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Editors

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Preface

Major horticultural crops produced in the world are vegetables and fruits such as tomatoes, watermelons, bananas, and apples. The production of tomatoes, watermelons, and bananas is more than 100 million tons and that of apples is more than 70 million tons. The production of vegetables and fruits is increasing with population growth. Although the production is less than that of major cereals such as corn, rice, and wheat, vegetables and fruits are essential as food and food supplements in a way different from cereals. That is, vegetables and fruits are necessary for the maintenance of body functions, whereas cereals are foods that mainly provide energy. Therefore, horticultural production is important for promoting a healthy life and for preventing hidden hunger.

The purpose of this publication is to elucidate the biological aspects of the abiotic stress response in horticultural plants from the field to the molecular level. There are few books focusing mainly on the abiotic stress response in horticultural plants, although many books concerning the basic aspects of abiotic stress biology and research progress at the molecular level in model plants are available. Many readers who are interested in plant abiotic stress biology are aware of the application of the latest findings to agricultural production; hence, this book will appeal to those readers especially.

Stress tolerance mechanisms in horticultural crops are gaining attention because most agricultural regions are predicted to experience considerably more extreme environmental fluctuations due to global climate change. Furthermore, because of recent progress in next-generation sequencing technologies, the emergent postgenomic era has enabled advances not only for model plants and major cereal crops but also for horticultural crops, which comprise a great diversity of species. In this postgenomic era, translational and transcriptional research on model plants has provided a large amount of valuable information on many horticultural species. This book provides information on the physiological aspects of the abiotic stress response in horticultural plants, which is considered essential for postgenomic research.

The book comprises four parts. Part I concerns the physiological and molecular aspects of stress response in horticultural plants, and Part II deals with the effects of abiotic stress on horticultural plants and production, mainly at the production stage.

These parts also describe quality improvement and freshness retention as a unique phenomenon in horticulture. Part III describes physiological disorders as a unique phenomenon, while Part IV provides updated information on genetic engineering and omics as a biotechnological aspect. Collectively, the parts of this book provide a detailed description of abiotic stress biology in horticultural plants from the field to the molecular level and discuss the latest findings mainly with regard to major fruit crops in which readers worldwide are interested.

We sincerely thank the authors of the chapters for their time spent on this book. We also thank Springer and especially Dr. Mei Hann Lee for the editorial assistance that made this publication possible. We hope our audience will enjoy reading this new work on horticultural plants and will find useful information in this book.

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Part I
Stress Physiology and Molecular Biology in Horticultural Plants

Chapter 1
Effect of Salt Stress on the Growth and Fruit Quality of Tomato Plants

Takeshi Saito and Chiaki Matsukura

Abstract During the past several decades, salt injury has arisen as one of the most serious problems in agriculture worldwide, especially in arid and semiarid areas. Generally, excessive exposure of crops to salinity stress leads to yield reduction and loss of quality. However, for tomato crops, moderate salt stress improves the fruit quality, increasing nutritional components but decreasing fruit yield. In the current Japanese market, such fruits are referred to as "fruit tomatoes" and are sold at a higher price compared with normally cultivated tomatoes because of their high Brix (sugar content) and excellent flavor. Previously, the mechanism underlying this phenomenon was referred to as a "concentration effect" because fruit enlargement was suppressed by limited water uptake as a result of salt stress. However, recent studies have suggested that, in addition to the "concentration effect," certain metabolic and molecular genetic responses to salinity are also involved in the development of fruit tomatoes. Here, we introduce metabolic alterations in major fruit components such as sugars, amino acids, organic acids, and carotenoids in high-Brix fruit, and we describe the physiological changes observed in tomato plants exposed to salt stress. We also discuss possible molecular mechanisms underlying the production of fruit tomatoes.

Keywords ADP-glucose pyrophosphorylase • Amino acid • Assimilate transport • Fruit quality • GABA • Invertase • Organic acid • Salt stress • Starch • Tomato

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1.1 Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops in agriculture, both domestically and worldwide. Because this vegetable can be adapted for cultivation in various environments ranging from tropical to nearly alpine regions, its production area is now expanding worldwide. On the other hand, in the traditional cultivation area, which is concentrated around the Mediterranean Sea and in the southern and western parts of the United States (USA) because of the warm and dry climate that is favorable for tomato cultivation, yield loss from salt injury has arisen as a serious problem in 19.5 % of the irrigated land area and in the irrigation water (Flowers and Yeo 1995; Cuartero and Fernandez-Munoz 1999; Foolad 2004). Additionally, 2 million hectares (ha), equivalent to 1 % of the world's tomato cultivation areas, are lost every year because of environmental factors such as salinity, drought, and soil erosion. In some cases, poor cultivation techniques can also promote the damage caused by environmental stresses (Ashraf and Foolad 2007).

It has been suggested that moderate salinity and drought stress improve the nutrient quality in tomato fruits by increasing sugars, amino acids, carotenoids, and other substances (Adams 1991; Gao et al. 1998; Ho et al. 1987; Krauss et al. 2006; Tal et al. 1979; De Pascale et al. 2001; Saito et al. 2008a). Recently, these cultivation conditions have been introduced in hydroponic cultivation techniques such as the nutrient film technique (NFT), and high-Brix tomatoes are being produced in Japan (Sakamoto et al. 1999; Saito et al. 2006) and in northern European countries (Adams 1991). Such high-Brix fruits are referred to as "fruit tomatoes" and have become popular in the Japanese consumer market because the fruit flavor is much better than that of a tomato cultivated under ordinary conditions. Until recently, the development of "fruit tomatoes" was thought to result from a "concentration effect" caused by the suppression of fruit size caused by salt stress (Ehret and Ho 1986; Ho et al. 1987; Sakamoto et al. 1999). However, evidence uncovered during the past decade indicates that other physiological and molecular alterations might be involved in this phenomenon.

This chapter describes the effects of salt stress on various aspects of tomato growth and on the content of the nutritive components of the fruit, with a specific focus on sugars, and discusses the possible mechanisms that are responsible for the formation of high-Brix fruit.

1.2 Effect of Salinity on Plant Growth and Yield

In Mediterranean countries, tomatoes have also been studied as a model crop in salinized or dry land agriculture, and a large amount of data about the effects of salinity on the growth of tomato plants has been produced. Most commercial cultivars are moderately sensitive to salinity at all stages of plant development, including seed germination, vegetative growth, and fruit production (Ashraf and Foolad 2007).

Even at NaCl concentrations as low as 80 mM, salt stress suppresses seed germination (Cuartero and Fernandez-Munoz 1999), vegetative growth, leaf area, and root weight (Papadopoulos and Rendig 1983; Van Ieperen 1996). Predictably, fruit yield is also negatively affected by salt stress because of a decrease in average fruit weight or the number of fruits produced by a single plant (Cuartero and Fernandez-Munoz 1999). At relatively low electrical conductivity (EC), fruit weight rather than fruit number tends affect the fruit yield. In contrast, at an elevated EC, fruit yield is mainly affected by reduction in fruit number (Van Ieperen 1996). In salt-stressed fruit, fruit enlargement is suppressed during the cell expansion phase because water uptake into the fruit, which is the motive force for cellular expansion, is suppressed by the increased salinity (Ehret and Ho 1986). Regarding marketable yield, increased blossom-end rot (BER) hampers tomato production. It has been suggested that BER is a physiological disorder caused by calcium deficiency in certain parts of the fruit (Adams and Ho 1992; Chretien et al. 2000; Franco et al. 1994; Willumsen et al. 1996) because Ca^{2+} uptake by the roots and its transport through the xylem to the fruits are both decreased under severe salt stress conditions (Belda et al. 1996; Ho et al. 1993). However, the detailed mechanism underlying BER is still unclear, and an effective method for combating BER has remained elusive.

1.3 Effect of Salt Stress on Fruit Qualities

As described in Sect. 1.2, salt stress has unfavorable effects on both plant growth and fruit enlargement in tomatoes. On the other hand, it has been reported that moderate salinity improves fruit quality by affecting the levels of soluble solids, such as sugars and acids, as well as the pH value; these are key factors in quality evaluations of fruit sold in markets, and salt stress generally improves fruit quality by increasing the content of those substances. This phenomenon has been attributed to a "concentration effect" that results from the suppression of fruit enlargement in plants exposed to salt stress. However, during the past decade, increasing evidence has indicated that alterations in assimilatory metabolism and the translocation of assimilates into the fruit are likely to be involved in the increase in soluble solids and other components.

1.3.1 Organic Acids

Acids largely affect fruit flavor by interacting with sugars and by creating acidity (Stevens et al. 1977), and organic acids account for approximately 13 % of the fruit dry matter (Davies and Hobson 1981). Malic acid and citric acid are the most abundant organic acids in fruit (4 % malic acid and 9 % citric acid on a dry matter basis); citric acid is more important for the sour flavor of the fruit than malic acid. Moderate salt stress (EC 8.0 dS m^{-1}) enhances the accumulation of citric and malic acids by

1.7- and 2.5 fold, respectively, compared to control conditions (EC 2.5 dS m^{-1}) at the red-ripe stage (Saito et al. 2008a). Transcriptional analyses showed that the expression of genes involved in organic acid metabolism, such as phosphoenolpyruvate carboxykinase (PEPCK), malate dehydrogenase (MDH), malic enzyme (ME), and pyruvate kinase (PK), is upregulated by both moderate (EC 8.0 dS m^{-1}) and severe (EC 15.0 dS m^{-1}) salt stress in ripening fruit (Saito et al. 2008a; Yin et al. 2010a). These results suggest that a shunt of the tricarboxylic acid (TCA) cycle (malate–oxaloacetate–PEP–pyruvate–citrate) is involved in organic acid metabolism and is stimulated by salt stress. Davies (1964) suggested that the accumulation of organic acids in the fruit counterbalances excessive cations to maintain the fruit pH. The difference between the cation and anion level tends to be greater in salt-stressed fruits, which leads to a higher concentration of organic acids in these fruits (Cuartero and Fernandez-Munoz 1999).

1.3.2 Carotenoids

Tomato fruits contain various carotenoids that are a major source of the antioxidant lycopene in the human diet, although the quantity, distribution, and antioxidant effects vary among cultivars (Minoggio et al. 2003). Fresh tomato fruit and its processed products provide approximately 85 % of the lycopene in the human daily diet (Canene-Adams et al. 2005). Tomatoes are also a notable source of other carotenoids, such as β-carotene, lutein, phytoene, phytofluene, and ζ-carotene. Because the carotenoid content is an important trait of tomato, many studies have reported the effects of salinity on the carotenoid content in tomato fruits. Generally, salt stress enhances lycopene and β-carotene accumulation; however, some reports indicated that the increase was observed only on a fresh weight and not a dry weight basis. Thus, these authors concluded that the increase should be attributed to a concentration effect caused by the suppression of fruit expansion (Krauss et al. 2006; Dumas et al. 2003; Shi and Le Maguer 2000). On the other hand, De Pascale et al. (2001) reported that the total carotenoid and lycopene content increased on both a fresh weight and dry weight basis under moderate salt stress (EC 4.0 dS m^{-1}) and suggested that metabolic alteration is involved in the increase along with the concentration effect. Our previous study also supports this observation. A key enzyme involved in carotenoid biosynthesis that is produced by the phytoene synthase gene was upregulated by moderate salinity stress (Saito et al. 2008a). Krauss et al. (2006) suggested that reduced leaf area caused by the growth suppression under salt stress and the increased exposure of fruit to sunlight resulted in increased carotenoid accumulation because carotenoid biosynthesis is regulated by light and increased exposure of the fruit to sunlight. Further investigation is required to elucidate the mechanisms underlying the effect of salinity on carotenoid accumulation.

1.3.3 Amino Acids

Tomato fruit flavor largely depends on the types and quantity of free amino acids present in the fruit. Several amino acids, such as glutamate, γ-aminobutyric acid (GABA), glutamine, and aspartic acid, account for approximately 80 % of the total free amino acids in the fruit (Kader et al. 1978). Among these amino acids, glutamate is the principal free amino acid in red-ripe fruit in commercial cultivars and it confers the characteristic "umami" flavor to tomato (Sorrequieta et al. 2010). It has been reported that salt stress increases the total amino acid content in tomato fruit, and the effect of the stress is greater in the pericarp than in the columella tissue (Zushi and Matsuzoe 2006; Yin et al. 2010a). Importantly, the proline content is increased markedly in both pericarp and columella tissue. Proline is a well-known indicator that responds to abiotic stresses such as salinity and drought in higher plants and most likely functions as an osmoprotectant in fruit exposed to salt stress (Ashton and Verma 1993; Claussen 2005).

Glutamate and GABA are the most abundant amino acids in tomato fruits (Inaba et al. 1980; Rolin et al. 2000). Tomato accumulates higher amounts of GABA in its edible parts than most other vegetable crops (Matsumoto et al. 1997). GABA is a four-carbon, nonprotein amino acid commonly found in diverse organisms, and it serves as a major inhibitory neurotransmitter in vertebrates (Zhang and Jackson 1993). It has been known to play a role in reducing blood pressure in the human body (Inoue et al. 2003). GABA is synthesized from glutamate by glutamate decarboxylase (GAD), which is activated by Ca^{2+}/CaM (Aurisano et al. 1995; Snedden et al. 1995; Turano and Fang 1998) and acidic pH (Johnson et al. 1997; Snedden et al. 1996). Salt stress also enhances GABA accumulation in the fruit at the red stage (Saito et al. 2008a, b). However, although salinity enhances fruit acidity and stimulates cellular Ca^{2+} in plant cells (Sanders et al. 1999), we cannot determine whether these factors directly promote GABA accumulation in tomato fruit because our previous data showed that glutamic acid decarboxylase (GAD) activity was unchanged in salt-stressed fruit; in fact, our data indicated that only the protein content increased (Yin et al. 2010a). Consequently, substrate availability and an increased concentration of GAD would promote GABA accumulation in salt-stressed tomato fruit. In fact, the GABA metabolic pathway is TCA cycle shunt (Bouchè and Fromm 2004). GABA accumulation reaches a maximum in mature green fruit and is rapidly degraded during fruit ripening (Rolin et al. 2000; Akihiro et al. 2008). Tracer analyses utilizing [^{14}C]-labeled GABA demonstrated that GABA in mature green fruit is converted to malate, citrate, *cis-aconitate*, isocitrate, and 2-oxoglutarate in red-ripe fruit; furthermore, [^{14}C] was also detected in the CO_2 gas fraction during ripening (Yin et al. 2010a). Those results indicate that dissimilated GABA flows back into the TCA cycle, is metabolized to organic acids, and is utilized as a substrate for respiration during climacteric ripening of fruit.

It was also reported that GABA accumulation in the fruit was enhanced 1.6- to 1.9 fold during the postharvest storage of red-ripe fruit under anaerobic (low O_2) conditions compared with normal air conditions, regardless of the CO_2 concentration (Mae et al. 2012). This increase was caused by increased GAD activity and decreased GABA transaminase (GABA-T) activity.

1.3.4 Ascorbic Acid

Ascorbic acid (ASA), which is present in tomato fruit, has an important role in the maintenance of human health by serving as an antioxidant that scavenges reactive oxygen species (ROS). In addition, ASA-related antioxidant systems, including the ascorbate–glutathione cycle (in which ascorbate peroxidase is involved), prevent the accumulation of toxic ROS levels under environmental stress conditions (Zhang 2013). Fruit ASA content is affected by cropping season, cultivar, and environmental stress (Dumas et al. 2003). However, the effect of salinity on fruit ASA content is actually not as clear as its effects on other metabolites such as sugars and amino acids. Several studies have shown positive results (Krauss et al. 2006), negative results (Zushi and Matsuzoe 2009), or no change (Fanasca et al. 2007) in the ASA content, and an interaction with other factors such as cultivars and growth conditions (e.g., temperature and light intensity) was suggested. Zushi et al. (2014) examined the combined effect of salt stress and light intensity on ASA content and the activity of ASA-related antioxidant enzymes in tomato fruits using tissue culture experiments. The results revealed that changes in the ASA content and the activity of antioxidant enzymes under salt stress conditions could not be explained only by salinity, because light intensity could also be involved in the regulation of antioxidant enzyme activity.

1.4 Effect of Salinity on Carbohydrate Allocation to Fruits

Salinity stress inhibits water uptake by the roots and water influx into the fruits as a result of high osmotic pressure around the root system, resulting in a decrease in fruit size and an increase in quality constituents. As described in Sect. 1.3, enhanced metabolite accumulation in salinity-stressed fruit has been attributed to a "concentration effect." However, our previous studies revealed that the ratio of sucrose to the total sugar content in salt-stressed fruits was increased compared with that of the control (Saito et al. 2006), and the effect of salt stress on the sugar concentration was apparently more severe than its suppressive effect on fruit weight (Yin et al. 2010b). Gao et al. (1998) reported that salinity enhanced the transport of assimilates from leaves to adjacent fruits. These results indicate that the increase in sugar content from salinity is not simply a result associated with the suppression of fruit growth; rather, it is also caused by alterations in sugar metabolism or sugar translocation into the fruit.

To examine alterations in carbohydrate partitioning in plants exposed to salt stress, Saito et al. (2009) investigated the distribution of carbohydrates under moderate salt stress conditions (EC 8.0 dS m^{-1}) in tomato plants by feeding [^{13}C]-labeled CO_2 to source leaves at 16 days after anthesis, which is the time when a tomato plant shows the greatest transport activity of photoassimilates from the source leaves to the fruits (Ho 1986). Although both photosynthetic and transpiration ratios were reduced by approximately 60 % under salt stress, the content of [^{13}C]-labeled assimilate to

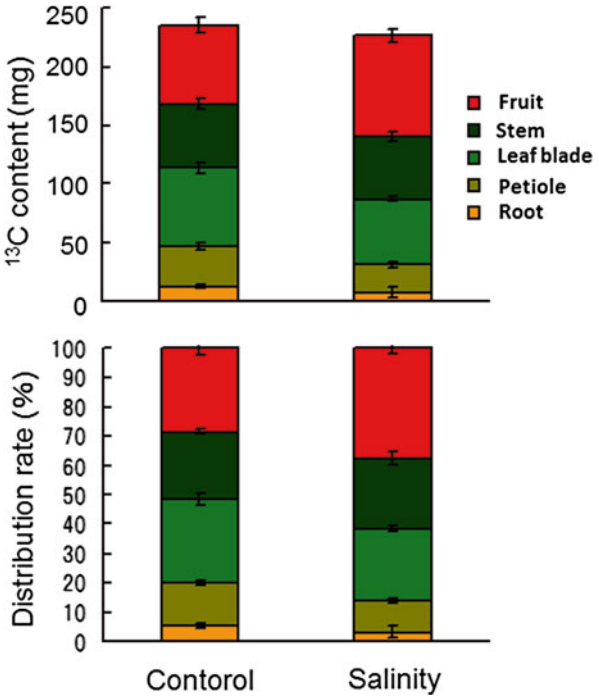

Fig. 1.1 Effect of salinity treatment on ^{13}C content and distribution in tomato plants 48 h after feeding $^{13}CO_2$. *Bars* indicate SE ($n = 3$)

fruits was 28 % higher compared with that observed in the control (Fig. 1.1). The distribution ratio of [^{13}C]-labeled assimilate in fruits was 32 % higher under saline conditions than under control conditions (Fig. 1.1). A similar result was also observed in the [^{13}C] tracer analyses performed under different salt stress conditions (EC 15.0 dS m^{-1}), in which the carbohydrate influx into the fruit was strongly enhanced by salinity in immature and mature green fruits, even though the stress severely suppressed plant growth (Yin et al. 2010b). These results indicate that salt stress alters the allocation of photoassimilate in the entire plant and enhances the sink strength of fruits.

1.5 Salinity Stress Enhances Starch and Sugar Accumulation in Fruit

1.5.1 Sugar Metabolism

In addition to the enhancement of photoassimilate translocation and the sink strength of fruit, salt stress increases the sugar content in fruit. This sugar mainly consists of hexoses produced by the cleavage of sucrose. Interestingly, sucrolytic activity is

also thought to be an index of sink strength (Yamaki 2010). There are two notable enzymes involved in the cleavage of sucrose to monosaccharides: sucrose synthase and invertase. Sucrose synthase (Susy) converts sucrose into fructose and UDP-glucose. By contrast, invertase irreversibly catalyzes the hydrolytic cleavage of sucrose into glucose and fructose. In tomato, Susy was considered to be a major factor determining the fruit sink strength because a strong correlation among Susy activity, ADP-glucose pyrophosphorylase activity, and starch accumulation in early developing fruit was found (Robinson et al. 1988; Yelle et al. 1988). Moreover, antisense transgenic plants in which Susy activity was suppressed displayed reduced fruit setting and sucrose import capacity in young fruit (D'Aoust et al. 1999). However, the suppression of the Susy-encoding gene in tomato did not lead to remarkable alterations in starch and sugar accumulation in the fruit (Chengappa et al. 1999). There is minimal evidence that sucrose synthase is directly involved in the control of fruit sugar content and composition in tomato.

On the other hand, during the past two decades, increasing evidence has indicated that invertase is an essential factor involved in the regulation of sugar content in tomato fruit. In plants, invertases are classified into three isozyme types according to their solubility, subcellular localization, isoelectric point (pI), and optimal pH (Sturm 1999): cell wall invertase (CWIN), vacuolar invertase (VIN), and cytoplasmic invertase (CIN). Among these isozyme types, CWIN and VIN are characterized as acid invertases because of their acidic optimal pH, whereas CIN is characterized as a neutral invertase because of its neutral optimal pH. Several studies have revealed the diverse roles of invertases in the plant life cycle, including their participation in various responses to abiotic and biotic stresses such as drought, hypoxia, high temperature, wounding, and pathogen infection (Roitsch and González 2004).

The relationship between fruit sugar content and abiotic stress suggests that the most important invertase is CWIN. During the past decade, CWIN has attracted attention as an essential enzyme for determination of the total soluble solids level in tomato (Fridman et al. 2002, 2004; Zanor et al. 2009). Among the genes encoding CWIN (*LIN*s), *LIN6* responds to various biotic and abiotic stimuli, including wounding, pathogen infection, and sugars (Godt and Roitsch 1997; Ohyama et al. 1998; Sinha et al. 2002). Additionally, *LIN7* was suggested to be involved in heat stress tolerance because its expression was specifically promoted by heat stress in heat-tolerant varieties (Li et al. 2012). Li et al. (2012) also suggested that the increased ability of young fruits to import sucrose contributes to the heat tolerance of the variety and is likely to be governed by *LIN7* expression. However, reports that fruit VIN and CWIN activities were not affected by salt stress (Carvajal et al. 2000; Saito et al. 2009) suggest that these two isozymes are most likely not involved in the phenomenon. In contrast, there was a positive correlation between CIN activity and hexose levels in the fruit of plants exposed to salinity stress (Balibrea et al. 1996, 2006). However, minimal information is available on CIN in tomato, and its physiological function has yet to be elucidated. These results suggest that CIN functions under specific environmental conditions such as salinity stress.

1.5.2 Starch Biosynthesis

Several studies have reported that salt stress enhances starch accumulation in early-developing tomato fruit (Balibrea et al. 1996; Gao et al. 1998; Yin et al. 2010b). In fact, this phenomenon involves an increase in ADP-glucose pyrophosphorylase (AGPase) activity. AGPase plays a role in the regulation of starch accumulation in early-developing fruit (Schaffer and Petreikov 1997; Schaffer et al. 2000). This enzyme catalyzes the synthesis of ADP-glucose from glucose-1-phosphate and ATP, which is the first regulatory step in starch biosynthesis in plants (Tsai and Neleson 1966; Lin et al. 1988; Stark et al. 1992). Plant AGPase is a heterotetrameric enzyme composed of two small and two large subunits (Morell et al. 1987). In tomato, four AGPase-encoding genes were isolated: one encodes the small subunit (*AgpS1*) and the other three genes encode the large subunit (*AgpL1, L2*, and *L3*) (Chen et al. 1998; Park and Chung 1998). Among these genes, *AgpL1* and *AgpS1* are predominantly expressed in fruit, and both genes show the highest expression during early developmental stages (Petreikov et al. 2006; Yin et al. 2010b). It has been reported that plant AGPase-encoding genes are regulated at the transcriptional level by phosphates, nitrates, and sugars (Müller-Röber et al. 1990; Scheible et al. 1997; Nielsen et al. 1998; Sokolov et al. 1998; Li et al. 2002). Additionally, our previous work showed that *AgpS1* and *AgpL1* expression was specifically enhanced by salt stress in early developing fruits in an ABA- and osmotic stress-independent manner (Yin et al. 2010b). Detailed expression analyses utilizing detached fruits revealed that the response of *AgpL1* expression to salt stress is a sugar-mediated response. This observation was indirectly supported by the [^{13}C] tracer analyses, in which the carbohydrate influx into the fruit was notably enhanced under salinity stress conditions in immature green fruits (Yin et al. 2010b). The observation that starch biosynthesis in fruit is dependent on the sugar supply is consistent with the results of N'tchobo et al. (1999). It has been reported that total soluble sugar contents and sucrose phosphate synthase (SPS) activity were increased (Carvajal et al. 2000) and the expression of the sucrose transporter gene *LeSUT1* was enhanced (Yin et al. 2010b) by salinity stress in leaves. Considering the results of the tracer analyses, which showed that the allocation of photosynthetic [^{14}C]/[^{13}C] to fruits and roots was increased under saline conditions (Gao et al. 1998; Saito et al. 2009), those responses are most likely a result of a systemic response to salt stress that promotes assimilate accumulation in sink organs. Salt stress expands carbohydrate availability in the developing fruits and promotes AGPase gene expression and consequent starch biosynthesis, followed by starch breakdown during ripening, which results in a high sugar content in red-ripe fruit. Similar phenomena were observed in different germplasms, such an *Solanum pennellii*-derived introgression line possessing the *Brix9-2-5* allele, in which enhanced starch accumulation in young fruit results in a higher content of total soluble solids in red-ripe fruit compared with normal tomato cultivars (Robinson et al. 1988; Baxter et al. 2005). These observations support the view that salt stress enhances sugar accumulation in red-ripe fruit through activation of starch biosynthesis in immature green fruit.

1.6 Conclusion

In this chapter, we introduced the concept of active alteration of fruit metabolism in response to salinity stress and discussed the proposed molecular mechanisms underlying the development of the "fruit tomato." As described in Sect. 1.5 and elsewhere, increased sugar levels cannot be explained only by a "concentration effect" caused by the suppression of fruit enlargement. This insight indicates that some metabolic pathways, at least those related to sugar and starch biosynthesis, have functions that are independent from that of the suppression of fruit enlargement. Generally, moderate salt stress simultaneously causes a reduction in fruit yield with a concomitant improvement in fruit quality. However, utilizing current biotechnological techniques, we are interested in producing a high-Brix fruit without yield reduction; this could be accomplished, for example, by manipulating the expression of starch biosynthesis genes. Another possibility entails the use of a reverse genetic approach to obtain a mutant in which a metabolic pathway is modified by knockout of a protein that degrades a particular metabolite (for example, GABA-T) utilizing a targeted mutant screen, such as the TILLING (targeting-induced local lesions in genomes) technique. Additionally, starch accumulation under salt stress would be a useful marker for identifying candidates that can be used in breeding varieties with high-Brix fruit. We hope that the information described in this chapter will be useful for researchers studying salt stress responses and those who are interested in the development of novel, high-value-added fruit crop varieties.

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Chapter 2
Ethylene, Oxygen, Carbon Dioxide, and Temperature in Postharvest Physiology

Yasutaka Kubo

Abstract Ethylene is biosynthesized from methionine by *S*-adenosyl-L-methionine (SAM) synthetase, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), and ACC oxidase (ACO) and plays a crucial role in ripening and senescence of horticultural crops. Ethylene first binds with ethylene receptors (*ETR/ERS*) localized on the endoplasmic reticulum (ER) membrane, and the signal is transmitted through a pathway involving *CTR1* → *EIN2* → *EIN3/EIL* → *ERFs* and then to ethylene-responsive genes, leading to ethylene response. Aminoethoxyvinylglycine (AVG) and 1-methylcyclopropene (1-MCP) are potent inhibitors of ACS activity and ethylene perception, respectively, and are commercially utilized for the control of ethylene in horticultural crops. Temperature is the most influential environmental factor for postharvest control of crops because low temperature suppresses most metabolic process, including respiration, thereby dramatically extending storage and shelf life. In chilling-susceptible crops such as bananas, pineapples, and avocados, exposure to temperatures below a critical limit imposes stress and results in chilling injury, with symptoms of pitting and browning of tissues. In pears and kiwifruit, low temperature modulates fruit ripening in an ethylene-dependent and -independent manner, respectively. Elevated CO_2 and reduced O_2 atmospheres have both beneficial and harmful effects on physiology and quality of crops, depending on concentration, duration, temperature, species, and other factors. Low O_2 conditions reduce rates of respiration and ethylene production in crops and extend storage life. When O_2 concentration drops to a certain limit, anaerobic respiration is induced, resulting in physiological damage. The respiratory response of horticultural crops to elevated CO_2 differs among species and may be mediated by the effect of CO_2 on the synthesis or action of ethylene. Excessive exposure to high CO_2 atmosphere induces a stress response, including a shift from aerobic to anaerobic metabolism in sensitive crops, resulting in CO_2 disorder.

Keywords 1-MCP • Controlled atmosphere • Ethylene • Fruit ripening • Low temperature

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2.1 Ethylene and Fruit Ripening

The gaseous plant hormone ethylene regulates several important aspects of plant growth and development as well as responses to the environment, such as responsiveness to stress and pathogen attack. In postharvest physiology and technology of horticultural crops, ethylene control is one of the critical points, as crops respond to both endogenous and exogenous ethylene in most conditions.

Fruit are divided into two groups: climacteric and nonclimacteric. Climacteric fruit ripen with increased respiration under the control of ethylene, and nonclimacteric fruit ripen independently of ethylene (Fig. 2.1). In climacteric fruit, such as

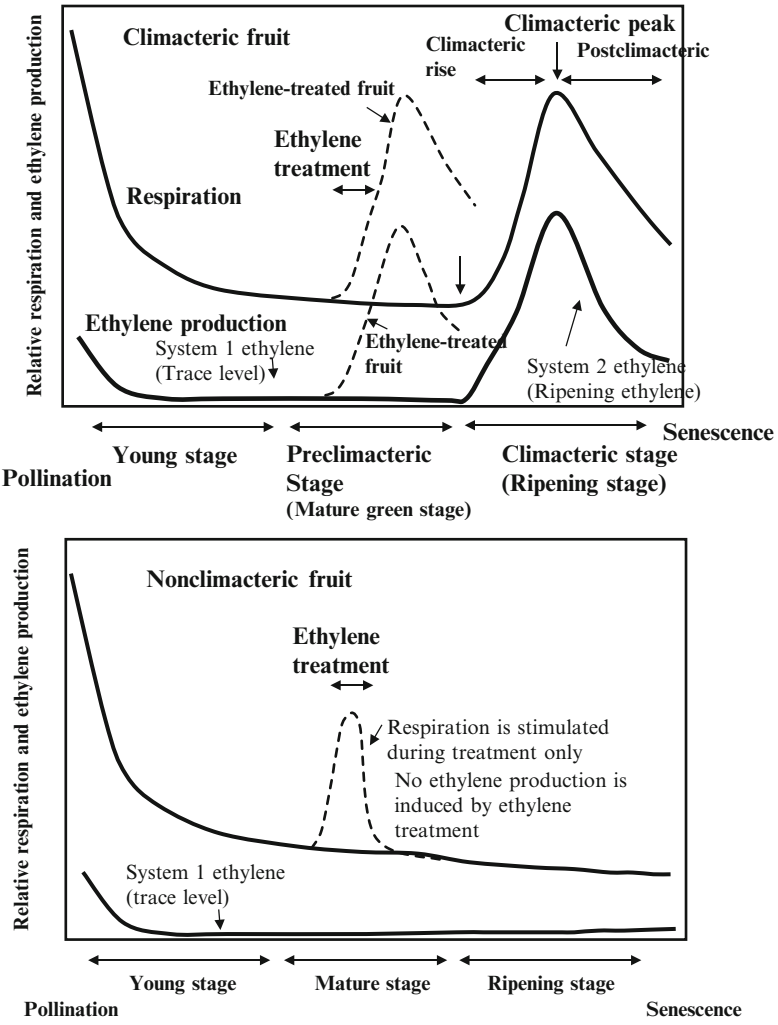

Fig. 2.1 A model of respiration and ethylene production in climacteric and nonclimacteric fruit

Table 2.1 Ethylene production rates of climacteric and nonclimacteric fruits

Type	Ethylene production rate at peak during ripening (nl/g/h)	Species ("cultivar")
Climacteric fruit	>100	Kiwifruit, Japanese apricot, Chinese pear, passion fruit, Cherimoya
	10–100	Apple, apricot, avocado, peach, pear, nectarine, papaya, plum, Feijoa
	1.0–10	Banana, fig, melon, mango, tomato, persimmon, Japanese pear ("Kohsui," "Kikusui," "Chohjuroh")
Nonclimacteric fruit	<0.2	Grape, lemon, orange, grapefruit, strawberry, pomegranate, cherry, watermelon, pineapple, cucumber, eggplant, Japanese pear ("Nijisseiki", "Niitaka")

tomatoes, melons, apples, bananas, and most stone fruits, the onset of ripening coincides with a climacteric increase of respiration and a burst of ethylene production, which is a so-called system 2 ethylene (Gapper et al. 2013). Ethylene production rates during ripening exceed 100 nl/g/h in kiwifruits, Japanese apricots, and passion fruits, whereas those in bananas, persimmons, and tomatoes range between 1.0 and 10.0 nl/g/h (Table 2.1). On the other hand, nonclimacteric fruit produce ethylene at levels lower than 0.2 nl/g/h during ripening. Japanese pears include climacteric-type cultivars such as "Kohsui," which has a short shelf life, and nonclimacteric type cultivars such as "Nijisseiki", which can be stored for more than 2 months. Trace levels of ethylene (system 1 ethylene, less than 0.1 nl/g/h) are produced in both climacteric and nonclimacteric fruit throughout fruit life (Fig. 2.1); however, their physiological roles have not been elucidated. Exogenous ethylene administered to fruit at the preclimacteric stage (mature green stage) stimulates respiration and induces endogenous ethylene biosynthesis, resulting in fruit ripening (Fig. 2.1). In nonclimacteric fruit, such as oranges, grapes, and watermelons, exogenous ethylene increases respiratory activity as in climacteric fruit and stimulates some ripening and senescence processes. Coloration of lemon is accelerated by ethylene treatment, and the technology is used commercially. Ethylene exposure of watermelons causes serious deterioration of flesh texture with little change in outward appearance. In both climacteric and nonclimacteric fruit, fruit abscission is stimulated by ethylene. However, no ethylene biosynthesis is induced in nonclimacteric fruit in response to exogenous ethylene; therefore, after removal of the ethylene, respiratory activities return to the same level as that before treatment (Fig. 2.1). Given that both climacteric and nonclimacteric fruit respond to ethylene, the physiological distinction between climacteric and nonclimacteric fruit depends on whether endogenous ethylene is induced in response to exogenous ethylene.

2.2 Ethylene Biosynthesis and Signal Transduction Pathway

Ethylene is synthesized from methionine in three steps (Fig. 2.2): (1) conversion of methionine to *S*-adenosyl-L-methione (SAM), catalyzed by SAM synthetase, (2) formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM by ACC synthase (ACS), and (3) conversion of ACC to ethylene by ACC oxidase (ACO). In addition, the formation of ACC leads to the production of 5′-methylthioadenosine (MTA), which is recycled via the methionine salvage pathway, which is the so-called Yang cycle, to yield a new molecule of methionine (Barry and Givannoni 2007; Lin et al. 2009; Wang et al. 2002; Yang and Hoffman 1984). The salvage pathway preserves the methyl group for another round of ethylene production. In this manner ethylene can be continuously synthesized without requiring an increasing pool of methionine. The sulfur group on the methionine is also conserved. Finally, ACC is oxidized by ACO to form ethylene, CO_2, and cyanide, which is detoxified to β-cyanoalanine by β-cyanoalanine synthase, preventing the toxic effects of accumulated cyanide. SAM is an important methyl donor and is used as substrate for multiple metabolic reactions, including polyamines and ethylene biosynthesis.

The rate-limiting steps of ethylene biosynthesis are conversion of SAM to ACC by ACS and ACC to ethylene by ACO. The first is a more important constraint, given that ACO activity and its gene expression are detected even in tissues with trace levels of ethylene production, such as climacteric fruit at the preclimacteric stage and the nonclimacteric fruit. However, the enzyme activity and gene expression of ACS are absent in tissues without ethylene biosynthesis and dramatically increase with ethylene-producing events such as fruit ripening and stresses such as

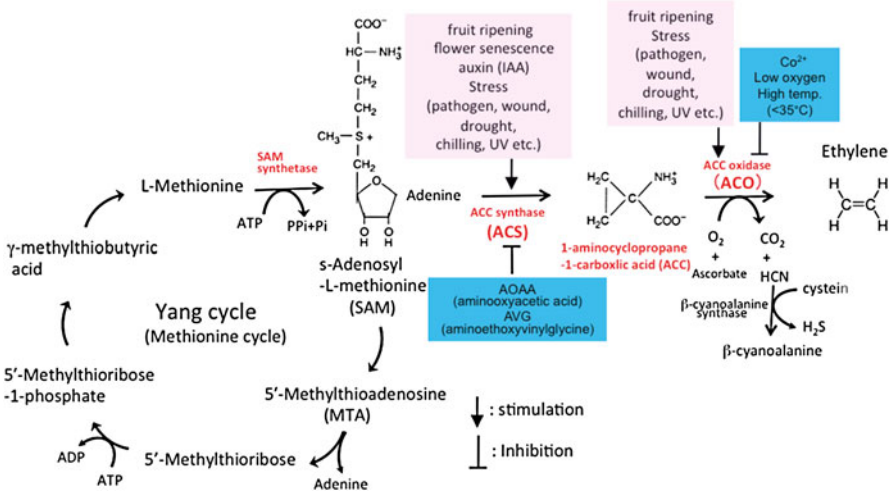

Fig. 2.2 Ethylene biosynthesis pathway and its regulation

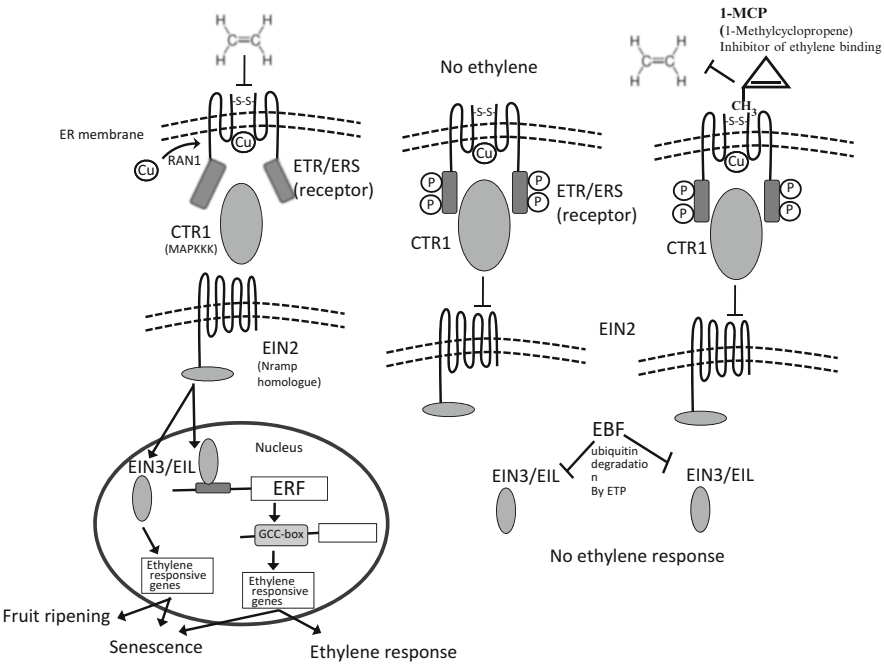

Fig. 2.3 Ethylene signal transduction pathway model

wounding and pathogen attack. ACS and ACO are encoded by multigene families in higher plants and are differentially regulated at the transcription level by developmental and environmental signals in response to both internal and external stimuli (Lin et al. 2009). The gene expression of ACS is stimulated during fruit ripening and flower senescence and in response to various stresses such as wounding, touch, chilling, and water stress (Fig. 2.2). In addition, application of high-level auxin induces specific ACS gene expression, resulting in a burst of ethylene production. Aminoethoxyvinylglycine (AVG) and aminooxyacetic acid (AOAA) have been shown to be inhibitors of ACS enzyme activity, and AVG is used as "ReTain," which is a practical plant growth regulator in apples and stone fruit. Gene expression and enzyme activities of ACO are also accelerated during fruit ripening and in response to stress. The enzyme activity of ACO is suppressed by the cobalt ion, temperatures higher than 35 °C, and low oxygen.

On the basis of ethylene-insensitive mutant analysis in *Arabidopsis*, ethylene signaling has been proposed to occur through a pathway of ethylene receptors (*ETR/ERS*) → *CTR1* → *EIN2* → *EIN3/EIL* → *ERFs* → ethylene-responsive genes (Fig. 2.3) (Adams-Phillips et al. 2004; Lin et al. 2009; Gapper et al. 2013; Seymour et al. 2013). The ethylene receptors are ancestrally related to two component histidine kinases localized to the endoplasmic reticulum membrane and act as negative regulators of ethylene signaling. In the absence of ethylene, a receptor directly interacts with CTR1, a Raf kinase-like protein, suppressing EIN2 and resulting in no ethylene

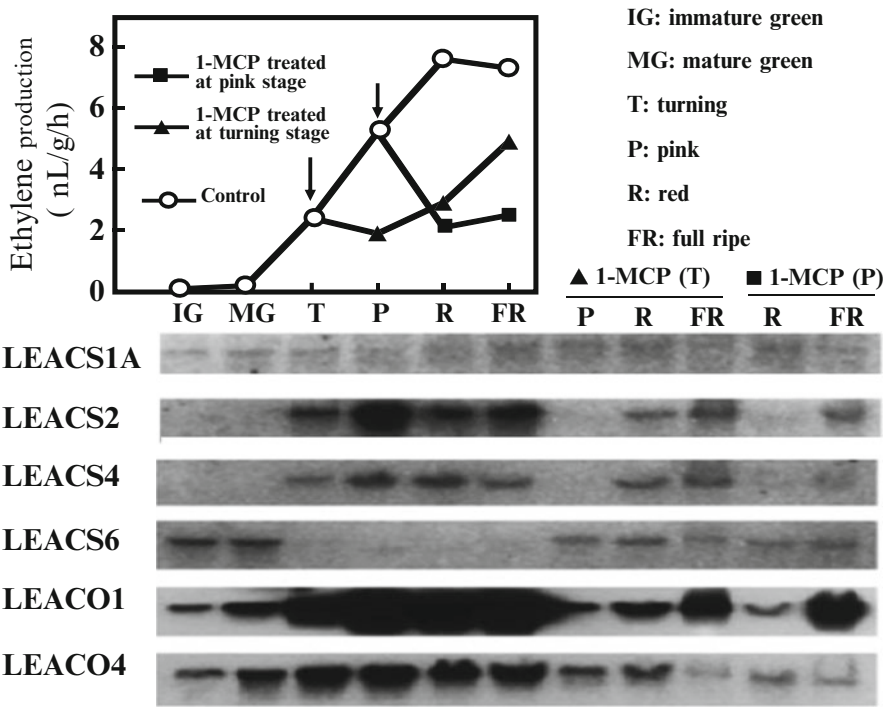

Fig. 2.4 Expression of LE-ACO and LE-ACS genes in tomato during fruit ripening and the effect of 1-methylcyclopropene (1-MCP). (From Nakatsuka et al. 1998)

response (Fig. 2.3) (Adams-Phillips et al. 2004; Tieman et al. 2000). When the receptor binds ethylene, the inhibition of EIN2 is relieved by degradation of the receptor–CTR1 complex, and a signaling process leads to the ethylene response.

In tomato, there are seven ethylene receptors, and the transcript levels of *LEETR3/Nr*, *LEETR4*, and *LEETR6* increase during fruit ripening (Kevany et al. 2007). The receptors are targets of protein turnover mediated by the 26S proteasome-dependent pathway, and exogenous ethylene enhances receptor degradation. 1-Methylcyclopropene (1-MCP), a potent inhibitor of ethylene perception, functions as receptor blocker and inhibits ethylene signals even in the presence of ethylene (Fig. 2.4). Recently, Kamiyoshihara et al. (2012) demonstrated that LEETR4 is multiply phosphorylated in vivo, and that ethylene treatment reduces the phosphorylation level whereas 1-MCP treatment increases it. This finding suggests that the phosphorylation state of receptors is implicated in ethylene signal output.

EIN2 level is likely to be controlled by protein turnover via the 26S proteasome. Qiao et al. (2009) identified two F-box proteins, ETP1/ETP2 (EIN2 TARGETING PROTEIN) for ubiquitin degradation in *Arabidopsis*. Thus, ETP acts as a negative regulator in ethylene signaling. In the later steps of ethylene signaling, the transcription factors *EIN3/EILs* and *ERFs* serve as positive regulators in ethylene signaling (Tieman et al. 2001; Yokotani et al. 2003). There are four *EIN3-like* (*EIL*) genes in

tomato, and suppression of their gene expression reduces ethylene sensitivity, resulting in a nonripening phenotype (Tieman et al. 2001; Yokotani et al. 2009). *ERFs* are important in the regulation of downstream ethylene-responsive genes by binding to a GCC box promoter element (Fujimoto et al. 2000).

2.3 Control of Ethylene and Fruit Ripening

In tomato, there are at least 7 *ACO* and 12 *ACS* genes in the genome, of which at least 3 *ACO* and 4 *ACS* genes are differentially expressed in fruit (Barry et al. 2000; Seymour et al. 2013). *LEACO1* and *LEACO4* were expressed at low levels in mature green fruit with trace levels of system 1 ethylene, but the transcripts of each increased at the onset of ripening as the fruit transitioned to system 2 ethylene production (Fig. 2.4) (Nakatsuka et al. 1998). The expression of *LEACS2, LEACS4, LEACO1*, and *LEACO4* was largely blocked by 1-MCP treatment, indicating that these genes are positively regulated by ethylene (Fig. 2.4). *LEACS6* transcripts accumulated in the fruit at the green and mature green stages and disappeared in the ripening stages, but 1-MCP treatment restored its accumulation. These observations indicate that *LEACS6* is involved in system 1 ethylene and negatively regulated by ethylene, whereas *LEACS2* and *LEACS4* are responsible for system 2 by positive feedback regulation.

In pear fruit, 1-MCP treatment either at harvest or after the initiation of ripening markedly suppressed ripening-related events such as ethylene production, flesh softening, and quality deterioration (Fig. 2.5) (Hiwasa et al. 2003). These results clearly indicate that ethylene is not merely a by-product but has a crucial role in both initiation and maintenance of regulation of the softening process during ripening. 1-MCP treatment can extend both storage and shelf life of climacteric fruit. In fact, a large part of the commercial apple fruit worldwide is treated with 1-MCP to maintain flesh firmness.

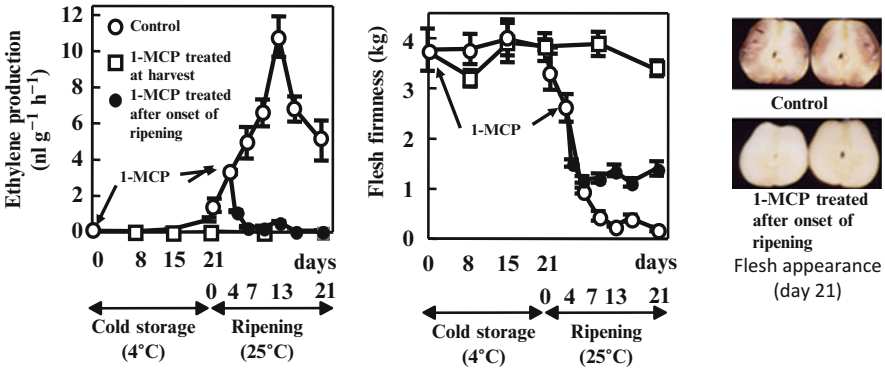

Fig. 2.5 Effects of 1-MCP treatment on ethylene production and flesh firmness in "La France" pear fruit. (From Hiwasa et al. 2003)

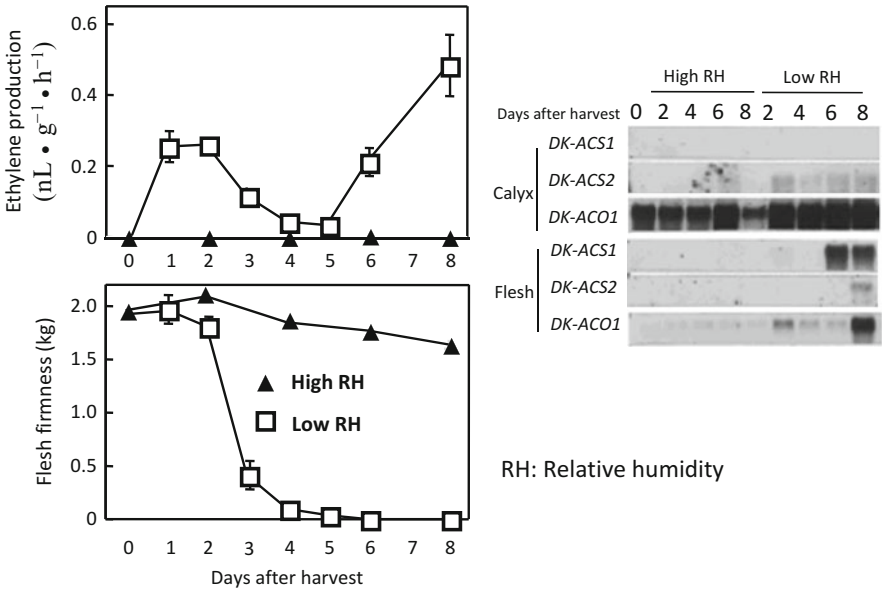

Fig. 2.6 Effects of humidity on ethylene production, flesh firmness, and gene expression of ACS and ACO in persimmon fruit stored at room temperature. (From Nakano et al. 2002)

As already mentioned, ethylene biosynthesis is induced by various stresses. When harvested persimmon fruits were held under ambient low humidity (40–60 % RH), ethylene production was induced with gene expression of *ACS* and *ACO* in the calyx followed by that in the flesh, resulting in rapid softening and deterioration of fruit quality (Fig. 2.6) (Nakano et al. 2002, 2003). High humidity (>95 %) prevented induction of both ethylene production and flesh softening. These observations indicate that fruit softening occurs because of the action of ethylene produced in response to water stress. Alleviation of the stress by wrapping the fruit with a plastic film could extend storage and shelf life in persimmon fruit.

