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Comparative analysis of chilling requirement and cold hardiness in peach (*Prunus persica*) cultivars

복숭아 품종의 저온 요구도와 내한성 비교

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DEPARTMENT OF AGRICULTURE, FORESTRY, AND BIORESOURCES

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ABSTRACT

Deciduous fruit trees temporarily suspend their growth through dormancy and increase their cold hardiness through cold acclimation to adapt low temperature in winter. Here, physiological changes during dormancy, cold acclimation, and deacclimation were compared among peach cultivars to elucidate different responses to freezing temperatures. Dormancy completion of peach cultivars ranged from early January to late February. Chilling requirements were calculated by using Chill Hours, Utah, Dynamic, North Carolina, and Low Chilling models and ranged from 263 to 2123 chill hour, 377 to 1134 chill unit, and 21.3 to 74.8 chilling portion. Low-chill cultivars might be readily deacclimated when exposed to warm spells and sudden temperature drop during late winter or early spring

which might induce freezing damage. Changes in soluble sugar content, related enzyme activity, and gene expression were monitored in the shoots of a cold-hardy 'Soomee' (SM) and a cold-sensitive 'Odoroki' (OD) peach cultivar during cold acclimation (CA) and deacclimation (DA). Although both cultivars had similar seasonal patterns of cold hardiness, SM was cold-hardier than OD from December to March. During this period, total soluble sugar content was higher in SM than in OD, but starch content was significantly lower in SM, along with higher total amylase activity and related gene expression. Of the detected soluble sugars, sucrose was predominant and its content was most significantly correlated with cold hardiness. Fructose and glucose contents in SM, but not in OD, increased during CA and then decreased during DA concurrently with increased acid invertase activity and its gene expression. Raffinose and stachyose were detectable only from November to April. Their contents and the expression of the genes involved in their synthesis were also significantly correlated with cold hardiness. To compare DA resistance and sugar metabolism between the cultivars, changes in sugar content and transcriptome of SM and 'Kanoiwa Hakuto' (KH) were compared after accelerated DA at 15ºC and delayed DA at –5ºC in February. During the accelerated DA for 4 weeks, KH lost cold hardiness more rapidly than SM, and starch content decreased more in SM than KH. The most enriched pathway with differentially expressed genes (DEGs) was 'carbohydrate metabolism' in which 'starch and sucrose metabolism' was also most enriched with 43 DEGs encoding 20 related enzymes. Transcripts encoding catabolic enzymes, including β-galactosidase (LOC18769018 and LOC18783479) and β-glucosidase (LOC18770931, LOC18779132, and LOC18779303) were up-regulated in SM relative to KH, while those encoding anabolic enzymes such as

galactinol synthase (LOC18789982) and raffinose synthase (LOC18770520) were down-regulated. Changes in gene expression and enzyme activities related to soluble sugar metabolism were different among cultivars which consequently cause different cold hardiness of peach trees.

Key words: chilling requirement, cold acclimation, cold hardiness, deacclimation, deciduous fruit species, dormancy, global warming, peach, *Prunus persica*

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACG	acid α-galactosidase
ACI	acid invertase
ALG	alkaline α-galactosidase
ALI	alkaline invertase
ANOVA	analysis of variance
bHLH	basic helix-loop-helix
BSA	bovine serum albumin
CA	cold acclimation
CBF	C-repeat binding factor
cDNA	complementary DNA
CH	chill hour
COR	cold-responsive
CP	chilling portion
CU	chill unit
CV	coefficient of variance
DA	deacclimation
DEG	differentially expressed gene
DOY	day of year
DREB	dehydration-responsive element binding factor
EC	enzyme code
EL	electrolyte leakage
ERF	ethylene responsive factor
FPKM	fragments per kilobase of transcript per million mapped reads

GA	gibberellin
GO	gene ontology
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-ethane-sulphonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
KH	Kanoiwa Hakuto
LT	lethal temperature
MYB	myeloblastosis transcription factor
NAC	no apical meristem, *Arabidopsis* transcription activation factor, and cup-shaped cotyledon
NIHHS	National Institute of Horticultural and Herbal Science
OD	Odoroki
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RAS	raffinose synthase
RFO	raffinose family oligosaccharide
SM	Soomee
SPS	sucrose phosphate synthase
STS	stachyose synthase
SUS	sucrose synthase
TAM	total amylase
TF	transcription factor
TTC	2,3,5-triphenyltetrazolium chloride
UDP	uridine disphosphate

GENERAL INTRODUCTION

Deciduous fruit trees survive unfavorable winter season through dormancy and the development of cold hardiness (Kalberer et al., 2006; Tanino et al., 2010; Wisniewki et al., 2014). To survive freezing temperatures during winter, the trees develop their cold hardiness by adjusting the metabolism during cold acclimation (CA) (Gu et al., 2001; Pagter et al., 2008; Wisniewski et al., 2018). The opposite process, deacclimation (DA), occurs when trees were exposed to warm temperatures in the following spring and refers to a loss of the cold hardiness (Vyse et al., 2019; Xin and Browse, 2000; Yu et al., 2017).

Freezing causes significant productivity and yield losses during the cultivation of the fruit trees. Cold hardiness can reflect the winter survival and adaptability of the fruit trees. Rate and magnitude of CA mostly determine the maximal cold hardiness level during mid-winter (Espevig et al., 2014; Gu et al., 2001). Freezing injury during winter usually occurs when CA is not rapid enough or not sufficient before damaging temperature occurs. DA resistance, a characteristics of cold hardiness loss under warm temperature, is also important to determine the cold hardiness (Espevig et al., 2014). DA is necessary for the growth resumption in spring, but premature transition to DA can severely injure the trees. Especially, DA has increasingly been emphasized in a global warming context which results in warmer weather conditions and frequent erratic climate events.

Cold hardiness is a multigenic trait which is influenced by extensive reprogramming of metabolic changes regulated by gene expressions and enzyme activities. Among the numerous metabolic changes during CA and DA, carbohydrates are involved in the changes in cold hardiness. Starch is degraded to

soluble sugars during CA, and sugar contents reach the highest levels during mid-winter in most cases (Fürtauer et al., 2019; Lee et al., 2013). The accumulated soluble sugars can be used for physical protection (Yano et al., 2005) and respiration (Bruelheide and Lieberum, 2001).

Peach is the widely grown fruit tree species and known to be vulnerable to freezing stress (Jun et al., 2016). Commercial plantings of peach cultivars with unknown cold hardiness might be risky in areas with cold winters and result in serious economic losses. Therefore, cold hardiness has often been a major selection criterion in peach breeding programs. In addition, cultivars that will persist under harsh winter conditions should be identified for their successful cultivation (Kalberer et al., 2007).

In this study, time of dormancy break and chilling requirement of peach cultivars were evaluated and changes in chilling accumulation spectrum were monitored based on the last 100-year data. Sugar metabolisms in a cold-hardy and a cold-sensitive peach cultivar were compared with respect to sugar content, enzyme activity, and gene expression during CA and DA. Changes in transcriptomes were also compared between the two cultivars during accelerated and delayed DA. Integrated regulation on sugar metabolism were discussed for explaining the changes of cold hardiness during CA and DA.

LITERATURE REVIEW

Dormancy and cold hardiness

Dormancy can be defined as the inability of a meristem to resume growth under favorable conditions (Lang, 1987; Rohde and Bhalerao, 2007). The dormancy states can be classified as paradormancy, endodormancy, and ecodormancy (Lang, 1987; Lang et al., 1987). Paradormancy can be defined as an inhibited growth by distal organs, while ecodormancy is provoked by unfavorable environmental conditions. Endodormancy induced by endogenous signaling cue which is originated solely within the meristem-containing tissue. The exposure to a specific amount of chilling, chilling requirement, is known to critically induce the shift of endodormancy to ecodormancy. Ecodormancy is a state brought about by the limitation of growth-promoting factors, such as warm temperatures, sufficient water and nutrient supply. Dormancy is the result of plant adaptation to the environment and the variation in intensity is due to the different pressure exercised at different latitudes and altitudes. Among the same species, the climatic variations generated different genotypes with a distinct dormancy intensity (Heide, 2008).

Dormancy and CA are essential for deciduous fruit species in order to survive freezing temperatures (Pagter et al., 2008; Vyse et al., 2019; Wisniewski et al., 2014). Dormancy and CA overlap and both are induced by low temperatures and short daylength (Kalberer et al., 2006; Weiser, 1970; Welling and Palva, 2008; Wisniewski et al., 2014). Freezing injuries which occur in early spring has been increased because the warm spells after endodormancy promote the premature DA which makes trees vulnerable to sudden temperature drop (Gu et al., 2008).

Dormancy usually inhibits DA and growth resumption (Kalberer et al., 2006). For example, grape and cherry buds resisted DA during dormancy while that buds readily deacclimated as spring approached (Callan, 1990; Wolf and Cook, 1992). Endodormant dogwood stems less deacclimated than non-dormant stems under identical conditions (Litzow and Pellett, 1980). Cold hardiness and dehydrin proteins decreased more rapidly in a non-dormant peach genotype than in an endodormant deciduous genotype during DA (Arora et al., 1992; Artlip et al., 1997).

