraded for energy and intermediary metabolites.

The introduction of this pathway to plants was carried out in an attempt to exploit the abundant phenylpropanoid pool in plants for the formation

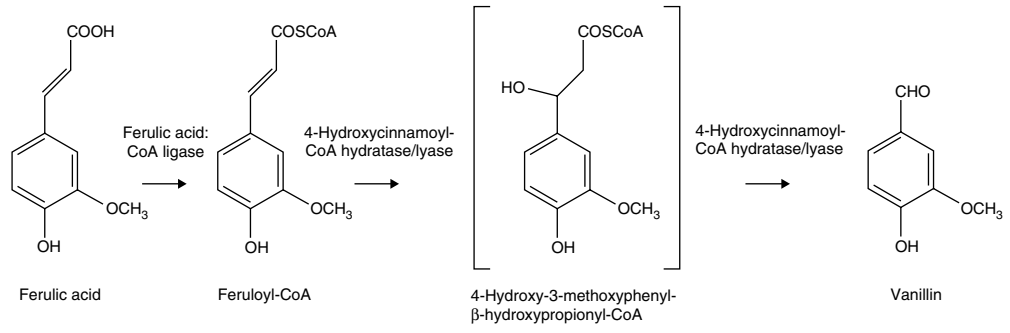

Fig. 3.3 The route from ferulic acid to vanillin in *Pseudomonas* strains. 4-Hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA is presumed to be an enzyme-bound intermediate. (Modified from Walton *et al.* (2003); Copyright Elsevier 2003.)

of vanillin. Expression of HCHL in *Nicotiana tabacum* plants (Mayer *et al.* 2001) and in hairy root culture of *Datura stramonium* L. (Mitra *et al.* 1999, 2002), resulted in major redirection of phenylpropanoid metabolism. These extensive and thorough studies, covering the work on the introduction of HCHL into model plants, are worth visiting. However, the transformed plants were found to accumulate new products, including β-D-glucosides and glucose esters of 4-hydroxybenzoic acid, small amounts of vanillic acid glucoside, and 4-hydroxybenzyl alcohol glucoside. Vanillin, 4-hydroxybenzaldehyde, or their glucosides were not formed. These results resemble the behavior of vanilla tissue culture (Herz 2000; Havkin-Frenkel and Belanger 2007), suggesting that plant tissues not specializing in vanillin biosynthesis cannot go beyond the stage of *p*-hydroxybenzyl alcohol or *p*-hydroxybenzoic acid.

It appears, then, that engineering microorganisms or higher plants each presents distinct problems. Conversion of ferulic acid to vanillin, using microorganisms, can lead to vanillin formation, as evidenced by commercial biotechnological production of vanillin (Desmurs *et al.* 2004), although previous attempts to commercialize the process resulted in failure. Aside from the problem of sourcing ferulic acid, the main issue is the degradation of the vanillin formed during the conversion from ferulic acid to vanillic acid or to vanillyl alcohol. Enzymes that oxidize or reduce vanillin are non-specific and are difficult to control. Moreover, cell-free systems for ferulate-to-vanillin conversion must contain co-factors (indicated above) and might be too expensive, unless a chain-shortening enzyme system is utilized which does not require co-factors.

The other microbial route from ferulic acid to vanillin is a CoA-dependent oxidative chain-shortening enzyme catalyzing the degradation of ferulate to vanillic acid. Obviously, this pathway is of limited use as a biotechnological approach for vanillin production, since vanillic acid is not the desired product.

In vanilla pods, vanillin is glycosylated and immediately secreted to the cell exterior, where it appears to accumulate in intercellular spaces around the

seeds (Havkin-Frenkel *et al*. 2005). Vanillin or its glycosylated form does not appear to accumulate in the interior of cells, perhaps to avert the reactivity and possible toxicity of the carbonyl group. This view is supported by our studies on feeding vanilla tissue cultures with 3,4-dihydroxybenzaldehyde, which resulted in the accumulation of glycosylated vanillyl alcohol. Feeding vanillin also resulted in glycosylated vanillyl alcohol. It may not be a coincidence that vanillin biosynthesis in *Vanilla* species occurs in specialized cells, where vanillin is glycosylated and expelled from the cellular interior. This perspective suggests that biological cells not equipped to deal with the cellular turnover of vanillin might be ill equipped to handle vanillin production. It brings to the fore the particular suitability of green vanilla beans for vanillin production. Proper agronomic production of vanilla vines and beans and selection for lines producing high levels of vanillin could result in efficient vanilla pods, especially since the present vanilla in cultivation show that glucovanillin can accumulate to levels of up to 20% of the tissue dry weight. With proper control of the curing process, it might be possible to obtain cured vanilla beans containing 10% of vanillin. This corresponds roughly to 100 tons of cured beans required to obtain 10 tons of purified vanillin. This vanillin could be economically competitive with biotechnologically produced vanillin and is much simpler to obtain. Moreover, it could, without a doubt, be labeled as 'natural vanillin'.

Several studies report on the *in vitro* enzymatic degradation of C_6–C_3 compounds to C_6-C_1 products, such as the production of benzoic acids and aldehydes from hydroxycinnamic acids (Funk and Brodelius 1990a,b; Yazaki *et al*. 1991; Schnitzler *et al*. 1992; Yalpani *et al*. 1993; Löscher and Heide 1994; Verberne *et al*. 1999). However, there is still considerable disagreement as to the nature of the biochemical mechanisms for side-chain shortening of hydroxycinnamic acids (Podstolski *et al*. 2002). One study on the synthesis of 4-hydroxybenzoate in *Lithospermum erythrorhizon* indicates that the pathway entails oxidation and cleavage of the parent compound, 4-coumaroyl-CoA, to 4-hydroxybenzoic acid and acetyl-CoA in a thiolase-type reaction with a requirement for nicotinamide adenine dinucleotide (NAD) (Löscher and Heide 1994). This mode of enzyme action, involving oxidative chain shortening, may account for the formation of vanillic acid as an oxidative cleavage product from ferulic acid and perhaps from other phenylpropanoid compounds.

Our studies, by comparison, provide confirmation for a non-oxidative mechanism involving a hydrolyase activity coupled to hydration of the side-chain 2,3 double bond of 4-coumaric acid, with subsequent cleavage of the side chain to yield acetate and 4-hydroxybenzaldehyde. Podstolski *et al*. (2003) purified a chain-shortening enzyme from *V. planifolia*, and noted 4-hydroxybenzaldehye synthase, which catalyzes the cleavage of coumaric acid to 4-hydroxybenzaldehyde (Fig. 3.4). The authors also observed that vanilla tissue converts ferulic acid to vanillin in a similar manner, although at only 2% of the activity noted with coumaric acid. This mode of action is also supported by our studies showing that ^{14}C-labeled tyrosine or phenylalanine was converted by vanilla

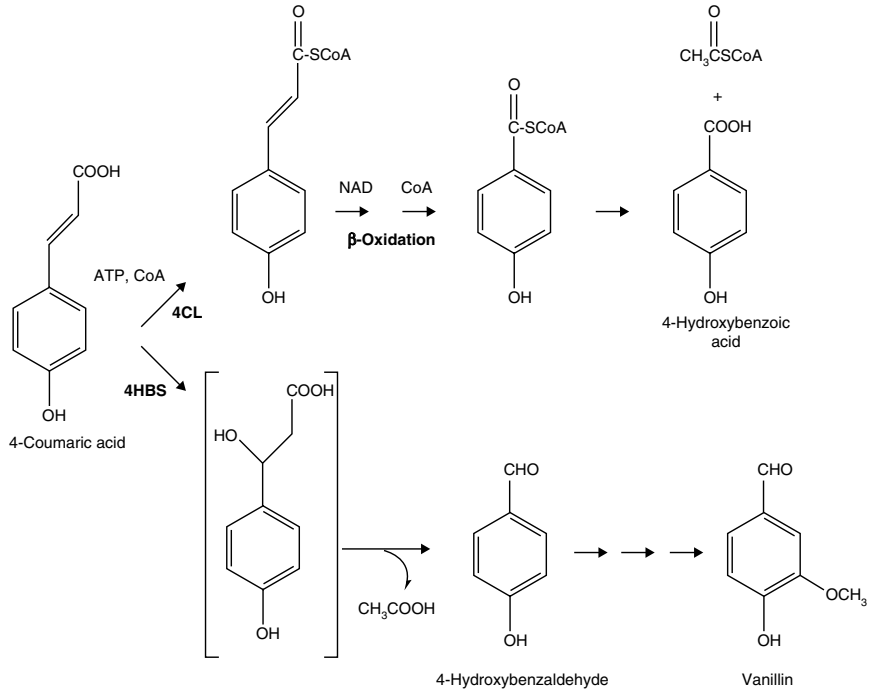

Fig. 3.4 Oxidative and non-oxidative pathways for the chain shortening of cinnamic acid derivatives in plants. The upper pathway shows the oxidative pathway to 4-hydroxybenzoic acid via the coenzyme A ester. The lower pathway shows the non-oxidative pathway to 4-hydroxybenzaldehde via an intermediary product, 4-hydroxyphenyl-β-hydroxypropionic acid. (From Podstolski *et al.* (2002); Copyright Elsevier 2002.)

pod tissue to 4-hydroxybenzaldehyde and vanillin (Havkin-Frenkel and Belanger 2007). Cloning enzymes from this family might be a potential route to the biotechnological production of vanillin in cell-free extracts. A recent study found that a similar mechanism applies to the cleavage of 2-coumaric acid to salicylic aldehyde (Malinowskia *et al.* 2007).

It appears that the mode of action of a putative chain-shortening enzyme is critical for the production of the desired end product. This reaction, which previously was proposed as the first step in formation of 4-hydroxybenzoic acid in *L. erythrorhizon* (Yazaki *et al.* 1991) and carrot cell cultures (Schnitzler *et al.* 1992) is presumed to involve the formation of an unstable 4-hydroxyphenyl-1-hydroxypropionic acid as an intermediate. A non-oxidative chain-shortening enzyme, which catalyzes an aldolase-like process, leads to the formation of aldehydic products, such as 4-hydroxybenzaldehyde that could then be methylated to form vanillin. This type of enzyme activity is found in plants (Podstolski *et al.* 2002; Malinowskia *et al.* 2007; Havkin-Frenkel and Belanger 2007). In contrast, microbial or fungal chain-shortening enzymes, involving the activity of a CoA thioester, appear to catalyze the conversion of phenylpropanoid compounds to acid products, including vanillic acid or hydroxypropionic acid.

The non-oxidative chain-shortening mechanism, involving a hydrolyase-like activity, which proceeds by hydration of the side-chain 2,3 double bond of 4-coumaric acid, is a likely reaction occurring in vanilla plant tissue leading to the cleavage of the side chain to yield acetate and 4-hydroxybenzaldehyde, an intermediate in the vanillin biosynthetic pathway (Havkin-Frenkel *et al*. 1996). We proposed to make use of an enzyme with a similar mode of action to degrade ferulic acid to vanillin. Accordingly, there is a patent (Havkin-Frenkel *et al*. 2004), which overcomes the problems described above and proposes the following: (1) To overcome the scarcity of ferulic acid and the difficulty involved in releasing the compound from cell walls, as well as interference by diferulates, we propose to use rosmarinic acid as the starting material. The rosmarinic acid will be hydrolyzed with esterase to caffeic acid. (2) The caffeic acid will be methylated using a caffeic acid *O*-methyltransferase (Pak *et al*. 2004) to generate ferulic acid. (3) A plant chain-shortening enzyme (Podstolski *et al*. 2002) will be used to cleave ferulic acid to vanillin and acetic acid.

Use of plant tissue culture

The metabolic potential of plants could be used in plant tissue or cell culture to synthesize useful compounds (see Chapter 4 of this book 'Plant cell culture as a source of valuable chemicals', by Chee-kok Chin). Several studies have attempted to exploit this potential for the production of vanillin in cultured cells and organs of *V. planifolia* (Knuth and Sahai 1988a,b; Funk and Brodelius 1990b; Knorr *et al*. 1993; Westcott *et al*. 1994; Havkin-Frenkel *et al*. 1996, 1998, 2003; Ramachandra and Ravishankar 2000). An inherent problem with plant tissue culture is a slow growth rate and low yields of desired products, and this problem was also encountered in attempts to produce vanillin. These issues put in doubt the commercial future of this concept, although future developments, particularly in biotechnology, might restore economic viability to the plant tissue culture approach.

When plant tissue culture is used as a biotransformation system, feeding of vanillin precursors, such as 3,4-dihydroxybenzaldehyde resulted in complete uptake of applied compounds from the media and close to complete conversion to vanillyl alcohol (Havkin-Frenkel and Pederson 2000; Herz 2000). It might be possible to couple the plant system to methano-bacteria, which could convert the vanillyl alcohol to vanillin. However, the precursor, 3,4-dihydroxybenzaldehyde, is not readily accessible in a natural form. In addition, coupling to yet another system (microbial) is an additional complexity, which might make the working concept economically prohibitive. For example, *Methylosinus trichosporium* OB3b oxidizes methanol to formaldehyde (Lee *et al.* 2004). We used this organism to convert vanillyl alcohol to vanillin (Havkin-Frenkel, unpublished data). Importantly, this microorganism did not metabolize vanillin further. Microorganisms such as those that do not metabolize aldehydes should be further investigated for the biotechnological production of vanillin.

Use of enzymes

Knowledge of the vanillin biosynthetic pathway and the gene products (enzymes), which catalyze successive steps in the process, might furnish an *in vitro* enzyme-based system for the production of vanillin. Biotechnology could potentially be applied to clone genes for relevant enzymes that could be used for the production of vanillin or vanillin intermediates. This system offers control over defined steps in the production process, for example, avoidance of vanillin conversion to vanillyl alcohol or vanillic acid, which often occurs when microorganisms are fed with precursors.

An example of one of the microbial enzymes used for vanillin production is that of Kamoda *et al*. (1989) who exploited lignostilbene αβ-dioxygenase (EC 1.13.11.43), isolated from *Pseudomonas* sp. TMY1009, to catalyze the oxidative release of vanillin from stilbenes, commonly found in wood bark. Synthetic enzymes, produced by DNA cloning and used in transformed microorganisms, were also exploited for the production of coniferyl alcohol, coniferyl aldehyde, ferulic acid, vanillin, and vanillic acid (Markus *et al*. 1992).

Vanillin can also be released from vanillylamine in capsaicin. Van den Heuvel *et al*. (2001) used the flavoprotein vanillyl alcohol oxidase (VAO) to convert both creosol and vanillylamine to vanillin with high yield. The VAO-mediated conversion of creosol proceeds via a two-step process in which the initially formed vanillyl alcohol is further oxidized to vanillin. This route to vanillin has questionable biotechnological potential because of the low amount of capsaicin in pepper or other plant sources. Creosol, also found to be converted to vanillin by the same enzyme, is a major component obtained from heating wood or coal tar but may not be considered a natural precursor.

Beta-glucosidase, though a degradative enzyme, is another enzyme that can be used to enhance the yield of vanillin. Beta-glucosidase catalyzes the hydrolytic release of vanillin from glucovanillin, the natural parent compound that accumulates in vanilla beans. The enzyme is important for the release of vanillin during the curing process, and commercial enzyme preparations (from almonds) have been used to enhance the production of vanillin in curing beans (Havkin-Frenkel *et al*. 2005; Dignum *et al*. 2001).

Use of physical and mild chemistry

Many naturally occurring compounds harbor the structure of vanillin – for example, capsaisin found in red peppers, ferulic acid which is abundant in plant cell walls, eugenol which is a major constituent of clove oil, curcumin which is found in turmeric, and phenolic stilbenes found in spruce bark. Many attempts have been made to release vanillin form these parent compounds using enzymatic methods, as discussed above, and also by chemical or physical methods. Usually, eugenol is a preferred compound for vanillin production, since it is found in large amounts in clove oil (up to 85%), as indicated by

many publications and patents dating back to the late nineteenth century. Vanillin released from parent compounds by chemical alterations may be considered a synthetic product, for example, vanillin released from lignin or eugenol.

In the case of eugenol, there are two main steps in vanillin production. The first steps involve isomerization of eugenol to isoeugenol under alkaline conditions followed by side-chain cleavage to vanillin and a two-carbon moiety under acid conditions (Fiecchi *et al*. 1967). However, in the past few years, modified protocols for vanillin production from eugenol have been implemented using high-heat high-pressure conditions, regarded by some as mild chemistry and, therefore, the resulting vanillin product is viewed as a natural product (see details in the section on Regulation). This source of vanillin is produced at the rate of 50 tons annually.

Synthetic vanillin

Most of the vanillin used in foods or by other industries is synthetic. Synthetic vanillin is produced at an estimated rate of 13 000 tons annually. In many applications, vanillin is used in combination with ethyl vanillin, which is not found in nature. For many years, vanillin was obtained from lignin by alkaline hydrolysis, since conifer wood contains up to 30% of its lignin as coniferyl alcohol derived from ferulic acid (Hocking 1997). Today, most synthetic vanillin is chemically synthesized from guaiacol in a cleaner process that has reduced the environmental impact compared with production from lignin. Only 10–15% of synthetic vanillin is produced from lignin.

Although lignin is a natural polymer generated as a by-product of the paper industry, extensive chemical modifications are required to generate vanillin, and so vanillin produced from lignin is considered to be synthetic. Guaiacol is a petrochemical product and, as with wood-derived vanillin, extensive chemical alterations render a product that is considered synthetic.

Vanillin from vanilla beans

Curing of green vanilla beans usually yields 2.5–4.5% vanillin or less, on a dry-weight basis of cured beans (Bala 2003; Ranadive 2003), corresponding roughly to 1.75–2.1% of vanillin in cured beans containing 30% moisture. By controlling factors that lead to the loss of vanillin, we found that the content of vanillin may reach around 7–8%, on a dry-weight basis, using an appropriate curing protocol (Frenkel and Havkin-Frenkel 2006). The content of vanillin in cured vanilla beans is an important index for the value and price of commercial vanilla beans. Owing to their high price, it is economically prohibitive to use vanillin-rich beans for the large-scale production of vanillin. However, there is such a product on the market for very specific applications, representing a minor portion of the vanillin market. Another product on the market is vanilla

CO_2 extract, considered to be a source of natural vanillin. This product, however, contains many impurities such as lipids and waxes, and commands a high price.

Vanillin is soluble mainly in organic solvents, but also exhibits some water solubility (up to 1% in water). Alkaline conditions greatly promote the solubility of vanillin, apparently because vanillin is a weak electrolyte resembling the behavior of other aromatic compounds (Heck *et al*. 1997), due to weak dissociation of hydrogen ions. Enhanced hydrogen-ion dissociation, by alkaline conditions and, subsequently, formation of vanillin in an ionized state may account for the solubility of the compound. However, solubility may result also from cation binding to the π (electron) face of aromatic structures, termed cation–π interaction. Cation–π interaction by an electrostatic attraction, through non-covalent force, is viewed as a process lending polarity to hydrophobic matrices (Dougherty 1996) and an additional force that might further increase the water solubility of vanillin upon the introduction of bases.

These conditions provide several strategies for manipulating the extraction, concentration, and purity of vanillin. Thus, vanillin is ordinarily extracted from vanilla beans in water–alcohol mixtures. The alcohol can then be removed and the remaining aqueous solution is brought to alkaline conditions, in which vanillin solubility is high. This mixture is next extracted in a non-polar solvent to remove impurities, such as lipids, followed by acidification to attenuate the affinity of vanillin to the solvent. Under these conditions, in which vanillin is not soluble, it can be removed by sublimation, resulting in a highly purified product.

Regulations

The two bodies for flavor regulation are the US Food and Drug Administration (regulation CFR 21) and the European regulatory body (regulation EC88/388); the EU regulation follows principally the direction taken by the French regulatory authority (the DGCCRF). According to the US FDA regulation, vanilla has a standard of identity, and is the only flavor that has this status. Accordingly, the only vanillin that can be labeled as 'natural vanillin' is vanillin that comes from vanilla beans. Any other vanillin, regardless of the source, and even when obtained from a natural source and by a natural process, cannot be labeled as 'natural vanillin'. This type of vanillin can be labeled as 'natural flavor' in a non-vanilla product. The last sentence is the interpretation of the law, as understood by the authors, and is shared by others in the industry.

In the EU, unlike the USA, natural vanillin has the same status as any other natural flavor, that is, it is produced from natural sources and by natural processes. For example, ferulic acid obtained by enzymatic hydrolysis from grain and converted to vanillin by enzymes or microorganisms complies with the EU definition of natural vanillin. The DGCCRF in France (on whose regulation the EU regulation is based) ruled that natural vanillin must have an

isotopic deviation equal to or greater than –21.2 0/00 PDB. The only current source of natural vanillin conforming to this regulation is the one obtained by fermentation from natural ferulic acid isolated from rice (Desmurs *et al*. 2004). Vanillin obtained from eugenol by a high-heat high-pressure process and carrying a certificate of 'natural' status is also a controversial issue. The issue of vanillin labeling will not be settled until the US FDA issues a clear ruling on this matter. Until then, interpretation of the law will vary with the different parties.

Conclusions and future outlook

In this chapter, we have explored the potential for biocatalytic processes, engineered by biotechnology, to offer control over specific metabolic reactions and to be exploited for the production of specific chemicals. This approach is constrained, however, by the following:

(1) Ferulic acid, used as base material for biocatalytic conversion to vanillin, is not abundant in natural sources (consisting of plant cell wall-bound ferulate).
(2) Intracellular conversion of ferulic acid yields mostly vanillic acid, vanillyl alcohol, and related compounds but very little vanillin. The authors believe that biological cells alter vanillin to other metabolites, because vanillin is perceived by various cells as a xenobiotic compound. This view is consistent with the mode of vanillin biosynthesis and accumulation in the vanilla pod, whereby vanillin is glycosylated, released to the cell exterior, and accumulated in intercellular spaces.

This perspective suggests that future efforts for the biotechnological production of vanillin must address the incompatibility of vanillin with cellular metabolism. This problem could perhaps be addressed by developing means for the rapid expulsion of newly synthesized vanillin to the cell exterior. Alternatively, vanillin could be produced by cell-free systems, where vanillin is not subjected to cellular metabolic alterations.

Finally, according to the US FDA regulation, biotechnologically produced vanillin may not be labeled as 'natural vanillin' and, in addition, may meet with consumer resistance due to the mode of production by genetically modified organisms. Vanillin obtained from cured vanilla beans is truly the only 'natural vanillin'.

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Chapter 4
Plant cell culture as a source of valuable chemicals

Chee-Kok Chin

Introduction

Plants are a rich source of flavor compounds, many of which have been exploited to enhance the pleasure and gratification of food and drink. Many of the plants that produce desirable flavor compounds are being cultivated, but the yields of the flavor compounds are variable and often are quite low. Generally, many of the flavor compounds are not produced by all plant tissues, or at all stages of the plant development. Rather, they are usually produced only in specific tissues found in specific organs such as fruit, flower, leaf, or root, and only at certain stages of the organ and tissue development, e.g. benzaldehyde from apricot is primarily produced in mature seeds and not by other tissues. These developmental and tissue specificities greatly limit the yield of a flavor compound from a plant. Another constraint is that cultivation of many of the plants that produce valuable flavor compounds, e.g. vanilla and cinnamon, is limited to certain geographical and climatological locations. In the light of these constraints associated with extraction from cultivated plants, attempts have been made to explore alternative approaches for production of valuable secondary plant metabolites. Chemical synthesis is an effective alternative for a large number of flavor compounds. However, many consumers still prefer natural, rather than synthetic, compounds.

Currently, advances in plant cell physiology and tissue culture technology have allowed plant cells to be grown in large volumes and, in many ways, this approach is similar to that of large-scale microbial culture. Since the industry has successfully used fermentors and bioreactors to produce flavor and pharmaceutical compounds derived from microorganisms, the practicality of producing plant flavor compounds by cultured plant cells has attracted considerable attention. However, even though much progress has been made with this approach, significant biological and technical issues need to be overcome before this production method can become commercially feasible. This chapter will provide an overview of plant cell culture and a review of the progress and challenges of using plant cell culture to produce flavor compounds.

Biotechnology in Flavor Production: A Case-based Approach. Edited by Daphna Havkin-Frenkel, Faith C. Belanger.
 ISBN: 978-1-4051-5649-3

Establishment of callus culture

Cultured plant cells are typically maintained in suspension in liquid nutrient medium under agitation. Hence, it is commonly called a suspension cell culture. A culture can be derived from a variety of plant organs, including leaf, flower, stem, root, etc. However, a suspension cell culture is usually not initiated directly from plant tissues or organs, as cells in these materials do not readily proliferate and separate in liquid medium. Thus, a common practice is to first generate callus tissue from a plant organ on a solid nutrient medium and then to use the callus tissue to initiate the suspension cell culture.

Although callus tissue is generally characterized as a mass of undifferentiated cells, the morphology and physiology of calli can be highly variable. Calli developed from a plant can be soft, firm, smooth, nodular, or fluffy, and their cells can vary in size, shape, cytoplasm density, and cellular vacuolation. These differences evidently reflect differentiation, even though callus tissues and their cells may not resemble commonly recognizable differentiated plant tissues and cells. In spite of its name, plant cell cultures do not consist only of individual cells. Instead, they typically contain a mixture of single cells as well as cell colonies of various sizes. The presence of large colonies or clumps of cells is not desirable, as they are heavier and tend to sink to the bottom of the medium where the oxygen level is lower, resulting in a lower growth rate. Large colonies also reduce the diffusion of oxygen and nutrients to the cells at the interior of the colonies. In contrast, suspension cell cultures consisting primarily of single cells and small cell colonies have faster growth rates and can be readily maintained as with many microbial cultures.

An ideal suspension cell culture is one that consists primarily of single cells and very small cell colonies. The characteristics of a cell culture have been found to have a close correlation with the morphology of the callus tissue from which the cell culture was derived. Thus, the morphology of the callus is very pertinent to initiation of a high-quality suspension cell culture. Generally, firm calli with cells that are difficult to separate are poor materials for cell culture initiation. On the other hand, friable and fluffy calli with cells that are loosely held together readily produce cultures of well-separated cells. Hence, for the initiation of suspension cell cultures, loose and friable calli are strongly preferred.

Many factors affect callus growth and development, the major ones being genetic background and physiological status of the source tissue, chemical composition and physical state of the culture medium, and cultural conditions. Young leaf or stem tissue is usually used as a source tissue in callus induction. Nevertheless, it has been noted that calli generated from different organs could be very different and calli derived from leaf tissue may not be the best tissue for cell culture initiation. Thus, experimentation to identify the appropriate organ from which to generate callus of the desired morphology is highly recommended. In addition to tissue source, the developmental stage of a tissue

also usually has an effect. As a rule, juvenile tissue is more conducive than mature tissue for callus induction. Hence, seedling and meristematic tissues are good choices for source tissues.

Culture medium plays an important role in callus development. Plant culture medium typically contains mineral salts, a carbon source, vitamins, and plant growth regulators. The salts supply the essential elements for the cells. The major elements for a medium (in mM ranges) include N, P, K, S, Ca, Mg, and the minor elements (in μM ranges) include B, Co, Cu, Fe, Mn, Mo, I, and Zn. Over the years, numerous media with different compositions of salts have been formulated. While many plant cells may adapt to a range of media, the response of tissues to different media can be very notable. Therefore, selection of an optimum medium is important. Some of the media used in callus initiation are the Murashige and Skoog (1962), Gamborg B5 (Gamborg *et al*. 1968), and White (1943) media. Generally, herbaceous tissues respond well to a medium with a higher salt concentration such as the Murashige and Skoog medium, and tissues of woody plants respond well to a medium with a lower salt concentration such as the White medium. All the constituents of a medium may affect callus growth and development, but the ones that have the most pronounced effects are plant growth regulators.

Among the different classes of growth regulators, usually only two classes of growth regulators, namely auxin and cytokinin, are used. Auxin is known for its ability to promote root formation, while cytokinin is known to promote shoot formation. At appropriate concentrations, a combination of auxin and cytokinin can induce callus formation. The auxins that are commonly used include indoleacetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), diclorophenoxyacetic acid (2,4-D), dicamba, and picloram. Commonly used cytokinins are kinetin, benzyladenine, zeatin, and 2iP (N6-(3-methylbut-2-enyl) adenine).

Although plant growth regulators belonging to the same class generally have similar effects on tissues, the potency varies with individual growth regulators. For examples, the cytokinin benzyladenine is generally more potent than kinetin, and the auxin 2,4-D is usually more potent than IAA. Needless to say, concentrations play an important role in the effects of growth regulators on growth and development. Optimal concentration depends on both the growth regulator and the plant tissue. In most cases, the effective growth regulator concentrations are in the range from 0.1 to 20 μM. In addition to the concentration of either the auxin or cytokinin, the relative concentrations of the two growth regulators can also have profound effects. Systematic studies may be required to identify the most favorable growth regulators and their concentrations for callus induction.

Other than the chemical composition, the physical state of a culture medium can also affect callus development. Medium for callus culture is solidified using gelling agents such as agar, gellan gum, agarose, etc. These substances are added to the medium and, after autoclaving and cooling, they polymerize into a gel state. Concentration of the gelling agent will affect the hardness of

the gel, which in turn can affect callus development. Normally, 0.5–0.8% of agar or 0.4–0.6% of gellan gum such as Gelrite is used.

Cultural conditions such as temperature, quality, and intensity of light and duration of photoperiod can also affect callus development. For example, callus developed in the light can be considerably different – both morphologically and physiologically – from that developed in the dark. There is no definitive guideline for appropriate cultural conditions for optimal callus development. Such conditions often depend on the genetic background and physiological state of the plant tissue.

As described above, a multitude of factors can affect callus growth and development. These factors vary with plant genotype and tissue source. Certain genotypes are amenable to a broad range of medium composition and/or cultural conditions, whereas others have a very narrow tolerance to medium compositions and cultural conditions. Hence, it is not possible to provide consistent specific guidance for optimal conditions. Tedious and systematic identification of optimal conditions is necessary when a source tissue does not produce callus with the desired characteristics.