2.4 Temperature Effects on Postharvest Physiology in Horticultural Crops

Temperature is the environmental factor that most influences the deterioration rate of harvested crops (Kader 2002; Wills et al. 1998). For each 10 °C increase above the optimum temperature, the deterioration rate increases two to three times. Exposure to undesirable temperatures results in many physiological disorders. In addition, temperature influences the rate of ethylene production, the sensitivity of products to ethylene, and the effects of reduced oxygen and elevated carbon dioxide.

Table 2.2 Chilling injury in some fruits and vegetables (Kader 2002, modified)

Species	Lowest safe temperature (°C)	Symptom of chilling injury
Avocado	5–12	Pitting, browning of pulp
Banana	13	Brown streaking on skin
Cucumber	7	Pitting, water-soaked area
Eggplant	7	Pitting, surface scald
Lemon	10	Pitting, red blotches
Mango	5–12	Dull skin, browning
Papaya	7–15	Pitting, water-soaked area
Sweet potato	10	Pitting, rotting
Pumpkin	10	Rotting
Tomato	10–12	Pitting, rotting
Pineapple	5–7	Browning of core
Chinese pear	5–7	Browning of pulp and core

The range of temperature indicates variation between cultivars in susceptibility to chilling injury

Storage of crops at low temperatures is beneficial because the rates of respiration and of general metabolism are reduced. However, low temperature does not always suppress all aspects of metabolism to the same extent (Wills et al. 1998). Some reactions are sensitive to low temperature and completely cease below a critical temperature. In addition, exposure to below-critical temperatures may induce changes in the physical properties of cell membranes owing to changes in the physical states of membrane lipids (the so-called lipid hypothesis of chilling). These changes may, in turn, result in metabolic changes with eventual disruption of cellular transport of metabolites. If the imbalance and disruptions become sufficiently severe, the cells will cease to properly function, resulting in development of symptoms of chilling injury and induction of ethylene biosynthesis, particularly on transfer to ambient temperature. Chilling injury is a disorder resulting from the exposure of susceptible tissues to temperatures between approximately 15 °C and 0 °C. It often occurs in crops of tropical or subtropical origin such as bananas, pineapples, and avocados, but is also observed in several species and varieties originating in the temperate zone. Susceptibility to chilling injury, critical temperature, and symptoms vary widely among different crops and varieties (Table 2.2). Major symptoms are pitting and browning of skin or flesh and in most tissues affected by chilling injury.

However, in some fruit, exposure to low temperature has a beneficial effect by accelerating ripening. European pears that ripen on the tree typically develop poor texture, lack succulence, and may lack the characteristic flavor of the cultivar (Mitchell 1992). “Bartlett” pears harvested early in the season slowly and unevenly ripen in room temperature without time in cold storage. “Anjou” pears can remain unripe for more than a month at ambient temperature. However, fruits ripen quickly and uniformly if they are first stored at low temperatures for several weeks. “Passe-Crassane” pears require a 3-month chilling treatment at 0 °C to produce ethylene and autonomously ripen after subsequent rewarming. The chilling treatment strongly stimulates gene expression of *ACO* and *ACS*, whereas fruit stored at room

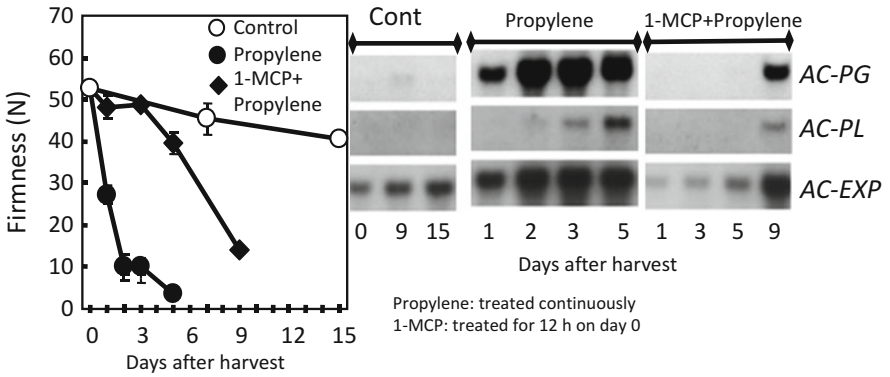

Fig. 2.7 Effects of propylene and 1-MCP treatment on firmness and expression of cell wall-modifying genes in "Sanuki Gold" kiwifruit. (From Mworia et al. 2012)

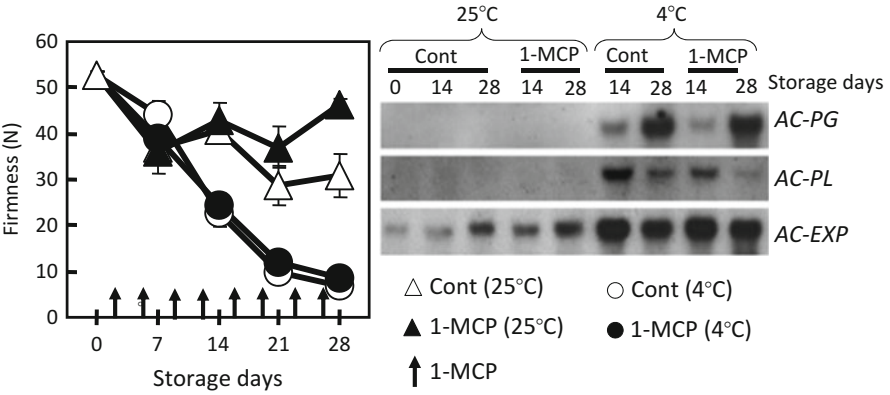

Fig. 2.8 Effects of storage temperature and 1-MCP treatment on flesh firmness and expression of cell wall-modifying genes in "Sanuki Gold" kiwifruit. (From Mworia et al. 2012)

temperature immediately after harvest exhibits no change (Lelièvre et al. 1997). During low-temperature storage, ethylene precursors accumulate within the pear tissue, so that fruits transferred to room temperature increase their ethylene production and uniformly ripen (Fig. 2.5) (Hiwasa et al. 2003).

Recently, modulation of fruit ripening by low temperatures in kiwifruit has been reported (Mworia et al. 2012). As kiwifruit are a climacteric fruit and are harvested at the preclimacteric stage, ethylene treatment is used to induce fruit ripening. Treatment with an ethylene analogue, propylene, immediately induced rapid fruit softening with increased gene expression of polygalacturonase (*PG*), expansin (*EXP*), and pectate lyase (*PL*) (Fig. 2.7). 1-MCP treatment before exposure to propylene delayed fruit softening and suppressed the expression of cell wall-modifying genes. In contrast, in kiwifruit stored at room temperature without exogenous ethylene, flesh firmness remained high during 4 weeks of storage (Fig. 2.8). Fruit stored at low temperature exhibited marked fruit softening, mRNA accumulation of *AC-PG*, *AC-PL*, and

AC-EXP without ethylene production, and repeated (twice weekly) 1-MCP treatments failed to inhibit the changes that occurred in low-temperature storage. These observations indicate that low temperature modulates the ripening of kiwifruit in an ethylene-independent manner, suggesting that kiwifruit ripening is inducible by either ethylene or low temperature.

2.5 Beneficial and Harmful Effects of Elevated CO_2 and Reduced O_2 Atmosphere

The gas composition of the atmosphere surrounding horticultural crops can affect their storage and shelf life. Controlled atmosphere (CA) storage usually refers to reduced O_2 and elevated CO_2, in comparison with ambient atmosphere. CA implies continuous and precise control of the gases, whereas the term "modified atmosphere" (MA) is used when the composition of the gases is not actively controlled, similar to that in plastic film packaging.

In general, the effects of reduced O_2 or elevated CO_2 on reducing respiration rate has been assumed to be the primary reason for the beneficial effects of CA on fruit and vegetables (Kader 1986; Kubo 1993), given that O_2 is a substrate and CO_2 is a product of respiration. This assumption is an oversimplification, given that postharvest deterioration of horticultural crops can be caused by several factors, such as metabolic changes, physical or physiological injuries, and pathogen attack, and that elevated CO_2 and reduced O_2 do not always result in suppression of respiration. Lowering the O_2 level around fruit and vegetables to a certain limit reduces O_2 uptake and CO_2 output by respiration in proportion to the O_2 concentration (Fig. 2.9). When the O_2 concentration drops to the extinction point, anaerobic respiration begins. As the concentration of O_2 further decreases, anaerobic respiration becomes predominant, CO_2 output starts to increase after reaching its lowest value at the

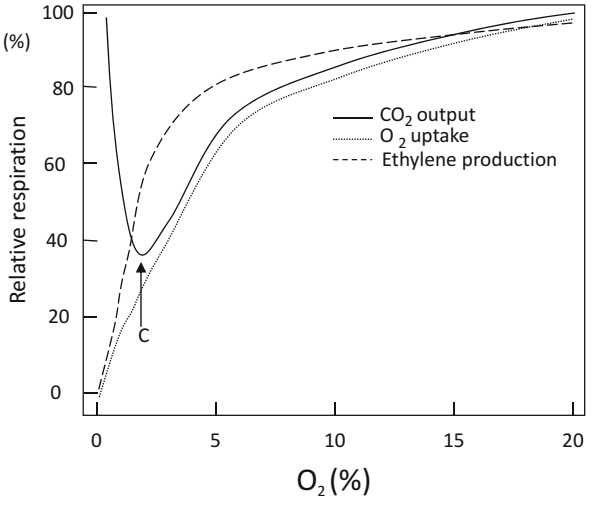

Fig. 2.9 Schematic diagram of the effects of the O_2 concentration, O_2 uptake, CO_2 output, and ethylene production by harvested crops. *E* extinction point, the lowest concentration of O_2 at which anaerobic respiration begins; *C* critical O_2 concentration, the O_2 concentration at which CO_2 output is lowest. (Calculated on measurement in peach fruit; Kubo 1993)

critical point, and the glycolytic pathway replaces the tricarboxylic acid (TCA) cycle as the main source of energy needed by plant tissues. Pyruvic acid is no longer oxidized but is decarboxylated to form acetaldehyde, CO_2, and ultimately ethanol, resulting in the development of off flavors and tissue breakdown. For these reasons, the minimum O_2 level needed to ensure anaerobic metabolism and prevent fermentation is optimal for the storage atmosphere of horticultural crops. This concentration depends on crop species, maturity, temperature, duration of storage, and other factors, but is usually 2 % to 5 %.

The conversion of ACC to ethylene by ACO is oxygen dependent (Fig. 2.2), and under anaerobic conditions ethylene formation is completely suppressed (Lin et al. 2009). The concentration of O_2 yielding half the maximum ethylene production rate ranges between 5 % and 7 % in various tissues, so that the 2 % to 5 % O_2 atmosphere usually used in CA storage directly interferes with ethylene synthesis (Fig. 2.9, Table 2.3). The action of ethylene on plant tissue at 3 % O_2 is approximately 50 % of that in the air. On the other hand, a reduced O_2 concentration that induces anaerobic stress can result in induction of ethylene biosynthesis after transfer to ambient conditions.

Respiration rates of horticultural crops have been thought to decrease as CO_2 concentration in the atmosphere increases, because CO_2 is a final product of respiratory metabolism and an increased CO_2 atmosphere can extend the storage life of several horticultural crops, such as apples and pears (Table 2.3). In pears under an elevated CO_2 atmosphere, phosphofructokinase, a key enzyme of glycolysis, was inhibited with accumulation of fructose-6-phosphate and decrease of fructose-1,6-phosphate, suggesting the suppression of glycolysis (Kerbel et al. 1988). In the Krebs cycle, exposure to an elevated CO_2 atmosphere caused accumulation of succinic acid and reduced succinic dehydrogenase in pear fruit. In studies regarding mitochondria isolated from apples, the elevated CO_2 suppressed the oxidation of several intermediates of the Krebs cycle, suggesting a disturbance of the cycle (Shipway and Bramlage 1973).

In avocados and bananas, a 5 % to 10 % CO_2 atmosphere showed little effect on the basic level of respiration at the preclimacteric stage, but delayed the onset of respiratory climacteric rise, triggered by endogenous ethylene (Fig. 2.10) (Biale 1960). This observation suggests that an elevated CO_2 atmosphere delays the burst of ethylene production. In peaches and broccoli, the O_2 uptake markedly decreased in response to 60 % CO_2 + 20 % O_2 condition along with suppression of ethylene production (Fig. 2.11) (Kubo et al. 1989, 1990). Similar responses were observed in ripening tomatoes; however, fruit at the mature green stage showed no change in O_2 uptake even in a 60 % CO_2 atmosphere, which is an extremely high concentration. In addition, in nonclimacteric fruit such as Satsuma mandarins, grapes, and carrots, no decrease of O_2 uptake was detected in the 60 % CO_2 atmosphere (Figs. 2.11, 2.12). In contrast, when eggplant, cucumber, and lettuce were exposed to 60 % CO_2, O_2 uptake increased with induction of ethylene biosynthesis (Fig. 2.12). These observations suggest that the respiratory response of horticultural crops to the elevated CO_2 condition is mediated by the effect of CO_2 on the synthesis or action of ethylene. Thus, retardation of ripening or senescence and associated biochemical

Table 2.3 Summary of recommended controlled atmosphere (CA) or modified atmosphere (MA) conditions during storage and transport of horticultural crops (Kader 2002, modified)

Species	Temperature (°C)	O_2 (%)	CO_2 (%)	Potential benefit[a]	Remarks
Climacteric fruit					
Apple	0–5	1–2	0–3	A	Wide commercial use
Banana	12–16	2–5	2–5	A	Some use during marine transport
Pear	0–5	1–3	0–3	A	Considerable commercial use
Kiwifruit	0–5	1–2	3–5	A	Some commercial use for transport and storage
Persimmon	0–3	2–5	5–8	A	Some commercial use of MA packaging
Avocado	5–13	2–5	3–10	B	Some use during marine transport
Tomato	10–15	3–5	3–5	B	Limited commercial use
Nonclimacteric fruit					
Lemon	10–15	5–10	0–10	C	No commercial use
Orange	5–10	5–10	0–5	C	No commercial use
Grapefruit	10–15	3–10	5–10	C	No commercial use
Grape	0–5	2–5	1–3	D	Incompatible with SO_2 fumigation
Strawberry	0–5	5–10	15–20	A	Some commercial use for short storage and transit
Vegetables					
Broccoli	0–5	1–2	5–10	B	Limited commercial use
Cabbage	0–5	2–3	3–6	B	Some commercial use
Cauliflower	0–5	2–3	3–4	C	No commercial use
Spinach	0–5	7–10	5–10	C	No commercial use
Potato	4–12	None	None	D	No commercial use
Carrot	0–5	None	None	D	No commercial use

[a]*A* excellent, *B* good, *C* fair, *D* slight, *D* slight or none

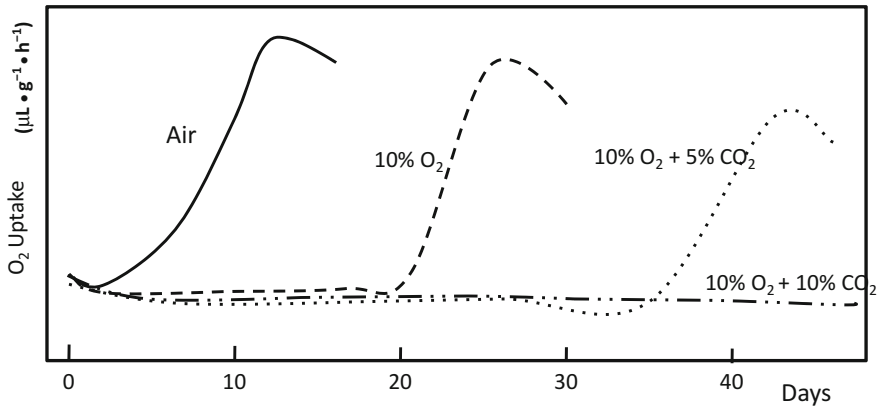

Fig. 2.10 Effects of CO_2 on the respiration of banana at a constant tension. (Modified from Biale 1960)

and physiological changes induced by the elevated CO_2 would depend on the effect of CO_2 on the action or synthesis of ethylene compared with that on direct effects on respiratory metabolism. CO_2 has been suggested to compete with ethylene for binding sites, and the relative affinity to the site of 15 % CO_2 is assessed equivalent to that of 1 ppm ethylene (Burg and Burg 1967). The potential benefits and extent of commercial use are shown in Table 2.3, which also shows the recommended CA and MA conditions during storage and transport. In general, climacteric fruits or vegetables producing ethylene, for example, broccoli, have more potential to benefit than nonclimacteric fruit and vegetables not producing ethylene. The recommended concentration of CO_2 for most crops to avoid harmful effects ranges from 0 % to 10 % CO_2. Strawberry is an exception, with 15 % to 20 % CO_2 used for a short time to suppress the growth of microbes on the surface of the fruit.

Excessive exposure to high CO_2 can induce a stress response in sensitive crops, leading to physiological disorders. Induced ethylene production and increased respiration in lettuce, spinach, and cucumber under a 60 % CO_2 atmosphere are examples of stress response and may be involved in development of CO_2 disorders (Fig. 2.12). Lettuce is known to be sensitive to high CO_2 condition and to develop "brown stain" symptoms, which is a typical CO_2 disorder. "Brown heart" is a CO_2 disorder in pears and apples. Under very high CO_2 conditions, even if there is sufficient O_2, several crops form aldehyde and ethanol, a phenomenon called CO_2 zymosis, suggesting a shift from aerobic to anaerobic metabolism under elevated CO_2, such as that which occurs under a very low O_2 atmosphere. The exposure of persimmon to 100 % CO_2 is utilized to accumulate acetaldehyde, which functions as a crosslinker in polymerization of tannin molecules, leading to reduction in astringency.

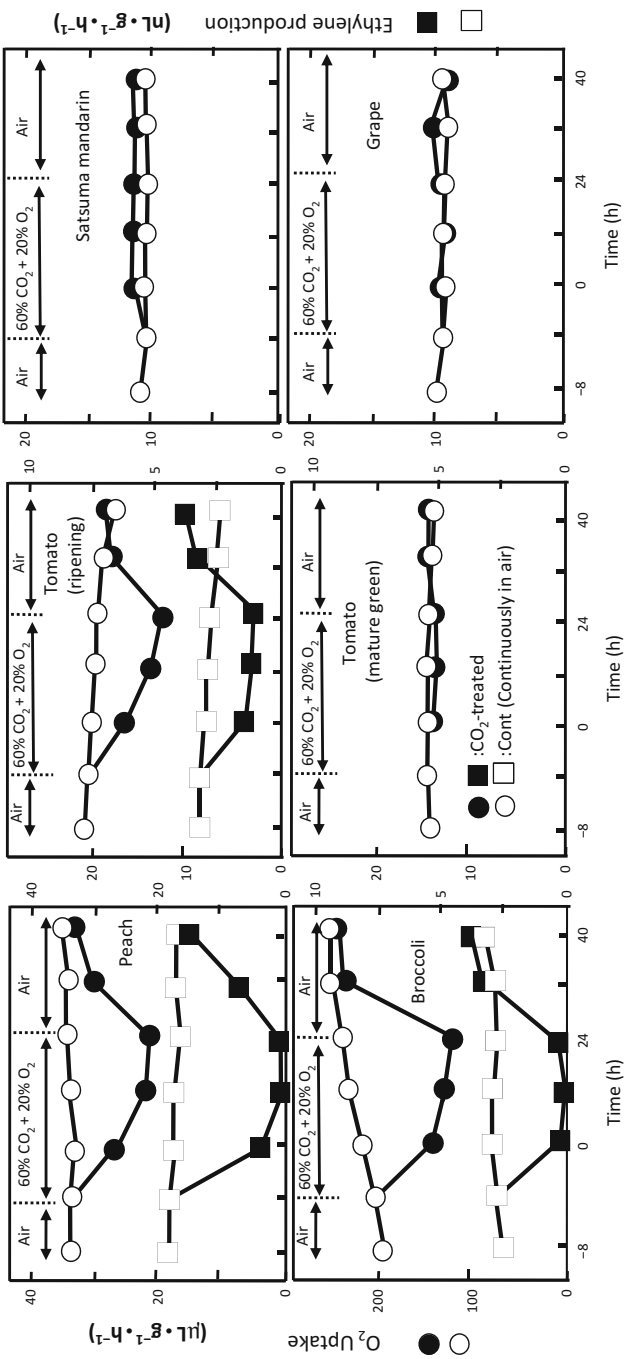

Fig. 2.11 Effects of exposure to 60 % CO_2 + 20 % O_2 on the rate of O_2 uptake and ethylene production in several fruits and broccoli. (From Kubo et al. 1989, 1990)

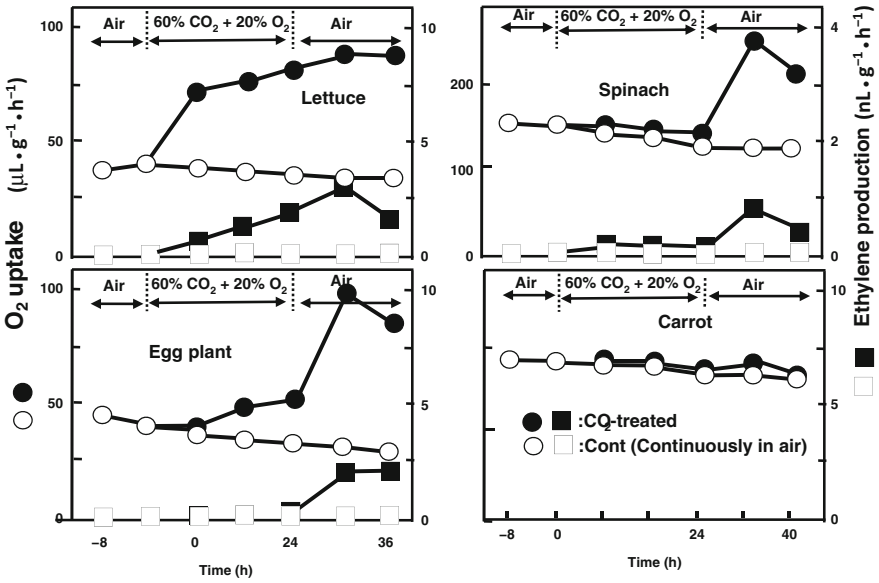

Fig. 2.12 Effects of exposure to 60 % CO_2 + 20 % O_2 on the rate of O_2 uptake and ethylene production in several vegetables. (From Kubo et al. 1990)

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Chapter 3
Role of Polyamines in Stress Response in Horticultural Crops

Xiaopeng Wen and Takaya Moriguchi

Abstract Polyamines (PAs) are low molecular weight aliphatic cations that are ubiquitous in all organisms, including plants. PA accumulation occurs under stress in plants, and modulation of the PA biosynthetic pathway confers tolerance to stresses. Over the past two decades, many reports have unraveled significant functions of PAs in the regulation of abiotic stress tolerance in plants. Here, we focused on the involvement of PA pathways in plants, including those of horticultural crops, in ameliorating abiotic stresses such as salt, drought, heat, chilling, and heavy metals. The possible mechanisms of PA functions on stress tolerance have also been summarized. In addition, the current research trends and future perspectives, especially in horticultural crops, are discussed.

Keywords Abiotic stress • Genetic transformation • Horticultural crop • Polyamine • Tolerance

3.1 Introduction

Polyamines (PAs) are ubiquitous low molecular weight aliphatic cations found in all living organisms. Because of their cationic nature, they can readily bind to the negatively charged head groups of phospholipids or other anionic sites on membranes, thus affecting the stability and permeability of such membranes. PAs are also able to bind to cellular polyanions such as DNA, RNA, and protein, thereby affecting the synthesis, structure, and function of these macromolecules. Through such possible interactions with other cell components, the involvement of PAs in a wide range of plant growth and developmental processes, as well as responses to environmental stresses, has been demonstrated. Furthermore, PAs can function as direct or

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indirect free radical scavengers (Ha et al. 1998) and have an antioxidative property by limiting the accumulation of O_2^-, probably through the inhibition of NADPH oxidase (Papadakis and Roubelakis-Angelakis 2005).

Previous studies have described the correlation between changes in PA levels and the protective effects of PAs from environmental stresses (Alcázar et al. 2006). However, the effect of PA concentration upon stress was known to show variations, and even sometimes to be contradictory, which was ascribed to the differences in the plant species/cultivars and treatment methods. The creation of several transgenic plants with modified PA biosynthetic genes and the utilization of mutants has been carried out to clearly demonstrate their functions on abiotic stresses; however, those of horticultural crops were quite limited (Wen et al. 2008, 2009, 2010, 2011; Hazarika and Rajam 2011). In this chapter, we intend to summarize the metabolism and functions of PAs on stresses in plants, including those in horticultural crops.

3.2 Polyamine Biosynthesis and Catabolism

With some variations, biosynthetic pathways for PAs are generally conserved from bacteria to animals and plants (Fig. 3.1). Putresine (Put) is synthesized via ornithine by arginase (EC 3.5.3.1) or ornithine decarboxylase (ODC, EC 4.1.1.17), which are known as the arginine and ornithine pathways, respectively. In the arginine pathway, Put is formed indirectly via the decarboxylation of arginine by arginine decarboxylase

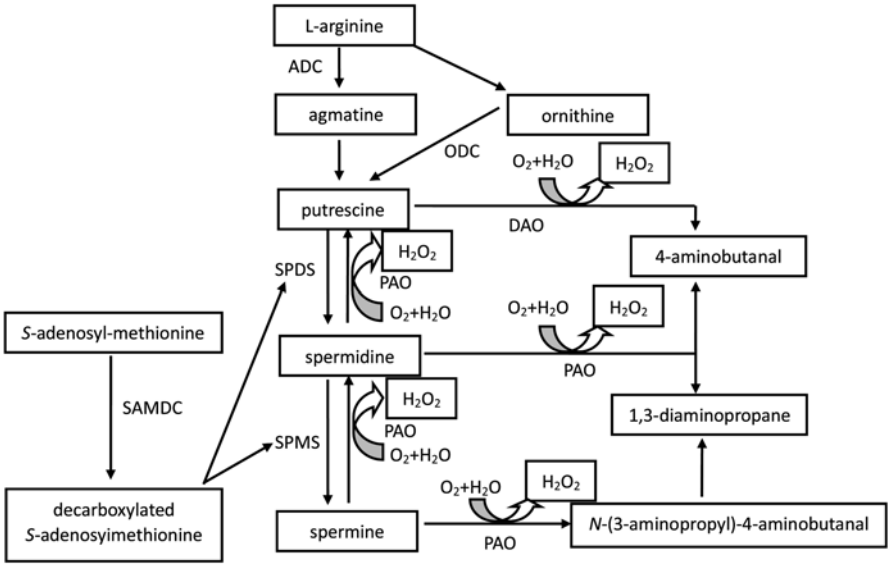

Fig. 3.1 Polyamine (PA) biosynthesis and degradation pathways. *ADC* arginine decarboxylase, *DAO* diamine oxidase, *ODC* ornithine decarboxylase, *PAO* polyamine oxidase, *SAMDC* *S*-adenosylmethionine decarboxylase, *SPDS* spermidine synthase, *SPMS* spermine synthase

(ADC, EC 4.1.1.19), whereas in the ornithine pathway, Put is formed directly from the decarboxylation of ornithine by ODC. Spermidine (Spd) and spermine (Spm) are formed by the successive transfer of an aminopropyl moiety from decarboxylated *S*-adenosylmethionine (dcSAM) to Put and Spd, respectively; the processes are catalyzed by the aminopropyl transferases Spd synthase (SPDS, EC 2.5.1.16) and Spm synthase (SPMS, EC 2.5.1.22), respectively. In *Arabidopsis*, ACAULIS5 (ACL5), which is required for stem elongation, was also identified as spermine synthase (Hanzawa et al. 2000), but ACL5 synthesizes thermospermine, an isomer of spermine rather than spermine (Knott et al. 2007). dcSAM is produced from the decarboxylation of *S*-adenosylmethionine (SAM) by SAM decarboxylase (SAMDC, EC 4.1.1.50). SAM is also the precursor for ethylene biosynthesis; thus, PA and ethylene biosynthesis may compete for the utilization of SAM pools in the cell.

Put is oxidatively deaminated by copper-containing diamine oxidases (DAO, EC 1.4.3.6), and Spd and Spm are oxidized by flavoprotein-containing PA oxidase (PAO, EC 1.5.3.11). DAO preferentially oxidize Put and other diamines. The function of DAO is to convert Put into 4-aminobutanal and hydrogen peroxide (H_2O_2). PAO oxidizes Spd to 4-aminobutanal, 1,3-diaminopropane (DAP), and H_2O_2, and Spm to *N*-(3-aminopropyl)-4-aminobutanal, DAP, and H_2O_2. DAP can be catabolized to alanine; 4-aminobutanal can be further converted into γ-aminobutyric acid (GABA). GABA is transaminated and oxidized to form succinic acid, which may then enter the Krebs cycle, ensuring the recycling of the carbon and nitrogen from Put. The model plant *Arabidopsis* contains five PAO-like genes, among which AtPAO1 prefers T-Spm and norspermine to Spm, but does not recognize Spd; AtPAO2 and AtPAO3 preferably recognize Spd; and AtPAO4 is likely to be Spm specific (Takahashi et al. 2010). PA catabolism is not simply a degradative process but also a contributing process for PA homeostasis.

Considering the PA concentrations in the transgenic plants with PA biosynthetic genes, the increase in the target PA concentration was not as much as expected, despite the usage of the constitutive 35S promoter. For example, Spd concentrations in *SPDS*-overexpressing European pears (Wen et al. 2008) and *Arabidopsis* (Kasukabe et al. 2004) were only 1.5- to 2.0 fold higher than the concentrations in the wild type. It could be that the transgenic plantlets with high PA concentrations cannot be regenerated in the regeneration medium, so only transgenic plants with mild PA concentrations successfully regenerated and developed into whole plants. This observation may indicate that the PA concentrations in the cells are kept under homeostasis by fine regulation.

3.3 PA and Salt and Drought Stresses

When plants are exposed to salt stress, such as high NaCl, ionic homeostasis is disturbed. High levels of ionic molecules cause hyperosmotic stress and activate the synthesis of abscisic acid (ABA), which can then upregulate the vacuolar Na^+/H^+ exchanger, NHX (Shi and Zhu 2002). To maintain a high concentration of K^+ and a low concentration of Na^+ in the cytosol on induction of salt stress, plants exert a

transport system, including an ion exchanger and an ion sensor via Ca^{2+} signaling (Zhu 2003). So far, many reports have suggested the involvement of PAs in salt stress alleviation through comparisons of salt-tolerant and salt-sensitive cultivars exposed to the exogenous application of PA. However, reports on the relationship between PA and transporters were quite limited. Yamaguchi et al. (2006) reported that an Spm-deficient *Arabidopsis* mutant (*spms*) was sensitive to salt without affecting the transcriptions of salt-overly-sensitive (*SOS*), *NHX*, and high-affinity K^+ transporter (*HKT*), but calcium channel inhibitors could reduce the salt sensitivity of this mutant, demonstrating the importance of calcium signaling modulated by Spm. Instead, there were several reports of the antioxidant aspect of salt stress alleviation. It was shown that transgenic European pears, which had an overexpression of an apple *SPDS*, showed salt and osmotic stress alleviation (Wen et al. 2008). The induction of Spd concentration and antioxidant enzyme activities such as superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR) upon salt treatment resulted in salt stress alleviation (He et al. 2008). Conversely, antisense inhibition of an apple *SPDS* in European pears showed an increased sensitivity to salinity by the reduction of Spd concentration, as well as SOD and GR activities, which confirmed the crucial role of PAs in stress alleviation (Wen et al. 2011). In *SPDS*-overexpressing transgenic tomato plants, APX activity was significantly higher in transgenic plants compared to the wild type under saline conditions, which might contribute largely to the protection against oxidative stress generated by NaCl treatments, and thus confer salinity tolerance in the transgenic tomato plants (Neily et al. 2011). By contrast, a *DS* insertion mutant of *Arabidopsis ADC2* indicated a reduction of Put concentration by about 25 % and was more sensitive to salt stress than the wild type, but the sensitivity was recovered by the addition of Put. This result showed the importance of Put to the ADC pathway for salt stress sensitivity (Urano et al. 2004). Taken together, these results demonstrated the importance of PA for salt stress alleviation, although the key PA molecule may vary depending on the plant species or experimental design.

On the other hand, the regulatory signaling cascade in drought stress responses has been well clarified, and both ABA-dependent and ABA-independent regulatory systems are involved in this stress response (Shinozaki and Yamaguchi-Shinozaki 2000). As mentioned previously, salt stress may induce osmosis, and lead to ABA synthesis, consequently resulting in the ABA-dependent signaling cascade. The integration of transcriptomic and metabolomic data sets has revealed that drought-induced ABA-dependent transcriptional regulation had a pivotal role in PA biosynthesis (Fujita et al. 2011). There were several reports on the relationships between stomata dynamics and PAs, possibly through ABA signaling. Alcázar et al. (2006) reported that transgenic *Arabidopsis* lines that constitutively expressed *ADC2* showed different degrees of drought tolerance paralleled with Put concentrations, wherein these lines contain high levels of Put upon drought stress without obvious changes in Spd and Spm. Interestingly, there were no significant differences in the number of stomata between wild-type and transgenic plants, but a reduction in the transpiration rate and stomata conductance was observed in the transgenic lines.

Thus, a mechanism underlying drought tolerance is controlled by a reduction in water loss modulated by Put (Alcázar et al. 2006). PAs, including Spm, inhibited stomatal opening and induced the closure of *Vicia faba* guard cells (Liu et al. 2000). In tomato seedlings, exogenous application of Spd enhanced the photosynthetic rate (Zhang et al. 2010). It was also documented that ABA was at least partially responsible for the induction of PA accumulation and exodus into the apoplast. There, they were oxidized by the apoplastic amine oxidases, producing H_2O_2, which signaled secondary stress responses in grapevines (Toumi et al. 2010).

3.4 PA and Temperature Stress

Throughout the world, many crops are frequently exposed to severe high temperatures during their life cycle, resulting in a reduction in quality and quantity of biomass. To protect themselves from severe damage and survive high-temperature stress, plants adopt a set of responsive mechanisms characterized by the elevated synthesis of heat-shock proteins (HSPs). It was postulated that PAs might directly affect HSP production at the level of protein synthesis. HSP synthesis was detected up to 46 °C in the cells of the heat-tolerant tobacco BY2, although it ceased at 40 °C in cells of a heat-susceptible alfalfa line. Higher leakage of soluble PAs was observed from the alfalfa cells than the tobacco cells at high temperatures (Königshofer and Lechner 2002). The inhibition of Put biosynthesis in alfalfa cells reduced PA leakage at high temperatures, and thus improved HSP synthesis, indicating that PA might influence the extent of HSP synthesis under heat stress (Königshofer and Lechner 2002). Transgenic tomato plants overexpressing yeast *SAMDC*, which produce 1.7- and 2.4-fold-higher levels of Spd and Spm, respectively, compared with the wild type, became thermotolerant by remarkable enhancement of the antioxidant enzyme activities and protecting membrane lipid peroxidation (Cheng et al. 2009). Recently, it was reported that Spm concentration positively correlated with the levels of heat stress tolerance accompanying an increase in the genes encoding HSPs and heat-shock transcription factors using transgenic *Arabidopsis* plants overexpressing *SPMS* and Spm-deficient *Arabidopsis* mutants, *spms*. Thus, Spm increases the heat-shock (HS) response at transcriptional and translational levels and protects host plants from HS-induced damage (Sagor et al. 2013).

On the other hand, low temperature is also one of the most severe environmental stresses, which inhibits the growth and distribution of plants. Several scientists have reviewed the involvement of PAs in low-temperature stress (Theocharis et al. 2012). In general, differential accumulation of PAs during low-temperature stress in a number of plant species has been reported and seems to have potential for counteracting chill-induced injuries (Alcázar et al. 2011). Shen et al. (2000) reported that Spd prevented the chill-induced increases in H_2O_2 content in cucumber leaves, as well as activities of NADPH oxidases and NADPH-dependent superoxide generation in microsomes; thereby conferring chilling tolerance in cucumbers. It was also shown that exogenous application of Spd in cucumbers improved the chilling

tolerance of the photosynthetic apparatus (He et al. 2002), with increases in SAMDC activity in the tolerant cultivar. The *SPDS* from *Cucurbita ficifolia* was introduced to *Arabidopsis*, and the transgenic lines exhibited a significant increase in SPDS activity and Spd content in leaves, together with enhanced tolerance to various stresses, including chilling and freezing, compared with the wild type (Kasukabe et al. 2004). Recently, transcriptome analysis suggested a positive feedback regulatory mechanism between ABA and Put, clearly suggesting that Put modulates ABA biosynthesis at the transcriptional level in response to low temperatures, thus uncovering a novel role for PA as a regulator of hormone biosynthesis (Cuevas et al. 2009).

3.5 PA and Heavy Metal Stress

Heavy metals, which are one of the major environmental pollutants, can cause serious problems for all organisms when present in the atmosphere, soil, and water, even in trace concentrations. Plants develop systems for metal tolerance, such as cell wall binding, chelation with phytochelatin, and compartmentalization (Zhang and Shu 2006). However, overaccumulation of heavy metals causes the generation of reactive oxygen species, which modifies the antioxidant defense and balance of redox status (Sharma and Dietz 2008), and elicits oxidative stress. Groppa et al. (2001, 2003) observed that Spm or Spd completely recovered the activity of GR, which had been impaired by copper (Cu) or cadmium (Cd) stress. Our previous work also revealed that Spd levels were implicated in the alleviation of heavy metal stresses such as Cd, lead (Pb), zinc (Zn), or a combination thereof in an *MdSPDS1-overexpressing* transgenic European pear by exerting SOD and GR activities (Wen et al. 2010). Thus, enhancement of antioxidant enzyme activities could be at least one reason for the mitigation of heavy metal stress. Indeed, overexpression of SOD and APX mitigated heavy metal stress, including Cu, Cd, and arsenic, in tall fescue transgenic plants (Lee et al. 2007). By contrast, Choudhary et al. (2012) provided the effects of exogenously applied brassinosteroids and PAs on radish plants exposed to toxic concentrations of Cu. The combined application of 24-epibrassinolide and Spd modulated the expression of genes encoding PA enzymes and genes that impact the metabolism of indole-3-acetic acid and ABA, resulting in enhanced tolerance to Cu stress.

In addition, several cases concerning the mitigation of other metal stress such as nickel (Ni) and aluminum (Al) have been also documented. Shevyakova et al. (2011) reported that *Amaranthas* leaves treated with Put or Spd did not show any signs of injury despite an increase in the amount of Ni; thus, they assumed that PAs manifested their protective action as Ni chelators and detoxicants. Wen et al. (2009) revealed that the activities of SOD and GR and the accumulation of proline or malondialdehyde acted upon Al stress, resulting in the *SPDS*-overexpressing European pear transgenic line toward a more favorable survival status than the wild type.

3.6 PAs and Other Molecules Related to Abiotic Stress

As mentioned previously, increased ABA concentrations brought on by abiotic stress could trigger the expression of multiple abiotic stress-adaptive genes in plants, possibly including PA biosynthesis genes (Hussain et al. 2011). PA catabolism produces H_2O_2 (Fig. 3.1), which has often been considered a toxic molecule. Recently, there has been a surge of reports highlighting the nature of H_2O_2 as a signal molecule, capable of diffusing into neighboring cells and tissues from the site of its production, and activating responses against stresses (Rhee 2006). Tun et al. (2006) reported on PA-induced nitric oxide (NO) biosynthesis in *Arabidopsis*. Subsequently, Palavan-Unsal and Arisan (2009) further confirmed that in addition to H_2O_2, PAs induced the production of NO in various tissues in seedlings of *Arabidopsis*. Wang et al. (2012) have recently elucidated the involvement of Put and NO in Al tolerance by modulating citrate secretion from the roots of red kidney beans. The evidence obtained so far strongly indicates the involvement and interaction of PA, ABA, H_2O_2, and NO during stress responses to be a complex network. Additionally, the interplay among PAs, ABA, H_2O_2, and NO in plant stomatal regulation is worth mentioning (Yamasaki and Cohen 2006). Cona et al. (2006) reported that the generation of H_2O_2 by amino oxidases was tightly linked to PA catabolism and was associated with plant abiotic stress responses. The regulation of stomatal movements in response to ABA was documented to be dependent on both H_2O_2 and NO, in that NO generation depends on H_2O_2 production (Paschalidis et al. 2010). Therefore, PAs may regulate stomatal closure by being directly involved in the biosynthesis of signaling molecules such as ABA, H_2O_2, and NO. Conclusively, plant responses to unfavorable environmental conditions, and the crosstalk between these signaling molecules, have a vital role in its defense against harsh environments (Bitrián et al. 2012).

3.7 Perspectives

During the past decade, analyses of metabolic adjustments of plants with different stress tolerances and transgenic research have provided important evidence for better understanding the role of PAs in response to abiotic environments; thus, our knowledge of the involvement of PA on tolerance to environmental stresses has increased considerably. Overexpressing PA biosynthetic genes confers enhanced abiotic stress tolerance in many transgenic plants. It is worthy to note that transgenic plants overexpressing one PA biosynthetic gene showed tolerance to a wide range of abiotic stresses such as salt, osmosis, cold, freezing, and even heavy metals (Kasukabe et al. 2004; Wen et al. 2008). It was apparent that wild-type European pear showed some wilting of leaves and necrosis of apical shoots even after a short period of stress treatment, although these phenotypic features were alleviated in the transgenic line (Fig. 3.2). One of the important PA functions could be the alleviation

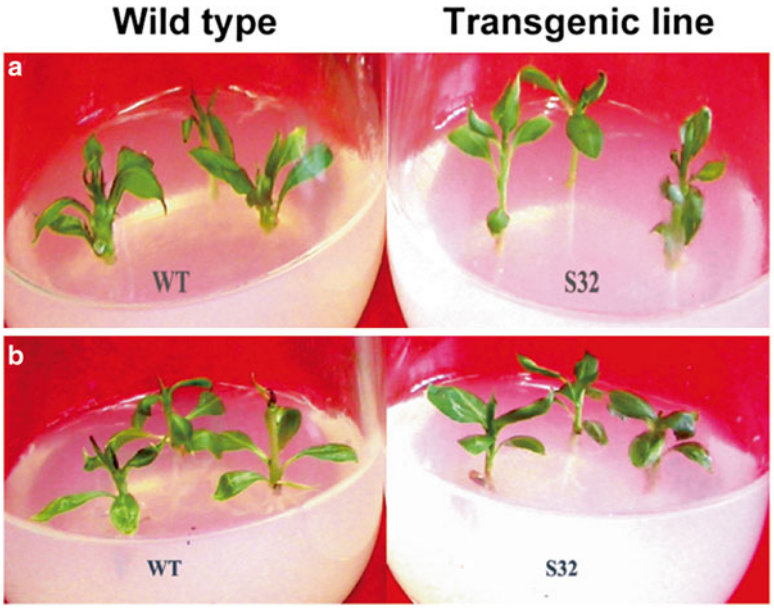

Fig. 3.2 Morphological features of the wild type (*WT*) and transgenic line after 10 days of mannitol (300 mM) treatment (**a**) and after 15 days of $CuSO_4$ (500 **μ**M) treatment (**b**). (Modified from Wen et al. 2008)

of oxidative stress through the enhancing of antioxidant molecules and antioxidant enzyme activities, possibly protecting cell membranes, proteins, and DNA/RNA from degradation, because all abiotic stresses eventually cause oxidative stress. At present, the detailed molecular mechanism of PA in these processes remains enigmatic, but intensive research using modern biological technologies, such as genetics, molecular biology, proteomics, and metabolomics, could facilitate the clarification of detailed PA functions in plant development, including abiotic stress response.

Horticultural crops are of high importance among the agronomic crops. Unfortunately, information regarding the involvement of PAs in the abiotic stress tolerance of horticultural crops, as well as improvements in survival rates in harsh environments via transformation approaches with PA biosynthetic genes, have thus far been very limited. Abiotic stress tolerance is especially important with respect to fruit trees, which cannot be easily rotated as can annual crops to avoid stress, because they are generally exposed to environmental stress over several years, once planted. In addition to abiotic stress, damage to plants and loss of crop production caused by biotic stresses such as plant disease and insects should be also considered. Interestingly, overexpression of an apple *SPDS* in a sweet orange reduced canker susceptibility (Fig. 3.3), indicating the possibility for both abiotic and biotic stress alleviation with PAs. Thus, the use of PA should be explored for stable and sustainable production of horticultural crops toward global warming conditions.

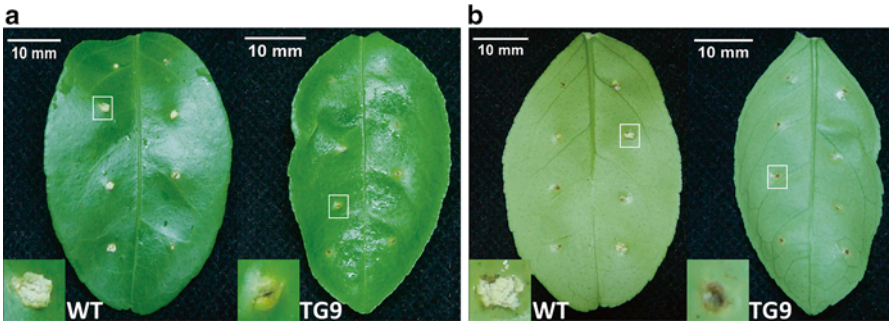

Fig. 3.3 Canker disease tolerance of the wild type (*WT*) and the transgenic line (*TG9*). Photographs show symptoms on the adaxial (**a**) and abaxial (**b**) sides of the leaves from WT and TG9. Selected inoculation sites of the leaves were zoomed in and shown below the corresponding photographs. (Photographs kindly provided by Drs. Xing-Zheng Fu and Ji-Hong Liu)

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Chapter 4
Effect of Temperature on Fruit Color Development

Yoshiko Koshita

Abstract Temperature is one of the most important factors affecting fruit coloration. Because of recent effects of global warming, inferior coloration has become a serious problem in many fruits, especially those for which coloration is important for the commercial appearance and value, such as grapes, apples, citrus fruits, and persimmons. Coloration in these fruit results from the synthesis and accumulation of pigments. The presence of anthocyanins or carotenoids is responsible for the color of fruit peels. The biosynthetic pathways of anthocyanins and carotenoids in these fruit have been characterized, and these studies have provided valuable information for understanding the occurrence of poor coloration in response to high temperature. In general, peel color development is enhanced by low temperatures and inhibited by high temperatures. Several techniques have been described to improve the peel color of fruit, and recent studies have identified the mechanisms underlying the effectiveness of these techniques.

Keywords Anthocyanin • Apple • Carotenoid • Citrus fruit • Global warming • Grape • Persimmon • Temperature

4.1 Introduction

Fruit development is influenced by many environmental conditions and the availability of nutrients. Although nutritional or soil conditions can be controlled by the application of fertilizers and soil management, environmental conditions, such as temperature, solar radiation, and humidity, are more difficult to control.

Recently, global warming has become a worldwide problem. For fruit cultivation, the ambient temperature during fruit development is an important factor that affects fruit quality. High temperatures during fruit development suppress fruit color development. For example, poor coloration caused by high temperature has been

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reported for grapes (Tomana et al. 1979; Naito et al. 1986), apples (Creasy 1968; Yamada et al. 1988; Arakawa 1991), Satsuma mandarin (Utsunomiya et al. 1982), and Japanese persimmon (Taira et al. 2000; Isobe and Kamada 2001). Efforts to reduce greenhouse gas levels in the atmosphere are underway; however, until such efforts are successful, fruit production must occur under undesirable conditions.

Poor fruit coloration often reduces the market price. Therefore, cultivation techniques that overcome high temperature-mediated color suppression are valuable to commercial producers. To facilitate the development of such cultivation methods, the relationship between temperature and color development, the molecular basis of pigment synthesis, and the mechanisms of color suppression at high temperature must be elucidated.

The aim of this chapter is to summarize the effect of high temperature on fruit color development and the cultivation technique to overcome poor coloration.

4.2 Effect of Temperature on the Fruit Coloration Process

Temperature affects fruit color development. For fruit production, most trees are grown in open fields, although some are cultivated under structures. Therefore, it is difficult to control atmospheric conditions such as temperature, solar radiation, or humidity. High temperatures cause poor fruit coloration in many types of fruit trees.

Fruit skin color is determined by secondary metabolites, such as phenolic compounds or terpenoids. For example, anthocyanin, a phenolic compound, is responsible for the pigmentation of apple and grape peels (Tsao et al. 2003; Shiraishi et al. 2007). Terpenoid compounds, including carotenoids, are present in the peel of the citrus fruits (Daito et al. 1975), persimmon (Veberic et al. 2010), and loquat fruit (Zhou et al. 2007). The quantities of anthocyanin or carotenoids present in fruit peels are determined by their biosynthesis, accumulation, and degradation. Temperature influences these processes, and high temperature can result in poor color development in certain types of fruit trees, particularly grape, apple, citrus, and persimmon.