Chilling requirement and models

Various models have been proposed to estimate chilling requirements in deciduous fruit trees. Among them, the three most widely used models are Chill Hours (Weinberger, 1950), Utah (Richardson et al., 1974), and Dynamic (Fishman et al., 1987a, b) models. Chill Hours model is regarded as the oldest and simplest model, as it simply registers the number of hours at temperatures below 7.2°C as accumulated chill hour (CH). This model, however, does not consider the negative effect of high temperatures on chill accumulation. This deficiency was overcome by Utah model (Richardson et al., 1974) through introduction of a chill unit (CU) with different weighing values for different ranges of temperature. This model performed well under cool or cold temperate climate (Dennis, 2003), although it is less adequate under subtropical or tropical conditions owing to the large amount of negative chill values (Linsley-Noakes and Allan, 1994). North Carolina (Shaltout and Unrath, 1983), Low Chilling (Gilreath and Buchanan, 1981), Positive Chill Unit (Linsley-Noakes et al., 1995), and Taiwan (Lu et al., 2012; Milech et al., 2018) models were all modifications derived from Utah

model to adjust to varying climate conditions. These models use CU values of different weight for different temperature ranges, but the calculation process used in each case the same as in Utah model (Cesaraccio et al., 2004). In turn, Dynamic model also adapted the negative weighing values at high and low temperatures. This model assumes that chilling portion (CP) can accumulate through two-step processes: 1) formation of an intermediate product by cold temperature and 2) transformation of a certain amount of the intermediate product into an irreversible CP.

CA

CA refers to an increase in freezing tolerance over time in response to short-days and low temperatures during fall (Guy, 1990; Thomashow, 1999; Wisniewski et al., 2018). Based on extensive reprogramming of transcriptomes, CA accompanies numerous physiological and biochemical changes (Gusta and Wisniewski, 2013; Strimbeck et al., 2015; Theocharis et al., 2012). Diverse strategies have been employed to CA such as accumulation of soluble sugars, amino acids, and soluble proteins (Fürtauer et al., 2019; Griffith et al., 2005; Kasuga et al., 2007); modification of protein metabolism including levels and activities of enzymes from various metabolic pathways (Chen et al., 2012; Guy, 1990; Janská et al., 2010); alteration of membrane lipid composition (Kawamura and Uemura, 2014; Uemura et al., 2006). Most of these processes are involved in membrane stabilization and protection against stresses including dehydration and mechanical wounding during inter- and intracellular ice formation caused by freezing (Thomashow, 1999; Wisniewski et al., 2018).

As a signal of cold perception, the change in membrane fluidity leads to

opening of Ca^{2+} channels which induce signaling cascades and comprehensive transcriptomic changes (Ding et al., 2019; Guo et al., 2018). Low temperatures also activate C-repeat binding factor (CBF) signaling via activating plasma membrane-located kinases (Liu et al., 2017). Induced CBFs alter the expression of more than 100 cold-regulated (*COR*) genes (Zhao et al., 2016). For example, CBF overexpression in apple (Wisniewski et al., 2015) and blueberry (Walworth et al., 2012) successfully enhanced the cold hardiness in their respective non-acclimated plants. This suggests that the CBF system can be manipulated to improve freezing tolerance in fruit tree species, although it is probably not the only pathway involved (Wisniewski et al., 2014).

DA

DA refers to a loss of freezing tolerance (Kalberer et al., 2006; Vitasse et al., 2014; Xin and Browse, 2000) and is affected by temperature conditions rather than photoperiod (Kjær et al., 2019; Renaut et al., 2008). The rate of DA increases with increasing both temperature and the duration of warm period (Hoffman et al., 2014). Although DA is necessary for the growth resumption the following spring, premature transition to DA might induce the freezing damage by sudden temperature drop during late winter or early spring (Shin et al., 2015; Vyse et al., 2019).

DA is simply regarded as the reverse process of CA, but all the DA reactions are not proceeded inversely (Kalberer et al., 2006). Stress- or defense-related proteins distinctly decrease during DA, while proteins related to growth and development increase (Pagter et al., 2014). DA usually occurs much faster than CA and the differential kinetics between CA and DA might be related to energy

requirement (Pagter and Arora, 2013). While CA involves structural and functional changes with large energy consumption, DA might be relatively less energy-intensive process (Browse and Lange, 2004).

Reacclimation

Reacclimation is process that plants regain some or most of lost cold hardiness when temperatures drop again (Byun et al., 2014; Kjær et al., 2019; Shin et al., 2015). Along with the DA resistance, reacclimation capacity in late winter or early spring is also an important factor determining the survival of plants under sudden or severe cold temperature conditions. Reacclimation is common in many overwintering woody perennials, but not always possible (Kalberer et al., 2006). As the DA can be reversible or irreversible depending on the temperature and duration of DA (Rapacz, 2002), reacclimation capacity necessarily depends on the state of DA.

Cold hardiness determination

Cold hardiness can be measured by plant survival in field trials or by artificial freezing treatment (Yu and Lee, 2020). Assessment of plant survival following severe winter hit lacks reproducibility and the freezing conditions cannot be controlled. Therefore, artificial freezing methods which are fast and correlated with plant survival have commonly been used (Espevig et al., 2011; Lindén et al., 2000). After artificial freezing treatment, damages are evaluated based on tissue browning, electrolyte leakage (EL), or 2,3,5-triphenyltetrazolium chloride (TTC) reduction (Yu et al., 2017). The principle of EL analysis is that membrane injuries causing ion leakage and the percentage of electrolytes leaked from damages cells

are evaluated. As a redox indicator, colorless TTC is reduced to red 1,3,5-triphenylformazan by the action of various dehydrogenases in living tissues (Yu and Lee, 2020). As the data from EL or TTC reduction analysis are quantifiable, the temperature at which 50% injury occurred, LT_{50}, can be calculated. Freezing injury can also be measured by detecting exotherms which are abrupt temperature increases in tissues when temperature gradually drops and ice forms interceullarly and intracellularly (Burke et al., 1976). As the last exotherm represent the temperature where the intracellular ice forms which is irreversibly lethal, thermal analysis can also be used to evaluate cold hardiness (Volk et al., 2009).

Mechanisms of freezing injury

Freezing injury in plant cells usually results from membrane damage caused by intracellular ice formation and cellular dehydration (Thomashow, 1999). When plant cells freeze, ice formation initiates at intercellular space as the freezing point of intercellular is higher than intracellular space (Rodrigo, 2000). Although intercellular freezing is not lethal to plant cells, the intercellular water potential decreases with ice formation (Xin and Browse, 2000). Consequently, cytoplasmic water moves to intercellular space through the plasma membrane, leading to cellular dehydration. Therefore, plants undergo not only the low temperature stress but also the dehydration stress. When freezing continues or the cooling is rapid, ice formation also occurs inside the cell which is irreversible and lethal (Burke et al., 1976).

Sugar metabolism and cold hardiness

Carbohydrate states and cold hardiness have been studied in a wide range of

plant species. Sugar metabolism is closely related to temperature conditions and soluble sugars usually accumulate in response to low temperature (John et al., 2016). Relationship between cold hardiness and sugar accumulation have been established in various fruit tree species including blueberry (Lee et al., 2012, 2013), citrus (Guy et al., 1980), peach (Yu et al., 2017), and raspberry (Palonen, 1999). Soluble sugars, such as glucose, fructose, sucrose, raffinose, and stachyose, accumulate during CA in plant species. Soluble sugar accumulation is correlated with cold hardiness and the soluble sugars are species-specific (Yang et al., 2015).

Along with the changes of soluble sugar contents, various enzymes involved in the sugar metabolism are associated with cold hardiness. As a central part of energy metabolism, biosynthesis and degradation of sucrose are vital to the development of cold hardiness (Nägele et al., 2012). Sucrose synthase (SUS), sucrose phosphate synthase (SPS), and invertases are directly involved in sucrose synthesis and degradation. SUS catalyzes the synthesis of sucrose using fructose and uridine disphosphate (UDP)-glucose as substrates. On the other hand, SPS catalyzes the synthesis of sucrose-6-phosphate, which is then hydrolyzed to sucrose by sucrose phosphatase. The activities of SUS and SPS showed positive correlations with cold hardiness in cabbage (Sasaki et al., 2001) and poplar (Schrader and Sauter, 2002). Furthermore, *Arabidopsis* overexpressing *SPS* gene showed an enhanced freezing tolerance (Strand et al., 2003).

Sucrose is degraded by the action of SUS or invertases. SUS reversibly split the sucrose into UDP-glucose and fructose, while invertase degrade the sucrose into glucose and fructose irreversibly (Stein and Granot, 2019). Invertases during CA are differently regulated depending on plant species. Transcript levels of invertase family genes and enzyme activity were suppressed under low

temperature in *Arabidopsis* (Usadel et al., 2008) and cabbage (Sasaki et al., 2001). On the contrary, invertase activity has been reported to increase during CA in pear (Ito et al., 2012) and tomato (Artuso et al., 2000). Although repeated sucrose breakdown and synthesis appear to be energetically futile, the sucrose cycling allows precise control over carbohydrate partitioning (Ruan, 2014). Sucrose, the first free sugar formed during photosynthesis, can be used as a substrate for the synthesis of different types of monosaccharides (fructose and glucose) or oligosaccharides (raffinose and stachyose). Furthermore, energy balancing mechanism that reacts efficiently to environmental changes can be served from sucrose cycling (Fürtauer et al., 2019).

Raffinose family oligosaccharides (RFOs) such as raffinose and stachyose are also involved in the responses to abiotic stresses (Sengupta et al., 2015). RFOs accumulate during CA or seed desiccation and they stabilize the membrane by replacing water molecules (Hincha et al., 2003). The transcriptional up-regulation of RFO pathway under low temperature has been reported in various plant species (Sengupta et al., 2015). One of key enzymes in the RFO pathway is galactinol synthase which catalyzes the first step in raffinose synthesis. Along with the raffinose synthase, transcription of galactinol synthase is induced during CA (Usadel et al., 2008). Overexpressing a myelobalstosis transcription factor (MYB)-related transcription factor from grapevine induced the gene expression of galactinol synthase and enhanced cold hardiness in *Arabidopsis* (Sun et al., 2018).