Initiation and maintenance of cell culture

Once friable callus with cells loosely held together is obtained, the callus tissue can be used for initiation of cell culture. Just as for callus culture, various culture media can be used for cell culture. Among the popular ones are Murashige and Skoog (1962), Gamborg B5 (Gamborg *et al*. 1968), and White (1943) media. The culture medium should also contain the plant growth regulators auxin and cytokinin. While several auxins can promote cell division of suspension cell cultures, in many cases 2, 4-D has been found to be most effective. Thus, it is widely used in plant cell culture medium. Cell culture medium does not contain a gelling substance so the medium remains liquid to facilitate cell separation. Initiation of culture involves transferring highly friable callus to the liquid medium. For less friable callus, the callus may require teasing apart to separate the cells. Cells can be cultured in various culture containers, e.g. a Petri dish, T-flask, Erlenmeyer flask, fermentor, etc. Approximately 0.5–1 g of callus tissue is transferred to every 10 mL of culture medium. Typically, cell culture goes through lag, acceleration, exponential, plateau, and decline phases.

Plant cells have the ability to synthesize many essential organic molecules such as organic acids, amino acids, and vitamins from simple medium constituents. During cell culture, initiation time is required for these essential organic molecules to accumulate to sufficient levels, resulting in a lag phase in the growth. High cell density will accelerate the build up of these organic nutrients. Hence, a low inoculum-to-medium ratio will lead to a longer lag phase, whereas a high inoculum-to medium-ratio will shorten or even eliminate the lag phase. As the culture grows, the cells will deplete the medium, and when the cells senesce they produce by-products that inhibit growth. To maintain

an active culture, it should be subcultured prior to reaching the plateau stage. This is done by transferring a volume of the culture to fresh medium at the exponential growth phase. The volume of the culture used for subculture is dependent on cell density, growth rate of the cells, and the desirable subculture interval time. Usually, the subculture inoculum is adjusted to fit into a weekly or biweekly subculture routine.

Cell cultures maintained in Petri dishes and T-flasks are convenient for cell biology studies as these vessels allow cells to be monitored using an inverted microscope. However, Petri dishes and T-flasks can accommodate only small culture volumes. For larger volumes, Erlenmeyer flasks or fermentors are used. Cultures of larger volumes require agitation to facilitate oxygen diffusion and to promote cell separation. Thus, plant cell culture is usually referred to as suspension cell culture. Agitation of the cell culture in an Erlenmeyer flask can be attained by various types of laboratory shakers. Orbital shakers are by far the most popular. Shaking speeds ranging from 60–200 rpm are routinely used. Plant cells may appear fragile and very susceptible to shear forces. In reality, they are reasonably tolerant to gyratory shaking. Higher shaker speeds, with orbiting speeds ranging from 200–300 rpm, may actually produce higher growth rates. To provide room for the culture to swirl around the flask, culture volume is best kept at 40–50% of the flask volume, e.g. 50–60 mL of culture in a 125-mL flask. While cultures of up to 5 L can be efficiently agitated with a shaker, it can be difficult to keep cultures of larger volumes in suspension with a shaker. Hence, large-scale cell cultures are usually grown in fermentors similar to those used for microorganisms.

As plant cells are heavier than microbial cells, as well as more sensitive to shear, agitation mechanisms are an important consideration in selecting or designing plant cell fermentors. Two common agitation approaches are airlift and stirring with an impeller. Large-scale fermentor systems for plant cells, including a two-stage 950 L airlift fermentor for the production of shikonin (Tabahashi and Fujita, 1991), a 5000 L fermentor for the production of *Catharanthus* alkaloids (Schiel and Berlin, 1987), a 20000 L fermentor for culture of *Panax ginseng* cells for ginseng production (Nitto Denko Corp, Japan) and a 75000 L fermentor for production of taxol (Phyton Biotech news release) have been successfully operated.

Production of valuable chemicals by cultured plant cells

Cultured plant cells have been shown to be able to produce a variety of valuable plant chemicals, including flavor compounds such as *cis*-3-hexenal (Chou and Chin 1994) and raspberry ketone (Borejsza-Wysocki and Hrazdina 1994; Pedapudi *et al*. 2000). However, for most valuable plant chemicals, there are significant biological obstacles to using cell cultures for commercial production. A common problem is that the cultured cells produce only low levels of the desired chemical, or they do not produce the chemical at all. Causes of this problem can be at the gene-expression, pathway-regulation, or precursor-availability levels.

The production levels may sometimes be enhanced by adjusting the culture medium composition, including the salts (Zenk *et al*. 1977; Ikeda *et al*. 1977; Fujita *et al*. 1981; Bohm and Rink 1988), carbon source (Misawa 1985; Berlin *et al*. 1983; Chou and Chin 1994), growth regulators (Fetto-Neto *et al*. 1993; Deus and Zenk 1982; Nakagawa *et al*. 1986; Kim *et al*. 1990; Meyer and van Staden 1995), and vitamins. Production of secondary chemicals could also be manipulated by altering cultural conditions such as pH (Roos *et al*. 2006), temperature (Ikeda *et al*. 1976; Courtois and Guren 1980; Zhang and Fevereiro 2007), light (Seitz and Hinderer 1988; Mulder-Krieger *et al*. 1984; Pedersen *et al*. 1992; Chou and Chin 1994), aeration (Kreis and Reinhard 1989; Thanh *et al*. 2006), and agitation (Kim *et al*. 1991; Pedersen *et al*. 1992). Unfortunately, optimization approaches generally work only with cells exhibiting low productivity caused by deficient biosynthetic-pathway activity, but are ineffective when the lack of production is due to the absence of expression of the genes for the pathway.

Many high-value plant chemicals of interest are secondary metabolites which are not required to maintain cellular house-keeping activities. Instead, these compounds are produced for specific purposes, including attracting insects for pollination or birds for seed dispersal, and for defense against pathogens, insects, or predators. Generally, secondary metabolites are not produced by all the cells of a plant, but rather are produced in specific differentiated cells. One example is the production of the chemicals picrocrocin, safranal, and crocin of saffron by *Crocus sativa* which are limited to the cells of the stigma and style. The differentiation process leads to the expression of genes for the biosynthetic pathways for the metabolites. The quandary of using cultured cells to produce chemicals can be traced to the process of cell culture establishment and maintenance. Generally, cells of the source tissue are mostly differentiated cells that do not readily divide and proliferate. The standard procedure to promote cell division of the source tissue is by inducing the cells to 'de-differentiate' with appropriate growth regulators. Subsequently, the cells in culture are kept in a de-differentiated state to maintain sustained growth. However, de-differentiation will turn off many of the genes associated with differentiation, including those involved in the production of secondary metabolites. Therein lies the conundrum: differentiated cells do not readily proliferate, but de-differentiation of the cultured cells turns off the genes for the biosynthetic pathways of interest.

One of the approaches to remedy this problem is to separate the cell culture into proliferation and production phases. The cultured cells are maintained in a de-differentiated state until a desired cell mass is reached; the cells are then induced with a suitable signal to turn on the biosynthetic pathway for the chemical of interest. The signal could be a component of the culture medium, exogenous hormone, and/or cultural conditions. One example is production of *cis*-3-hexenal by alfalfa cell culture. *Cis*-3-hexenal is produced at relatively low levels in green leaves of plants, and its production is associated with the chloroplasts. Chloroplasts usually do not develop in cultured plant cells, and

therefore the cells do not produce *cis*-3-hexenal. Induction of chloroplast development with high light intensity has been found to promote *cis*-3-hexenal production in alfalfa cells (Chou and Chin 1994).

Various secondary metabolites are produced by plants in response to plant pathogens, including fungi, bacteria, and viruses. Synthesis of these compounds can be triggered by the presence of pertinent pathogens. This phenomenon has been successfully exploited to elicit secondary metabolite synthesis with killed (autoclaved) pathogens. Live pathogens are not used because they may proliferate and contaminate the medium. Examples of elicitor induction include: (1) an increase in diosgenin, a bioactive saponin, production in cell suspension cultures of *Dioscorea deltoidea* by autoclaved mycelia of non-host-specific fungi (Zenk 1977); (2) an increase in sanguinarin production by a homogenate of the fungus *Dendryphion penicillatum* (Cline and Coscia, 1988); (3) synthesis of psoralen in cultured parsley cells by a preparation of *Phytophthora megasperma* (Tietjen *et al*. 1983); and (4) furanocoumerin phytoaleines in cells of *Petroselinum crispum* by *Phytophthora parasitica* (Fellbrich *et al*. 2000). Elicitation could also be obtained with constituents of the pathogens, such as cell wall fragments, oligosaccharides, glycoproteins, chitosan, fatty acids, and glycolipids. In fact, in many cases, even constituents of non-pathogenic organisms have been found to be able to elicit synthesis of secondary metabolites. Table 4.1 lists some examples of enhancement in metabolite production by elicitation with constituents from different organisms.

When cultured plant cells detect the presence of relevant pathogens or their constituents, the cells switch on pertinent signal transduction pathways, which in turn activate the biosynthetic pathways for specific secondary metabolites. Hence, in addition to microbial constituents, signal molecules found in plant

Table 4.1 Elicitation of secondary metabolite synthesis in cell culture by constituents of microorganisms.

Species	Elicitor	Product	Reference
Solanum tuberosum	Eicosapentaenoic and arachidonic acids from *Phytophthora infestans*	Sesquiterpenoid phytoalexin	Bostock *et al*. 1982
Ruta graveolens	*Rhodotorula rubra* cell wall	Acridone alkaloid	Eilert *et al*. 1984
Oryza sativa	*Fusarium mononiforme* mycelial cell wall	Diterpene	Ren and West 1992
Oryza sativa	Chitin	Diterpene	Ren and West 1992
Mentha piperita	Chitosan	Menthol	Chang *et al*. 1998
Taxus canadensis	Chitosan	Taxol	Linden and Phisalaphong 2000
Taxus chinesis	Oligosaccharide from *Fusarium oxysporum*	Taxol	Yuan *et al*. 2002; Li *et al*. 2003
Oryza sativa	Cerebroside from *Magnaporthe grisea* and *Rhizoctonia* spp.	Phytoalexins	Umemora *et al*. 2002
Silybum marianum	Yeast extract	Flavonolignan silymarin	Sanchez-Sampedro *et al*. 2005
Echinacea purpurea	Yeast extract	Phenolic glycosides	Li and Barz 2006

signal transduction pathways activated by pathogen or stress, such as jasmonic acid (Ali *et al*. 2006; Mueller *et al*. 1993), salicylic acid (Ali *et al*. 2006), ethylene (De and De 2005), and lipid second messengers have been successfully exploited to elicit secondary metabolite synthesis. Table 4.2 shows some examples of elicitation of production of secondary metabolites by signal molecules.

In addition to biological molecules, elicitation could also be obtained by abiotic molecules that include heavy metals such as Cu, Se, and Ag and stress factors such as ultra-violet light and heat shock. Examples of abiotic elicitation are listed in Table 4.3.

Depending on the compounds involved, some secondary metabolites are accumulated in the cells while others are excreted into the medium. The latter category includes sanguinarine, berberine, and anthraquinone. Accumulation of these compounds in the media can produce a feedback inhibition on their continuous production (Byun *et al*. 1990). To prevent feedback inhibition, the use of an inert second phase with high affinity to the metabolite in the medium, to partition off the metabolite, has been explored. The second phase can be solid or liquid and can also serve to concentrate the metabolic product.

Table 4.2 Elicitation of plant cell cultures with signaling compounds.

Species	Elicitor	Product	Reference
Solanum tuberosum	Eicosapentaenoic and arachidonic acids from *Phytophthora infestans*	Sesquiterpenoid phytoalexin	Bostock *et al*. 1982
Rubus idaeus	Ethephon (2-chloroethyl-phosphonic acid)	*p*-Hydroxyl-2-butanone	Pedapudi *et al*. 2000
Hypericum perforatum	Salicylic acid	Xanthone	Conceicao *et al*. 2006
Hypericum perforatum	Jasmonic acid	Hyperin (a bioactive napthodianthrone)	Walker *et al*. 2002
Nicotiana tobacum	Palmitoleic acid	*cis*-3-Hexenal	Hong *et al*. 2004
Uncaria tomentosa	Jasmonic acid	Ursolic acid, oleanolic acid	Feria-Romero *et al*. 2005
Dioscorea floribunda	Ethephon	Diosgenin	De and De 2005
Silybum marianum	Methyl jasmonate	Flavonolignan silymarin	Sanchez-Sampedro *et al*. 2005
Panax ginseng	Methyl jasmonate	Ginsenosides	Thanh *et al.* 2005; Wang *et al*. 2005; Ali *et al*. 2006
Panax ginseng	Salicylic acid	Ginsenosides	Ali *et al*. 2006
Taxus spp.	Phytosylfokine-α	Taxol	Kim *et al*. 2006
Azadirachta indica	Salicylic acid	Azadirachtin	Satdive *et al.* 2007

Table 4.3 Abiotic elicitation of secondary metabolite production in plant cell cultures.

Species	Elicitor	Product	Reference
Taxus yunnanensis	Lanthanum	Taxol	Wu *et al*. 2001
Portulaca oleracea	Cu^{2+}	Betacyanin	Bhuiyan and Adachi 2003
Lavandula vera	Vanadyl sulfate	Rosmarinic acid	Georgiev *et al*. 2006
Medicago truncatula	Ultra-violet light	Amino acids	Broeckling *et al*. 2005

For example, Amberlite ion-exchange resins, XAD-2, XAD-4, XAD-7, and XAD-8 have been used successfully as solid extraction phases to significantly increase (1) the accumulation of anthroquinones from suspension cell cultures of *Cinchona ledgeriana* (Robins and Rhodes 1986); (2) the production of ajmalicine, catharanthine, and serpentine by *Catharanthus roseus* (Lee-Parsons and Shuler 2002); and (3) the production of benzophenanthridine alkaloids from cell cultures of *Eschscholtzia californica* (Kalvana *et al*. 2005). Byun *et al*. (1990) used a silicone fluid (demethyl siloxane polymer) as a liquid second phase to adsorb the benzophenanthridine alkaloids from suspension cell culture of *E. californica*. A concentration of the silicone fluid of up to 23% (vol/vol) of the culture resulted in a very large increase in alkaloid production without inhibiting growth of the culture. Moreover, recovery of benzophenanthridine alkaloids was greatly simplified as almost all were accumulated in the silicone fluid second phase (Byun *et al*. 1990).

Typically, biosynthetic pathways of secondary metabolites of interest involve multiple steps. Occasionally, production of a chemical is limited by the availability of precursors or intermediates of the pathway. Thus, attempts have been made to increase production by precursor feeding, and this approach has been found to be effective for some chemicals. For this approach to be successful, the enzymes in the biosynthetic pathway must be present and active. Table 4.4 lists some of the examples of precursor or intermediate feeding to increase the downstream production of metabolites. It should be noted, however, that although, downstream products of a precursor are obtained in many cases, the products are not necessarily the targeted ones. More importantly, while in most cases the magnitudes of the increases are notable, e.g. a few fold, the

Table 4.4 Increase in secondary metabolite production by precursor feeding of plant cell cultures.

Species	Precursor/intermediate	Product	Reference
Glycyrrhiza glabra	Papaverine	Papaverinol	Dorisse *et al.* 1988
Eschschotzia california	Tyrosine	Sanguinarin	Byun *et al*. 1990
Holarrhena antidysenterica	Cholesterol	Conessine	Panda *et al*. 1992
Catharanthus roseus	Secologanin, loganin, loganic acid	Ajmalicine, strictoside	Moreno *et al*. 1993
Medicago sativa	Linolenic acid	*cis*-3-Hexenal	Chou and Chin 1994
Rodiola rosea	*trans*-Cinnamyl alcohol	Rosavin	Furmanowa *et al*. 1999
Rubus idaeus	L-Phenylalanine	*p*-Hydroxyl-2-butanone	Pedapudi *et al*. 2000
Alliums	Allylthiol, allyl cysteine	Alliin	Huges *et al*. 2005
Fragaria x ananassa	L-Phenylalanine	Anthocyanin	Edahiro *et al*. 2005
Catharanthus roseus	Geraniol, loganin	Ajmalicine	Lee-Parsons and Royce 2006
Ginkgo biloba	Geranyl pyrophosphate, geranylgeranyl pyrophosphate, isopentenyl pyrophosphate, dimethylalyl pyrophosphate, farnesyl pyrophosphate	Ginkgolides	Kang *et al.* 2006

increases are still insufficient to make the process competitive with production by extraction from field-grown plant tissues.

Concluding remarks

Plant suspension cell culture technology has advanced to such an extent that plant cell culture can now be scaled up to very large volumes. Yet, challenges still remain in exploiting the cultured plant cells to economically produce valuable chemicals on a large scale. The key challenge is the lack of chemicals of interest or low production levels of those chemicals by the cultured cells. Notwithstanding that various manipulations, including adjusting medium composition and cultural conditions, elicitation, precursor feeding, and two-phase culture have been found capable of improving chemical production to various degrees, production of many chemicals of interest by cell culture remains economically non-competitive when compared with extraction from cultivated plants. While, in certain cases, the low production rates are the result of insufficient precursors or negative biochemical control, in most cases the principal constraint can be traced to an absence of, or low expression of, the genes for the biosynthetic pathway. In these instances, future research should target the expression of the genes that limit chemical production. To do so will require full elucidation of the biosynthetic pathway and its genetic control. At present, only very few of the biosynthetic pathways for the huge pool of secondary metabolites have been elucidated. Another complication is that many of the biosynthetic pathways are not necessarily linear, i.e. there may be diversions to other pathways and products. Nevertheless, by identifying and cloning the genes for the pathway, the expression of these genes can be monitored, and the step or steps of the constraint can be recognized.

Regulation of gene expression can be engineered to allow for induction or constitutive expression of the genes in the pathway. Information on the biosynthetic pathways may also be of help in engineering to silence the genes controlling the pathway shunts to other products. Transgenic cells that can fully express the genes for the synthetic pathway constitutively or with an inducer should allow the cell culture to produce the chemical of interest competently and economically.

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Chapter 5
Tomato aroma: Biochemistry and biotechnology

Rachel Davidovich-Rikanati, Yaniv Azulay, Yaron Sitrit, Yaakov Tadmor and Efraim Lewinsohn

The major aroma impact volatiles in tomato and their biosynthetic pathways

The tomato (*Solanum lycopersicum*, formerly *Lycopersicon lycopersicum*, Solanaceae), a plant native to the Americas, is cultivated worldwide for its edible fruits. The unique flavor of tomato fruit renders it as one of the favorite ingredients of both popular and high-cuisine dishes. The tomato flavor, as with the flavors of many other fruits and vegetables, is due to a complex mixture of volatile and non-volatile compounds (Buttery *et al.* 1971; Petro-Turza 1986; Baldwin *et al.* 2000). Extensive studies of the tomato aroma volatile composition have yielded the identification of more than 400 different compounds (Buttery *et al.* 1971, 1987, 1990; Petro-Turza 1987; Linforth *et al.* 1994; Baldwin *et al.* 2000). Of these, about 30 compounds appear in concentrations greater than 1 nL/L and therefore might have a significant impact on tomato flavor and aroma. However, since the human nose can be very sensitive to one odorant and insensitive to another, some volatiles are perceived as strong odorants in very small concentrations. For example, β-ionone can be detected by the human nose at concentrations of only 0.007 nL/L, while other odorants, such as pentanol, are detected only at concentrations greater than 4000 nL/L.

To establish a more realistic list of volatiles important to tomato aroma, Buttery *et al.* (1971) determined the odor thresholds (the minimal concentration of a compound required for detection by the human nose) of 30 compounds and determined their log odor unit, the log of the ratio of the concentration of a component in a food to its odor threshold. It was shown by Buttery *et al*. (1971) that only 16 compounds have a positive log odor unit value and are thus likely to have an impact on the fruit aroma. According to this cutoff, Buttery *et al*. (1971) suggested that the aroma of fresh ripe tomato is attributed mainly to *cis*-3-hexenal, *cis*-3-hexenol, hexanal, 1-pentene-3-one, 3-methylbutanal, *trans*-2-hexenal, 6-methyl-5-hepten-2-one, methyl salicylate, 2-isobutylthiazole, and β-ionone at the appropriate concentrations (see Fig. 5.1). These compounds are biosynthetically derived from the degradation of larger compounds such as fatty and amino acids and carotenoids as well as formed through other biosynthetic routes (see the following page).

Biotechnology in Flavor Production: A Case-based Approach. Edited by Daphna Havkin-Frenkel, Faith C. Belanger.
 ISBN: 978-1-4051-5649-3

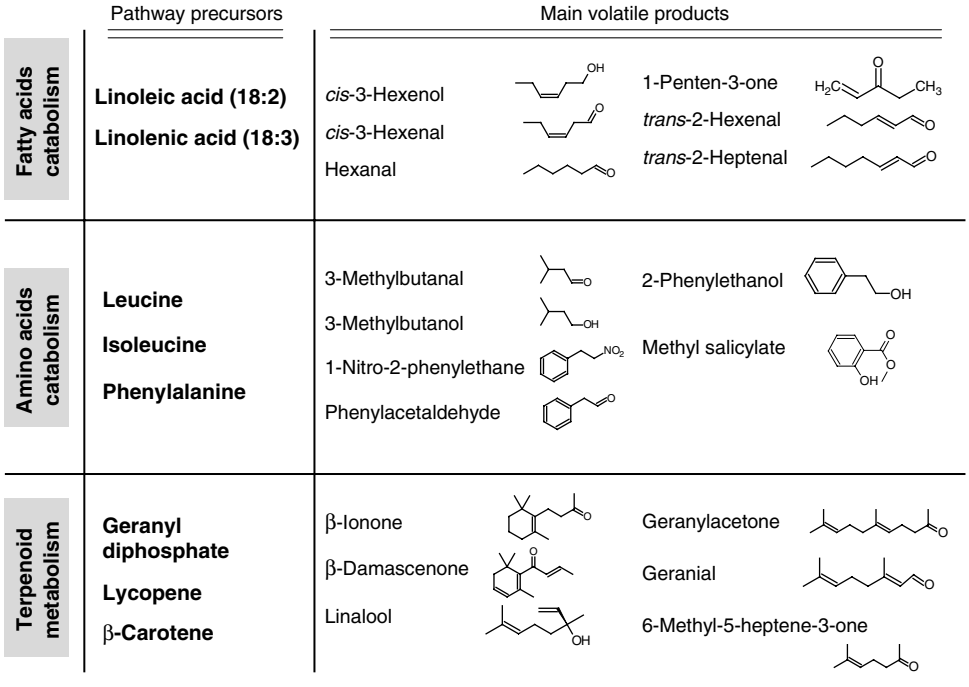

Fig. 5.1 Distribution of the main volatiles present in fresh tomato by their metabolic pathway and main precursors. Volatiles present at levels >1 nL/L and considered to have a contribution to tomato aroma (based on Buttery and Ling 1993) are shown together with their chemical structure.

Biosynthesis of tomato volatiles

Degradation of fatty acids

The most abundant volatiles in tomato fruits are short-chain aldehydes and alcohols such as *cis*-3-hexenol and *cis*-3-hexenal (Galliard *and Matthew* 1977; Buttery and Ling 1993). These volatiles are associated with flavors described as 'tomato-like', 'green', or 'grassy', and are the products of lipid degradation (see Fig. 5.1). During fruit ripening or upon tissue disruption, fatty acids come in contact with catabolic enzymes, and the volatiles are subsequently released (Baldwin *et al.* 1991). Thus, short-chain aldehydes are formed from lipid degradation by the action of lipoxygenases (LOX) and hydroperoxide lyases (HPL), converting fatty acids such as linoleic (18:2) and linolenic (18:3) acids to hexanal and *cis*-3-hexenal. The aldehydes can be further reduced into alcohols by the action of alcohol dehydrogenases (ADH) (Sieso *et al.* 1976; Bicsak *et al.* 1982; Longhurst *et al.* 1990). The biosynthetic pathway of volatiles derived from fatty acids is illustrated in Fig. 5.2. Further isomerization of *cis*-3-hexenal to *trans*-3-hexenal might occur either enzymatically or non-enzymatically. The activity of the enzymes that participate in lipid breakdown

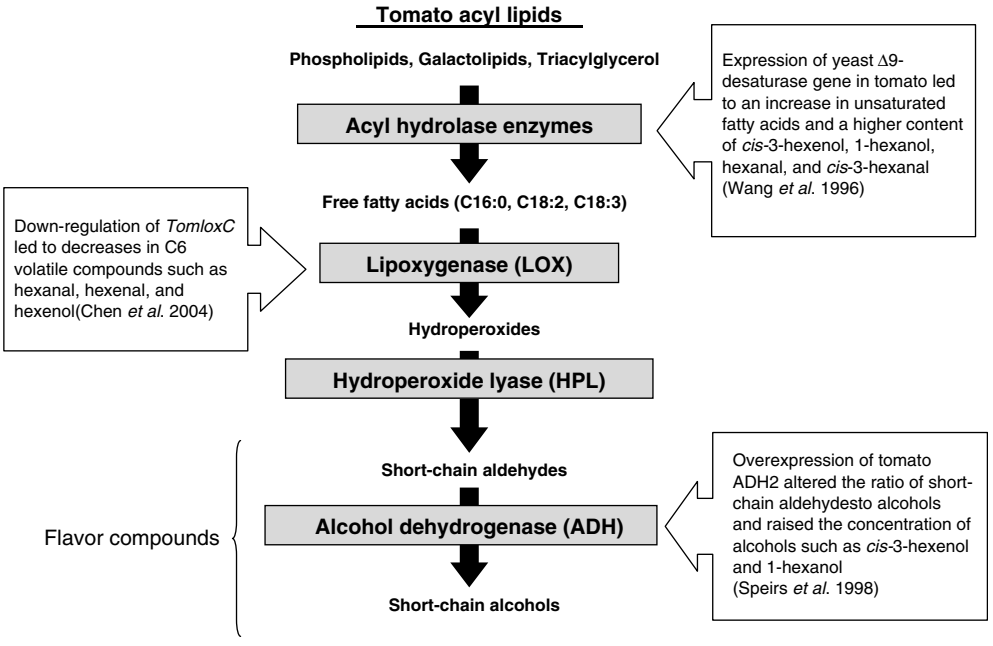

Fig. 5.2 Short-chain aldehydes and alcohols produced from the degradation of fatty acids in tomato fruits via the lipoxygenase (LOX)/hydroperoxide lyase (HPL) pathway. Genetic manipulations of the fatty acid catabolism pathway in three key points of the pathway are described.

increases during ripening, causing the levels of hexanal and *cis*-3-hexenal to rise (Baldwin *et al.* 1991).

Volatiles derived from amino acids

A second group of volatiles that contribute to tomato flavor is derived from the essential amino acids leucine, isoleucine, and phenylalanine (Buttery *et al.* 1971; Petro-Turza 1987; Baldwin *et al.* 2000). These volatiles include 2- and 3-methylbutanal, 3-methylbutanol, phenylacetaldehyde, 2-phenylethanol, and methyl salicylate, and are important flavor constituents of many other fruits, including strawberries and apples as well as processed foods such as breads, cheese, wines, and beer. The exact pathways of the biosynthesis of the individual compounds are poorly understood, but a few key enzymes have been shown to participate in these pathways. Short branched-chain derivatives probably evolve from branched amino acids by decarboxylation and deamination through a shortened acyl-coenzyme A derivative (Croteau and Karp 1991). For example, leucine is a precursor of 3-methylbutanol and 3-methylbutanal in tomato (Yu *et al.* 1968). The major pathway for the synthesis of 2-phenylethanol from phenylalanine in tomato has recently been reported. Tieman *et al.* (2006a,b) identified a family of aromatic amino acid decarboxylases (AADCs) and showed that these enzymes decarboxylate phenylalanine to phenethylamine. Phenethylamine is presumably converted by an amine

oxidase, dehydrogenase, or transaminase to 2-phenylacetaldehyde, which in turn is converted to 2-phenylethanol by 2-phenylacetaldehyde reductase (Tieman *et al.* 2006a,b). However, in petunia flowers, it appears that the same decarboxylating enzyme is also able to deaminate phenylalanine to directly release phenethyl aldehyde (Kaminaga *et al.* 2006), but this has not been proven for tomato.

Phenylalanine is also the precursor of eugenol in sweet basil (Koeduka *et al.* 2006). Eugenol is a phenylpropene compound reminiscent of cloves' aroma that is present in tomato at low levels. A recently described enzyme termed 'eugenol synthase' converts *trans* cinnamic acid (the product of the ubiquitous plant enzyme phenylalanine ammonia lyase, PAL) into eugenol. This phenylpropene-forming enzyme belongs to a structural family of NADPH-dependent reductases (Koeduka *et al.* 2006). Methyl salicylate is present in tomatoes, but is also an important component of the essential oil of wintergreen. Methyltransferases that act specifically on salicylic acid are known in snapdragon and petunia (Zubieta *et al.* 2003), but the pathways for the biosynthesis of eugenol and methyl salicylate in tomato are still unknown.

Terpenes

Terpene volatiles in tomato may derive directly from geranyl diphosphate (GDP) by the action of monoterpene synthases or from the breakdown of larger terpenes such as the carotenoid pigments (Stevens 1970; Buttery and Ling 1993; Lewinsohn *et al.* 2005a,b; Goff and Klee 2006; Pichersky *et al.* 2006). Carotenoids and monoterpenes share a common biosynthetic origin, both starting from the condensation of isopentenyl diphosphate through a plastid-localized pathway initiated by the condensation of pyruvate and glyceraldehyde 3-phosphate. Isopentenyl diphosphate is converted into GDP, farnesyl diphosphate (FDP), and geranylgeranyl diphosphate (GGDP) en route to carotenoid biosynthesis. GDP can also serve as the precursor for monoterpenes which are important contributors to many floral and fruit fragrances (Pichersky *et al.* 2006). Although the terpenoid pathway is highly active in ripening tomato fruits, leading to the production of the red tetraterpene pigment lycopene, ripe fruits contain only minute amounts of monoterpenes (Buttery *et al.* 1971; Petro-Turza 1987; Baldwin *et al.* 2000). Among the monoterpenes found in tomato are citral, a mixture of the *cis* and *trans* acyclic monoterpene aldehyde isomers (neral and geranial, respectively), which possesses an agreeable scent, reminiscent of lemon and linalool, an acyclic monoterpene alcohol which has an aroma that is reminiscent of flowers (Pichersky *et al.* 1995).