4.2.1 Relationship Between Temperature and Grape Coloration and the Mechanism Underlying the Effects of Temperature

The grape is one of the most common fruit trees for which color development is suppressed by high temperature. Of all fruit under cultivation, grape production is the highest in the world, and the fruit are used for a variety of purposes, including wine production, table fruits, and for consumption of dry and fresh fruit. Poor coloration of the skin causes prices to decline, particularly with respect to sales for wine production and table fruit. Therefore, several investigations have explored the

Table 4.1 Research about temperature response of grape coloration under controlled temperature

Cultivar	Treated organ	Reference
Cardinal, Pinot Noir	Whole vine	Kliewer (1970)
Cardinal, Pinot Noir, Tokay Cabernet Sauvignon	Whole vine	Kliewer and Torres (1972)
Kyoho	Clusters, whole vine	Tomana et al. (1979)
Kyoho	Whole vine	Mori et al. (2004a)
Kokuo	Whole vine	Mori et al. (2004b)
Darkridge	Whole vine	Mori et al. (2005)
Aki Queen	Whole vine	Yamane et al. (2006), Yamane and Shibayama (2006b)
Pino Noir	Whole vine	Mori et al. (2007b)
Aki Queen	Clusters	Koshita et al. (2007)

relationship between temperature and anthocyanin content in grapes; further, the coloration response to temperature under controlled conditions has been the focus of intense research (Table 4.1). Kliewer (1970) and Kliewer and Torres (1972) first examined the relationship between temperature and anthocyanin content. Kliewer (1970) demonstrated that the anthocyanin concentration of the Cardinal and Pinot Noir varieties grown at an ambient temperature (68 °F) was significantly higher than in grapes grown at 86 °F. They also investigated the difference in grape tolerance to elevated temperature (Kliewer and Torres 1972). Tomana et al. (1979) separately controlled the ambient temperature around grape clusters and the whole vine, showing that low temperature around clusters enhanced anthocyanin content in the "Kyoho" grape. Mori et al. (2004a, b, 2005, 2007a, b) investigated the relationship between temperature and anthocyanin content in many cultivars. They reported that high temperatures at night decreased anthocyanin content in "Kyoho" (Mori et al. 2004a), "Kokuo" (Mori et al. 2004b), and "Darkridge" (Mori et al. 2005) grapes, as determined using a phytotron, on whole vines.

In "Kyoho" and "Aki Queen" grapes, the effect of the temperature surrounding clusters from early maturation to the harvest stage was investigated. These studies demonstrated that high temperatures around clusters inhibit anthocyanin accumulation, whereas low temperatures accelerate anthocyanin accumulation (Tomana et al. 1979; Koshita et al. 2007).

The effect of temperature on grape coloration has been demonstrated in many reports, and these investigations have led to research on the regulatory mechanisms of anthocyanin biosynthesis in grape skin under different temperature conditions.

Coombe (1973) reported that abscisic acid (ABA) and sugar content increased concomitant with grape maturation, and they speculated that ABA participates in anthocyanin biosynthesis. Studies on the relationship between ABA content and coloration (Tomana et al. 1979; Yamane et al. 2006; Koshita et al. 2007) revealed that anthocyanin content and ABA content are higher in the skin when these cultivars are grown at lower temperatures compared with those grown at higher temperatures. ABA is an important phytohormone for regulating anthocyanin

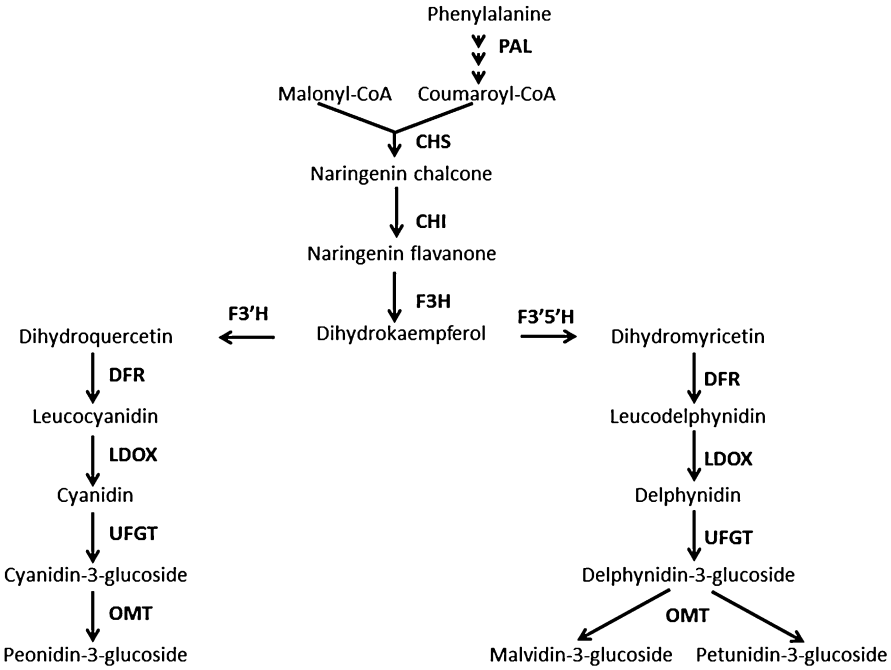

Fig. 4.1 Scheme of the anthocyanin biosynthetic pathway in grape skins. PAL phenylalanine ammonia lyase, CHS chalcone synthase, CHI chalcone isomerase, F3H flavanone 3-hydroxylase, F3′H flavonoid 3′-hydroxylase, F3′5′H flavonoid 3′-5′-hydroxylase, DFR dihydroflavonol 4-reductase, LDOX leucoanthocyanidin dioxygenase, UFGT UDP-glucose flavonoid 3-*O*-glucosyltransferase, OMT *O*-methyltransferase

accumulation in the grape, and application of ABA treatments to grape clusters improves coloration (Matsushima et al. 1989). Sparvoli et al. (1994) and Boss et al. (1996) isolated grape genes related to the anthocyanin biosynthetic pathway, and they reported that UDP-glucose flavonoid 3-*O*-glucosyltransferase (UFGT) is a key enzyme for anthocyanin biosynthesis in the grape skin (Fig. 4.1). Transcriptional regulators of anthocyanin biosynthesis contain a MYB domain, a basic helix–loop–helix (bHLH) domain, or WD40 repeats, and these protein complexes determine the expression of the anthocyanin biosynthesis genes (Ramsay and Glover 2005). In grapes, *Myb*-related genes control the anthocyanin biosynthetic pathway (Kobayashi et al. 2005), (Kobayashi et al. 2002). Therefore, expression of the *Myb*-related genes and anthocyanin biosynthesis-related genes regulates anthocyanin content, and these pathways may have an important role in grape coloration. The expression of these genes is closely related to anthocyanin content. Mori et al. (2004b, 2005) demonstrated the importance of anthocyanin biosynthesis-related gene expression and enzyme activity, showing that activity was higher in cultivars grown under low night temperature conditions compared to those grown under high night temperatures. Tomana et al. (1979) and Koshita et al. (2007) reported that low temperatures around clusters increased both anthocyanin levels and ABA content.

Thus, ABA content is higher in the skin of grapes grown under lower temperature conditions, resulting in better coloration. Exogenously applied ABA also activates the expression of the anthocyanin biosynthetic genes and enhances grape coloring (Ban et al. 2003; Jeong et al. 2004). Therefore, ABA also is important in determining grape coloration.

4.2.2 Temperature During Maturation Affects Citrus Peel Coloration

The peel color of the yellow- or orange-colored citrus fruit primarily consists of carotenoids. In general, high ambient temperature during the citrus maturation period causes poor coloration of the citrus peel. The peel color of the "Redblush" grapefruit was a deeper hue of yellow when trees were exposed to cooler temperatures during development (Young et al. 1969). In Satsuma mandarin fruits, lower ambient temperatures during fruit development resulted in earlier accumulation of carotenoids (Utsunomiya et al. 1982). Manera et al. (2012) demonstrated that the autumnal temperature decline was associated with degreening of lemon peel. Sugiura et al. (2007) suggested that global warming may delay the onset of coloration or cause poor coloration of the Satsuma mandarin peel. The carotenoid biosynthetic pathway in citrus is well characterized (Ikoma et al. 2001; Kato et al. 2004, 2006) (Fig. 4.2). Ikoma et al. (2001) demonstrated that phytoene synthase has an important role in carotenoid accumulation in citrus fruits. Kato et al. (2004) showed that changes in the peel color from green to orange were accompanied by a decline in lycopene ε-cyclase and an increase in lycopene β-cyclase. Utsunomiya et al. (1982) quantified ABA content in the peels of Satsuma mandarin fruit grown at temperatures of 15 °C, 23 °C, and 30 °C; they observed higher ABA content and better coloration in the peels of fruit grown at the lowest temperature. These data suggest that ABA is involved in carotenoid synthesis in Satsuma mandarin fruits.

4.2.3 Relationship Between Temperature and Apple Coloration

The red skin color of an apple is the result of anthocyanin and many environmental factors, including light, temperature, and soil nutrition (reviewed by Saure 1990). The skin color of the apple fruit is an important factor for determining market prices, and it has been suggested that high temperature inhibits coloration. Yamada et al. (1988) controlled the ambient temperature around fruit during its development, and they demonstrated that the anthocyanin content of the skin was higher at 10 °C than at 17 °C in the "Fuji" apple; furthermore, anthocyanin accumulation was inhibited at 24 °C. Arakawa (1991) investigated the relationship between temperature and anthocyanin accumulation in several apple varieties, including "Jonathan," "Fuji,"

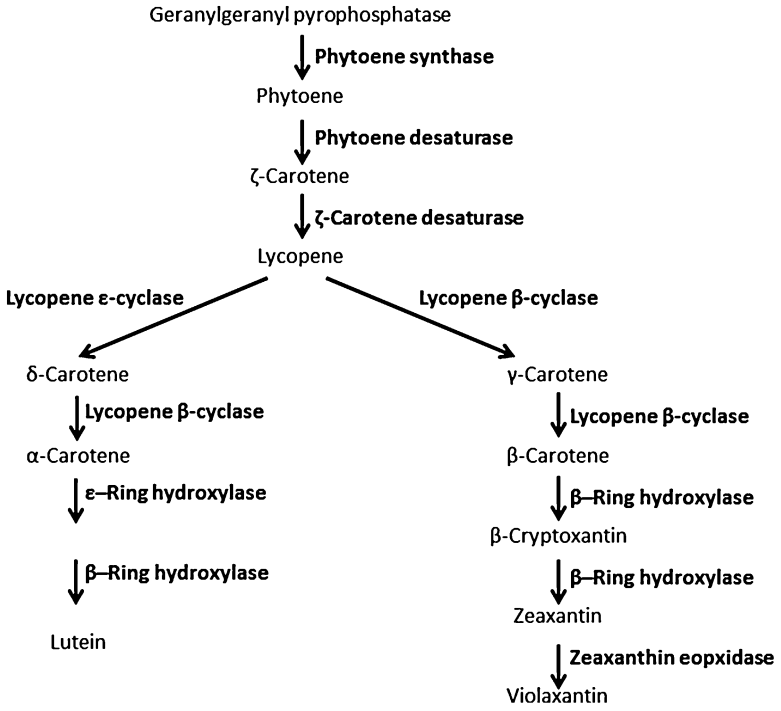

Fig. 4.2 Scheme of the main carotenoid biosynthetic pathway in the peels of citrus and persimmon

"Jonagold," and "Tsugaru" and reported the coloring response to temperature varied among cultivars. In apple skin, anthocyanin biosynthetic genes are responsible for the synthesis of anthocyanin, and the predominant anthocyanin in the apple skins is cyanidin-3-galactoside (Kondo et al. 2002). The expression of anthocyanin biosynthetic genes during red color development in apples was first reported by Honda et al. (2002). They isolated genes related to anthocyanin biosynthesis, including chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UFGT (Fig. 4.3), and demonstrated that these genes are expressed coordinately during fruit development; there is a positive correlation between anthocyanin content and the expression of these genes. Subsequently, the effects of temperature on the expression of anthocyanin biosynthetic genes were investigated. Ubi et al. (2006) reported that low-temperature treatment during exposure to UV-B light enhanced both the expression of anthocyanin biosynthetic genes and the anthocyanin content of apple skin. Ban et al. (2007) suggested that the MYB transcription factor gene, *MdMYBA*, is a key regulatory gene in anthocyanin biosynthesis in apples, and they also proposed the importance of the bHLH transcription factor gene. Indeed, increased expression of the MdbHLH3 was confirmed at 17 °C, whereas lower transcription was detected under 27 °C (Xie et al. 2012).

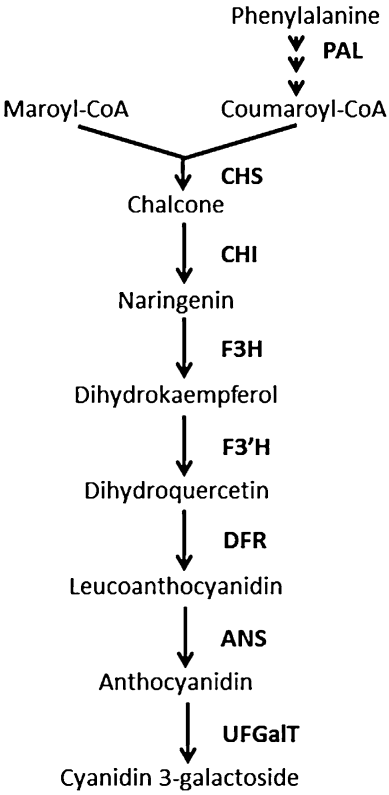

Fig. 4.3 Scheme of the main anthocyanin biosynthetic pathway in apple skins. Abbreviations of the pathway (CHS, CHI, F3H, F3′H, DFR) are defined in the legend of Fig. 4.1. ANS anthocyanin synthase, UFGalT UDP galactose flavonoid 3-*O*-galactosyltransferase

4.2.4 Effect of High Temperatures in Autumn on the Coloration of the Japanese Persimmon

Previous reports documented a delay in the harvest season in the Japanese persimmon. Taira and Itamura (1989) noted that the timing and rate of peel coloration in the "Hiratanenashi" persimmon varied considerably on a yearly basis, and they suggested that this variation might be caused by temperature changes during color development. In the "Maekawa Jiro" persimmon, higher temperatures during the day or night were associated with slower rates of peel coloration during fruit development (Isobe and Kamada 2001). Sugiura et al. (2007) classified several fruit trees according to the response of fruit development to climate change, and the Japanese persimmon was categorized as having an accelerated flowering period but not an accelerated maturation period. Niikawa et al. (2014) investigated the relationship between coloration of the "Fuyu" persimmon at harvest time and the monthly mean temperature during the past 20 years. Their data suggested that skin coloration is influenced by temperature during the months of September and October. The peel color of the persimmon is derived from carotenoids (Veberic et al. 2010), and the

red coloration is related to the lycopene content of the peel (Chujo 1982). Chujo and Ashizawa (1973a) investigated the relationship between temperature and the reddish color of the peel. They demonstrated that lycopene content in the peel of the detached "Fuyu" persimmon fruit was highest when temperatures were 25 °C during mid- to late October, and 15 °C from beginning to middle of November. They also compared the development of "Fuyu" persimmon peel color to the reddish-colored cultivars, "Beniemon" and "Nigorokonariba," and they found that a gradual decrease in temperature was favorable for red color development as the fruit matured (Chujo and Ashizawa 1973b).

The biosynthetic pathway of carotenoids in the Japanese persimmon has been described, and the processes involved in carotenoid accumulation have been characterized (Fig. 4.2) through carotenoid biosynthetic gene expression and the quantification of carotenoids during maturation (Niikawa et al. 2007). Additional information obtained through molecular studies and gene expression experiments will be available in the future.

4.3 Techniques for Overcoming Poor Coloration Caused by High Temperature

Several cultivation techniques to overcome poor fruit coloration have been proposed. If these techniques are effective at high temperature, it will be possible to produce fruits with good coloration under warm conditions. In grapes, girdling treatment (Peacock et al. 1977; Yamamoto et al. 1992), low crop load (Kitamura et al. 2005), and water stress (Fukui et al. 2004) enhance coloring. Of these, the application of girdling treatment to the trunk is a popular technique for enhancing grape coloration. To make this approach feasible, many investigators have sought to identify the most effective treatment and the appropriate growth stage for its application to enhance color development. Yamamoto et al. (1992) investigated the application of girdling treatment during different growth stages and determined that girdling treatment applied 30 days after full bloom was most effective for color development in the "Kyoho" grape. Yamane and Shibayama (2007) determined that the application of girdling treatment 30 and 35 days after full bloom was most effective for enhancing anthocyanin accumulation in "Aki Queen" grapes. They suggested the concentration of total soluble solids present in the berry 30 days after full bloom is critical for anthocyanin accumulation. The effect of girdling treatment is also influenced by crop load, as higher crop loads reduced the effectiveness of girdling (Yamamoto et al. 1992; Yamane and Shibayama 2006a). These investigations indicate that determining the appropriate crop load and girdling stage are important for enhancing fruit color using this approach.

Grape cultivation under restricted rooting volume has the potential to produce better quality fruit. Imai et al. (1991) established an effective watering program for grapevines that are grown with restricted rooting volume. Fukui et al. (2004)

investigated the relationship between water-stressed and non-water-stressed conditions: the color index value was higher for grapes grown under water-stressed conditions than those grown under non-water-stressed conditions. Because of global warming, the production of grapes with good coloration will be challenging, even in areas where grapes have historically been produced. However, the integration of girdling treatment and the appropriate management of crop load and water stress may enable the production of grapes with good coloration, even in areas affected by global warming.

Apple coloration is enhanced by light (Arakawa et al. 1985; Ubi et al. 2006); therefore, improving light conditions by pruning or training is essential. In addition to these fundamental management techniques, leaf thinning, use of reflective mulch, and application of paper bags are often used to produce red-colored fruit. These strategies are performed to enhance the exposure of apple fruit to light. Soil nitrogen level is another important factor influencing apple coloration, although the effects are indirect. In "Golden Delicious" apples, the application of high levels of nitrogen increased the production of green-colored fruit (Williams and Billingsley 1974). Komamura et al. (2000) reported that excessive nitrogen application inhibited apple skin coloration and did not detect a relationship between fruit yield and nitrogen levels.

The peel color of citrus fruit is enhanced by water stress and nitrogen content. In navel oranges, peel coloration is improved in the fruits that received less water than fruits that were fully watered by an irrigation system (Kallsen et al. 2011). Tachibana and Yahata (1998) showed that the color indices of the "L," "a," and "b" decreased as nitrogen increased, and they also reported that lower nitrogen content in leaves was associated with better fruit quality. Takagi et al. (1989) suggested that reduced nitrogen levels in the peel improved the development of Satsuma mandarin peel color. Therefore, the nitrogen content of fruit peels is an important indicator of the nutritional condition of citrus fruits.

Fruit breeding programs have aided in the development of several new cultivars with enhanced color. The introduction of these cultivars into the current cultivation system will be beneficial for fruit producers.

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Chapter 5
Polyol Metabolism and Stress Tolerance in Horticultural Plants

Yasuo Suzuki

Abstract Various horticultural plants synthesize polyols as major products of photosynthesis in addition to sucrose and starch and use polyols and sucrose as translocated sugars. The parallel presence of two translocated sugars and their metabolic pathway is specific and complicates the comprehension of their roles in physiology and response to stress. This review first describes the metabolism of sorbitol, focusing on sorbitol-specific metabolizing proteins and their physiological roles in Rosaceae fruit trees. In addition, research on sorbitol as a signal molecule and sorbitol-metabolizing proteins regulated by sugar is discussed. A series of studies regarding various Rosaceae fruit trees has revealed the relationship of sorbitol accumulation with abiotic stresses, including drought, salt, cold, and micronutrient deficiency stresses. On the basis of acknowledging the metabolism of sorbitol, the biochemical mechanism of sorbitol accumulation in response to abiotic stress has been investigated. Furthermore, recent molecular analyses are providing direct evidence of the correlation of the proteins.

Keywords Abiotic stress • Compatible solute • Polyol • Rosaceae • Sorbitol • Sugar signaling

5.1 Introduction

Typically, most plant species synthesize sucrose and starch as major products of photosynthesis and use sucrose as a translocated sugar. However, various horticultural plants synthesize polyols as the major products in addition to sucrose and starch and use polyol and sucrose as translocated sugars. For examples of such polyols, sorbitol is synthesized and transported in the Rosaceae, mannitol in the Apiaceae, Combretaceae, Oleaceae, and Rubiaceae, and galactitol in the Celastraceae (Loescher and Everard 1996; Zimmermann and Ziegler 1975). The parallel presence of two translocated sugars and their metabolic pathway complicates the

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comprehension of their roles in physiology and response to stress and regulation of the metabolism. Research on clarification must advance because the plants are economically important. This review focuses on a series of studies on the metabolism of sorbitol in Rosaceae fruit trees and its role in abiotic stress tolerance.

5.2 Metabolism of Sorbitol

Sorbitol is a major product of photosynthesis and a primary translocated sugar in Rosaceae fruit trees. Sorbitol accounts for more than 80 % of newly fixed carbohydrates during the light period in apples (Wang et al. 1997). It also accounts for 60 % to 90 % of the carbon exported from source leaves (Loescher 1987), and its concentration in phloem sap reaches approximately 560 mM, whereas that of sucrose is about 140 mM (Moing et al. 1997). A sorbitol-specific metabolism is mainly the result of four proteins, including sorbitol-6-phosphate dehydrogenase (S6PDH), sorbitol-6-phosphate phosphatase (S6PP), sorbitol transporter (SOT), and sorbitol dehydrogenase (SDH). Briefly, sorbitol is synthesized by S6PDH and S6PP in source leaves, translocated through phloem, and catabolized by SDH in fruit. SOT functions in the translocation of sorbitol across the plasma membrane in these processes. Recently, apple genomic information of these proteins has been shown; it has been reported that there are 16 *S6PDH* or *S6PDH-like* genes, 17 *SDH* or *SDH-like* genes, and 38 *SOT* or *SOT-like* genes (Velasco et al. 2010). Also, genomic information is available for the peach; manual annotation has identified two *S6PDH*, seven *SDH*, and ten *SOT* (International Peach Genome Initiative 2013). Genomic information shows that those of apple and peach comprise large gene families, suggesting the importance of the specific sorbitol metabolism in Rosaceae fruit trees.

5.2.1 Sorbitol-Metabolizing Proteins

5.2.1.1 NADP-Sorbitol-6-Phosphate Dehydrogenase (S6PDH) (EC 1.1.1.200)

S6PDH is a key enzyme in the process of synthesizing sorbitol in source organs (Hirai 1981; Loescher et al. 1982; Yamaki and Ishikawa 1986). It synthesizes sorbitol-6-phosphate (S6P) via reduction of glucose-6-phosphate (G6P), which is also a precursor of sucrose as another photosynthetic product. S6PDH has been purified and characterized from mature leaves of loquat and apple seedlings (Hirai 1981; Kanayama and Yamaki 1993). S6PDH activity is in both directions, reduction of G6P and oxidation of S6P (Kanayama and Yamaki 1993). The maximum velocity of the former is much higher than that of the latter, and the oxidation of S6P proceeds very slowly at a neutral pH. These biochemical results support the belief that S6PDH has a major function in the biosynthesis of sorbitol.

The cDNA encoding *S6PDH* is first cloned from apple seedlings (Kanayama et al. 1992). It has been shown that in peach and pear the level of *S6PDH* mRNA is higher in source mature leaves than in sink young leaves, concomitant with the alteration of enzyme activity and protein level (Sakanishi et al. 1998; Suzue et al. 2006). Tobacco, which does not have the potential to produce sorbitol, when transformed with apple cDNA encoding *S6PDH* synthesizes sorbitol (Tao et al. 1995), and, in transgenic apple plants silenced for *S6PDH*, the sorbitol content in leaves drastically decreases (Kanamaru et al. 2004; Teo et al. 2006). These results confirm that S6PDH is a key enzyme in the sorbitol biosynthetic pathway in Rosaceae fruit trees.

Subcellular localization of S6PDH was revealed in apple cotyledons using differential centrifugation and linear sucrose density gradient centrifugation; it is localized in both cytosol and chloroplast (Yamaki 1981). Immunogold electron microscopy analysis confirms the localization of S6PDH in apple mature leaves (Liang et al. 2012). These results are consistent with S6PDH localized in source organs, including mature leaves, primarily being synthesizers of sorbitol as a major product of photosynthesis.

The roles of S6PDH in apple trees and fruit have definitely been revealed using apple transformed with *S6PDH* (Teo et al. 2006). *S6PDH*-suppressed apples show a decrease in the vegetative growth and acid content of fruit and an increase in the total soluble solid content of fruit, whereas *S6PDH* overexpression shows the opposite result. Thus, S6PDH is a critical enzyme for determining the vegetative growth and fruit quality through the degree of sorbitol synthesis.

5.2.1.2 Sorbitol-6-Phosphate Phosphatase (S6PP) (EC 3.1.3.50)

S6PP synthesizes sorbitol via dephosphorylation of S6P, which is the final step in synthesizing sorbitol. Although Grant and ap Rees (1981) first suggested the presence of S6PP in leaves of apple seedlings, it had remained undetermined. The necessity of the enzyme for synthesizing sorbitol was questionable because there are nonspecific phosphatases in cells and it could dephosphorylate S6P to sorbitol. Actually, transgenic plants, which do not have the potential to produce sorbitol, introduced for the *S6PDH* gene, including tobacco and persimmon, can synthesize sorbitol without the *S6PP* gene (Gao et al. 2001; Sheveleva et al. 1998; Tao et al. 1995). Zhou et al. (2003) purified and characterized S6PP from mature apple leaves, which confirms the presence and the necessity of S6PP. S6PP is highly specific for S6P and is regulated by sorbitol through negative feedback inhibition.

5.2.1.3 Sorbitol Transporter (SOT)

A sugar transporter is necessary for the functional transport of sugars across membranes. SOT has important roles in unloading sorbitol in sink organs, including fruit, young leaves, and flowers (Gao et al. 2003, 2005). cDNAs of *SOT* were

first isolated from sour cherry fruit: *PcSOT1* and *PcSOT2*. Using heterologous expression of the genes in yeast, it has been proved that sorbitol transporters actually act as plasma membrane sorbitol/H^+ symporters. The expression of *PcSOT1* during the development of fruit is high when the growth and sugar accumulation rates of fruit are high, suggesting the sorbitol transporter is important in sugar accumulation and sink strength. On the other hand, *PcSOT2* is mainly expressed only early in fruit development, suggesting that isogenes seem to all have their own role in the development of fruit such as apples (Li et al. 2012c; Teo et al. 2006). In addition, the genes are also expressed in young leaves and are low in mature leaves. These facts show that SOT works in sink organs (Gao et al. 2003). SOT is also suggested to be related to the occurrence of a watercore in apples. Expressions of *MdSOT1* and *MdSOT2* are typically high in sink tissues but low in watercore-affected fruit tissues. Decreased ability to transport sorbitol into fruit parenchyma tissues because of the decreased expression would result in sorbitol accumulation in the intercellular space and occurrence of watercore (Gao et al. 2005). In apple source leaves, the expression of *MdSOT3*, *MdSOT4*, and *MdSOT5* has been identified, suggesting that these MdSOTs have different functions (Watari et al. 2004).

5.2.1.4 NAD-Sorbitol Dehydrogenase (SDH) (EC 1.1.1.14)

SDH is a key enzyme of sorbitol catabolism in sink organs, including the fruit and immature leaves, which converts sorbitol to fructose (Loescher et al. 1982; Yamaki and Ishikawa 1986; Yamaki and Moriguchi 1989). It has been shown that the activity of SDH is positively correlated with sink strength of fruit throughout the development of peaches (Lo Bianco and Rieger 2002). SDH has been purified to homogeneity and characterized from Japanese pear (Oura et al. 2000). The K_m values for sorbitol are much lower than for fructose. This biochemical result supports the belief that SDH favors the conversion of sorbitol to fructose.

cDNA-encoding *SDH* has been cloned from fruit of Rosaceae fruit trees, including loquats, peaches, pears, plums, and apples (Bantog et al. 2000; Guo et al. 2012; Yamada et al. 1998, 2001, 2006). Expression analyses of *SDH* during fruit development show that the activity is regulated at the transcriptional level because the gene expression pattern corresponds to the SDH activity and confirms that SDH is important in fruit maturation and sugar accumulation (Bantog et al. 2000; Yamada et al. 2001, 2006). SDH is important in fruit set and early fruit development as well as maturation in apples (Nosarzewski et al. 2004). In those cases, SDH is expressed not only in the cortex but also in the seeds and is derived from *SDH* genes, which are differentially expressed in seeds and the cortex (Nosarzewski and Archbold 2007). In other sink organs, young leaves of pear, the expression of the *SDH* mRNA level is not coincident with the activity, although the activity is high (Suzue et al. 2006).

In source leaves of apples, expression of the *SDH* isogene, *MdSDH1*, has also been detected (Nosarzewski et al. 2004; Park et al. 2002). Immunohistochemical analysis shows that SDH is distributed both in the flesh and in the vascular tissue of the fruit and in the vascular tissue and mesophyll tissue of the young and old leaves (Wang et al. 2009), suggesting that SDH is localized not only in sink organs but also in source organs. Immunogold electron microscopy analysis revealed the subcellular localization of SDH. It is localized mainly in the cytoplasm and chloroplast of the fruit and leaves, although, interestingly, SDH is also localized in vacuoles in young and mature leaves (Wang et al. 2009). The fact that sorbitol is a primary soluble carbohydrate that can be widely metabolized strongly supports the importance of sorbitol in Rosaceae fruit trees.

The importance of SDH in sink organs is directly confirmed using transgenic apple trees with reduced SDH activity, which show vegetative disorders, such as shorter growth, precocious spring leaf loss, loss of apical dominance, and excessive growth of axillary shoots close to the apex because of an altered fructose:sorbitol ratio in immature leaves. These results suggest that reduced SDH activity in immature leaves, a sink organ, could affect sugar partitioning and, as a result, vegetative growth (Martinelli et al. 2011).

5.2.2 Phloem Loading and Unloading

Loading strategies into the minor vein in plants are categorized in three pathways and mechanisms: passive loading, polymer trapping, and active transport (Rennie and Turgeon 2009). In apples, which use sorbitol and sucrose as translocated sugars, there are abundant plasmodesmata at all interfaces in the minor vein phloem (Rennie and Turgeon 2009) and much higher concentrations of sorbitol and sucrose in leaves (Cheng et al. 2005). Radiolabeled sorbitol, sucrose, or CO_2 is not detected in the minor veins when apple leaf tissues are exposed to them because of ready diffusion. These facts show that the movement of sugar alcohol from the mesophyll into the phloem in apples is symplastic and passive (Reidel et al. 2009). Additionally, Reidel et al. (2009) point out that the presence of an active uptake mechanism for a solute in the phloem does not, in itself, prove that the phloem-loading route is apoplastic and that sorbitol transporters in apple leaves are involved in retrieving sorbitol that leaks from phloem cells into the apoplast.

In the fruit of apples, the presence of plasmodesmata between the sieve element and the companion cell and between parenchyma cells but not between the companion and parenchyma cells suggests that phloem unloading of sorbitol and sucrose is related with an apoplastic step between the sieve element–companion cell complex and parenchyma cells (Zhang et al. 2004). The presence of a sorbitol transporter on the plasma membrane, which transports sorbitol into the cytosol of parenchyma cells using the proton motive force, also supports apoplastic unloading (Gao et al. 2003, 2005).

5.3 Regulation of Sorbitol Metabolism

5.3.1 Regulation of Sorbitol Metabolism by Environmental Factors

Partitioning of photoassimilates into sorbitol is dependent on environmental conditions that affect photosynthesis, including the concentration of CO_2 and light intensity. In mature apple leaves, when photosynthesis increases with an increase in CO_2, sorbitol and starch concentrations increase, but sucrose concentrations are stable; this means that the photoassimilate is partitioned into sorbitol rather than sucrose (Pan et al. 1998; Wang et al. 1999). In contrast, in mature peach leaves, when photosynthesis increases with an increase in light intensity, sucrose and starch concentrations increase more drastically than does that of sorbitol. In this case, the photoassimilate seems to be partitioned into sucrose rather than sorbitol (Escobar-Gutiérrez and Gaudillèr 1997). The photoperiod affects carbon partitioning in Rosaceae fruit trees. In mature apple leaves, as the photoperiod increases, sorbitol concentrations increase concomitant with glucose, fructose, and starch concentrations, and the relative partitioning of ^{14}C into only sorbitol increases. However, sucrose concentrations and that into sucrose decrease. It is suggested that longer photoperiods favor sorbitol over sucrose accumulation whereas shorter photoperiods favor sucrose over sorbitol synthesis (Wang et al. 1997). These changes affect other organs, including sink leaves, stems, and roots, as a result of the transport of translocated sugars from the source leaves (Wang et al. 1998). Sorbitol and starch content show diurnal changes at regular intervals throughout a natural day–night cycle. S6PDH activity also shows diurnal changes; these changes seem to be related to endogenous rhythms, although sucrose phosphate synthase (SPS) activity is not (Zhou et al. 2001).

5.3.2 Sugar Signaling in Rosaceae Fruit Trees

In plants, sugars not only are a carbon resource but also function as signal molecules; they modulate gene expression, in which way they could play roles in development, growth, and differentiation (Koch 1996; Rolland et al. 2006; Smeekens 2000). However, most research has focused on sucrose and hexoses, although research on polyols and the plants using them as translocated sugars is limited.

5.3.2.1 Regulation by Sorbitol

SDH activity is decreased by girdling treatment, which interrupts the assimilate supply in fruit (Berüter and Studer Feusi 1997; Morandi et al. 2008), whereas SDH activity of fruit cortex sections from the fruit treated with defoliation and girdling is

increased by incubating in a sorbitol solution (Archbold 1999). In transgenic apple trees with decreased sorbitol synthesis, both SDH activity and transcripts are decreased in shoot tips and fruit (Teo et al. 2006; Zhou et al. 2006). Conversely, by exogenously feeding sorbitol to shoot tips, both *SDH* transcription and activity are stimulated (Zhou et al. 2006). Partial defoliation treatments, which cause more carbohydrate demand upon the remaining source leaves, increase S6PDH activity, although girdling treatment does not affect it (Zhou and Quebedeaux 2003). Furthermore, exogenously feeding sorbitol does not affect the activity and transcripts of sucrose synthase, a key enzyme of sucrose metabolism, in the shoot tips of apple trees (Zhou et al. 2006), whereas it decreases the transcript levels of *S6PDH*, *SPS*, and *ADPGPPase large subunit* in mature leaf-petiole cuttings of loquats (Suzuki and Dandekar 2014a). These facts indicate that sorbitol regulates gene expression as a signal molecule in Rosaceae fruit trees, and, as a result, sorbitol affects vegetative growth and fruit quality. In fact, the effects have been revealed with analyses on transgenic apple plants silenced or upregulated for *S6PDH* (Teo et al. 2006) and cDNA microarray analyses of fruit and leaves of transgenic apples; drastic changes in expression of various genes were shown (Dandekar et al. 2008; Suzuki and Dandekar 2014b). Alteration of the phenotype thus could result from regulation of gene expression by sorbitol.

A mechanism for sorbitol uptake of sorbitol transporter, isolated from the apple, being regulated with sorbitol level around cells, has been revealed: interaction of the sorbitol transporter, MdSOT6, with cytochrome b5, MdCYB5, in response to low sorbitol supply leads to enhancing the affinity of MdSOT6 to sorbitol, stimulating sorbitol uptake (Fan et al. 2009). Because the sugar regulation of sugar transporters has been reported to be at transcriptional level, this posttranslational regulation is proposed as a novel mechanism by Fan et al. (2009).

5.3.2.2 Regulation of Sorbitol-Metabolizing Enzymes by Sugars

Sugars are metabolized to various sugars and their derivatives by enzymes in plants. For example, in sink organs of Rosaceae fruit trees, sorbitol could first be converted to fructose by SDH, fructose to fructose-6-phospahte (F6P) by fructokinase, and then F6P to G6P, sucrose-6-phosphate, and fructose-1,6-phosphate by phosphoglucoisomerase, SPS, and phosphofructokinase, respectively, and then further metabolized. Thus, gene expression regulated by sugars is complicated, and the regulation of sorbitol-metabolizing enzymes by various sugars has been researched to comprehend that. In apples, *SDH* transcripts in shoot tips are upregulated by sorbitol, downregulated by sucrose, and not affected by nonmetabolized sucrose analogues (palatinose and turanose), glucose, and fructose (Zhou et al. 2006), whereas those in sliced tissues of the fruit of Japanese pears are upregulated by sorbitol, glucose, sucrose, mannitol, and fructose (Iida et al. 2004). This inconsistency might be dependent on the differences of organ and physiological condition. In mature loquat leaves, *S6PDH* transcripts are, interestingly, increased by sucrose but decreased by sorbitol (Suzuki and Dandekar 2014a). These trees might have mechanisms to

positively keep sorbitol as the dominant translocated sugar, suggesting that sorbitol has an important role in their survival strategy. In addition, *S6PDH* transcripts are increased by palatinose, a sucrose analogue, and mannose and 3-*O*-methylglucose, glucose analogues, but not by glucose, and are decreased by fructose. Understanding the function of sorbitol as a signal molecule and sugar-signaling system in Rosaceae fruit trees contributes to the improvement of fruit quality and stress tolerance.

5.4 Sorbitol and Stress Tolerance

Compatible solutes, which are of low molecular weight, highly soluble, and non-toxic at high concentrations, are accumulated in response to abiotic stress and include proline, betaine, and polyols (Chen and Murata 2002). A series of studies has revealed sorbitol accumulation in response to stress and the biochemical mechanism of its accumulation in Rosaceae fruit trees. Furthermore, recent molecular analyses are providing direct evidence of the correlation of proteins.

5.4.1 Drought Stress

In Rosaceae fruit trees, including apples, cherries, and peaches, sorbitol is the soluble carbohydrate primarily accumulated in response to drought stress to decrease osmotic potential and maintain turgor pressure (Arndt et al. 2000; Escobar-Gutierrez et al. 1998; Lo Bianco et al. 2000; Ranney et al. 1991; Wang et al. 1995; Wang and Stutte 1992). Additionally, in mature leaves, increase in sorbitol concentration is observed in young leaves, stems, and roots. Although the increase does not occur in some cases, such as peach seedlings and the root of potted apple trees based on glasshouse experiments (Escobar-Gutierrez et al. 1998; Wang et al. 1995), research on field-grown peach trees experiencing drought periods confirmed that sorbitol is significantly accumulated, resulting in active osmotic adjustment (Arndt et al. 2000). The contribution of sorbitol to osmotic adjustment is reported to be more than 50 % and from 60 % to 80 % in the mature leaves of apples and peaches, respectively (Lo Bianco et al. 2000; Wang and Stutte 1992). On the other hand, in response to drought stress, the roles of other sugars, including glucose, fructose, and sucrose, seem to be limited; increase of those concentrations is not necessarily observed, and the contribution to osmotic adjustment is small (Escobar-Gutierrez et al. 1998; Lo Bianco et al. 2000; Ranney et al. 1991). These results suggest that sorbitol has an important role in osmotic adjustment in Rosaceae fruit trees when they are exposed to drought stress.

It appears to be generally accepted that sorbitol accumulation in response to drought stress is principally caused by an increase in S6PDH activity because S6PDH is an essential enzyme of sorbitol synthesis. Sorbitol accumulation may result from the preferential conversion of glucose to sorbitol rather than to sucrose

and starch when the apple is under osmotic stress (Wang et al. 1996), suggesting that the accumulation is caused by S6PDH. In peach seedlings in response to short-term drought stress, S6PDH activity in mature leaves significantly increases linearly with the severity of the stress and correlates with the increase in sorbitol content in the phloem sap (Escobar-Gutierrez et al. 1998). However, in micropropagated apple exposed to water stress and accumulating sorbitol in mature leaves, S6PDH activity increases; still, its positive effect on sorbitol accumulation is limited because the correlation between sorbitol content and the activity is not significant. SDH has a direct effect on sorbitol accumulation in response to water stress because the negative correlation between sorbitol content and SDH activity is significant (Li and Li 2005, 2007). In potted peaches exposed to drought stress, both S6PDH and SDH activities are reduced in both mature leaves and shoot tips, respectively, during drought, suggesting that osmotic adjustment via sorbitol accumulation results from the decrease in the metabolism of sorbitol by SDH in shoot tips, not the increase in sorbitol synthesis by S6PDH in the mature leaves (Lo Bianco et al. 2000). On the basis of these biochemical analyses, it is suggested that sorbitol accumulation is related not only to an increase in sorbitol synthesis by S6PDH but also to a decrease in sorbitol catabolism by SDH.

Molecular approaches provide evidence of the contribution of S6PDH to sorbitol accumulation in response to drought stress. In the leaves of micropropagated apples exposed to osmotic stress, the *S6PDH* gene is induced, and the level of expression of the *S6PDH* gene is positively correlated with the severity of the stress. The gene expression level almost coincides with the enzyme activity and sorbitol accumulation, suggesting that S6PDH has an important role in the response of the apple to osmotic stress and that the regulation is at gene level. Furthermore, promoter analysis of the *S6PDH* gene shows that a positive regulatory region is present between −361 and −221 and causes a key response to osmotic stress, which contains two ABA-responsive elements and a putative MYB-recognition sequence (Zhang et al. 2011). In addition, it is suggested that SOTs are related to the response to drought stress. In micropropagated apple exposed to water stress, mRNAs of *SOTs* in roots, phloem tissues, and leaves are upregulated, and the sorbitol content is increased in those organs. Increased SOTs contribute to loading more sorbitol into the phloem and roots. The apple adapts to drought stress via increasing in sorbitol transport as well as sorbitol synthesis (Li et al. 2012b). Truncation analysis reveals that *MdSOT3* and *MdSOT5* promoters contain a number of *cis*-acting elements related to drought stress (Li et al. 2012a), which also supports the contribution of the sorbitol transporter to the response to drought stress.

5.4.2 Salt Stress

The relationship between sorbitol accumulation and salt stress has been often documented using *Plantago* (Ahmad et al. 1979; Gorham et al. 1981; Lambers et al. 1981). In apples, it is shown that leaf disks treated with high-salinity stresses

accumulate sorbitol and, concomitantly, the expression of the *S6PDH* gene increases (Kanayama et al. 2006). The Japanese persimmon, which does not have potential to produce sorbitol, when transformed with apple cDNA encoding *S6PDH* accumulates sorbitol and shows enhanced tolerance of salt stress (Deguchi et al. 2004; Gao et al. 2001). These results indicate that sorbitol enhances salt stress tolerance and that the accumulation of sorbitol in response to salt stress in Rosaceae fruit trees is the result of transcriptional regulation of S6PDH. In addition, although tobacco transformed with apple cDNA encoding *S6PDH* accumulating sorbitol is growth inhibited with necrosis (Sheveleva et al. 1998), transgenic persimmons show dwarfism; however, that is not severe, and has no necrosis, suggesting that persimmons seem to be tolerant to sorbitol and tolerance to sorbitol varied with plant species (Deguchi et al. 2004).

5.4.3 Cold Stress

Total soluble carbohydrates are associated with increased cold hardiness in fruit trees (Palonen and Buszard 1997). As temperature decreases, sorbitol content increases in apples (Raese et al. 1978; Williams and Raese 1974) and loquats (Hirai 1983). The increase in sorbitol content in mature leaves of the loquat, which is an evergreen tree, in an orchard in winter is correlated with an increase in S6PDH activity (Hirai 1983). The increased activity is caused by the induction of *S6PDH* expression by low temperatures (Kanayama et al. 2006). Low-temperature treatment of apple leaf disks induces *S6PDH* expression and ABA content, which means that expression of *S6PDH* is under the control of ABA when the apple responds to cold stress (Kanayama et al. 2006). Similar induction by low temperature is observed in leaf disks of peaches and Japanese pears; the response of apples as described here is one of the common mechanisms to achieve cold hardiness in Rosaceae fruit trees (Deguchi et al. 2002a, b). S6PDH induction by low temperature has been confirmed by promoter analyses; the promoter region of apple S6PDH can be induced by cold and abscisic acid treatment, and the abscisic acid-responsive *cis*-element has been identified in the gene promoter (Liang et al. 2012).

5.4.4 Micronutrient Deficiency Stress

In peaches, iron (Fe)-deficiency chlorosis largely reduces fruit yields and leads to firmer fruits with higher acidity, total phenolics, and carboxylates. In such a situation, the sorbitol content in the fruit increases, although the content of other major sugars, including sucrose, fructose, and glucose, does not change; this might be an adaptive response to Fe-deficiency stress (Álvarez-Fernández et al. 2011). Sorbitol contributes to boron transport through the phloem in Rosaceae fruit trees by the formation of boron–sorbitol complexes (Brown and Hu 1996, 1998; Hu et al. 1997).

In transgenic apple silenced for the *S6PDH* gene, because of a lack of sorbitol, cracking and necrotic spot occurred in fruit and shoot growth was inhibited (Suzuki and Dandekar 2014b). These facts suggest that sorbitol could potentially cause boron-deficiency stress, if boron is inadequate.

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Part II
Effects of Abiotic Stress on Horticultural Crop Production and Storage

Chapter 6
Influence of Drought and High Temperature on Citrus

Keiko Sato

Abstract In the future, citrus culture will be influenced by climate change. Here I discuss the influence of drought and high temperatures on fruit set, fruit pigment, and fruit disorders in citrus. Drought and high temperatures during the fruit-drop season cause heavy fruit drops and reduce yield. High temperatures during maturation delay the start of the color break. Drought and high temperatures cause much fruit splitting and creasing and reduce yield. If abnormal drought and high temperatures occur often, measures for prevention of these problems should be taken. I also discuss water stress and irrigation in the citrus culture. In Japan, mulch cultivation has been increased to produce high-quality Satsuma mandarins by preventing the absorption of excessive water by roots. Experiments have been performed to determine the effective timing and degree of water stress to produce high-quality citrus fruit and the way to easily measure water stress. In the Mediterranean area, sufficient water use for irrigation is important because water is a scarce resource. RDI (deficit irrigation), which is based on the idea of reducing water application during stages of crop development when yield and fruit quality have low sensitivity to water stress and providing normal irrigation during the rest of the season to maintain production and fruit quality at adequate levels, has been tested on citrus.

Keywords Citrus • Creasing • Fruit drop • Fruit pigment • Fruit splitting • Satsuma mandarin • Water stress

6.1 Introduction

Based on many studies covering a wide range of regions and crops, negative impacts of climate change on crop yields have been more common than positive impacts (IPCC 2014). Decreasing food production and quality resulting from heat and drought stress are predicted as future risks in many areas (IPCC 2014). Citrus is grown in many countries of Asia, North and South America, Africa, Europe, and

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Oceania. In future, citrus culture will be influenced by climate change. Here I discuss the influence of drought and high temperature on fruit set, fruit pigment, and fruit disorders in citrus. I also discuss water stress and irrigation in citrus culture.

6.2 Fruit Set

Although citrus trees usually bloom abundantly, only a small proportion of flowers produce mature fruit, because many flower buds and flowers drop before fruit is set. Most of the fruitlet drops occur during spring and early summer, called "June drop" in the Northern Hemisphere (Erickson 1968; Reuther 1973). Fruitlet drop is caused by both internal and environmental factors. The primary internal factor is competition among flowers and competition between flowers and spring flush, resulting from carbonate and nutrition competition (Iwasaki 1966).

Environmental factors are lack of sunshine, drought, very high or low temperature, and lack of adequate soil moisture (Iwasaki 1966). Spurling (1951) stated that fruit drop was caused by improper frequency of irrigation. Trumble (1952) suggested that citrus roots are inefficient water absorbers, and therefore the soil must be kept fairly wet during this period to avoid stress, the presumptive cause of fruit drop. Furr (1955) found that drying the top 2 or 3 ft of soil to near the wilting point during the June drop period resulted in heavy fruit drop and reduced yield.

As the weather becomes hot in the early summer months, there is generally a period of accelerated fruit drop. Jones and Cree (1965) indicated that very high temperatures occurring during the May–June period are correlated with poor set. Below-average yields of the 'Washington' navel variety in California tend to occur if a maximum temperature above approximately 40 °C occurs on one or more days during May–June. Many reports suggested that temperatures artificially maintained in the 30–34 °C range for 12 h each day directly promote abscission of young fruitlets during stage 1 of development (Reuther 1973). In Japan, Inoue and Chien (1987) studied the effect of temperature on fruit drop in Satsuma mandarin. High temperatures of 25 °C caused an early peak of fruit drop, shortened the period of fruit drop, and increased the fruit-drop rate, whereas low temperatures below 20 °C retarded the peak of fruit drop, extended the period of fruit drop, and reduced the fruit-drop rate. Many reports have suggested that temperatures above 25–30 °C promote fruit drop in Satsuma mandarin (Inoue and Chien 1987; Ono et al. 1984; Taniguchi 1983; Yamamoto et al. 1972). In recent years, heavy fruitlet drops have sometimes occurred under abnormally high temperatures from May to July in Japan. We evaluated the influence of a 2 °C increase in air temperature on fruit drop in citrus using a growth chamber for 2 months after full bloom (Sato et al. 2010). Temperature treatment was conducted in a phytotron. One zone was maintained at mean temperature (control) and the other zone was maintained at 2 °C above mean temperature (+2 °C treatment). In some Satsuma mandarin varieties and 'Shiranui,' fruit drop occurred more intensively under +2 °C treatment than in the control after

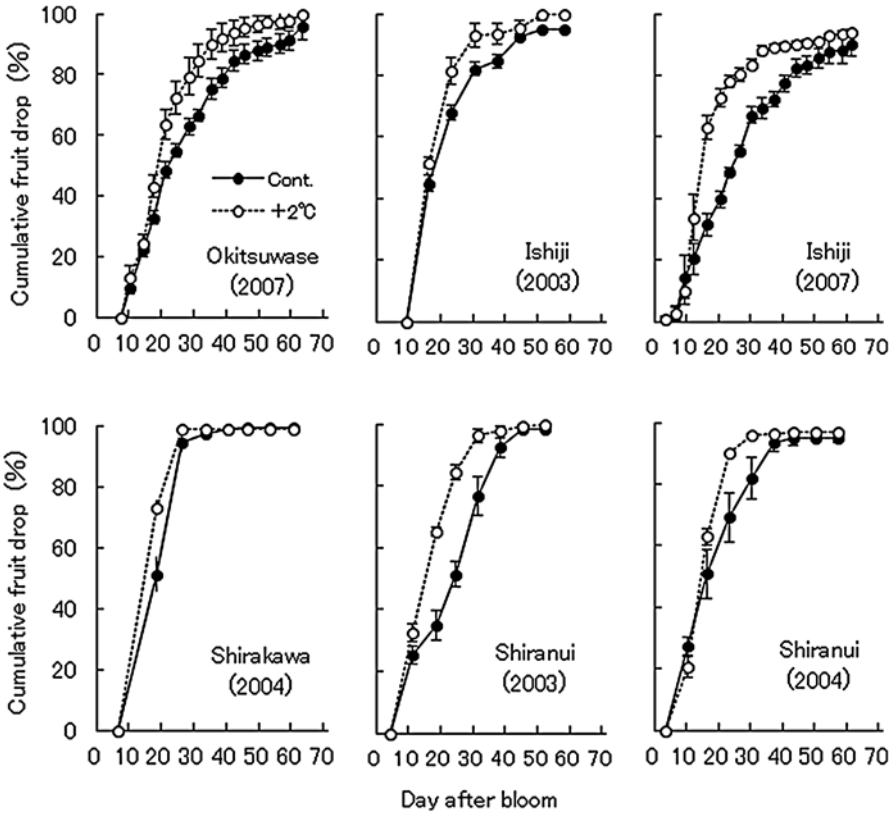

Fig. 6.1 Influence of 2 °C increase in air temperature on fruit drop in citrus. Early-bearing 'Okitsu wase,' middle-bearing 'Ishiji,' late-bearing 'Shirakawa' Satsuma mandarins, and 'Shiranui' are used

10 to 20 days of full bloom (Fig. 6.1). At the end of the experimental period, the physiological fruit drop ratio in plants receiving +2 °C treatment was higher (5–26 %) than that in controls. Recently, because of occasional abnormally high temperatures during fruit drop season, heavy fruit drops occurred in Japan. Because a large amount of fruit drop results in loss of fruit, measures for prevention of fruit drop should be taken.