Starch hydrolysis is also closely related to CA, because starch contents were negatively correlated with cold hardiness in apple (Raese et al., 1977), cloudberry (Kaurin et al., 1981), and peach (Lasheen and Chaplin, 1971). Starch was proposed to be hydrolyzed into soluble sugars in response to low temperature,

increasing intercellular water potential (Kaplan et al., 2006). Amylases, which catalyze starch breakdown, have been studied as key target genes related to cold hardiness in many plant species such as blueberry (Lee et al., 2012). Overexpression of β-amylase gene from trifoliate orange enhanced the cold hardiness along with the starch degradation and soluble sugar accumulation through the increased β-amylase activity in tobacco (Peng et al., 2014).

Functions of soluble sugars against freezing stress

Soluble sugar accumulation during CA allows plants to withstand low temperatures, and the soluble sugars have multiple functions such as osmoprotection, membrane stabilization, and protein protection from desiccation (Fürtauer et al., 2019; Guy, 1990; Xin and Browse, 2000). Molecules increasing the intracellular osmotic potential are referred to as osmolytes, osmoprotectants, or compatible solutes (Theocharis et al., 2012). During CA, plants accumulate a range of compatible solutes including soluble sugars, amino acids, polyamines, and betaines (Ruelland and Zachowski, 2010). The accumulated compatible solutes lower the freezing point and reduce the amount of dehydration during extracellular freezing (Xin and Browse, 2000).

Soluble sugars enhance the membrane stability under freezing conditions by hydrophilic interaction with the membrane interface (Sami et al., 2016). Amino acid (proline) and protein (dehydrin) were also reported to accumulate during CA and to enhance the membrane stability due to their hydrophilic nature (Kawamura and Uemura, 2014).

Soluble sugars also mitigate the desiccation stress induced by intercellular ice formation (Castonguay et al., 1995). For example, the hydroxyl groups of sucrose

may replace water molecules by hydrogen bonding to the phospholipid head groups of the membrane (Palonen, 1999). The hydrogen bonding between sugars and proteins might interfere the dehydration-induced protein unfolding (Allison et al., 1999). Similarly, sucrose and oligosaccharides have been reported to accumulate during the desiccation phase of maturing seeds and pollens (Chen and Buris, 1990), indicating that the sugars could play a key role in cell tolerance to desiccation.

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CHAPTER 1

Determination of dormancy break and chilling requirement of peach (*Prunus persica*) cultivars and changes in chilling accumulation spectra

ABSTRACT

Chilling requirements of 18 peach cultivars and chilling accumulation spectra over the last 100 years (1919-2019) were evaluated to determine phenological characteristics for dormancy break in Republic of Korea. The chilling requirements were calculated by using Chill Hours, Utah, Dynamic, North Carolina, and Low Chilling models. Chilling requirements of 15 cultivars ranged from 263 to 2123 chill hour, 377 to 1134 chill unit, and 21.3 to 74.8 chilling portion. Dynamic model showed the highest precision with the smallest variation among years, followed by Utah model. During the last 100 years, the initiation date of chilling accumulation had been delayed for 9-12 days in Chill Hours and Utah models. In contrast, Dynamic model showed no significant changes in chilling accumulation spectra. Although the chilling accumulation has been enough to fulfill the chilling requirement in Republic of Korea, reduced chills were monitored from 100-year data. Therefore, the chilling accumulation is needed to be continuously monitored or estimated in the future.

Key words: chilling accumulation, chilling requirement, deciduous fruit trees, dormancy, global warming, peach, *Prunus persica*, temperate climate

INTRODUCTION

Dormancy is a phenological mechanism evolved in deciduous fruit trees to survive under unfavorable environmental conditions, such as harsh winter (Arora et al., 2003; Baldocchi and Wong, 2008; Yamane, 2014). Dormancy minimizes low temperature damage to flowers or to the whole tree by halting bud break and flowering. Thus, to overcome endodormancy and resume growth, a certain amount of chilling must be accumulated prior to bud break (Campoy et al., 2011; Samish, 1954). After the fulfillment of chilling requirement, buds need heat accumulation for bud break and flowering (Campoy et al., 2012; Couvillon and Erez, 1985).

Chilling requirement varies among the fruit tree species or cultivars and determines their climatic distribution (Arora et al., 2003; El Yaacoubi et al., 2014). Therefore, quantifying the chilling requirement of cultivars is a key factor to determine their appropriate growth area for commercial production (Byrne et al., 2000; Valentini et al., 2001). For example, insufficient chilling in regions with warm winters can cause problems such as deformation of buds during their development, delayed bud break, and partial or uneven germination of vegetative and flower buds, reducing fruit set and yield (Couvillon and Erez, 1985; Erez and Lavee, 1971; Leite et al., 2004; Oukabli et al., 2003). Furthermore, according to Li et al. (2016a), aberrant fruit shape was caused by reduced chilling hours and delay of bloom date. This problem can be more severe in the future in the context of climate change, whereby studies are increasingly focusing on the impact of global warming on fruit-tree biology (El Yaacoubi et al., 2014; Luedeling, 2012). Furthermore, the demand of low-chill cultivars has been increasing (Campoy et al.,

2011) for the same reason, and low-chill cultivars have been developed in warm areas, such as the subtropics, for environmental adaptation of Asian plum, apricot, and peach (Byrne and Anderson, 2012; Byrne et al., 2000; Sherman and Lyrene, 2003; Topp et al., 2012).

On the other hand, low-chill cultivars can suffer frost and freezing damage in cold-winter areas owing to the ready fulfillment of chilling requirement and early blooming (Gao et al., 2012; Scorza and Okie, 1990). Freezing damage is also more likely to happen under global warming owing to the premature deacclimation caused by warm spells. In cold-winter areas, therefore, high-chill or late-flowering cultivars are major objectives in breeding programs with the evaluation of chilling requirements (Bailey et al., 1982).

Various models have been proposed to estimate chilling requirements in deciduous fruit trees. Chill Hours model (Weinberger, 1950) is regarded as the oldest and simplest model where the number of hours below 7.2ºC is accumulated as chill hour (CH). This model, however, does not consider the negative effect of high temperatures on chill accumulation, which was overcome by Utah model (Richardson et al., 1974) through introduction of a chilling unit (CU) with different weighing values for different ranges of temperature. North Carolina (Shaltout and Unrath, 1983), Low Chilling (Gilreath and Buchanan, 1981), Positive Chill Unit (Linsley-Noakes et al., 1995), and Taiwan (Lu et al., 2012; Milech et al., 2018) models were the modifications derived from Utah model to adjust to varying climate conditions and these models use CU values of different weights for different temperature ranges. Dynamic model (Fishman et al., 1987a, b) also adapted the negative weighing values at high and low temperatures. This model assumes that chilling portion (CP) can accumulate through two-step

processes: formation of an intermediate product by cold temperature and transformation of a certain amount of the intermediate product into an irreversible CP.

Chilling requirement is an important trait which should be considered to select suitable cultivation area. Despite the lack of information of chilling requirement values in Korean peach cultivars, no serious problems regarding insufficient chilling requirement have been reported in domestic cultivation. However, the decrease of winter chill was reported from long-term climatic records (Baldocchi and Wong, 2008) and the chilling requirement of cultivars is needed to be evaluated for safe cultivation in the near future. In this study, therefore, the chilling requirement values of Korean peach cultivars were evaluated. Five different chilling requirement models were also compared to select a proper chilling requirement model for Korean climatic conditions. Furthermore, to test the hypothesis that global warming is ongoing and is influencing on the chilling accumulation in Korea, the last 100 years of winter temperature data were analyzed.

MATERIALS AND METHODS

Plant materials

Eleven Korean peach cultivars and two leading cultivars were used in this study. Five cultivars whose chilling requirements were already known (Okie, 1998) were also included as a control group (Table I-1): 'TropicSnow' (200 CU), 'Springtime' (650 CU), 'Flavorcrest' (750 CU), 'Collins' (950 CU), and 'Garden State' (1050 CU). The chilling requirements of the cultivars were evaluated in the experimental orchards at the National Institute of Horticultural and Herbal Science (NIHHS), Suwon (37º2'N, 127º0'E), Republic of Korea during 2013-2015 and in Wanju (35º8'N, 127º0'E) during 2015-2017. Because of the relocation of the institute, the experimental orchards of the NIHHS were inevitably moved from Suwon to Wanju. Trees were 4-6 years old at the beginning of the study. The trees were planted at 6 × 4 m and maintained with the recommended cultural practices including irrigation, fertilization, and pest control. Temperature data were collected from the weather stations of the Korea Meteorological Administration located in Suwon (37º2'N, 126º6'E) and Jeonju (35º5'N, 127º1'E); these were the closest stations to the experimental orchards.

Chilling requirements

From October of each year, ten shoots (30 cm in length and 5 mm in diameter) were randomly collected every week from the field-grown trees. The shoots were inserted into wet expanded foam and placed in a growth chamber maintained at 25ºC under white fluorescent light with a photoperiod of 16/8 h, light/dark. The shoots were monitored every 3-4 days to determine bud break.

Table I-1. Parentages and origins of 18 peach cultivars used in the study. Previously reported chill unit (CU) of five control cultivars (Okie, 1998) were noted.