Of particular interest is a group of carotenoid-derived volatiles, the norisoprenes (apocarotenoids), produced from oxidative cleavage of carotenoids (Stevens 1970; Baldwin *et al.* 2000; Simkin *et al.* 2004; Lewinsohn *et al.* 2005a,b). Norisoprenoids are generally present at relatively low levels but possess strong effects on the overall human appreciation of the flavor of

tomato (Buttery *et al.* 1971, 1987; Baldwin *et al.* 1991, 2000), carrots (Kjeldsen *et al.* 2003), and watermelon (Lewinsohn *et al.* 2005a,b). Among these are β-ionone, geranylacetone (6,10-dimethyl-5,9-undecadien-2-one) and pseudoionone (6,10-dimethyl-3,5,9-undecatrien-2-one). Comparative genetic analysis of the carotenoid composition and the volatiles emitted from tomato fruits indicated that carotenoid pigmentation patterns have profound effects on the norisoprene and monoterpene aroma volatiles of tomato. In these fruits, geranial (*trans*-citral) is apparently derived from lycopene *in vivo* (see below). This biosynthetic pathway was further established by the isolation and functional expression of two tomato genes encoding for carotenoid cleavage dioxygenases (CCDs): *Le*CCD1A and *Le*CCD1B (Simkin *et al.* 2004). The encoding enzymes cleaved multiple linear and cyclic carotenoids resulting in the formation of a C14 dialdehyde and a variety of C13 products, depending on the substrate. Transgenic tomato plants constitutively expressing the carotenoid cleavage *LeCCD1B* gene in reverse orientation exhibited a 50% decrease in β-ionone (a β-carotene-derived C13 cyclohexone) and a 60% decrease in geranylacetone (a C13 acyclic product likely derived from a lycopene precursor), indicating an important role for CCDs in the production of carotenoid-derived volatiles.

Carotenoid pigmentation affects the flavor and volatile composition of tomato fruit

Tomato varieties displaying different colors due to different carotenoid compositions often differ in their flavor and aroma characteristics. In an early report by Stevens (1970) it was shown that the volatile profiles of tomato varieties differing in flesh color were closely related to the respective fruit carotenoid composition, a finding suggestive of carotenoid breakdown into aroma volatile molecules. Owing to insufficient knowledge of the carotenoid biosynthetic pathway and the lack of the appropriate genetic material, however, it was not possible at that time to show a conclusive direct relationship between color and aroma. More recent studies have taken advantage of advanced genetic material with near isogenic backgrounds to show such correlations between color and flavor.

Studies utilizing tomato near-isogenic lines, differing almost only in their carotenoid compositions, have revealed that the marked differences in carotenoid compositions do indeed correlate with differences in the composition of monoterpene and norisoprenoid volatiles (Lewinsohn *et al.* 2005a,b). Wild-type (red) tomatoes containing high concentrations of lycopene and lower concentrations of β-carotene also accumulate non-cyclic volatile norisoprenoids (such as 6-methyl-5-hepten-2-one, farnesyl acetone, (*E,E*)-pseudoionone, 2,3-epoxygeranial, 2,6-dimethyl hept-5-1-al, geranyl acetone, and dihydro-*apo*-farnesal) in addition to geranial, neral, and the cyclic norisoprenoid β-ionone (Lewinsohn *et al.* 2005a,b, see Plate 5.1). With the exception

of farnesyl acetone, such compounds are, for the most part, absent in the yellow flesh (*r*) mutant of tomato; this mutant lacks phytoene synthase activity and its fruit does not accumulate carotenoids (see Plate 5.1). The tomato *tg* mutant, which accumulates the orange pigment prolycopene (tetra-*cis*-lycopene) and higher levels of tetraterpene precursors (including phytoene, phytofluene ζ-carotene, and neurosporene) than the wild-type tomato (control), contains similar concentrations of non-cyclic norisoprenes and citral to those in the wild-type red tomato. The levels of 6-methyl-5-hepten-2-one (melonal), geranyl acetone, dehydro *apo*-farnesal, and farnesyl acetone are up to threefold higher in the *tg* mutant line than in the wild-type red tomato.

The tomato *Del* mutant, which accumulates high levels of δ-carotene, also produces α-ionone in addition to citral and the non-cyclic norisoprenes detected in the wild-type and *tg* tomatoes. The orange-colored *B* genotype accumulates high levels of β-carotene in addition to lycopene. Dihydroactinodiolide, β-ionone, and β-cyclocitral produced in the *B* line are apparently derived from β-carotene. Interestingly, although geranial is a monoterpene, it is consistently present in tomatoes and watermelons in a pattern that follows that of the non-cyclic norisoprenoid present in the fruit, indicative of its possible carotenoid cleavage origin.

In conclusion, lycopene, prolycopene, δ-carotene, and neurosporene give rise *in vivo* to the non-cyclic volatiles, neral and geranial, as well as to 6-methyl-5-hepten-2-one, 2,6-dimethyl hept-5-1-al, 2,3-epoxygeranial, and (*E*,*E*)-pseudoionone (Plate 5.1). β-Ionone, β-cyclocitral, and dihydroactinodiolide are apparently oxidative breakdown products of β-carotene. δ-Carotene appears to gives rise to α-ionone, probably by the cleavage of the ε-ionone ring of δ-carotene (Plate 5.1). Farnesyl acetone, dihydro-*apo*-farnesal, geranyl acetone, and 6-methyl hept-5-en-3-one are probably derived from phytoene or phytofluene. Thus, in essence, color and aroma compounds are highly associated in tomato and watermelon fruits, and this relationship is probably a function of the degradation of carotenoids into aroma volatiles. The correlation between carotenoid compositions and volatiles is also apparent in watermelon (Lewinsohn *et al.* 2005a,b) and melons (Ibdah *et al.* 2006), as well as in peppers and carrots (Y. Azulay *et al*., unpublished data), indicating that carotenoid degradation is an important route to the formation of aroma volatiles in many fruits and vegetables.

Owing to up to 1.5-fold elevated levels of lycopene and β-carotene, the 'high pigment' photomorphogenic mutants in tomato are characterized by increased pigmentation in fruits. Two types of such mutants are known: the *hp-2* mutants are due to different mutations in the gene encoding the tomato homologue of the *Arabidopsis thaliana DEETIOLATED1* (*DET1*) gene, a negative regulator of photomorphogenesis, while the *hp-1* mutants are characterized by mutations of the tomato homologue of the human and *A. thaliana* gene encoding the UV-damaged DNA-binding protein 1 (DDB1) (reviewed in Bino *et al.* 2005). Changes in the volatile levels of fruits of these mutants as compared to their wild-type counterparts have been noted (Bino *et al.* 2005).

However, these changes are apparently restricted to compounds derived from the lipid-degradation pathway, while the levels of carotenoid breakdown products such as β-damascenone and β-ionone seemed unchanged (Bino *et al.* 2005). At present, the biochemical and molecular mechanisms that bring about these differences in volatile compositions are unknown.

Genetic engineering of tomato aroma

Despite the advances in tomato flavor analysis, breeders and molecular biologists still lack a clear genetic target for selection and manipulation of tomato flavor quality (Galili *et al.* 2002; Tieman *et al.* 2006b). A few attempts have been made to modify the tomato aroma volatiles by genetic engineering. These attempts focused on overexpression or down-regulation of key enzymes involved in all three biosynthetic pathways mentioned above.

Manipulation of the fatty acid catabolism pathway was attempted in a few points of the pathway (shown in Fig. 5.2). Expression of a yeast Δ9-desaturase gene driven by CaMV 35S promoter in tomato led to changes in the levels of unsaturated as well as saturated fatty acids in tomato leaves and fruits (Wang *et al.* 1996, 2001). Increases in palmitoleic acid, 9,12-hexadienoic acid, and linoleic acid were observed along with a reduction in palmitic acid and stearic acid. Changes in the profile of fatty acids were associated with a significant increase in flavor compounds derived from fatty acids, most notably *cis*-3-hexenol, 1-hexanol, hexanal, and *cis*-3-hexenal. Certain flavor compounds not derived from fatty acids, *viz*. 6-methyl-5-hepten-2-one and 2-isobutylthiazole, also showed increases in transgenic fruits. These results show that changes in the levels of fatty acids in a plant could lead to changes in its profile of flavor compounds. However, the effect of these alterations in tomato volatiles on the flavor of the fruit was not evaluated.

The tomato gene *TomloxC* codes for a lipoxygenase that is involved in the generation of volatile C6 aldehyde and alcohol flavor compounds (Chen *et al.* 2004). Tomatoes with a reduced expression level of *TomloxC* mRNA possessed a marked reduction in the levels of known flavor volatiles, including hexanal, hexenal, and hexenol, to as little as 1.5% of those of wild-type controls following maceration of ripening fruit. Addition of linoleic or linolenic acid to fruit homogenates significantly increased the levels of flavor volatiles, but the increase with the *TomloxC*-depleted transgenic fruit extracts was much lower than with the wild-type controls. These results demonstrate that the *TomloxC* lipoxygenase isoform has a key role in the generation of the volatile C6 flavor compounds.

Overexpression of a tomato alcohol-dehydrogenase gene in a fruit-specific manner altered the ratio of short-chain aldehydes to alcohols (Speirs *et al.* 1998; Prestage *et al.* 1999). This manipulation resulted in small changes to the volatiles profiles of the fruits, and these were found to affect the perception of their aroma by a taste panel.

Genetic engineering of the phenylalanine catabolism pathway confirmed the biosynthetic route leading to the production of some phenylalanine volatile derivatives (Tieman *et al.* 2006a). Overexpression of either *Le*AADC1A or *Le*AADC2 (coding for enzymes that catalyze the conversion of phenylalanine to phenethylamine), resulted in fruits with up to ten-fold increased emissions of the products of the pathway, including 2-phenylacetaldehyde, 2-phenylethanol, and 1-nitro-2-phenylethane which are considered to have a negative effect on the flavor of tomatoes when in high concentration (Tadmor *et al.* 2002). Furthermore, antisense reduction of *Le*AADC2 significantly reduced emissions of these volatiles. These results show that it is possible to change phenylalanine-based flavor and aroma volatiles in tomatoes by manipulating expression of a single gene. However, in both cases, the effect of these alterations in the tomato volatiles on the flavor of the fruit was not evaluated.

The potential of genetic engineering to improve fruit aroma by modifying the early and downstream steps of the terpenoid pathway has also been demonstrated. The role of carotenoid cleavage deoxygenase (CCD) in the production of carotenoid-derived volatiles of tomato has been demonstrated by the down-regulation of the *LeCCD1B* gene (Simkin *et al.* 2004). Transgenic tomatoes exhibited a 50% decrease in β-ionone (a β-carotene-derived C13 cyclohexone) and a 60% decrease in geranylacetone (a C13 acyclic product likely derived from a lycopene precursor). However, the opposite effect of overexpression of CCDs in tomato is still absent. Unfortunately, the effect of these alterations in the tomato volatiles on the flavor of the fruit was not evaluated.

Monoterpenes are important contributors to many floral and fruit fragrances. These volatiles are synthesized from GDP, which is also an intermediate in the pathway leading to the biosynthesis of carotenoids (see Fig. 5.3). Therefore, this pathway is highly active in ripening tomato fruits, leading to the production of lycopene. The first attempt to genetically manipulate the tomato terpenoid pathway to produce monoterpenes was the ectopic expression of the *Clarkia breweri* linalool synthase (*LIS*) gene under the control of the tomato late-ripening E8 promoter (Lewinsohn *et al.* 2001). The expression of *LIS* in tomatoes led to the production and accumulation of detectable levels of linalool and 8-hydroxylinalool in ripening fruits, without noticeably affecting the accumulation of fruit carotenoids. These results indicated, for the first time, that it is possible to increase the levels of monoterpenes in tomatoes. However, the effect of this addition to the tomato aroma perception by consumers remained to be tested.

In a subsequent study, the tomato terpenoid pathway was modified by the ectopic expression of the geraniol synthase (*GES*) gene isolated from lemon basil (*Ocimum basilicum*) (Iijima *et al.* 2004) under the control of the fruit ripening-specific tomato polygalacturonase promoter (PG) (Nicholass *et al.* 1995; Davidovich-Rikanati *et al.* 2007). The volatile profiles of transgenic ripe tomato fruit showed high concentrations (5–1800 ng/g of fresh weight) of more than ten new monoterpene compounds that did not appear or appear in

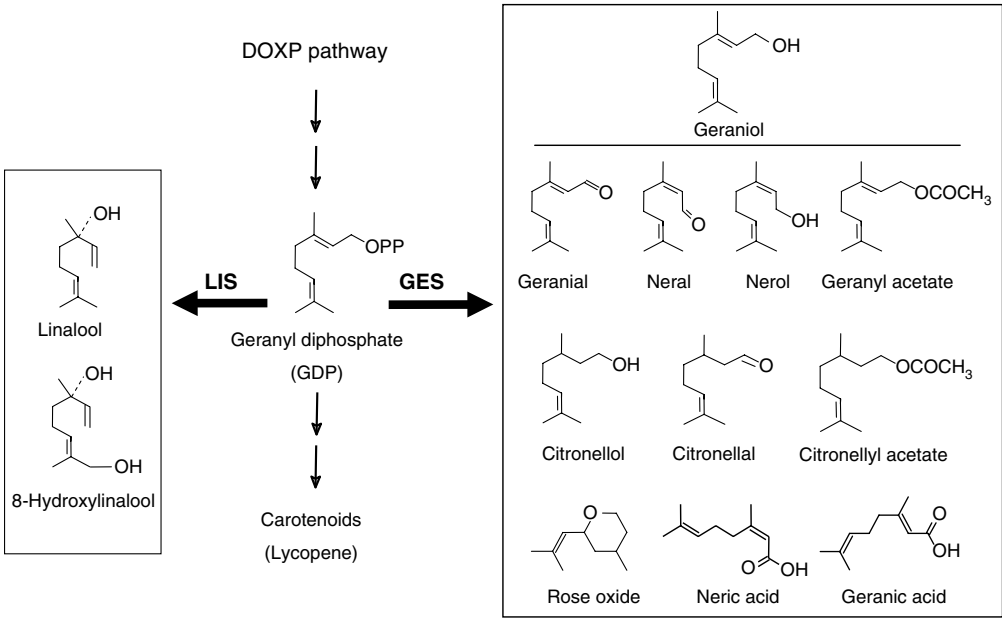

Fig. 5.3 Diversion of the existing plastid terpenoid pathway (DOXP, deoxy-D-xylulose 5-phosphate pathway), which leads to carotenoids, into the production of new monoterpenes in ripening transgenic tomatoes by the expression of the *C. breweri* linalool synthase (LIS) and the *O. basilicum* geraniol synthase (GES). Derivatives of linalool and geraniol produced in transgenic tomatoes were probably formed by the action of enzymes present in tomato fruit (Lewinsohn *et al.* 2001; Davidovich-Rikanati *et al.* 2007).

minute levels in the volatile profile of the control fruits. These compounds included the important aroma volatiles geraniol (possessing a strong rose scent) as well as the geraniol derivatives, citral (geranial and neral, lemon scented), citronellol (rose scented), geranic acid, and neric acid. A taste trial was conducted to study the effect of these added volatiles on the taste and aroma of the fruit. Indeed, taste panelists reported marked differences in the overall aroma and taste of the transgenic tomato fruits (Davidovich-Rikanati *et al.* 2007). The aroma of the transgenic fruits was often described to have an added 'flowery' or 'lemony' note.

Conclusion

The understanding of the chemistry of the aroma chemicals in tomato fruits has led to the elucidation of the biosynthetic routes to these compounds, which in turn has led to the identification of the genes involved. Genetic evidence has indicated a correlation between color patterns and aroma in tomato fruits. Genetic engineering has led to the manipulation of these pathways, confirming the biosynthetic routes and affording novel flavors in tomato fruits. With the identification of more genes that are amenable for transformation and a better understanding of the biosynthetic pathway to aroma

chemicals and regulation, it will be possible to generate tomatoes with varied and enhanced flavors. The generation of novelty and niche products is therefore feasible, but it is for the consumer to decide whether to accept such innovative tomatoes.

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Chapter 6
Flavor development in rice

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Introduction

There are many distinct yet subtle flavors and textures that influence rice eating quality. Rice consumers are aware of these flavors and often demand what they perceive to be the best quality rices. The most desirable combination of traits can be unique to particular markets. Some consumers prefer the flavors and qualities of older stored rice, while other consumers have a preference for fresh rice flavors (Zhou *et al*. 2002). There are many chemicals that contribute to the aroma and flavor of rice (Table 6.1) and these are often associated with long-term storage. Methods have been developed to remove or mask flavors that are characteristic of stored rice (Arai and Watanabe 1994; Fukai and Ishitani 2004) in order to meet consumer preferences.

Old flavors of rice

Differing levels and combinations of various chemicals explain the flavors and fragrances associated with long-term rice storage. Masumoto *et al*. (2004) determined that 1-butanal, 1-hexanal, 1-heptanal, methyl ethyl ketone, 1-pentanal and propanal are responsible for what is known as the 'old' or 'stale' aroma of stored rice, while 1-butanal and 1-heptanal are involved in the aroma of 'fresh' rice. Many of these old flavors or fragrances, particularly hexanal, have been noted elsewhere (Zhou *et al*. 2002; Lam and Proctor 2003; Masumoto *et al*. 2004). Methods such as the addition of aromatic rice to old rice have been developed in an effort to mask or dilute old rice flavors (Fukai and Ishitani 2004). Protease treatment followed by washing in water is another method of old flavor reduction in stored rice (Arai and Watanabe 1994).

The amount of hexanal in rice grain has been reported to be linearly proportional to the concentration of oxidised linoleic acid in the grain (Shin *et al*. 1986). When stored at 35°C for longer than 2 weeks, several rice varieties have been shown to have markedly reduced pentanal, hexanal and pentanol levels compared to other rice varieties (Suzuki *et al*. 1999). Analysis of these varieties found reduced levels of one of the three lipoxygenase (LOX) isozymes, LOX-3, an enzyme known to be involved in peroxidation of some polyunsaturated fatty acids including linoleic acid (Suzuki *et al*. 1999). Introduction of the LOX-3-deficient character into many rice varieties using molecular breeding methods is now occurring in many breeding programs (Suzuki *et al*. 1999) in an effort to reduce the levels of old flavors of rice.

Biotechnology in Flavor Production: A Case-based Approach. Edited by Daphna Havkin-Frenkel, Faith C. Belanger.
 ISBN: 978-1-4051-5649-3

Table 6.1 Concentration, thresholds and odour descriptions of significant volatile aroma compounds in fragrant and non-fragrant rices. Adapted from Wilkie *et al.* (2004).

			Jasmine	Basmati	Non-fragrant
Aroma compound	Threshold (ppb)	Odour description	Concentration (ppb)	Concentration (ppb)	Concentration (ppb)
2-Acetyl-1-pyrroline	0.1	Sweet, popcorn	49	7	3
Benzaldehyde	350	Nutty, sweet	36	27	49
Butanol	500	Medicinal	5	1	9
(*E*,*E*)-Deca-2,4-dienal	0.07	Fatty, citrus, powerful	13	8	31
(*E*)-Dec-2-enal	0.4	Fatty, green	11	9	15
2-Ethyl hexanol	—	Oily, sweet	0	—	44
Heptanal	3	Fruit, fatty	25	34	26
Heptan-2-one	140	Fruit, spicy	23	22	40
(*E*)-Hept-2-enal	13	Fatty, green	45	22	80
Hexanal	5	Green, grass	853	751	1960
Hexan-1-ol	2500	Sweet, green	51	45	59
(*E*)-Hex-2-enal	17	Green, fruity	7	5	15
Indole	140	Faecal, floral	12	3	17
6-Methylhept-5-en-2-one	50	Herby, green	11	3	3
Nonanol	1	Floral, fatty	28	25	42
(*E*)-Non-2-enal	0.08	Fatty, waxy	14	6	28
Octanal	0.7	Citrus, fatty	26	40	29
(*E*)-Oct-2-enal	3	Green, herby	47	27	95
Oct-1-en-3-ol	1	Herby, earthy	34	25	58
Pentan-1-ol	4000	Sweet, strong	84	139	104
2-Pentyl furan	—	Nutty, beany	35	21	78
4-Vinylguaicol	3	Spicy, fruity	15	23	42

Rice texture

While the texture of the rice grain may not be directly linked to flavor, it is one of the most important eating quality traits of rice. In addition, cooking temperature is known to affect the flavor of many foods (Bhandari *et al.* 2001), and so the flavor compound profile of cooked rice will be altered by reducing the cooking temperature. The texture and cooking temperature of rice is directly influenced by the properties of rice starch.

Starch is composed of a mixture of two forms of glucose polymer, amylose and amylopectin. Amylose is principally a linear polymer of α(1–4) linked glucose with some α(1–6) linkages. Amylopectin is a more complex mixture owing to the extensive branching introduced by it having many more α(1–6) linkages of the α(1–4) linked chains of glucose. Starch is synthesised by the activity of several enzymes, each of which occurs as a number of different isoforms that display tissue-specific expression (Ball and Morell 2003; Fitzgerald 2004).

In its native state, rice starch has a semi-crystalline structure which is disrupted by cooking, transforming the starch into a softer edible gel-like material. Because it is associated with the cooking time and texture of cooked rice, the temperature at which rice starch gelatinises is an important aspect of rice

eating quality (Maningat and Juliano 1978). A link between the gelatinisation temperature (GT) of rice starch and the enzymes of starch biosynthesis was made when it was found that the major gene controlling rice starch GT, via amylopectin structure, genetically maps to a genomic region that codes for soluble starch synthase IIa (SSIIa) (Umemoto *et al*. 2002).

Further support for the hypothesis that SSIIa is the enzyme responsible for natural variation in GT is provided by two sources:

(1) Analysis of near-isogenic rice lines (NIL), in which a narrow genomic region surrounding the SSIIa-encoding gene of the high-GT variety Kasalath was introgressed into the low-GT variety Nipponbare (Umemoto *et al*. 2004);
(2) Western blot analysis for SSIIa in two rice varieties that differed by starch disintegration in both urea and alkali (Jiang *et al*. 2004).

DNA sequence analysis of the gene that codes for SSIIa found a number of single nucleotide polymorphisms (SNPs) in the gene, two of which were associated with GT class (Umemoto and Aoki 2005; Waters *et al*. 2006). Rice cultivars belonging to the high-GT class had haplotype G/GC, while rice cultivars in the low-GT class were either A/GC or G/TT at the key SNP sites. The presentation of two GT classes is consistent with the finding that the fine structure of Asian rice varieties falls into one of two categories, either the L-type which has a greater number of long cluster chains or the S-type with a greater number of short cluster chains (Nakamura *et al*. 2002). In cultivated rice, it is possible that the combination of 'G' (valine) at SNP3 and 'GC' (leucine) at SNP4 is required for the production of L-type rice starch and that this has a higher GT relative to the S-type starch. Changing the valine to methionine or the leucine to phenylalanine may change starch from the L-type to the S-type which in turn reduces the GT of the starch.

Fragrant rice

The most important rice flavors are often considered to be the aromatic or fragrant flavors associated with the Basmati and Jasmine style rices. The demand for fragrant rice has increased markedly in recent years to the extent that consumers are willing to pay a premium price for fragrant rices. In 2006, premium non-fragrant rice traded for US$250 to US$300 per metric ton, premium Jasmine rice traded for US$400 per metric ton, while premium Basmati rice traded at US$850. The higher price obtained for Basmati rice is due not only to the demand for the aroma and unique characteristics of the rice grain, but also to the limited supply of the Basmati grain. Despite India, the largest supplier of Basmati rice (Bhattacharjee *et al*. 2002), contributing to about one-third of the world's rice acreage, production levels of Basmati rice are relatively low, reflecting the low yields of Basmati varieties in comparison

with non-Basmati rice varieties (Singh *et al*. 2000; Aggarwal *et al*. 2002; Bhattacharjee *et al*. 2002; Nagaraju *et al*. 2002; Garg *et al*. 2006).

Premium Jasmine rice also suffers from low yields (Berner and Hoff 1986; Sriboonchitta and Wiboonpongse 2005), although this is usually due to environmental as well as to genetic factors. Low yield in fragrant rice varieties is due in part to its susceptibility to insect pests (Lorieux *et al*. 1996; Ghareyazie *et al*. 1997; Tanasugarn 1998; Sriboonchitta and Wiboonpongse 2005; Toojinda *et al*. 2005) and to its poor disease resistance (Tanasugarn 1998; Sriboonchitta and Wiboonpongse 2005, Toojinda *et al*. 2005) and is compounded by management practices which exploit the enhanced aroma response of Jasmine rices to abiotic stresses such as drought and high salinity (Yoshihashi *et al*. 2004). Because fragrant rices exhibit higher aroma levels when grown in suboptimal conditions, virtually all premium-quality Thai Jasmine rice is grown in the Isan areas of north-eastern Thailand, a region characterised by saline sandy soils that are prone to flooding and drought (Rigg 1985; Itani *et al*. 2004 and references therein). The soil structure combined with the suboptimal rainfall of this area make the application of nitrogenous fertiliser economically impractical, further reducing yield in fragrant rice varieties (Rigg 1985).

The combined effects of environmental and genetic factors inducing both low yield and enhanced aroma in rice are the most likely reasons why the USA and other non-traditional rice-growing countries are unable to produce rice varieties with sufficient aroma. Most non-traditional rice-growing countries utilise intensive farming practices which are focused on high yield through irrigation and the addition of nitrogenous fertilisers, practices which probably enhance yield to the detriment of rice aroma. Many countries that are net exporters of rice, such as the USA, import large amounts of rice from Thailand, India and Pakistan because they can not produce fragrant varieties with grain qualities equal to those from these countries (Boriss 2006).

Since the start of the green revolution, most rice breeding programs have focused on improving disease and insect resistance and, most importantly, grain yield. Because fragrant varieties have low yields, farmers have stopped growing specific local fragrant varieties and have replaced them with the new, fast-growing, disease-resistant, high-yielding, non-fragrant varieties (Bhattacharjee *et al*. 2002; Itani *et al*. 2004). This has been to the detriment of the genetic diversity of fragrant rice varieties (Berner and Hoff 1986; Ghareyazie *et al*. 1997; Singh *et al*. 2000; Bhattacharjee *et al*. 2002; Garg *et al*. 2006) as many local fragrant types were out-competed and lost.

Owing to an increasing worldwide interest in fragrant rice, biotechnological and breeding efforts later focused on increasing the yield of these fragrant varieties while retaining their aromatic qualities (Ghareyazie *et al*. 1997; Bhattacharjee *et al*. 2002; Garg *et al*. 2006). This has been particularly difficult with Basmati styles as, in addition to aroma, there are many other qualities, such as grain elongation with little swelling in breadth on cooking, soft texture and fine cooking quality (Bhattacharjee *et al*. 2002) that make Basmati rice

distinctive and popular. These desirable traits may be linked to genes for, or may be the causal factor of, low yield in Basmati rice.

Basmati rices belong to a genetically distinct cluster, known as *group V* (Glaszmann 1987) or *aromatic* (Garris *et al*. 2005). Molecular genetic evidence suggests that the aromatic rices have been through a recent or severe bottleneck event (Nagaraju *et al*. 2002; Garris *et al*. 2005). The occurrence of a bottleneck in the fragrant Basmati rice group means that there is very little genetic diversity to be utilised for crop improvement within this group. Attempts at introgressing traits that influence yield into Basmati rice from the other rice groups often result in sterile progeny, owing to inter-group incompatability (Glaszmann 1987; Pinson 1994; Garris *et al*. 2005). Despite the inter-group sterility issues facing Basmati breeding programs, some breeding programs have enjoyed limited success using conventional breeding in enhancing yield, disease, insect and lodging resistance in Basmati varieties (Bhattacharjee *et al*. 2002; Shobha Rani *et al*. 2002; Khan *et al*. 2003). Others have resorted to mutation breeding (Awan and Cheema 1999; Soomro *et al*. 2003) or to using transgenic approaches (Ghareyazie *et al*. 1997; Garg *et al*. 2006), although no genetically modified fragrant rice breeding lines are currently available.

Fragrant Jasmine rices from Thailand fall within the *indica* group (Khush 2000), while a few fragrant cultivars from countries such as China and the Philippines fall into the *japonica* group. The green revolution initially greatly enhanced yields in the *japonica* group and then later in the *indica* group. Although limited in comparison to non-fragrant rices, efforts aimed at increasing yield in fragrant Thai Jasmine rices have enjoyed some success (Siangliw *et al*. 2003; Toojinda *et al*. 2005) relative to what has been achieved in the aromatic Basmati rices.

Breeding programs outside of traditional fragrant rice-growing countries which have attempted to introduce fragrance into adapted backgrounds (Reinke *et al*. 1991; Bollich *et al*. 1992; Marchetti *et al*. 1998; Wilkie and Wootton 2004) have met with limited success. The main barrier to breeding high-yielding fragrant rice cultivars, whether Basmati, Jasmine or in adapted backgrounds, is the accurate assessment and selection of the subtle, recessive trait of fragrance within individual plants. To this end, many sensory and chemical methods have been developed to enable rice breeders to determine whether or not rice plants or grains are fragrant, with each method having various positive and negative attributes.