6.3 Fruit Pigments

During maturation of oranges and mandarins, there is a loss of chlorophyll and an increase in carotenoids (Reuther 1973). Analytical studies with fruiting plants in controlled environments all demonstrate that cool temperatures during the 6 to 16 weeks

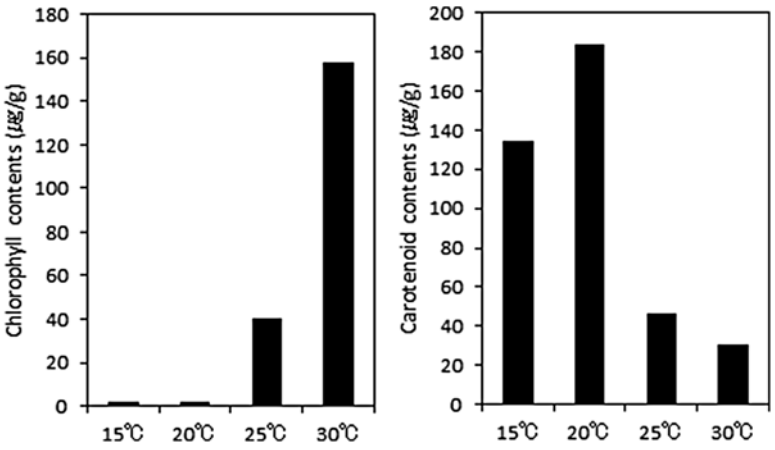

Fig. 6.2 Influence of temperature for chlorophyll and carotenoid contents of the peel in Satsuma mandarin. Temperature treatment was conducted from late September to early November. Chlorophyll and carotenoid content were measured on November 15 (Nii et al. 1970)

preceding harvest maturity promote more rapid decomposition of the chlorophylls than warm temperatures (Young and Erickson 1961; Erickson 1968; Kurihara 1969, 1971; Nii et al. 1970; Young et al. 1969; Meredith and Young 1969, 1971). Young and Erickson (1961) found that the greatest reduction in chlorophyll and greatest increase in carotenoids occurred in Valencia oranges exposed to a combination of cool day-air temperatures, cold night-air temperatures, and cold soil temperatures. When any of these temperatures was higher, the fruit tended to remain green in color. Stearns and Young (1942) reported that the color change did not begin until temperatures reached 12.8 °C. In Japan, Kurihara (1969) found that 18 °C/13 °C (day/night) temperatures advanced the coloring of the peel, whereas 28 °C/23 °C temperatures retained the green color of the peel during 2 months before harvest maturity. Nii et al. (1970) found that with only 1- to 1.5-month periods of differential preharvest treatment and constant day–night temperature regimes applied to fruits, carotenoids in the peel or flesh of Satsuma at harvest were greatest at 20 °C, only slightly lower at 15 °C, but very appreciably lower at 5 °C and 30 °C (Fig. 6.2). Nii et al. (1970) also suggested that warm night temperatures (25 °–30 °C) have a stronger effect than warm day temperatures on producing green fruit. Utsunomiya et al. (1982), applying 1.5-month periods of differential preharvest treatment and constant day–night temperature periods by enclosing fruits in transparent acrylic cylinders on a tree grown under natural conditions, showed that carotenoid accumulation and earlier chlorophyll degradation in the peel of Satsuma mandarin was the highest at 15 °C and 23 °C and lower at 30 °C. The findings all support the general conclusion that there is a strong relationship between temperature, particularly during maturation, and peel color at harvest maturity. Recently in Japan, because hot days have continued until October, the start of the color break is often delayed.

6.4 Fruit Disorders

6.4.1 Fruit Splitting

Fruit splitting is often seen in navel oranges such as 'Washington' and mandarin hybrids such as 'Murcott,' 'Ellendale,' 'Nova,' and 'Niva' (García-Luis et al. 1994, 2001) (Fig. 6.3). In Japan, some new varieties such as 'Reikou,' 'Ariake,' and 'Kanpei' are prone to splitting. Splits occur longitudinally starting at the navel end, where the rind is thinnest in September (Hoffmann 2009). Losses caused by fruit splitting may represent up to 50 % of the potential yield. Fruit splitting results from the physical pressures of the rapidly expanding juice vesicles on the thin, stretched peel (Erickson 1968). Fruit splitting may be caused by soil moisture, temperature, and relative humidity. Both drought and excessive watering induce splitting. To minimize it, moderate soil moisture should be maintained by watering during summer and autumn. Because oblate-shape fruits tend to split, fruits less oblate in shape should be left at fruit thinning (García-Luis et al. 1994, 2001; Hayashida 2012). Gibberellic acid (GA3) reduces fruit splitting when applied shortly after the end of the June drop in 'Nova' (García-Luis et al. 1994). The application of 2,4-dichlorophenoxyacetic acid (2,4-D) at full bloom also reduces fruit splitting in 'Nova' (García-Luis et al. 2001).

6.4.2 Creasing

Creasing is a defect of the peel, appearing as random grooves on the surface of the fruit (Erickson 1968). Creasing is often seen in oranges such as 'Valencia' and in other citrus varieties. Severely creased fruit is unattractive and its fragility presents

Fig. 6.3 Fruit splitting

packing and shipping problems (Erickson 1968). Matsumoto (1980) proposed that extreme drought during summer and autumn causes cracks in the white tissue (albedo) of peel, resulting in creasing. To minimize creasing, adequate watering during summer and autumn is recommended. July application of GA3 reduced creasing in 'Valencia' orange (Monselise et al. 1976; Jona et al. 1989). Creased fruit has higher soluble solids and acid, although it is unsuitable for the fresh market. Okuda et al. (2008) reported that creased Satsuma mandarin is attractive to Japanese customers for its sweeter taste and good sugar and acid balance.

6.5 Water Stress and Irrigation

It is well known that water deficit during summer and/or autumn increases the soluble solids (SSC) of fruit juice in citrus (Maotani and Machida 1980). The annual mean precipitation in Satsuma mandarin-growing areas in Japan ranges from 1,500 to 2,000 mm. When there are many rainy days in summer and autumn, it is difficult to produce high-quality Satsuma mandarin. In recent years, the use of mulch cultivation, which prevents the absorption of excessive water by roots, has been increased to produce high-quality Satsuma mandarin fruit in Japan. Mulch cultivation is generally practiced from the end of the rainy season to harvest. Maotani and Machida (1980) recommended that leaf water potential before sunrise is maintained below −0.7 MPa from July to August to produce high-quality Satsuma mandarins. Kaihara et al. (2008) recommended that leaf water potential before sunrise be maintained around −1.0 MPa from July to harvest to produce high-quality early-bearing Satsuma mandarins. Iwasaki et al. (2012) recommended that leaf water potential before sunrise be maintained around −0.7 to −1.0 MPa from mid-July to September to produce high-quality early-bearing Satsuma mandarins. Experiments aimed at identifying the period and degree of water stress necessary to produce high-quality fruit are undertaken not only for Satsuma mandarins but also for some new varieties. Iwasaki et al. (2011) reported that water stress at threshold values of stem water potential (−0.7 to −1.2 MPa) from mid-August to harvest in 'Harehime' [('Kiyomi' × 'Osceola') × 'Miyagawa wase'] increased soluble solids, citric acid, and amino acids in its juice.

Although the period and degree of leaf water potential necessary for producing high-quality Satsuma mandarins have been identified, it is difficult to measure leaf water potential in orchards owing to the cost and complexity of the measuring equipment. Thus, the development of a cheap and easy measuring equipment to replace leaf water potential as a sensitive water stress indicator is desirable. Hoshi et al. (2007) reported a correlation between diurnal change of transpiration and leaf water potential before sunrise. They developed an indicator to be attached to the abaxial side of the leaf as a patch test for water status in Satsuma mandarins from summer to early autumn. If the indicator turned light red within 5 min of

attachment, the water stress of the tree was moderate. If the indicator did not turn light red within 5 min, the water stress in the tree was high. Suzaki et al. (2011) showed that the morning maximum leaf water potential could be estimated from the fruit hardness on the evening of the previous day. They developed a set of three silicone rubber balls with different degrees of hardness as a reference indicator of irrigation management during July and August in mulched citrus orchards. Because the foregoing two methods are useful only in summer, new tools for measuring water stress from summer to harvest are under development.

In Mediterranean areas, sufficient water use for irrigation is important because water is a scarce resource. To cope with scarce water supplies, deficit irrigation (RDI) is an efficient tool to achieve the goal of reducing irrigation water use (Fereres and Soriano 2007). RDI is based on the idea of reducing water application during stages of crop development when yield and fruit quality have low sensitivity to water stress, and providing normal irrigation during the rest of the season to maintain production and fruit quality at adequate levels (Gonzalez-Altozano and Castel 1999). Because citrus is among the most important crops in the Mediterranean area, many RDI experiments have been performed with mandarin (Ballester et al. 2011, 2014; Conesa et al. 2014; Gonzalez-Altozano and Castel 1999; Romero et al. 2006), orange (Ballester et al. 2013; Castel and Buj 1990; Consoli et al. 2014; Pérez-Pérez et al. 2008, 2009), and grapefruit (Pérez-Pérez et al. 2014). The response of fruit trees to deficit irrigation depends mainly on the phenological stage when it is applied (Ginestar and Castel 1996). Water stress applied during phase 1 (flowering and the initial stage of fruit growth) reduces yield by increasing the June fruit drop of mandarin (Romero et al. 2006) and sweet orange (Pérez-Pérez et al. 2008; García-Tejero et al. 2010). Water stress applied during stage 2 (rapid fruit growth) affected fruit size and fruit quality in mandarin (Navarro et al. 2010), sweet orange (García-Tejero et al. 2010), and grapefruit (Pérez-Pérez et al. 2014). Finally, water stress during phase 3 (i.e., maturation) had a major effect on fruit quality in sweet orange, increasing total soluble solids and acidity (Pérez-Pérez et al. 2009). Although water stress is the same, the response is different among varieties. Mandarin is the most suitable for RDI because of its influence on fruit quality and load. Ballester et al. (2011, 2014) showed that the threshold values of midday stem water potential of −1.3 to −1.5 MPa from July to mid-September can save 15 % of irrigation water without detrimental effects on yield and fruit quality in commercial ‘Clementina de Nules’ mandarin orchards. The only limitation in using DI strategies is the development of cheap and easy measuring equipment to replace leaf water potential as a sensitive water stress indicator. Consoli et al. (2014) suggested that the canopy temperature of orange trees observed by an infrared thermometer may be a reliable and less laborious indicator of water stress. Conesa et al. (2014) suggested that proline is a good biomarker of deficit irrigation stress and could be used for monitoring the intensity of such deficit irrigation treatments.

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Chapter 7
Fruit Set and Temperature Stress

Yasushi Kawasaki

Abstract In fruit vegetables, inhibition of fruit set by both high and low temperatures decreases the yield. Because one of the main reasons for fruit abortion is a decrease in pollen viability, which is usually caused by suboptimal mean daily temperature, periodic checking of pollen viability may help to detect and prevent fruit abortion. Although observation of pollen grain germination or pollen tube elongation at the base of the style is a reliable way to evaluate pollen viability, staining with acetocarmine is recommended as a fast method in the production field. To improve fruit set under temperature stress, temperature management, heat- and chilling-tolerant cultivars, and parthenocarpy can be used. In greenhouse production, temperature management is the most popular method to improve fruit set. However, heating or cooling incurs high energy costs. Local temperature management offers a low-cost alternative. Fruit set was improved by heating flower trusses of tomato and basal stems of eggplant. Breeding of heat- and chilling-tolerant cultivars is being attempted. To develop chilling-tolerant tomato cultivars, breeders are exploring wild relatives. Parthenocarpy can be achieved by spraying plants with growth regulators, but this involves high labor costs. Therefore, parthenocarpic cultivars are being developed.

Keywords Fertilization • Greenhouse production • Parthenocarpy • Pollen viability • Pollination • Temperature management • Tomato

7.1 Introduction

For production of fruit vegetables influenced strongly by fruit set, maintaining fruit set is essential. Two main factors—assimilate deficiency and suboptimal (higher or lower than optimum) temperatures—inhibit fruit set in cultivated plants.

Fruit abortion caused by assimilate deficiency occurs under high fruit load (Aloni et al. 1996; Bertin 1995) or prolonged low-light conditions (Rylski and Spigelman 1986). Insufficient assimilate translocation to the flowers suppresses fruit set and

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development. Irregular fruit set, known as 'flushing,' is a serious problem in sweet pepper production that causes strong fluctuations in market supply and labor demand (Heuvelink and Körner 2001; Marcelis and Baan Hofman-Eijer 1997).

Fruit abortion caused by temperature stress occurs when temperatures are considerably higher or lower than the optimum. Some crops grown in greenhouse (e.g., tomatoes, sweet peppers, cucumbers) are often exposed to suboptimal temperatures because they are grown in various regions and temperature conditions. For example, crops can be exposed to low temperatures in winter in Europe and North America (Osborne and Went 1953; Picken 1984), whereas high temperatures can cause problems in summer in Asia (Abdul-Baki and Stommel 1995; Dane et al. 1991; Nkansah and Ito 1995; Sasaki et al. 2005). If fruit set could be maintained under such temperature stresses, significant fruit yield increases and cost reductions could be expected. Therefore, many studies are now in progress to improve fruit set under suboptimal temperatures.

In this chapter, I overview fruit abortion in vegetable crops affected by temperature stress in the production field, especially in greenhouse production of tomato. I also discuss some approaches to improve fruit set.

7.2 Suboptimal Temperature Stress Affects Fruit Set

Inhibition of fruit set by temperature stress occurs when temperature exceeds the optimal range for a particular crop. Fruit set is affected more by mean daily temperature than by temperature during the day or during the night, or by the day–night temperature differential (Peet et al. 1997). The mean daily temperature range for stable fruit set differs for different crops: 13–25 °C for tomato (Charles and Harris 1972; Osborne and Went 1953; Sato et al. 2000), 16–25 °C for eggplant (Kikuchi et al. 2008; Nothmann and Koller 1975; Passam and Khah 1992; Sun et al. 1990), 18–25 °C for sweet pepper (Cochran 1936; Rylski and Spigelman 1982; Shaked et al. 2004), and 20–30 °C for cucumber and melon (Maestro and Alvarez 1988; Hikosaka et al. 2008). However, these ranges can change with light intensity and cultivar.

7.2.1 High-Temperature Stress

A number of studies investigated the effects of high-temperature stresses on plant productivity. Many focused on the effects of acute, transient, high-temperature treatments (e.g., 40–50 °C for 2–4 h), known as heat shock (Banzet et al. 1998; Neta-Sharir et al. 2005; Nover et al. 1983). Although such studies are important, heat-shock conditions are rarely encountered in the production field, where fruit set is affected by prolonged, moderately high mean daily temperatures (e.g., 25–35 °C for several weeks). Sato et al. (2000) showed that fruit set percentage in tomato was decreased under such moderate heat stress, and suggested that this decrease was

related to a reduction in pollen grain release. They also determined that 8 to 13 days before anthesis is the period of the highest sensitivity to heat stress (Fig. 7.1) (Sato et al. 2002). A decrease in pollen grain viability and different composition in carbohydrate content in the androecium were shown (Sato et al. 2006).

Reduction in pollen viability is considered as the main factor preventing fruit set at high temperatures. In *Phaseolus vulgaris* L., this reduction was explained in relationship to the development of anther tissues (Suzuki et al. 2001). At the pollen tetrads stage, heat stress affects the structure and function of the tapetum, playing a crucial role in supplying nutrients to pollen mother cells and regulating release of pollen grain. Tapetum malfunction causes pollen sterility. In tomato, high temperature at the pollen tetrads stage leads to the enlargement of tapetal cells, resulting in pollen sterility (Iwahori 1965). In the field, tomato pollen viability drops drastically when mean daily temperature exceeds 27 °C (Kawasaki et al. 2009).

Fruit set can be suppressed by ovule sterility even if pollen is highly viable (Charles and Harris 1972; El Ahmadi and Stevens 1979). Ovule viability is also reduced by high-temperature stress. It was suggested that ovary development is more tolerant to high-temperature stress than pollen and ovule development, because parthenocarpic fruits induced by plant growth regulators develop under high temperature when normal fruit set is inhibited (Sasaki et al. 2005).

7.2.2 Low-Temperature Stress

Similar to high temperature, low temperature reduces fruit set because of a reduction in pollen viability. In tomato, normal pollen development, pollen germination, and pollen tube growth are suppressed under low-temperature conditions (Charles and Harris 1972; Dempsey 1970). Although no information about structural abnormalities in vegetable crops under low temperature could be found, reduction of pollen viability at low temperature was explained by a structural abnormality of the tapetum in rice (Gothandam et al. 2007). Ovule viability is less affected by low temperature (Charles and Harris 1972). In greenhouse production, low-temperature stress is not considered as great a problem as high-temperature stress, because it can be easily alleviated by heating.

7.2.3 Evaluation of Fruit Set and Pollen Viability

Monitoring pollen viability enables earlier evaluation of fruit set than visual observation (Fernández-Muñoz et al. 1994; Heslop-Harrison et al. 1984). If fruit abortion is predicted early, measures to improve pollen viability can be taken. Assessing pollen viability is also used in breeding for pollen tolerance to suboptimal temperatures (Fernández-Muñoz et al. 1995; Domínguez et al. 2005). Among the methods for testing pollen viability, counting pollen tubes at the base of the style

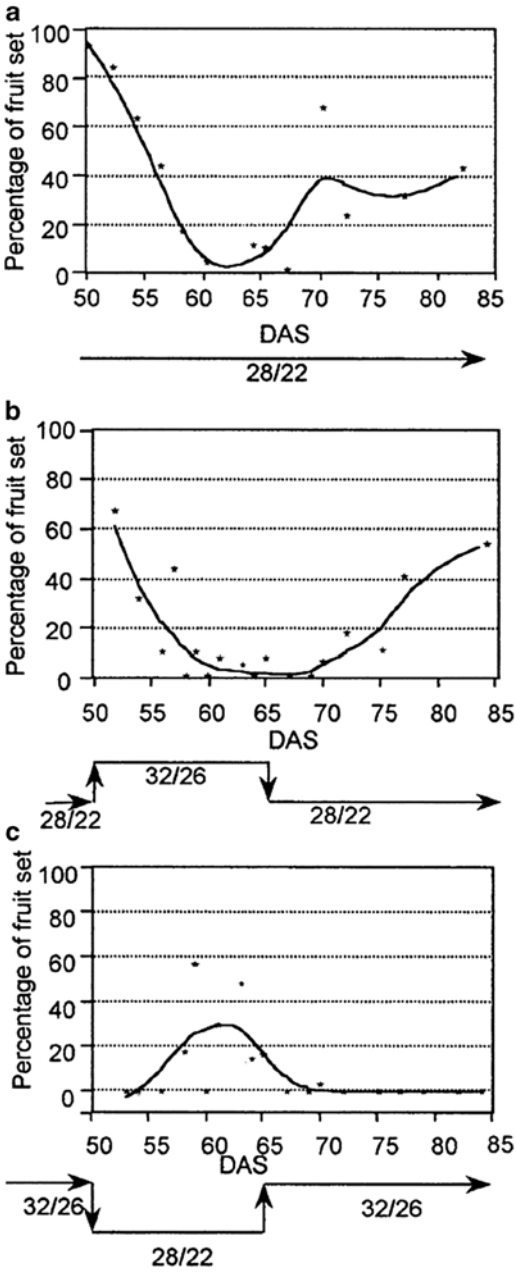

Fig. 7.1 Percentage of fruit set in control (day–night temperature, 28–22 °C) (**a**); 15 days at high temperature (32–26 °C) (**b**); 15 days high-temperature relief treatment (**c**). *DAS*, days after sowing. The decrease of percentage of fruit set in control is assumed to be caused by increasing fruit load. (From Sato et al. 2002, reprinted with permission from the publisher)

after artificial pollination are considered to be most reliable (Abdalla and Verkerk 1970; Charles and Harris 1972; Dempsey 1970). Because this method is laborious, many studies use a faster method: evaluation of pollen grain germination or pollen tube growth in vitro (Charles and Harris 1972; El Ahmadi and Stevens 1979; Sato et al. 2000, 2006). However, its potential ability to predict pollen performance

depends heavily on optimization of the germination medium and temperature (Abdul-Baki 1992; Heslop-Harrison et al. 1984). Staining pollen with vital stains (Abdul-Baki 1992; Charles and Harris 1972; Domínguez et al. 2005) readily detects pollen viability and can be used in the production field because only stains and a simple microscope are needed. Fernández-Muñoz et al. (1994) noted that pollen staining, especially with acetocarmine, is the best method for large-scale applications to monitor field production or breeding, whereas counting pollen tubes at the base of the style is most reliable and is best for precise assessment of small samples.

Parthenocarpy is not affected by pollen and ovule viability at fruit set. Therefore, the ability of the ovary to develop has to be assessed to evaluate fruit set (Kikuchi et al. 2008; Sasaki et al. 2005). This procedure requires 2 to 3 weeks after anthesis to obtain results, and there is no alternative method available in the production field. A new method is required for earlier evaluation of parthenocarpic fruit set in the field.

7.2.4 Use of Insect Pollinators

In greenhouse production of fruit vegetables, insect pollinators are often used to reduce the labor of pollination or hormone treatment (Peet and Welles 2005). Honey bees and bumble bees are used as pollinators. The optimal temperature range is 15–30 °C for honey bees and 10–30 °C for bumblebees (Heinrich 1975; Kwon and Saeed 2003), and suboptimal temperatures can affect their activities; in particular, honey bees are sensitive to low temperatures in winter.

7.3 Techniques for Improvement of Fruit Set

In greenhouse production, temperature management by climate control is essential for improvement of fruit set and yield. Another approach is breeding for cultivars that are better adapted to suboptimal temperatures; the availability of such cultivars could considerably reduce costs of temperature management and CO_2 emission (Van der Ploeg and Heuvelink 2005). Parthenocarpy, which is not dependent on pollen viability, is also available as a technique to improve fruit set under unfavorable conditions.

7.3.1 Temperature Management

To alleviate high-temperature stress, ventilation, shading, and cooling are used in greenhouses. Although ventilation and shading are commonly used because of their low cost, they are sometimes insufficient for alleviation of heat stress during summer days in low-latitude regions because they cannot decrease the temperature below that outside, which can be high enough to inhibit fruit set. For cooling, evaporative systems, such as fogging and fan-and-pad, and heat pumps are available and

are used by some advanced greenhouse farmers; these can decrease the temperature below that outside. On the other hand, fogging and fan-and-pad can, in principle, only decrease to wet-bulb temperature, and may be ineffective in humid climates (Peet and Welles 2005). Heat pumps are more effective in humid climates and demonstrated improvement of fruit set by night cooling (Willits and Peet 1998). However, both the setup and running costs of heat pumps are higher than those of other cooling systems.

In winter greenhouse production, thermal screens and heating are mainly used to prevent low-temperature stress. Peet and Welles (2005) wrote: 'Pulling thermal curtains of porous polyester or an aluminum foil fabric over the plants at night reduces heat loss by as much as 20–30 % a year,' and some lightweight retractable curtains are available not only as thermal curtains but also as shade. Heating systems that use fossil fuels and supply hot water or warm air are most popular among temperature management measures because they are easy to control, although they incur high energy costs. As Van der Ploeg and Heuvelink (2005) observed: 'With increasing public concern about environmental problems, including CO_2 emissions from fossil fuels, the greenhouse sector will have to improve its energy efficiency.'

To reduce energy costs, a local temperature control technique is being developed (Kawasaki et al. 2010, 2011, 2013). As fruit abortion caused by temperature stress is affected only by the temperature of the flower, fruit set can be maintained so long as flowers are kept at the optimal temperature. In winter production of tomato, supplying warm air around flower trusses from suspended air ducts (Fig. 7.2) improves fruit set in comparison with the absence of general heating (Table 7.1) even though the lower plant parts are not warmed (Kawasaki et al. 2010). Furthermore, this technique reduces fuel consumption by 26 % in comparison with conventional temperature treatment without loss of fruit set and yield (Fig. 7.3) (Kawasaki et al. 2011). In eggplant, fruit set and yield can be maintained when basal stems are heated locally with a reduction of the temperature set-point by 2 °C (Moriyama et al. 2011).

7.3.2 Breeding for Heat and Chilling Tolerance

Wide genotypic variation among tomato cultivars in sensitivity of fruit set to high temperature (Firon et al. 2006; Levy et al. 1978; Rudich et al. 1977; Sato et al. 2004) makes breeding for heat-tolerant cultivars feasible. Tomato lines originating from tropical Asia are frequently used as breeding materials (Abdul-Baki and Stommel 1995; Dane et al. 1991; Nkansah and Ito 1995). These lines and cultivars show high photosynthetic performance (Nkansah and Ito 1995) and high sugar content in pollen grains under high-temperature conditions (Firon et al. 2006), which raises pollen viability, fruit set, and yield in comparison with non-heat-tolerant cultivars (Table 7.2). In addition, small-fruited genotypes with many flowers are less affected by heat stress than are larger-fruited cultivars (Dane et al. 1991).

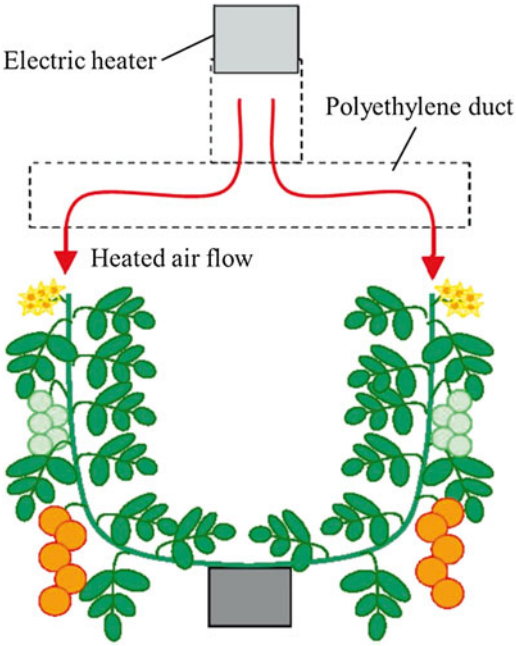

Fig. 7.2 Scheme of local heating that supplies heated air to flower trusses. (From Kawasaki et al. 2010, reprinted with permission from the publisher)

In contrast, the variation in low-temperature responses among current tomato cultivars is limited, which hampers breeding for enough levels of commercial yield at lower temperatures (Maisonneuve et al. 1986; Van der Ploeg and Heuvelink 2005). Therefore, breeders must look for alternative sources of variation in the low-temperature response of tomato. Two wild relatives of the cultivated tomato, *Solanum habrochaites* (*Lycopersicon hirsutum*) and *Solanum pennellii*, show high pollen viability and fruit set (Fernández-Muñoz et al. 1995; Zamir et al. 1981, 1982) and are considered as breeding materials for chilling tolerance.

7.3.3 Parthenocarpy

The defects of fruit set caused by temperature stresses can be mitigated to some extent if parthenocarpic fruits are available, because fruit set depends mostly on both the quality and quantity of pollen, which are not relevant to parthenocarpy. At very high temperatures, however, parthenocarpy is still inhibited (Kikuchi et al. 2008; Sasaki et al. 2005).

In commercial production, parthenocarpic fruits can be induced by plant growth regulators or can be a trait of parthenocarpic cultivars. Spraying flowers with auxins, gibberellic acid, or both can induce parthenocarpy. This technique is used in the commercial production of tomato and eggplant. In tomato, parthenocarpic fruits develop earlier than seeded fruits. In eggplant, parthenocarpic fruits have higher

Table 7.1 Effects of local heating around shoot apex and flower clusters on fruit set in two tomato cultivars

	Local heating (surface temperature of shoot apex)	No. of harvested trusses	No. of flowers	No. of fruits set	Fruit set (%)[a]	Flowering interval (day truss^{-1})[b]
Reiyo	High (13.0 °C)	9.3 A[c]	49.9 A	48.4 A	96.9 A	12.5 A
	Low (11.5 °C)	9.0 AB	48.7 A	46.5 A	95.5 A	13.2 AB
	None (9.6 °C)	8.7 B	53.6 A	47.9 A	89.3 B	13.6 B
Momotaro–Haruka	High (13.0 °C)	10.4 a	56.9 a	50.8 a	89.1 a	11.3 a
	Low (11.5 °C)	10.0 a	55.0 a	41.3 ab	75.1 b	11.7 b
	None (9.6 °C)	9.4 a	56.6 a	34.7 a	61.3 c	12.0 b
ANOVA[d]	Cultivar (C)	*	NS	*	**	**
	Local heating (H)	NS	NS	*	**	**
	C×H	NS	NS	*	**	NS

[a]Arcsine-transformed values were used for statistical analysis
[b]Mean interval between first flower anthesis on each truss
[c]Values within a column and cultivar followed by the same letter are not significantly different at $P<0.05$ by Tukey's multiple comparison test ($n=8$)
[d]* and ** indicate significant difference at $P<0.05$ and 0.01, respectively; *NS* no significant difference at $P<0.05$
Source: From Kawasaki et al. (2010); reprinted with permission from the publisher and translated from Japanese

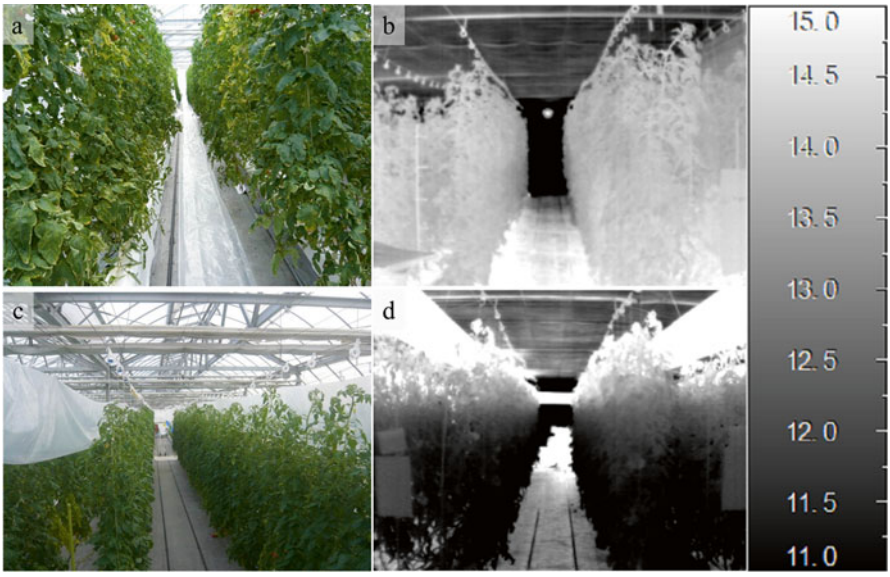

Fig. 7.3 Surface temperature of tomato plants under conventional heating (**a**, **b**) or local heating using air ducts arranged around flower trusses (**c**, **d**). The *gray-scale bar* indicates surface temperature measured with an infrared thermometer at midnight (Kawasaki et al. 2011)

value, because seedlessness prevents browning of fruit fresh upon cutting and lessens the content of saponin and solasonin compounds, which cause a bitter taste (Donzella et al. 2000). In parthenocarpic sweet pepper, yield fluctuation and blossom-end rot are reduced (Heuvelink and Körner 2001). However, spraying is as laborious as hand-pollinating and is difficult to implement in large-scale production.

Parthenocarpic cucumber cultivars are common. Some summer squash cultivars have the parthenocarpic trait (Robinson and Reiners 1999). Breeding for this trait is carried out in tomato (Takisawa et al. 2012; Philouze and Maisonneuve 1978) and eggplant (Kikuchi et al. 2008; Saito et al. 2007).

7.4 Conclusion

In greenhouse production of fruit vegetables, plants are often grown under suboptimal temperatures. High temperatures in summer and low temperatures in winter affect fruit set, especially in year-round production. To mitigate the fruit set defects, knowledge of optimal temperature ranges for particular crops and cultivars is important. Monitoring pollen viability allows fruit set to be predicted and necessary measures to be taken earlier than if fruit set is examined visually.

Maintaining optimum air temperature, which is most important for stable fruit set, is energy costly. Local temperature control can effectively reduce costs without

Table 7.2 Differences in fruit set between heat-tolerant and non-heat-tolerant tomato cultivars

Cultivar	Heat tolerance	Temperature (day/night, °C)	Fruit set[a] (%)	Reference
Duke	None	35/23	13 (54)	Abdul-Baki and Stommel (1995)
AVRDC-CL-1131	Tolerant		65 (71)	
Duke	None	29–36/17–24	6.6	Dane et al. (1991)
AVRDC-PT-3027	Tolerant		42.7	
Grace	None	31/25	26.7[b]	Firon et al. (2006)
FLA 7156	Tolerant		48.5[b]	
Hosen-Eilon	None	36.5–38.7/16.5–20.0	16.3	Levy et al. (1978)
Hotset	Tolerant		69.2	
F_4 (Hotset × Hosen-Eilon)	Tolerant × None		63.1	
Sataan	None	40/23[c]	44.6 (87.6)	Nkansah and Ito (1995)
Shuki	Tolerant		86.2 (70.0)	
Fresh Market 9	None	32/26	1.3 (56.1)	Sato et al. (2004)
FLA 7156	Tolerant		22.3 (46.8)	

[a]Values at optimal temperatures are in parentheses
[b]Pollinated with viable pollen of 'Grace'
[c]Root-zone temperature was 25 °C constant

loss of fruit set and yield. Use of available heat- and chilling-tolerant varieties, and parthenocarpic cultivars, remarkably reduces energy costs of cultivation under unfavorable temperature conditions.

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Chapter 8
Postharvest Chlorophyll Degradation and Oxidative Stress

Naoki Yamauchi

Abstract A loss of green color from chlorophyll (Chl) degradation in harvested green horticultural crops is one of the main quality problems occurring during transportation and storage. In crops such as leafy vegetables, broccoli, and lime, initial Chl *a* degradation was thought to lead to chlorophyllide *a* formation by chlorophyllase, pheophytin *a* formation by Mg-dechelation, or 13^2-hydroxychlorophyll *a* formation by oxidation. Oxidative degradation of Chl *a* could be caused by the oxidation of phenolics, which have a OH group of p-position in the benzene ring, or that of unsaturated fatty acids with senescence.

Postharvest stresses, immoderate temperature, light, and desiccation seem to induce the production of reactive oxygen species (ROS) in horticultural produce. Those environmental stresses can accelerate the advancement of senescence, including Chl degradation. In contrast, generation of an appropriate amount of ROS by stress treatments induces the activation of antioxidant enzymes and an ascorbate-glutathione cycle, which eliminates hydrogen peroxide in the cell, and in consequence, suppression of postharvest Chl degradation of green horticultural crops. Thus, oxidative stress could be seen as a double-edged sword in association with the advancement of quality deterioration throughout transportation and storage of green crops.

Keywords Antioxidant • Chlorophyll degradation • Quality deterioration • Reactive oxygen species • Senescence • Stress treatments • Transportation and storage

8.1 Introduction

Drastic quality changes in postharvest horticultural crops occur during transportation and storage. In green horticultural crops such as leafy vegetables, broccoli, and lime, yellowing, which entails a loss of green color, is one of the main quality deterioration factors. For quality maintenance, it is very important to hold those crops at low temperatures as much as possible throughout transportation and storage.

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However, keeping these crops consistently at low temperatures from the farm to the table is difficult, even now in developed countries. Complete maintenance of a cold-chain system for postharvest horticultural crops could be difficult.

Environmental stresses such as unsuitable temperature, humidity, and improper light conditions during transportation and storage are known to cause quality deterioration in postharvest horticultural crops by producing reactive oxygen species (ROS). In this way, the excessive oxidative stress condition could induce senescence and physiological disorders. On the other hand, oxidative stress could induce the enhancement of antioxidant level and activation of a ROS-scavenging system, such as an ascorbate-glutathione cycle, resulting in the suppression of chlorophyll (Chl) degradation with senescence in postharvest green fruits and vegetables. Thus, postharvest stress treatments, that is, heat treatment and UV irradiation, are thought to be useful methods for quality maintenance (Toivonen and Hodges 2011; Lurie and Mitcham 2007; Yamauchi 2013)

Chl degradation, which occurs with senescing postharvest green horticultural crops, has been suggested to have the following putative pathways. An early step of Chl *a* degradation seems to be the removal of phytol to form a chlorophyllide (Chlide) *a* by chlorophyllase (Chlase) (Amir-Shapira et al. 1987; Lee et al. 2010; McFeeters et al. 1971; Mínguez-Mosquera et al. 1994; Shimokawa et al. 1978; Tsuchiya et al. 1997). Next, the Chlide *a* is removed from an Mg atom to form a pheophorbide (Pheide) *a* by Mg-dechelating substances (MDS), which are heat-stable and low molecular weight compounds (Costa et al. 2002; Kunieda et al. 2005; Shioi et al. 1996; Suzuki et al. 2005). Finally, Pheide *a* is degraded to fluorescent Chl catabolites, which are primary colorless catabolites, via a red Chl catabolite by both Pheide *a* oxygenase and red Chl catabolite reductase (Matile et al. 1999; Hörtensteiner and Lee 2007), as shown in Fig. 8.1. These consecutive reactions, which are suggested to be the main pathway of Chl *a* degradation, occur in the chloroplast (Okazawa et al. 2006; Matile et al. 1997, 1999). An alternate pathway that differs in the first step is proposed by removing Mg instead of the phytol group from Chl *a* to form pheophytin (Phein) *a* by MDS (Hörtensteiner and Kräutler 2011; Tang et al. 2000). Phein *a*, afterward, could be dephytylated to form Pheide *a* by Chlase, which is noted to react with both Chl *a* and Phein *a* as substrates (Heaton and Marangoni 1996). Recently, pheophytinase (PPH), which is localized in the chloroplast, was also found to be a Phein *a*-specific dephytilation enzyme (Hörtensteiner and Kräutler 2011; Schelbert et al. 2009). In addition, Chl *a* is also degraded in vitro by peroxidase in the presence of phenolic compounds, which have a hydroxyl group at the p-position, to form 13^2-hydroxychlorophyll (OHChl) *a* as an intermediate (Kato and Shimizu 1985; Yamauchi and Minamide 1985; Yamauchi et al. 2004). In horticultural crops, OHChl *a* was also present as a Chl derivative, and the level usually decreased with senescence (Yamauchi and Watada 1991, 1993, 1998), suggesting that oxidation, as related to oxidative enzymes such as a peroxidase, could be involved in Chl degradation of postharvest green fruits and vegetables.

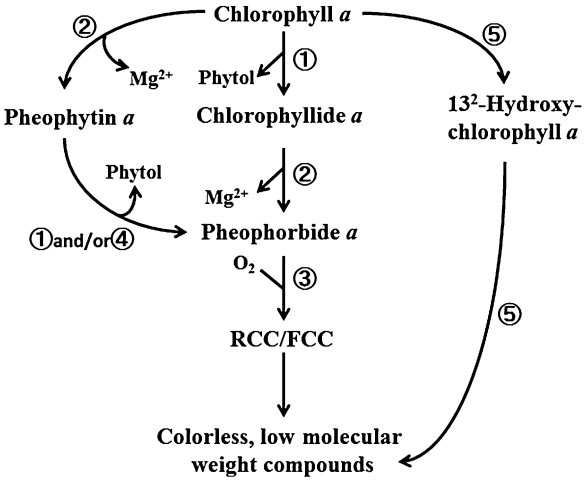

Fig. 8.1 Putative pathway of chlorophyll degradation in postharvest horticultural crops. ① chlorophyllase, ② Mg-dechelation, ③ pheophobide a oxygenase, ④ pheophytinase, ⑤ oxidation related to phenolics and/or fatty acids, RCC - red chlorophyll catabolite, FCC - fluorescent chlorophyll catabolite

This section discusses, first, Chl degradation of postharvest horticultural crops during storage, then the effects of oxidative stress on Chl degradation, and finally, the control of Chl degradation by stress treatments.

8.2 Chlorophyll Degradation During Storage in Postharvest Horticultural Crops

8.2.1 Chlorophyll Derivative Formation with Chlorophyll Degradation

Yellowing, the loss of green color, occurs quickly in postharvest fruits and vegetables during storage at ambient temperature. In those crops, the reduction of Chl *a* and *b* content during storage has been reported, and several kinds of Chl *a* derivatives—Chlide *a*, OHChl *a*, Pheide *a*, and Phein *a*—were detected in both fresh and senescing green fruits and vegetables (Gross 1987, 1991).

With leaf senescence, Chl content in parsley, spinach, and radish cotyledons decreased concomitantly with a temporary accumulation of Chlide *a* (Yamauchi and Watada 1991, 1993; Akiyama et al. 2000). Ethylene treatment in parsley and spinach leaves accelerated Chl degradation. On the other hand, CA storage in parsley leaves suppressed the decline of Chl levels and the increase of Chlide *a* levels. OHChl *a* was detected as a main Chl derivative in fresh and senescing leaves, but the level in those leafy vegetables decreased gradually during storage. Moreover, Amir-Shapira et al. (1987) demonstrated that Phein *a* was formed as a Chl *a* derivative with senescing in stored parsley leaves. In the Japanese bunching onion, Dissanayake et al. (2008) noted that Phein *a* also appears to be a main Chl derivative during storage at 25 °C.

In broccoli florets, Chl content decreased significantly during storage at 15 °C (Yamauchi and Watada 1998). Chl degradation was accelerated during storage in air containing ethylene, whereas the degradation was suppressed during CA storage. In contrast to Chlide *a* changes in stored leafy vegetables, Chlide *a* levels decreased with floret yellowing in broccoli during storage. The OHChl *a* level with ethylene treatment showed a decline after a temporary slight increase, indicating that Chl *a* derivative changes in broccoli florets could be different from those in leafy vegetables. Phein *a* was also found during storage in broccoli florets (Aiamla-or et al. 2010) and green asparagus (Tenorio et al. 2004).

Chl content in green citrus fruits decreased with degreening during storage. In Satsuma mandarin fruit, Chl content decreased markedly during storage with ethylene treatment as compared with the control fruit, whereas Chlide *a* levels in ethylene-treated fruit were shown to be higher than those in the control (Yamauchi et al. 1997; Maeda et al. 1998). In ethylene-treated green tangerine fruit (Amir-Shapira et al. 1987), Chlide *a* levels also increased during storage with degreening. Additionally, Phein *a*, Pheide *a*, and OHChl *a* in ethylene-treated Satsuma mandarin fruit decreased during storage.

Oxidized Chls such as OHChl *a* and 15[1]-hydroxylactone-chlorophyll (OH-lactone-Chl) *a* were reported to accumulate with the decline of Chl content during the development and ripening of olive fruit (Vergara-Domínguez et al. 2011). Janave (1997) also suggested that oxidized Chls were formed by enzymatic oxidation in the peel of banana fruit.

A possible explanation from these findings on the formation of Chl derivatives in postharvest horticultural crops is that an initial degradation of Chl *a* seems to be (1) Chlide *a* formation by Chlase, (2) Phein *a* formation by Mg dechelation, or (3) OHChl *a* formation by oxidation (Fig. 8.1).

8.2.2 Involvement of Chlorophyll-Degrading Enzymes in Chlorophyll Derivative Formation

In fresh and senescing green horticultural crops, Chlide *a*, Phein *a*, and OHChl *a* were mainly present as Chl *a* derivatives. Chlase seems to play an important role in Chl degradation, and many reports on the changes in the activities and gene expression of Chlase have been published (Amir-Shapira et al. 1987; Lee et al. 2010; McFeeters et al. 1971; Mínguez-Mosquera et al. 1994; Shimokawa et al. 1978; Tsuchiya et al. 1997). In spinach leaves, Chlase activity increased significantly with the formation of Chlide *a* during storage, and ethylene treatment accelerated the increase of the activity with leafy yellowing (Yamauchi and Watada 1991). Chlase activity in radish cotyledons (Akiyama et al. 2000) and mitsuba leaves (Yamauchi et al. 1995) also increased with the progress of yellowing during storage. In green citrus fruits, Chlase activity in ethylene-treated Satsuma mandarin fruit enhanced degreening during storage (Shimokawa et al. 1978). Similarly, Barmore (1975) reported that Chlase activity in ethylene-treated calamondin fruit increased with the

degradation of Chl during storage. In contrast, Chlase activity in stored broccoli florets, which showed the decline of Chlide *a* levels, did not show an increase, however; the Chlase genes BoCLH1, BoCHL2, and BoCHL3 (Chen et al. 2008; Lee et al. 2010) also were not upregulated with yellowing of the florets (Aiamla-or et al. 2012; Büchart et al. 2010).

MDS, which are heat-stable and low molecular weight compounds (Costa et al. 2002; Kunieda et al. 2005; Shioi et al. 1996; Suzuki et al. 2005), increased with yellowing during storage in broccoli florets and Japanese bunching onions (Aiamla-or et al. 2010; Dissanayake et al. 2008). The activity of Mg-dechelatase, an Mg-releasing protein (Suzuki and Shioi 2002), has also been reported to increase with senescing plants (Tang et al. 2000; Büchert et al. 2011; Vicentini et al. 1995). However, Mg-dechelatase seems not to be involved in Mg^{2+} removal from Chlide *a*, as the enzyme did not react with Chlide *a* but with chlorophyllin *a*, an artificial compound produced from Chl *a*, as a substrate.

Schelbert et al. (2009) recently demonstrated that PPH in *Arabidopsis thaliana* is a Phein *a*-specific dephytilation enzyme. They suggested that first Chl *a* was removed from Mg^{2+} by MDS to form Phein *a*, and then Phein *a* was dephytylated to Pheide *a* by PPH without forming Chlide *a*. In broccoli, the PPH gene (*BoPPH*) expression increased with floret yellowing (Aiamla-or et al. 2012). Büchert et al. (2011) reported that the expression of *BoPPH* was accelerated with rapid yellowing of broccoli florets by ethylene treatment but not with slow yellowing by cytokinin. The same tendency of *BoPPH* changes by plant hormone treatments was also observed in stored Chinese flowering cabbage leaves (Zhang et al. 2011). For an accurate assay of PPH activity, it was necessary to separate PPH protein from Chlase protein, because Chlase also reacted with Phein *a* as well as Chl *a* for dephytilation (McFeeters 1975; McFeeters et al. 1971) In broccoli florets, a simple analytical method of PPH activity was designed using ammonium sulfate precipitation; the protein precipitated by 45–60 % saturated ammonium sulfates was suitable for PPH determination, as almost no Chlase activity was included in those proteins. Using this analytical method, it was determined that PPH activity gradually increased with the yellowing of broccoli florets (Aiamla-or et al. 2012).

The appearance of OHChl *a* in most postharvest horticultural crops could be the result of the oxidation of Chl *a* during storage. Peroxidase was found to degrade Chl in vitro in the presence of phenolic compounds, and the activity increased significantly with yellowing or degreening during storage in several green horticultural crops (Dissanayake et al. 2008; Huff 1982; Johnson-Flanagan and McLachlan 1990; Johnson-Flanagan and Spencer 1996; Kato and Shimizu 1985; Ma and Shimokawa 1998; Martínez et al. 2001; Maunders et al. 1983; Yamauchi et al. 2004; Yamauchi and Minamide 1985). As is apparent in Fig. 8.2, peroxidase oxidizes phenolic compounds such as *p*-coumaric acid, apigenin, and naringin, which have a hydroxyl group at the p-position of the benzene ring to form a phenoxy radical, and Chl *a* could be degraded by the formed phenoxy radical to a colorless, low molecular weight compound through OHChl *a* as an intermediate (Kato and Shimizu 1985; Yamauchi et al. 2004; Yamauchi et al. 2012). In addition to the degradation of Chl, the formed phenoxy radical might also relate to the oxidation of carotenoids,

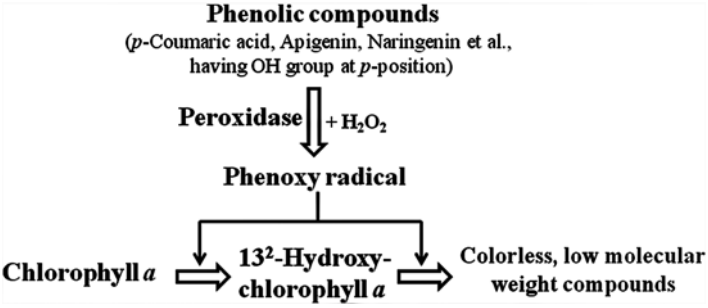

Fig. 8.2 Putative pathway of peroxidase-mediated chlorophyll degradation

phenolic compounds, and fatty acids (Ninomiya et al. 1987; Takahama and Oniki 1998). It was found that a cationic isoperoxidase (molecular size, 34 KDa) that was present in the chloroplast of broccoli florets was expressed with yellowing during storage (Aiamla-or et al. 2014). Kuroda et al. (1990) also noted that cationic isoperoxidase, whose increased activity was suppressed with kinetin treatment, was localized in barley leaf chloroplasts, implying that these cationic isoperoxidases might relate to the oxidative degradation of Chl *a*. OH-lactone-Chl *a* was also formed with peroxidase-mediated Chl *a* degradation as an intermediate (Janave 1997). Furthermore, both lipoxygenase (Buckle and Edwards 1970) and Chl oxidase (Blackbourn et al. 1990; Schoch et al. 1984), which degraded Chl involved with fatty acid oxidation, seem to be involved in oxidative Chl degradation during senescence. Thus, oxidative degradation of Chl might be involved in the occurrence of senescence in postharvest horticultural crops.