Cultivar	Parentage	Origin	CU
Cheonhong	Garden State OP	Suwon, Korea	
Collins	Jerseyland × NJ188	New Jersey, USA	950
Flavorcrest	P53-68 × FV89-14	California, USA	750
Garden State	Elberta OP OP	New Jersey, USA	1050
Hahong	Cheonhong × SunGlo	Suwon, Korea	
Hwanghoo	Changhowon Hwangdo OP	Suwon, Korea	
Jinmi	Hakuho × Nunomewase	Suwon, Korea	
Kanoiwa Hakuto	Bud mutation of Asama Hakuto	Yamanashi, Japan	
Mihong	Yumyeong × Chiyomaru	Suwon, Korea	
Misshong	Yumyeong × Chiyomaru	Suwon, Korea	
Odoroki	Bud mutation of Hakuho	Nagano, Japan	
Seonmi	Hakuho × Baekhyang	Suwon, Korea	
Soomee	Yumyeong × Chiyomaru	Suwon, Korea	
Springtime	(Luken's Honey × July Elberta) × Robin	California, USA	650
Suhong	SunGlo × Cheonhong	Suwon, Korea	
TropicSnow	Fla.7-11 OP	Florida, USA	200
Yumi	Yumyeong × Chiyomaru	Suwon, Korea	
Yumyeong	Yamatowase × Nunomewase	Suwon, Korea	

OP, open pollination.

Dormancy was considered to be completed when minimum 50% of flower buds protruded more than 1 mm after 14 days (Fan et al., 2010), which has widely been used to estimate chilling requirement (Campoy et al., 2019). Chilling requirement was calculated as sum of chills (CH, CU, or CP according to the models) from initiation date for chilling accumulation to date of dormancy completion. Initiation date for chilling accumulation was considered as the date when the temperature producing a negative effect on chilling accumulation was scarce (Erez et al., 1979). The equation for Chill Hours model (Weinberger, 1950) is as follows.

$$CH = \sum CH_t \quad \begin{cases} T_t > 7.2℃, & CH_t = 0 \\ T_t \leq 7.2℃, & CH_t = 1 \end{cases}$$

The equation for Utah, Low Chilling, and North Carolina models is as follows and the chill unit factors (CU_t) were shown in Table I-2.

$$CU = \sum CU_t$$

The equations for Dynamic model are as follows. The constants a_0, a_1, e_0, e_1, *slp*, and *tetmlt* were set to 1.395×10^5, 2.567×10^{18}, 4.1535×10^3, 1.28888×10^4, 1.6, and 277, respectively (Darbyshire et al., 2011).

$$x_t = \frac{e^{slp \times tetmlt \times (T_t - tetmlt)/T_t}}{1 + e^{slp \times tetmlt \times (T_t - tetmlt)/T_t}}$$

$$y_t = \frac{a_0}{a_1} \times e^{(e_1 - e_0)/T_t}$$

$$ak_t = a_1 \times e^{(-e_1/T_t)}$$

$$Inter_{Et} = y_t - (y_t - Inter_{St}) \times e^{-ak_1}$$

Table I-2. Temperature ranges and chill unit factors (CU_t) for Utah, North Carolina, and Low Chilling models (from Cesaraccio et al., 2004).

Utah		North Carolina		Low Chilling	
Temp.	CU_t	Temp.	CU_t	Temp.	CU_t
<1.5	0.0	≤1.5	0.0	≤1.7	0.0
1.5-2.4	0.5	1.6-7.1	0.5	1.8-7.9	0.5
2.5-9.1	1.0	7.2-12.9	1.0	8.0-13.9	1.0
9.2-12.4	0.5	13.0-16.4	0.5	14.0-16.9	0.5
12.5-15.9	0.0	16.5-18.9	0.0	17.0-19.4	0.0
16.0-18.0	–0.5	19.0-20.6	–0.5	19.5-21.4	–0.5
>18.0	–1.0	20.7-22.0	–1.0	≥21.5	–1.0
		22.1-23.2	–1.5		
		≥23.3	–2.0		

$$Inter_{St} = \begin{cases} t = 1, & 0 \\ t > 1 \ and \ Inter_{Et-1} < 1, & Inter_{Et-1} \\ t > 1 \ and \ Inter_{Et-1} \geq 1, & Inter_{Et-1} \ X \ (1 - x_{t-1}) \end{cases}$$

$$delt_t = \begin{cases} t = 1, & 0 \\ t > 1 \ and \ Inter_{Et-1} < 1, & 0 \\ t > 1 \ and \ Inter_{Et-1} \geq 1, & Inter_{Et} \ X \ x_t \end{cases}$$

$$CP_t = \begin{cases} t = 1, & delt_t \\ t > 1, & delt_t + CP_{t-1} \end{cases}$$

$$CP = \sum CP_t$$

Chilling accumulation over the last 100 years of climate records

Among the few locations that have more than 100 years of temperature records, Jeonju was selected for consistency of the study. The hourly temperature data from July 1919 to June 2019 were obtained from the Korea Meteorological Administration weather station located at Jeonju (35°5'N, 127°1'E). The temperatures were recorded every 4 h during 1919-1980, and the missing data were replaced by using the linear function. From 1981, temperatures were recorded every hour. The hourly temperatures were transferred to chilling accumulation using Chill Hours, Utah, and Dynamic models.

Statistical analysis

Differences among the cultivars and years were determined based on analysis of variance (ANOVA). The statistical analysis and Pearson correlation analysis among the models were performed using the R package (version 3.3.1) (R Core Team, 2016).

RESULTS AND DISCUSSION

Chilling requirements of peach cultivars

Chilling accumulation in Suwon and Wanju during 4 years were estimated by the five chilling requirement models (Fig. I-1). Chilling temperature accumulated from October to March of the following year. In Chill Hours model (Fig. I-1a), chilling accumulation was higher in Suwon than in Wanju. Because Suwon and Wanju are located in the northern and southern parts of Republic of Korea, respectively, the temperatures are usually lower in Suwon than in Wanju. Therefore, the CH accumulation in Suwon was more rapid than that in Wanju and the total accumulated CH at the end of March was also higher in Suwon. In Utah, North Carolina, and Low Chilling models (Fig. I-1b, d, and e), however, the CU accumulation was higher in Wanju than in Suwon after mid-December. Different chilling accumulation patterns were also found between the locations especially in North Carolina and Low Chilling models (Fig. I-1d and e), resulting from the higher and lower chilling accumulations in Wanju and Suwon, respectively, during December and January. In these models, there was no effective chilling accumulation at the temperature below 1.5°C. As the temperature decreased and the frequencies of subzero temperatures increased, the rates of chilling accumulation significantly decreased. On the other hand, chilling accumulation continuously increased during December in Wanju where the temperature about 5°C was frequently observed.

In Dynamic model (Fig. I-1c), no different chilling accumulation patterns were observed between the locations. As Dynamic model depends on the synergistic effect between moderate and low temperatures for chilling requirement

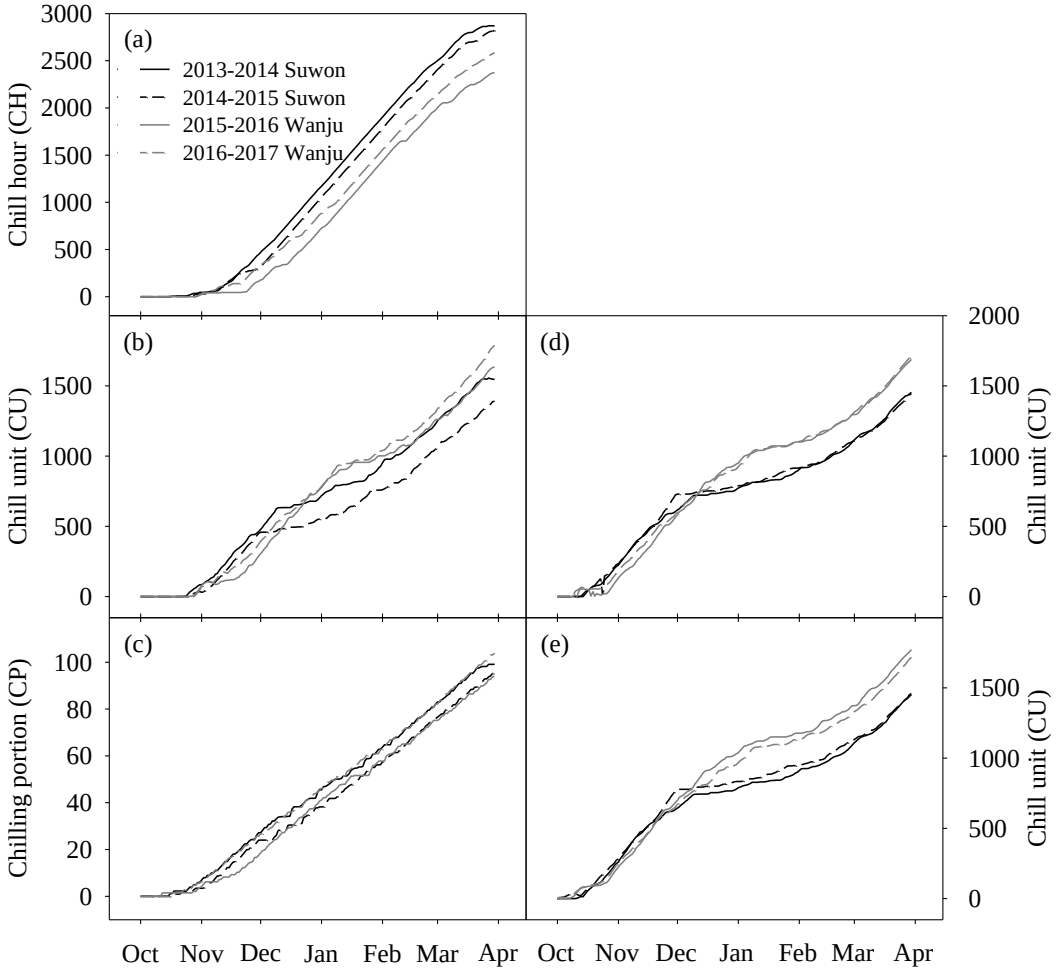


Fig. I-1. Chilling accumulation in (a) Chill Hours, (b) Utah, (c) Dynamic, (d) North Carolina, and (e) Low Chilling models from October to March in Suwon and Wanju, Republic of Korea.