At their most simple, these methods involve smelling or chewing individual grains (Ghose and Butany 1952; Reinke *et al*. 1991). Unfortunately, the objective evaluation of fragrance using these methods is labour-intensive and unreliable. A panel of analysts is required as the ability to detect fragrance varies between individuals. For any individual analyst, the ability to distinguish between fragrant and non-fragrant samples diminishes with each successive analysis, as the senses become saturated or physical damage occurs from abrasions to the tongue; such abrasions often result from chewing the hard grain

(Garland *et al*. 2000; Cordeiro *et al*. 2002). Semi-chemical methods for determining the fragrance status of grains or plants involve heating grain or leaf in water or adding solutions of KOH or I_2-KI to the grain or leaf before smelling the vapours (Sood and Sidiq 1978). These methods require an objective analysis from a panel of experts, but can saturate the senses of the analytical panellists and can cause damage to the nasal passages, leading to inaccurate and unreliable analysis of the fragrance status of an individual plant. The requirement for an accurate and reliable method for determining the fragrance phenotype of rice plants has led to many investigations into the chemical and genetic components of rice fragrance.

The chemistry of rice fragrance

Chemical analysis of a wide range of rice varieties has revealed many compounds that differ in concentration between fragrant and non-fragrant rice varieties (Yajima *et al*. 1978, 1979; Buttery *et al*. 1986; Paule and Powers 1989; Petrov and Lorieux 1996; Widjaja *et al*. 1996; Grosch and Schieberle 1997; Wilkie and Wootton 2004) (Table 6.1). Using a combination of sensory panels and gas chromatogram techniques Buttery *et al*. (1983b) determined that 2-acetyl-1-pyrroline (2AP), although only present in fragrant rice at low concentrations, was the primary chemical responsible for the characteristic aroma of Jasmine and Basmati rice. 2AP is also present in non-fragrant rice varieties but at a concentration in the range of 10 to 100 times lower than that of fragrant rices (Buttery *et al*. 1983b, 1986; Widjaja *et al*. 1996; Wilkie and Wootton 2004).

The threshold concentration at which 2AP can be detected by the human nose is around 0.1 ppb when diluted in water (Buttery *et al*. 1983b), but is probably somewhat higher in the complex rice grain. Fragrant rice grain has 2AP concentrations from about 3000 times this level and upwards, while non-fragrant rice has 2AP concentrations of only about 30 times this threshold level of 2AP in water (Buttery *et al*. 1983b, 1986; Wilkie and Wootton 2004) (Table 6.1). A wide range of 2AP concentrations have been observed in both fragrant and non-fragrant varieties in different studies. These differences may be due to the different rice varieties studied, differences in extraction procedure or quantification of 2AP, environmental influences on the level of fragrance such as temperature and salt and drought stress (Itani *et al*. 2004; Yoshihashi *et al*. 2004, 2005), harvest time or storage conditions of the rice (Bhattacharjee *et al*. 2002; Itani *et al*. 2004), whether the rice was milled or unmilled (Buttery *et al*. 1983b; Philpot *et al*. 2005) or timing/level of nitrogenous fertiliser application to the growing plants (Wilkie and Wootton 2004).

Buttery *et al*. (1983a) also discovered that 2AP was the main chemical cause of pandanus leaf fragrance. The aroma of fragrant rice is often described as pandanus-like and, in some Asian cultures, dried pandanus leaf is added to non-fragrant rice while cooking to impart a characteristic Basmati/Jasmine scent. Since the discovery that 2AP is the major chemical compound involved

in fragrance in both rice and pandanus, it has been found that the flavor of a range of foods, including, popcorn (Schieberle 1995), corn tortillas (Buttery and Ling 1995), baguettes (Zehentbauer and Grosch 1998), ham (Carrapiso *et al*. 2002), cheese (Zehentbauer and Reineccius 2002), mung bean (Brahmachary and Ghosh 2002), green tea (Kumazawa and Masuda 2002) and wine (Herderich *et al*. 1995) is associated with the presence of 2AP. Analysis of a diverse range of biological non-consumables such as tiger pheromone (Brahmachary *et al*. 1990) and select bacteria, moulds and yeasts (Rungsardthong and Noomhoom 2005) have found that the characteristic aroma of these sources is also due to the presence of 2AP.

Many objective methods of determining the level of 2AP using gas chromatography have been developed (Lorieux *et al*. 1996; Widjaja *et al*. 1996; Bergman *et al*. 2000). However, these methods often require large tissue samples and are expensive and extremely time consuming; in addition, the peak corresponding to 2AP is small compared to the peaks corresponding to other chemicals present in rice, making the results difficult to interpret.

The genetics of rice fragrance

Molecular markers are used widely as a selection tool for specific traits in breeding programs in many species, including rice. If the genetic cause of a trait is known or the genomic region that controls a trait is sufficiently narrow, genotypic tests using molecular markers offer many advantages relative to many direct phenotypic tests. The advantages include a requirement for small quantities of tissue, independence from the confounding effects of the environment and a single platform test for multiple traits.

The majority of studies which have focused on the genetics of fragrance in rice determined that fragrance is due to a single recessive gene (Sood and Sidiq 1978; Berner and Hoff 1986; Ahn *et al*. 1992; Bollich *et al*. 1992; Lorieux *et al*. 1996; Garland *et al*. 2000; Cordeiro *et al*. 2002; Jin *et al*. 2003; Bradbury *et al*. 2005a), while other studies have identified two, three or four genetic loci as having an influence on fragrance (Kadam and Patankar 1938; Dhulappanavar 1976; Geetha and Nadu 1994; Pinson 1994; Vivekanandan and Giridharan 1994; Lorieux *et al*. 1996). The contradictory nature of these reports may be due to either the different rice varieties studied or to the different methods used to evaluate fragrance. Some methods used a simple binary system of fragrant/non-fragrant, to categorise the fragrance phenotype (Sood and Sidiq 1978; Pinson 1994; Jin *et al*. 2003; Bradbury *et al*. 2005a,b). Other methods measured varying degrees of fragrance using sensory panellists scaling fragrance, usually on a scale of one to ten (Berner and Hoff 1986), while others used a combination of sensory panellists and gas chromatographic methods to measure the level of 2AP in plant samples (Lorieux *et al*. 1996).

While not ruling out the possibility of multiple genetic loci influencing the level of fragrance in rice, it appears there is one major genetic loci on

chromosome 8 that determines whether rice is fragrant or not – in essence an on/off switch for fragrance. Berner and Hoff (1986) determined that aroma in the fragrant cultivar Della was due to a single recessive gene. Ahn *et al*. (1992) then used 126 molecular markers to map the position of this gene, *fgr*, in aromatic Lemont (which derives its aroma from Della), and determined that the gene was 4.5 cM from the chromosome 8 RFLP marker (RG28). Linkage of RG28 to aroma was verified using an F_3 population, segregating for fragrance with fragrant and non-fragrant Lemont parents. Phenotyping the F_3 population was undertaken both by chewing seeds and by utilising the method developed by Berner and Hoff (1986) which involved placing leaf or grain samples in KOH before smelling the samples and scoring each sample as fragrant or non-fragrant in a binary fashion. Using both gas chromatography analysis of 2AP and sensory evaluation panellists in conjunction with linkage analysis of 16 polymorphic markers separated by no more than 25 cM across chromosome 8 in a population of 135 double haploid lines, Lorieux *et al*. (1996) further refined the map position of *fgr*, when they determined that *fgr* is flanked by the molecular markers RG28 and RG1 at a distance of 6.4 ± 2.6 and 5.3 ± 2.7 cM, respectively. Lorieux *et al*. (1996) also identified two other QTL on chromosomes 4 and 12 involved in fragrance. However, these QTL were detected only when the analysis accounted for a major gene for fragrance being located on chromosome 8.

In an effort to develop PCR-based markers for *fgr*, Garland *et al*. (2000) identified a 1-bp polymorphism within the RFLP clone RG28. This marker required the use of expensive capillary electrophoresis equipment to discriminate between PCR products that differed by 1 bp and, because it was physically removed from *fgr*, was not 100% accurate in discriminating between non-fragrant and fragrant plants. Corderio *et al*. (2002) utilised rice genome sequence data to identify an SSR marker which was approximately 4 cM from *fgr*. This loci was highly polymorphic and 13 alleles were identified; eight alleles were found in the fragrant plants and eight in the non-fragrant plants, with three alleles common to both fragrant and non-fragrant varieties. DNA sequence analysis of approximately 500 bp of the 5′ ends of 14 genes chosen based on their proximity to RG28 revealed only one SNP (RSP04) between Kyeema (fragrant cultivar) and Doongara (non-fragrant cultivar) (Jin *et al*. 2003).

By utilising a combination of the complete rice genome sequence and SSR markers in a population segregating for fragrance, Bradbury *et al*. (2005a) found that the major gene for fragrance in rice, *fgr*, is most likely a gene encoding a betaine aldehyde dehydrogenase paralog (BAD2). The work of Wanchana *et al*. (2005) and sequence analysis by Chen *et al*. (2006) of a 69-kbp region containing *fgr* identified three genes, one of which encoded for BAD2, supporting the contention that BAD2 is responsible for fragrance in rice. In comparison to the allele of the gene encoding BAD2 in non-fragrant rice plants, the allele in fragrant rice plants contains an 8-bp deletion and three SNPs that lead to the generation of a premature stop codon in

exon 7 (Plate 6.1). The premature stop codon leads to the production of a truncated BAD2 enzyme that is missing highly conserved regions which have been shown to be required for BAD catalytic activity in other plants, animals and microorganisms (Johansson *et al*. 1998; Incharoensakdi *et al*. 2000; Gonzalez-Segura *et al*. 2002; Li *et al*. 2003) (Plate 6.2).

Identification of the major gene for fragrance in rice allowed construction of a perfect marker for fragrance in rice. A single-tube competitive allele-specific PCR assay (Plate 6.3) that utilises four PCR primers and agarose gel technology was designed to discriminate between homozygous fragrant, homozygous non-fragrant or heterozygous non-fragrant individuals with 100% accuracy. The simplicity of this genetic test allows it to be performed in most laboratories (Bradbury *et al*. 2005b) (Plate 6.3).

Of the four primers used in this assay, two flank the area where the mutation occurs and anneal to both genotypes and the other two are specific to each one of the two alleles (Plate 6.3A,B). The two flanking primers act as an internal positive control amplifying a region of approximately 580 bp in both fragrant and non-fragrant samples. Individually, these flanking primers also pair with internal primers to give products of varying size. The internal primers, Internal Fragrant Antisense Primer (IFAP) and Internal Non-fragrant Sense Primer (INSP) (Plate 6.3A,B), will anneal only to their specified genotype, producing DNA fragments with their corresponding flanking primer pair, External Sense Primer (ESP) and External Antisense Primer (EAP), respectively. The result of using these four primers in a PCR gives three possible outcomes; in all cases, a large positive control band of about 580 bp is produced. In the first case, a band of 354 bp is produced, indicating a homozygous non-fragrant variety or individual. In the second case, a band of 255 bp is produced, indicating a homozygous fragrant variety or individual. In the third case, bands of both 354 bp and 255 bp are produced, indicating a heterozygous non-fragrant individual (Plate 6.3A, C, D).

The assay was validated using 168 field-grown F_2 individuals, segregating for fragrance derived from a cross between Kyeema (fragrant) and Gulfmont (non-fragrant) (Plate 6.3D) and a range of fragrant and non-fragrant varieties. The assay is robust, allowing the use of DNA derived from rice grains using a simple NaOH extraction protocol (Bergman *et al*. 2001) and leaves using a simple 10-minute boiling protocol.

BAD enzymes and 2AP synthesis

BAD enzymes were first recognised in plants that accumulate glycine betaine for their ability to catalyse the second step in the production of glycine betaine from choline, a compound that has been shown to protect plants from a range of abiotic stresses including drought, salt, cold and heat (Kishitani *et al*. 2000; Li *et al*. 2003; Gao *et al*. 2004). Rice is a non-accumulator of glycine betaine, so the native substrate of rice BAD enzymes is not obvious.

Although the biochemical pathway leading to 2AP accumulation has not been established, radio-labelling studies have shown that L-proline and to a much lesser extent glutamate and ornithine, are precursors of 2AP in rice (Yoshihashi 2002). Other studies demonstrated that proline is a precursor of 2AP in pandanus (Thimmaraju *et al*. 2005) and that both proline and glutamate can serve as precursors of 2AP in some *Bacillus sereus* strains (Romanczyk *et al*. 1995). This may go some way to explaining the increase of 2AP in rice in response to salt and drought stress, as both proline and glutamate are known to accumulate in salt-stressed rice (Lutts *et al*. 1999; Yang and Kao 1999). The catalytic activity of the BAD2 enzyme may lead to an increase or reduction of the 2AP level; however, fragrance is a recessive trait, suggesting that a loss of function is responsible for the accumulation of 2AP. Furthermore, the truncated version of the enzyme that is encoded by the fragrant genotypes is less likely to be functional and favours the later hypothesis. Some BADs have been shown to have wide substrate specificity for various omega-aminoaldehydes (Livingstone *et al*. 2003; Trossat *et al*. 1997), oxidising them into their corresponding omega-amino acids.

One of the recognised aldehyde substrates of BAD, known as gamma-aminobutyraldehyde, can spontaneously cyclise to form delta-1-pyrroline (Struve and Christophersen 2003), a compound which is structurally similar to 2AP, suggesting that 2AP or a precursor to 2AP may be a substrate of BAD. Once again, the later hypothesis (that a loss of function is responsible for the accumulation of 2AP) is more likely as 2AP is not an aldehyde and so BAD2 is unlikely to have affinity for 2AP. Bradbury *et al*. (2006) suggested a pathway in rice similar to that theorised in yeast (Costello and Henschke 2002) and in chemical reactions (Hofmann and Schieberle 1998) utilising delta-1-pyrroline, derived from proline via putrescine and polyamine metabolism, reacting with a compound such as methylglyoxal to form 2AP. The removal of delta-1-pyrroline or the linearised compound gamma-aminobutyraldehyde by a functional BAD2 enzyme would decrease the levels of the downstream compound 2AP (Plate 6.4). Gamma-aminobutyric acid, the oxidation product of gamma-aminobutyraldehyde, has been shown to have natural pesticide properties in plants (Ramputh and Bown 1996; Shelp *et al*. 2003). This may explain why some fragrant rice cultivars are particularly susceptible to insect pests (Lorieux *et al*. 1996; Ghareyazie *et al*. 1997; Tanasugarn 1998; Sriboonchitta and Wiboonpongse 2005; Toojinda *et al*. 2005) because the nonfunctional BAD2 enzyme in the fragrant plants would be unable to convert gamma-aminobutyraldehyde into gamma-aminobutyric acid so the plants may accumulate less of this natural pesticide than non-fragrant plants.

Many plants, including rice, have been shown to encode two paralogs of the BAD enzyme. BAD1 in rice is encoded by a gene on chromosome 4. BAD1 and BAD2 in sugar beet (*Beta vulgaris* L.) have been shown to have varying substrate specificities (Trossat *et al*. 1997), while other species such as *Avicennia marina* (Hibino *et al*. 2001) have been reported to have BAD paralogs with similar substrate specificities. Enzymes which appear to be the

bacterial equivalent of BAD1 and BAD2 include an enzyme with aminoaldehyde dehydrogenase (AAD) activity isolated from the bacteria *Pseudomonas putida*; this enzyme is active against a range of aminoaldehydes, while another has affinity specific to γ-guanidinobutyraldehyde (Yorifuji *et al*. 1986). BAD1 enzymes of higher plants usually contain a C terminal tri-peptide sequence, known to target proteins to peroxisomes (Olsen *et al*. 1993; Reumann 2004). BAD2s of higher plants do not usually contain this tri-peptide sequence and are therefore probably cytosolic in nature. Rice is an exception to this rule as both BAD1 and BAD2 of rice contain the tri-peptide signal sequence at the C terminus, suggesting that they are both targeted to the peroxisome. If this is the case, the formation of BAD heterodimers in rice plants is possible, which would allow a role for BAD1 in rice fragrance.

Since BADs have been shown to have wide substrate specificity for omega-aminoaldehydes, it could be argued that classifying them as BADs is potentially misleading and perhaps they should be classified as AADs. AADs have been discovered in higher plants (Matsuda and Suzuki 1984; Sebela *et al*. 2000) and although protein sequence availability is limited, the evidence suggests that some BADs should be reclassified as AADs (Sebela *et al*. 2000; Trossat *et al*. 1997). Further investigation into whether the rice BADs are BADs or AADs would greatly aid the understanding of the biochemical pathway that leads to production of 2AP.

The concentration of 2AP in rice is reported to be higher in plants subjected to salt and drought stress (Yoshihashi *et al*. 2004). BAD has been associated with stress tolerance in plants (Nakamura *et al*. 2001; Li *et al*. 2003; Livingstone *et al*. 2003). A BAD gene from a halophyte (*Suaeda liaatungenis*) improved salt tolerance when expressed in tobacco (Li *et al*. 2003). Overexpression of a barley BAD gene in rice has been shown to improve tolerance of the plants to salt, cold and heat (Kishitani *et al*. 2000). Although most of these studies suggest that the major activity of BAD is the synthesis of glycine betaine as a defence against stress (Kishitani *et al*. 2000, Li *et al*. 2003, Kumar *et al*. 2004), some studies have shown an increase in BAD mRNA (Nakamura *et al*. 2001). An increase has also been shown in enzyme activity (Cha-um *et al*. 2004) in rice and other non-accumulators of glycine betaine (Ishitani *et al*. 1993) in response to salt stress, suggesting another BAD activity which plays a role in the protection of plants from salt stress.

The mutation in the gene encoding BAD2 in fragrant rice varieties does not appear to be associated with any loss of plant performance and may have a positive effect under drought since the fragrant variety Khao Dawk Mali 105 was initially selected for its improved drought tolerance (Yoshihashi *et al*. 2004). It seems likely that the pathway which leads to 2AP synthesis is part of a stress-response pathway that is up-regulated in the highly fragrant and salt-tolerant variety Khao Dawk Mali 105. Removal of the activity of the BAD2 paralog, while leaving the BAD1 paralog functional, could mean that the stress pathway is intact and is partially active, allowing for the accumulation of 2AP.

If this is the case, then the *fgr* gene parallels the *sd1* gene (Sasaki *et al*. 2002) for semi-dwarf stature in that the trait is due to a mutation resulting in loss of function in one member of a gene family that is not essential for survival, while loss of function of other members of the gene family might be lethal.

There is strong evidence that cereals have been cultivated and consumed by humans for a very long time (Piperno *et al*. 2004), but the domestication path of cereals, including rice, is not understood. Key mutations associated with domestication of barley (Piffanelli *et al*. 2004) and maize (Wang *et al*. 1999) are associated with changes in gene expression at important loci. The presence of one allele of the gene in all fragrant rice genotypes examined by Bradbury *et al*. (2005a) is consistent with the trait being inherited from a common fragrant genotype, supporting the suggestion by Garris *et al*. (2005) that the aromatic group rices have been through a recent genetic bottleneck.

The future

Understanding the major genetic cause of fragrance will assist in elucidating the biochemical pathway that leads to accumulation of 2AP in rice. However, there is much to be learned about the production of 2AP in rice as well as in other organisms such as pandanus or bacteria. For example, the biochemical pathway leading to 2AP production is uncertain, and it is not known whether the final step in the production of 2AP is enzymatically or chemically driven. Further research may lead to the identification of enzymes and genetic loci responsible for this final step, which in turn may then lead to further enhancement of rice fragrance through selective breeding or genetic engineering of rice cultivars. Alternatively, once the biochemical pathway surrounding 2AP synthesis is known, it may be possible to select cultivars that have up-regulated expression of genes upstream of 2AP formation and to cross these with varieties that have the gene that encodes for the nonfunctional BAD2 enzyme, and this may lead to enhanced fragrance. Mutation breeding may generate a whole range of new fragrant foods, such as potatoes and corn with disrupted BAD2 activity, that have increased levels of 2AP.

Lorieux *et al*. (1996) identified a region on chromosome 4 linked to fragrance; this region contains the gene encoding BAD1. This suggests that BAD1 may also contribute to the accumulation of 2AP, at least in some varieties of rice. Assuming BAD1 is involved in 2AP accumulation, it is possible that down-regulation of the gene encoding BAD1 may lead to increased 2AP accumulation. Analysis of the gene encoding BAD1 and the BAD1 enzyme may determine that the substrate specificity of BAD1 is similar to that of BAD2. If so, knockout mutants of the gene encoding BAD1 in existing fragrant cultivars, or in cultivars crossed to existing fragrant cultivars, could lead to super-fragrant rice varieties, perhaps with a 2AP level similar to that of the pandanus leaf, which is ten times more than that of existing fragrant rice cultivars.

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Chapter 7
Breeding and biotechnology for flavor development in apple (*Malus* × *domestica* Borkh.)

Susan K. Brown

Tremendous progress has been made in the investigation and understanding of apple flavor, but the inherent complexity and variability of apple flavor will require intensive and sustained research to strengthen our ability to genetically enhance apple flavor.

Apples are extremely variable, even within a single fruit. Dever *et al*. (1995) used analysis of variation and multivariate relationship among analytical and sensory characteristics in whole-apple evaluations of 'McIntosh' and 'Jonagold', and found differences among apples sorted into three red categories: within apples (sun versus shade side) and from the top to bottom of the fruit. Apples are also variable within a tree, among cultivars, within sports of the same clone (Fellman *et al*. 2000), and among different environments and seasons (Hern and Dorn 2003). Apples are affected by many cultural manipulations, especially those affecting sunlight penetration, crop load and proper harvest timing (Song and Bangerth 1996; Echeverria *et al*. 2004).

There are many excellent publications on apple quality and flavor, including those by Dimick and Hoskin (1983), Yahia (1994), Dixon and Hewett (2000a), Fellman *et al*. (2000) and Knee (2002) that review our current state of understanding. The complexity of apple flavor and volatiles is evident. As methodology, the related aspects of texture and firmness and the effects of many cultural conditions on flavor are discussed in the aforementioned publications, this chapter will instead focus on the cultivars that have been characterized, the key treatments that affect flavor, the new tools in genomics that are adding to our knowledge and, finally, the ways in which transgenic work will elucidate the complexities of flavor.

Quality

Even simple traits that are important to apple quality have been elusive to researchers wanting to develop molecular markers to select for components of apple quality. The ratio or balance of sugar to acid is an important component of apple quality and may affect perception of flavor and texture. Sugar and acid can be measured and have been a focus of breeders for a long time. Aroma, flavor and texture are more challenging to characterize. Sweetness is usually measured using a refractometer to determine total soluble solids (TSS), expressed in degrees Brix. Degrees Brix was found to have a correlation of

Biotechnology in Flavor Production: A Case-based Approach. Edited by Daphna Havkin-Frenkel, Faith C. Belanger.
 ISBN: 978-1-4051-5649-3

only 0.41 for the median trained sensory panelist, so that a difference in sweetness was predicted only when apples differed by more than 1 Brix (Harker *et al*. 2002). The genetics of sweetness in apple is not yet fully understood.

Fruit acidity, primarily malic acid in apple, is under the control of a dominant gene (*Ma*), but other genes are also thought to be involved. Many apple cultivars are heterozygous (*Mama*). In crosses of heterozygotes, one quarter of the progeny will have unacceptably low acidity (*mama*). The *Ma* gene has been placed on linkage group (LG) 16 (Conner *et al*. 1997; Maliepaard *et al*. 1998). In sensory trials, titratable acidity was the best predictor of acid taste (a correlation of 0.86 for the median panelist) (Harker *et al*. 2002). Despite its characterization and linkage map location, at present there are no molecular markers for malic acid in apple.

Apple volatiles

Apple is a climacteric fruit, and the production of the characteristic aromas coincides with ethylene synthesis. Aroma compounds come from several different pathways in fatty acid, amino acid, phenolic and terpenoid metabolism (Baldwin 2002). Apple volatiles are important in apple flavor and in attracting seed dispersers (Goff and Klee 2006) but, unfortunately, often attract apple-infesting insects such as the codling moth, *Cydia pomonella* L. (Coracini *et al*. 2004). α-Farnesene is well known for its role in the characteristic 'green apple' flavor, but also for its role in the post-harvest disorder storage scald (Watkins *et al*. 1993).

Apples produce more than 200 volatile flavor compounds that include alcohols, aldehydes, esters, ketones and sesquiterpenes (Dimick and Hoskin 1983). A detailed account of volatiles extracted from apple is found in Paillard (1990) and Dixon and Hewett (2000a). The most abundant volatiles were even-numbered carbon chains and included combinations of acetic, butanoic and hexanoic acids with ethyl, butyl and hexyl alcohols.

The majority of volatiles in apple aroma are esters, and their formation is dependent on the availability of C2–C8 acids and alcohols (Dixon and Hewett 2000a). Twenty of these compounds are characterized as 'character impact' compounds that are important to typical apple aroma, while others contribute to aroma intensity or aroma quality. Esters are associated with the fruity attributes of fruit flavor and characteristically increase with ripening, while aldehydes, which impart the green or grassy taste/smell, decrease with ripening (Fellman *et al*. 2000).

Ester compounds and ester biosynthesis

Alcohol acyl-CoA transferase (AAT) from 'Royal Gala', MpAAT1, produces esters involved in apple fruit flavor (Souleyre *et al*. 2005). More than 34 esters

have been identified in 'Royal Gala' (Young *et al.* 1996; 2004), with hexyl acetate and 2-methylbutyl acetate important in sensory perception (liking). More than 12 acyltransferases were identified in apple; enzymes may have different substrate preferences and may thus produce different esters. The pool of available substrates is likely to dictate the esters that are formed.

Measurement techniques

Many different analytical techniques have been used to characterize apple flavor (Baldwin 2002). This section will focus on only a few methods that target apple odor or labeling.

In 1986, Cunningham *et al.* used charm analysis to describe apple flavor and apple volatiles. Of the 40 cultivars that were examined, no two cultivars were similar in their charm response. Odor activity was quantitatively associated with identified and unidentified components. Across samples, beta-damascenone, butyl, isoamyl and hexyl hexanoates were important to the odor of many cultivars. Ethyl, propyl and hexyl hexanoates were also important. Five of the odors that were detected were not associated with any known apple compound. This study showed that odor in different cultivars originates from different compounds.

Rowan *et al.* (1999) used deuterium-labeled precursors to investigate the biosynthesis of straight-chain volatiles in 'Red Delicious' and 'Granny Smith' apples, and found that straight-chain ester volatiles only arose from the lipoxygenase (LOX) pathway and suggested that their presence may be a useful indicator of LOX activity in apple.

Rapid analysis of flavor volatiles that are important in apple wine was accomplished using headspace solid-phase microextraction (HS-SPME) with gas chromatography-mass spectrometry (GC-MS), and parameters were defined (Wang *et al.* 2004). Ethanol concentrations from 0% to 12% had no effect on the analyses. This method can be used on wine and juice and in monitoring the volatiles that are present in the mash during fermentation.

Komthong *et al.* (2006) used gas chromatography olfactometry with headspace gas dilution analysis to evaluate the odor potency of volatiles in 'Fuji' apple. Eight compounds were identified as important odor contributors. Threshold values of compounds in air were estimated from the relationship between flavor dilution (FD) and the concentration of the compound.

Varietal and developmental differences

Given the tremendous diversity of apple characteristics and the existence of thousands of different apple varieties, apple flavor profiling has only scratched the surface. However, researchers are now using repositories – collections of apple clones – and are beginning to realize the diversity present within such

collections and within breeding programs. Of the 20 cultivars with the greatest commercial production in the world, less than half have been studied in any great detail, with the exception of 'Delicious', 'Fuji' and 'Gala', together with their sports. Fortunately, this trend is starting to change with post-harvest collaborators now being an important part of many breeding programs.

Higher-coloring sports of 'Delicious' were found to have lower aroma contents. Fellman *et al*. (2000) suggested that, owing to increased synthesis and localization of acetate moieties in anthocyanin in the peel, mutations that affect skin color might reduce acetate ester synthesis by limiting substrate availability.

GC-MS identified four compounds as being important contributors to the aroma and flavor of 'Royal Gala'. Analytic sensory panel results have indicated that 2-methylbutyl acetate, butanol and hexyl acetate had a greater causal effect on characteristic 'Royal Gala' flavor and aroma attributes than did butyl acetate (Young *et al*. 1996).

Mehinagic *et al*. (2006) studied 'Golden Delicious', 'Fuji' and 'Braeburn' at three maturity stages, and their influence on odor-active volatiles, and found that 24 odorant compounds were common to all extracts. They found that sensory-quality changes during maturation were cultivar-dependent.

In a study of 'Pacific Rose' apple volatile reductions in storage, Tough *et al*. (2001) found a 34% reduction in butyl acetate, a key aroma component, in controlled-atmosphere (CA) stored fruits compared with regular-atmosphere (RA) stored fruits at 4 weeks; by 26 weeks, there was virtually no butyl acetate in the CA stored fruits. The aroma volatile suppression was not reversible upon transfer to air storage. Tough *et al*. (2001) suggested that breeders should expose advanced breeding selections to CA conditions prior to their commercial release in order to ensure that they are not subject to high losses of aroma volatiles.

Kuhn and Thybo (2001) examined 22 scab-resistant apple cultivars using trained sensory panelists, and analyzed the results with principal component analysis. They used a rough grouping of 'apple flavor'.

Holland *et al*. (2005) compared 'Fuji' and 'Granny Smith' apples. 'Fuji' had 2-methylbutyl acetate (a fruity, banana-like aroma), while 'Granny Smith' lacked this compound. For alcohol acetyltransferase activities, 'Granny Smith' only had activity in the peel, while 'Fuji' had activity in the peel and flesh and accepted a broader range of substrates for the production of volatile esters.

The variation in ethyl ester enhancement among apple cultivars may be due to differential activity/synthesis of AAT or alcohol dehydrogenase (ADH), or separate isoforms of AAT and ADH, with each isoform having its own substrate specificity. Variation in alcohol precursors may also be cultivar-dependent. Dixon and Hewett (2000a) suggested that any of these could occur singly, or in combination.