In addition, the expression of Chl-degrading-associated genes, such as *SGR* (stay-green), *NYC* (Chl *b* reductase), and *CAB* (Chl *a*/*b* binding protein), seems to relate to Chl degradation (Cheng et al. 2012; Hörtensteiner 2013; Peng et al. 2013). SGR protein, which was localized to the chloroplast, has been particularly known to have a role in the interaction between Chl-degrading enzymes such as PPH, Pheide *a* oxygenase, and red Chl catabolite reductase, and thylakoid membrane (Hörtensteiner 2013).

8.3 Effects of Oxidative Stress on Senescence

During the growth and development of plants, ROS are continuously produced throughout the cell, including the photochemical reaction in the chloroplast, the electron transport system in the mitochondria, and the ROS-generating enzyme reactions such as xanthine oxidase and urate oxidase in the peroxisome (Gill and Tuteja 2010; Mittler 2002). The level of ROS in a plant could be regulated in balance with activation of a ROS-scavenging system, such as an ascorbate-glutathione cycle, and the level of antioxidants such as ascorbate, α-tocopherol, and glutathione.

ROS were highly reactive substances and could directly damage cell components, such as lipids, proteins, carbohydrates, and DNA, whereas they could play a physiological role in signaling in wide-ranging metabolic reactions in cells (Gill and Tuteja 2010). It was reported that hydrogen peroxide could activate a specific mitogen-activated protein kinase cascade in *Arabidopsis* (Kovtun et al. 2000). Postharvest stresses to horticultural crops such as high or low temperature, UV irradiation, and water deficiency seem to also induce the production of ROS during handling, storage, and distribution; those environmental stresses are thought to accelerate the advance of senescence (Hodges 2003; Mittler 2002). Simultaneously, ROS produced by oxidative stress in horticultural produce induces lipid peroxidation of cell membranes to form peroxide and radicals of polyunsaturated fatty acids such as linoleic acid and linolenic acid. Moreover, phospholipase-D, which is stimulated by Ca^{2+} or ethylene, was reported to be involved in lipid degradation of cell membranes with senescence, and the hydroperoxides of polyunsaturated fatty acids, which are formed by lipoxygenase, could also relate to the formation of ROS or free radicals.

As a result, changes in the permeability of cell membranes conduct homeostatic imbalance in cell metabolism and induce oxidative damage such as senescence, which could suggest that ROS produced by oxidative stress might be involved in the induction of physiological disorders in plants.

8.4 Control of Chlorophyll Degradation by Stress Treatments

Physical treatments such as high temperature, low temperature, and UV irradiation or chemical treatments such as ethanol vapor after harvest have been evaluated to control senescence in horticultural crops during storage (Lurie and Mitcham 2007; Terai et al. 1999; Funamoto et al. 2002; Aiamla-or et al. 2009; Fukasawa et al. 2010). Postharvest heat treatment, one of those stress treatments, was the original method traditionally used to control pests and pathogens related to decay in mango, papaya, and citrus fruits, and it has been applied to plant quarantine (Lurie and Mitcham 2007). The inhibitory effect of yellowing by hot-water or hot-air treatment has also been reported in broccoli florets (Kazami et al. 1991; Tian et al. 1996; Terai et al. 1999; Funamoto et al. 2002), green citrus fruits (Ogo et al. 2011), and leafy vegetables (Gómez et al. 2008).

As is shown in Fig. 8.3, hot-air treatment of broccoli at 50 °C for 2 h efficiently suppressed floret yellowing during storage; this could be the result of the inhibition of the activity and gene expression of Chl-degrading enzymes (Funamoto et al. 2002; Büchart et al. 2010). Moreover, the hydrogen peroxide level in heat-treated broccoli florets was higher than that in the control during storage, and antioxidative substances and enzyme activities in relation to the ascorbate-glutathione cycle also showed a higher level in heat-treated florets (Shigenaga et al. 2005). In spinach leaves, Gómez et al. (2008) demonstrated that the level of hydrogen peroxide

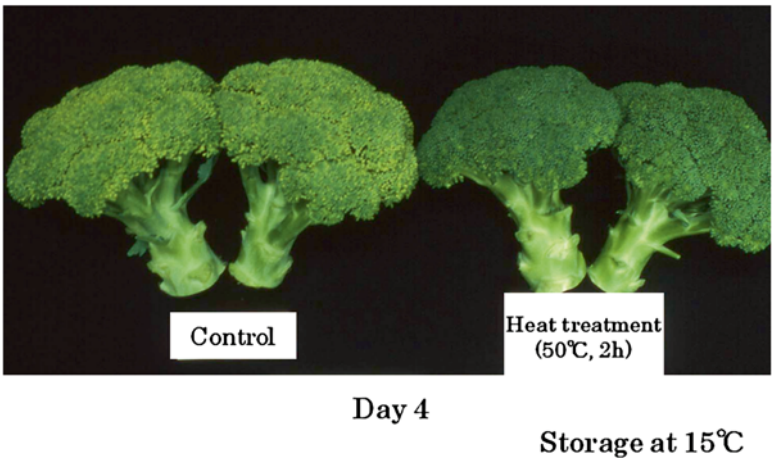

Fig. 8.3 Effects of heat treatment on floret yellowing in stored broccoli. Broccoli heads were loosely covered with perforated polyethylene film bags to reduce weight loss during treatment and held in an incubator in which hot air (50 °C) was circulated for 2 h. Heads were kept in a polyethylene film bag (0.03 mm thick) with top folded over and stored at 15 °C in the dark. (From Yamauchi 2013)

production in mitochondria enhanced by heat treatment concomitantly suppressed leaf yellowing, and heat treatment also inhibited the increase of the oxidized form/reduced form ratio of ascorbate and glutathione. These findings indicate that heat treatment induces the enhancement of reducing substances such as ascorbate and the ascorbate-glutathione cycle activity by generating ROS, and thereby its enhancement could lead to the suppression of senescence in horticultural produce.

There are many reports proving that UV irradiation, especially UV-C, suppresses the yellowing of green horticultural crops during storage (Chairat et al. 2013; Erkan et al. 2008). In broccoli, UV-C has been shown to delay the yellowing of florets with senescence, caused by the suppression of activity and gene expression of Chl-degrading enzymes, such as Chlase, PPH, MDS, and peroxidase (Büchart et al. 2010; Costa et al. 2006). UV-B irradiation also delayed floret yellowing in stored broccoli, whereas UV-A did not show the suppression of yellowing during storage (Aiamla-or et al. 2009, 2010, 2012). As with UV-C, the inhibitory effect of Chl degradation by UV-B was caused by suppression of the activity and gene expression of Chl-degrading enzymes (Fig. 8.4). In lime fruit, UV-B treatment also showed the inhibitory effect of Chl degradation. UV-B treatment induced an increase in the level of total peroxide, which is mainly hydrogen peroxide, and the levels in both UV-B-treated broccoli florets and lime fruit were higher than those in the control. Additionally, activity of the ascorbate-glutathione cycle increased, and leakage of K^+ ions from tissues was also reduced during storage, which could suggest that enhancement of ascorbate-glutathione cycle activity with the generation of ROS in UV-B-treated horticultural produce could be involved in retarding the advance of senescence because of maintaining the stability of cell membranes in stored horticultural produce. Thus, both UV-B and UV-C irradiation could be useful for controlling senescence in stored green horticultural produce.

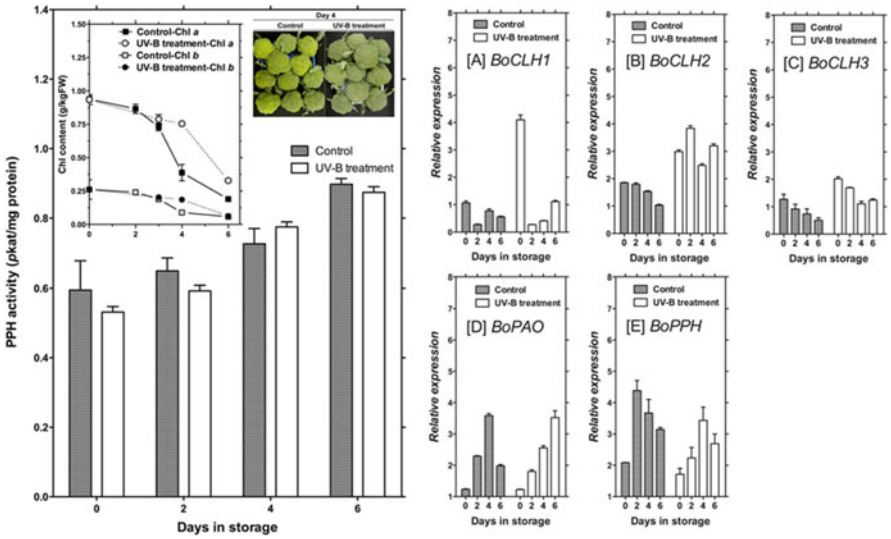

Fig. 8.4 Changes in pheophytinase (PPH) activity, chlorophyll content, and gene expression of chlorophyll-degrading enzymes of broccoli florets treated with or without UV-B (19 kJ m^{-2}) during storage at 15 °C. Relative expression as measured by qRT-PCR of *BoCLH1, BoCLH2, BoCLH3*, *BoPAO*, and *BoPPH*. (From Aiamla-or et al. 2012)

In addition, ethanol vapor treatment was observed to delay degreening of green citrus fruit (Noma et al. 2004) and yellowing of broccoli florets (Fukasawa et al. 2010; Mori et al. 2009). It was inferred that inhibiting the gene expression of Chl-degrading enzymes and activating the ascorbate-glutathione cycle could be involved in retarding senescence.

A possible explanation for these findings is that an appropriate amount of ROS generated by stress treatments could induce activation of the ascorbate-glutathione cycle and antioxidant enzymes, maintain homeostasis of the cell membrane, and, eventually, control Chl degradation in stored green horticultural produce. Furthermore, in stored green citrus fruits, the inhibitory effect of degreening by treatment with electrostatic atomized water particles containing ROS might be sufficient to support it (Yamauchi et al. 2014).

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Part III
Stress Biology in Physiological Disorders of Horticultural Crops

Chapter 9
Blossom-End Rot in Fruit Vegetables

Hiroki Ikeda and Yoshinori Kanayama

Abstract Calcium (Ca) is an essential element for plant growth, as calcium deficiency causes various disorders in some types of horticultural crops. The most significant calcium deficiency disorder is blossom-end rot (BER) of fruit vegetables. In tomato (*Solanum lycopersicum*), one of the most important vegetables in the world, the incidence of BER often becomes a serious problem in agricultural production and results in financial losses. The typical external symptoms of BER in tomato are water-soaked tissues, necrosis, and discoloring of tissues in the distal portion of the fruit. BER develops in the necrotic region of the parenchymal tissue surrounding young seeds and the distal placenta in the internal tissue of the fruit. The symptoms and causes of BER have been extensively studied, and BER is assumed to be related to Ca deficiency of the fruit. Here, we reviewed symptoms and physiological mechanisms of BER that are related to Ca concentration in fruit tissue and focus on recent molecular genetic research on tomato BER.

Keywords Blossom-end rot • Ca^{2+}-ATPase • Calcium • Cation exchanger • Fruit vegetable • Tomato

9.1 Introduction

Calcium (Ca) is an essential element for plants: it maintains the integrity of the plasma membrane, the structure of the cell wall, and is involved in intracellular signaling (White and Broadley 2003; Hepler 2005). Ca deficiency induces various physiological disorders such as bitter pit in apple (*Malus domestica*), black heart in celery (*Apium graveolens* var. *dulce*), tip burn in leafy vegetables, and cracking in tomato (*Solanum lycopersicum*), apple, and cherry (*Prunus* spp.) fruit (Kirkby and Pilbeam 1984; White and Broadley 2003). These physiological disorders induce severe economic and productivity losses. In fruit vegetables, such as tomato, blossom-end rot (BER) at the distal portion of the fruit is caused by Ca deficiency.

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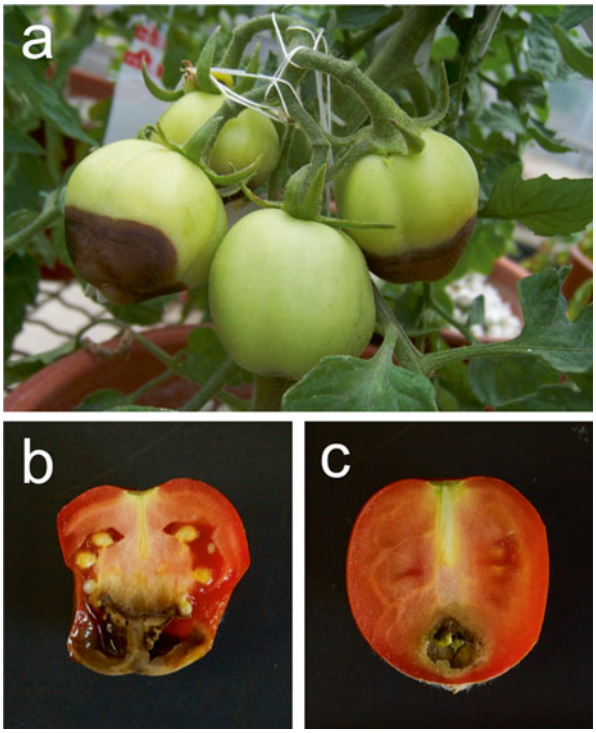

Fig. 9.1 Blossom-end rot (BER) in tomato fruit. **a** Induction of BER occurs within 2 weeks after fruit set, and the distal portion of BER fruit exhibits necrosis and discoloring during the early stages of fruit development. **b** BER enhances softening, causes premature ripening, results in small fruit, and exhibits water-soaked symptoms at the distal portion of the fruit. **c** A necrotic region also develops in the internal parenchymal tissue surrounding the young seeds and in the distal placenta

The typical symptoms of BER in tomato are necrosis and discoloring of tissues in the distal portion of the fruit (Fig. 9.1).

The incidence of BER is also observed in other fruit vegetables such as bell pepper (*Capsicum annuum*), watermelon (*Citrullus lanatus*), and eggplant (*Solanum melongena*) (Taylor and Locascio 2004; Aktas et al. 2005; Silber et al. 2005). BER symptoms in bell pepper are enhanced by reactive oxygen species (ROS) production in the apoplast at the most sensitive stage to BER. BER symptoms in bell pepper are suppressed by manganese (Mn), which inhibits ROS production (Aktas et al. 2005). A negative correlation between the incidence of BER and fruit Mn concentrations has been reported in bell pepper (Silber et al. 2005). Therefore, Mn concentration may be related to the incidence of BER in bell pepper fruit, but further studies are needed to demonstrate this hypothesis (Silber et al. 2005).

Several previous studies about BER have focused on tomato fruit. Tomato is one of the most important vegetables in the world, and its global production is increasing. The incidence of BER results in a 50 % loss in the worldwide tomato production (Taylor and Locascio 2004), and thus induces serious problems in agricultural productions and results in financial losses. The incidence of BER is related to environmental factors such as high salinity and high temperature (Taylor and Locascio 2004). Salinity treatment or water deficiency to increase the soluble solids content of tomato often induces BER in fruit (Saito et al. 2006). BER is believed to be a Ca deficiency disorder in tomato fruit because of the high incidence of BER during plant growth under low-Ca conditions, the low tissue Ca content in fruit with BER, and the reduced incidence of BER after spraying plants with Ca (Ho et al. 1993; White and Broadley 2003; Ho and White 2005). However, fruits with BER occasionally contain equal or higher Ca content than that of healthy fruits (Saure 2001). Therefore, high Ca content in the fruit tissue is not always critical in the prevention of the incidence of BER. In this chapter, we describe the physiological mechanisms of BER incidence that relate to Ca concentration in the fruit tissue and the molecular genetics of tomato BER.

9.2 BER Symptom Development

Initial BER symptoms are membrane leakage of cell solutes, cell plasmolysis, and membrane breakdown (Saure 2001; Ho and White 2005; De Freitas et al. 2011). Subsequently, the fruit surface exhibits water-soaked symptoms, and the tissue at the distal portion of the fruit becomes discolored and necrotic. BER enhances fruit softening in tomato and bell pepper, causes premature ripening, and results in small fruit (Aktas et al. 2005). In the internal tissue of the fruit, BER develops in the necrotic region of the parenchymal tissue surrounding young seeds and in the distal placenta (Adams and Ho 1992; Ho and White 2005).

BER of tomato fruit is considered to be the result of Ca deficiency, and especially the concentration of Ca is reportedly low in the distal portion of the fruit (Bradfield and Guttridge 1984; Adams and Ho 1992). BER is induced within 2 weeks after fruit set when fruit cell expansion is most rapid (Saure 2001; De Freitas et al. 2011). The results suggest that, when the fruit grows rapidly and demands Ca, an insufficient Ca supply to cells in rapidly developing tissue causes BER despite a sufficient Ca concentration in the whole fruit (Ho and White 2005). The incidence of BER is related to the daily irradiance and temperature, which controls fruit cell expansion (Ho et al. 1993). A previous study showed that plum tomatoes are more susceptible to BER than round tomatoes, and BER is never observed in cherry tomatoes or wild relatives of tomato (Ho and White 2005). Therefore, it is important to consider the genetics of BER incidence as well as the environmental effects on the induction of BER.

9.3 Calcium Movement and the Incidence of BER in Tomato Fruit

As already stated, BER in tomato fruit is induced by Ca deficiency in cells of the distal portion of the fruit within 2 weeks after pollination. Ca is transported through the xylem (Michael and Kirkby 1979; Kirkby and Pilbeam 1984; Jeschke and Pate 1991). Tomato cultivars susceptible to BER have a lower capacity for Ca transport through the fruit xylem network than nonsusceptible cultivars (Belda et al. 1996). As fruit cell expansion advances, the density of xylem vessels decreases in the distal portion of fruit where there are fewer and narrower xylem vessels compared to the proximal end (Belda and Ho 1993; Ho et al. 1993; Belda et al. 1996). The xylem/phloem ratio also decreases toward the distal end of the fruit (Ho and White 2005). These findings strongly suggest that low Ca transport capacity to the distal end of fruit may cause BER.

Environmental factors that reduce Ca fluxes into the developing fruit may induce BER, such as high canopy transpiration rates diverting the xylem stream preferentially to leaves and high electrical conductivity (EC) impairing xylem development within the fruit (Ho and White 2005). BER is also induced by other growing conditions such as low Ca or low phosphorus (P) supply, high magnesium (Mg), high nitrogen (N), high potassium (K), or high salinity, drought or waterlogging in the root zone, and low humidity or high light and temperature in the shoot environment (Ho and White 2005). These conditions that induce fruit BER limit absorption and transition of Ca (or Ca^{2+}) from the root zone.

Some Ca^{2+} transport proteins occur in plant cells (Fig. 9.2), and studies on these proteins have progressed in *Arabidopsis thaliana* (Mäser et al. 2001). Cation exchangers (CAXs) are the integral membrane proteins that transport Ca^{2+} using the

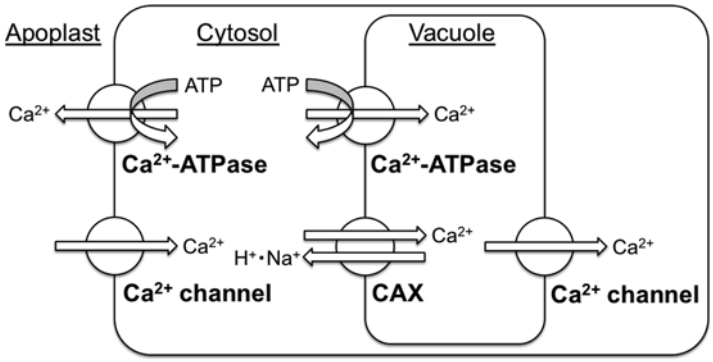

Fig. 9.2 Summary of Ca^{2+} transport system in plant cells based on White and Broadley (2003) and Manohar et al. (2011). Cation exchangers (*CAX*s) are integral membrane proteins that transport Ca^{2+} using the H^+ or Na^+ gradient. Plant CAXs are mainly localized in the vacuole membrane and transport Ca^{2+} into the vacuole. Ca^{2+}-ATPases are mainly localized in the plasma membrane and actively transport Ca to the apoplast against substantial concentration gradients using ATP. Calcium-permeable channels (Ca^{2+} channel) are localized in the vacuole and plasma membrane

proton (H^+) or sodium (Na^+) gradient generated by primary transporters. They are mainly localized in the vacuole membrane and transport Ca^{2+} into the vacuole (Hirschi et al. 1996; Cheng et al. 2005; Shigaki et al. 2006; Manohar et al. 2011). Ca^{2+}-ATPases are integral membrane proteins mainly localized in the plasma membrane and actively transport Ca^{2+} to the apoplast against substantial concentration gradients in plant cells using ATP (Axelsen and Palmgren 2001; White and Broadley 2003). Calcium-permeable channels (Ca^{2+} channels) are localized in the plasma membrane (White 2000; White and Broadley 2003). Knowledge about these Ca^{2+} transport proteins in fruit crops is poor, and further genetic and physiological studies of these transport proteins in fruits may reveal novel findings regarding the incidence of BER that relates to Ca^{2+} movement.

9.4 Stabilization of the Plasma Membrane with Ca^{2+}

A cellular mechanism for the induction of BER by Ca^{2+} deficiency is described in this section. The largest Ca^{2+} pool in plant tissue is in the cell wall, where at least 60 % of the total Ca^{2+} content is found (Demarty et al. 1984). Higher membrane leakage is observed during an early stage of BER incidence and is responsible for the deficiency of apoplastic free Ca^{2+} (Clarkson and Hanson 1980; Kirkby and Pilbeam 1984; Hirschi 2004). Apoplastic free Ca^{2+} stabilizes the plasma membrane by bridging phosphate and carboxylate groups of phospholipids and proteins at the membrane surface (Clarkson and Hanson 1980; Kirkby and Pilbeam 1984; Hirschi 2004). The apoplastic level of Ca^{2+} is maintained at certain thresholds to prevent excessive membrane leakiness and damage (Kirkby and Pilbeam 1984; Picchioni et al. 1998).

Transgenic tomato that overexpresses *CAX* (*sCAX1*) from *A. thaliana* shows an enhanced BER development compared to that of nontransgenic plants (Park et al. 2005a; De Freitas et al. 2011). This study revealed that the *sCAX1*-expressing fruit reduces cytosolic and apoplastic Ca^{2+} concentrations, affecting the plasma membrane structure, and leads to the development of BER symptoms in the fruit tissue, although water-soluble Ca concentrations in *sCAX1*-expressing fruit tissue are higher than those in a nontransgenic control (De Freitas et al. 2011). Pectin methylesterases (PMEs), which increases the Ca^{2+} bound to the cell wall, also affect apoplastic Ca^{2+} concentrations. Apoplastic water-soluble Ca^{2+} was increased, and membrane leakage and BER incidence were decreased by suppressing PME gene expression in transgenic tomato fruit (De Freitas et al. 2012). These reports indicate that BER could be triggered by low concentration of apoplastic Ca^{2+}, which stabilizes the plasma membrane.

9.5 Genes Affecting the Incidence of BER

The incidence of BER can be regulated by changing apoplastic Ca^{2+} concentrations in transgenic tomato plants. However, few studies have been performed on the isolation of endogenous genes affecting the incidence of BER. As already stated, the

incidence of BER could be related to rapid cell expansion, resulting in a low concentration of apoplastic Ca^{2+} in the distal portion of tomato fruit within 2 weeks after anthesis. Because this early stage of fruit development is regulated by auxins and gibberellins (Vriezen et al. 2008), the analysis of auxin- and gibberellin-related genes such as cell wall-modifying proteins would be useful for indentifying the relationship between early fruit development and BER incidence (Catalá et al. 2000).

Ca^{2+} transport proteins such as CAXs and Ca^{2+}-ATPase (White and Broadley 2003) could be involved in the incidence of BER. Overexpression of the *Arabidopsis* CAX gene in tomato, carrot, and potato increases calcium content in edible parts (Park et al. 2004, 2005a, b; De Freitas et al. 2011), and that in petunia enhances cadmium tolerance and accumulation (Wu et al. 2011). Overexpression of the *Arabidopsis* CAX gene in tomato also increases the incidence of BER, as described. These studies used the *Arabidopsis* CAX gene; however, the information on endogenous CAX gene in fruit crops is inadequate. Because whole-genome sequencing has been previously reported in tomato (The Tomato Genome Consortium 2012), the relationship between tomato CAX genes and BER incidence can be investigated efficiently based on the *Arabidopsis* CAX family functional classification information (Mäser et al. 2001).

Because wild species of tomato are generally stress tolerant and the occurrence of BER has not been reported (Ho and White 2005), wild species may have genes that inhibit the incidence of BER. Genetic variation in wild species is valuable for breeding modern cultivars, in which natural biodiversity has been lost from domestication. However, it is difficult to evaluate the agriculturally important traits of wild species because most of the useful traits are quantitative, and genetic variation in wild species has a negative effect on agricultural productivity.

To resolve these problems, introgression lines (ILs), which contain genomic segments of *Solanum pennellii* LA716 replaced by homologous regions in the background of the cultivated tomato *S. lycopersicum* "M82", have been developed (Eshed et al. 1992; Eshed and Zamir 1994). *Solanum pennellii*, a wild species with small green fruit, has some useful traits (Eshed and Zamir 1995; Eshed et al. 1996). Seventy-six tomato ILs, which cover the entire genome, lack most negative traits of wild species and are useful for evaluating individual quantitative trait loci (QTLs) (Lippman et al. 2007). Some useful genes that relate to fruit sugar concentration and fruit weight have been isolated using these lines (Frary et al. 2000; Fridman et al. 2004; Gur et al. 2010). ILs and DNA markers are also available for pepper QTL analyses (Thabuis et al. 2004; Zygier et al. 2005; Eggink et al. 2014). Thus, ILs are potentially useful for genetic studies on the incidence of BER, which is reportedly controlled by a QTL.

The incidence of BER in IL8-3, one of the ILs that carries a *S. pennellii* chromosome segment on chromosome 8 of M82, is reportedly lower than that in independently cultivated M82 (Fig. 9.3) (Uozumi et al. 2012). The gene locus affecting the incidence of BER has been mapped to a narrow region on chromosome 8, which is covered by two BAC clones. The isolation of BER tolerance genes using ILs is expected in the near future. It is also important to explore further genetic material other than ILs using the *S. pennellii* chromosome.

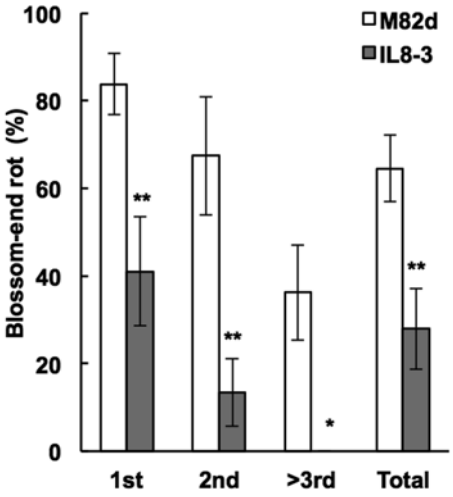

Fig. 9.3 Incidence of blossom-end rot (BER) in *Solanum lycopersicum* "M82" and the tomato introgression line "IL8-3" (Uozumi et al. 2012). Percentage of fruit with BER per total number of fruits is shown for the M82 and IL8-3 lines. About 65 % of the total fruits exhibited BER symptoms in M82; IL8-3 exhibited <30 %. Each value represents the mean and standard error (SE; $n = 11$) in the first, second, third, and total inflorescences. Values with ** and * of IL8-3 are significantly different compared with M82 at $P < 0.01$ and 0.05, respectively, by the *t* test

9.6 Conclusion

BER is observed in various fruit vegetables such as tomato, bell pepper, watermelon, and eggplant. Although BER is a well-known physiological disorder of tomato fruit induced by calcium deficiency, it may occur despite a sufficient Ca content in the fruit because BER is related to Ca movement determined by xylem vessels in the fruit. BER is induced by insufficient Ca in the distal portion of the fruit tissue and occurs within 2 weeks after anthesis when fruit cell expansion is most rapid. Therefore, the incidence of BER is affected by daily irradiance and temperature, which affect fruit cell expansion as well as Ca supply. Studies using transgenic tomato indicated that BER could be triggered by a low concentration of apoplastic Ca^{2+}, which stabilizes the plasma membrane. A QTL for BER tolerance has been identified using ILs that contains a *S. pennellii* chromosome segment. The tomato genome project has recently been completed, and the entire genome sequence is now available (The Tomato Genome Consortium 2012). A huge quantity of information on tomato and its wild relatives has been revealed with next-generation sequencing technology (Koenig et al. 2013). Therefore, progress on genetic analysis and isolation of genes related to BER is expected in Solanaceae fruit crops.

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Chapter 10
Watercore in Fruits

Akihiro Itai

Abstract Watercore is a physiological internal disorder affecting apples and pears, in which the intercellular air spaces of the flesh become filled with liquid, resulting in tissues with translucent appearance. Watercore is associated with fruit maturity as well as the presentation of varietal differences in susceptibility in apples and pears. Susceptibility is thus considered a heritable character. Watercore is promoted by low or high air temperatures during the preharvest period, large fruit, poor calcium concentration, high nitrogen and boron nutrition, a high leaf-to-fruit ratio, excessive fruit thinning, high or low light exposure, growth in volcanic ash soil, ethrel (ethephon) and gibberellin treatment, and girdling of the trunk and limbs. Mild watercore symptoms can disappear in storage, but when severe, internal browning and large cavities can develop. The fleshy tissue of apples with watercore has a higher sorbitol and sucrose concentration and lower glucose concentration than tissue without watercore. Watercore is also accompanied by changes in membrane permeability during maturation and ripening. A decrease in the expression of sorbitol transporter, leading to sorbitol accumulation in the intercellular spaces and subsequent flooding of tissues, has also been suggested.

Keywords Apple • Membrane integrity • Pear • Sink–source relationship • Sorbitol • Sugar transporter

10.1 Introduction

Watercore is a fleshy physiological disorder seen in pears, apples, and sugarbeet (Marlow and Loescher 1984), although the term watercore seems to no longer be used for sugarbeet. The term watercore was first applied to apples in the 1890s, although the disorder itself was already known by other names (Marlow and Loescher 1984). Watercore is believed to be associated with fruit maturity, and in apples can be divided into two types, early and late (Carne and Martin 1934; Yamada et al. 2005).

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Fig. 10.1 Watercore in a "Fuji" apple. The photo provided by courtesy of Dr. Murayama, Yamagata Univ

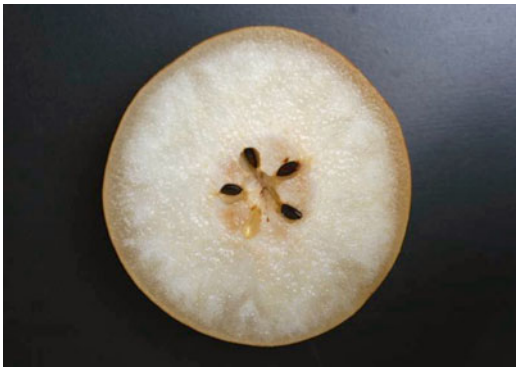

Fig. 10.2 Watercore in a "Hosui" pear

Early watercore develops in immature apples during hot summers, disappearing slowly with cooler weather in the autumn (Yamada et al. 2005, 2006). The late type, on the other hand, is very problematic in the apple industry. Apple and pear fruits consist of a large amount of intercellular air spaces (Westwood et al. 1967). Recent analysis of "Braeburn" apples revealed that intercellular air space represents 7–20 % of the tissue volume, depending on the tissue tested (Drazeta et al. 2004). Watercore symptoms suggest that these air spaces fill with liquid, reducing the light-scattering ability of the tissues and creating a more translucent appearance. The specific gravity of the fruit also increases and this has been used for the nondestructive detection of watercore. In apples, water-soaked areas are usually found near the core and around the primary vascular bundles (Fig. 10.1), although other tissues, including the cortex all the way to the epidermis, can be affected in severe cases. Kajiura et al. (1976) identified five different types of appearance based on the distribution of affected tissues in fruit dissected transversely at the equator. In pears, symptoms are usually found near the cortex, becoming visible around the core when severe (Fig. 10.2).

However, in both apples and pears, watercore can occur anywhere and involve the entire fruit. Development of large cavities has been observed in cortex tissue in severely watercore-affected pear (Kajiura et al. 1976).

Watercored apples and pears have been described as having a sweet or sweetish fermented flavor (Marlow and Loescher 1984; Kajiura et al. 1976); however, in some pear cultivars the affected area is slightly brown and tastes bitter. If the symptoms are mild, watercored apples are often preferred over unaffected apples in Japan and New Zealand (Atkinson 1971). In contrast, watercored pears are not deemed acceptable for consumption in Japan. Watercore often shows a rapid increase with overmaturation and does not come to a stop during postharvest. If mild, symptoms can dissipate completely during storage; however, in severe cases flesh browning can occur.

10.2 Detection

Unless the watercored tissue is severely affected or located near the epidermis, it is not visually detectable in intact fruit. The most reliable method of detection is to cut open the fruit for visual examination; however, not only does this method destroy the fruit but it also renders it useless from an economic point of view. Nondestructive technologies that allow for detection of watercored fruit have been developed, the oldest of which uses the specific gravity of the fruit (Marlow and Loescher 1984) because the presence of liquid in the intercellular air spaces increases the specific gravity. Non-watercored apples have a specific gravity ranging from 0.699 in those with large intercellular spaces to 0.850 in those with small intercellular spaces (Smith 1937). As watercore develops, the specific gravity increases to approximately 1.10 in apples (Filder et al. 1973). In pears, a relationship between the degree of pithiness and the specific gravity has also been observed (Kajiura et al. 1976). According to this report, specific gravities of healthy fruit, fruit with slight pithiness, and fruit with severe pithiness were 1.015, 1.004, and 0.988, respectively. This difference has thus been employed to distinguish between non-watercored and watercored apples and pears by simply floating the fruit in solutions of appropriate density. However, this method requires modification depending on the cultivar, fruit size, and temperature fluctuations. Recently, several new technologies have been developed for detection of watercore, for example, optical density (Throop et al. 1994), X-ray imaging (Schatzki et al. 1997; Kim and Schatzki 2000), magnetic resonance imaging and magnetic resonance (MR) spectroscopy (Clark et al. 1998; Wang et al. 1988), mass density (Cavallieri 1997), and dynamic thermography (Baranowski et al. 2008) methods. A low-field MR sensor for online detection of watercore in apples has also been developed (Cho et al. 2008).

10.3 Genetic Factors

There are large cultivar differences among apples and pears in susceptibility to watercore (Kajiura et al. 1976; Marlow and Loescher 1984) (Tables 10.1 and 10.2, respectively). Compared to the number of resistant cultivars, there are a large number that are susceptible. Three major species of pear, *Pyrus communis* L. (pear or European pear), *P. bretschneideri* Rehd. or *P. ussuriensis* Maxim. (Chinese pear), and *P. pyrifolia* Nakai (Japanese pear, "*nashi*"), are commercially cultivated in the temperate zone, and of these, Asian pears (Japanese and Chinese pears) are at highest risk of watercore because they are picked at a more mature stage than European pears (Kajiura et al. 1976).

Despite the cultivar differences in susceptibility among apples and pears, little is known about the genetic inheritance of watercore. When susceptible apple cultivars such as "Delicious" were used as parents, the resulting offspring tended to develop watercore (Aomori Apple Experiment Station 1953). Furthermore, O'Loughlin and Matthews (1968) compared strains of "Delicious" and found marked differences in watercore susceptibility. Fruit morphological differences are related to genetic differences. In line with this, susceptible cultivars such as "Delicious" show cellular breakdown and proliferation near the vascular bundles, although resistant cultivars such as "Golden Delicious" do not (Marlow and Loescher 1984). Vascular arrangement was also found to be quite different between susceptible and resistant types, and thus, the relationship with susceptibility should also be considered.

In Japanese pear, "Nijisseiki," "Chojuro," and their progenies have been used repeatedly as parents for breeding programs. Almost all are susceptible, as well as being closely related to widespread commercial cultivars (Kajiura and Sato 1990). Abe et al. (1995) reported that the narrow-sense heritability of flesh firmness might derive from pyramiding genes controlling low flesh firmness. Terakami et al. (2009) constructed a genetic linkage map of the watercore-susceptible cultivar "Hosui" and revealed three particular genomic regions (LG 4, LG 5, LG 12) that were homozygous and increased in Japanese pear, possibly because of biased crossing and selection of seedlings during breeding. Watercore in a number of pear cultivars may therefore be the result of narrow genetic diversity. Further work involving segregation of populations is therefore needed to determine the inheritance of watercore susceptibility in apples and pears.

10.4 Environmental Factors

10.4.1 Temperature

A wide range of environmental and physiological factors have been implicated in the incidence of watercore (Marlow and Loescher 1984). Watercore in apples was previously divided into two types, early or immature and late or mature watercore

Table 10.1 Watercore-susceptible and watercore-resistant apple cultivars

Cultivar	Watercore[a]	Cultivar	Watercore	Cultivar	Watercore
Alfriston	S	King	S	Shinsekai	S
Allington Pippin	S	King David	S	Spitzenberg	S
Antonovka	S	Kinnard	S	Stark	S
Arkansas	S	Lady	S	Starking	S
Baldwin	S	Lalla	S	Starkrimson	S
Ballarat	S	Lane's Prince Albert	S	Statesman	S
Beacon	S	London Pippin	S	Stayman	S
Ben Davis	S	Lord Derby	S	Stayman Winesap	S
Blenheim	S	Lord Wolesley	S	Stewart's seedling	S
Braeburn	S	Margil	S	Stone Pippin	S
Bramley Seedling	S	McIntosh	R	Sturdeespur	S
Breton Henry	S	Mela Carlo	S	Sturmer Pippin	S
Calville Blanc	S	Miller's Seedling	S	Suntan	S
Cleopatra	S	Morgendoft	S	Tasmans Pride	S
Commerce	S	Mutsu	R	Tompkins King	S
Cortland	R	Newton	S	Tolman	S
Cox's Orange Pippin	S	Northern Spy	S	Transparent	S
Delicious	S	Northwest Greening	S	Tsugaru	R
Democrat	S	Oldenburg	S	Turner Red	S
Devonshire Quartredon	S	Ontario	S	Twenty Ounce	S
Dougherty	S	Orin	S	Virginia Summer Rose	S
Duchess	S	Paulared	S	Wagener	S
Dunns	S	Pioneer	S	Wealthy	S
Early Harvest	S	Pound Sweet	S	White Astrachan	S
Fall Pippin	S	Pumpkin Sweet	S	Willow Twig	S
Fameuse	R	Rambo	S	Winesap	S
Freedom	S	Red Canada	R	Winter Banana	S
French Crab	S	Red Delicious	S	Winter Golden Pearmain	S
Fuji	S	Red Miller	S	Wolf River	S
Gano	R	Red St. Lawrence	S	Worcester	S
Gardner Red	S	Reinette d'Angleterre	S	Worcester Pearmain	S

(continued)

Table 10.1 (continued)

Cultivar	Watercore[a]	Cultivar	Watercore	Cultivar	Watercore
Glori Mundi	S	Rhode Island Greening	S	Yates	S
Gloster	S	Ribston Pippin	S	Yellow Bellflower	S
Golden Delicious	R	Richared	S	Yellow Newton	S
Granny Smith	S	Rival	S	Yellow Transparent	S
Gravenstein	S	Rogers Red	S	York Imperial	S
Grimes Golden	S	Rokewood	S	Zurich Transparent	S
Himekami	S	Rome	S		
Holstein Cox	S	Rome Beauty	S		
Irish Peach	S	Royal red	S		
Jacobs Sweet	S	Russian	S		
James Grieve	S	Sansa	S		
Jardin Red	S	Scarlet Nonpareil	S		
Jonagold	S	Shinano Gold	R		
Jonathan	S	Shinano sweet	R		

[a]*S* susceptible, *R* resistant
Source: Modified from Marlow and Loescher (1984)

(Carne and Martin 1934; Yamada et al. 2005). In Japan, late watercore was shown to be more severe in colder regions among fruit harvested at a similar stage of maturity (Tomana and Yamada 1988). Harker et al. (1999) also reported that "Fuji" watercore developed earlier and with greater severity in colder regions of New Zealand. These reports suggest that climatic factors as well as fruit maturity are involved in watercore development. Harker et al. (1999) compared preharvest development of watercore in "Fuji" apple from northern (warmer) and southern (colder) regions of New Zealand, finding that watercore was more severe and developed earlier in the south compared to the north. They also showed that symptoms differed between growing regions, with block-type and radial-type watercore predominating in the south and north, respectively. Yamada et al. (1994) also investigated the temperature effect on watercore development in "Fuji" apple by controlling fruit temperature. They found that temperatures about 25 °C completely inhibited watercore whereas lower temperatures (7–10 °C) resulted in a high incidence. Williams and Billingsley (1973) examined watercore development across three seasons and revealed development of symptoms at a minimum temperature approaching 4 °C. Yamada et al. (2004) also examined the relationship with water availability in watercored and non-watercored apples induced by fruit temperature treatment, and suggested that the effect of preharvest fruit temperature on the water status of the fruit

Table 10.2 Watercore-susceptible and watercore-resistant pear cultivars

Cultivar	Watercore[a]	Cultivar	Watercore
Aikansui	R	Mikasa	S
Akaho	HS	Mishirazu	R
Akiakari	S	Nanseichabo	S
Akibae	HS	Nekogoroshi	R
Akizuki	S	Niitaka	S
Amanogawa	S	Nijisseiki	HS
Aoyagi	R	Osa Gold	HS
Atago	S	Osa Nijisseiki	HS
Azumanishiki	HS	Oushu	S
Choju	HS	Rikiya	S
Chojuro	HS	Rokugatsu	R
Chosen	R	Seigyoku	S
Ci Li	S	Seiryu	S
Doitsu	R	Sekaiichi	R
Edoya	S	Shimokaburi	R
Fukushima	R	Shinchu	HS
Gion	S	Shinko	S
Gold Nijisseiki	HS	Shinseiki	HS
Gozennashi	R	Shinsetsu	S
Hakataao	HS	Shinsui	S
Hakko	S	Shugyoku	S
Hakuteiryu	R	Shurei	S
Hatsushimo	HS	Suisei	HS
Heishi	R	Suishu	S
Hokkainashi	R	Sotoorihime	R
Hong Li	S	Taihei	R
Hosui	HS	Taiheiyo	S
Ichiharawase	R	Tama	S
Imamuraaki	S	Tanzawa	S
Inugoroshi	R	Toho	S
Ishiiwase	HS	Tosanishiki	S
Kansaiichi	R	Waseaka	S
Kikusui	S	Wasetaicho	R
Kinchaku	S	Yahatanishiki	R
Kogetsu	S	Yakumo	S
Konpeito	R	Yali	S
Kosui	S	Yatasori	S
Kozo	S	Zuishu	HS
Kuninaga	R		

[a]*HS* highly susceptible, *S* susceptible, *R* resistant
Source: Yoshida and Itai, unpublished data and Kajiura et al. (1976)

via evapotranspiration resulted in differing rates of watercore (Yamada et al. 2004). Extremely high or low temperatures were also shown to induce a watercore-like interior (Bir and Bramlage 1972; Marlow and Loescher 1984).

Despite the foregoing evidence, early watercore can develop in immature apples during the summer, disappearing slowly in the autumn. Moreover, in warmer regions, a higher incidence of early watercore is observed during the summer in certain cultivars (Yamada et al. 2005). "Fuji" is highly susceptible to late watercore but resistant to the early type. A higher fruit temperature induced by higher air temperatures and exposure to sunlight has been suggested as a possible causal factor for development of early watercore (Yamada et al. 2004).

In Japanese pear, the cause of watercore development in the susceptible cultivar "Hosui" has been studied extensively. Watercore in pears is more likely to occur in years with a cool summer (Inomata et al. 1993a), with low air temperature in July increasing the incidence. Inomata et al. (1993a) compared the development of watercore in potted pear trees exposed to July temperatures of 23/15 °C (day/night) and 33/25 °C and found that the former treatment caused more severe watercore symptoms, also suggesting that there was a high degree of correlation between the development of watercore and exposure of pear trees to relatively cool temperatures 80 to 100 days after full bloom. In addition, a higher incidence of watercore was observed in fruit trees covered with polyvinyl chloride film during the early period of fruit development (Sakuma et al. 1995). Fruit covered with a polyvinyl chloride bag during early fruit development also showed more severe watercore (Sakuma et al. 2000). These data indicate that the incidence of watercore is positively correlated to high fruit temperatures during the early stage of development.

The effect of fruit bagging and covering treatments on the relative humidity and transpiration rate should also be considered. The effects of controlling transpiration using water-soaked bagging and wax spray, an anti-transpiring agent, on the occurrence of watercore have been examined, revealing more severe watercore with transpiration inhibition compared to the control (Sakuma et al. 2000). These data suggest that fruit temperature while on the tree also affects the water status via evapotranspiration from the fruit surface, thus affecting the incidence of watercore.

Recently, the effects of fruit temperature on the incidence of watercore in another susceptible variety, "Niitaka," were also investigated (Hayama et al. 2014). Watercore was found to increase significantly under higher air temperatures during fruit maturation. Moreover, shade treatment slightly reduced fruit temperature and watercore incidence. These findings suggest that "Hosui" and "Niitaka" respond differently to high temperatures during fruit maturation. In addition, the affected regions in "Niitaka" fruit differed from those in "Hosui," which were mainly affected in the flesh near the epidermis (Hayama et al. 2014). They concluded that the mechanisms causing watercore in "Hosui" and "Niitaka" pear cultivars are therefore different.

10.4.2 Mineral Nutrition

Calcium appears to have a fundamental role not only in fruit quality and senescence but also in physiological disorders, affecting basic structural features and physiological processes. A number of physiological disorders associated with low fruit calcium content can develop in the orchard or become evident after a period of storage in both apples and pears (Jackson 2003). Of these disorders, apple bitter pit is one of the most important. Sharples (1967) reported that watercored apples had a low calcium content, with high potassium and magnesium. Some reports have further suggested that watercore is indeed correlated with low calcium levels (Perring et al. 1974: Perring and Pearson 1979). On the other hand, Bowen and Watkins (1997) found that calcium concentration decreased with mild watercore, but increased with moderate to severe types in "Fuji" apples. Perring (1968) further suggested that the ratio of nitrogen to calcium might be important, but others failed to find a correlation (Tong et al. 1980).

Differences in the severity of watercore symptoms between trees grown in volcanic and alluvial soil have also been observed in "Hosui" pears. Gemma et al. (2002) studied watercore occurrence in "Hosui" pears grown in volcanic and alluvial soil and found that the severity and frequency in fruits from volcanic soil were much higher than those from alluvial soil. No differences in calcium content were found between soil types, even though volcanic soil has a much higher level of calcium. Differences in calcium content in different parts of the fruit are well documented. In apples, for example, the core has a higher calcium concentration than the inner cortex, which in turn, has a higher concentration than the outer cortex (Ferguson and Watkins 1989). The relationship between calcium concentration and watercore occurrence is therefore not always apparent and in some cases is contradicted.

Despite this, many reports have shown a reduction in watercore incidence with calcium spraying and by dipping fruits in calcium chloride solution while still on the tree (Bangerth 1973; Fukuda 1977; Marlow and Loescher 1984; Tanaka et al. 1992; Inomata et al. 1999). Moreover, vacuum infiltration of sorbitol solution into apple fruit was shown to lead to development of watercore, although simultaneous infiltration with $CaCl_2$ prevented symptoms (Bangerth 1973). Vacuum infiltration of apple fruits with $CaCl_2$ also greatly reduced subsequent fruit softening (Siddiqui and Bangerth 1993). The occurrence of watercore in "Hosui" pear has also been studied by applying calcium compounds (calcium chloride, calcium acetate, calcium citrate, calcium gluconate, and calcium EDTA) and calmodulin inhibitor paste to the pedicel (Tanaka et al. 1992). The frequency of watercore decreased with all calcium compounds, Ca-EDTA being most effective. Calmodulin inhibitor, on the other hand, increased the occurrence. Inomata et al. (1999) also found that calcium carbonate treatment decreased the occurrence of watercore, not through recovery of calcium deficiency, but through promotion of transpiration rate through calcium carbonate. Calcium is implicated in a large number of plant physiological disorders including several disorders in apples (Marlow and Loescher 1984). Understanding the role of calcium in watercore development is therefore not easy.

A few reports have suggested that watercore can develop during storage as a result of excessive boron treatment of apple trees, regardless of whether the application method was foliar or to the soil (Bramlage and Thompson 1962). Boron is considered to be phloem immobile or to have limited phloem mobility in higher plants. However, boron has also been shown to form a stable complex with sorbitol and can be mobile in apples and pears (Brown and Hu 1996). Hu and Brown (1994) also demonstrated that the majority of insoluble boron in the cell wall is bound to pectin. Although the function of boron in apples and pears remains unknown, it is becoming increasingly important to understand the significance of the formation of sorbitol-boron complexes in watercore development.

10.5 Source–Sink Relationship

While on the tree, fruits compete for available resources and competition between sinks is a major factor controlling growth. Both fruit and tree growth are determined by complex carbohydrate source–sink relationships between source leaves and vegetative and reproductive sinks (Wunsche and Ferguson 2005). If the source-to-sink ratio is high, watercore is more likely to occur (Marlow and Loescher 1984). In line with this, no watercore was found on trees with fewer than ten leaves per fruit (Harley 1938). These findings led to the conclusion that the source-to-sink ratio is the most important factor in watercore development. Palmer (1931) also found that watercore and internal breakdown increased whenever the source-to-sink ratio was high. Moreover, Ballard et al. (1922) observed an absence of watercore in defoliated limbs, whereas girdled limbs showed an increase.