(Fishman et al., 1987a, b), CP was usually continuously accumulated without fluctuations which make differences between the locations or years. This result is accordance with the other reports that variation in Dynamic model was smaller than that in the other models (Campoy et al., 2012; Viti et al., 2010).

Chilling accumulation in Suwon (October 16) initiated approximately 14 days earlier than that in Wanju (October 30) in Chill Hours model (Fig. I-1a). Because the average temperature is lower in Suwon than in Wanju, the temperatures below 7.2°C in Suwon started to accumulate in mid-October. However, the opposite pattern was observed in North Carolina and Low Chilling models, indicating that chilling accumulation initiated earlier in Wanju (October 8) than in Suwon (October 14). The late accumulation in cold area was resulted from the unusual high temperature during early October in Suwon. During this period, temperatures over 20°C were more frequent than normal years, a data with previous 10 years. North Carolina and Low Chilling models have 0.5 CU at the 13.0-16.4°C and 14.0-16.9°C, respectively, while Utah model have zero or negative effect at those temperature ranges. In Wanju, temperatures near 15°C were frequent during early October and the positive CU factors started to accumulate in North Carolina and Low Chilling models. Because the temperature in Suwon was too high (> 20°C) to positively affect chilling requirement, chilling accumulation of these two models started earlier in Wanju than in Suwon.

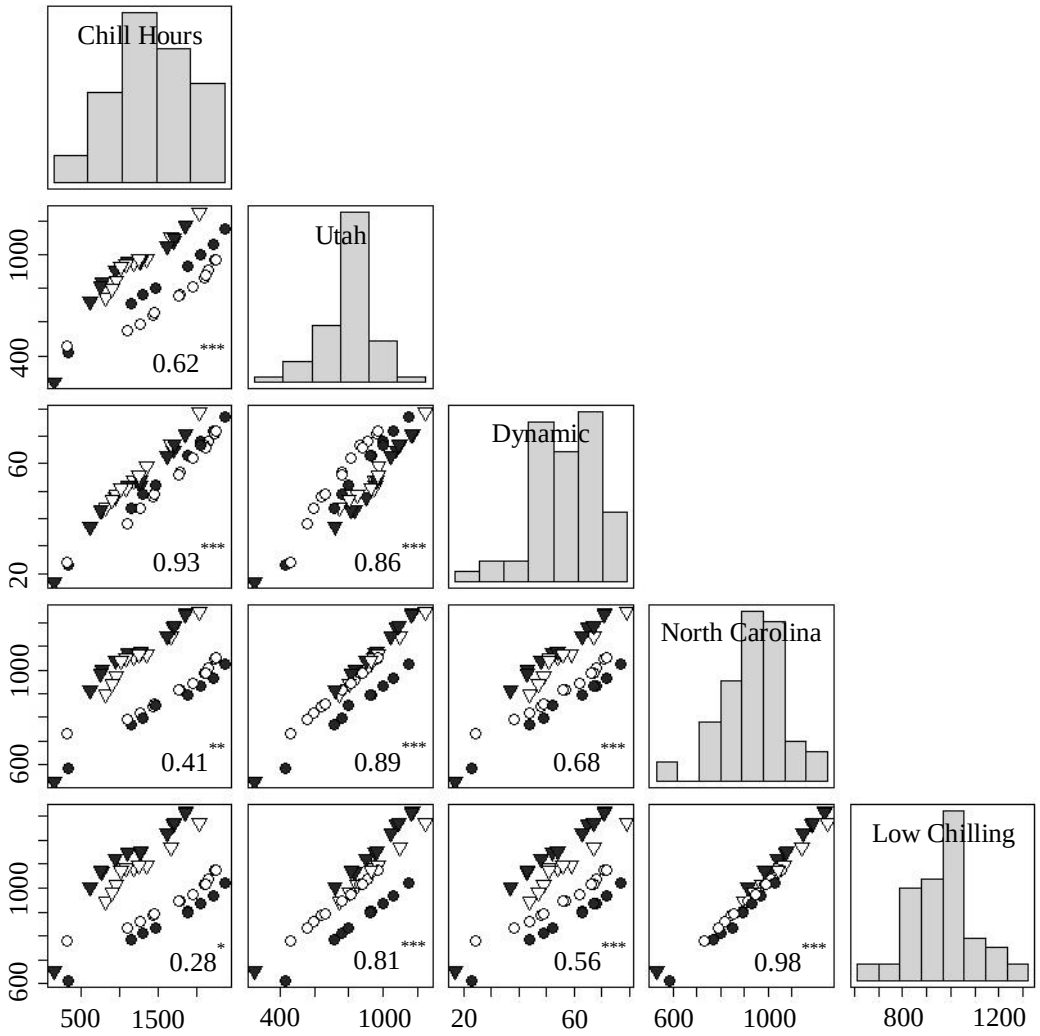
The differential initiation of chilling accumulation was also compared to that in other studies. Utah model revealed that chilling accumulation was initiated in late October, and this timing was similar to the results reported for Seneca, South Carolina (Fan et al., 2010) and earlier than that in Murcia, Spain, where chilling accumulation started in November (Alburquerque et al., 2008; Egea et al., 2003).

Similarly, Richardson et al. (1974) reported earlier chilling accumulation at Ogden, Utah than that at St. George, Utah, south of Ogden. The earlier initiation in the colder area is reasonable, because the frequency of low temperature at the colder location is higher in early fall, whereby chill accumulation starts earlier.

Relationships among the chilling requirement models are shown in Fig. I-2. Chill Hours model showed different patterns depending on the locations. Utah model and its modifications, North Carolina ($r = 0.89^{***}$) and Low Chilling ($r = 0.81^{***}$) models, showed close relationships. North Carolina and Low Chilling models were most closely related ($r = 0.98^{***}$).

Calculated chilling requirements of 18 peach cultivars are shown in Table I-3. The control cultivars, 'Springtime' (754 CU), 'Flavorcrest' (796 CU), 'Collins' (940 CU), and 'Garden State' (1019 CU), showed CUs similar to those previously reported (Okie, 1998). Chilling requirement of 'TropicSnow' was estimated as 377 CU which is slightly higher than previously reported (200 CU). Nonetheless, this low-chill cultivar showed the earliest dormancy breaking date (November 28) and the lowest chilling requirement. Dates of dormancy completion of Korean cultivars were distributed in January and February. 'Suhong' and 'Hahong' showed the lowest chilling requirement with 733 CU and 742 CU among the Korean cultivars, respectively. Meanwhile, 'Seonmi' completed dormancy after mid-February with the highest chilling requirement (1134 CU) in this study.

In the present Korean climate, chilling accumulation in most locations is enough to fulfill the chilling requirement of peach cultivars, but frost damage or freezing injury occasionally impacts peach trees in Korea (Jun et al., 2016). Due to these problems, high-chill or late-flowering cultivars are still preferred over low-chill cultivars. On the other hand, low-chill cultivars, especially in early



I-2. Scatter plot matrix and Pearson correlation coefficients between pairs of chilling requirement values estimated by Chill Hours, Utah, Dynamic, North Carolina, and Low Chilling models. Closed circle, open circle, closed triangle, and open triangle are 2013-2014 (Suwon), 2014-2015 (Suwon), 2015-2016 (Wanju), and 2016-2017 (Wanju), respectively. *, **, ***Significant at $P < 0.05$, 0.01, or 0.001 respectively.

Table I-3. Date of dormancy break and chilling requirement estimated by Chill Hours, Utah, Dynamic, North Carolina, and Low Chilling models for 18 peach cultivars. Data were obtained in Suwon (S) during 2013-2014 and 2014-2015 winters; in Wanju (W) during 2015-2016 and 2016-2017 winters; the mean (M) of the two locations.

			Chilling requirement									
		Dormancy break	Chill Hours		Utah		Dynamic		North Carolina		Low Chilling	
Cultivar		(DOY)	(CH)	CV	(CU)	CV	(CP)	CV	(CU)	CV	(CU)	CV
Cheonhong	M	13	1224	39	788	15	49.5	18	910	7	951	9
	S	24	1653	19	785	26	55.4	19	924	4	895	6
	W	1	795	4	792	8	43.5	1	915	8	1006	9
Collins	M	28	1576	36	940	5	59.3	17	1009	6	1045	8
	S	43	2095	3	953	7	67.8	1	1025	6	986	7
	W	14	1057	16	927	4	50.8	9	926	1	1103	2
Flavorcrest	M	12	1193	35	796	5	49.5	12	919	10	961	12
	S	19	1535	23	762	1	52.9	11	951	10	880	11
	W	4	852	17	831	3	46.0	9	972	5	1042	4
Garden State	M	49	1871	21	1019	6	66.8	9	1067	8	1101	11
	S	49	2221	1	1013	7	71.5	1	1116	6	1020	7
	W	50	1521	16	1026	7	62.0	7	933	7	1182	10
Hahong	M	5	1051	24	742	14	45.7	6	885	11	931	13
	S	8	1275	18	677	1	46.4	7	901	2	835	4
	W	3	826	13	808	1	45.0	6	917	3	1027	6
Hwanghoo	M	25	1497	23	897	11	55.4	10	983	10	1020	12
	S	31	1819	14	843	5	59.4	8	994	2	923	4
	W	19	1175	11	951	1	51.3	1	973	3	1116	4

Table I-3. Continued.