Effect of storage

CA storage and long-term air storage have long been known to result in the loss of apple flavor and aroma (Bangerth and Streif 1987). 'Gala' aroma, in particular, appears to be short-lived in storage and has been the subject of many studies. 'Gala' aroma and flavor were characterized by a trained sensory panel after CA and RA storage. The intensity of fruity (pear, banana and strawberry) and floral descriptors decreased in CA as opposed to RA storage (Plotto *et al*. 1999). When Osme analysis, a gas chromatography-olfactometry technique, was used to characterize changes in 'Gala' aroma following storage, several compounds were identified as contributing to 'fresh' (4-week RA storage) 'Gala' aroma even though the compounds do not have an apple odor (Plotto *et al*. 2000).

Solid-phase microextraction (SPME) was coupled with GC-MS to study variability of volatile release in 13 apple varieties (Young *et al*. 2004). Principal component analyses clustered the varieties into three groups based on their skin color (red, green and red-green or bicolor). Total esters were highest in the red group, and the green group had the highest α-farnesene. When fruits were removed from CA to room temperature, the total esters increased 25-fold and α-farnesene increased five-fold. After storage at room temperature for 11 days, the increase was inversely proportional to ester size, with the smallest esters increasing the most.

Tough *et al*. (2001) reported rapid reduction in the aroma volatiles of 'Pacific Rose' apples in CA storage. 'Pacific Rose' has 'Gala' as a parent – a cultivar that is also prone to aroma loss in storage.

Lara *et al*. (2006) examined 'Fuji' apples exposed to air storage or to three CA storage regimes and suggested that for volatile production after storage, substrate availability may be more important than AAT enzyme activity. There were higher emissions of straight-chain esters in air-stored fruit.

Effect of processing

Elss *et al*. (2006) examined the influence of technological processing on apple aroma using high-resolution GC-MS and on-line gas chromatography-combustion/pyrolysis-isotope ratio mass-spectrometry. They looked at single-strength juices and the concentrates and aromas made from them and found no qualitative changes in apple aroma profiles from juice to aroma. However, they observed 3-methyl-1-butanol and its acetate, neither of which are genuine apple constituents, but are believed to occur due to fermentative effects in industrial juice production.

Effect of 1-methylcyclopropene treatment

Watkins (2006) reviewed the use of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, to extend the storage and shelf-life of fruits and vegetables (including apple). Examination of the effect of 1-MCP on apple storage and volatile production has been studied intensively, but only on a limited number of commercial cultivars.

The effects of 1-MCP and methyl jasmonate (MeJA) on volatile production in 'Delicious' and 'Golden Delicious' apples were examined by Kondo *et al*. (2005). Volatiles of 1-MCP-treated fruit were lower than those of untreated controls. The effect of MeJA on volatiles was cultivar-dependent, related to its effect on internal ethylene concentration, and may be dependent on the growth stage of the fruit when treated.

Bai *et al*. (2005) examined the response of four apple cultivars ('Gala', 'Delicious', 'Granny Smith' and 'Fuji') to 1-MCP treatment prior to air or CA storage. The treatments (CA plus/minus 1-MCP) that were most effective at retaining titratable acidity and firmness retained the least volatiles. These same treatments delayed loss of soluble solids content for 'Gala', but not for the other cultivars tested.

'Gala' apples that were either treated with 1-MCP and stored in CA or RA, or were stored in CA without an MCP treatment, had reduced volatile production compared with apples stored in RA and not treated with 1-MCP. Ester production decreased in CA storage regardless of 1-MCP treatment. When CA or 1-MCP-treated fruit were stored at 20°C and exposed to ethylene, esters and alcohol production were stimulated but ethylene had no effect on production of aldehydes or acetic acid (Mattheis *et al*. 2005).

Hypoxia

The effect of hypoxia, an environment where the oxygen concentration surrounding tissues or organs is insufficient to support aerobic metabolism, was tested on nine apple cultivars using an atmosphere of 100% carbon dioxide for 24 hours at 20°C (Dixon and Hewett 2000b). Response, in this case volatile compounds, was cultivar-dependent, with 'Fuji' and 'Delicious' having the greatest increase in ethyl esters. In general, concentrations of acetaldehyde, ethanol, ethyl acetate and ethyl esters were enhanced and those of acetate esters and aldehydes were depressed. Dixon and Hewett (2000a,b) suggested mechanisms for the effects of hypoxia and questioned whether the use of hypoxia to mitigate the decrease in volatiles that occurs in storage would be perceived as significant in sensory testing of quality.

Gene isolation

Pechous and Whitaker (2004) cloned and examined functional expression of an (*E,E*)-α-farnesene synthase (AFS) cDNA from apple peel tissue of

'Law Rome'. AFS1 transcripts increased almost four-fold in apple peels during the first 4 weeks of storage (at 0.5°C), but treatment with 1-MCP resulted in a decline in AFS1.

Souleyre *et al.* (2005) isolated the alcohol acyltransferase gene M*AAT1* from 'Royal Gala'. This gene produces a protein that shares features with other plant acyltransferases. Distinct flavor profiles of different varieties may be due in part to the kinetic characteristics of AATs. Md*AAT1* may produce many esters found in 'Royal Gala' fruits but prefers to produce hexyl esters (of C3, C6 and C8 CoAs). Production of acetate esters depends upon substrate concentrations, with 2-methylbutanol favored at low concentrations of alcohol substrates and hexanol used at high substrate concentrations. The authors suggest that many factors influence the production of distinctive apple aromas that are specific to cultivars, and propose that these may include substrate availability, the number of AATs, their regulation and the kinetic characteristics of the enzymes under different substrate concentrations. More than 20 acyltransferase genes have been identified from the Hort Research apple EST (expressed sequence tags) database in New Zealand.

Li *et al.* (2006b) cloned Md*AAT2* alcohol acyltransferase from 'Golden Delicious' and found that in this cultivar Md*AAT2* is expressed only in the apple peel. Tissue disc assays of fruit peel and the use of added alcohols also suggested the importance of substrate availability for ester formation.

Genetic studies, linkage maps and marker-assisted selection

Genetic approaches to studying flavor offer hope in furthering our knowledge in this area. However, there is still an unmet need to reduce the inherent variability by choosing to establish experiments with reduced variability, so as to make genetic backgrounds and environmental effects as uniform as possible. Examples include the examination of genetic sports, which are as close to genetically similar material as we can achieve in apple. The trees producing the apples under study need to be in replicated randomized plantings on the same rootstock, of the same age. Replicated trees should be used to assess variation between trees. Proper sampling and maturity assessment are essential.

Progress in apple genetic linkage map construction and saturation was reviewed in Brown and Maloney (2005). Genetic maps exist for the following cultivars and selections: 'Braeburn', 'Discovery', 'Fiesta', NY 75441-58, NY 75441-67, 'Prima', 'Rome Beauty', 'Telamon', 'White Angel' and 'Wijcik McIntosh'.

Map saturation and alignments are being aided by the release of more markers such as the 140 microsatellites of Liebhard *et al.* (2002) and the saturated reference map of apple with 840 markers (Liebhard *et al.* 2003). Eighty-six SSRs that cover 85% of the apple genome, with a marker every 15 cM, have also been released (Silfverberg-Dilworth *et al.* 2006). Mapping of ESTs will add markers to regions not yet covered.

As map saturation continues, regions associated with quality traits are more likely to be identified. Zini *et al*. (2005) used proton transfer reaction-mass spectrometry (PTR-MS) to measure the headspace composition of volatile organic compounds (VOCs) from apples of progeny from the mapping population 'Fiesta' × 'Discovery'. Quantitative trait loci (QTLs) were identified, as was a relationship between apple skin color and peaks related to carbonyl compounds.

ESTs

The Genome Database for Rosaceae (GDR) is a resource for those working in genetic improvement or on genomic studies within the Rosaceae family (Jung *et al*. 2004). This is a curated and integrated web-based relational database with all publicly available Rosaceae sequences, including apple.

Park *et al*. (2006) analyzed apple EST sequences in such public databases. These analyzed ESTs were from 20 contributors, represented 70 cDNA libraries and were generated from nine cultivars. Genes suspected of participating in the generation of flavor and aroma compounds in mature apple fruits were identified. Numerous LOX-related genes were revealed, but one was identified as potentially important in ester formation. The authors provided a good discussion of some of the constraints of working with current EST resources in apple.

Newcomb *et al*. (2006) analyzed more than 150 000 ESTS developed at the Horticulture and Food Research Institute of New Zealand Limited (HortResearch). These ESTs originated primarily from 'Royal Gala'. Clustering of sequences resulted in sets of almost 43 000 nonredundant sequences – 17 460 tentative contigs and more than 25 000 singletons. Many genes present were involved in disease resistance, and biosynthesis of flavor and phenolic compounds. Evidence for duplication of biosynthetic and regulatory genes expressed in fruit was also presented. All the enzymes involved in the mevalonate pathway, which is important for the production of terpenoids such as α-farnesene, were present as were enzymes that are important in ester biosynthesis from fatty acids. The HortResearch group has filed patent applications for the use of the genes involved in α-farnesene synthase (green apple scent).

A genome-wide physical map of apple is closer to reality given the bacterial artificial chromosome (BAC) library of the scab-resistant cultivar 'Goldrush'. In concert with this, more than 160 719 ESTs were analyzed and more than 2000 SSRs were identified. EST-SSR markers are being developed for use with the 'Goldrush' BAC library to integrate the physical and genetic maps of apple (Han *et al*. 2006).

Transgenic approaches

Dandekar *et al*. (2004) examined the effect of down-regulation of ethylene biosynthesis on fruit flavor complex in transgenic 'Greensleeves' apple fruit.

Fruit from plants silenced for either ACS (ACC synthase; ACC-1-aminopropane-1-carboxylic acid) or ACO (ACC oxidase) – both key enzymes for ethylene biosynthesis – had reduced autocatalytic ethylene production. While sugar and acid composition were not affected, there was a significant suppression of synthesis of volatile esters but no significant suppression of the aldehyde or alcohol precursors of these esters. There was a decrease in total ester production in transgenic fruit of 65–70%, with a 90% inhibition of hexyl acetate and a 60–80% inhibition of butyl 2-methylbutanoate, hexyl propanoate and hexyl butanoate.

Transgenic 'Greensleeves' apples with a high suppression of ethylene were used to examine volatile-related enzymes and amino acids and fatty acids as precursors to aroma. LOX was independent of ethylene. Isoleucine, an important precursor, increased in the peel with ripening and responded to ethylene (Defilippi *et al*. 2005a).

In transgenic apples suppressed for ACS or ASO, there was a major reduction in ester production (Defilippi *et al*. 2005b). The activity of AAT, known to be important in ester biosynthesis, showed an ethylene-dependent pattern of regulation. Activity and expression of ADH were not affected by changes in endogenous ethylene levels.

Ethylene production and softening (ACS-ACO)

The first markers for traits related to fruit quality, ethylene production and softening have been developed (Costa *et al*. 2005a). ACS, ACO, expansin (EXP) and polygalacturonase (PG) were studied. Md-ACO1 was mapped on LG 10 on the border of a known QTL for firmness (King *et al*. 2001), and MdACS1 was mapped on LG 15. Offspring homozygous for Md-ACS1-2 were found to have low ethylene synthesis and good retention of storage and shelf-life (Costa *et al*. 2005b). Md-EXPDCA1 mapped on LG 1 approximately 9 cM from V_f, a gene for resistance to the apple scab fungus, and which correlated with a known QTL for crispness and juiciness. Md-PG1 also mapped on LG 10, and single nucleotide polymorphisms (SNPs) were associated with fruit softening. In addition, Sato *et al*. (2004) found that an allelotype of a ripening-specific 1-ACS gene related to the rate of fruit drop in apple.

Consumer perceptions and sensory testing

Research on consumer perceptions of, and responses to, apple quality demonstrates the need for integration of research across disciplines (Harker *et al*. 2003). A better understanding of quality and consumer perception of quality will be beneficial to the area of sensory testing and to the industry as a whole.

There are often low correlations between instrumental measurements and sensory analysis of apples. This is not surprising given the heterogeneity of apples, the difficulty in providing uniform samples at the proper stage of

maturity to panels and the fact that sensory testing and instrumental measurements of quality are rarely conducted on the exact same apple. There are many theories as to optimum panel size, panel training, systems of evaluation, sample preparation and analysis (Lawless and Heymann 1999). The use and misuse of certain discrimination tests for assessing sensory properties of fruits is a concern addressed by Harker *et al*. (2005).

There is a need to ensure that sensory testing is conducted in a reproducible manner and that it takes into account sample heterogeneity, sample preparation and the impact of each sample on subsequent samples tested. Development of a uniform vocabulary and descriptors for trained panels would be useful.

Kuhn and Thybo (2001) evaluated the sensory quality of 22 scab-resistant apple cultivars using a trained sensory panel. Unripe flavor and sourness were present in many early-season cultivars, perhaps due to aldehydes. Panelists assessed the intensity of flavor, the intensity of perfumed or aromatic flavor, the intensity of overripe flavor (which was defined as negative) and the duration of flavor after chewing.

Variation in sensory attributes among the cultivars 'Elshof', 'King Jonagold' and 'Topaz', was correlated with the concentration of volatile compounds. The highest correlations between aroma compounds and sensory flavor attributes were for apple flavor ($r = 0.89$) and perfumed flavor ($r = 0.84$) (Thybo *et al*. 2005).

In 1994,Yahia outlined areas in apple flavor that merited more research and, while progress has been made, his list still holds true today: (1) breeders need to pay more attention to fruit quality; (2) the flavor profiles of different cultivars must be classified and research should differentiate primary from secondary odors; (3) the biosynthesis and metabolism of odor-active volatiles must be examined; (4) further study is needed on the effects of pre- and post-harvest treatments on flavor; (5) studies are needed on the physiology and biochemical basis of human flavor perception; and (6) research on off-odors and off-flavors and their generation should continue. Research during the past decade has set the stage for even greater future advances in our understanding of apple flavor and in our ability to genetically improve flavor in apple.

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Chapter 8
Aroma as a factor in the breeding process of fresh herbs – the case of basil

Nativ Dudai and Faith C. Belanger

The importance of selecting for aroma in breeding of aromatic plants

The aroma of plants is determined by the composition of volatile components. Currently, we are witnessing a rapid growth in the production of fresh herbs, which is in response to the increasing consumer demand. The intensive cultivation required for year-round availability of fresh herbs has stimulated the development of innovative growing methods and a greater interest in breeding (Dudai *et al*. 2002). Naturally, as has happened in other crops such as vegetables, fruits and flowers, there is a danger that this process is leading to high-yielding, disease-resistant and attractive varieties – but with a loss in their aroma and flavor quality. This process has already started, since the market has been requesting breeders to provide rapid solutions to urgent problems – such as resistance to diseases or tolerance to chilling. Aroma and flavor quality are often not selected for and may be lost or reduced in newer varieties. This chapter presents the case of basil, which is an important well-known herb. Here, we present the identification of the aroma factors and discuss the ways in which we can select and genetically manipulate the aroma quality.

The importance of genetic factors regarding the essential oil composition in aromatic plants

Genetic factors greatly influence both the content and the composition of the essential oils in plants and, irrespective of whatever other factors may be involved, genetic factors are the main reason for variations in the essences of various species (Guenther 1965; Lincoln and Langenheim 1978, 1981; Werker *et al*. 1985a,b). Thus, aromatic plants such as *Origanum vulgare*, *Origanum syriacum*, *Salvia fruticosa* and *Artemisia judaica*, which were collected from wild populations in various locations and transferred to experimental plots, did not show substantial changes in their essential oil composition (Putievsky and Ravid 1984a,b; Ravid and Putievsky 1985a,b; Putievsky *et al*. 1992).

The genetic effect is strong enough to create essential oils that differ widely in their composition, even among varieties within the same species. For instance, in *Salvia sclarea* (clary sage) of Russian origin, the main components of the

Biotechnology in Flavor Production: A Case-based Approach. Edited by Daphna Havkin-Frenkel, Faith C. Belanger.
 ISBN: 978-1-4051-5649-3

oil are linalool and linalyl acetate, whereas a variety of the same species that originated in Israel contains citral, geraniol and geranyl acetate. Cross-breeding of those two varieties yielded a hybrid whose oil comprised components that are present in both the parent plants (Elnir *et al*. 1991).

This genetic variability and diversity within herb species provides great opportunities for breeding programs. Classic breeding is still the main tool used in these programs, and has the power to produce new varieties with a wide range of characteristics to suit the demands of various markets, and to select chemotypes according to a variety of quality requirements. For instance, the commercial hybrid sage of *Salvia officinalis* × *S. fruticosa*, named cv. Newe Ya'ar No. 4, yields an aroma that has been found desirable in the marketplace (Putievsky *et al*. 1990; Dudai *et al*. 1999a). In a similar way, there are two identified chemotypes of *O. syriacum* (za`atar): one contains mainly carvacrol (60–80%) and a small amount of thymol, whereas the other contains mainly thymol, and only a little carvacrol (Ravid and Putievsky 1985b).

Since the oil composition may be critical for commercial purposes, it is a great advantage to be able to control genetic factors. The Australian tea tree (*Melaleuca alternifolia*) provides an example of the potential commercial importance of the variations in essential oil composition. The market requires a variety rich in terpinen-4-ol, which has useful medicinal (bactericidal and fungicidal) properties. However, the oils from some chemotypes of *M. alternifolia* contain low concentrations of terpinen-4-ol, some as little as 1%. They therefore lack the desired antimicrobial activity, and this situation presents quality-control problems for the industry (Russel and Southwell 2002).

In this chapter, we focus on sweet basil as an example of an aromatic plant whose essential oil components are important flavor compounds. We discuss the importance of extensive chemical analyses, biochemical dissection of biosynthetic pathways and genetic analysis of inheritance in efforts to improve and control the flavor of aromatic plants.

Sweet basil and the *Ocimum* genus

Sweet basil is an annual aromatic herb, grown for use fresh or dry, or as a source of essential oil and oleoresin for manufacturing perfumes, food flavors and aromatherapy products. The aroma arises from the essential oils, composed of various volatiles that are contained in specialized tissues or glands. The genus *Ocimum* belongs to the Labiatae family and contains between 30 and 160 species (Paton *et al*. 1999). Most of these species originated in tropical and subtropical regions of Africa, Asia and South America. Most of them are aromatic plants due to their essential oil, which accumulates in functional trichomes on various parts of the plant (Paton *et al*. 1999). The most well-known edible basil varieties belong to the species *Ocimum basilicum*.

Some species of *Ocimum* are commonly referred to as 'basil' even though they do not belong to the species *O. basilicum.*

The most commonly used basil in the western world is *O. basilicum*, which is well known for its importance in Italian cuisine. The chromosome numbers reported in the literature vary from $2n = 52$ to $2n = 72$ in different varieties (Grayer *et al*. 1996). This variability may be the result of crossing with related species, which created new sub-species and varieties (Putievsky and Galambosi 1999). There is huge morphological variation within commercial basil varieties (Plate 8.1).

Uses of sweet basil

Commercially, basil's importance is mainly due to its role in Italian food, primarily as the principal component of Italian pesto sauce. It is also used as a fresh or dry spice. Sweet basil is also used for oleoresins and essential oil production for the food and fragrance industries (Simon 1990). Its essential oil and extract have been found to have biological activity against fungi (Reuveni *et al*. 1984; Oxenham 2005), bacteria (Thoppil *et al*. 1998; Suppakul *et al*. 2003), viruses (Chiang *et al*. 2005) and nematodes (Chatterjee *et al*. 1982). The essential oil can also inhibit seed germination (Dudai *et al*. 1999b) and has high anti-oxidant activity (Juliani and Simon 2002; Javanmardi *et al*. 2003). The bioactivity of basil is usually ascribed to its essential oil components, mainly eugenol, methyl chavicol and linalool.

Sweet basil is one of the main herbs grown in the Mediterranean region. In response to the need for a year-round supply, intensive production in climate-controlled greenhouses has recently been established. This has been accompanied by an increase of disease infection and the need to develop tools to avoid it. The sophisticated market today limits the pesticides that may be used and the levels of their residues that are allowed in the final product.

All of this has resulted in an intensive breeding process that has yielded some new cultivars, with an emphasis on desirable appearance, resistance to diseases, especially *Fusarium*, and an improved shelf life (Dudai *et al*. 2002). There is now the need to learn more about the aroma factors, mainly their biochemistry, analysis methods and mode of inheritance in order to facilitate maintenance of aroma qualities during the breeding process. In many cases during the breeding process, the breeder does not know how to identify the desirable aroma in chemical terms or does not have the facilities for doing so. In this way, the long and complicated process of breeding may eventually change the aroma qualities (Galili *et al*. 2002). This is particularly important in aromatic plants, since their role is to add to and improve the flavors of food. Genetic improvement of herb species has been thoroughly discussed by Bernath and Nemeth (2000) and Franz and Novak (2002).

The chemistry of the aroma factors of plants: The essential oil

In aromatic plants, the term 'essential oil' usually refers to the total volatile compounds in a given plant. Essential oils are made up of many different chemical components. These various chemical components combine in many different ways to create specific oils; some oils contain a few volatiles, while others have hundreds of volatile components. Essential oils are produced industrially and in the laboratory by pressing or by extraction, or by water or steam distillation (Tyler *et al.* 1976; Walradt 1982). Most oil components are volatile terpene derivates (mono and sesquiterpenes), which are produced in the isoprenoidic pathway (Croteau *et al.* 2000). Other aromatic substances are produced in the biosynthetic pathway originating from shikimic acid (Croteau and Karp 1991). There are many functional groups that characterize essential oil components; they include hydrocarbons, alcohols, ketones and esters (Walradt 1982).

The quality of an aromatic plant is determined by the content and the composition of its essential oil. The essential oil content is expressed as a percentage of the plant weight, whereas the oil quality depends on the oil composition, i.e. the chemical substances it contains, and their relative quantities. Another important parameter in commercial agriculture is the essential oil yield, which is the amount of essential oil obtained from a plant or an area. Whereas the yield of essential oils is an important parameter for the growers, users are more interested in the quality and aroma of the oil or the plant.

Essential oil profiles of common commercial basil varieties

Interspecific hybridization during the course of evolution produced many polyploid *Ocimum* species with variable chemical compositions of their essential oils (Paton 1999). Even within any *Ocimum* species, there are different chemical types (Simon 1990). The essential oil of *Ocimum* species typically contains monoterpenes, sesquiterpenes and phenylpropanoids (Hiltunen and Holm 1999).

The original Pesto sauce, which is a very important culinary component of Italian cuisine, may contain various chemical aroma factors, depending on the basil varieties grown in Italy. The most common variety for Pesto sauce is the *O. basilicum* cv. Genovese Gigante (Miele 2001). The relative content of eugenol and methyl eugenol, which are the main components in this cultivar, varies with plant development. However, this cultivar, as well as other varieties grown in Italy, contains additional main components such as (–)linalool, 1,8-cineole and methyl chavicol (also called estragole) (Miele *et al.* 2001). The structures of some of the most abundant aroma compounds are shown in Fig. 8.1. However, the total aroma of basil originates from many compounds, including microcomponents, and these may play an important role in the final aroma composition. One of the reasons for this might be the low sensory threshold of some of the microcomponents.

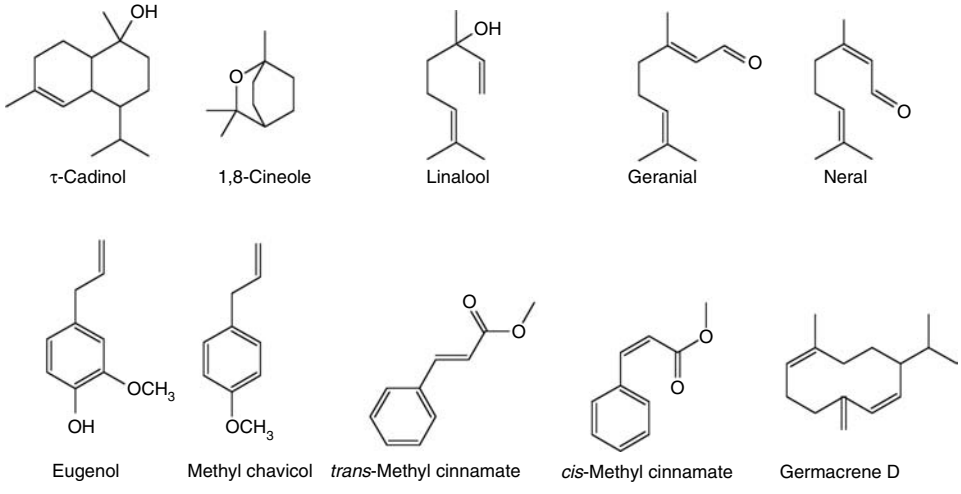

Fig. 8.1 Structures of some of the main components of the essential oil of basil.

Many studies of essential oil composition in basil have been carried out on commercial germplasm sources as well as on sources that are not commercially available (De Masi *et al*. 2006; Grayer *et al*. 1996; Hiltunen and Holm 1999; Lachowicz *et al*. 1996; Marotti *et al*. 1996; Simon *et al*. 1999; Vina and Murill 2003; Vieira and Simon 2006). Our approach is to focus on commercial varieties and to also include analysis of microcomponents that may have a critical role in the overall aroma of the plant. Table 8.1 shows the essential oil composition of some commercially available varieties, and all of them are produced and listed in the catalogue of Genesis Seeds Co. (N. Dudai, unpublished data). The plants were grown at an experimental station in Israel during the summer of 2005. The essential oil was distilled by using a Clevenger apparatus and was analyzed by a gas chromatograph-mass spectrometer system (GC-MS) (Dudai *et al*. 2003). These data illustrate the variation in the chemical composition of commercial sweet basil types in common use.

We also present a categorization of basil varieties according to chemotypes. Previous categorizations of basil chemotypes have been related to many exotic basil varieties, most of which are not available commercially (Hiltunen and Holm 1999). To our knowledge, our division of basil chemotypes is unique in representing commercially available basil varieties. These cultivars represent the main chemical types of edible and ornamental basil in the western world market. They can be divided into five main chemical types, according to their major components:

(1) Eugenol plus linalool type,
(2) 'Lemon' types containing citral (a mixture of neral and geranial) and sometimes also methyl chavicol,
(3) Methyl chavicol type ('exotic basil'),
(4) Methyl chavicol plus linalool type,
(5) Methyl cinnamate type.

Table 8.1 Essential oil profiles of various commercial basil varieties. Values given are the percentage of the essential oil. The major components are highlighted in bold.