High crop densities during the growth period may cause a deficit in carbohydrate availability for the developing fruit, potentially leading to decreased growth and reduced final weight (Lakso 1994). In contrast, light cropping results in increased rates of fruit development and larger fruit with a higher soluble solid content. Light cropping has also been shown to result in heavier fruits and a higher incidence of watercore (Marlow and Loescher 1984). It is also notable that young bearing trees producing a small yield showed an increased incidence of watercore (Marlow and Loescher 1984).

Some reports have suggested a link between excess fruit thinning and increased watercore (Palmer 1931; Perring 1971). Fruit thinning also increases the source-to-sink ratio. Fruit size is the result of a combination of cell number, cell size, and the volume of intercellular air spaces. In general, the timing and severity of fruit thinning has a marked influence on fruit size. For example, the rate of cell division on thinned trees is higher from 3 to 4 weeks after full bloom, whereas earlier thinning results in a significant increase in fruit size at harvest with increased cell numbers in the cortex, rather than an increase in cell size (Jackson 2003). Meanwhile, late thinning produces larger cells. This characteristic has already been implicated in several apple disorders (Marlow and Loescher 1984).

The pear cultivar "Akibae" shows an increase in watercore when trees develop many water sprouts (Tamura et al. 2003). Moreover, the incidence showed reduction after summer pruning, with removal of the water sprouts on primary and lateral branches. Development of numerous water sprouts results in competition for with the fruits for available carbohydrates, and thus removal may reduce the source-to-sink ratio.

10.6 Plant Hormones and Growth Regulators

Plant growth regulators control many physiological processes including stem elongation, seed germination, fruit set, development, and ripening. Ethylene is generally thought to accelerate ripening and senescence. Ethylene or ethrel, which is an ethylene-releasing compound, increases watercore incidence and watercore-related internal breakdown in apples (Amezquita and Dewey 1971; Couey and Wiliams 1973; Greene et al. 1977). Moreover, fruit with watercore produces more ethylene and contains greater amounts of putrescine, spermidine, and 1-aminocyclopropane-1-carboxylic acid (ACC) (Wang and Faust 1992a). In contrast, the internal ethylene concentration showed little correlation with watercore severity in "Fuji" apples (Harker et al. 1999). However, ethrel treatment accelerated fruit maturity and led to a higher incidence of watercore in Japanese pear (Inomata et al. 1993a).

During Japanese pear production, exogenous gibberellin (GA) is widely used to increase fruit size and promote ripening (Kajiura 1994). However, exogenous application of GA_4 paste to young "Hosui" fruit up until 40 days after full bloom resulted in an increase in fruit size and watercore incidence, and early ripening by about 1 week, as characterized by a decrease in flesh firmness (Table 10.3). Several reports have documented the relationship between GA and increased watercore incidence in Japanese pear (Inomata et al. 1993b; 1996; Sakuma et al. 1995).

As for other growth regulators, the occurrence of watercore was previously shown to be suppressed in pear by paclobutrazol, a GA inhibitor (Sakuma et al. 1995). A reduction in the incidence of watercore in pear was also achieved with *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU), a synthetic cytokinin compound (Zhang et al. 2009), whereas in apples, daminozide delayed or reduced watercore incidence (Bartram 1969; Williams 1969; Greene et al. 1977).

Table 10.3 Effect of gibberellin (GA) paste treatment on fruit quality and the incidence of watercore in "Hosui" pear (2008)

Treatment	Fruit weight (g)	Fruit length (mm)	Fruit width (mm)	Brix	pH	Flesh firmness (*N*)	Incidence (%)
Cont	420.1	82.54	94.9	12.5	4.8	14.7	2.5
GA	541.5	89.15	104.07	12.6	4.9	10.9	16.6
ANOVA[a]	*	*	*	ns	ns	*	*

[a]*ns* nonsignificant, *significant at $P<0.05$

10.7 Maturation and Ripening

10.7.1 Cell Wall Composition

Many reports have noted the relationship between watercore occurrence and maturation in apples (Marlow and Loescher 1984), although some describe the incidence of early watercore (Yamada et al. 2005, 2006). Watercore in Japanese pears is also related to maturity (Kajiura et al. 1976). The connection between watercore occurrence and maturation can be derived from studying the effects of growth regulators, as mentioned in the previous section. Treatment that promotes maturation results in accelerated watercore development. Maturation, ripening, and softening can result from degradation of starch, cell wall degradation, and weakening of the cohesive forces between cells (Jackson 2003). Cell walls of apple and pear fruit consist mainly of cellulose, hemicellulose, and pectin, with some lignin in pears. Yamaki and Kajiura (1983) reported that watercore tissues have a lower hemicellulose and cellulose content than non-watercored tissues. In addition, watercored tissues show increased activity of endocellulase, polygalacturonase, β-galactosidase, xylanase, and arabinase, which participate in pectin, hemicellulose, and cellulose production (Yamaki et al. 1976; Yamaki and Kajiura 1983). Chun et al. (2003a, b) investigated chemical degradation and structural changes in the cell walls of watercored tissues in Japanese pear "Akibae" and "Hosui." The amount of CDTA-soluble pectin increased, whereas that of Na_2CO_3-soluble pectin, 4 % KOH-soluble hemicellulose, and 24 % KOH-soluble hemicellulose decreased as watercore progressed in both cultivars. They also found that depolymerization of high molecular mass CDTA-soluble pectin occurred in severely watercored tissues, although the partial molecular mass downshifted in 4 % hemicellulose fractions with considerable changes in xyloglucan in "Akibae" (Chun et al. 2003b). However, it is unclear whether these changes in cell wall components of affected tissues were directly induced by watercore or indirectly by other factors associated with maturity.

10.7.2 Membrane Integrity

Fruit maturation and ripening are associated with changes in membrane integrity (Burg et al. 1964; Wade et al. 1980). Kollas (1968) used ^{14}C-labeled sucrose to investigate membrane integrity during maturation of apple fruit, and found that mature watercored tissue leaks at a higher rate than immature non-watercored tissue (Marlow and Loescher 1984). Glycolipids, phospholipids, and sterols have also been examined in normal and watercore-affected "Delicious" apples (Wang and Faust 1992b). Watercored fruit was shown to contain higher amounts of all three, with a lower ratio of unsaturated to saturated fatty acids in watercore-affected tissue compared to normal tissue. Moreover, the ratio of free sterols to phospholipids was higher, whereas that of phosphatidylcholine (PC) to phosphatidylethanolamine (PE)

was lower in watercore-affected apples. It was concluded that these membrane lipids are altered in watercore-affected fruit, thus resulting in accelerated senescence.

Inomata et al. (1993b) examined the potassium ion efflux through the membranes of fruit with watercore compared to non-watercored Japanese pear "Hosui." They revealed that watercored tissues leaked potassium ions faster than non-watercored tissues, and membranes exposed to cooler temperatures in July were more permeable than those exposed to warmer temperatures. They also found that GA and ethrel treatment accelerated potassium ion leakage. Despite the evidence showing changes in membrane integrity in watercored tissues, it is very difficult to interpret whether these alterations cause watercore or vice versa. Once membrane leakage occurs, cells experience a loss of turgor (Marlow and Loescher 1984). Membranes also have limited repair capability; however, mild watercore symptoms often disappear during storage. Taken together, these findings suggest that the leaky membrane theory is unlikely.

10.8 Sugar Metabolism and Transport

In the family Rosaceae, which includes apples and pears, sorbitol is a major carbohydrate form of translocation photosynthates (Yamaki et al. 1976; Bieleski 1977; Loescher 1987). More than 80 % of the sugars exported from leaves are sorbitol, constituting the main form of translocated carbohydrate in the phloem. Sorbitol 6-phosphate dehydrogenase (S6PDH) is a key enzyme in sorbitol biosynthesis in source leaves (Kanayama 2009). S6PDH has been purified and characterized, and cDNA encoding S6PDH also has been cloned from apple (Kanayama et al. 1992). In fruit, sorbitol is converted into fructose and glucose through NAD^+-dependent sorbitol dehydrogenase (SDH) and sorbitol oxidase (SOX), respectively. Sucrose is then synthesized or degraded by sucrose phosphate synthase (SPS), sucrose synthase (SS), and acid invertase (AIV) (Yamaki and Moriguchi 1989; Moriguchi et al. 1992; Tanase and Yamaki 2000).

The nature of the fluid in the intercellular spaces during watercore has generated much attention. Williams (1966) discovered that watercored had a consistently higher sorbitol content than non-watercored apples, and this observation has been supported repeatedly in both apples (Kollas 1968; Perring 1971; Williams and Billingsley 1973) and pears (Yamaki et al. 1976). Thus, the accumulation of sorbitol in the intercellular spaces has been speculated to be a possible cause of watercore. There are two main theories behind this accumulation of sorbitol: one is the inability to metabolize translocated sorbitol, whereas the other is increased leakage of sorbitol into intercellular spaces (Yamada et al. 2006). With regard to the former theory, a key enzyme in the sorbitol metabolic pathway in fruit is believed to be SDH, which converts sorbitol to fructose (Kanayama 2009). Sorbitol increases SDH concentration and activity in apple fruit tissue whereas fructose causes a decrease (Iida et al. 2004). Although some older reports support this theory, Marlow and Loescher (1985) suggested no evidence of a relationship between SDH and

watercore in several resistant and susceptible cultivars. In addition, it has been suggested that watercore incidence is higher in cooler regions, although no correlation was detected between sorbitol content and preharvest temperature (Tomana and Yamada 1988). Yamada et al. (1994) also reported that the onset of watercore was markedly affected by fruit temperature without any related changes in sorbitol content. They therefore concluded that the role of sorbitol metabolism was not a causative factor in the development of late watercore occurring during maturation (Yamada et al. 2006). Chun et al. (2003a) also reported that sorbitol metabolism was unlikely to be the primary cause of watercore development in the Japanese pear "Akibae."

To examine the second theory, comparison of permeability across the tonoplast and plasma membrane with each sugar in both watercored and non-watercored apples is needed. Yamaki and Ino (1992) measured the distribution of sugars in vacuoles, the cytoplasm, and intercellular spaces by using the compartmental analysis method. They found no increase in leakage of sorbitol compared to other sugars from cells in the tonoplast and plasma membrane of mature apples (Yamaki and Ino 1992). Yamada et al. (2006) also reported no significant differences in the distribution rate of sorbitol to each compartment by comparing early watercored to non-watercored "Orin" fruit. These data suggest that accumulated sorbitol in the intercellular spaces might be primarily caused by active unloading from the phloem and not increased leakage from cells.

Through structural analysis, Zhang et al. (2004) provided evidence that phloem unloading in apple fruit is apoplasmic. They revealed that a functional monosaccharide transporter localized on the plasma membrane of both sieve elements and parenchyma cells, driven by H^+-ATPase activity, is involved in phloem unloading of sorbitol. Furthermore, Gao et al. (2005) isolated two sorbitol transporter genes, *MdSOT1* and *MdSOT2*, from apples. Based on sequence analysis and characterization of heterologous expression in yeast, *MdSOT1* and *MdSOT2* were shown to be involved in the apoplastic step during unloading processes (Gao et al. 2005). They also confirmed gene expression in all sink tissue tests, with the exception of watercored tissues, and concluded that a decrease in the ability to transport sorbitol into fruit storage parenchyma tissues through lowered expression of sorbitol transporter genes would result in sorbitol accumulation in the intercellular spaces, and thereby flooded tissues with a transparent appearance. These findings support the hypothesis that accumulation of sorbitol in the intercellular spaces itself may reduce uptake into cells during maturation through a decrease in gene expression of sorbitol transporters.

10.9 Concluding Remarks

Physiological and agronomical evidence of watercore occurrence has been accumulating; however, the actual cause remains unknown, largely because of the lack of genetic studies using populations segregated into susceptible and resistant seedlings.

Studies on the mode of inheritance are also required to help define narrow- and broad-sense heritability of watercore traits. Recently, the whole-genome sequence of pears and apples was released (Velasco et al. 2010; Wu et al. 2013; Chagné et al. 2014), expanding the potential of genomic studies. Although evaluation of watercore is further complicated by fluctuations in incidence from season to season, and the fact that breeding objectives related to watercore are not highly ranked, studies utilizing the newly available genome information are expected.

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Chapter 11
Water Uptake Through the Surface of Fleshy Soft Fruit: Barriers, Mechanism, Factors, and Potential Role in Cracking

Moritz Knoche

Abstract Rain cracking of soft, fleshy fruits is thought to result from excessive water uptake through the wetted fruit surface and also through the vascular systems of the fruit pedicel. Significant new information has become available in recent years, particularly for sweet cherries and grapes. This information is reviewed with a particular focus on the mechanisms and pathways of water ingress and egress through the fruit skin and the vasculature of the fruit pedicel. The pathways of water movement through the cuticle, stomata, and lenticels are described in detail. The presence of cuticular microcracking on the fruit surface, and also of tiny areas of periderm that form in the scars created by floral part abscission (sepals, petals, anthers, styles), are discussed in relationship to their significant influences on overall fruit water balance.

Keywords Cuticle • Lenticels • Microcrack • Penetration • Periderm • Splitting • Stomata • Transpiration

11.1 Introduction

Rain cracking (syn., splitting) represents a common, and at times devastating, problem to commercial producers of soft, fleshy fruit of a number of species. Well-known examples include the cracking of sweet cherries (Christensen 1996), plums (Mrozek and Burkhardt 1973), grapes (Considine and Kriedemann 1972), tomatoes (Hankinson and Rao 1979), gooseberries, jostaberry, and black currants (Khanal et al. 2011), and blueberries (Marshall et al. 2009). However, cracking is also occasionally observed in apple (Verner 1938), orange (Garcia-Luis et al. 2001), and pepper (Moreshet et al. 1999). Most cracking occurs during and after rain. Cracked

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fruit is subject to rapid microbial spoilage and is usually not acceptable in the marketplace. In sweet cherries, overall cracking percentages of 25 % or more can make any attempt at harvest uneconomical (Christensen 1996). Much lower cracking percentages significantly impact crop quality and thus its market value.

Cracking is thought to result from surface water uptake (Christensen 1996) and possibly vascular uptake by the fruit. This uptake increases both fruit turgor and fruit volume with concomitant increases in fruit surface area. It is suggested that if fruit turgor exceeds a critical value, the skin fails ("critical turgor pressure concept:" Considine and Kriedemann 1972; Measham et al. 2009; Sekse 1995; Sekse et al. 2005). Two mechanistically unrelated sets of factors are believed to be involved in fruit cracking: category 1 factors relate to the movement of water into or out of the fruit via the fruit skin and via the vascular systems of the pedicel, and category 2 factors relate to the mechanical properties of the skin itself.

This review focuses on category 1 factors (i.e., those affecting water movements). Much of the published research on rain cracking relates to sweet cherries and to grapes, because both are major crops and also because they are particularly susceptible to cracking, often with crippling commercial consequences. Although this review focuses on these two crops, other cracking-susceptible fruit crops are mentioned where appropriate.

11.2 Barriers to Water Movement

11.2.1 The Cuticle

The cuticular membrane (CM) is a lipophilic biopolymer deposited on the outer epidermal cell walls of all fruits and leaves (Fig. 11.1). The CM functions as a barrier to the movement of water (Kerstiens 1996a; Riederer and Schreiber 2001) and in pathogen defence (Köller 1991). The thickness, structure, and chemical

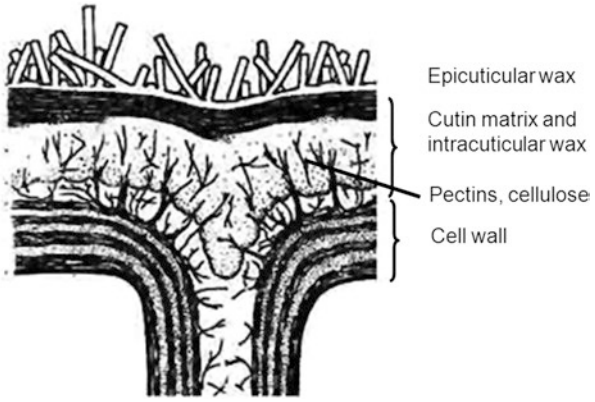

Fig. 11.1 Schematic drawing of a cross section through the cuticular membrane (CM) deposited on the outer cell wall of epidermal cells. The CM is shown in regions of the periclinal and anticlinal cell wall. (Modified from Jeffree 1986)

composition of the CM vary among species, organs, and developmental stages (Jeffree 1996; Kollatukudy 1996) (Table 11.1).

The primary constituents of the cuticle are cutin (possibly also cutan), wax, and cell wall carbohydrates (Heredia 2003). Cutin is a biopolyester comprising oxygenated C16 and C18 fatty acids, crosslinked by ester bonds (Heredia 2003). The two most abundant constituents of sweet cherry cutin are 9(10),16-dihydroxyhexadecanoic acid (53.6 %) and 9,10,18-trihydroxy-octadecanoic acid (7.8 %) (Peschel et al. 2007). In some species, cutan (a nonhydrolyzable polymethylene polymer) may also be present (Jeffree 1996; Bargel et al. 2006).

Waxes occur both as epicuticular waxes (ECW) on the surface of the cutin matrix and as intracuticular waxes (ICW) embedded within it (Fig. 11.1). The primary effect of the ECW is to modify the wetting of the surface. Wetting and, hence, wetness duration (and thus the opportunity for osmotic water uptake) depend on the physical arrangement of the superficial wax crystals (i.e., the micro-roughness) and

Table 11.1 Physical characteristics of cuticular membranes isolated from fruit of a range of plant species

Fruit type	Mass per unit area (g m^{-2})		Wax (%)	Calculated thickness (μm)	Strain release upon isolation (%)	Permeability in osmotic water uptake (P_f) (m s^{-1})	References
	Cuticle	Wax					
Apple	26.7	11.9	44.6	22.1	5.7	*n.d.*	Khanal et al. (2013)
Black currants	5.1	1.0	18.9	4.2	8.2	7.7×10^{-8}	Khanal et al. (2011)
European plum	4.7	2.3	48.7	3.9	47.1	*n.d.*	Knoche and Peschel (2007)
Gooseberry	5.6	0.6	10.5	4.6	23.8	5.2×10^{-8}	Khanal et al. (2011)
Grape	4.6	1.4	30.7	3.8	18.4	0.4×10^{-8}	Becker and Knoche (2012a), Becker and Knoche (2011)
Jostaberry	4.8	1.0	21.4	4.0	19.5	3.3×10^{-8}	Khanal et al. 2011
Sweet cherry	1.3	0.3	25.8	1.1	76.7	6.7×10^{-8}	Peschel and Knoche (2012)
Tomato	21.2	1.1	5.2	17.5	*n.d.*	*n.d.*	Petracek and Bukovac (1995)

Thickness estimate calculated from mass per unit area divided by the specific gravity of 1,210 kg m^{-3} (Petracek and Bukovac 1995)
n.d. not determined

also on the functional groups exposed on the surface (Holloway 1969, 1970). The ICW, and particularly the crystalline portion thereof, is thought to affect the water permeability of the CM (Riederer and Schneider 1990). This fraction is located within the cuticle and is considered impermeable to water. Therefore, there is no simple relationship between the amount of wax in a CM, or CM thickness and the CM water permeability. For example, fruit CMs are often much thicker than leaf CMs but they are generally more water permeable (Becker et al. 1986). In sweet cherry, ECW and ICW account for 72 % and 26 %, respectively, of the resistance in transpiration (Knoche et al. 2000).

Wax is a complex mixture of long-chain C20–C40 alcohols, aldehydes, fatty acids, and alkanes (Kunst and Samuels 2003; Samuels et al. 2008). In sweet cherry, triterpenes, alkanes, and alcohols account for 75.6 %, 19.1 %, and 1.2 % of total wax, respectively (Peschel et al. 2007). The most abundant constituents are triterpenes (ursolic, 60.0 %; oleanolic acid, 7.5 %), alkanes (nonacosane, 13.0 %; heptacosane, 3.0 %), and a secondary alcohol (nonacosan-10-ol, 1.1 %).

The inner surface of the CM is rich in cell wall carbohydrates whose mass can constitute as much as 18–22 % of the CM (Schreiber and Schönherr 1990) (Fig. 11.1).

In a number of fruit crops, including sweet cherries, plums, grapes, tomatoes, peppers, apples, and pears, the CMs are sufficiently robust that they may be separated enzymatically (pectinase and cellulase) from the underlying cell wall and handled (carefully) in the laboratory as isolated units (Orgell 1955; Yamada et al. 1964).

Rates of cutin and wax deposition in fruit are usually high during early in development but decrease toward maturity (Becker and Knoche 2012a; Khanal et al. 2011). In sweet cherry there is essentially no deposition of CM in the later stages because the genes involved in cutin and wax synthesis are downregulated (Alkio et al. 2012).

When CM deposition keeps pace with surface-area expansion, CM thickness remains constant. Increases in CM thickness during development (as in apple) indicate that deposition rates exceed surface-area expansion rates (Casado and Heredia 2001; Knoche et al. 2011). However, when the CM deposition rate is low or negligible, as in sweet cherries (Knoche et al. 2004) and European plums (Knoche and Peschel 2007), surface expansion strains the CM, resulting in considerable thinning. The inability of a CM polymer to accommodate a fruit surface-area expansion rate can result in the formation of CM microcracks (Becker and Knoche 2012a, b; Knoche and Peschel 2007; Peschel and Knoche 2005). In addition, surface wetness or exposure to high humidity can also induce microcracking in sweet cherries (Knoche and Peschel 2006), grapes (Becker and Knoche 2012b), and apples (Knoche and Grimm 2008). Thus, regions of a fruit surface that for geometric reasons experience extended periods of wetness following rain or dew, such as a sweet cherry stylar scar (a hanging droplet) or its stem cavity (cup shaped), usually exhibit increased densities of microcracks (Peschel and Knoche 2005). Conversely, cherries grown in greenhouses or under rain shelters usually have reduced densities of microcracking.

The skin of sweet cherries (Peschel et al. 2003), apples (Blanke 1987), currants (Blanke 1993), and grapes (Blanke and Leyhe 1987), but not that of tomatoes or peppers, is stomatous. Stomata on young fruit are usually functional (i.e., they can open and close) but later on they often become fixed in a partially open state (Peschel

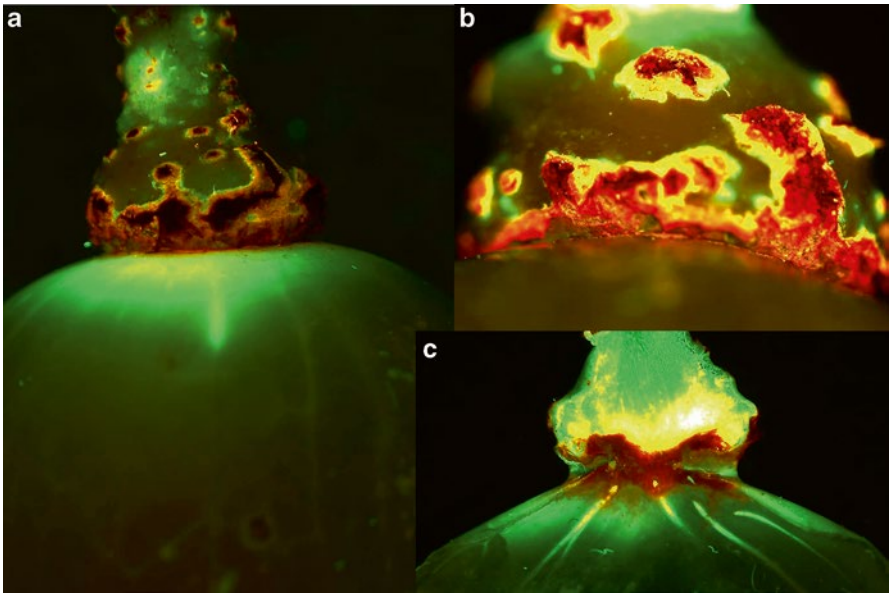

Fig. 11.2 Fluorescence micrographs of "Riesling" grape berries incubated in an aqueous solution of acridine orange. Water and dye uptake was restricted to the undamaged surface of pedicel and fruit by sealing the cut pedicel end. **a** Overview. **b** Receptacle with abscission zones of sepals, petals, and anthers. **c** Cross section through berry and pedicel in proximal region. **a**, **c** from Becker et al. (2012); **b** from E. Grimm (unpublished data)

et al. 2003). They may also become clogged with waxes (Bukovac et al. 1999) or be torn apart by excessive growth strains (Knoche and Peschel 2007). Stomatal densities on fruit, in contrast to leaves, are usually very low. In sweet cherries, for example, stomatal density ranges from 0.12 to 2.13 mm^{-2} (Peschel and Knoche 2012), whereas in the leaves stomatal densities were two to three orders of magnitude greater (458–543 mm^{-2}) (Goncalves et al. 2008). An internal cuticle lines the stomatal pore, the inner surface of the guard cells, and the substomatal chamber (Franke 1967; Norris and Bukovac 1968).

11.2.2 Periderms

A periderm is a secondary dermal tissue. Periderms occur over the entire fruit surface in some cultivars of some fruit crops (e.g., "Beurre Bosc" pears) or, more commonly, over parts of the surface as a response to wounding. Wounds include those caused by mechanical damage such as wind rubbing, impact, and the attentions of predators (also sometimes from spray damage). Wounds also arise as a result of normal growth and development wherein the integrity of the cuticle is breached following the abscission of a flower organ (styles, stamens, petals, sepals), such as in grape (Fig. 11.2) or the stem scar of a tomato. A periderm formed when a stomatum

is torn apart by normal growth is referred to as a lenticel (Shaheen et al. 1985). Lenticels occur on the surfaces of apples and grapes but not of cherries, currants, gooseberries, or jostaberries. With russeting, a larger area of periderm is formed that is rough in texture and is visible to the naked eye as a dull red-brown color. Russeting is usually preceded by microcracking of the cuticle (Faust and Shear 1972a, b).

Periderms are composed of phellogen, phellem, and phelloderm. Because suberin incrusts the cell walls, a phellem (syn., cork tissue) presents a significant barrier to water transfer (Franke and Schreiber 2007). Transition regions between cuticle and periderm often exhibit significant microcracking, possibly because of the different rheological properties (stiffness) of the CM and periderm or differences in strain concentration (Becker et al. 2012; Brown and Considine 1982; Khanal et al. 2013).

11.3 Mechanism of Water Transfer

Water transfer into and out the fruit may occur through the surface as transpiration or surface uptake (from liquid and vapor phase) or through the vasculature of the pedicel.

11.3.1 Transpiration

Transpiration is a physical process and is independent of metabolic energy. It is commonly described using Fick's law of diffusion (Eq. 11.1), wherein F_{trans} represents the flow rate (kg s^{-1}), J_{trans} (kg m^2 s^{-1}) the flux density, A (m^2) the surface area available for transpiration, ΔC (kg m^{-3}) the difference in water vapor concentration between the interior of the fruit (C_{fruit}; kg m^{-3}) and the surrounding atmosphere (C_{out}; kg m^{-3}), and P_{trans} (m s^{-1}) the permeance to water vapor of the fruit surface. Because a pressure difference across the cuticle is absent, F_{trans} is purely diffusional.

$$F_{trans} = J_{trans} \bullet A = A \bullet P_{trans} \bullet \Delta C = A \bullet P_{trans} \bullet \left(C_{fruit} - C_{out} \right) \tag{11.1}$$

Because the concentration of water vapor may be expressed in various units (e.g., as a mol fraction, a partial pressure, or an absolute concentration), the units of F also vary. A liquid water-based permeance for transpiration may also be calculated: this is lower than the vapor-phase permeance by a factor of 43,212 at 25 °C [the ratio of the density of liquid water at 25 °C (996.9 kg m^{-3}) divided by the saturated water vapor concentration at that temperature (23.07 g m^{-3})]. Whatever the basis of calculation, the material constant P_{trans} reflects the characteristics of a particular surface and is largely independent of the experimental setting, provided that a boundary layer is absent. Such material constants are useful in making comparisons among species, cultivars, systems, and experiments.

To determine P_{trans}, the steady state F_{trans} is established under defined conditions. The A of a fruit is usually estimated from measurements of either its volume or its

orthogonal dimensions and assuming a simple geometric model (a sphere, ellipsoid, truncated cone, etc.). Under experimental conditions, the driving force can be standardized by incubating the fruit (or diffusion cell with epidermal segment or isolated cuticle) in a closed container at constant temperature and humidity (and low light intensity) above a water receiver such as dry silica gel or a saturated salt slurry (Beyer et al. 2005; Knoche et al. 2000; Wexler 1995). To minimize the in-series boundary layer (and so measure the pure skin property), the vapor phase should be vigorously stirred or the distance between the transpiring surface and the silica gel (or salt, etc.) minimized. Obviously, it is important that the sample does not make direct physical contact with the water donor/receiver material. Weight loss is usually established gravimetrically by repeated weighings. From the cumulative weight loss with time, F_{trans} is calculated by linear regression. Because the humidity inside the container is known and the internal atmosphere of the fruit is assumed to be saturated with respect to water vapor, ΔC can be calculated. When incubating above dry silica, the RH is practically zero (Geyer and Schönherr 1988) and ΔC is essentially equal to the water vapor concentration at saturation at the relevant temperature. These standard values are tabulated in textbooks (Nobel 1999).

Because the CM represents the primary barrier to transpiration, the flow rate determined will not depend on whether it is established on a whole fruit, an excised epidermal segment, or (for astomatous surfaces) an isolated CM mounted in a diffusion cell. The latter systems have the potential advantage of focusing the measurement on a particular region of the surface. However, whole-fruit systems are easier and they also maintain any natural strain in the cuticle (Knoche et al. 2004) or underlying cell layers (Grimm et al. 2012a). Also, they are more robust, particularly in species having thin, delicate CMs. Suitable designs for diffusion cells have been described by Geyer and Schönherr (1988). These cells may be used with isolated CMs or excised epidermal segments (Harz et al. 2003). The P_{trans} established represents the weighted mean of all parallel water pathways contributing to transpiration through the sample.

11.3.2 Uptake of Liquid Water Through the Fruit Surface

Osmotic water uptake occurs through the wetted fruit surface following dew or rain. As with transpiration, water uptake proceeds along the same parallel pathways.

In analogy to transpiration, the rate of osmotic water uptake (F_{uptake} in kg s^{-1}) through the fruit surface may be calculated using a transport coefficient and a driving force. In uptake, this transport coefficient is the hydraulic conductivity coefficient (L_w in m s^{-1} MPa^{-1}). The ρ_w equals the density of water (kg m^{-3}). The driving force equals the difference in water potential ($\Delta\Psi$ in MPa) between the fruit (Ψ_{fruit} in MPa) and that of the water on the surface (Ψ_{water} in MPa; Eq. 11.2) (Beyer et al. 2005). Because the water potential of the water adhering to the fruit surface is zero ($\Psi_{water}=0$ MPa), the water potential of the fruit drives uptake ($\Delta\Psi=-\Psi_{fruit}$; Eq. 11.2). Fruit water potential in turn represents the sum of fruit osmotic potential

and turgor. In the presence of porous pathways such as polar pathways, cracks, stomata, lenticels, or periderm scars, F_{uptake} may have a viscous component (through the pores) in addition to a diffusive component through the nonporous part of the cuticle.

$$F_{uptake} = A \bullet J_{uptake} = A \bullet L_w \bullet \rho_w \bullet \left(\Psi_{water} - \Psi_{fruit}\right) = A \bullet L_w \bullet \rho_w \bullet \left(-\Psi_{fruit}\right) \quad (11.2)$$

To convert the hydraulic conductivity coefficient L_w (m s^{-1} MPa^{-1}) into an osmotic water permeability coefficient or filtration permeability constant (P_f in m s^{-1}) Eq. (11.3) may be helpful (House 1974; Schönherr 1982):

$$P_f = L_w \bullet \frac{RT}{V_w} \quad (11.3)$$

In this equation, RT represents the product of the universal gas constant (R) and the absolute temperature (T; $RT = 2.479 \times 10^{-3}$ m^3 MPa mol^{-1} at 25 °C), and V_w the partial molal volume of water (1.8 cm^3 mol^{-1}).

Estimates for Ψ_{fruit} are difficult to obtain and only a few results have been published (for sweet cherry, see Andersen and Richardson 1982; Beyer and Knoche 2002). Different procedures have been employed to determine Ψ_{fruit}. First, the pressure bomb has been used to quantify Ψ_{fruit} (Andersen and Richardson 1982; Measham et al. 2009). However, the pressure bomb measures apoplast pressure (not apoplast water potential; Scholander et al. 1965). The measured pressure is numerically equal to the water potential only when the osmotic potential of the apoplast is zero. This special condition is not necessarily met (indeed, it is unlikely) in a fruit's apoplast where solutes commonly accumulate (Matthews and Shackel 2005). Second, Ψ_{fruit} has been quantified by determining F_{uptake} (usually by serial weighings) from osmotica in a stepped concentration series, followed by regression and interpolation to estimate the osmotic potential for zero water exchange (Weichert and Knoche 2006a). At this point, the $\Delta\Psi$ for water uptake is taken to be zero and thus the osmotic potential of the incubation solution (easily measured) should be equal to Ψ_{fruit}. This interpretation assumes the absence of significant diffusion of solutes either into the fruit from the osmoticum or into the osmoticum from the fruit.

To quantify P_f, a fruit is incubated in deionized water. At selected time intervals it is removed, quickly dried, weighed, and reincubated for a further period. The fruit surface area is quantified as described earlier and the driving force is taken as being equal to the fruit water potential. Alternatively, for fruit surfaces of very high water permeability (such as sweet cherries), a diffusion cell (such as described by Beyer et al. 2002) may be employed. These systems are inherently more difficult to operate in uptake mode than transpiration mode because water uptake occurs into an essentially closed volume.

Three important consequences of the differing transfer mechanisms for water uptake and transpiration are worth noting. First, the skin permeance for water uptake (P_f) is more variable than that for transpiration (P_{trans}). Second, surface defects have a markedly larger effect on P_f than on P_{trans} and this is likely to account for the higher

variability. Third, there is little effect of temperature on osmotic water uptake (Beyer and Knoche 2002), whereas the temperature dependence of transpiration is much higher. Under field conditions, this is primarily caused by the increase in the water vapor concentration deficit and, hence, in driving force as temperature increases.

Data on P_f are summarized in Table 11.1.

11.3.3 Surface Uptake of Water Vapor

In theory, at least, water uptake may also occur from the vapor phase. For a sweet cherry fruit having an osmotic potential of −2.8 MPa, the equilibrium humidity above which water vapor uptake would occur is about 98 % (Beyer et al. 2005). Above this humidity, fruit will take up water from the vapor phase even though the surface is dry. However, the rates of vapor uptake from a saturated atmosphere will be low and negligible compared to water uptake from the liquid phase (Beyer et al. 2005). Moreover, air humidity rarely exceeds 99 % under field conditions (even in the rain; Nobel 1999). Thus, vapor uptake is unlikely to be an important factor in cracking.

11.3.4 Vascular Water Uptake Through the Pedicel

The vascular system of the pedicel is a parallel route for water entry into the fruit. Considerable effort has been devoted to the analysis of vascular flow, particularly into grapes and, to a lesser extent, into sweet cherries.

In grapes, it is assumed that transport through the xylem and phloem occurs during development stages I and II, but that through the xylem ceases at around veraison (beginning of stage III development), leaving the phloem as the primary route for water import through the pedicel (Coombe and McCarthy 2000). Decreased xylem transport may be related to (1) mechanical rupture of the xylem as a result of growth strain (Düring et al. 1987), (2) increased resistance of the xylem in the brush region of the fruit (Rogiers et al. 2001; Tyerman et al. 2004), or (3) a lack of driving force for xylem transport, as suggested by Bondada et al. (2005). The decrease in xylem water transport coincides with a reduced tension in the berry xylem, probably because solutes accumulate in the apoplast. These solutes prevent the buildup of turgor as carbohydrates accumulate in the grape (Matthews and Shackel 2005; Wada et al. 2009) and most likely also in sweet cherry (Knoche et al. 2014).

Because driving forces and cross-sectional areas for vascular transport in the two conducting systems of the pedicel are difficult to quantify and because of the complex interaction with whole-tree/vine–water relationships, analysis of vascular flows is largely descriptive. The experimental systems employed for studying pedicel transport include tracer studies in which the berry pedicel is fed with dyes and the dye distribution subsequently monitored (Keller et al. 2006), potometric techniques where a calibrated glass capillary is attached to the fruit pedicel and the movement

of a meniscus driven by transpiration is monitored over time (Hovland and Sekse 2004a, b), and pressure probes that allow direct quantification of xylem tension or the monitoring of flow rates at defined driving forces (Tyerman et al. 2004). A highly original and elegant approach was used by Lang and Thorpe (1989) in grape where fruit volume was measured with high resolution using Archimedes' principle allowing quantification of xylem, phloem, and transpiration flows in the attached grape berry. More recently, Measham et al. (2010) used heat pulse sensors to quantify net vascular flows in sweet cherries. The flow rates reported ranged from 0.8 and 1.5 mg h^{-1} (Measham et al. 2010) to 3.0 to 11.6 mg h^{-1} (Hovland and Sekse 2004a, b). For comparison, the net increase in fruit volume as estimated from the maximum rate of sweet cherry growth amounted to about 11.2 to 14.6 mg h^{-1} (Peschel and Knoche 2005).

11.4 Pathways for Water Movement

Transpiration and water uptake can occur along several possible pathways which are aligned in parallel; these include through the fruit–pedicel vasculature, the cuticle, the pedicel–fruit junction, stomata, lenticels, microcracks, or the stylar scar–fruit junction. The algebraic sum of all the flows along these pathways equals the net inflow, and this the rate of fruit volume growth. It is important to note that because transport mechanisms differ between transpiration and uptake, so will the relative contributions of flows along the individual pathways to total flow.

In the laboratory, the pathways for many of these flows may be isolated experimentally using selective applications of a sealant. For water uptake, a rapid-curing, nonphytotoxic silicone rubber is useful, whereas for transpiration a two-component epoxy adhesive is superior.

In the subsequent sections, results on water flows through the fruit surface and along noncuticular pathways are reviewed.

11.4.1 Polar Pathways in the CM

The concept of polar pathways was originally developed to account for the penetration of polar substances through a lipophilic CM (Franke 1967). This concept, although still controversial (Fernandez and Eichert 2009), has received renewed interest (Schönherr 2006). Polar pathways are dynamic transient structures in the CM of some species that result from orientation and hydration of polar functional groups (Schönherr 2006). Because the aqueous continuum forms only on hydration, polar pathways allow rapid water uptake by viscous flow along a hydraulic continuum, but they have no influence on transpiration rate. Polar pathways also occur in sweet cherry fruit (Weichert and Knoche 2006a). First, the permeability of (osmotic) water uptake P_f exceeds that in self-diffusion, that is, that of ^3H_2O or D_2O in H_2O

(House 1974). Second, uptake of polar substances depends on the size of the penetrant. Third, water uptake decreases as viscosity increases. Fourth, the activation energy for penetration (E_a) is lower for dissociated and, hence, more-polar organic acids than for the nondissociated less-polar species. Fifth, polar pathways may be plugged by precipitation reactions, causing P_f to decrease (Weichert and Knoche 2006b). It is interesting to note that the change in E_a and the decrease in P_f obtained by plugging polar pathways were both linear functions of stomatal density. These observations are consistent with an increase in the density of polar pathways at, or around, stomata.

11.4.2 Pedicel–Fruit Junction

There is no published information on transpiration at the pedicel–fruit junction. However, one may expect junction transpiration to be small because (1) the cross-sectional area of the gap is small and (2) diffusion through the gap (in transpiration) is slow as compared to viscous flow through the gap (in osmotic water uptake).

Dissimilar to transpiration, the pedicel–fruit junction serves as a site for preferential water uptake in sweet cherries (Beyer et al. 2002) and grapes (Becker et al. 2012; Fig. 11.2). In sweet cherries, junction penetration was found to increase during development and to be inversely related to the force required to remove the pedicel from the fruit. In addition, junction penetration is highly variable (Beyer et al. 2002). On average, at maturity, 46 % (range, 27–73 % depending on cultivar) (Weichert et al. 2004) of the uptake into a sweet cherry submerged in deionized water (with the cut pedicel end sealed) occurred along the pedicel–fruit junction. From a practical point of view, preferential uptake in this region is particularly important in sweet cherry because dew and rainwater tend to collect in the pedicel cavity, resulting in extended durations of wetness.

In “Riesling” grapes, penetration rates at the pedicel–fruit junction averaged 24 % (range, 18–29 %) of those into a fruit submerged in deionized water (Becker et al. 2012).

11.4.3 Stomata and Lenticels

Even when closed, stomata in leaves have increased water vapor permeance compared to the surrounding CM (Kerstiens 1996b). However, because stomatal density is usually low in fruit, their contribution to whole-fruit transpiration is low. In sweet cherries, transpiration is weakly but significantly related to stomatal density ($r^2 = 0.09^{***}$, $n = 918$) (Peschel and Knoche 2012). From these relationships the contribution of stomatal transpiration to whole-fruit transpiration for an average sweet cherry is estimated at only about 25 %, whereas the remaining 75 % would be cuticular transpiration (Peschel and Knoche 2012).

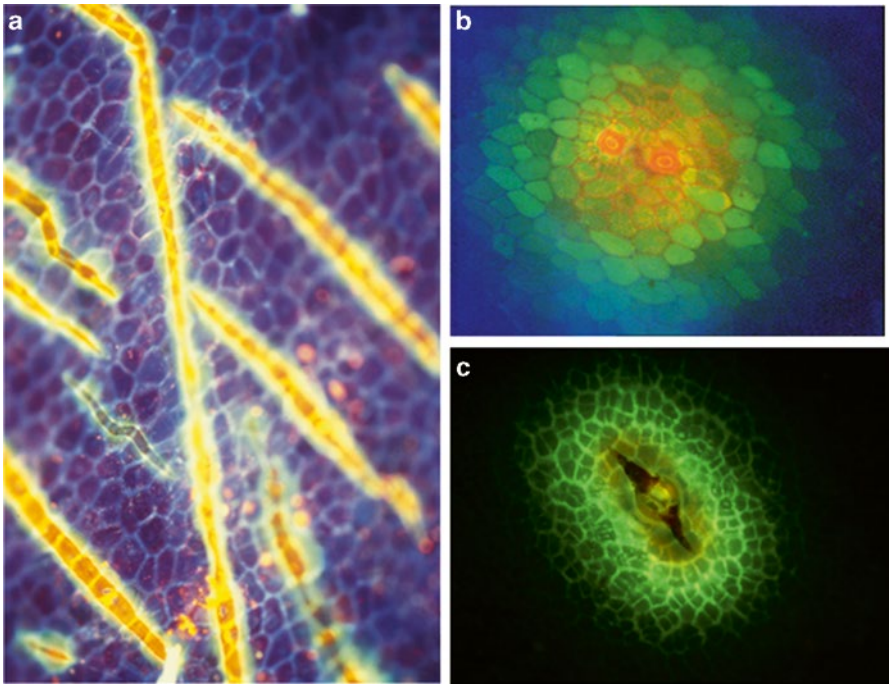

Fig. 11.3 Microscopic cracks (**a**) and stomata (**b**) on the surface of a mature sweet cherry fruit. **c** A microcrack traversing a stomatum on the surface of European plum. In this species most cracks are initiated at or near a stomatum. Fruit were incubated in an aqueous solution of acridine orange, removed after 10 min, blotted dry, and then viewed using a fluorescence microscope. The *yellow*/*red*/*greenish* areas represent infiltration zones where the dye penetrated. (All images from S. Peschel, unpublished data)

When sweet cherries are incubated in a solution of acridine orange, a fluorescent infiltration zone around the stomatal guard cell complex is apparent (Fig. 11.3).

Although stomatal pores represent significant openings in the fruit surface, the geometry and dimensions of the pore, the wetting characteristics of the surface, and the high surface tension of rainwater prevent the entry of liquid water through an open stomatum (Schönherr and Bukovac 1972). Eichert and Goldbach (2008) argue that some penetration may occur along water films lining the stomatal pore.

No evidence has been presented for viscous flow of liquid water through the stomatal pore except when a significant external pressure has been imposed or when an aqueous solution of very low surface tension has been applied. The critical surface tension (γ_{crit}) for the surface of a sweet cherry fruit averages 24.9 mN m^{-1} (Peschel et al. 2003). Only when a fruit is incubated in a solution having a surface tension less than γ_{crit} will significant stomatal penetration be expected (Schönherr and Bukovac 1972; Zisman 1964). Under these conditions, stomatal penetration is readily detectable from the coloring of incubation solutions caused by the leaching of anthocyanins. Despite these observations, the permeability of osmotic water

uptake is weakly but significantly correlated with stomatal density ($r^2 = 0.04^{**}$, $n = 236$) (Peschel and Knoche 2012). Because viscous flow through the open stomatal pore is unlikely, the positive relationship between permeability and stomatal density may be accounted for by an increased density of polar pathways in the vicinity of the stomatal apparatus (Beyer et al. 2005; Schönherr 2006).

11.4.4 Sepal, Petal, Anther, and Stylar Scars, Lenticels

When flower parts abscise during early fruit development, tiny areas of periderm form at the points of abscission. In some fruit crops, such as currants, jostaberry, and gooseberry, the remnants of the dried flowering organs remain attached to the fruit apex. These structures may serve as preferential routes for water uptake and can account for about 30 % of the uptake of a submerged berry (Khanal et al. 2011). The role of these organs in transpiration does not seem to have been assessed.

In grapes, the lenticels on the surfaces of the berry, on the receptacle, and on the pedicel are involved in free water uptake, as is indicated by preferential staining in these areas following incubation in aqueous fluorescent dye solution (Becker et al. 2012; Fig. 11.2). For mature "Riesling" berries submerged in water, of the total water uptake, that through the stem surface including the pedicel–fruit junction averaged 55 %, whereas that through the berry surface averaged only 45 % (data for 96–144 h; Becker et al. 2012). Fluorescence microscopy further indicates that this stem uptake occurs primarily through lenticels on the pedicel, the periderms of the abscission zones of the flowering organs, and through microcracks within and in the immediate vicinity of the periderms (Becker et al. 2012). Microcracking in these regions occurs at high frequency, probably as a result of growth stresses along the boundaries between tissues of differing elasticity (Brown and Considine 1982).

There is no indication of preferential water uptake or transpiration through the stylar scar of a sweet cherry. However, as a result of extended periods of surface wetness and stress concentrations, the stylar scar region often exhibits severe microcracking that will result in increased water uptake (Peschel and Knoche 2005).

11.4.5 Microcracks

Microcracks are sites of preferential water movement (Fig. 11.3). They increase transpiration and are causal in shriveling symptoms such as in neck shriveling in European plums or the shriveling associated with skin-spot disorder in "Elstar" apples (Grimm et al. 2012b).

Microcracks are even more important in uptake, because they allow water to bypass the cuticle as a penetration barrier and to enter the fruit by viscous flow. Simulating microcracking in the cuticle of mature "Huxel" grape berries increased water uptake 47 fold but transpiration only 8 fold compared with intact controls

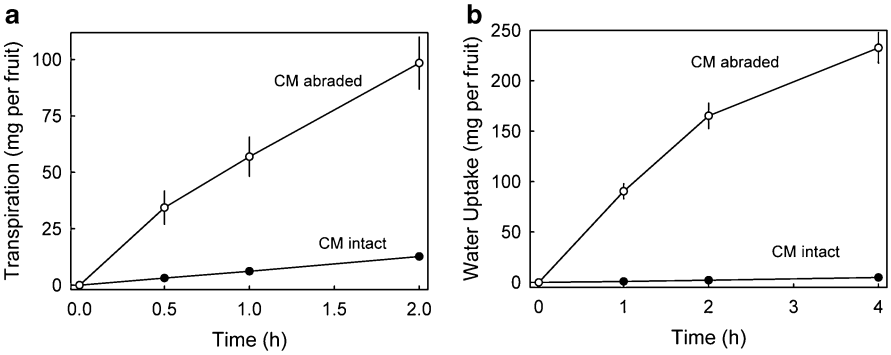

Fig. 11.4 Effect of abrading the cuticular membrane (*CM*) on the time course of transpiration from (**a**) and water uptake into (**b**) mature "Huxel" grape berries. (From Becker, unpublished data)

(Fig. 11.4). Similarly, water uptake rates through exocarp segments of sweet cherry fruit that had microcracking were almost double those in control segments (Peschel and Knoche 2005).

It is important to note that microcracking also facilitates infection with pathogens (Blaich et al. 1984; Borve et al. 2000; Knoche and Peschel 2007).

11.5 Water Uptake and Fruit Cracking

The category 2 factors that affect cracking susceptibility relate to those affecting the mechanical properties of the fruit. This aspect is technically challenging and has received little attention till now. Experimental approaches used to address the mechanical properties include fruit pressure probe techniques (Bernstein and Lustig 1985; Lang and Düring 1990) and biaxial tensile tests (Bargel et al. 2004; Brüggenwirth et al. 2014). Using the fruit pressure probe technique, bursting pressures, strains, and volumetric moduli of elasticity were quantified in different grape berry cultivars (Bernstein and Lustig 1985; Lang and Düring 1990). Biaxial tensile tests were employed by Bargel et al. (2004) and Brüggenwirth et al. (2014) to quantify the modulus of elasticity, fracture strains, and fractures pressures of excised sweet cherry fruit skin. No attempts to relate a fruit skin's mechanical characteristics to its anatomical and physiological one seem to have been published.