			Chilling requirement									
		Dormancy break	Chill Hours		Utah		Dynamic		North Carolina		Low Chilling	
Cultivar		(DOY)	(CH)	CV	(CU)	CV	(CP)	CV	(CU)	CV	(CU)	CV
Jinmi	M	27	1540	31	923	4	58.1	13	1001	8	1037	10
	S	39	1989	8	896	5	64.9	5	1029	7	957	8
	W	15	1091	1	951	1	51.3	1	973	2	1116	4
Kanoiwa	M	38	1753	18	977	7	62.9	7	1042	11	1075	14
Hakuto	S	44	2150	4	944	6	68.2	3	1075	6	989	7
	W	32	1306	9	958	4	52.2	4	953	4	1149	7
Mihong	M	29	1587	22	920	8	58.7	7	996	9	1032	11
	S	35	1915	2	871	10	62.4	1	1012	4	938	6
	W	22	1259	1	969	1	54.9	3	980	2	1126	4
Misshong	M	42	1874	9	1016	15	66.9	5	1066	15	1101	17
	S	39	1989	8	896	5	64.9	5	1113	7	957	8
	W	45	1759	8	1136	4	68.9	4	1085	6	1245	9
Odoroki	M	36	1735	15	986	10	62.8	6	1013	11	1104	13
	S	37	1956	9	859	5	64.5	3	957	3	964	5
	W	35	1427	5	1009	3	59.1	2	1053	2	1164	4
Seonmi	M	53	2123	11	1134	11	74.8	5	1142	11	1172	13
	S	53	2305	3	1059	12	74.5	5	1145	2	1049	4
	W	54	1941	6	1209	5	75.1	7	1008	1	1294	3
Springtime	M	6	1052	33	754	17	45.4	14	895	14	939	14
	S	9	1294	17	684	5	46.7	7	885	8	839	9
	W	3	810	35	824	18	44.2	16	920	9	1039	14
Soomee	M	45	1985	7	1026	16	62.8	7	1068	12	1038	12
	S	44	2085	5	952	6	68.6	3	1105	5	958	7
	W	46	1604	12	1018	5	64.5	4	1090	4	1251	8

Table I-3. Continued.

			Chilling requirement									
		Dormancy break	Chill Hours		Utah		Dynamic		North Carolina		Low Chilling	
Cultivar		(DOY)	(CH)	CV	(CU)	CV	(CP)	CV	(CU)	CV	(CU)	CV
Suhong	M	4	1010	22	733	17	44.2	11	879	12	925	14
	S	5	1195	22	658	11	43.4	17	888	1	823	2
	W	3	826	13	808	1	45.0	6	761	3	1027	6
TropicSnow	M	-33	263	40	377	30	21.3	19	614	17	680	13
	S	-33	323	8	442	5	23.7	6	630	16	695	3
	W	-33	143	na	248	na	16.6	na	849	na	652	na
Yumi	M	29	1569	26	924	14	59.0	14	1017	14	1048	17
	S	30	1781	26	837	6	59.1	17	1088	11	924	14
	W	28	1358	36	1011	11	58.8	19	987	9	1172	12
Yumyeong	M	40	1870	12	952	13	64.1	4	1008	12	1045	15
	S	39	1997	3	904	15	64.5	5	938	1	955	3
	W	41	1616	na	1049	na	63.3	na	1147	na	1227	na
Mean	M	25	1420		861		54.6		959		999	
	S	29	1692		819		57.2		976		912	
	W	21	1135		902		51.8		956		1092	
Max	M	53	2123		1134		74.8		1142		1172	
	S	53	2305		1059		74.5		1145		1049	
	W	54	1941		1209		75.1		1147		1294	
Min	M	-33	263		377		21.3		614		680	
	S	-33	323		442		23.7		630		695	
	W	-33	143		248		16.6		761		652	

DOY, day of year; CH, chill hour; CU, chill unit; CP, chilling portion; CV, coefficient of variance; na, not applicable to the data only from 2015-2016 winter.

ripening cultivars, can be useful if cultivated in greenhouses or in warm areas. Although low chilling requirements were observed in ‘Suhong’ and ‘Hahong’, new peach cultivars with less chilling requirement are needed to be developed for forcing culture or for coping with climate change.

The models showed different coefficient of variance (CV) values depending on the cultivars and locations (Table I-3). In Suwon and Wanju, the highest CV values were observed most frequently in Chill Hours model. For example, ‘Flavorcrest’, ‘Hahong’, ‘Hwanghoo’, ‘Springtime’, and ‘Yumi’ showed the highest CV values in Chill Hours model in both locations. Dynamic model was well fitted in both locations followed by Utah model. Low CV values were observed in cultivars such as ‘Jinmi’ and ‘Misshong’ in Dynamic model, while Utah model showed high precision in ‘Hahong’.

‘TropicSnow’ showed lower CV values in Low Chilling model than in the other models. Interestingly, this low-chill cultivar originated in a subtropical area showed high accuracy with Low Chilling model, which is suitable for the subtropics. The differences between Low Chilling and Utah models are: 1) the lower chill unit factor (0.5 CU) at 2.5-7.1ºC in the former than in the latter (1.0 CU), and 2) the higher value (1.0 CU) at 9.2-12.4ºC in the former than in the latter (0.5 CU). In November, the frequency of those ranges of temperatures is high and most of the effective chills of ‘TropicSnow’ accumulated in November because dormancy break of ‘TropicSnow’ occurs at the end of November. Thus, the weighing values of Low Chilling models might be more suitable for ‘TropicSnow’ which was originated from Florida, a subtropical region.

In most studies comparing chilling requirement models, Dynamic model showed the good performance for many *Prunus* species in various regions

(Benmoussa et al., 2017; Ruiz et al., 2007; Zhang and Taylor, 2011). Until recently, Utah model had been more widely and practically used in field cultivation than Dynamic model. Due to the easy calculation and good predictions, Utah model has been adopted to a certain extent in cold-winter climates. Furthermore, Utah model has been used in many of studies in genetics (Bielenberg et al., 2015; Fan et al., 2010), cytology (Yooyongwech et al., 2008), and estimation of flowering date (Sawamura et al., 2017). As Chill Hours and Utah models overestimate winter chill losses, Dynamic model was found to be more accurate than the other models in temperate and subtropical climates (Erez and Fishman, 1998; Linsley-Noakes and Allan, 1994). Dynamic model has been adopted in California (Luedeling et al., 2009a). At least for some time, using both Dynamic and Utah models can be an option in Korean climate; in fact, using more than one model might avoid potential miscalculation of chilling requirement. However, Dynamic model which was developed for warmer conditions in Israel (Erez et al., 1990) is likely to be highly accurate even in warm temperature areas.

Chilling accumulation over the last 100 years of climate records

Chilling accumulation spectra from the winters of 1919-1920 to 2018-2019 were expressed using three chilling requirement models (Fig. I-3). The 100-year span was divided into five terms at 20-year intervals. In accordance with many reports regarding global warming and rising temperature (IPCC, 2014), the annual average temperature of Jeonju also increased from 12.1ºC in 1919 to 13.9ºC in 2018 (Korea Meteorological Administration, 2019).

Effective chilling accumulated usually from October, and the delay of the initiation date was observed in Chill Hours and Utah models. In Chill Hours

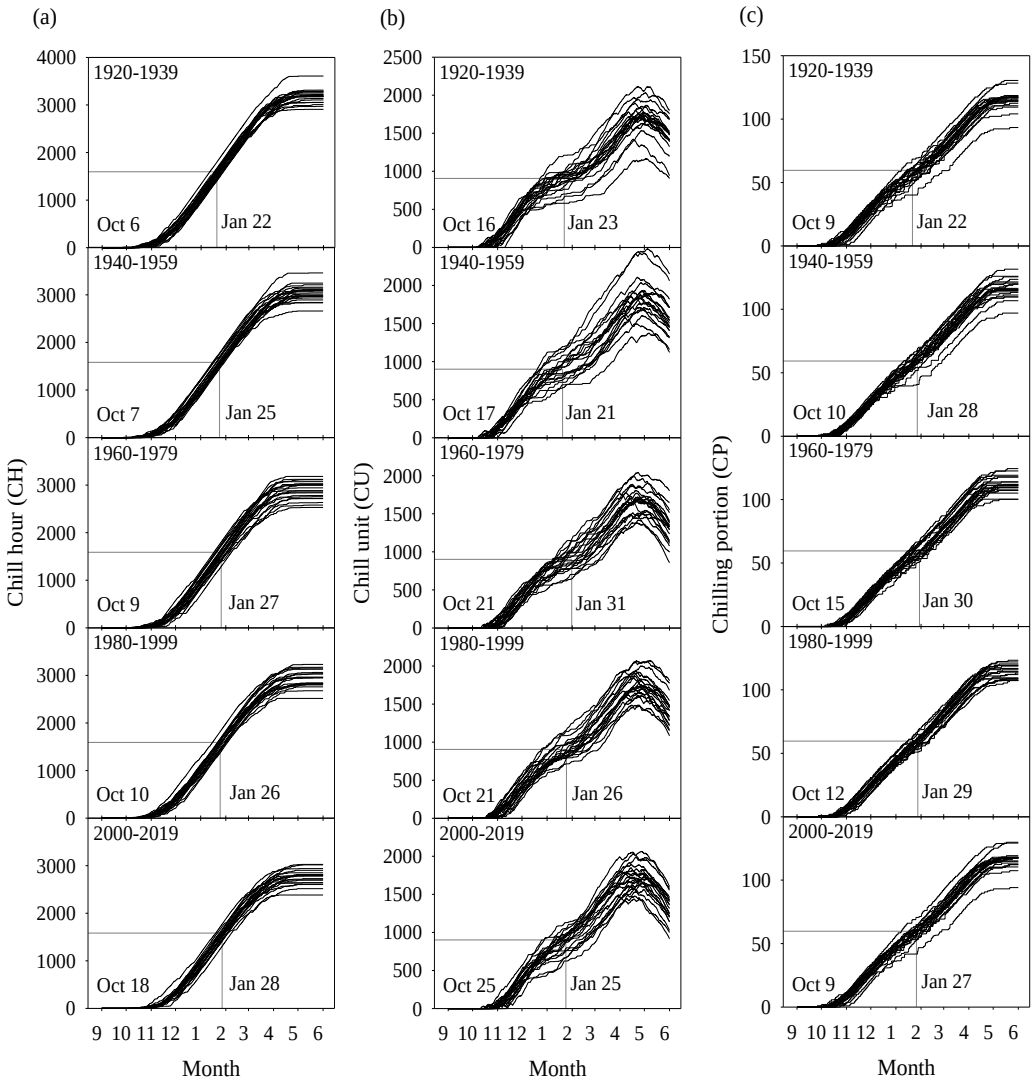


Fig. I-3. Chilling accumulation spectra over the last 100 years using (a) Chill Hours, (b) Utah, and (c) Dynamic models in Jeonju, Republic of Korea. Each spectrum shows the range of chilling accumulation in 20 years. Dates located on the left and right of each spectrum represent the average initiation date of chilling accumulation and the estimated dormancy completion date of Korean peach cultivars with reference line.