	Cultivar Name															
	Petra	Aroma 1	Aroma 2	Aroma 3	Greek	Genovese	Sweet Swiss	Fino Verde	Cinnamon	Lime	Lemon tall	Sweet Nufar	Sweet Chen	Sweet Mammoth	Ararat	Cardinal
Percentage of oil in FW	0.14	0.27	0.21	0.30	0.49	0.25	0.24	0.17	0.24	0.33	0.25	0.20	0.22	0.24	0.11	0.26
1,8-Cineole	**4.20**	**6.98**	**6.35**	**4.72**	**4.13**	**2.25**	**3.31**	0.41	0.26	0.17	ND	**4.29**	**4.26**	**3.35**	0.37	**3.86**
Linalool	**42.68**	**17.92**	**22.12**	**27.42**	**11.25**	**16.70**	**22.26**	**19.07**	**15.83**	2.93	Tr	**35.31**	**30.26**	**13.52**	0.82	**14.46**
Neral	0.03	0.02	ND	ND	ND	ND	ND	ND	ND	**30.96**	**7.55**	ND	ND	ND	ND	ND
Geranial	0.04	0.02	ND	ND	ND	ND	ND	ND	Tr	**38.29**	**9.37**	ND	ND	ND	ND	ND
Methyl chavicol	Tr	Tr	ND	Tr	ND	ND	ND	ND	3.38	0.21	**55.66**	**19.71**	**39.90**	**46.49**	**71.03**	**59.23**
Eugenol	**25.77**	**47.87**	**27.96**	**46.30**	**52.36**	**46.87**	**47.27**	**48.91**	0.18	0.23	ND	11.84	4.29	0.12	ND	0.95
(*Z*)-Methyl cinnamate	ND	ND	ND	ND	ND	ND	ND	ND	**9.36**	ND	ND	ND	ND	ND	ND	ND
(*E*)-Methyl cinnamate	ND	ND	ND	ND	ND	ND	ND	ND	**53.82**	ND	ND	ND	ND	ND	ND	ND
τ-Cadinol	**3.12**	**5.07**	**7.70**	**5.32**	**5.88**	**8.62**	**6.42**	**8.64**	**3.97**	ND	Tr	**6.10**	**4.14**	**8.73**	**6.17**	**6.71**
Germacrene D	**3.35**	**2.81**	**5.17**	**2.73**	**2.49**	**3.79**	**3.44**	**1.98**	**1.80**	**1.92**	**5.17**	**2.60**	**1.33**	**1.89**	**1.98**	**1.13**
α-Thujene	Tr	Tr	Tr	ND	Tr	ND	ND	Tr	0.01	ND	ND	Tr	0.02	ND	ND	Tr
α-Pinene	0.16	0.28	0.42	0.21	0.22	0.06	0.05	0.04	0.01	0.02	ND	0.17	0.18	0.12	ND	0.10
Camphene	0.05	0.04	0.08	0.03	0.05	0.01	0.01	Tr	0.01	ND	ND	0.02	0.02	0.02	ND	0.02
Sabinene	0.24	0.51	0.50	0.34	0.30	0.11	0.11	Tr	0.01	ND	ND	0.27	0.26	0.20	ND	0.22
β-Pinene	0.41	0.84	0.91	0.56	0.51	0.19	0.20	Tr	0.02	Tr	ND	0.47	0.44	0.37	ND	0.37
1-Octen-3-ol	0.01	0.02	0.01	0.04	0.01	0.02	0.01	Tr	Tr	Tr	ND	0.05	0.05	0.02	ND	0.01
6-Methyl-5-hepten-2-one	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.08	Tr	ND	ND	ND	ND	ND
Myrcene	0.50	0.74	0.79	0.51	0.37	0.22	0.25	0.05	0.11	ND	ND	0.54	0.53	0.23	ND	0.44
α-Phellandrene	0.02	0.01	0.01	ND	0.01	ND	ND	Tr	Tr	ND	ND	Tr	Tr	Tr	ND	Tr
δ-2-Carene	0.01	0.01	ND	0.01	0.02	ND	ND	ND	Tr	ND	ND	ND	Tr	ND	ND	ND
α-Terpinene	0.02	0.02	0.05	0.03	0.01	0.01	Tr	Tr	0.01	ND	ND	ND	0.06	0.02	ND	Tr
para-Cymene	Tr	Tr	Tr	Tr	Tr	ND	ND	Tr	0.04	ND	ND	Tr	Tr	Tr	ND	Tr
Limonene	0.33	0.31	0.35	0.20	0.22	0.10	0.13	0.10	0.09	0.03	ND	0.16	0.22	0.15	Tr	0.26
(*Z*)-β-Ocimene	0.00	0.00	0.09	0.00	0.02	0.04	0.04	ND	0.07	Tr	ND	0.05	0.04	0.01	ND	0.05
(*E*)-β-Ocimene	0.00	0.00	1.68	0.00	0.38	0.90	0.98	ND	1.46	ND	ND	1.06	0.78	0.14	0.20	1.19
γ-Terpinene	0.03	0.03	0.08	0.04	0.02	0.01	Tr	0.18	0.11	ND	ND	0.02	0.22	0.04	ND	0.02

(*E*)-Sabinene hydrate	0.14	0.28	0.06	0.11	0.17	0.10	0.13	0.33	0.17	ND	ND	0.18	0.19	0.02	Tr	0.15
n-Octanol	ND	ND	ND	ND	ND	Tr	Tr	ND	ND	0.05	ND	0.12	0.07	ND	ND	0.04
Terpinolene	0.26	0.17	0.27	0.12	0.22	0.08	0.11	0.16	0.06	ND	ND	0.09	0.14	0.08	0.20	0.09
Fenchone	0.63	ND	ND	ND	ND	ND	ND	ND	0.10	0.16	0.08	ND	ND	ND	0.21	ND
exo-Fenchol	0.07	ND	ND	ND	ND	ND	ND	ND	Tr	ND	ND	ND	ND	ND	ND	ND
Photocitral 1	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.13	0.07	ND	ND	ND	ND	ND
(*E*)-Myroxide	ND	ND	0.36	Tr	0.07	0.09	Tr	ND	0.17	0.12	ND	Tr	Tr	ND	0.34	ND
Photocitral 2	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.69	0.24	ND	ND	ND	ND	ND
δ-Terpineol	0.18	0.33	0.24	0.18	0.21	0.15	0.15	0.02	Tr	ND	ND	0.19	0.18	0.12	0.05	0.18
Borneol	0.11	0.04	0.24	0.11	0.11	0.35	0.12	0.44	0.15	ND	ND	0.07	0.10	Tr	ND	0.33
Terpinene-4-ol	0.09	0.10	0.23	ND	0.08	0.04	0.05	2.67	1.47	ND	Tr	0.07	1.32	0.12	0.03	0.09
α-Terpineol	1.13	1.65	1.39	0.97	1.29	0.86	0.89	0.30	0.20	0.47	ND	0.81	0.40	0.11	0.34	0.13
Octanol acetate	ND	ND	0.23	Tr	0.22	0.15	0.12	Tr	ND	ND	0.51	0.05	0.02	0.01	ND	0.05
endo-Fenchyl acetate	0.28	ND	ND	ND	ND	ND	ND	Tr	Tr	ND	ND	ND	ND	ND	ND	0.73
Nerol	0.03	0.01	ND	ND	ND	ND	ND	Tr	ND	3.76	2.10	ND	ND	ND	ND	ND
Citronellol	ND	ND	ND	ND	ND	ND	ND	Tr	ND	6.43	ND	ND	ND	ND	ND	ND
Geraniol	1.33	0.07	ND	ND	ND	ND	ND	ND	ND	1.59	0.66	ND	ND	ND	ND	ND
Chavicol	Tr	Tr	ND	Tr	Tr	Tr	ND	ND	ND	ND	ND	0.43	0.49	ND	ND	0.60
Bornyl acetate	0.39	0.32	2.93	0.30	1.19	1.19	0.66	1.04	0.48	ND	ND	0.43	0.37	0.21	0.16	0.99
δ-Elemene	ND	ND	ND	Tr	ND	ND	Tr	ND	ND	ND	ND	0.03	0.02	0.02	ND	0.03
α-Cubebene	0.03	0.02	0.05	ND	0.02	0.02	0.02	ND	Tr	Tr	Tr	0.03	0.01	0.02	Tr	ND
Neryl acetate	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.49	0.92	ND	ND	ND	ND	ND
α-Copaene	ND	Tr	0.25	ND	0.08	0.09	0.10	Tr	0.06	0.30	0.47	0.17	0.09	0.14	0.10	0.07
Geranyl acetate	0.28	0.02	ND	ND	ND	ND	ND	ND	ND	0.29	0.92	ND	ND	ND	ND	Tr
β-Bourbonene	0.07	0.05	0.15	0.07	0.05	0.09	0.06	0.04	ND	ND	Tr	0.10	0.05	0.04	0.06	0.03
β-Elemene	2.38	1.00	3.14	1.30	1.51	1.62	1.65	1.47	ND	0.08	Tr	1.61	0.86	1.97	3.93	0.88
Methyl eugenol	0.23	0.26	0.30	0.12	2.96	0.26	0.22	0.11	ND	ND	Tr	0.21	0.14	0.07	0.36	0.14
Sesquisabinene	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.09	ND	ND	ND	ND	ND	ND
(*E*)-Caryophyllene	1.05	0.65	0.23	0.13	0.16	0.15	0.15	0.11	Tr	2.39	6.35	0.36	0.18	0.18	0.54	0.11
β-Copaene	ND	ND	Tr	ND	ND	ND	Tr	ND	0.11	ND	ND	0.04	0.02	0.17	0.09	0.04
(*E*)-α-Bergamotene	0.40	3.84	3.31	0.03	3.70	4.72	2.34	4.93	0.74	1.05	1.53	0.17	1.38	7.57	1.50	0.13
α-Guaiene	0.84	0.25	0.93	0.46	0.45	0.35	0.39	0.38	0.27	ND	ND	0.47	0.24	0.52	0.91	0.21
(*Z*)-β-Farnesene	0.27	0.21	0.15	0.10	0.35	0.24	0.17	0.15	0.08	Tr	Tr	0.21	0.18	0.17	0.20	0.14
α-Humulene	0.50	0.47	0.68	0.48	0.60	0.52	0.53	0.36	0.28	0.75	2.22	1.93	0.80	0.62	1.10	0.47
(*E*)-Muurola-4(14),5-diene	0.21	0.34	0.67	0.37	0.34	0.52	0.39	0.34	0.20	ND	ND	0.38	0.26	0.56	0.31	0.30

(Continued)

Table 8.1 Continued

	Cultivar Name															
	Petra	Aroma 1	Aroma 2	Aroma 3	Greek	Genovese	Sweet Swiss	Fino Verde	Cinnamon	Lime	Lemon tall	Sweet Nufar	Sweet Chen	Sweet Mammoth	Ararat	Cardinal
(*E*)-β-Farnesene	ND	Tr	0.18	ND	0.32	0.36	0.19	0.37	Tr	0.16	Tr	ND	0.08	0.44	Tr	ND
Bicyclogerma crene	2.22	1.66	2.29	1.99	1.59	1.75	1.95	2.11	0.84	ND	ND	3.80	1.86	1.37	2.18	1.73
α-Bulnesene	1.32	0.43	1.45	0.62	0.83	0.73	0.70	0.60	0.35	ND	Tr	0.71	0.38	0.98	1.45	0.34
β-Bisabolene	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.16	0.20	ND	ND	ND	ND	ND
Germacrene A	0.99	0.44	0.53	0.46	0.64	0.48	0.38	0.53	0.37	ND	ND	0.63	0.35	0.76	0.79	0.26
γ-Cadinene	0.83	1.40	2.86	1.55	1.54	2.36	1.79	1.66	1.27	ND	ND	1.58	1.06	2.44	1.42	1.43
δ-Amorphene	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.12	ND
δ-Cadinene	0.10	0.08	0.24	0.09	0.12	0.13	0.13	0.12	0.08	0.14	0.23	0.10	0.09	3.19	Tr	0.11
(*Z*)-Calamenene	0.03	0.04	0.08	0.04	0.06	0.07	0.05	0.04	0.11	ND	ND	Tr	Tr	Tr	Tr	Tr
β-Sesquiphellandrene	0.01	0.20	0.12	ND	0.17	0.27	0.10	0.21	Tr	ND	ND	ND	0.07	0.41	ND	ND
(*Z*)-Muurol-5-en-4-a-ol	ND	ND	ND	ND	ND	ND	ND	ND	0.10	ND	ND	ND	ND	ND	ND	ND
(*Z*)-α-Bisabolene	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.81	4.50	ND	ND	ND	ND	ND
10-epi-Cubebol	0.07	0.12	0.09	0.10	0.11	0.19	0.14	0.15	0.06	ND	ND	0.10	0.05	0.05	ND	Tr
(*E*)-Nerolidol	0.21	0.12	0.14	0.12	0.14	0.15	0.16	0.20	0.15	ND	ND	0.61	0.26	0.16	ND	Tr
Spathulenol	ND	ND	ND	ND	ND	ND	ND	ND	0.14	ND	ND	ND	ND	ND	ND	ND
Caryophyllene oxide	ND	ND	ND	ND	ND	ND	ND	ND	Tr	0.33	0.75	ND	ND	ND	ND	ND
1,10-di-epi-Cubenol	0.52	0.81	1.22	0.89	0.89	1.46	0.98	1.19	0.64	ND	ND	0.90	0.59	1.21	0.78	0.91
α-Cadinol	0.18	0.12	0.30	0.14	0.15	0.18	0.17	0.05	0.22	ND	ND	0.33	0.19	0.41	ND	0.23
β-Eudesmol	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.14	ND	ND	ND	ND	0.34	ND
α-Bisabolol	ND	Tr	0.13	ND	0.15	0.25	Tr	0.19	Tr	0.24	Tr	Tr	Tr	Tr	Tr	Tr
Total %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	97.66	99.50	99.98	100.00	100.00	100.00	100.00

FW, fresh weight; ND, not detected; Tr, trace.

Comparison of chemical analysis methods

Today, the composition of volatiles is usually analyzed by GC-MS (reviewed by Marriott *et al*. 2001). Component identification is done using linear retention indexes (LRI) and mass spectrometry data. Basil volatiles are a medium-complexity mixture and can be sufficiently resolved on a single stationary phase in a reasonable time (30–40 mins). The most popular column type is apolar (5% diphenyl, 95% polydimethylsiloxane). Mass spectrometry with electron ionization (EI) and quadrupole mass analyzer is used in many applications because of the availability of the commercial libraries of Willey and Adams, which are built on similar conditions. The GC-MS technique also provides acceptable quantitative information, even though correlation coefficients are generally not applied. However, the effects of technical conditions, such as the type of column or the detector, on the output data have to be considered. Sampling and extraction methods also seriously influence the volatile content and composition (Mardarowiczl 2004).

For quality control in industrial essential oil/extract production, methods of analysis are adopted that simulate large-scale processes. Different requirements exist for analysis of fresh herbs during the breeding process. The methods used should permit the analysis of variable sample sizes, such as a single leaf or flower as well as a whole plant, and should allow examination of many samples within a reasonable time. Furthermore, the analysis method used should capture the herb aroma as perceived by the user.

Two methods are commonly used for the analysis of edible herbs: solvent extraction and hydrodistillation (Marriott *et al*. 2001; Mardarowiczl 2004). Recently, head-space analysis using solid-phase microextraction (SPME), which absorbs and concentrates the volatiles from the gaseous phase, has become popular in essential oil research. The disadvantage of this method is that it is not as quantitative as other methods. The advantage of SPME, however, is that it makes possible the analysis of extremely small samples, such as the amount from an individual gland (Johnson *et al*. 2004). We have investigated the effect of the extraction methods (as described in Larkov *et al*. 2005) on the volatiles composition in basil (N. Dudai, unpublished data). The comparison of results from distillation, cold solvent extraction and SPME is presented in Table 8.2. The results show a great variation among the methods in composition and content of volatiles. The major aroma compound, eugenol, varies greatly between 10% and 49%. The τ-cadinol percentage drops from 6% in the distilled oil to undetectable levels in two SPME analyses. Owing to different affinities of volatiles to the specific absorptive material used, large variations are also seen within the same SPME method using different fiber matrices. The large effect of the extraction method used on the content and composition of volatiles requires the researcher to adopt a uniform analysis method according to the investigation's goal.

Our experience with basil breeding showed that solvent extraction is the most reliable method for consistent analyses. Using solvent extraction reduces

Table 8.2 The effect of the extraction method on the volatiles composition in basil. Values given are the percentage of the essential oil.

Compound	Solvent[a] extraction	Hydrodistillation	SPME fiber matrix		
			DVB/ PDMS	PDMS	DVB/Carboxen/ PDMS
2-(*E*)-Hexenal	ND[b]	ND[b]	2.4	1.1	4.6
β-Pinene	0.7	0.8	1.5	2.0	1.2
Myrcene	0.6	0.9	1.9	1.3	1.4
1,8-Cineole	9.0	9.5	13.2	31.1	21.1
(*E*)-β-Ocimene	2.0	1.7	7.5	3.9	3.7
Linalool	19.9	30.5	33.0	28.6	45.9
Eugenol	49.4	27.4	10.0	13.9	11.8
β-Elemene	2.8	1.7	1.3	1.5	0.3
α-Guaiene	0.7	0.8	1.7	0.7	0.2
Germacrene D	2.7	4.3	6.2	4.5	0.9
Bicyclogermacrene	0.6	1.8	2.5	1.8	0.5
α-Bulnesene	0.2	1.3	1.6	0.8	0.3
γ-Cadinene	1.8	2.0	3.3	1.8	0.7
τ-Cadinol	3.4	6.2	0.3	ND	ND
Essential oil content	0.37	0.13	Cannot be determined by SPME method		

[a] *tert*-Butyl methyl ether.
[b] Compound is coeluted with a solvent.
DVB, divinylbenzene; PDMS, polydimethylsiloxane; SPME, solid-phase microextraction.

the potential interference from distillation artifacts (Dudai *et al.* 2003). We adopted the method of Lewinsohn *et al.* (1993) using the solvent *tert*-butyl methyl ether (Dudai *et al.* 2001, 2003). The main advantages of this method to the breeder are the consistent qualitative and quantitative results, and the ability to extract many samples simultaneously, using very small samples such as a single leaf or flower. In the next section, we use this method to analyze the composition of the aroma factors in different tissues of the same plant.

Variation of the volatile compound composition within the plant

It is well known that the plant developmental stage, as well as leaf age and position, have a strong effect on the volatiles composition in aromatic plants (McConkey *et al.* 2000; Gershenzon *et al.* 2002; Szabo and Bernath 2002). There may be significant variation between organs or leaves of a given plant (Dudai *et al.* 2001) and, in the case of basil, it is also an important issue. Werker *et al.* (1993) showed morphological and chemical changes related to leaf age and position of a methyl chavicol chemotype of basil. Deschamps *et al.* (2006) reported the biochemical and molecular changes that were observed during the plant development of this chemotype. Here we present our analysis of different leaves from a commercial eugenol chemotype, a Genovese-type cultivar (Fig. 8.2). The main conclusion is that there are dramatic differences

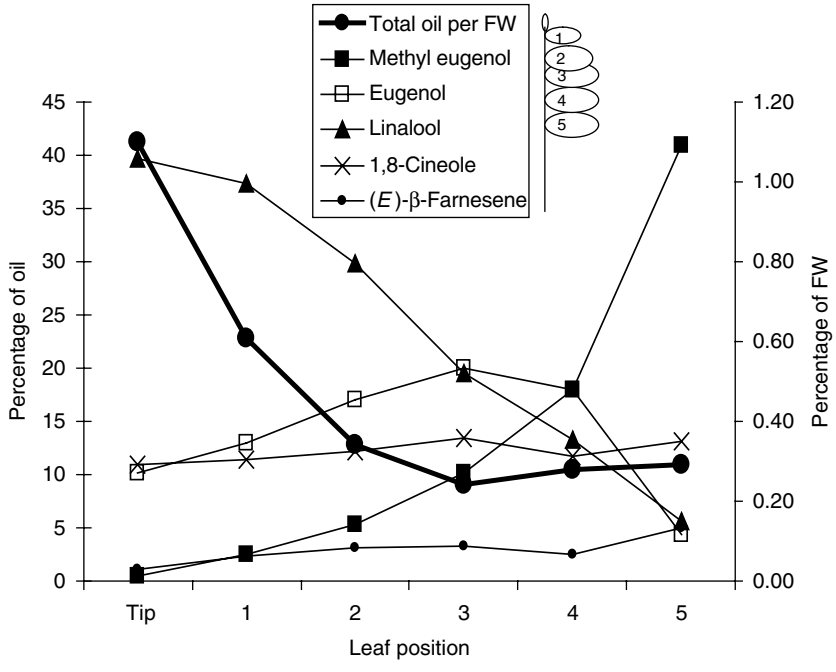

Fig. 8.2 Variability in the levels of aroma compounds in leaf extracts from a single plant. The percentages of the total oil for the aroma compounds methyl eugenol, eugenol, linalool, 1,8-cineole and (*E*)-β-farnesene are shown as well as the total percentage of oil based on the fresh weight (FW) of the sample. Inset is a diagram illustrating the positions of the leaves used for analysis.

between leaves on the same plant, depending on their age and position. The lower leaves contain a much lower total concentration of volatiles than the upper young leaves, and their composition is also significantly different. Methyl eugenol is a main component of the lower leaves, while the upper leaves contain more linalool and eugenol. So, using the upper leaves of a young plant for analysis will result in a different profile of aroma compounds than when using the older leaves. These results agree with those of Miele *et al.* (2001) who distilled whole plants of various ages, and showed the effect of the plant age and height on the relationships between eugenol and methyl eugenol. This phenomenon illustrates the importance of consistent sampling for chemical aroma analysis. For reliable comparisons, sampling must be of the same leaf position of the same plant age. This issue is very important for selection during breeding for the aroma properties.

Variation of aroma compounds within cultivars and the potential for selection

In many studies, the composition of aroma factors of varieties of basil are determined by distillation of samples consisting of a number of plants or

from biomass harvested from a given unit of area. This type of sampling provides information on the composite composition of aroma compounds of the plants sampled. However, analysis of single plants can reveal considerable variability of essential oil composition within commercial varieties, even if the variety appears morphologically homogeneous. This is important because, in the past, basil was mainly investigated for use dry or for production of essential oil. For both of these uses, total biomass is important and variability among individual plants is not so critical. With the increasing use of basil as a fresh herb, the aroma of a single plant is very important.

This variability among individual plants of a single cultivar is illustrated by the data presented in Table 8.3. Here the major essential oil components of 12 plants of Variety 22 are compared. In this analysis, in order to minimize the variability in essential oil composition due to plant age or to leaf position on the plant, these plants were grown in controlled conditions in pots. After 6 weeks, when the plants had five nodes, the third leaf pair from the top was sampled. This uniform sampling strategy makes the differences in essential oil composition among the 12 plants even more striking. From the data in Table 8.3, the plants can be divided into two main types according to the phenylpropene composition, the methyl chavicol type and the eugenol/methyl eugenol type. In this case, all the plants containing methyl chavicol lack eugenol/methyl eugenol and vice versa. One unusual plant of the 12 had a high level of methyl eugenol as well as of eugenol. As discussed above, methyl eugenol is typically found mainly in the lower leaves, while eugenol is found in the upper leaves. Since the third leaf from the top was analyzed, no methyl eugenol was expected, even in the eugenol types. Such an unusual plant could be useful for further breeding, since it may have an altered regulation of expression of critical enzymes.

Table 8.3 Content of selected aroma components of 12 randomly selected single sweet basil plants of Variety 22. The analysis was done by extraction of the third leaf pair from plants with five nodes above the cotyledons. Values are the percentage of total essential oil.

	Plant number											
Components	1	2	3	4	5	6	7	8	9	10	11	12
Sabinene	0.7	ND	0.6	ND	0.9	0.7	1.2	0.6	1.2	0.8	1.0	1.0
1,8-Cineole	11.5	16.1	10.9	14.6	8.9	12.0	8.1	9.8	8.5	7.5	7.4	8.7
Linalool	29.6	27.9	32.4	11.8	26.8	23.8	8.2	18.0	16.7	26.7	8.6	6.7
Camphor	ND	0.3	ND	0.7	ND	ND	0.3	0.3	ND	ND	0.3	0.4
Borneol	0.3	0.2	0.2	0.4	0.3	0.2	ND	0.3	0.4	0.4	0.2	0.2
Methyl chavicol	ND	ND	ND	ND	30.3	32.1	58.1	39.8	49.0	33.3	58.1	57.6
Chavicol	ND	ND	ND	ND	4.7	2.7	ND	2.3	ND	2.6	ND	ND
Bornyl acetate	0.4	0.3	0.5	0.7	0.3	0.4	ND	0.4	ND	0.4	0.2	0.2
Eugenol	20.3	22.9	22.3	8.9	ND	ND	ND	ND	ND	ND	ND	ND
Methyl eugenol	2.2	0.6	1.2	27.7	0.3	0.3	0.2	0.6	ND	0.4	0.3	0.2
Oil content (%)	0.5	0.8	0.8	0.4	0.8	0.8	0.7	0.4	0.8	0.8	0.8	1.0

ND, not detected.

In addition to the phenylpropenes, there was also variation in the levels of the various terpenes among the 12 plants. The largest variation was seen in linalool, which varied from 8% to 32% of the total oil. For the overall aroma, the microcomponents of the essential oil could also be of significance. There were also differences in the levels of sabinene, camphor, borneol and bornyl acetate, where some plants did not contain one or more of these components. In Table 8.3, only the main components are presented but there was also high variability between single plants in some of the microcomponents, such as citral and various terpene-acetates.

Variety 22 was developed by selection for low temperature tolerance. No attention was given to the aroma properties through the breeding process. At the current stage, further breeding efforts should focus on stabilization of the aroma factors as well as on maintenance of the low temperature tolerance. The variability of aroma compounds found among plants from this variety is a good example of what can happen when the aroma factors are not taken into account during the development of new varieties. In fact, all of the cultivars that are common in the marketplace have variable levels of aroma compounds among individual plants within the cultivar. The cultivars differ from each other in the degree of the variation. Figure 8.3 shows the coefficient of variance of three cultivars for the levels of the main aroma compounds. In addition to the overall aroma composition of a specific variety, it can also be characterized by the extent of internal variability of a particular aroma compound. As seen in Fig. 8.3, Variety 22 shows high variability compared with the moderate and low variability shown by the other commercial varieties, Aroma 4 and Aroma 2, respectively. Uniformity is very important in commercial varieties that are produced for the fresh market, because the end customer is purchasing the basil in small bunches of 10–20 g. The goal of the breeder is therefore to produce uniform varieties, not only in morphological and agronomically important traits, but also in the presence of the desired

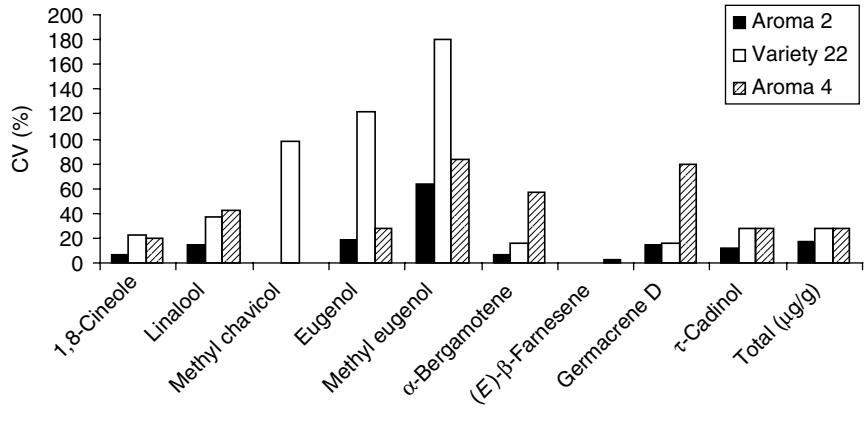

Fig. 8.3 Coefficient of variation (CV) of selected volatiles content in the essential oil of 24 randomly selected single plants of three basil cultivars.

aroma compounds. Analysis of single plants for essential oil composition, as discussed here, will be critical for breeding and selection for the desired aromas.

Biosynthetic pathways of basil aroma components

The biosynthetic pathways of some of the major components of basil aroma are currently active areas of research, and some of the enzymes and genes involved have been characterized. The major aroma compounds of basil are synthesized in peltate glandular trichomes on the leaf surface (Gang *et al*. 2001). The mRNAs and enzymes responsible for the synthesis of the aromatic compounds are also synthesized in the trichomes and are found in relatively high abundance. Intact trichomes can be isolated from the plant, and this has greatly facilitated the discovery of enzymatic activities as well as the cloning of genes involved in the synthesis of the aromatic compounds.

A considerable amount of work has been done on the biosynthetic pathways of the phenylpropenes eugenol and chavicol and their methylated derivatives, methyl eugenol and methyl chavicol. The phenylpropenes originate from phenylalanine and have the same early phenylpropanoid precursors as lignin. A review of these pathways has been published (Gang 2005). The proposed biosynthetic pathway, beginning with the steps that are specific for the phenylpropenes, is shown in Fig. 8.4. Enzymes from the peltate glandular trichomes can catalyze each of these biosynthetic steps and have been characterized. A recombinant coniferyl alcohol acetyltransferase (CAAT) has been

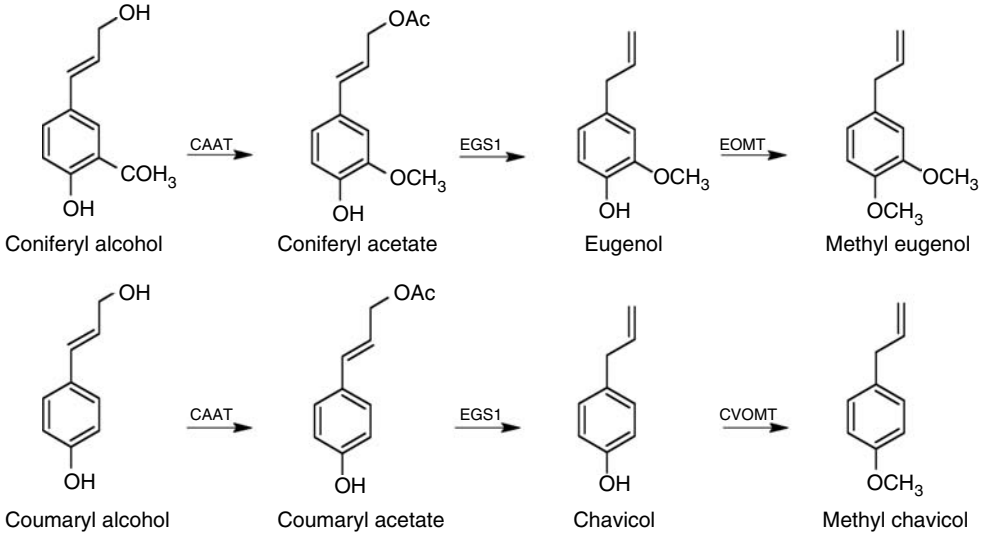

Fig. 8.4 Biosynthetic pathway for methyl eugenol and methyl chavicol (from Gang *et al*. 2002; Harrison and Gang 2006; Koeduka *et al*. 2006; and Vassao *et al*. 2006).

characterized; CAAT actually prefers *p*-coumaryl alcohol, but it also acts on coniferyl alcohol (Harrison and Gang 2006). A novel reduction reaction, catalyzed by eugenol synthase 1 (*EGS1*), results in the conversion of coniferyl acetate to eugenol (Koeduka *et al*. 2006).

A similar NAD(P)H-dependent reductase activity, which converts *p*-coumaryl acetate to chavicol, has been reported; a corresponding cDNA clone has not yet been characterized, however (Vassao *et al*. 2006). The activity of recombinant *EGS1* with *p*-coumaryl acetate was lower than with coniferyl acetate (Vassao *et al*. 2006). It will be interesting to see whether there is an enzyme that prefers *p*-coumaryl acetate as a substrate. Eugenol and chavicol are converted to methyl eugenol and methyl chavicol by the enzymes eugenol *O*-methyltransferase (EOMT) and chavicol *O*-methyltransferase (CVOMT), respectively. Analysis of *O*-methyltransferase activities in leaf extracts from several basil chemotypes has indicated that the chemotype is controlled, at least in part, by the specificities of the *O*-methyltransferases present (Lewinsohn *et al*. 2000). The amino acid sequences of EOMT and CVOMT are 90% identical, and molecular modeling and site-directed mutagenesis have revealed that a single amino acid difference between the two enzymes was responsible for the differences in substrate preferences (Gang *et al*. 2002).

The steps in the biosynthetic pathways of methyl eugenol and methyl chavicol are catalyzed by similar enzymes, although the relationships of these enzymes to each other are not known. Are they allelic variants with distinct substrate preferences, or are they encoded by similar genes at different loci? For breeding for specific combinations of aroma compounds, these are important questions that could be answered by genetic linkage mapping. As discussed below, inheritance studies suggest that at least one step in the pathways to methyl chavicol and methyl eugenol is controlled by variant alleles.

In addition to the phenylpropenes, terpenes are often major aroma compounds in basil and, in some varieties, are the major components of the aroma. The biosynthesis of various terpenes has been studied extensively in many plant species (for reviews see Dudareva *et al*. 2004, 2006). Several terpene synthases from basil have been characterized (Iijima *et al*. 2004a) and the biosynthetic pathway of citral has been investigated. Citral is a term that refers to a mixture of geranial and neral. As the name implies, citral imparts a lemon-like aroma. Cultivars rich in citral constitute the Lemon-type cultivar discussed above.