A robust way to relate differential cracking susceptibility to the mechanical properties of a fruit skin is to quantify its "intrinsic cracking susceptibility" (Weichert et al. 2004). In this assay, time courses of water uptake and cracking are determined on the same batch of fruit. The percentage of cracked fruit is recorded at each time interval and expressed as a function of the cumulative water uptake to that point. This assay automatically normalizes cracking for differences in water uptake rate between batches. In analogy to the LD_{50}, the amount of water uptake at

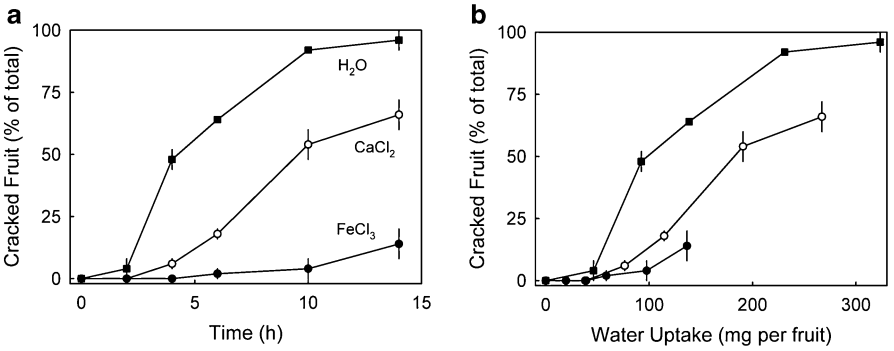

Fig. 11.5 (**a**) Time course of cracking of mature Summit sweet cherries when incubated in deionized water, 25 mM $CaCl_2$ or 25 mM $FeCl_3$. (**b**) Same data as in **a**, but redrawn as a function of cumulative water uptake from the respective solutions. Cumulative water uptake was calculated from rates of water uptake (F_{uptake}) multiplied by the incubation time. The F_{uptake} values were 23, 19, and 10 mg h^{-1} for fruit incubated in H_2O, $CaCl_2$, and $FeCl_3$, respectively. (Data redrawn from Weichert et al. 2004)

50 % cracking is calculated using regression and interpolation and is expressed as a WU_{50} (mg per fruit). This parameter reflects the mechanical constitution of the fruit, that is, its intrinsic cracking susceptibility.

An example of the effects of $CaCl_2$ and $FeCl_3$ on the cracking of sweet cherry fruit and the intrinsic cracking susceptibility is given in Fig. 11.5. Both $CaCl_2$ and $FeCl_3$ decrease fruit cracking compared to control fruit incubated in water. However, $CaCl_2$ ($F_{uptake} = 19$ mg h^{-1}) and $FeCl_3$ ($F_{uptake} = 10$ mg h^{-1}) also decreased the rates of water uptake as compared to the controls ($F_{uptake} = 23$ mg h^{-1}). Therefore, it may be argued that decreased cracking results simply from decreased water uptake compared to the controls. However, by expressing the percentage of cracking as a function of the amount of water taken up it can be demonstrated that $CaCl_2$, and to an even greater extent $FeCl_3$, also decrease the intrinsic cracking susceptibility. Thus, sweet cherries incubated in $CaCl_2$ ($WU_{50} = 183$ mg per fruit) or in $FeCl_3$ ($WU_{50} \gg 183$ mg per fruit) are able to sustain more water uptake for 50 % cracking compared to fruit incubated in water ($WU_{50} = 95$ mg per fruit; Fig. 11.5). The decreased intrinsic cracking susceptibility was attributed to increased crosslinking of cell wall components (Weichert et al. 2004).

11.6 Conclusions

Significant progress has been made in understanding and analyzing water movement through the fruit surface and along the vascular system of the pedicel of soft and fleshy cracking-susceptible fruit. Compared to water movement through the fruit skin, movement through the vascular system is more complex because (1) two

conducting systems are involved and (2) cross-sectional areas for movement and driving forces are usually unknown and thus are difficult to quantify experimentally. Furthermore, driving forces are affected by whole tree/vine–water relationships and, therefore, subject to rapid changes, making realistic mechanistic modeling difficult. Nevertheless, some descriptive data on net water flows have been published that allow the establishment of simple mass balances for water movement. Further progress will be made when a way is found to quantify the water potential of an individual fruit. Meanwhile, because turgor pressure is essentially absent in a mature grape or sweet cherry (and probably in other cracking-susceptible fruit crops also), fruit osmotic potential should be a useful indicator of whole-fruit water potential. Knowledge of fruit water potential would allow skin permeability for osmotic water uptake to be quantified on an individual (whole) fruit basis. The absence of significant turgor pressure and the complex interaction between fruit and tree/vine–water relationships through the vascular continuum also imply that a simple relationship between water uptake and cracking as predicted by the critical turgor pressure concept may not be present.

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Part IV
Genetic Engineering Technologies and Omics in Stress Tolerance

Chapter 12
Promoters for Transgenic Horticultural Plants

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Abstract Gene engineering provides an opportunity to obtain plants with either silenced or overexpressed target genes. This approach is frequently used to study various aspects of the biochemistry, physiology, and stress tolerance of horticultural plants. Selection of appropriate promoters to govern transcription of a transgenic construct is an important step in the development of efficient genetic models. Here we present a brief overview of available literature data on the promoters investigated with the transgenic horticultural plants and some valuable information resources in this filed.

Keywords Gene engineering • Horticulture • Promoter • Stress-responsive • Tissue-specific • Transgenic plant

12.1 Introduction

The usefulness of transgenic plants for both pure and applied research is beyond question. Genetic constructs can be introduced into plant genomes to make selective changes in the expression patterns of the genes under investigation, to influence various plant physiological and biochemical characteristics, and to increase production of recombinant proteins and valuable secondary metabolites. Indeed, an efficient genetic modification demands an application of well-designed genetic construction typically

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containing one or more transgenes (coding for either proteins or microRNA) or inducing RNA interference. In many cases, the pattern of a transgene expression should be restricted by certain tissues or stages of development to meet the specific criteria of the experiment. Thus, an important step in the transgenic construct design concerns the selection of appropriate promoters. However, the number of commonly used promoters is rather small and is defined by available vector systems. Indeed, it is possible to clone promoters with appropriate characteristics of an experiment to make the genetic construct more efficient for the particular purposes. A large volume of experimental data concerning plant promoter functional activities is available in the literature because these investigations help to reveal the expression patterns of genes under study. In most cases the promoter (full-sized and variants with deletions) controls transcription of a reporter gene such as *GUS* (β-glucuronidase) or *GFP* (green fluorescent protein), and its activity can easily be visualized. Thus, it is possible to find a promoter with desirable characteristics by searching through these publications. However, this process is time consuming (more than a thousand plant promoters are described in the literature, and this number is rapidly increasing). Several reviews about virus and plant promoters used for overproduction of recombinant proteins in plants (Egelkrout et al. 2012), metabolic engineering of plant defense pathways (Jirschitzka et al. 2013), genetic transformation of fruit trees (Gambino and Gribaudo 2012), and multigene transformation (Peremarti et al. 2010) are available. We have developed the TransGene Promoters Database (TGP; Smirnova et al. 2012) to annotate plant promoters with known characteristics. TGP accumulates data on the promoter nucleotide sequences and their specific activities in transgenic plants. This instrument (periodically updated but still far from being complete) could also be useful for selection of candidate promoters with characteristics appropriate for particular experimental needs. In this chapter, we present a brief overview of available literature data concerning a wide range of plant promoters of which activities were experimentally evaluated on the experiments with transgenic horticultural plants.

12.2 Apple

Fruit trees have a relatively long juvenile growth period to reach the flowering stage, which makes experiments with transgenic plants time consuming.

AFL (Apple FLORICAULA LEAFY) 1 and 2 gene promoters of *Malus domestica* clearly displayed GUS reporter staining in transgenic semi-dwarf apple trees cv. JM2. The *MdAFL1* promoter is expressed at a much lower level than *MdAFL2* promoter, but the expression pattern was almost the same (the meristem of the shoot apices, lateral axils, and leaf primordia). The expression levels of both *AFL1* and *-2* genes in vegetative tissues were much lower than that in floral buds (Wada et al. 2009).

MdTFL1 (TERMINAL FLOWER1) transcription is strongly related to juvenility/vegetative and flower initiation. The *MdTFL1*:*GUS* expression was induced strongly throughout newly generated young shoots of cytokinin-treated transgenic apple plants and in apices of shoots on the medium containing cytokinin and auxin. These results suggest that cytokinin may induce *MdTFL1* expression (Mimida et al. 2011).

Ribulose bisphosphate carboxylase small subunit (*RBCS*) gene promoters from tomato (*Lycopersicon esculentum*) and soybean (*Glycine max*) were highly active in the leaves of apple (*Malus pumila*), whereas much lower GUS activity was found in petioles, green stems, and roots. In leaf tissue the mean GUS activity for the *LeRBCS3C* and *GmRBCS1* promoters was approximately half that of the promoter of 35S RNA of cauliflower mosaic virus (*35S*) and was localized to the mesophyll and palisade cells of the leaf. The activity of the *GmRBCS1* promoter was strictly dependent on light, whereas that of the *LeRBCS3C* promoter appeared not to be. Both promoters would be suitable for the expression of transgenes in green photosynthetic tissues of apple (Gittins et al. 2000).

The *Brassica napus* extensin A (*ExtA*) gene is highly expressed in root tissue of oilseed rape. A-940 *BnExtA* promoter was active in all tissues of young transgenic apple plants (*Malus pumila* cv. Greensleeves). Specific GUS activity was highest in the stems and was comparable with standard *35S* promoter. Although the *BnExtA* promoter is clearly not root specific in apple, it could target the expression of transgenes to the developing stem tissues. It was proposed that this promoter could be used to drive expression of technologically valuable transgenes resulting in dwarfing, or altering lignin composition; both belong to major goals of tree biotechnology (Gittins et al. 2001).

The ability of the potato (*Solanum tuberosum*) glutathione *S*-transferase gene (*Gst1*) promoter to drive expression of the *GUS* reporter gene was determined in two genotypes of apple: the fruit cultivar Royal Gala and the M.26 rootstock. In both genotypes, the *StGst1* promoter was systematically activated in apple following infection with a fungal pathogen. It was also activated by salicylic acid, but not by wounding. Although the *StGst1* promoter is less active than the *35S* promoter in apple, its characteristics could be useful to drive the expression of transgenes involved in resistance to diseases (Malnoy et al. 2006).

The stilbene synthase (*Vst1*) gene of *Vitis vinifera* under control of its own promoter was transferred into the commercially important apple cultivars Elstar and Holsteiner Cox. *Vst1* transcription was detected after induction of the *VvVst1* promoter by wounding or UV irradiation (Szankowski et al. 2003). Molecular analysis revealed that stilbene synthase is expressed in transgenic plants and in the skin and flesh of transgenic apple fruit. Accumulation of resveratrol glucoside in transgenic apple fruit slightly decreased the flavonol content. The fertility of stilbene synthase transgenic plants was demonstrated (Rühmann et al. 2006).

12.3 Pear

To improve pear resistance against fire blight caused by *Erwinia amylovora*, a search for promoters driving expression of transgenes specifically in response to this bacterial pathogen has been undertaken. The *str246C* promoter from *Nicotiana tabacum* can induce high levels of *GUS* expression in transgenic pear upon infection by *E. amylovora*. Its systemic expression could also be beneficial, as it may help the plant to mount an active defense in advance of bacterial spread in the tissues.

However, its inducibility by wounding, salicylic acid, jasmonic acid, and several elicitors may cause unwanted accumulation of the transgene product in fruits. The *Ntsgd24* promoter (184-bp fragment of the *Ntstr246C* promoter) presents a more specific pattern of induction in response to pathogen attack and exhibited a low level of expression after wounding, elicitors, and phytohormones, which could avoid these potentially undesirable effects. Although the *NtStr246C* and *NtSgd24* promoters are less active than the *35S* promoter in pear, their pathogen responsiveness would permit them to be used to drive the low expression of transgenes to promote bacterial disease resistance (Malnoy et al. 2003).

12.4 Papaya

The *Vitis vinifera* stilbene synthase promoter was induced in papaya (*Carica papaya* L.) shortly after inoculation with the pathogen *Phytophthora palmivora* (Zhu et al. 2004). Abscisic acid activated transcription from tomato *Asr2* (*ABA/water stress/ripening-induced*) promoter in papaya (Rossi et al. 1998).

12.5 Peach

Peaches (*Prunus persica*) that are subjected to long periods of cold storage to delay fruit ripening may develop chilling injury symptoms (woolliness and internal breakdown). To better understand the relationship between cold acclimation and chilling injury in peaches, promoters from cold-inducible basic endochitinase (*Ppbec1*), dehydrin (*Ppxero2*), and thaumatin-like protein (*Pptha1*) genes were isolated and functionally characterized for cold inducibility. It was found that the *Ppbec1* and *Ppxero2* promoters were cold inducible (Tittarelli et al. 2009).

12.6 Cherry

Calmodulin (CaM) genes, tightly associated with the Ca^{2+} regulatory pathway, could be involved in plant defense mechanisms against various pathogens. A 981-bp-long upstream sequence of the apple (*Malus domestica*) *CaM* promoter was fused to the *GUS* gene and introduced into cherry (*Prunus incisa* × *serrula*). The expression of *GUS* mRNAs was upregulated under abiotic stresses (low- and high-temperature, application of salicylic acid, wounding). The *MdCaM* promoter, responsible for the expression of a gene in vascular tissues, may offer interesting perspectives for plant defense programs (Maghuly et al. 2008).

12.7 Plum

Gastrodia antifungal protein (GAFP-1) is a mannose-binding lectin that can confer increased disease resistance. Transgenic plum lines were created with *gafp*-1 under the control of the *Solanum bulbocastanum* polyubiquitin promoter *bul409. bul409* transgenic plums were more resistant to *Phytophthora* root rot and equally resistant to root knot nematode compared with the cultivar Stanley-derived 4 J line, which expresses *gafp-1* under the control of the *35S* promoter. Inoculation with neither *P. cinnamomi* nor *Meloidogyne incognita* increased GAFP-1 synthesis in transgenic plums, suggesting that there was no inducibility of *bul409:gafp-1* gene expression (Kalariya and Schnabel 2011).

12.8 Mulberry

Transgenic plants with the *AtRd29A* promoter were found to be more tolerant to salt and drought stress than those with the constitutive *35S* promoter. Fungal challenge undertaken with three fungal species known to cause serious losses to mulberry cultivation (*Fusarium pallidoroseum*, *Colletotrichum gloeosporioides*, and *Colletotrichum dematium*) revealed that transgenic plants with osmotin under control of the constitutive promoter had a better resistance than those with the osmotin gene under the control of the stress-inducible promoter (Das et al. 2011).

12.9 Strawberry

Many plants are susceptible to diseases caused by phloem-limited pathogens. A series of vascular-specific promoters has been studied in different species. A plasma-membrane sucrose transporter protein is essential for transporting sucrose long distances and is expressed specifically in the phloem companion cells. A promoter of *Arabidopsis* sucrose transporter 2 gene (*AtSUC2*) addressed *GUS* expression specifically in phloem of leaves, petioles, and roots of transgenic strawberries (*Fragaria* × *ananassa*) and may be used for engineering the phytoplasma-resistant transgenic strawberry cultivars (Zhao et al. 2004).

The genetic engineering approach was applied to control the *Botrytis cinerea* infection that is believed to penetrate at the flowering stage of strawberry via the filaments of the stamen. To achieve production of resveratrol in the infection route of the grey mold fungus, a filament-specific *Fil1* promoter from *Antirrhinum majus* was used to express the *NS-Vitis3* gene encoding stilbene synthase (STS) from frost grape (*Vitis riparia*). Also, the *AmFil1* promoter gives filament-specific expression in *A. majus* but in strawberry (*Fragaria*×*ananassa* cv. Jonsok) *STS* expression was also found in the

leaves, albeit at lower levels than with the *35S* promoter. Interestingly, the *STS* transgene expression resulted in changes in phenylpropanoid metabolism and downregulation of endogenous chalcone synthase (Hanhineva et al. 2009).

Strawberry endo-*b*-1,4-glucanase gene (*Cel1*) under the control of fruit-specific strawberry ascorbate peroxidase promoter (*FaAPX*) was used to increase fruit firmness (Lee and Kim 2011). An antisense cDNA of ADP-glucose pyrophosphorylase (AGPase) small subunit under the control of the *FaAPX* promoter had increased sugar and reduced starch content in strawberry fruits (Park et al. 2006).

Interestingly, in some cases the well-known heterologous promoters were found to be inefficient. A transient assay has been used to study the activity of the tomato polygalacturonase and the pepper fibrillin promoters in strawberry fruits. Although slight activity was observed with the fibrillin promoter, no significant activity was found with the polygalacturonase promoter (Agius et al. 2005).

In strawberry, the *FaRB7* gene is expressed predominantly in roots, with very low additional expression in petioles. The *FaRB7* promoter was shown to direct strong root-specific expression in transgenic strawberry plants comparable to that observed with *35S* promoter. Light-blue GUS staining was observed in petioles, and no staining was observed in leaves, floral organs, and fruit of transgenic strawberry plants (Vaughan et al. 2006).

12.10 Sweet Orange

The *StGst1* promoter is activated in transgenic citrus leaves by both wounding and inoculation with pathogens. A genetic transformation of sweet orange (*Citrus sinensis*) using a pathogen-inducible *StGst1* promoter and the *hrpN* gene from *Erwinia amylovora* has been made. *hrpN* encodes a hairpin protein, which elicits a hypersensitive response and systemic acquired resistance in plants. It was demonstrated that the *hrpN* gene reduces the susceptibility of citrus plants to the canker disease (Barbosa-Mendes et al. 2009).

A 4.2-kb full-length promoter of a Satsuma mandarin (*Citrus unshiu*) type 3 metallothionein-like gene (*CuMT45*) was fused to the *GUS* gene and expressed in Valencia orange (*Citrus sinensis* Osbeck) juice sacs, young leaves, and embryogenic suspension calli in a transient essay. In juice sacs and suspension calli, the *CuMT45* and *35S* promoters showed similar levels of GUS activities. In young leaves, the activity of *CuMT45* promoter was about 10 % of *35S* promoter. These results suggest that *CuMT45* contains a *cis*-element for preferential expression in juice sacs. Thus, the *CuMT45* promoter could be used for manipulation of fruit quality by genetic engineering in *Citrus* (Endo et al. 2007).

Citrus sinensis phloem protein 2 (*CsPP2*), *Arabidopsis thaliana* phloem protein 2 (*AtPP2*), and *AtSUC2* gene promoters can drive phloem-specific *GUS* expression in sweet orange leaves. Expression of specific genes in the phloem might affect bacteria growth and development and may be an adequate strategy to improve citrus resistance to Huanglongbing (Miyata et al. 2012).

The *AtPP2* promoter was used to govern transcription of a gene for synthetic antimicrobial peptide *D4E1* to control the HLB-associated bacteria in citrus (Attílio et al. 2013). Among the main diseases, citrus variegated chlorosis (CVC), caused by *Xylella fastidiosa*, is characterized by colonization and clogging of the xylem vessels. A promoter of a phenylalanine ammonia-lyase (*PAL*) gene cloned from *C. sinensis* "Madam Vinous" was characterized in "Valencia" sweet orange. GUS staining was detected in superficial layers of cells in leaf petioles but not in the vascular bundle of transgenic sweet orange. Further investigation is needed to identify the PAL isoform mainly expressed in citrus xylem (de Azevedo et al. 2006).

12.11 Lime and Citrus Rootstock US-802

"Mexican" lime (*Citrus aurantifolia* Swingle) was transformed with constructs containing either phloem-specific promoter *AtSUC2* or sucrose synthase 1 (*Ss1*) gene promoter of *Oryza sativa*. Sucrose synthase is involved in sucrose utilization and catalyzes a conversion of sucrose and uridine diphosphate (UDP) to fructose and UDP-glucose. Both *AtSUC2* and *OsSs1* promoters provided vascular-specific *GUS* expression in citrus root, stem, and leaves. GUS activity driven by the *AtSUC2* promoter was observed in young and older lime leaves, contradicting that reported in transgenic strawberries, where GUS activity was absent in young leaves (Zhao et al. 2004). *GUS* expression driven by the *AtSUC2* promoter was about 30 % as compared with *35S*. The *OsSs1* promoter could drive only low levels of *GUS* expression in citrus (Dutt et al. 2012).

Citrus rootstock US-802 is a hybrid of pummelo with trifoliate orange. Transgenic US-802 citrus plants were produced using phloem-specific *AtSUC2* and *C. sinensis* sucrose synthase (*CsSUS*) promoters or constitutive *bul409S* promoter. Histochemical GUS staining was observed throughout leaf and stem tissues for the constitutive *bul409S* promoter, whereas phloem-specific promoters largely showed the expected tissue-specific staining. Both phloem-specific promoters, *AtSUC2* and *CsSUS*, showed much lower *GUS* transcript levels than the *bul409S*. The level of expression seen in "Mexican" lime (*Citrus aurantifolia* Swingle) transformed with *AtSUC2*:*GUS* (Dutt et al. 2012) was much lower (~2 %) than the level of expression observed with the same construct in citrus rootstock US-802 (Benyon et al. 2013).

12.12 Grapefruit

A rate-limiting cytokinin biosynthetic isopentenyl transferase (*IPT*) gene from *Agrobacterium tumefaciens* was placed under the control of flower-specific *Arabidopsis APETALA3* promoter. Transgenic *APETALA3*:*IPT* grapefruit plants (*Citrus paradise* cv. "Duncan") have higher relative expression level of the *IPT* gene and cytokinin content than the wild-type plants. Despite the presence of a

tissue-specific promoter, the expression was not restricted by flower tissue. Altered expression of the pathogenesis-related genes in transgenic grapefruit plants suggested a possible role of cytokinins in pathogen resistance (Pasquali et al. 2009).

12.13 Trifoliate Orange

D-Limonene synthase gene (*MTSE2*) promoter was isolated from *C. unshiu*. Seeds and leaves from trifoliate orange (*Poncirus trifoliata*) and juice sacs and peel from *C. unshiu* were used for the transient assay. The *CuMTSE2* promoter could be utilized for regulating the quantitatively preferential expression in peel (Nishikawa et al. 2009). A *FLOWERING LOCUS T* homologue gene (*MFT1*) promoter was isolated from satsuma mandarin (*C. unshiu* "Miyagawa-wase"). A transient expression assay revealed that the *CuMFT1* promoter directed the quantitatively preferential expression in trifoliate orange seeds. *CuMFT1* may be useful for studies of seed development and manipulation by genetic engineering in *Citrus* (Nishikawa et al. 2008).

12.14 Banana

Northern hybridization analysis indicated that banana actin (ACT1) transcript was present in all tissues examined (petals/sepals, stigma/stamens, leaves, and roots). Expression studies in transgenic banana plants showed the *MusaACT1* promoter drove strong reporter gene expression in both leaves and roots. Deletions of the promoter suggested that all the necessary regulatory elements required for strong (twofold greater than *35S*) and near-constitutive expression were located within a 1.2-kb segment (Hermann et al. 2001).

Embryogenic cells of banana cv. Rasthali (*Musa AAB Group*) have been transformed with the "s" gene of hepatitis B surface antigen (HBsAg) under either ubiquitin promoter of *A. thaliana* (*AtUbq3*) or ethylene-forming enzyme promoter of banana (*MusaEFE*). Maximum expression level [38 ng/g fresh weight (F.W.) in leaves] was found in plants with banana *EFE* promoter grown under in vitro conditions, whereas *AtUbq3* transformed plants grown in the greenhouse showed the maximum expression level at 19.92 ng/g. In both cases the *HBsAg* expression was increased in wounded leaves as well as after auxin or abscisic acid treatment (Sunil Kumar et al. 2005).

A genome-wide T-DNA tagging strategy was therefore performed for the identification and characterization of novel banana (*Musa AAB Group*) promoters. The isolated sequence 17-1 possesses a reproducible promoter activity inducible by cold in dessert banana "Grande Naine" leaf and root tissue. A maize (*Zea maize*) ubiquitin (*ZmUbi1*) promoter was highly active in banana. In leaf and root tissue *ZmUbi1*:*GUS* activity was slightly up- and downregulated, respectively, after cold treatment. The full-length *Musa17-1* promoter was less active than the *ZmUbi1* promoter at both temperatures (26 °C and 8 °C) tested (Santos et al. 2009).

Dehydrins are highly hydrophilic proteins involved in tolerance to abiotic stress conditions. A novel banana SK(3)-type dehydrin MusaDHN-1 was expressed in banana under the control of *ZmUbi1* promoter. Transgenic banana plants were phenotypically normal and displayed improved tolerance to drought and salt stress treatments. Enhanced accumulation of proline and reduced malondialdehyde levels in drought- and salt-stressed *ZmUbi1*:*MusaDHN-1* plants further established their superior performance in stressed conditions (Shekhawat et al. 2011). ACC synthase and ACC oxidase genes specifically expressed in banana fruit were used to isolate promoter regions able to direct fruit-specific transgene expression (Wang and Peng 2001a, b).

12.15 Grapevine

Cinnamoyl-CoA reductase (CCR) of *Eucalyptus gunnii* catalyzes the first step in the biosynthesis of monolignols, the monomeric units of lignins. *EgCCR* promoter showed high expression in the newly formed vascular tissues in stems, leaves, and petioles of transformed grapevine that could be useful for engineered resistance against vascular pathogens (Gago et al. 2011).

Some *Vitis* species, including Chinese wild *Vitis pseudoreticulata*, possess powdery mildew (PM) resistance. Stilbene synthase (STS) participates in the biosynthesis of resveratrol taking part in the defense mechanisms. Transcription patterns provided by deleted variants of *VpSTS* promoter were analyzed in grapevine leaves after infection by *Uncinula necator* and *Alternaria alternata*. It was detected that the proximal 162 bp from the transcription initiation site were enough to keep the specific transcription pattern. Alignment of the stilbene synthase promoter sequence from a PM-resistant Chinese wild *V. pseudoreticulata* showed only a 56.4 % identity to stilbene synthase promoter from a PM-susceptible cultivated grapevine *V. vinifera* (Xu et al. 2010).

Type I lipid transfer proteins (LTPs) are believed to be involved in plant defense mechanisms. The 5′-truncated versions of *V. vinifera LTP1* promoter were cloned in front of the *GUS* reporter gene and introduced in grapevine cell suspensions. *VvLTP1* promoter activity was enhanced by a nonspecific fungal elicitor ergosterol (Laquitaine et al. 2006).

RING-finger protein (RFP1) has a key role in responses to biotic and abiotic stresses in *V. pseudoreticulata. VpRFP1* promoter isolated from *V. pseudoreticulata* was strongly induced by pathogen infection and high temperature in grapevine leaves. By contrast, the *VvRFP1* promoter isolated from *V. vinifera* showed only weak induction. The expression differences between *VpRFP1* and *VvRFP1* may be related to the observed difference in disease resistance between these two *Vitis* species. Deletion analysis of the *VpRFP1* promoter revealed the 148-bp region was fully responsible for the transcription pattern (Yu et al. 2013).

A promoter of the *V. vinifera* alcohol dehydrogenase (*VvAdh2*) gene, which was highly expressed in ripening berries and responded to anaerobic conditions, was transiently assayed in *V. vinifera* suspension cells (Verries et al. 2004).

An anthocyanin-based color histogram analysis method was utilized to evaluate quantitatively a large number of promoters for their ability to activate transgene expression in *V. vinifera*. Promoter fragments were used to drive the *VvMybA1* gene of "Merlot" for anthocyanin production in nonpigmented somatic embryo (SE) grapevine explants by transient GUS quantitative assay. Several ubiquitin *VvUb* promoters showed a pigmentation level comparable to that of standard *35S* promoter. In particular, *VvUb6* and *VvUb7* promoters provide a good tool to support constitutive expression. On the other hand, relatively lower anthocyanin pigmentation was detected in SE transformed with pathogenesis-related *VvPR1* and phenylalanine ammonialyase *VvPAL1* promoters under normal conditions. Anthocyanin expression of a relatively low intensity was strictly localized in cotyledonary tissues in SE transformed with 2S albumin protein (*VvAlb1*) promoter, confirming the seed-specific expression pattern of the 2S albumin gene. The *VvAlb1*:*VvMybA1* construct can be used in studies where cotyledons are used as target tissue for transformation. Transformed plants, once recovered, will expectedly have no growth-interfering anthocyanin overexpression and accumulation in vegetative tissues. The nondestructive anthocyanin reporter system should offer a useful tool for high-throughput evaluation of plant promoters (Li et al. 2012).

12.16 Ornamentals

Because of the slow growth of anthurium plants, development of their transgenic forms takes years. Plasmids containing the *Ubiquitin 2* (*UBQ2*), *Actin 1* (*Act1*), and *Cytochrome C1* (*Cc1*) gene promoters from rice (*Oryza sativa*) and *Ubiquitin 1* (*Ubi1*) gene promoter from maize fused to a *GUS* reporter gene were bombarded into the lamina, somatic embryos, and roots of *Anthurium andraeanum* Hort. "Marian Seefurth" grown in vitro. *OsUbi2* and *ZmUbi1* promoters provided the highest numbers of expressing cells in all tissues examined and may be used for expression of antibacterial genes and other desirable transgenes. However, it may also be useful to consider the use of the *35S* or *OsAct1* promoters for moderate expression of selectable markers to prevent the occurrence of chimeric transgenic plants. The failure of the *OsCc1* construct to confer substantial expression in anthurium may have several potential causes: lacking the needed *cis*-elements, lacking a 5′-intron, or the use of a relatively large binary vector plasmid for the bombardments. It will be interesting in the future to determine how these promoters perform in stably transformed *Anthurium andraeanum* transgenic plants (Matsumoto et al. 2013).

The *ZmUbi1* promoter performed better than the *35S* in lily (*Lilium longiflorum* cv. Snowqueen), but poorly in tulip (*Tulipa gesneriana* cv. Lucky Strike) leaf explants. *OsAct1* expressed as well as the *35S* promoter in tulip and lily leaf explants (Wilmink et al. 1995).

A polyubiquitin promoter (*UBQ1*) including its 5′-UTR and intron was isolated from the floral monocot *Gladiolus grandiflorus* cv. Jenny Lee. Transient expression showed that relative levels of GUS activity with the *GgUBQ1* promoter were

comparable to that of the *35S* promoter in *Gladiolus* cv. Jenny Lee, rose (*Rosa hybrida* cv. Classy), and a floral monocot *Freesia* cv. Blue Lady. There were very small blue spots on calla lily (*Zantedeschia eliotiana* cv. Flame) and Easter lily (*Lilium longiflorum* cv. Nellie White) cells following bombardment with the *GgUBQ1* promoter, and there were very light blue spots on *Gladiolus* cells following bombardment with the *ZmUbi1* promoter. The full-length *GgUBQ1* promoter including 5′-UTR and intron were necessary for maximum *GUS* expression in *Gladiolus*. The *GgUBQ1* promoter should be useful for genetic engineering of disease resistance in *Gladiolus*, rose, and freesia, if high levels of gene expression are important (Joung and Kamo 2006).

Chrysanthemum (*Dendranthema grandiflorum*) is the second largest cut flower market next to rose. For chrysanthemum, pest resistance, longer vase life, and the development of new colors are important breeding targets. Several efficient promoters for transgene expression in the leaf tissues of chrysanthemum have been proposed with higher expression than the *35S* promoter can provide: these include a potato *StLhca3.1* promoter (Annadana et al. 2001), tobacco translation elongation factor 1α promoter (Aida et al. 2005), and a *Cab* gene promoter isolated from the chrysanthemum wild species *D. japonicum* Makino (Aida et al. 2004). *GUS* expression driven by a chrysanthemum *DgRbcS1* promoter resulted in accumulation of, at maximum, 0.88 % of total soluble protein (Outchkourov et al. 2003).

Growth retardation is an important breeding target in horticultural plant production. The potential of transferring the *Arabidopsis* short internode (*shi*) mutant phenotype was explored by expressing the *AtSHI* gene under native *AtSHI* promoter in the popular ornamental plant *Kalanchoë*. The *AtSHI*:*SHI* transgenic plants showed dwarfing phenotypes similar to *35S*:*SHI* lines and also resulted in a longer flowering period. Two *AtSHI*-like genes were identified in *Kalanchoë*, indicating a widespread presence of this transcription factor. These findings are important because they suggest that transformation with the *AtSHI* gene could be applied to several species as a tool for growth retardation, and that this approach could substitute for the use of conventional chemical growth regulation in plant production (Lütken et al. 2010).

Expansins are cell wall proteins required for cell enlargement and cell wall loosening during many developmental processes. *Petunia hybrida* expansin A1 (*PhEXPA1*) promoter activity was evaluated in transgenic petunia plants. In flowers, GUS activity was detected in petals, sepals, ovaries, styles, and stigmas, and was coincident with areas of cell expansion during limb development. No GUS activity was detected in anthers. The GUS staining was restricted to the nodes at the base of axillary meristems, suggesting that PhEXPA1 has a role in promoting stem elongation and possibly axillary bud outgrowth. Studying of the promoter activity suggests that PhEXPA1 may acts downstream of phytohormones in balancing meristem maintenance and organ initiation (Zenoni et al. 2011).

Many bacteria, plants, and animals accumulate betaine under water or salt stress conditions. Betaine protects cells from salt stress by maintaining an osmotic balance and by stabilizing the structure of proteins. Because many crop plants do not have a betaine synthetic pathway, genetic engineering of betaine biosynthesis represents a potential way to improve plant stress tolerance. A betaine-accumulating

plant *Amaranthus tricolor* has vibrant, ornamental yellow, red, and green foliage that lends a tropical effect to the garden. Promoter of choline monooxygenase (*CMO*) gene involved in betaine synthesis was investigated. It was found that a 410-bp segment of *AmCMO* promoter directed relatively high reporter activity in agro-infiltrated leaves of *A. tricolor* plants under normal conditions, and after salt stress onset this activity was increased by fourfold (Bhuiyan et al. 2007).

12.16.1 Flower-Specific Promoters in Ornamentals

Flower-specific promoters can enable transgenic enhancement of valuable ornamental traits, including flower shape and color. *D. grandiflorum Ubiquitin Extension Protein* (*UEP1*) gene promoter confers the highest levels of expression in the chrysanthemum petal tissues of ray florets; threefold and ninefold lower expression levels were observed in the disc florets and leaves, respectively. Comparison of the *DgUEP1* promoter to the heterologous promoters, such as *eceriferum* from *Arabidopsis*, chalcone synthase from petunia, *EPF2-5* zinc-finger transcription factor from petunia, and *multicystatin* from potato, showed that *DgUEP1* provided two- to tenfold higher expression in ray and disc florets (Annadana et al. 2002).

A *Petunia hybrida* chalcone synthase (*PhCHSA*) promoter 220-bp segment contains *cis*-acting elements conferring flower-specific and UV-inducible expression in transgenic plants (van der Meer et al. 1990). A 307-bp fragment of a chalcone synthase (CHS) promoter from *Lilium orential* "Sorbonne" (*LoCHS*) could direct flower-specific expression in *P. hybrida* "Dreams Midnight" (Liu et al. 2011). To obtain enhanced flower-specific promoters, chimeric promoters combining the *35S* or *OCS* enhancer fused to a 302-bp *PhCHSA* core promoter segment from petunia or a 307-bp *LoCHS* core promoter fragment from lily were constructed. Tissue specificity of *GUS* expression was examined in the transgenic *Torenia fournieri*. Among the four chimeric promoters, *OCS-PhCHSA* exhibited stronger activity only in colored corollas, making it useful for transgenic enhancement of floral traits, such as expressing "blue genes" in lily to produce new lines with blue flowers (Du et al. 2014).

To establish an efficient way to create novel floral traits in horticultural flowers, a chimeric repressor MYB24-SRDX of *Arabidopsis* MYB24 transcription factor was introduced into *Torenia* under the control of a floral-specific *Arabidopsis APETALA1* (*AtAP1*) promoter. *AtAP1*:*MYB24-SRDX*-bearing torenias exhibited wavy petals with a characteristic configuration; this is a good example of the utilization of a floral organ-specific promoter for creating distinct flower phenotypes without causing unfavorable morphological and physiological changes in other organs (Sasaki et al. 2011).

Chromoplastogenesis during flower development and fruit ripening involves the dramatic overaccumulation of carotenoids. Expression analyses of cucumber (*Cucumis sativus*) a chromoplast-specific carotenoid-associated protein gene

(*CsCHRC*) revealed that it accumulates in floral tissues up to anthesis. No expression was detected in leaf tissues. Transactivation of *CsChrC*:*GUS* construct specifically by MYB-like *trans*-activator (MYBYS) was obtained when flowers of petunia (*P. hybrida*), carnation (*Dianthus caryophyllus*), and gypsophila (*Gypsophila paniculata*), which do not accumulate chromoplasts, were used in the transient expression assays. In leaves, MYBYS was not sufficient for *CsChrC*:*GUS* transactivation (Leitner-Dagan et al. 2006).

The *Antirrhinum majus* DEFH125 MADS-box protein is expressed in maturing pollen and thus likely participates in the regulation of pollen development. *AmDEFH125* promoter drives *GUS* expression in pollen and pollen tubes in *Antirrhinum* (Lauri et al. 2006). A lilium (*Lilium longiflorum*) generative cell-specific gene promoter (*LGC1*) is sufficient to regulate reporter gene expression in the mature pollen of *L. longiflorum* (Singh et al. 2003). *Pisum sativum* ENDOTHECIUM 1 (*END1*) is a pea anther-specific gene that displays a very early expression in the anther primordium cells. A chimeric construct with *PsEND1* promoter fused to a cytotoxic ribonuclease gene (*barnase*) from *Bacillus amyloliquefaciens* was used to induce the selective ablation of the anther cells at early stages of development and, subsequently, efficient male sterility in both *Kalanchoe blossfeldiana* (García-Sogo et al. 2010) and *Pelargonium zonale* (García-Sogo et al. 2012) plants. In addition, seed set was completely abolished in all the transgenic *Kalanchoë blossfeldiana* plants obtained (García-Sogo et al. 2010). Thus, the *PsEND1* and *LGC1* promoters can be used to produce engineered male-sterile plants.

12.16.2 Promoters That Drive Isopentenyl Transferase (IPT) Gene Expression in Ornamentals

Cytokinins have been implicated in several aspects of plant development, including plant senescence. Concentrations of endogenous cytokinins decline in plant tissues as senescence progresses. The ability to delay leaf senescence has potential for horticultural plant improvement. Overexpression of the rate-limiting cytokinin biosynthetic isopentenyl transferase (*IPT*) gene in transgenic plants led to elevated foliar cytokinin concentrations and delayed leaf senescence, but high cytokinin levels have been reported to be detrimental to growth and fertility. To circumvent these effects, a strategy exploited a senescence-specific *Arabidopsis* (*SAG12*) gene promoter fused to the *IPT* gene. Very low *IPT* transcript level was detected in young leaves of the *AtSAG12*:*IPT* transgenic petunia, and drought stress resulted in a greater than 50-fold increase in *IPT* transcript abundance in the leaves. Elevated cytokinin levels in nonsenescence transgenic plant tissues compared to wild type confirmed that the *AtSAG12* promoter is tightly regulated in petunia. The transgenic petunia plants grow and develop similar to wild-type plants in terms of vegetative propagation, branching habit, and flowering timing. These characteristics allow the production of delayed leaf senescence without compromising a desired trait

performance of the plants (Clark et al. 2004). *AtSAG12*:*IPT* transgenic petunia cv. V26 lines with extended flower longevity were used to study the effects of elevated cytokinin content on ethylene synthesis and sensitivity and abscisic acid accumulation in petunia corollas. Floral senescence in these lines was delayed for 6 to 10 days relative to the wild-type flowers. *IPT* transcripts were increased in abundance after pollination and were accompanied by increased cytokinin accumulation. Endogenous ethylene production was induced by pollination in both wild-type and *AtSAG12*:*IPT* corollas, but this increase was delayed in *AtSAG12*:*IPT* flowers. Flowers from *AtSAG12*:*IPT* plants were less sensitive to exogenous ethylene and required longer treatment times to induce endogenous ethylene production, corolla senescence, and upregulation of the senescence-related protease. Accumulation of abscisic acid, another hormone regulating flower senescence, was significantly greater in wild-type corollas, confirming that floral senescence was delayed in *AtSAG12*:*IPT* plants (Chang et al. 2003). Introduction into petunia of the *IPT* gene and homeobox knotted1 (*Kn1*) gene from maize, linked to the senescence-associated *AtSAG12* and *AtSAG13* promoters, was able to inhibit senescence of the lower leaves, although transgenic lines showed stronger symptoms of nutritional deficiency (Jandrew and Clark 2001).

Pelargonium is a very popular garden plant. The *AtSAG12*:*IPT* transgenic *P. zonale* plants showed delayed leaf senescence, increased branching, and reduced internodal length, as compared to control plants. Leaves and flowers of the *P. zonale* *AtSAG12*:*IPT* plants were reduced in size and displayed a more intense coloration (García-Sogo et al. 2012).

Leaf senescence is often caused by water deficit. Increases in activities of antioxidant enzymes were responsible for the delay of leaf senescence induced by osmotic stress in the *AtSAG12*:*IPT* transgenic commercially important flower *Gerbera jamesonii* "Yuyan" (Lai et al. 2007).

To prevent leaf senescence of young transplants or excised shoots during storage under dark and cold conditions, the *IPT* gene was placed under the control of an *Arabidopsis* stress-inducible promoter *COR15* and introduced into *Petunia hybrida* "Marco Polo Odyssey" and *Doronicum grandiflorum* "Iridon". Transgenic *AtCOR15*:*IPT* petunia and chrysanthemum plants and excised leaves remained green and healthy during prolonged dark storage (4 weeks at 25 °C) after an initial exposure to a cold-induction period (4 °C for 72 h). However, *AtCOR15*:*IPT* chrysanthemum plants and excised leaves that were not exposed to a cold-induction period senesced under the same dark storage conditions. Regardless of cold-induction treatment, leaves and plants of nontransformed plants senesced under prolonged dark storage. Analysis of *IPT* expression indicated a marked increase in gene expression in intact transgenic plants and isolated transgenic leaves exposed to a short cold-induction treatment before dark storage. These changes correlated with elevated concentrations of cytokinins in transgenic leaves after cold treatment. *AtCOR15*:*IPT* transgenic plants showed a normal phenotype when grown at 25 °C (Khodakovskaya et al. 2005).

12.17 Conclusion

Here we would like to demonstrate the wide range of opportunities provided by promoters tested in horticultural plants. Some promoters were found to be as strong as *35S* and nearly constitutive, other are tissue-, stage-, or stimuli specific. This wealth of regulatory elements in the gene engineering toolbox makes possible the decisions of many actual tasks of horticultural plants genetics, biotechnology, and bioengineering.

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Chapter 13
mRNA Translational Enhancers as a Tool for Plant Gene Engineering

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Abstract Plant gene engineering is widely used for both pure and applied investigations. It is commonly considered that a promoter is a key element controlling the expression pattern of the transgenes. However, the level of protein synthesis is also regulated at the posttranscriptional stage, and successful experiments need a careful design of the genetic constructs. Here, we briefly discuss the basic principles of plant mRNA organization influencing translation initiation efficiency under normal and stress conditions and some translational enhancers available in the gene engineering toolbox.

Keywords Gene engineering • mRNA features • Stress tolerance • Transgenic plant • Translation

13.1 Plant mRNA Features Influencing General Translation Efficiency

13.1.1 Linear Scanning is a Eukaryotic Mechanism of Translation Initiation Used "By Default"

There are two major initiation mechanisms (linear scanning and internal ribosome entry sites, IRES) and a number of various deviations (e.g., ribosome shunt sites, cap-independent translation enhancers) (Kozak 2005; Jackson et al. 2010). It should be noted, however, that the linear scanning is a mechanism used "by default" and all

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other variants demand the presence of special signals within the mRNA. In most typical genetic constructs (especially for fundamental research), no specific signals are commonly used and translation of transgenic mRNA is made through a linear scanning way.

According to the scanning model, 40S ribosomal subunits are recruited to the 5′-terminal cap structure, scan mRNA in a 5′-to-3′ direction, and can initiate translation at the first AUG they encounter. The recognition of an AUG triplet as translation initiation site (TIS) depends on its nucleotide context: if context is optimal, most 40S ribosomal subunits will recognize AUG and initiate translation. However, if the context is suboptimal, some 40S ribosomal subunits still recognize it as a TIS, but others may skip it, continue scanning in the 3′-direction, and initiate translation at downstream AUG ("leaky scanning" mechanism). The initiation/scan through ratio depends on the AUG context.

13.1.2 Nucleotide Context of Start Codon

It is well known that the nucleotide frequencies in positions near the start AUG codon are biased in comparison with the average nucleotide content in the 5′-untranslated region (5′-UTR) and coding sequence (CDS). It is suggested that this nucleotide sequence corresponded to the perfect context, that is, the context recognized by all the ribosomes loaded onto mRNA as a TIS. In mammalian translation systems the consensus of the start AUG codon is GCCRCCAUGG (R = A or G). Relative importance of some context positions was experimentally verified. In brief, the positions −3 and +4 were found to be the most important (Kozak 2005). Contexts AnnAUGn and GnnAUGG are considered to be optimal (i.e., they are strong enough to be recognized by most of the scanning ribosomes); context YnnAUGH (Y = U or C; H = not G) is considered to be the most leaky. An efficiency of recognition of other context variants is hard to predict. It is generally assumed that if the positions −3 is occupied by a pyrimidine, the "strength" of context correlates with its closeness to the consensus sequence (Kozak 2005; Jackson et al. 2010; Volkova and Kochetov 2010).

TIS organization in mRNAs of monocot plants resembles that of mammals (consensus: GCRGCARCCAUGGC) whereas mRNAs of dicot plants differ considerably (consensus: AAAAAAAAMAUGGC (M = C or A)) (Cavener and Ray 1991). It was demonstrated that the most important positions in plant mRNAs are also −3 and +4; the other important positions are −2, −1, and +5. GCCAUGGC and RAAAUGGC were shown to be the most efficient TISs in maize and tobacco cells, respectively (Lukaszewicz et al. 2000). There are some examples of detailed analysis of relative importance of AUG context positions. Evaluation of a set from 64 reporter mRNAs containing various combinations of nucleotides in positions −3 to −1 upstream from the start AUG codon revealed the optimal consensuses (A/G)(a/c)(a/g)AUG in *Arabidopsis thaliana* and (A/G)(u/C)(g/C)AUG in O. sativa cells (capital letters correspond to relatively more optimal nucleotide). It was demonstrated

that the nucleotides in positions −2 and −1 make an additive contribution to the overall context "strength"(Sugio et al. 2010). Another research demonstrated that *A. thaliana* 5′-UTRs greatly differ in translation efficiencies (up to 200 fold). Adenines in positions −5 to −1 were the most favorable nucleotides (uridines were least efficient). Translation efficiency also correlated positively with the presence of adenines in positions −21 to −1 (Kim et al. 2014).

13.1.3 5′-UTR Size

There is a limitation to the minimal 5′-UTR length: if the leader is less than 15–20 nt it can be too short for efficient AUG recognition. This limitation results from the 40S ribosomal subunit structure: the anticodon of initiator Met-tRNA is located about 13–15 nt from the leading edge and it could miss the start AUG codon because of steric restrictions (Kozak 2005; Jackson et al. 2010). There are no upper limitations on 5′-UTR size: if it forms no stable secondary structure and contains no AUG triplets (upstream AUG, uAUG) it will provide a suitable platform for the initiation of translation. Indeed, a larger 5′-UTR tends to form a more stable secondary structure and AUG triplets could appear more frequently because of occasional mutations. It was considered that a 5′-UTR length between 50 and 75 nt is most favorable for efficient translation and that leaders larger than 175 nt are frequently inhibitory (Kawaguchi and Bailey-Serres 2005).

13.1.4 RNA Secondary Structure

Stem-loop structures even of moderate stability can inhibit translation strongly if located close to the 5′-end of mRNA (Kozak 2005). It is assumed that these structures can interfere with interaction between the 5′-cap and translation initiation factors (eIF-4F). Stable hairpins in more distal 5′-UTR segments can decrease translation intensity by slowing down the leaky scanning: 40S ribosomal subunits form a queue waiting for hairpin melting by associated RNA helicases (Kozak 2005; Jackson et al. 2010). Although this model looks simple, the prediction of both the RNA secondary structure and its inhibitory effect is still not an easy task. mRNAs are situated in the cytoplasm as RNA–protein complexes. It seems likely that each type of mRNA molecules occurs as a set of dynamically interconverted conformations and that interactions with proteins and ribosomes can change their ratio and stabilize certain segments in (mostly) unfolded state. This field of biology needs further development based on high-throughput techniques (Kertesz et al. 2010).

It is generally assumed that 5′-UTRs should be depleted in G+C because these nucleotides make a stronger contribution to the secondary structure stability (Kozak 2005; Kawaguchi and Bailey-Serres 2005). However, this observation is not completely correct because the nucleotide sequence could be characterized with a high

G+C content but also with imbalance in G to C or vice versa: it was demonstrated that eukaryotic 5′-UTRs are specifically characterized by imbalance in the contents of complementary nucleotides (Kochetov et al. 2002a, b, 2005), and more efficiently translated mRNAs were characterized by higher G/C and A/U imbalance (Kochetov et al. 1998, 1999).

13.2 Typical Reasons Making Translation of Transgene mRNA Inefficient

To conclude, the checklist of important 5′-UTR features includes size larger than 30 nt (preferably between 40 and 80 nt), no uAUG, no strong secondary structures (at least, in a close proximity to the 5′-end), and optimal context of the start codon (at least, purine in position −3 and, if possible, guanine in position +4); if this is not possible because of the second amino acids constraints, aaaAUG is preferable for dicots and a/gccAUG for monocots).

5′-UTR features should be thoroughly designed. In some cases transgenic constructs were based on the vectors with nonoptimized leader regions. For example, pBi121 was often used for direct cloning of mRNA protein-coding segments that commonly resulted in both a too-short 5′-UTR and a suboptimal context of start codon. In addition, 5′-UTR composed of polylinker segments frequently form stable hairpins because most restriction sites are inverted repeats. It is no wonder that an improved version of pBI121 with modified 5′-UTR provided a tenfold increase in the reporter protein production (De Amicis et al. 2007).

Another reason of translation problems could concern alternative translation start sites. If 5′-UTR is too short or the start codon is located in a suboptimal context, translation can also be initiated at the downstream AUG(s). If the next AUG is located in the same reading frame as the CDS, translation of N-end shortened protein isoform can be initiated. For example, DNA ligase 1 (AtLIG1) is the only essential DNA ligase activity in *Arabidopsis thaliana*. The mitochondrial and nuclear forms of DNA ligase 1 are translated from a single mRNA species through translation initiation from either the first or second in-frame AUG codons, respectively. The nucleotide context around alternative start codons in the AtLIG1 transcripts affects translation initiation to ensure a balanced synthesis of both nuclear and mitochondrial AtLIG1 isoforms, probably via a context-dependent leaky scanning (Sunderland et al. 2004). Alternative TISs and leaky scanning are responsible for synthesis of plastidic and cytoplasmic/nuclear isoforms of tRNA ligase of *Arabidopsis thaliana* and *Oryza sativa*, plastidic and mitochondrial isoforms of spinach protoporphyrinogen oxidase, and *Arabidopsis* DNA polymerase (Christensen et al. 2005; Englert et al. 2007; Watanabe et al. 2001). However, if the next AUG situated in the +1 or +2 reading frame, it typically starts translation of a small ORF encoding unrelated polypeptide. Actually, it is becoming evident that a large fraction of eukaryotic mRNAs contains alternative start codons and encode several functional proteins (Kochetov 2008; Bazykin and Kochetov 2011; Ingolia et al. 2009, 2011). Small proteins are

considered as an important class of regulatory and bioactive affectors (Andrews and Rothnagel 2014; Marmiroli and Maestri 2014).