model, the initiation dates were October 6 in 1920-1939 and October 18 in 2000-2019, indicating a delay of approximately 12 days. Initiation date in Utah model was also delayed 9 days, from October 16 in 1920-1939 to October 25 in 2000-2019. The initiation date might be delayed continually in the future with global warming because the frequency of low temperature which is effective for chilling requirement is decreasing during early fall.

Among the three chilling requirement models, Chill Hours models showed the smallest inter-annual difference (CV = 6.3), while Utah model showed the largest one (CV = 13.7). Due to the simple estimation in Chill Hours model, CHs are equally accumulated with 1 CH as long as the hourly temperature is below 7.2ºC. Considering the average temperature between October and February when the most of the chilling accumulated is 4-6ºC, the CH continually accumulates through the winter. Therefore, the accumulation in Chill Hours model showed the smallest variation among years. On the other hand, Utah model segmented the temperature range into seven grades with different CU. Especially, this model assign 0 CU when hourly temperature is below 1.5ºC. Because the distribution of low temperature (< 1.5ºC) varies among years, the accumulation patterns are also different between years in Utah model. For example, high frequency of low temperatures (< 1.5ºC) can be observed on late December, early January, or late January depending on the years. Furthermore, graded CU values such as 0.5 and 1.0 CU cause the large yearly differences.

Chilling accumulation was scarce after April in all models, and there was a decrease in the CU of Utah model owing to the chill negation of high temperature (> 16ºC). Chill Hours model does not consider the chill negation effect under warm temperature, and warm temperature does not erase previously accumulated

CP in Dynamic model. Therefore, CH in Chill Hours model or CP in Dynamic model did not decrease under warm temperature in April. In Chill Hours model, chilling accumulated up to 3182 CH during 1920-1939 and this value significantly decreased to 2753 CH during 2000-2019 (Table I-4). The average of maximum chilling accumulation unit over the last 100 years was 1755 CU in Utah model and has not significantly changed. The average of maximum chilling accumulation in Dynamic model was approximately 114 CP, with no significant change.

In this study, the chilling requirements of Korean peach cultivars tested were evaluated at 1010-2123 CH, 733-1134 CU, and 44.2-74.8 CP by the three different models, respectively. Although the maximum chilling accumulation in CH decreased over the last 100 years, the amount fully suffices for breaking dormancy in Korean peach cultivars up to date. However, chilling accumulation might decrease under global warming conditions, which was noticeable using Chill Hours model in this study. In California, for example, CH in some regions had decreased by 30% from 1950 to 2000 and such a severe decrease might be a threat to some crops in the future (Luedeling et al., 2009a, b).

To monitor the changes in chilling accumulation, the dates of dormancy completion were inversely estimated using the average chilling requirement of Korean peach cultivars which were 1600 CH, 900 CU, and 60 CP (Fig. I-3). Chill Hours model revealed that the average dormancy break dates were January 22 in 1920-1939, and January 28 in 2000-2019, suggesting a significant delay. However, dormancy completion was not significantly different between Utah and Dynamic models, and the average dates were January 25 and 28, respectively.

Among the three chilling requirement models, Chill Hours model showed the

Table I-4. Maximum chilling accumulation by 20-year intervals over the last 100 years using Chill Hours, Utah, and Dynamic models.

Period	Chill Hours (CH)	Utah (CU)	Dynamic (CP)
1920-1939	3182 a	1766	115
1940-1959	2828 b	1774	115
1960-1979	2883 b	1698	112
1980-1999	2927 b	1790	114
2000-2019	2753 c	1748	116
Mean	2914	1755	114

Means within Chill Hours column followed by different letters are significantly different by Duncan's multiple range test at $P < 0.05$. Means within Utah and Dynamic columns are not significantly different.

most significant changes in chilling initiation, dormancy completion, and maximum chill accumulation. On the other hand, Dynamic model showed the highest stability, with no specific changes in the spectra over the 100 years. Guo et al. (2014) reported that chilling accumulation was not significantly reduced during the last 50 years using Dynamic model in Beijing, China. Winter chill, however, is likely to decrease in the future, which will impact fruit production. Based on 100-year synthetic weather records, a decrease in CH at Davis, California (Luedeling et al., 2009a), and a decreased in CU at Zhengzhou, China (Li et al., 2016b) were also reported. Due to the decline of chilling accumulation, cultivation areas for the cultivars with high chilling requirement might be relocated in the future.

Even the chilling requirement varied among Korean peach cultivars (700-1100 CU), chilling accumulation in Suwon or Wanju is higher than 1200 CU. Due to the enough chilling accumulation in Korean climate so far, low-chill peach cultivars are not demanding in Korea. Furthermore, frost damage and death of whole trees by freezing injury are the more pressing adversities faced by peach growers in Korea (Jun et al., 2016). However, reduced CH (Table I-4) and delayed initiation of chilling accumulation were observed from the historical data (Fig. I-3). Global warming is undoubtedly accelerating, chilling accumulation is changing gradually owing to climate change (Guo et al., 2013; Luedeling et al., 2011). ‘Seonmi’, which showed the highest chilling requirement in this study, might soon face a problem of insufficient chilling in future warmer climate. Chilling accumulation should be monitored in peach cultivation area and chilling requirements of new peach cultivars are needed to be estimated.

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CHAPTER 2

Comparative sugar metabolism in the shoots of a cold-hardy and a cold-sensitive peach (*Prunus persica*) cultivar during cold acclimation and deacclimation

ABSTRACT

Sugar metabolism affects cold hardiness in overwintering woody plants. In this study, the changes in soluble sugar content, related enzyme activity, and gene expression were monitored in the shoots of 'Soomee' (SM) and 'Odoroki' (OD) peach trees during cold acclimation (CA) and deacclimation (DA). Both cultivars had similar seasonal patterns of cold hardiness. However, SM was cold-hardier than OD from December to March based on the LT_{50} values, the temperature at which 50% injury occurred. During this period, total soluble sugar content was higher in SM than in OD, but starch content was significantly lower in SM, along with higher total amylase activity and related gene expression. Of the detected soluble sugars, sucrose was predominant and its content was most significantly correlated with cold hardiness. Fructose and glucose contents in SM, but not in OD, increased during CA and then decreased during DA concurrently with increased acid invertase activity and related gene expression. Raffinose and stachyose were detectable only from November to April. Their contents and related synthase gene expressions were also significantly correlated with cold hardiness. Anabolic enzymes such as sucrose synthase (in the synthesis direction),

sucrose phosphate synthase, raffinose synthase, and stachyose synthase showed increased activity and their related gene expressions were up-regulated during CA. In contrast, catabolic enzymes such as sucrose synthase (in the hydrolysis direction), acid α-galactosidase, and alkaline α-galactosidase showed increased activity during DA. These results suggest that the genes and enzymes regulating soluble sugar content during CA and DA are responsible for the differences in cold hardiness between the two peach cultivars.

Key words: cold acclimation, deacclimation, freezing tolerance, peach, *Prunus persica*, soluble sugars

INTRODUCTION

Cold hardiness is the ability of plants to withstand freezing temperatures. It determines the geographic distribution, growth, and productivity of deciduous fruit trees (Wisniewski et al., 2018). For winter survival, deciduous fruit trees change their cold hardiness by adjusting the metabolism during cold acclimation (CA) and deacclimation (DA) (Pearce, 2001; Thomashow, 1999; Wisniewski et al., 2014). CA, which is triggered by the low temperatures and short days of fall, is the genetic ability to enhance the cold hardiness of trees (Renaut et al., 2008; Thomashow, 1999). DA, conversely, is the loss of acquired cold hardiness in response to warm temperatures (Arora and Rowland, 2011; Kalberer et al., 2006). CA and DA accompany numerous physiological and biochemical changes, including changes in sugar metabolism (Fürtauer et al., 2019; Kasuga et al., 2007), membrane lipid composition (Kawamura and Uemura, 2014), protein metabolism and enzyme activity (Chen et al., 2012), antioxidant metabolism (Bolouri-Moghaddam et al., 2010), and gene expression (Kaplan et al., 2007; Yu et al., 2020). Especially, sugar metabolism fluctuates wildly during CA and DA. As cryoprotective molecules, soluble sugars are well known to reduce cellular dehydration by lowering the osmotic potential (Thomashow, 1999; Wolkers et al., 2001), to enable supercooling, which prevents ice formation to be lethal to plant cells, and to stabilize membranes through hydrophilic interactions (Kawamura and Uemura, 2014).