The biosynthetic pathway for geranial is shown in Fig. 8.5. Specific to the glandular trichomes of a Lemon-type cultivar, a geraniol synthase (GES), which converts geranyl diphosphate to geraniol, has been characterized (Iijima *et al*. 2004b). Two distinct basil enzymes that can carry out the reversible oxidation of geraniol to geranial have also been functionally characterized (Iijima *et al*. 2006). One of these enzymes, cinnamyl alcohol dehydrogenase (CAD1), was found to actually prefer cinnamyl alcohol as a substrate, but is highly expressed in the glandular trichomes relative its expression in leaves. The other enzyme, geraniol dehydrogenase (GEDH1), was found to prefer

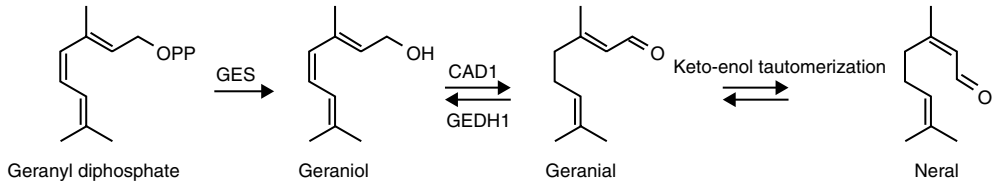

Fig. 8.5 Biosynthetic pathway for geranial and neral (from Iijima *et al.* 2006).

geraniol rather than cinnamyl alcohol as a substrate, but is only weakly expressed in the glandular trichomes. However, the expression patterns of both CAD1 and GEDH1 were found to be similar in a cultivar producing citral, but were also found to be similar in two cultivars that did not produce citral. While both these enzymes can catalyze the reversible oxidation of geraniol, their expression patterns suggest that another, more specific, dehydrogenase that is highly expressed in the glands of citral-producing cultivars may exist (Iijima *et al.* 2006). Neral is formed non-enzymatically from geranial through keto-enol tautomerization.

Inheritance of aroma compounds in basil

As shown earlier in Table 8.1, there is enormous variability among basil cultivars regarding the composition and amounts of aroma compounds. This variability can be exploited through breeding to develop new cultivars with new combinations of aroma factors (Putievsky *et al.* 1999). Although this approach offers tremendous opportunities for modification of the aroma of basil, most basil breeding to date has focused on important agronomic traits, such as low temperature tolerance and disease resistance (Dudai *et al.* 2002). There have only been a few reports of the inheritance of secondary metabolites in basil (Gupta 1994; Vieira 1999). Targeted breeding for specific aroma compounds is just beginning to be practiced.

As an example of the possibilities that are achievable through breeding, we present results from a cross of a Thai-type cultivar, whose essential oil contains high methyl chavicol, no chavicol and no eugenol, and a Genovese-type cultivar, whose essential oil contains high eugenol and no chavicol or methyl chavicol. Thirty-two individual F2 plants resulting from the selfing of an F1 plant were analyzed for 53 essential oil components (Dudai *et al.* unpublished data). As expected, many of the F2 individuals exhibit an essential oil profile that differs from either of the plants used in the original cross.

To understand the inheritance of the phenylpropene composition in the F2 plants, we compared the inheritance of total eugenol + methyl eugenol (total E) and total chavicol + methyl chavicol (total C). The rationale for this approach was based on what is known regarding the biosynthetic pathways of these compounds (Fig. 8.4). It is well established that chavicol and eugenol are the precursors of methyl chavicol and methyl eugenol, respectively (Gang *et al.* 2002).

Table 8.4 Gene model for inheritance of phenylpropenes in F2 progeny from a cross between a Genovese-type cultivar and a Thai-type cultivar.

	Predicted parental genotypes		F2 progeny			
Gene model, phenotype	Thai-type	Genovese-type	Observed	Expected	X^2	*P*
Single gene, partial dominance	S^CS^C	s^Es^E			3.12	0.20
S^CS^C, High total C + MC			5	8		
S^Cs^E, High total C + MC, moderate E			15	16		
s^Es^E, High E, no C + MC			12	8		

The F2 plants could be grouped into three categories based on levels of total C and of total E. One group contained high levels of total C and very low levels (<1%) of total E. Another group contained high levels of total E and very low levels (<1%) of total C. The third group had high levels of total C and moderate levels (1–8%) of total E. Individuals having both high levels of total E and high levels of total C were not observed. The lack of segregation on selfing of the Genovese-type and Thai-type parental cultivars suggested that both were homozygous for the genes controlling the overall phenylpropene composition. Segregation of chemotypes in the F2 progeny and the absence of any individuals having both high levels of total E and high levels of total C suggested an allelic basis for the observed total phenylpropene composition. The segregation ratios suggested that the inheritance was controlled by a single gene with partial dominance, exhibiting the expected 1:2:1 phenotypic ratio (Table 8.4). This gene, designated as S^C, could be any gene in the pathway upstream of the *O*-methyltransferase genes. Gupta (1994) also suggested that there is an allelic basis to the inheritance of chavicol or eugenol biosynthesis. Candidate genes in the biosynthetic pathway upstream of the methylation step are eugenol synthase and CAAT (Harrison and Gang 2006; Koeduka *et al*. 2006), or transcription factors regulating their expression.

Interspecific hybridization among *Ocimum* species

The example presented above illustrates that, within the species *O. basilicum*, there is considerable variation of essential oil profiles that can be tapped through targeted breeding strategies. Additional variability is found between and within other species from the genus *Ocimum* that could be utilized to generate novel essential oil profiles. The essential oil profiles of several *Ocimum* spp., and accessions within these species, have been compared (Simon *et al*. 1999; Vieira and Simon 2006). The major aroma compounds of some of these other *Ocimum* spp. were distinct and uncharacteristic of *O. basilicum* accessions. For example, thymol and *p*-cymene were prominent in *Ocimum gratissimum*

and β-carophyllene was prominent in *Ocimum tenuiliflorum* (Simon *et al*. 1999).

Interspecific hybridization between *Ocimum* spp. therefore may offer additional possibilities for targeted breeding for producing new combinations and proportions of aroma compounds. There have been a few studies on interspecific hybridization of various *Ocimum* spp. (Sobti and Pushpangadan 1995; Paton and Putievsky 1996; Rabinovich 2002). Khosla (1995) divided *Ocimum* spp. into two groups: the 'basilicum group', which contains *O. basilicum*, *O. americanum*, *O. kilimandscharicum* and *O. canum*, and the 'sanctum group', which contains *O. sanctum*, *O. gratissimum*, *O viride* and *O. suave*. Successful interspecific crosses were more frequent between species belonging to the same group.

Rabinovich (2002) followed several generations of plants originating from interspecific hybridization of several *Ocimum* spp. The F2 progeny of the successful interspecific crosses were found to be highly variable and exhibited a wide range of morphological characteristics and a variable chemical composition in the essential oil. Among the F2 plants, there were many new types regarding leaf size and color, inflorescence morphology, color, aroma and taste. Analysis of the F3 generation suggested that it may be possible to achieve stable new varieties in only three to four generations.

Applications of biotechnology-based approaches to modification of basil aroma

Biotechnology, using the approaches of genomic analysis and plant transformation, is beginning to be applied to the improvement of basil. *Agrobacterium*-mediated transformation of basil has been reported (Deschamps and Simon 2002). In that study, two species of basil, *O. basilicum* and *O. citriodorum*, were transformed with the *Escherichia coli* β-glucuronidase (GUS) marker gene. This work demonstrates that basil can be transformed and that phenotypically normal plants can be recovered. This methodology opens up the possibility for future transformation of basil with genes that may modify the aroma profile. The transgenic approach would be most appropriate for the transfer of genes between species from the two hybridizing groups discussed above, where there are genetic barriers to gene transfer through breeding. The transgenic approach has been successfully applied to numerous plant species for the modification of food and feed quality (Galili *et al*. 2002). To fully utilize the transgenic approach with basil will require the elucidation of biosynthetic pathways for additional important aroma compounds and isolation of the genes involved.

Basil is also a rich source of genes that may be useful for transformation into other plant species for the modification of aroma. For example, the GES gene from basil has been used to transform tomato (Davidovich-Rikanati *et al*. 2007). The volatiles isolated from the transgenic fruit were markedly different to those isolated from nontransgenic control fruit. The components of citral, geranial and neral were present at six times the levels seen in

the controls. In addition, owing to the action of endogenous enzymes on geraniol, some geraniol derivatives, such as citronellal, citronellyl acetate, geranyl acetate and rose oxide, were found only in the transgenic fruit. These results indicate that the addition of a single new gene in a biosynthetic pathway may result in unexpected products owing to the conversion of excess substrate by endogenous enzymes. Many plant enzymes can act on numerous substrates, including some substrates that are not normally present. For example, Dudai *et al*. (2000) found that germinating wheat seeds were able to metabolize exogenously applied monoterpenes, even though the wheat seeds themselves did not contain any monoterpenes.

In addition to transformation technology, one of the most broadly useful applications of biotechnology regarding agronomically important plants is likely to be based on genomic analysis encompassing genetic linkage mapping, QTL analysis of important phenotypic traits and marker-assisted selection. Such approaches are currently being used in the major crops to accelerate the development of new varieties that have the desired phenotypic characters. Such an approach has not yet been applied to basil aroma improvement, but some of the required resources are in place. Dudai *et al*. (unpublished data) have demonstrated that populations of individuals segregating for morphological traits and essential oil profiles can readily be generated from crosses between divergent parental plants. Such populations would be ideal for genetic linkage mapping. Expressed sequence tag (EST) databases of several basil cultivars are available (Gang *et al*. 2001; Iijima 2004b), and a new method of marker development based on EST sequences has been reported (Rotter *et al*. 2007). We therefore expect that a genetic linkage map of basil will be generated in the near future; the availability of such a map would facilitate the application of marker-assisted selection for the development of improved basil varieties with specific combinations of aroma compounds.

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Chapter 9
Increasing the methional content in potato through biotechnology

Rong Di

Flavor compound methional in foods

Volatile methional (3-methylthio propanal) is the characteristic flavor compound responsible for the particular aroma of cooked potato (Lindsay 1996). Potato snacks, especially potato chips, are among the most popular food in the snack-food market. An extrusion process has been used extensively to produce low-fat and fat-free potato snacks while retaining the desirable texture and flavor (Majcher and Jelen 2005). Potato cultivars and storage time greatly affect the volatile flavor components of baked potato (Duckham *et al.* 2002).

This potato-like flavor is also present in other foods. Black tea and green tea are the most consumed beverages in the world owing to their health benefits and flavor (Kumazawa and Masuda 2001, 2002). Methional was found to be one of the major potent odorants in tea during heat processing measured by gas chromatography-mass spectrometry in aroma extract dilution analysis. In heat-processed coffee drink, however, reduced methional was shown compared with coffee samples before heat treatment (Kumazawa and Masuda 2003), resulting in a reduction of the 'roasty' odor of coffee drink. Methional is also the key aroma-active compound in roasted wild mango seeds (Tairu *et al*. 2000), cooked mussel (Le Guen *et al*. 2001) and cooked pine-mushrooms (Cho *et al.* 2006). Methional is found to accumulate in beer over time during storage, and its level is dependent on the temperature of storage (Soares Da Costa *et al*. 2004). Since methional is negatively correlated with the aroma quality of beer, information on its concentration is helpful to ensure the flavor stability of beer.

Formation of methional

Methional is a thermally induced flavor compound (Lindsay 1996) (Fig. 9.1). Heat processing induces the Maillard reaction with reducing sugars and amino acids (BeMiller and Whistler 1996). This reaction is also called non-enzymic browning, leading to the ultimate formation of brown pigments. Methional is formed through the interaction of α-dicarbonyl compounds (intermediate products in the Maillard reaction) with methionine (Met) by the Strecker degradation reaction (Lindsay 1996). Methional readily decomposes to yield

Biotechnology in Flavor Production: A Case-based Approach. Edited by Daphna Havkin-Frenkel, Faith C. Belanger.
 ISBN: 978-1-4051-5649-3

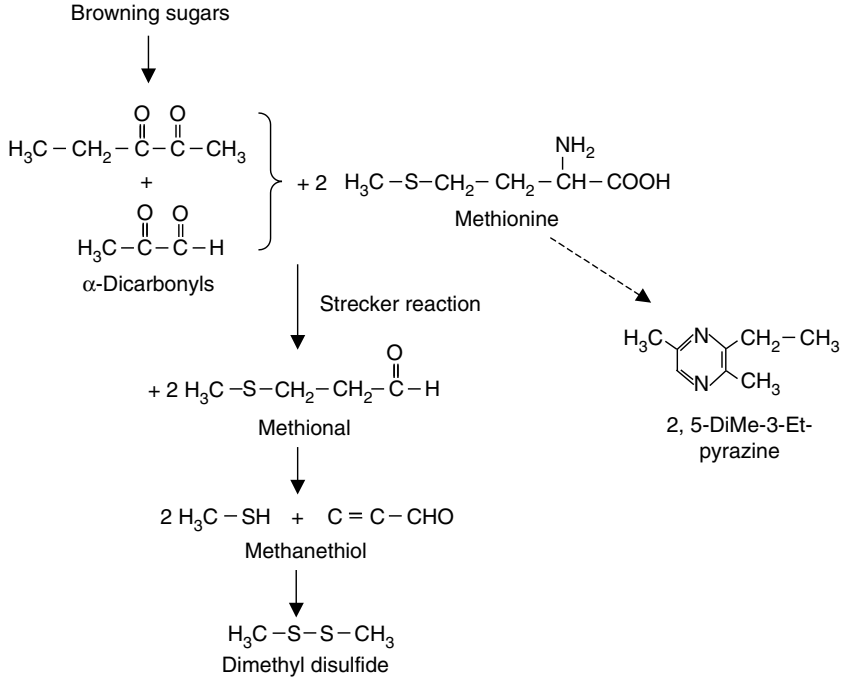

Fig. 9.1 Formation of flavor compound methional.

methanethiol which oxidizes to dimethyl disulfide. Dimethyl disulfide is the source of reactive sulfur compounds which contribute to the overall flavor development. The Strecker degradation reaction involving Met also produces alkyl pyrazines which are important flavor contributors in roasted, toasted or similarly thermally processed foods.

Since methional is heat labile and readily decomposes to methanethiol and dimethyl disulfide, a large percentage of methional is lost during potato processing (Di *et al*. 2003). Owing to costly production, methional and its precursor Met are not added back during food processing. To enhance the flavor of heat-processed potato foods, we have taken the biotechnological approach to increase the Met level in potato tubers (Di *et al*. 2003).

Synthesis of Met in plants

Met is the only sulfur-containing amino acid that is essential for mammals and needs to be derived entirely from the diet (Ravanel *et al.* 1998). Met is not only a building block for protein synthesis, it is also the immediate precursor of *S*-adenosyl-L-methionine (SAM) (Ravanel *et al.* 1998). Apart from being the major methyl donor in transmethylation reactions, SAM serves as the intermediate in the biosysnthesis of polyamines and of the phytohormone ethylene. Figure 9.2 shows the pathway for Met biosynthesis and metabolism

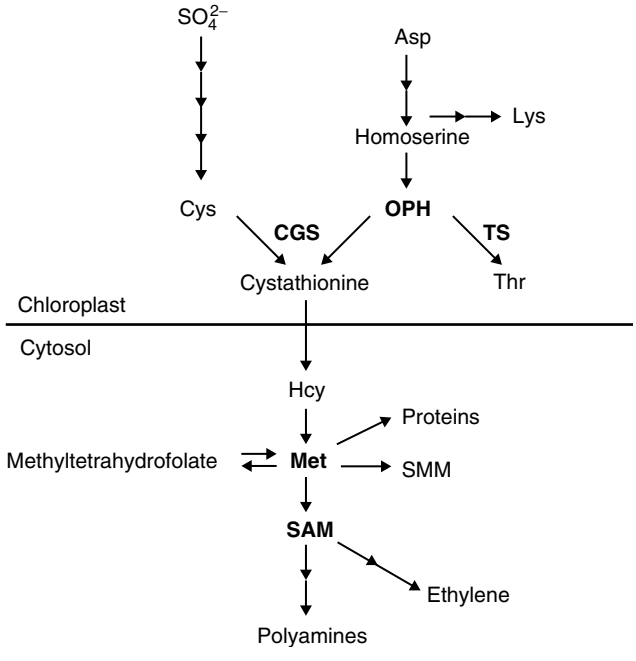

Fig. 9.2 Met biosynthesis pathway and metabolism in plants. Cys, cysteine; Asp, aspartate; Lys, lysine; OPH, *O*-phosphohomoserine; Thr, threonine; Hcy, homocysteine; Met, methionine; SMM, *S*-methylmethionine; SAM, *S*-adenosyl-L-methionine; CGS, cystathionine γ-synthase; TS, threonine synthase.

in plants (Di *et al.* 2003). Met is synthesized from three independently derived compounds (Giovanelli *et al*. 1980). The carbon skeleton is derived from aspartic acid (Asp), and the intermediate *O*-phosphohomoserine (OPH) is used for both Met and threonine (Thr) synthesis. The sulfur moiety of Met is derived from cysteine (Cys), the end-product of the sulfate-assimilation pathway. The transfer of the thiol group from Cys to OPH, forming cystathionine and homocysteine (Hcy), is irreversible and commits both compounds toward Met synthesis. The reactions are catalyzed by two enzymes, cystathionine γ-synthase (CGS) and cystathionine β-lyase. All of these reactions occur in the chloroplast. The methyl group of Met is derived from methyltetrahydrofolate. Hcy is methylated in the cytosol to form Met. Met is converted to SAM, *S*-methylmethionine (SMM) and proteins.

CGS is suggested to be the key enzyme regulating the Met biosynthesis pathway in plants (Ravanel *et al.* 1998; Kim and Leustek 2000). The enzymatic activity of CGS is negatively auto-regulated by Met and SAM in several plants (Thompson *et al*. 1982; Rogens *et al.* 1986; Chiba *et al*. 2003). The *Arabidopsis thaliana* CGS gene encodes a 563-amino acid, 60-kDa protein and is interrupted by ten introns (Kim and Leustek 1996). Met and SAM down-regulate CGS gene expression by a post-transcriptional mechanism that destabilizes CGS mRNA (Chiba *et al*. 1999; Onouchi *et al*. 2004). A short highly conserved

amino acid sequence encoded in exon 1 of the CGS gene was identified as the functional region required for the post-transcriptional auto-regulation of the CGS gene in *Arabidopsis* (Ominato *et al*. 2002). An *Arabidopsis* mutant, *mto1*, has a point mutation in CGS exon 1 that results in the abolition of the Met-dependent destabilization of CGS mRNA, causing the CGS mRNA and protein to accumulate (Inaba *et al*. 1994).

Threonine synthase (TS) competes with CGS for the branching point intermediate OPH for the synthesis of Thr (Ravanel *et al.* 1998). An *Arabidopsis* mutant, *mto2-1*, has a mutation in the TS gene, and this results in decreased Thr biosynthesis and marked over-accumulation of Met (Bartlem *et al*. 2000). The phenotype of *mto2-1* suggests that OPH is channeled into the Met biosynthesis pathway owing to the suppression of Thr synthesis. While CGS expression is negatively regulated by SAM (Chiba *et al*. 2003), TS activity is stimulated by SAM (Curien *et al*. 1998). More recently, it was demonstrated that the synthesis of Met and Thr is limited by the availability of homoserine, the precursor of OPH (Lee *et al*. 2005).

Understanding the biosynthesis pathway of Met and the regulation of gene expression has been most useful when devising strategies to increase the levels of Met and methional in plants.

Biotechnology to enhance Met and methional

Since CGS catalyzes the committing step in Met synthesis, the *Arabidopsis* CGS (AtCGS) gene was overexpressed in *Arabidopsis* under the cauliflower mosaic virus (CaMV) 35S promoter to determine whether this would result in the accumulation of soluble Met (Kim *et al*. 2002). It was found that Met and its metabolite SMM were accumulated by as much as 8- to 20-fold above the wild-type *Arabidopsis* plants in sink organs – seedling tissues, flowers, siliques, and roots of mature plants. When AtCGS-transgenic *Arabidopsis* seeds were germinated on medium containing ethionine (a toxic Met analogue), they demonstrated resistance, implying the overexpression of Met in seeds. Considering the autogenous control mechanism from Met-dependent down-regulation of CGS expression, these results indicate that the autogenous control can be overcome by increasing the level of CGS mRNA through transcriptional control. However, some of the AtCGS-transgenic *Arabidopsis* plants showed silencing of CGS resulting from the introduction of the homologous copy of CGS, leading to deformed plants with a reduced reproductive growth.

The AtCGS gene was overexpressed in alfalfa (*Medicago sativa* L.) under the *Arabidopsis* rubisco small subunit promoter (Avraham *et al*. 2005). When compared to wild-type plants, the levels of Met, SMM (the transport form of Met) and Met incorporated into the water-soluble protein fraction in leaves were increased by up to 32-, 19-, and 2.2-fold, respectively. When the AtCGS

gene was introduced into alfalfa under the CaMV 35S promoter, there was a significant increase in the level of free Met and SMM, but not in the bound fraction in the transgenic alfalfa plants (Bagga *et al*. 2005). However, when both AtCGS and maize β-zein (which is high in Met) genes were co-expressed in alfalfa, the levels of the β-zein transcript and protein were increased and the level of free Met was decreased (Bagga *et al*. 2005), suggesting that β-zein is post-transcriptionally regulated by free Met in alfalfa. These data provide model systems for improving the nutritional quality of legume forage crops.

As shown in Fig. 9.2, TS competes with CGS to use the common substrate OPH for the synthesis of Thr. To investigate the regulation of this branching point, transgenic potato (*Solanum tuberosum* cv. Desiree) plants were engineered to express the antisense copy of TS under the constitutive cauliflower mosaic virus 35S promoter (Zeh *et al*. 2001). Thr levels were reduced to 45% of wild-type controls in the leaves of these transgenic potato plants, whereas Met levels increased up to 239-fold. Interestingly, the increased Met content did not affect the mRNA or protein levels or the enzymatic activity of CGS in these TS antisense-transgenic potato plants as was observed in *Arabidopsis*, suggesting that an autogenous control may not exist in certain plant species such as potato. In the tubers of these transgenic potato plants, however, the Met level was increased up to 30-fold while there was no reduction in Thr content. The methional level was not measured in these TS antisense-transgenic potato tubers.

The efforts to over-produce Met using the potato CGS gene in transgenic potato plants were not as successful as with the TS antisense-transgenic potato plants (Nikiforova *et al*. 2002; Kreft *et al*. 2003). The CGS-transgenic potato plants exhibited either high transgene RNA levels and elevated CGS activities but unchanged soluble Met level, or decreased transcript amounts and enzyme activities. It is very likely that the lack of overexpression of the potato CGS gene in transgenic potato plants was due to gene silencing, which has been observed in transgenic plants when sequences corresponding to endogenous genes are introduced into the genome (Ingelbrecht *et al*. 1994; Matzke *et al*. 1994). As shown previously, some transgenic *Arabidopsis* plants transformed with the *Arabidopsis* CGS gene displayed a gene-silencing phenomenon (Kim *et al*. 2002).

In order to enhance the flavor compound methional level in potato through the increase of Met content, we genetically engineered potato (*S. tuberosum* cv. Russet Burbank) with AtCGS driven by cauliflower mosaic virus 35S promoter using *Agrobacterium*-mediated transformation (Di *et al.* 2003). All AtCGS-transgenic potato lines were phenotypically normal and were indistinguishable from the untransformed potato plants, indicating that gene silencing did not occur in these transgenic potato plants. Furthermore, the AtCGS cDNA insert did not hybridize to the DNA from the wild-type potato plants, demonstrating that the AtCGS can be distinguished from the endogenous potato CGS. This is due to the fact that there is only 70–71% homology

between *Arabidopsis* and potato CGS (Campbell *et al*. 2000; Hesse *et al.* 1999) which provided the opportunity of avoiding gene silencing.

The AtCGS-transgenic potato plants displayed AtCGS gene expression in all tissues including leaves, roots, and tubers. AtCGS enzymatic activity was higher in the leaves and roots of all the transgenic potato lines compared to the wild-type plants. In the leaves, roots, and tubers of transgenic potato lines, the Met level was enhanced as high as six-fold compared to wild-type potato plants. In addition, it was found that the Met level in the tubers of transgenic lines was an order of magnitude higher than the Met level in the leaves of the same lines. Since the AtCGS protein level was not significantly higher in the tubers than in the leaves, these differences could only suggest that Met synthesized in the leaves of transgenic potato plants may have been transported into the tubers. Indeed, the level of SMM, the transport form of Met, was shown to be enhanced in leaves and roots of all transgenic potato plants, but was barely detectable in the tubers. These results supported the observation, first observed in wheat plants, that Met is converted to SMM in the leaves, translocated as SMM in the phloem and reconverted into Met in the sink tissues (Bourgis *et al*. 1999). These data also supported the results in transgenic *Arabidopsis* overexpressing AtCGS in that Met is increased in specific sink tissues and during specific stages of development (Kim *et al*. 2002). As a result of the increase in Met levels in the AtCGS-transgenic potato plants, the roots of these plants demonstrated resistance to ethionine (the toxic analogue of Met) in tissue culture medium, which was similar to the resistance shown by the AtCGS-transgenic *Arabidopsis* seeds (Kim *et al*. 2002).

Volatile methional was extracted from the field-grown AtCGS-trangenic potato tubers after baking and was analyzed by gas chromatography. The methional level was shown to increase between 2.4- and 4.4-fold in some of the transgenic potato lines. The methional increase correlated with the soluble Met level in the tubers from the same lines. This study provides a model for enhancing the flavor production in potato through biotechnology.

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Chapter 10
Regulatory aspects of flavor development – traditional versus bioengineered

Sabine Teske and James C. Griffiths

This chapter offers a perspective on the regulatory status of flavor biotechnology in the USA.

Bioengineered food products

Biotechnology, the process whereby scientists are able to modify a cell's genetic structure using recombinant DNA (rDNA), recombinant RNA (rRNA), and cell fusion techniques has been touted as offering the potential for beneficial advances in medicine, agriculture, and the processed food industries. However, the technology may pose substantial ecological and health risks, conceivably from the migration of genetic material from the engineered organism to an indigenous species. For example, the engineered trait, such as herbicide resistance, could potentially be transferred to weeds, or unintended antibiotic resistance could be transferred to pathogens from intentional marker genes.

Food biotechnology, in its broadest sense, employs recombinant techniques to genetically engineer (also termed 'bioengineer') entirely new food ingredients (e.g. enzymes, yeasts, vitamins, and flavors), or to modify existing food ingredients or whole foods (e.g. cereal crops, vegetables, and fruits). Traditional techniques, consisting primarily of manually manipulative genetic hybridization (such as ultraviolet irradiation or mutagens), have resulted in random change. In contrast, the use of sophisticated recombinant techniques allows researchers to target specific genes of animals, trees, vegetables, fruits, yeasts, and bacteria (Bren 2003). As a result of the genetic manipulation, the host organism encodes an altered or, in some cases, an altogether novel molecule. This altered or new molecule could confer a variety of new characteristics to food products, including improved shelf life, increased resistance to insects, higher nutritional value, or improved texture. The US Food and Drug Administration (FDA) approved the first genetically produced food ingredient, chymosin, for use as a milk-clotting enzyme to produce cheese and other dairy products in 1990 (US Center for Food Safety and Applied Nutrition [CFSAN] 1995). Four years later, the first genetically modified whole food, the Flavr Savr™ tomato, a genetically modified whole fresh tomato engineered to have a slower rate of ripening, was introduced to the US market.

Biotechnology in Flavor Production: A Case-based Approach. Edited by Daphna Havkin-Frenkel, Faith C. Belanger.
 ISBN: 978-1-4051-5649-3

This means that the Flavr Savr™ tomato can ripen for longer on the vine than other tomatoes in order to more fully develop its flavor, and it also has a longer shelf life because an enzyme responsible for pectin degradation and spoilage has been eliminated (CFSAN 1995). Since then, many other bioengineered products have entered the US market, including corn, soybean oil, sugar beets, squashes, potatoes, and papayas, each with an altered trait conferring an advantage over the naturally occurring counterpart (Thompson 2000; Bren 2003 and CFSAN 2005).

The FDA has published several policy statements and regulatory guidelines on the use of biotechnology, including the 'Proposal for a Coordinated Framework for Regulation of Biotechnology' (Federal Register 1984). It is obvious thus far, that the federal government intends to regulate biotechnology under existing laws and regulations, rather than to develop new statutory and regulatory standards. The FDA unequivocally states 'Upon examination of the existing laws available for the regulation of products developed by traditional genetic manipulation techniques, the working group concluded that, for the most part, these laws as currently implemented would address regulatory needs adequately' (Federal Register 1986). Therefore, the products of biotechnology, including food ingredients, and thus flavors, will be subjected to regulations and statutory standards which originally were developed to address products produced through processes other than through genetic engineering (Ausubel 1989).

Part of the logic of this approach is that the use of existing health and safety laws would provide immediate protection to consumers and certainty to industry without the need to develop new, potentially multi-Agency, legislation. Furthermore, existing laws and regulations have been proven to be adequate.

Although many may point to increased uncertainty (and thus toxicity), or at least the potential for unknown toxicity, from foods and food ingredients developed via biotechnology, there are examples of increased toxicity being introduced through traditional breeding, for example the increased solanines in potatoes that have been developed for pest and disease resistance. The Lenape potato, after launching and commercial incorporation into the food supply, was found to have unusually high levels of solanine, such that it was unacceptable from a human safety standpoint and was removed from the market. Celery cultivars that were bred through traditional methods to deter insect infestation, and thus have more aesthetic appeal to consumers, were found to have accomplished this feat via toxic levels of the human skin irritant, psoralen.