This opportunity should be taken into account if transgenic plants are used as a model for investigation of a particular gene (this topic is beyond our discussion). However, alternative translation can result not only from the intrinsic features of ORF under analysis but also from inappropriate vector design (e.g., because the context of a start codon was suboptimal: leaky scanning will not only significantly decrease the synthesis of a protein of interest but also provide translation of truncated protein isoforms or small unrelated peptides unpredictably influencing cellular processes).

13.3 General Translational Enhancers

Many RNA-positive viruses developed mechanisms providing their mRNAs with efficient translation in plant cells (Nicholson and White 2011; detailed description is outside the scope of this chapter). It was reported that some 5′-UTRs of (commonly) viral or (sometimes) cellular origin could enhance translation of heterologous proteins if used as a leader region in the recombinant mRNA. These so-called translational enhancers should be taken into consideration in the genetic construct design. Below, we briefly present some useful examples.

A 67-nt-long 5′-UTR derived from the Tobacco mosaic virus (TMV) RNA ("Omega leader") (Gallie et al. 1987a) is the most widely used translational enhancer in plant gene engineering. It reliably provides a significant increase in mRNA translation efficiency in dicot plant cells (Fan et al. 2012). The underlying mechanisms are not fully clear: it was shown that Omega could interact with translation initiation factors eIF4G and eIF3 as well as with heat-shock 101-kDa protein and functionally overlaps with the 5′-cap and poly(A)-tail (Gallie 2002). Sequence elements responsible for interaction with these *trans*-factors remain unclear: probably, a central (CAA)n segment able to form a stable spatial non-Watson–Crick structure is involved in the translation factor binding (Agalarov et al. 2011). In our opinion this enhancer can be used by default if increased level of recombinant protein production in dicot plants is needed [it does not concern the bioproduction (biofarming) because more sophisticated approaches were developed for this field of biotechnology (e.g., Nopo et al. 2012; Akua and Shaul 2013; Meshcheriakova et al. 2014)]. Another viral leader sequence known to be a general translational enhancer is that of the alfalfa mosaic virus RNA4 (AMV; Gallie et al. 1987b). It is shorter (and less efficient) than Omega and was successfully used in many experiments to improve translation of transgenic mRNA. These two 5′-UTRs compile a classic set of cap-dependent translational enhancers in the plant gene engineering toolbox.

There are a number of other 5′-UTRs reported to be general translational enhancers in plant cells. It should be however noted that in some cases an increase in the reporter mRNA translation associated with the 5′-UTR under investigation was revealed in a comparison with vector sequences characterized by very poor efficiency

(e.g., with pBI121: see foregoing comment). It is likely that some of these leaders represented ordinary plant 5′-UTRs with sequence features optimized for efficient interaction with general translational factors (i.e., there are no specific *trans*-factors or mechanisms underlying their initiation efficiency). Indeed, these 5′-UTRs could also be used in the genetic constructs design as efficient leader regions.

It was found that a 65-nt-long 5′-UTR of soybean glutamine synthetase strongly enhanced reporter mRNA translation (Ortega et al. 2012; the control CAMBIA 2301 vector 5′-UTR was 20 nt long).

5′-UTRs from HSP18.2, HSP17.4, HSP81-1, HSP81-2, and HSP81-3 enhanced translation of the reporter mRNA in tobacco and *Arabidopsis* (pBI121 vector 5′-UTR was used as a control) (Dansako et al. 2003). It was reported that 5′-UTRs of alcohol dehydrogenases (ADH) from *Arabidopsis*, tobacco, and rice strongly increased translation efficiency of reporter mRNAs in tobacco (in comparison with pBI121 vector 5′-UTR) and rice ADH 5′-UTR could specifically enhance translation in *Oryza sativa* cells (Satoh et al. 2004; Sugio et al. 2008; Matsui et al. 2009). A 28-nt-long synthetic 5′-UTR (synJ) was found to be a potent translation enhancer in tobacco and cotton, and in certain tissues (cotton calli and tobacco roots) it was even more efficient than Omega and AMV (Kanoria and Burma 2012) (the experimental design there was also not perfect: in some constructs the context of the reporter ORF start codon was suboptimal, whereas in synJ and Omega constructs it was optimal). The list of 5′-UTRs reported as translation enhancers includes a 23-nt-long 5′-UTR of *Nicotiana sylvestris* photosystem I gene (Yamamoto et al. 1995), a 87-nt-long 5′-UTR of the *Vigna radiata* aminocyclopropane-1-carboxylate synthase (Wever et al. 2010), and a 66-nt-long chlorophyll *a/b* binding gene leader sequence from petunia (Cab22L) (De Loose et al. 1995).

13.4 Specific Translational Enhancers

mRNA translation efficiency can be regulated in a tissue- or stage-specific manner by specific translational enhancers (Mustroph et al. 2009; Liu et al. 2012). For example, ferredoxin-1 (Fed-1) mRNA contains an internal light response element (iLRE) that mediates a rapid decline in translational efficiency: iLRE-containing mRNAs dissociate from polyribosomes within 20 min after plants are transferred to darkness (Hansen et al. 2001). Transcriptome-wide analysis in *Arabidopsis* showed that the response reaches far beyond just photosynthetic proteins (Juntawong and Bailey-Serres 2012; Liu et al. 2012). The 5′-UTR of tobacco ntp303 gene strongly enhances translation during pollen tube growth but not during pollen maturation (Hulzink et al. 2002). If an experiment demands specific expression of a transgene, commonly only the promoter is taken into consideration. However, in some cases it could be useful to acquire UTR sequences from a host gene efficiently expressed in the tissue or at the development stage investigated. For instance, if a transgene expression should be strictly light regulated, Fed-1 iLRE can be applied (Hansen et al. 2001). UTRs of amaranth ribulose 1,5-bisphosphate carboxylase (RbcS1)

mRNA mediate bundle sheath cell-specific gene expression in leaves of C4 photosynthesis plants (Patel et al. 2006). In other words, specific translation enhancers could be instrumental in complex experiments with transgenic plants.

13.5 Keeping Transgenic mRNA Translated Under Stress Conditions

Translation of specific plant mRNAs is differentially regulated under certain abiotic stress conditions such as heat, cold, dehydration, and oxygen deprivation. The majority of transcripts exhibit varying degrees of translational repression, whereas a subset of transcripts escapes such repression and remains actively translated (Munoz and Castellano 2012; Ueda et al. 2012; Echevarría-Zomeño et al. 2013; Roy and von Arnim 2013). Somewhat less dramatic shifts in translation state have been detected in response to virus infection (Moeller et al. 2012). The underlying mechanisms that mediate this control, and in particular the identities of the regulatory RNA elements involved, remain poorly understood (Matsuura et al. 2010).

It should be noted that plants are frequently affected by a stressful environment. Even model investigations on the transgenic plants aimed to reveal functions of particular proteins can involve pathogen attacks, drought, or temperature deviations. If a transgene expression should continue after the stress onset, it is important to pay attention to the construct design. Certain 5′-UTRs were found to provide stress-selective translation (even though the signals and mechanisms remain unclear) and can be used for this purpose (a few examples are given below).

the 5′-UTR of *Arabidopsis* FAD3 protein mediated twofold increase in the reporter mRNA translation efficiency upon low temperature induction (from 22 °C to 10 °C) (Wang and Xu 2010). Maize alcohol dehydrogenase 5′-UTR provides efficient translation of the reporter mRNA in *Nicotiana benthamiana* cells under hypoxia and heat shock (Mardanova et al. 2007). Omega leader provides the reporter mRNA translation under heat shock (Gallie 2002). Some signals of heat-shock-selective translation were recently found in the 5′-proximal portion of plant mRNAs (Matsuura et al. 2008, 2013).

13.6 Conclusion

In this chapter we directed attention to the importance of proper design of genetic constructs used for generation of transgenic plants for pure and applied research. Translation of transgene mRNA is a critical stage in the protein synthesis process, and its characteristics should be taken into account to increase the reliability of the experiments.

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Chapter 14
RNA-Seq Data Analysis for Studying Abiotic Stress in Horticultural Plants

V.V. Mironova, C. Weinholdt, and I. Grosse

Abstract Initiating the project on sequencing the *Arabidopsis thaliana L.* genome at the end of the twentieth century, researchers one day wished to expand the accumulated knowledge on *Arabidopsis* genetics to horticultural plants. The future arrived with the appearance of high-throughput sequencing technologies that allowed the investigation of transcriptomes of non-model plants at an unprecedented pace. RNA-seq experiments provide a unique opportunity of studying in depth the molecular-genetic basis for plant response to environmental cues. Here we substantiate the potential of RNA-seq experiments in applications to horticultural plants. The basic steps in RNA-seq data analysis and available software packages are presented in the first section. Examples of RNA-seq data analyses, including studies of gene expression changes under various stresses in horticultural plants, and transcriptome analyses of the tolerance to abiotic stresses in horticultural plants are given in the second section.

Keywords Genomics • Horticultural plants • RNA-seq • Stress response • Transcriptomics

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14.1 Introduction

The transcriptome is the full set of RNA molecules generated by a cell or a population of cells. The transcriptome includes mRNAs, tRNAs, miRNAs, and other noncoding RNAs. In contrast to the genome, the transcriptome varies significantly with cell type, developmental stage, biotic and abiotic conditions. There are two sources of transcriptome variability: variability of the transcribed sequences and variability of expression levels (Wang et al. 2009). Several technologies have been developed for studying transcriptomes, but only the application of next-generation-sequencing methods to transcriptomes (RNA-seq) has provided the opportunity of studying both sources of variability simultaneously and with high resolution (McGettigan 2013).

RNA-seq experiments have shown that both the transcribed sequences and their expression levels vary significantly under different biotic and abiotic stresses. Alternative splicing, alternative transcription start sites, alternative transcription stop sites, and RNA editing are widely present in transcriptomes of well-studied species (Zavolan et al. 2003; Trapnell et al. 2010). Moreover, a great variety of previously uncharacterized noncoding RNAs has been found by RNA-seq experiments, and sequencing of small RNAs (miRNA-seq) is nowadays as popular as standard sequencing of mRNAs (Motameny et al. 2010; Lee et al. 2010).

RNA-seq experiments provide additional benefits for species without sequenced genomes or with poor genome annotations. For horticultural plants, the availability of information about transcribed sequences has a fundamental impact on many areas of plant biology such as plant phylogenetics, reverse genetics, DNA fingerprinting, and marker-assisted selection. Here, we review applications of RNA-seq techniques to study abiotic stresses in horticultural plants. The review consists of two sections. The first section describes technical aspects of handling RNA-seq data. The second section describes examples of applications of RNA-seq techniques to horticultural plants subjected to various abiotic stresses such as drought, salinity, flood, cold, and mineral deficiency.

14.2 Analysis of RNA-Seq Data

Different next-generation sequencing technologies exist that convert input RNA material into millions of short reads. Modern sequencing platforms are based on the sequencing-by-synthesis technology with either a DNA polymerase (e.g., Roche 454, Illumina, Helicos, Pacific Biosciences) or a ligase (e.g., Life Technologies SOLiD, Complete Genomics) as a key component. The sequencing platforms can be further categorized as either *single-molecule-based* such as Helicos and Pacific Biosciences or *ensemble-based* such as Illumina and SOLiD (Metzker 2010). So far Illumina and SOLiD have the smallest error rates, less than 1 % per base, which is of special importance for the analysis of miRNAs. Both platforms are widely used because of their high sequencing capacity, which makes it possible to measure low-abundance transcripts (Metzker 2010).

RNA-seq data analyses consist of a sequence of steps that must be adapted and optimized depending on the goal of the experiment, the RNA material, and the species. The analysis can be divided in two main parts: (i) general steps that must be performed in each RNA-seq analysis (Sect. 14.2) and (ii) specific steps that vary from analysis to analysis for long RNAs (Sect. 14.2.2) and small RNAs (Sect. 14.2.3).

14.2.1 General Steps of RNA-Seq Data Analysis

General steps of RNA-seq data analysis involve quality control and filtering of reads (Sect. 14.2.1.1) and assembling the filtered reads and connecting them with a reference genome or transcriptome by mapping or *de novo* assembly (Sect. 14.2.1.2).

14.2.1.1 Quality Control and Filtering

Reads obtained from a sequencing platform must be quality controlled and filtered for possible sequencing errors, artifacts, or contaminations. Typically, the following three types of reads or sub-reads are eliminated: (i) low-quality reads or sub-reads including a high percentage of low-quality bases or a high percentage of uncalled bases; (ii) reads or sub-reads including sequencing artifacts such as duplicate reads, adapter sequences, barcodes, or a strong bias in the GC content; and (iii) reads or sub-reads including DNA contamination or RNA contamination from other species.

The tool *fastQC* (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) can be used for visualizing the read qualities, the distribution of read-lengths, which is important for the analysis of RNA-seq data of small RNAs, or the GC content. An extensive comparison of methods addressing errors of Illumina data, published by Del Fabbro et al. (2013), shows that quality control and filtering reduce the need for disk space and computation time for subsequent mapping and de novo assembly steps.

Table 14.1 provides an overview of different tools for quality control and filtering. Column 2 of Table 14.1 provides information about the underlying algorithmic approach such as the *running sum* approach utilized by Cutadapt (Martin 2011) or different *window-based* approaches utilized by other tools such as *FASTX quality trimmer* (hannonlab.cshl.edu/fastx_toolkit), *PRINSEQ* (Schmieder and Edwards 2011), *Trimmomatic* (Lohse et al. 2012), and *sickle* (github.com/najoshi/sickle). Column 3 provides information about the capability of handling paired-end reads. Here, tools such as *sickle* or *Trimmomatic* can process paired-end reads simultaneously, whereas *Cutadapt*, *FASTX quality trimmer*, or *PRINSEQ* process the two read sets independently. Columns 4 and 5 provide information about the capability of handling color-space reads relevant for processing SOLiD data and about the capability of adapter removal. Clipping of sequencing adapters by tools such as *Cutadapt* or *Trimmomatic* is indispensable for the analysis of RNA-seq data of small RNAs, because the typical read length of 50–200 bp is greater than the typical length of small RNAs so that adapters or barcodes are also sequenced.

Table 14.1 Selection of tools for quality control and filtering grouped by their algorithmic approach and other useful features

Tool	Algorithmic approach	Processing of paired-end reads	Processing of SOLiD data	Adapter removal
Cutadapt	Running sum	No	Yes	Yes
FASTX quality trimmer	Window based	No	Yes (for fixed-length trimming)	Yes (by FASTX Clipper)
PRINSEQ	Window based	No	No	No
Trimmomatic	Window based	Yes	No	Yes
Sickle	Window based	Yes	No	No

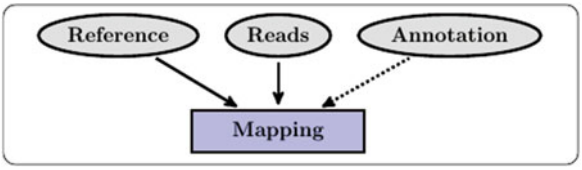

Fig. 14.1 Read mapping for case (i) where a reference genome or transcriptome and an annotation are available. Reads can be mapped to the reference genome or transcriptome using the annotation

14.2.1.2 Assembly of Reads and Connecting them to a Reference Genome or Transcriptome

Three situations can occur when analyzing RNA-seq data of horticultural plants: (i) an annotated reference genome or transcriptome is available, (ii) a reference genome or transcriptome is available, but it is not annotated, and (iii) no reference genome or transcriptome is available. We address typical approaches for these three cases in the sections "Mapping Assembly," "Transcriptome Assembly," and "De Novo Transcriptome Assembly."

Mapping Assembly

In case (i), in which both a reference genome or transcriptome and an annotation are available, mapping of quality-filtered and trimmed reads can be performed directly without additional preprocessing steps (Fig. 14.1). Mapping is the process of determining the position of each read in the reference genome or transcriptome. Hence, mapping is a fundamental step of each RNA-seq data analysis. To date more than 80 mapping tools are available, which differ strongly in their algorithmic strategies and their mapping behavior (Fonseca et al. 2012). Mapping tools can be grouped by several criteria (Li and Homer 2010; Alamancos et al. 2014), and we concentrate on the following two criteria in this review.

Table 14.2 Selection of mapping tools from Li and Homer (2010) and Alamancos et al. (2014)

Tools	Algorithmic approach	Alignments reported	SOLiD data	Splice-aware mapper	Use of annotation
MAQ	Hash table	B,R	Yes	No	–
SHRiMP	Hash table	B, U, S	Yes	No	–
SOAP	Hash table	B, R, S	No	No	–
Bowtie	Suffix/prefix tree	A, B, R, S	Yes	No	–
Bowtie 2	Suffix/prefix tree	A, B, R, S	No	No	–
BWA	Suffix/prefix tree	R, S	Yes	No	–
PALMapper	Suffix/prefix tree	A, B	Yes	Yes	No
segemehl	Suffix/prefix tree	A, B	No	Yes	No
STAR	Suffix/prefix tree	A, B, S	No	Yes	Yes
TopHat 2	Suffix/prefix tree	B, S	Yes	Yes	Yes

Mapping tools are grouped by their algorithmic approach (column 2), by their treatment of reads that map to multiple locations (column 3), and by their capability of handling color-space reads from SOLiD (column 4), handling split reads (column 5), and using the annotation (column 6) in case of splice-aware mapping tools. If a read maps to multiple locations (column 3), all alignments can be reported (A), only the best alignment can be reported (B), a randomly selected alignment can be reported (R), only unique alignments can be reported (U), or a user-defined number of alignments can be reported (S).

First, mapping tools can be grouped according to their algorithmic approach. They can be based on hash tables, such as *MAQ* (Li et al. 2008a), *SOAP* (Li et al. 2008b), and *SHRiMP* (Rumble et al. 2009), or on suffix or prefix tries, such as *Bowtie* (Langmead et al. 2009), *Bowtie 2* (Langmead and Salzberg 2012), *BWA* (Li and Durbin 2009), *segemehl* (Hoffmann et al. 2014), and *TopHat 2* (Trapnell et al. 2009) (Table 14.2).

Second, mapping tools can be grouped by their capability of handling reads that span exon–exon junctions. Such reads, which are called split reads, can be neglected by *unspliced mappers* such as *PALMapper* (Jean et al. 2010), segemehl, *STAR* (Dobin et al. 2013), or *TopHat 2* (Table 14.2). *Splice-aware* mappers make it possible to map reads to splicing junctions and thereby increase the number of mappable reads and provide helpful information for detecting novel isoforms. Mappers such as *TopHat 2* and *STAR* (Table 14.2) can optionally use the annotation for increasing the accuracy of mapping to known splice junctions.

Transcriptome Assembly

In case (ii), in which the reference genome or transcriptome is available but an annotation is missing, read mapping can be performed as in section "Mapping Assembly", but mapping tools cannot benefit from the optional use of the annotation. However, an annotation can be generated by one of the following two approaches (Fig. 14.2).

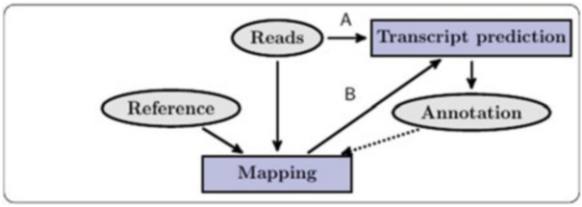

Fig. 14.2 Missing annotation can be substituted by computational transcript prediction directly from the reads (A) or using the mapped reads for a genome-guided assembly of the genes (B)

Evidence-based transcript prediction tools such as *mGene* (Schweikert et al. 2009) or *Augustus* (Keller et al. 2011) can be used for obtaining an annotation of transcripts or exon-intron structures. *Augustus* can integrate evidence from expressed sequence tags (EST), tandem mass spectrometry (MS/MS) data, protein alignments, and genomic alignments (Stanke et al. 2008) and is capable of identifying coding exons even from transcripts with very low abundance (Steijger et al. 2013).

Alternatively, *genome-guided* transcriptome assembly tools such as *Cufflinks* (Trapnell et al. 2010), *Scripture* (Guttman et al. 2010), and *GRIT* (Boley et al. 2014) use reads mapped by splice-aware mappers to a reference genome or transcriptome for identifying transcript models. One advantage of using genome-guided transcriptome assembly tools is that these tools make it possible to discover novel transcripts and to assemble low-abundance transcripts (Martin and Wang 2011).

De Novo Transcriptome Assembly

In case (iii), in which a reference genome or transcriptome is not available, a reference transcriptome must first be generated by a de novo assembly tool (Fig. 14.3). This step is often necessary when working with non-model species, and in this case it is advisable to have a sufficiently high sequencing depth and to perform paired-end sequencing.

Historically, *de novo* assembly tools such as *ABySS* (Birol et al. 2009), *SOAPdenovo* (Luo et al. 2012), and *Velvet* (Zerbino and Birney 2008) were developed for assembling genomes, but specialized tools for performing de novo assembly of transcriptomes have also been developed in the meantime. Examples of de novo transcriptome assembly tools are *OASES* (Schulz et al. 2012), *Rnnotator* (Martin et al. 2010), and *SOAPdenovo-trans* (Xie et al. 2014), *Trans-ABySS* (Simpson et al. 2009), and *Trinity* (Haas et al. 2013).

Table 14.3 shows several features of these five *de novo* transcriptome assembly tools. Specifically, column 2 shows the algorithmic approach, column 3 the capability of detecting alternative isoforms, and column 4 the capability of quantifying isoform-specific expression. From column 2 of Table 14.3 we see that *Trinity* has a k-mer size fixed to k = 25 and that the k-mer sizes for the tools *Oases*, *Rnnotator*, *SOAPdenovo-trans*, and *Trans-AbySS* are variable. We see from column 3 that

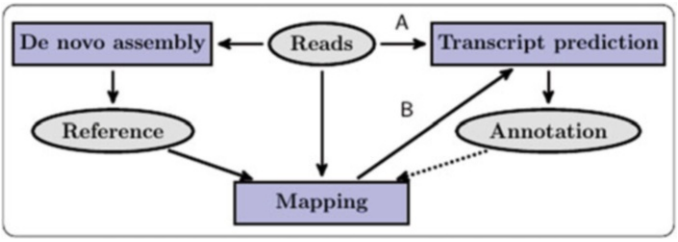

Fig. 14.3 The missing reference genome or transcriptome can be substituted by de novo transcriptome assembly. Subsequent mapping to the *de novo* assembled reference transcriptome can be performed and the annotation can be predicted as described by (Fig. 14.2)

Table 14.3 Selection of de novo transcriptome assembly tools

Tool	Algorithmic approach	Detection of alternative isoforms	Quantification of isoforms
OASES[a]	Variable k-mer	No	No
Rnnotator[a]	Variable k-mer	No	No
SOAPdenovo-trans	Variable k-mer	Yes	No
Trans-Abyss	Variable k-mer	Yes	Isoform read coverage
Trinity	Single k-mer	Yes	Yes[b]

De novo transcriptome assembly tools are grouped by their algorithmic approach (column 2) and by their capability of detecting alternative isoforms (column 3) and of quantifying isoform-specific expression (column 4)

[a]Uses *Velvet* for de novo transcriptome assembly

[b]Uses *RSEM* for calculating *RKPM* values (see Sect. 14.2.2.1)

SOAPdenovo-trans, *Trans-AbySS*, and *Trinity* are capable of detecting alternative isoforms. De novo assembly tools can consume hundreds of gigabytes of RAM and can run for weeks even on a high-performance-computing cluster (Martin and Wang 2011). Rapaport et al. (2013) provides further details on and a comprehensive review of de novo transcriptome assemblers.

Additionally, the tool *scaffold_builder* (Silva et al. 2013) has been developed for scaffolding preassembled contigs against a genome from an evolutionarily related species with a sufficiently high degree of sequence similarity.

After de novo transcriptome assembly has been performed and a resulting reference transcriptome is available, reads can be mapped to the de novo assembled reference transcriptome as described in section "Mapping Assembly" and illustrated by Figs. 14.1 and 14.2. Additionally, *evidence-based* transcript prediction tools or genome-guided transcriptome assembly tools can be applied for annotating exons in the reference transcriptome (Fig. 14.2).

14.2.2 Specific Steps of RNA-Seq Data Analysis of Long RNAs

Mapped reads can be used for addressing numerous tasks such as detecting differentially expressed transcripts (*Sect. 14.2.2.1*) or calling single-nucleotide polymorphisms (*Sect. 14.2.2.2*).

14.2.2.1 Detecting Differentially Expressed Transcripts

In this section we describe the detection of differentially expressed transcripts, which consists of three steps called quantification, normalization, and statistical testing (Fig. 14.4).

Quantification

As starting point for the detection of differentially expressed transcripts, RNA abundances corresponding to each transcript must be quantified: this can be accomplished by (i) *count-based* approaches or by (ii) *model-based* approaches.

Count-based approaches simply quantify the relative RNA abundance per transcript by counting the number of mapped reads per annotated transcript. Count-based approaches can be divided in two groups. Only uniquely mapped reads are counted by tools of group (a), whereas all mapped reads are counted by tools of group (b). Examples for tools of group (a) are *HTSeq-count* (Anders et al. 2014) and *GenomicRanges* (Lawrence et al. 2013). Examples for tools of group (b) are *IRanges* (Lawrence et al. 2013) and *BEDTools* (Quinlan and Hall 2010).

Several tools such as *featureCounts* (Liao et al. 2014) belong to both groups as they allow counting both uniquely mapped and all mapped reads. Approaches for quantifying the relative RNA abundance based on all mapped reads often lead to a biased quantification from cross-mapping of reads from close homologs (Anders et al. 2013). whereas, approaches based on counting only uniquely mapped reads

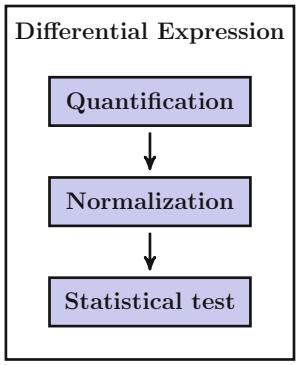

Fig. 14.4 Detection of differentially expressed transcripts. Current approaches for detecting differentially expressed transcripts consist of three steps quantification, normalization, and statistical testing

typically lead to a less biased quantification of the relative RNA abundance and thus are preferable for detecting differentially expressed transcripts.

Model-based approaches such as *Cufflinks* (Trapnell et al. 2010) and *RSEM* (Li and Dewey 2011) combine the quantification step with the subsequent normalization step. This combination leads to normalized values for relative RNA abundances per transcript. Examples are the RPKM normalization for single-end reads, where RPKM stands for *reads per kilobase of exon per million mapped reads* (Pang et al. 2013), or the FPKM normalization for paired-end reads, where FPKM stands for *fragments per kilobase of exon per million mapped reads* (Trapnell et al. 2010).

Normalization

Subsequent to the quantification step, a normalization step is required for obtaining normalized values of relative RNA abundances that are comparable across different samples and different libraries. Raw counts obtained from count-based quantification approaches should not be compared to each other without normalization for reasons of different library sizes, different technical biases of library preparation, and different nucleotide compositions (Kvam et al. 2012; Rapaport et al. 2013).

A comparison of several normalization approaches published by Dillies et al. (2013) shows that RPKM normalization does typically not improve the results of count-based quantification approaches and should thus be replaced by *upper quartile normalization*, *median* normalization (Bolstad et al. 2003), *DESeq* normalization (Anders et al. 2012), or *TMM* normalization (Robinson et al. 2010). According to Dillies et al. (2013), the normalization tools *DESeq* and *TMM* yield the most robust results with respect to different library sizes and different library compositions.

Statistical Testing

Pipelines for the detection of differentially expressed transcripts require normalized values of relative RNA abundances in at least two groups of samples as input, then compute some test statistics for each of the transcripts from these normalized input data, and finally rank the transcripts by their computed test statistics.

Popular software packages for the detection of differentially expressed transcripts are *baySeq* (Hardcastle and Kelly 2010), *DESeq* (Anders and Huber 2010), *edgeR* (Robinson et al. 2010), and *PoissonSeq* (Li et al. 2012) (Table 14.4). Each of these software packages includes several normalization methods for which the statistical test is optimized. For example, the Bioconductor (Gentleman et al. 2004) packages *DESeq* and *edgeR* use a variation of Fisher's exact test adapted to the negative binomial (NB) distribution for calculating the significance of the change of the normalized relative RNA abundances between two conditions, whereas the software package *PoissonSeq* uses a statistical test based on the Poisson distribution. *DEXseq* (Anders et al. 2012) is a special software package devoted to the detection of differentially expressed exons, and a detailed description of *DESeq* and *edegR* is published by Anders et al. (2013).

Table 14.4 Selection of software packages for detection of differentially expressed transcripts

Tools	Input	Normalization	Model distribution	Test statistic	Differential expression
DESeq	Gene counts	DESeq normalization (normalization factor by median of scaled counts)	NB	Adapted Fisher's exact test for NB	Genes
edgeR	Gene counts	TMM (weighted trimmed mean of log expression ratios)	NB	Adapted two-sided Fisher's exact (binomial test)	Genes
baySeq	Gene counts	TMM, Quantile	NB	Empirical Bayes approach	Genes
PoissonSeq	Gene counts	Quantile, TMM	Poisson	Score statistic on the basis of a Poisson log-linear model	Genes
EBSeq	Isoform/ gene counts or RSEM-EBSeq pipeline	Median, Quantile	NB	Bayesian method: estimate posterior probability	Genes and isoform
DEXseq	Exon counts	DESeq	NB	NbinomTest	Exons
Cuffdiff 2	FPKM	FPKM	NB	*t* test-like statistics for FPKM	Genes and isoforms

The software packages are grouped by the input data from the quantification step (column 2), by the supported normalizations (column 3), by the statistical model for the normalized values of the relative RNA abundance (column 4), by the statistical test (column 5), and by the type of transcript that can be handled such as genes, exons, or isoforms

Soneson and Delorenzi (2013) compare 11 different software packages for detecting differentially expressed transcripts. They find that different software packages are optimal for different situations and that the resulting sets of differentially expressed genes can differ strongly between the software packages. As a result, the authors recommend using more than one software package for detecting differentially expressed transcripts.

An alternative approach for detecting differentially expressed transcripts is based on model-based methods for quantification and normalization. One popular example is the *Tuxedo suite* pipeline, which is composed of the mapping tool *TopHat 2*, the quantification and normalizing tool *Cufflinks*, and the tool *Cuffdiff 2* for the detection of differentially expressed genes or isoforms (Trapnell et al. 2013). A detailed description of the TopHat-Cufflinks pipeline has been published by Trapnell et al. (2010).

Detecting differentially expressed transcripts in cases where no reference genome or transcriptome is available, but where a de novo transcriptome assembly is performed for generating a reference transcriptome, is an important task for the analysis of RNA-seq data of horticultural plants and other non-model species. In this case, the software package *RSEM* is often used, which provides the RSEM-EBSeq pipeline and the tool *EBSeq* for the detection of differentially expressed transcripts (Leng et al. 2013) (Table 14.4).

14.2.2.2 SNP Calling

Calling single-nucleotide polymorphisms (SNPs) is a very important task in many RNA-seq experiments including studies on plants. SNP calling can be performed directly on the sorted output of mapped reads (Sect. 14.2.1). One limitation of calling and analyzing SNPs from RNA-seq data is that only SNPs in exonic regions can be detected. Three popular SNP callers are *Freebayes* (https://github.com/ekg/freebayes), *GATK* (DePristo et al. 2011), and *Samtool* (Li et al. 2009). An extended list of SNP callers can be found at http://seqanswers.com/wiki/SNP_discovery. The standard output format is the variant calling format (vcf), so most SNP callers write their output in vcf files. vcf files can then be filtered for significant SNPs by *vcftools* (Danecek et al. 2011), and their content can be visualized, for example, by the *Integrative Genomics Viewer* (IGV) (Thorvaldsdóttir et al. 2013).

14.2.3 Specific Steps of RNAseq Data Analysis of Small RNAs

Mapped reads from a pool of small RNAs can be used for addressing numerous tasks such as detecting differentially expressed small RNAs, predicting novel miRNAs, or predicting miRNA targets, and we address these tasks in Sects. 14.2.3.1–14.2.3.3. Detailed reviews of RNA-seq data analyses of small RNAs have been published by Motameny et al. (2010) and Gomes et al. (2013).

14.2.3.1 Detecting Differentially Expressed Small RNAs

One main task of the analysis of small RNA-seq data is the detection of differentially expressed miRNAs, siRNAs, snoRNAs, tRNA, or rRNAs. This task can be accomplished by the same steps as described in Sect. 14.2.2.1 with the only addition that the reference genome or transcriptome or the annotation should be related to the class of small RNAs to be analyzed. In case of miRNAs, such information is available from the database *miRBase* (Kozomara and Griffiths-Jones 2011), which allows using the miRNA hairpin structure as reference for mapping or using genome-related miRNA annotation files for quantification.

14.2.3.2 Predicting Novel Plant miRNAs by *plantDARIO*

The tool *plantDARIO* (http://plantdario.bioinf.uni-leipzig.de/), an extension of the tool *DARIO* (Fasold et al. 2011), can be used for the prediction of novel miRNAs, tRNAs, C/D-box snoRNAs, and H/ACA-box snoRNAs in plants. *PlantDario* is specifically tailored to plants and uses the tool *NOVOMIR* (Teune and Steger 2010) for the prediction of novel miRNAs and the tool *SnoReport* (Hertel et al. 2008) for predicting novel snoRNAs. *PlantDARIO* provides basic features including quality control, quantification, and normalization in addition to predicting novel small RNAs. Currently, *plantDARIO* allows analyses of small RNAs in *Arabidopsis thaliana*, *Beta vulgaris*, and *Solanum lycopersicum*.

14.2.3.3 Predicting miRNA Targets

The prediction of miRNA targets in mammals is reviewed by Witkos et al. (2011). For plants, however, there are only a few tools available for the prediction of miRNA targets. Two noteworthy examples are *psRNATarget* (Witkos et al. 2011) and *TAPIR* (Bonnet et al. 2010). Additionally, software packages for the analysis of miRNA such as *miRDeep-P* (Yang and Li 2011), *miREvo* (Wen et al. 2012), *MirTools 2.0* (Wu et al. 2013), *seqBuster* (Pantano et al. 2010), or *miRanalyzer* (Hackenberg et al. 2011) can be used for the prediction of plant miRNA targets. These software packages can contain additional tools for predicting differentially expressed miRNAs (Table 14.5, column 3), novel miRNAs (Table 14.5, column 6), isoforms (isomiRs) (Table 14.5, column 5), and point mutations (Motameny et al. 2010; Git et al. 2010).

Table 14.5 Selection of software packages for the analysis of miRNAs

Tool	Quantification	Differential expression	Prediction of miRNA targets	Prediction of miRNA isoforms	Prediction of novel miRNAs	Organism
miRanalyzer	Yes	Yes	Yes	Yes	Yes	A, P
MirTools 2.0	Yes	Yes	Yes	No	Yes	A, P
psRNATarget	Yes	No	Yes	No	No	P
TAPIR	Yes	No	Yes	No	No	P
miREvo	Yes	No	Yes	No	Yes	A, P
miRDeep-P	Yes	No	No	No	Yes	P
plantDARIO	Yes	No	No	No	Yes	P
SeqBuster	Yes	No	No	Yes	Yes	A, P

Several of these software packages are not tailored to plants. The software packages are grouped by their capability of quantifying relative miRNA abundance (column 2), of detecting differentially expressed miRNAs (column 3), of predicting miRNA targets (column 4), miRNA isoforms (column 5), and novel miRNAs (column 6), and of analyzing animal (A) and/or plant (P) miRNAs (column 7)

14.3 RNA-Seq Data on Abiotic Stresses of Horticultural Plants

Although abiotic stresses in model plants have been studied mainly by microarray techniques, nowadays we observe a dramatic growth of transcriptome profiling by RNA-seq experiments in non-model plants. Here we review recently published results on RNA-seq derived transcriptome data analyses for plants under abiotic stresses such as cold, drought, or salinity. One can classify the experiments in two groups: (i) the study of transcriptional response in horticultural plants and (ii) the study of transcriptomes of plants that are well adapted to abiotic stresses such as endemics or wild cultivars. Several studies have been also performed to investigate the molecular basis of adaptation under selection processes, for example, in wheat (Jia et al. 2013) and in tomato (Koenig et al. 2013). In some studies (Massa et al. 2013; O'Rourke et al. 2013), cross-species comparisons of stress-induced transcriptomes have uncovered differentially expressed orthologs and defined evolutionary conserved genes. Using the example of cold-responsive transcriptome studies, we show which bioinformatics methods researchers have used for transcriptome assembly and annotation and for the detection of differentially expressed genes (Table 14.6). For other abiotic stresses studies, we only give an overview of the RNA-seq data analysis tasks.

14.3.1 Cold

Recently, a number of studies have been published on the analysis of cold-responsive transcriptomes based on RNA-seq experiments. Two different questions that have been studied are (i) plant cold resistance and acclimation and (ii) the harmful effect of low temperatures. In the first case, the transcriptomes of plants known for their adaptability to cold have been studied. Examples are *Ammopiptanthus mongolicus*, an evergreen broadleaf legume shrub, distributed in Mid-Asia where the temperature can be as low as −30 °C during winter (Pang et al. 2013), the sheepgrass *Leymus chinensis*, an important perennial forage grass across the Eurasian Steppe (Chen et al. 2013), or the extremophile Antarctic hairgrass *Deschampsia antarctica*, the only natural grass species in the maritime Antarctic (Lee et al. 2013). In the second case, researchers studied the transcriptomes of tropical or other cold-sensitive plants useful in biotechnology or horticulture. Among them has been *Jatropha curcas* L., an oil-rich tropical shrub with multiple uses, including biodiesel production (Wang et al. 2013b), *Anthurium andraeanum*, one of the most popular tropical flowers (Tian et al. 2013), and the tea plant *Camellia sinensis* (Wang et al. 2013d).

In the studies, the transcriptomes have been assembled *de novo* (Table 14.6). Standard transcriptome annotation included BLAST alignments against the NCBI nonredundant (NR) database and the COG database as well as GO and KEGG annotations. However, in some works an extensive annotation has been done also by alignments against EST databases (Wang et al. 2013b, d; Lee et al. 2013), the

Table 14.6 Summary on materials and methods in studies of cold-induced plant transcriptomes

Species	Samples	Sequencing platform	Transcriptome assembly and detection of differentially expressed genes (DEG)[a]	Transcriptome annotation	Links
Jatropha curcas	Leaves 12, 24, 48 h at 12 °C	Illumina Hiseq 2000	*Assembly*: *Trinity platform* *DEG Detection*: Digital Gene Expression	*BlastX*: NR, wiss-Prot, EST *BlastN*: Nt GO in *map2slim*	SRR653198 (Wang et al. 2013b)
Anthurium andraeanum	Leaves 1, 2, 3, 10, 24 h at 6 °C	Illumina Hiseq 2000	*Assembly*: *SOAPdenovo* *DEG Detection*: Digital Gene Expression	*BlastX*: NR COG GO in *Blast2GO* KEGG pathways	E-MTAB-1955 (Tian et al. 2013)
Camellia sinensis	Leaves non-, fully-, and de-acclimated plants	Illumina Hiseq 2000	*Assembly*: *Trinity platform* *DEG Detection*: RSEM software package; DESeq package; Digital Gene expression	*BlastX*: NR, UniRef90, TAIR10, EST, COG *HMMER 3.0* *Blast2GO* KEGG pathways	SRA061043 (Wang et al. 2013d)
Ammopiptanthus mongolicus	Leaves and roots, 14 days at –4 °C	Illumina Hiseq 2000	*Assembly*: *Trinity platform*; TGICL[b] *DEG Detection*: RPKM method	*BlastX*: NR, Swiss-Prot, COG *Blast2GO*, WEGO KEGG pathways	SRA064010 (Pang et al. 2013)
Leymus chinensis	Buds –40; –15; 25 °C Spikes	454 GS FLX	*Assembly*: Newbler 2.5. *DEG Detection*: RPKM method; DESeq package	EMBOSS package *BlastP*: Swiss-Prot, NR, COG GO in *GoPipe* *KEGG* pathways *PlantGDB* for Poacea-Specific gene analysis	SRA065691 (Chen et al. 2013)
Deschampsia antarctica	Leaves at 4 °C	454 GS FLX	Assembly: TGICL[b] *DEG Detection*: Permutation *t* test after normalization of raw read counts	*BlastX*, *BlastN*: *Nr*, *tBlastX*: *EST* *Blast2GO*, *AgriGO* *KEGG pathways*	SRA051881 (Lee et al. 2013)

[a]Details about the programs and algorithms are described in Sect. 14.2
[b]From Pertea et al. (2003)

TAIR10 database (Wang et al. 2013d), or the PlantGDB database (Chen et al. 2013). For *Leymus chinensis* and *Deschampsia antarctica*, the transcriptome assembly allowed performing phylogenetic analyses (Chen et al. 2013; Lee et al. 2013). In each of these studies, thousands of genes were found to be differentially expressed under cold. Their functional annotation allowed revealing (i) pathways that were significantly affected under cold and (ii) cold-sensitive genes which were specific for the analyzed species. Specific attention has been paid to cold-sensitive transcription factors by Chen et al. (2013), Tian et al. (2013), and Wang et al. (2013d).

An interesting study of the cold-induced mRNA degradome in *Brachypodium distachyon* has been performed based on RNA-seq experiments and a parallel analysis of RNA ends (PARE) in (Zhang et al. 2013a). The authors identified specific patterns of mRNA decay in cold response. Uncapped transcripts changed significantly after cold treatment, whereas their transcript abundance remained unchanged. MiRNA-seq experiments of the similar samples showed some miRNA–mRNA pairs associated with cold response. In addition to miRNA-directed internal cleavage, the authors also revealed 90 transcripts that undergo an endogenous cleavage by an unknown mechanism through a specific and conserved motif.

14.3.2 Drought

RNA-seq analysis has been used for identifying genes that mediate the tolerance to water-limiting environments, which in the long term will contribute to improvement of plant productivity under drought. Transcriptome profiles under drought response have been analyzed in horticultural plants that are vulnerable to drought such as potato (*Solanum tubersosum*) (Massa et al. 2013; Zhang et al. 2014), rice (*Oriza sativa*) (Zong et al. 2013), and common beans (*Phaseolus vulgaris*) (Müller et al. 2013). Similarly, several drought-tolerant species such as *Agave deserti* and *Agave tequilana* have been studied based on RNA-seq experiments (Gross et al. 2013). Two varieties of quinoa (*Chenopodium quinoa* Willd.), the allotetraploid grain crop with an impressive drought tolerance and nutritional content, have been studied by RNA-seq (Raney et al. 2014).

To identify differentially expressed genes under drought stress in cotton, RNA-seq experiments have been performed in the tetraploid *Gossypium hirsutum* cotton (Bowman et al. 2013) and the diploid *Gossypium arboretum* cotton (Zhang et al. 2013c). RNA-seq experiments have also provided insight into transcriptional drought responsive in trees such as poplars (Cossu et al. 2013; Tang et al. 2013) and eucalyptus (Villar et al. 2011).

Drought stress during flowering and grain-filling stages of growth contributes to serious yield loss in common bean (Kakumanu et al. 2012; Müller et al. 2013), and the dehydration stress response of the transcriptome of *Chrysanthemum* have been studied by Xu et al. (2013b).

In some works, RNA-seq experiments have been performed for studying specific gene families that play an important role in drought response. Examples are studies

of expression patterns of ten AP2/EREB-like transcription factors in two soybean genotypes (Marcolino-Gomes et al. 2013) or the detection and analysis of LEA proteins in the tropical legume *Castanospermum austral* (Delahaie et al. 2013).

Comprehensive analyses of drought response mechanisms were performed for *Oryza sativa* by Zong et al. (2013). The authors have performed ChIP-seq and RNA-seq analyses for studying the relationships between epigenomic and transcriptional regulation in response to drought and have been found associations between the distribution pattern of histone H3K4-tri-methylation and gene expression profiles.

Drought- and salinity-responsive miRNAs have been analyzed for the emerging biofuel crop switchgrass (*Panicum virgatum*) by Xie et al. (2013). Differentially expressed miRNAs and their predicted targets have been functionally annotated and a number of interesting targets have been selected to aid in designing next-generation switchgrass for biomass and biofuel.

14.3.3 Heat and Light

In most cases, heat stress is associated with drought, but the study of heat stress defense mechanisms has a primary importance also for some of the cold-temperate species. For example, one of the most crucial factors that limits the cultivation of the Pacific Ocean kelp *Saccharina japonica* in China is its sensitivity to high temperature. The response of the *S. japonica* transcriptome to heat have been studied based on RNA-seq experiments (Liu et al. 2013), and the functional annotation of differentially expressed genes under primary heat response has showed that algae respond to heat stress by a complex network of genes rather than by a few specific stress-related genes.

The lack or excess of light can be also stressful for some plant species. An interesting comparison of the transcriptomes of an allotetraploid *Glycine* and its diploid progenitors has been published by Coate et al. (2013). Allopoliploidy is often associated with increased photosynthetic capacity as well as enhanced stress tolerance. In this work it has been shown that, under chronic excess of light, a photoprotective mechanism was higher in an allopolyploid *Glycine dolichocarpa* than in its diploid progenitors *G. tomentella* and *G. syndetika*.

14.3.4 Soil Pollutants

Toxic heavy metals in the soil can be absorbed and accumulated by plant roots, significantly suppressing their growth and making them a potential source for human health risks, especially in vegetables with edible roots. The radish response to lead stress has been studied based on RNA-seq experiments by Wang et al. (2013e). In this work, the radish transcriptome has been *de novo* assembled, and thousands of differentially expressed genes between control roots and roots

subjected to lead stress have been detected. Their functional annotation revealed that the upregulated genes have been predominately involved in defense responses in the cell wall and glutathione metabolism, whereas downregulated genes have been mainly related to carbohydrate metabolism pathways.

Al^{3+} tolerance mechanisms in rice roots have been studied by Arenhart et al. (2013). The central role of the ASR5 transcription factor for regulating the transcriptional response to Al^{3+} has been demonstrated by the fact that most of the genes differentially expressed under Al^{3+} stress have not been differentially expressed in plants with suppressed ASR5.

Boron-induced transcriptomes in barley have been studied by Tombuloglu et al. (2013), wherein the authors identified critical boron-induced transcription factors of the MYB family, which are well-known regulators of stress response. Boron-induced transcriptome changes in barley have been also studied based on miRNA-seq experiments by Ozhuner et al. (2013), whereby boron-induced miRNAs and their potential targets have been identified and partially validated by quantitative polymerase chain reaction (qPCR).

14.3.5 Mineral Deficiency

Response to deficiency of macro- and micronutrients has been also studied by RNA-seq experiments in plants. One example is the study of the response to potassium starvation in two watermelon genotypes (Fan et al. 2014).

The response to phosphate deficiency has been intensively studied by RNA-Seq experiments in plants. Plants utilize different morphological and physiological strategies to adapt to phosphate starvation in the soil. Studies of phosphate-deficient transcriptomes or the transcriptomes of plants highly tolerant to the lack of phosphate may elucidate the molecular basis of the response to phosphate starvation, and such studies have been performed by RNA-seq experiments in four rice cultivars (Oono et al. 2013). As a result, a set of core transcripts responsive to phosphate deficiency in the four rice cultivars has been identified.

The response to phosphate deficiency was studied in white lupin (O'Rourke et al. 2013), which has evolved unique adaptations for growth in phosphate-deficient soils, including the development of cluster roots to increase the root surface area. As a result, 12 genes have been found differentially expressed in response to phosphate deficiency in *Arabidopsis thaliana*, potato, and white lupin, making these genes ideal candidates to monitor the phosphate status of plants.

The expression of mRNA of ribosomal proteins has been studied based on RNA-seq experiments in phosphate- and iron-deficient plants of *A. thaliana* (Wang et al. 2013c), and three and 81 differentially expressed genes have been identified, respectively. At the protein level, many more ribosomal proteins were accumulated in response to phosphate than in response to iron, suggesting that phosphate and iron starvation provoke an altered composition of ribosomes and a biased translation, which can be an important mechanism of adaptation to changing environmental conditions.

14.3.6 Salinity

Salinity is one of the major abiotic factors affecting productivity of horticultural plants. Recently RNA-seq experiments have been performed for studying the response to salinity stress in lucerne (alfalfa) (Postnikova et al. 2013), potato (Massa et al. 2013), and barley (Ziemann et al. 2013). Among cereal crops, barley is considered as notably salt tolerant, but an interesting task is to analyze the transcriptomes of halophytic plants. For example, global transcriptome profiling has been performed in *Salicornia europaea*, an edible plant well adapted to extreme saline environments (Ma et al. 2013). Among other halophytic plant species, RNA-seq experiments have been also performed on the desert poplar *Populus euphratica* (Tang et al. 2013) and the Inner Mongolia endemic shrub *Reaumuria trigyna*, which has unique morphological characteristics that allow it to tolerate stress imposed by semidesert saline soil (Dang et al. 2013).

Salinity stress has been also studied in wild halophyte relatives of horticultural plants such as *Porteresia coarctata,* a wild rice that is capable of tolerating high salinity and submergence (Garg et al. 2014), and *Gossypium* species with a remarkable tolerance to saltwater immersion such as *Gossypium aridum* (Xu et al. 2013a).

Effects of saline-alkaline soils have been studied by RNA-seq experiments in several plant species. Time series of transcriptomes of roots of the halophyte wood *Tamarix hispida* stressed by $NaHCO_3$ have been studied by Wang et al. (2013a). Early transcriptomic adaptation to sodium carbonate in maize has been studied by Zhang et al. (2013b), where the authors analyzed shared and distinctive targets in $Na_2CO_3^-$, $NaCl^-$, and high-pH-induced transcriptomes.

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