To understand the potential for genetic engineering to play a role in both the production of novel flavors and in the development of new processes for traditional flavors, it is important to briefly review exactly what flavors are.

Conventional flavors

A flavor(ing), flavor(ing) agent, or flavor adjuvant is a substance that is added to food to impart a taste or aroma, as defined in FDA regulation 21CFR170.3 (12)[1].

Currently, the food industry uses thousands of flavors, many as complex mixtures, to impart characteristic tastes/aromas to food products. *Fenaroli's Handbook of Flavor Ingredients* (Burdock 2005) is one readily available resource that provides a comprehensive summary of these flavors, including annual consumption and regulatory approval. Flavors can be broadly classified as either 'artificial' or 'natural'. Artificial flavors are defined as 'any substance, the function of which is to impart flavor, which is not derived from spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, fish, poultry, eggs, dairy products, or fermentation products thereof' (21CFR101.22(a)(1)). On the other hand, natural flavors are defined as an 'essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis, which contains the flavoring constituents derived from a spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof' (21CFR101.22(a)(3)).

Regardless of whether food manufacturers add artificial or natural flavors to their finished products, the presence and type of flavor (i.e. artificial or naturally derived) must be declared on the principal display panel of the label (21CFR101.22(b)). For example, the following statement, 'flavored with artificial vanilla' is acceptable for finished food products containing one distinct flavor (21CFR101.22(i)(2)). If a finished food product (e.g. fruit punch) contains a mixture of several artificial flavors, manufacturers can accommodate this on the label using a simple statement, such as 'artificially flavored fruit punch', without having to list each individual flavor on the label (21CFR101.22(i)(3)(iii)) (also known as the 'bulk flavor labeling exemption). Likewise, even for finished food products containing naturally derived flavors, manufacturers are required to declare this fact on the principal display panel of the label (21CFR101.22(g)(3)). Therefore, although flavors are added in very small quantities to food products (generally less than 100 ppm [Burdock 2002a]), manufacturers are, nevertheless, required to disclose their presence in detail on the food label.

The use of microbes as vectors of food ingredients and flavors

There are many examples of bacteria and yeast that are used to manufacture food ingredients, as well as several cases where the dried yeasts themselves are considered food ingredients. For example, the dried yeasts, *Saccharomyces cerevisiae*, *Saccharomyces fragilis*, and *Candida utilis* (21CFR172.896), as well as baker's yeast extract from *S. cerevisiae* (21CFR184.1983), are microbial food ingredients approved as direct food ingredients. Bacteria are utilized to make, after appropriate purification, discrete food ingredients and flavors. Examples include xanthan gum isolated and purified from *Xanthomonas campestris*, yogurt (and yogurt flavors), cheeses (and cheese flavors), and

lactic acid (21CFR184.1061), many derived from *Lactobacillus* spp. Acetic acid (21CFR183.1005) and propionic acid (21CFR184.1081) are additional examples of microbially derived direct food ingredients which, among other uses, are approved as flavors. Citric acid, one of the highest-volume food ingredients (more than 1 million pounds in weight used per year) (Burdock 2005), and a key flavor (Flavor and Extract Manufacturers Association [FEMA] No. 2306) is, for the most part, fermentatively produced from the yeasts *Candida guilliermondii*, *Candida lipolytica* and *Aspergillus niger* (21CFR184.1033; 21CFR173.160; 21CFR173.165; 21CFR173.280). Vitamin B_{12} from *Streptomyces griseus* (184.1945) and vitamin D produced by ultraviolet irradiation of yeast- and fungi-derived ergosterol (21CFR184.1950) are approved as direct food additives. All these food additives have been approved as safe for human consumption and are part of CFSAN's *Partial list of Microorganisms and Microbial-Derived Ingredients that Are Used in Foods* (CFSAN 2002).

Bioengineered flavors

Biotechnology is not only used to modify whole foods (e.g. crops), but also to produce food ingredients derived from rDNA, primarily using bacterial or other microbial vectors. Two examples of bioengineered enzymes approved as direct food additives are lipase from *Rhizopus niveus* for inter-esterification of fats and oils (21CFR184.1420) and lactase from *Kluyveromyces lactis* for hydrolysis of lactose in milk (21CFR184.1388). When the list of food ingredients submitted to CFSAN as Generally Recognized as Safe (GRAS) ingredients is examined, it is apparent that there are many examples of biotechnologically developed and approved ingredients. Examples include

(1) *S. cerevisiae* strain ML01, which carries a gene encoding malolactic enzyme from *Oenococcus oeni* and a gene encoding malate permease from *Schizosaccharomyces pomb* (GRAS Notice 120)[2];
(2) *alpha*-amylase enzyme preparation from *Pseudomonas fluorescens* Biovar I expressing a gene encoding a hybrid *alpha*-amylase derived from three microorganisms within the order *Thermococcales* (GRAS Notice 126)[3];
(3) phospholipase A1 (PLA1) enzyme preparation from *Aspergillus oryzae* expressing a gene encoding a PLA1 from *Fusarium venenatum* (GRAS Notice 142)[4];
(4) phospholipase A2 enzyme preparation from *A. niger* expressing a gene encoding porcine phospholipase A2 (PLA2, porcine) enzyme preparation from *A. niger* (GRAS Notice 183)[5].

In addition to these currently approved microbially derived food ingredients, food manufacturers and researchers are continuously using biotechnology to develop novel food ingredients, including flavors, as well as methods to produce these ingredients. One such application is the use of bioengineered

yeasts to alter the hop flavor of beer (King and Dickinson 2003). During the fermentation process of beer, terpenoids are generated, which impart aroma (King and Dickinson 2003). Using bioengineered yeasts would allow brewers to modify terpenoids and, thereby, to generate beer (as well as wine) with customized aroma notes (Dequin 2001). In addition to bioengineering terpenoid flavors for beer and wine, biotechnology is also used to in the development of other flavors, such as vanillin. In the future, biotechnology will also be used increasingly to produce fruit flavors (Singer 2006). To generate these fruit flavors, genes encoding a particular fruit flavor will be isolated from the fruit during the ripening process, when the expression of these genes is enhanced, and will be introduced into bacteria or yeast to 'mass-produce' a certain flavor. According to the 'Grocery Manufacturers Association', currently 70–75% of all processed foods sold in grocery stores contain bioengineered ingredients (Bren 2003).

Safety standards of food products, ingredients, and flavors

FDA requires that 'articles used for food and drink in man or animals, chewing gum, and articles used for components of any such article be not ordinarily injurious to man or animals' (Federal Food, Drug and Cosmetic Act, 21CFR 402). This subjects foods to the highest standard of safety, ensuring that food manufacturers and distributors only market safe products (i.e. 'not ordinarily injurious' food products) for consumption by the public. Similar to the 'not ordinarily injurious' standard for food products, food ingredients are subject to the strict 'reasonable certainty of no harm' standard (21CFR190.6). This places both whole-food products and food ingredients (e.g. flavors) at the upper end of the safety spectrum compared to dietary supplements and prescription drugs. The latter two classes of products are subject to the lower 'reasonable expectation of no harm' and to the yet lower 'risk–benefit' safety standards, respectively (CFSAN 2001a; 21CFR190.6).

In today's society, the ordinary consumer does not generally expect to succumb to food-related illnesses due to consumption of foods (e.g. bread, fruit, and juice) and, to keep it that way, the Agency mandates and reputable manufacturers adhere to the 'not ordinarily injurious' high safety standard for foods. Unlike foods, the lay consumer is generally willing to assume a certain risk with consumption of a prescription drug as long as the overall benefits of that particular drug outweigh the risks associated with that drug; i.e. the greater the benefits derived from a prescription drug (e.g. treatment of a debilitating or life-threatening condition), the more willing a patient becomes to assume potential health risks (e.g. 'side effects'). This makes using a sliding risk–benefit safety standard acceptable for drugs, ranging from quite substantive side effects with cancer chemotherapy to much more benign effects with treatments for gastric reflux, for example.

Likewise, the ordinary person electively consumes or does not consume dietary supplements, thus assuming some degree of risk (i.e. a theoretically

lower safety standard of 'reasonable expectation of no harm') to be acceptable with consumption of supplements (CFSAN 2001a). Furthermore, unlike foods and food ingredients, there are recommended daily intakes associated with consumption of dietary supplements, such as 'take one tablet twice daily' or 'serving size: four capsules per day'.

Unlike prescription drugs or dietary supplements, a consumer does not have the option of deciding whether or not to ingest a certain food ingredient, such as a specific flavor, usually listed as 'this beverage contains natural and artificial flavors'. This is especially true as today's food products contain a myriad of ingredients, many of which are used to impart one or more of the 32 total technical effects to food (Burdock and Carabin 2004). Hence, to ensure overall safety of a food product, it is imperative to subject each individual food ingredient of that food product, including flavors, to the 'reasonable certainty of no harm' safety standard.

Similar to conventionally derived food ingredients, bioengineered food ingredients (e.g. flavors) are also consumed by the consumer as part of a food product. Hence, manufacturers, which add bioengineered food ingredients to their products have to ensure that these comply with the 'reasonable certainty of no harm' safety standard (CFSAN 1995 and Rowlands 2002). Subjecting bioengineered food ingredients to the same safety standard (i.e. 'reasonable certainty of no harm') allows regulators and experts to determine that the safety of bioengineered products is no less than that of the conventional food ingredients, and allows the use of existing food safety standards and regulations.

Determination of 'reasonable certainty of no harm' safety standard

The 'reasonable certainty of no harm' safety standard for food ingredients, irrespective of whether their manufacture is via either traditional or biotechnologically derived processes, is determined via a food additive petition (FAP) or the GRAS process. Although the step-by-step safety determinations for a FAP or GRAS are different, both procedures require the objective review of scientific data by qualified experts to ascertain whether a food ingredient fulfills this high safety standard (Burdock and Carabin 2004). The primary technical intricacies that are different between a FAP and a GRAS are the nature of the scientific data and reviewing of these data by the experiencd (CFSAN 2004a). For a FAP, proprietary data from the manufacturer regarding the proposed food ingredient, i.e. non-published safety data, are allowed to be used in conjunction with published data and are reviewed by experts within the FDA. On the other hand, for a GRAS, only publicly available data are reviewed by a number of independent experts, who are qualified by scientific training and experience to evaluate the safety of the ingredient in question (Burdock and Carabin 2004). These experts are referred to as the 'Expert Panel' or 'GRAS Panel'. Because virtually every flavor has undergone the GRAS process, the remainder of this chapter will not further discuss the FAP process.

The GRAS process first involves the preparation of a comprehensive dossier addressing the safe use of the ingredient. The dossier must include published, peer-reviewed, scientific data regarding (1) metabolism (i.e. biological fate); (2) toxicity data obtained from appropriate acute and sub-chronic studies in rodents; and (3) geno- and cytotoxic effects in bacteria and cells (Burdock 2003; CFSAN 2004a). Furthermore, dependent upon higher intakes and/or likely target consumers (unique sub-populations, such as children, pregnant or nursing mothers, the elderly, etc.) longer-term repeat dose studies, reproductive and developmental toxicity studies, and cancer studies may be needed to demonstrate safety. If these data are not publicly available, because an ingredient is completely novel, the manufacturer has the burden of proof to generate the outstanding data by conducting appropriate *in vivo* and *in vitro* experiments, which follow FDA guidelines, as outlined in the *FDA Redbook*[6].

In addition to data from laboratory tests, the Expert Panel also takes anticipated exposure to the food ingredient into account. To evaluate this, anticipated human exposure to a particular food ingredient is calculated based on consumption of food categories (e.g. beverages, dairy products, etc.) to which this food ingredient will be added (Burdock 2003). The anticipated human intake is then compared to the No Observed Adverse Effect Level (NOAEL) determined from rodent-feeding studies. If, even after dividing by safety or uncertainty factors, the anticipated consumption of the 'new' food ingredient is significantly below the rodent NOAEL, the ingredient is likely to be recognized as GRAS by the Expert Panel.

Once the ingredient has been found to be GRAS by the independent Expert Panel, a food manufacturer can, but is not required to, notify the FDA of the Expert Panel's decision (CFSAN 2006). The FDA then reviews the findings of the Expert Panel and publishes a 'no objection letter' on the FDA website, which is publicly accessible, if the Agency agrees with the conclusion of the Expert Panel (Griffiths and Borzelleca 2005). This 'no objection letter', which is not an FDA approval per se, can state 'based on the information provided by Company X, as well as other information available to FDA, the Agency has no questions at this time regarding the conclusion of Company X that Flavor Y is GRAS under the intended conditions of use'. This food ingredient can then be used in the food industry without pre-market or any other FDA approval (CFSAN 2004b). On the other hand, if the FDA has safety concerns regarding the food ingredient, it will issue an objection letter, which is also published on the FDA website.

Despite being granted GRAS status, food ingredients, such as flavors, cannot be freely added at any amount to any food across the board, unless the safety data were persuasive to allow such broad penetration and unless the Expert Panel was in agreement (Federal Register 39:34194-34195, 1974). GRAS status permits addition of a food ingredient only to certain specified food categories for a specific intended purpose – i.e. based on the 43 total food categories (21CFR170.3(n)) and on the 32 total technical food effects

(21CFR 170.3(o)) – and only at specified amounts, to provide an exposure intake that is acceptable compared with the known toxicity at much higher levels in appropriate rodent safety studies. For example, if a food ingredient is granted GRAS status only for the intended purpose as a flavor ingredient or adjuvant (i.e. technical food effect No. 12), it cannot be added to food as a formulation aid (i.e. technical food effect No. 14) unless approved for this new use. Likewise, if it is given approval only as a flavor ingredient for baked goods (food category No. 1), it cannot be used as a flavor in non-alcoholic beverages (food category No. 3), unless both food categories and the exposure to the consuming public were evaluated and approved by the Expert Panel. The use level is also part of the GRAS decision, as the risk assessment compared total exposure, estimated daily intake (EDI) to the safe use, and acceptable daily intake (ADI). For example, if a flavor is approved for use in alcoholic beverages at 10 ppm, and in baked goods at 23.5 ppm, it cannot be used at higher levels in either category. Importantly, there is no allowance to trade diminished uses in one category for increased uses in an alternative category.

The GRAS process ensures that ingredients added to food have undergone adequate safety evaluation prior to being available for consumption by the public. Even though the GRAS process could be managed and conducted by anyone with the appropriate credentials as having the relevant training and expertise, there are distinct advantages to using neutral third-party consultants. For most food ingredients, the GRAS process is relatively transparent, wherein scientific consultants prepare a comprehensive dossier using publicly available data, and this is often followed by notification to the FDA. Flavors can be granted GRAS status through this same process but, in reality, few are. Most flavor companies belong to the US trade group FEMA, which represents flavor manufacturers, flavor users, and flavor ingredient suppliers, and provides the administrative and technical support to the safety evaluation of flavor ingredients. This is accomplished by the trade group supporting the role of the FEMA Expert Panel who review, deliberate, and conclude 'yes or no' on the safety-in-use of flavor ingredients via the FEMA-GRAS program.

Since the foundation of the FEMA-GRAS paradigm in 1960, the FEMA-GRAS Expert Panel has evaluated approximately 2000 flavors (Smith *et al.* 2005a). Additionally, the FEMA-GRAS Expert Panel also re-evaluates previous FEMA-GRAS flavors in a cyclical fashion, and this re-evaluation is important – particularly if new data or new risk assessment factors warrant a new safety assessment (Smith *et al.* 2005a). To conduct its evaluations of both new flavor ingredients and to re-evaluate previously FEMA-GRAS flavors, the FEMA-GRAS Expert Panel meets approximately four times a year; and the flavor ingredients that are determined to be FEMA-GRAS are published, in list form, in *Food Technology* approximately every 2 years. The latest of these lists, FEMA-GRAS No. 22, was published in August 2005 (Smith *et al.* 2005b). Although FEMA provides the list of flavors for which the FEMA-GRAS Expert Panel has made a decision to the FDA, this differs

from other GRAS determinations as the data reviewed are not made public nor do the flavors undergo the GRAS Notification procedure employed for non-flavors.

As traditional GRAS determinations follow a regulatory prescribed pathway, both on pivotal safety data and in the cohesive, persuasive presentation of the facts, the FEMA-GRAS Expert Panel approves flavors for use in the food industry by (1) exposure to the substance in specific foods; (2) natural occurrence in foods; (3) chemical identity and chemical structure; (4) metabolic and pharmacokinetic characteristics; and (5) animal toxicity (Woods and Doull 1991; Schrankel 2004; Smith *et al.* 2005a). The FEMA-GRAS Expert Panel review also includes safety data of structurally similar flavoring substances and proprietary data from safety and toxicity studies provided by the submitter (Smith *et al.* 2005a). The FEMA-GRAS Expert Panel evaluates only flavors, flavor ingredients, and flavor adjuncts (i.e. no other food ingredients are reviewed). Furthermore, in order for a submitter (i.e. a flavor manufacturer, flavor user, or flavor ingredient supplier) to engage the FEMA-GRAS Expert Panel, the submitter must be a member of FEMA, as the trade group and the FEMA-GRAS process are a member-supported activity. In fact, 90% of the flavors originating in the USA are produced by companies that are members of FEMA (Smith *et al.* 2005a).

Following the review and evaluation of a flavor ingredient by the FEMA-GRAS Expert Panel, FEMA notifies the petitioner that (1) the flavor is GRAS (for its specific technical effect); (2) that the flavor is placed on 'hold' due to insignificant data submitted by petitioner; or (3) that the flavor cannot be GRAS (for its specific technical effect) (Smith *et al.* 2005a). In the latter two cases, the petitioner usually withdraws the FEMA-GRAS application to take corrective action.

The 1992 Policy – Substantial equivalence of bioengineered food ingredients

Determination of the safety of bioengineered food products and ingredients is based on the concept of 'substantial equivalence'. This is applicable to novel bioengineered food molecules, which are substantially equivalent to their conventional counterparts (CFSAN 1995; Burdock 2002b). For example, a terpenoid flavor produced by a yeast using rDNA techniques would be compared to the terpenoid flavor produced by the conventional, non-bioengineered yeast. Likewise, vanillin produced using a biotechnological process would be compared to a conventionally derived vanillin. In both cases, the substantial equivalence of the novel molecule is determined by comparison to its conventional counterpart, and not via the evaluation of the rDNA, which encodes for this flavor. According to the 1992 Policy, introduction of nucleic acids, including rDNA and antisense RNA, into food does not pose safety concerns (Federal Register 57:22984-22998, 1992). Instead, only the proteins and/or organic products encoded by rDNA, or the inhibition of proteins encoded by

use of antisense RNA, are to be reviewed for safety. In contrast, nucleic acids used to produce bioengineered food products and ingredients are considered GRAS to begin with and, therefore, are exempt from any safety evaluation (Federal Register 57:22984-22998, 1992).

Plant-derived bioengineered foods – A special case

Although the production of all bioengineered foods or food products involves the use of rDNA techniques, the FDA has established separate guidelines for plant-derived bioengineered food products and ingredients derived from these products. Currently, plant-derived bioengineered products, such as genetically modified cereal crops and vegetables, undergo a voluntary consultation process with the FDA (CFSAN 1997). As part of this consultation process, manufacturers of bioengineered plant-derived food and feed products provide the FDA with data regarding safety and the nutritional value of their products. In particular, food manufacturers provide the FDA with detailed information, including the name of the bioengineered food/feed; applications in the food industry/use for animal feed; the name of the host crop or plant; the name of the host gene/gene product/gene fragment targeted by recombination techniques; the name and source of the donor gene introduced into the host plant; the intended effect of the genetic modification in the host plant; and the expression of potential allergenic or toxic proteins by the host plant; as well as any other significant differences mediated by the genetic modification (e.g. nutrient value, texture) (CFSAN 1994; CFSAN 1997).

In response, the FDA assigns a Biotechnology Notification File (BNF) number to each submitted consultation. The Biotechnology Evaluation Team (BET) evaluates each consultation and, if approved, adds the approval notice to the FDA's online list of 'completed consultations on plant-derived bioengineered foods' (FDA 2005). This list contains bioengineered food and feed products, dating back to the Flavr Savr™ tomato in 1994, and concludes with the herbicide-resistant Roundup Ready® cotton (seed meal) for animal feed in 2005. Bioengineered crops, which encode gene products conferring pesticidal or herbicidal resistance to the host crop, are designated with an asterisk on this list (FDA 2005). The asterisk denotes that these products are regulated by the US Environmental Protection Agency (EPA), and not the FDA, since they encode a plant-incorporated protectant (PIP); i.e. a pesticidal/herbicidal substance (FDA 2005). The EPA (2006) regulates pesticides and herbicides, as well as establishing tolerable levels of pesticides/herbicides permitted in food products.

Although the FDA consultation process for plant-derived bioengineered food products (e.g. cereal crops and vegetables) remains voluntary up to this date, the FDA proposed guidelines for mandatory pre-market approval of these products in 2001 (Federal Register 66:4706-4738, 2001). These pre-market approval guidelines proposed that manufacturers of plant-derived

bioengineered food products/ingredients would be required to notify the FDA 120 days prior to introducing food products from bioengineered plants into commerce (Federal Register 66:4706-4738, 2001; Formanek 2001). To be granted pre-market approval, manufacturers would have to prove that the bioengineered products are as safe as their conventional counterparts. In addition to bioengineered crops and plants, the FDA recommends that food manufacturers should consult with the Agency prior to introducing any bioengineered food products into commerce (CFSAN 1997).

Labeling of bioengineered food products

As previously stated, bioengineered food ingredients are subject to the same safety standard of 'reasonable certainty of no harm' as conventional food ingredients. Therefore, general labeling requirements for food products containing bioengineered ingredients do not differ from those governing conventional food products (CFSAN 1995). The labeling of both bioengineered and conventional food products has to be truthful and not misleading to avoid the product being 'misbranded'. To ensure that labeling of food products fulfills these criteria, all ingredients have to be listed in the ingredient statement located in the principal display panel of the label (21CFR101). However, the FDA does not require food manufacturers to specifically declare which, if any, of these ingredients are derived as a result of bioengineering (Thompson 2000; CFSAN 2001). In fact, even if a manufacturer chooses to voluntarily disclose information about a food ingredient derived from biotechnology (bioengineering/genetic manipulation), this information is not permitted in the ingredient statement present in the principal display panel of the label. A food manufacturer, who chooses to disclose that a food ingredient (e.g., a flavor) is bioengineered, must declare this information outside of the ingredient statement (21CFR101.2(e); only terms directly part of the ingredient statement are allowed to be included). Therefore, should a food manufacturer decide to make a statement related to the fact that a food ingredient, including a flavor was derived via bioengineering or genetic engineering it would have to be made outside the ingredient statement, along the lines of "this product contains —— produced via genetic engineering" or "this product contains —— flavor that was produced using biotechnology".

If a bioengineered food product is significantly different compared to its conventional counterpart (i.e. does not fulfill the substantial equivalence paradigm), FDA requires that this difference is indicated on the label (CFSAN 2001b). Food products which are considered significantly different are products with altered nutritional values (e.g. high oleic acid soybean oil or products thereof, which are derived from bioengineered soybeans) and altered texture (e.g. tomatoes that resist bruising and softening). Additionally, bioengineered foods with potential allergenic properties also require food manufacturers to disclose the presence of allergenic proteins on the label (CFSAN 2001b). For example, as a result of bioengineering, an allergenic protein could be

encoded in what is otherwise considered a non-allergenic food (Thompson 2000; CFSAN 2001b). Interestingly, although the FDA requires food manufacturers to declare alterations, such as in nutrient content, texture, and allergenicity, manufacturers are not required to reveal that these alterations are caused as a result of bioengineering.

The advent of bioengineered food products in the 1990s has not so much posed any particular labeling issues to manufacturers who market these foods, but it has prompted some conventional food manufacturers to modify their food labels. Some manufacturers of conventional food products were compelled to distinguish their products from bioengineered counterparts. Provided they are not misleading, these manufacturers can comply with FDA requirements by using statements to the effect that foods or food products/ingredients were produced without the use of biotechnology (CFSAN 2001b), with labels stating that, for example, they 'do not use ingredients that were produced using biotechnology' or 'this oil is made from soybeans that were not genetically engineered'. However, these statements can only be purely factual and cannot have a connotation; for example, they must not imply that 'bioengineered-free' food products are of superior quality compared to bioengineered products. In addition to prohibiting superiority statements, the FDA does not allow nonsensical statements such as 'free of genetically modified organisms (GMOs)' on conventional food products (e.g. fruits or vegetables), since these foods do not contain microorganisms (CFSAN 2001b).

Statements regarding the lack of use of biotechnology/bioengineering to produce foods apply only to conventional food products and not to organically produced foods (CFSAN 2001b). The US Department of Agriculture (USDA) has established rules outlining production criteria for organic foods and ensures that food manufacturers comply. The rules include the default mandate that manufacturers of legitimate organic food products are not allowed to use bioengineering (Federal Register 65:80548-80550, 2000). In turn, products from these manufacturers are allowed to bear the USDA 'certified organic' statement, which precludes the use of biotechnology. This illustrates the fact that, although all food products are subject to a uniform safety standard of 'reasonable certainty of no harm', labeling issues governing different food products, including flavors, are rather complex, and often require a case-by-case evaluation to determine whether a label is misbranded.

Allergenicity of bioengineered foods

Although more than 160 foods have been shown to cause allergies in sensitive individuals, only eight foods or food ingredients derived from these foods account for more than 90% of all food allergies (FDA 2006). These eight major food allergens are: milk, eggs, fish (e.g. trout, flounder, and bass), crustaceans (e.g. lobster, shrimp, and crab), tree nuts (e.g. walnuts, almonds, and pecans), peanuts, wheat, and soybeans (FDA 2005, 2006). Therefore, as an amendment to the 1958 Food Additives Amendment (to the 1938 Federal

Food, Drug and Cosmetic Act), the FDA developed the Food Allergen and Labeling and Consumer Protection Act (FALCPA) in 2004. As a result of FALCPA, which took effect on January 1, 2006, all manufacturers are required to declare ingredients that are classified as one of the eight major food allergens, or that contain protein from these major food allergens, on the food label (FDA 2006). To fulfill FALCPA labeling requirements, manufacturers list the common/usual name of the ingredient (e.g. lecithin) followed by the food source (e.g. soy) in parenthesis in the ingredient statement located in the principal display panel of the label (FDA 2006). Alternatively, manufacturers can add statements, such as 'contains soy', at the end of or adjacent to the ingredient list in the principal display panel. FALCPA applies to all food ingredients, including flavors (e.g. flavors containing peanut-derived protein).

Because bioengineered food ingredients, including flavors, are subject to the same safety standard as their conventional food ingredient counterparts, the presence of allergenic proteins in these bioengineered food ingredients has to be declared on the label (Maryanski 1997). Although the 1992 Policy, which describes safety standards pertaining to bioengineered foods, preceded FALCPA by more than a decade, interestingly it already provided some advice to food manufacturers regarding allergenicity (Federal Register 57:22984-22998, 1992). For example, the introduction of rDNA from a donor plant (e.g. peanut) into a generally considered non-allergenic host plant (e.g. corn) could encode an allergenic protein (e.g. peanut) in the host plant (e.g. corn). Therefore, the original 1992 Policy advises food manufacturers to consider all newly encoded bioengineered proteins as potential allergens and to determine their potential allergenicity using *in vitro* and *in vivo* tests (Federal Register 57:22984-22998, 1992; suitable rodent models to test food allergens are reviewed by Matsuda *et al.* 2006). Following the implementation of FALCPA in 2006, manufacturers of bioengineered food products which contain proteins derived from the eight major food allergens are now required to declare the presence of the allergenic protein on the label according to FALCPA standards (FDA 2006).

To determine the potential allergenicity of any novel bioengineered protein, the FDA (2005) advises that at least six, preferably eight, contiguous amino acids of novel, bioengineered proteins are compared to the amino acid sequences of known allergenic proteins (i.e. linear amino acid sequence where the IgE antibody epitope binds). If the sequence of the novel, bioengineered protein is homologous or highly similar to the amino acid sequence of the IgE epitope of a known allergenic protein, the potential for allergenicity is high (FDA 2005). Using an eight amino acid-long sequence, instead of a shorter six amino acid-long sequence, reduces the number of false-positive allergenic predictions in this comparison. On the other hand, amino acid sequences should not be significantly longer than six to eight amino acids, because sequences longer than these are more prone to lead to false negatives (i.e. the allergenicity of novel, bioengineered proteins could be erroneously missed) (FDA 2005).

Although food regulations are highly complex and, occasionally, are very case-specific, the underlying regulatory principles are similar, because all food ingredients are governed by a single safety standard (i.e. 'reasonable certainty of no harm'). This safety standard is determined by generally, well-understood, consistent, and proven procedures (i.e. FAP and GRAS), which are sufficiently versatile to allow the adequate safety evaluation of vastly different food products and ingredients. Therefore, even as the food industry continuously improves current products or develops new products to satisfy consumer demands, existing regulations are flexible and robust in allowing safety-in-use evaluation of all novel food ingredients, including bioengineered flavors.

Notes

(1) All CFR regulations available at: http://www.gpoaccess.gov/cfr/index.html (Accessed September 1, 2006).
(2) Available: http://www.cfsan.fda.gov/~rdb/opa-g120.html (Accessed September 2, 2007).
(3) Available: http://www.cfsan.fda.gov/~rdb/opa-g126.html (Accessed September 2, 2007).
(4) Available: http://www.cfsan.fda.gov/~rdb/opa-g142.html (Accessed September 2, 2007).
(5) Available: http://www.cfsan.fda.gov/~rdb/opa-g183.html (Accessed September 2, 2007).
(6) Available: http://www.cfsan.fda.gov/~redbook/red-toca.html (Accessed September 2, 2007).

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Biotechnology in Flavor Production: A Case-based Approach. Edited by Daphna Havkin-Frenkel, Faith C. Belanger.
 ISBN: 978-1-4051-5649-3