Soluble sugars, which are formed by degradation of starch, accumulate in plant tissues during CA. Their content is commonly highest in mid-winter and decrease during DA (Kalberer et al., 2006; Welling and Palva, 2008). Sucrose, a

dominant sugar in many plant species, accumulates during CA in peach (Yu et al., 2017a), blueberry (Lee et al., 2012, 2013), and red raspberry (Palonen et al., 2000). Sorbitol, a major mobile sugar in Rosaceae (Loescher and Everard, 1996), also accumulates during winter season in apple (Raese et al., 1978) and pear (Ito et al., 2013). However, the sugars that are correlated with cold hardiness differ among different plant species, and the abundance of a sugar is not always correlated with the degree of cold hardiness (Lee et al., 2012, 2013). Even though raffinose family oligosaccharides (RFOs) are present in relatively minor quantities, their contents are significantly correlated with cold hardiness in many plant species including apple (Stushnoff et al., 1993) and blueberry (Lee et al., 2012), In pine trees, raffinose content increases over 20-fold during CA, while other sugars remain in the less than 2-fold range (Chang et al., 2015).

Changes in sugar content during CA or DA are due to the alteration of gene expression and enzyme activity. For example, starch degradation genes such as β-amylase, encoded by *VcBMY* in blueberry (Lee et al., 2012) and by *CsBAM* in tea (Yue et al., 2015), and dual specificity protein phosphatase 4 in chestnut (Berrocal-Lobo et al., 2011) were associated with changes in sugar content. Activity of sucrose phosphate synthase (SPS) reached high levels during winter season in acacia (Hauch and Magel, 1998) and poplar (Schrader and Sauter, 2002). Since sugar metabolism involves multiple steps in complex pathways, numerous genes and enzymes appear to be involved in determining cold hardiness. However, little or no information is available regarding enzyme activity and gene expression related to sugar metabolism in association with cold hardiness, especially woody plants.

The involvement of sugar metabolism in cold hardiness regulation has been

reported in various plant species (Ito et al., 2013; Lee et al., 2012; Palonen et al., 2000; Welling and Palva, 2008; Yu et al., 2017a). However, the previous reports have mostly dealt with the metabolism during CA, but not with that during DA. The responses to cold temperatures under natural conditions vary greatly among deciduous fruit tree species, cultivars, and tissues (Lee et al., 2013; Moran et al., 2011; Yu and Lee, 2020; Yu et al., 2017a). Compared to other deciduous fruit trees, peach trees are more vulnerable to cold temperatures (Jun et al., 2016) due to their lower cold hardiness and earlier blooming. In addition, recently, unseasonable warm spells have frequently appeared in late winter and early spring, causing premature DA, which endangers trees that are exposed to subsequent temperature drops (Jönsson et al., 2004; Pagter et al., 2011). In this study, the seasonal changes in cold hardiness were examined in the shoots of a cold-hardy 'Soomee' (SM) and a cold-sensitive 'Odoroki' (OD) peach cultivar along with changes in soluble sugar contents. Activities of selected enzymes and relative expressions of the genes involved in sugar metabolism were also determined. The findings in the present study will provide the information to understand different cold hardiness in peach cultivars in association with differential sugar metabolism.

MATERIALS AND METHODS

Plant materials

Four-year-old SM and OD peach trees grown at the experimental orchard of the National Institute of Horticultural and Herbal Science, Wanju (35°8'N, 127°0'E), Republic of Korea were used in this study. Shoots longer than 30 cm from six different SM and OD trees each were collected monthly from August, 2017 to April, 2018 to provide three biological replicates. One replication consisted of two trees. Ten and six shoots from each tree on each sampling date were used for cold hardiness determination and sugar metabolism analysis, respectively. The shoots for analyzing cold hardiness were wrapped in moist paper and placed in a plastic bag to prevent dehydration, while the shoot internodes for determining sugar and starch contents, enzyme activity, and gene expression were immediately frozen in liquid nitrogen, ground using a bead mill (TissueLyser II, Qiagen, Düsseldorf, Germany), and stored at –80°C until use.

Cold hardiness determination

Cold hardiness was estimated by electrolyte leakage analysis as described by Yu et al. (2017a). To avoid the damage caused by bud separation from the shoots, bud-attached shoots having 7-9 mm in diameter were cut into 5-cm pieces, rinsed under cold distilled running water for 15 s, and then placed in a 50-mL conical test tube containing 1 mL of distilled water. The tubes were incubated in a circulating water bath (VS-1203P4S-3C, Vision Scientific, Daejeon, Republic of Korea) equipped with a temperature controller (UP351E, Yokogawa Electric Korea Co., Seoul, Republic of Korea). The tubes were cooled at a rate of 5°C h^{-1}

until the target temperatures were reached, maintained for 2 h at each target temperature, and thawed at a rate of 5°C h^{-1} to 4°C. The temperatures were monitored with a copper-constantan thermocouple and recorded using a data logger (CR-1000, Campbell Scientific Inc., Logan, UT, USA). After freezing treatment, the internodes from the bud-attached shoot segments were cut into 1-cm long pieces, placed in a 15-mL tube containing 8 mL deionized water, and then vacuum infiltrated for 2 min. The tubes were incubated at room temperature for 20 h on an orbital shaker (VS-8480, Vision Scientific) at 120 rpm. Electrical conductivity of the aliquots was measured using an electrical conductivity meter (COND 6+, Thermo Fisher Scientific, Waltham, MA, USA). After autoclaving at 120°C for 30 min, electrical conductivity was measured again. Using the percent-adjusted injury data, the temperature at which 50% injury occurred (LT_{50}) was calculated as described by Lim et al. (1998).

Determination of sugar and starch contents

Soluble sugar content was determined as described by Yu et al. (2017a). Briefly, 100 mg of shoot internode powders were incubated in 1 mL of 80% ethanol at 85°C for 15 min. After centrifugation at 6,300 *g* for 5 min, the supernatant was collected and the pellet re-extracted twice as described above. The supernatants from each extraction were combined and evaporated using an evaporator (N-EVAP^{TM}, Organomation Associates, Inc., West Berlin, MA, USA) with N_2 at 60°C, and the pellets were saved for further starch analysis. The evaporated extracts were dissolved in 3 mL of distilled water, and passed through a nylon syringe filter (0.45 μm pore size, Whatman 6789-1304, GE Healthcare, Little Chalfont, UK) and a C18 Sep-Pak cartridge (Waters Corp., Milford, MA,

USA). The soluble sugars were analyzed using an HPLC (Dionex UltiMate 3000, Thermo Fisher Scientific) equipped with a Sugar-Pak 300 × 6.5 mm column (Waters Corp.) and a Shodex RI-101 detector (Showa Denko K.K., Kawasaki, Japan). Ten microliters of the filtered extract were injected into a Sugar-Pak column stored at 75°C. Distilled water was used as a solvent at a flow rate of 0.5 mL min^{-1}. Starch in the remaining pellets was determined using a Total Starch Assay Kit (Megazyme, Wicklow, Ireland) according to the manufacturer's instructions. Briefly, starches in the pellets were degraded with 3,000 U mL^{-1} thermostable α-amylase (Sigma-Aldrich, St. Louis, MO, USA) and the amount of released glucose was determined at 510 nm using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific).

Determination of enzyme activities

Approximately 500 mg of shoot internode powders were suspended with 3 mL of extraction buffer consisting of 50 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane-sulphonic acid (HEPES, pH 7.5), 10 mM $MgCl_2$, 2 mM EDTA-Na_2, 3 mM dithiothreitol, 0.1% (v/v) Triton X-100, and 0.1% (w/v) bovine serum albumin (BSA). After centrifugation at 3,000 × *g* at 4°C for 20 min, supernatants were desalted by filtration on Sephadex™ G-25 columns (PD-10, GE Healthcare, Uppsala, Sweden) that were equilibrated with the extraction buffer without Triton X-100 and BSA. All extraction steps were done at approximately 4°C and protein content was determined using a Bicinchoninic Acid Protein Assay Kit (B9643 and C2284, Sigma-Aldrich).

Sucrose synthase (SUS, EC 2.4.1.13), SPS (EC 2.4.1.14), acid invertase (ACI, EC 3.2.1.26), and alkaline invertase (ALI, EC 3.2.1.26) were assayed as the

enzymes responsible for sucrose synthesis and hydrolysis. SUS and SPS activities were determined as described by Hubbard et al. (1989) with slight modifications. The SUS activity was determined in both synthesis and hydrolysis directions. For assaying the synthesis activity of SUS, the enzyme extract was incubated in a reaction mixture consisting of 100 mM HEPES (pH 7.5), 25 mM $MgCl_2$, 20 mM fructose, and 4 mM uridine diphosphate (UDP)-glucose at a ratio of 1:1 (v/v) at 30°C for 45 min and terminated by heating at 100°C for 5 min after the addition of 0.5 N NaOH. After cooling at room temperature, 0.02% anthrone in 75% (v/v) H_2SO_4 was added to the reaction product and the reaction mixture was incubated at 80°C for 15 min. After cooling, color development was measured at 620 nm using a spectrophotometer. For assaying the hydrolysis activity of SUS, the enzyme extract was incubated in a reaction mixture consisting of 100 mM HEPES (pH 5.5), 5 mM NaF, 5 mM UDP, and 100 mM sucrose. After incubation at 30°C for 45 min, the reaction product was treated with 1% 3,5-dinitrosalicylic acid reagent (Miller, 1959) at a ratio of 1:1 (v/v) and boiled at 100°C for 5 min to stop the reaction. After cooling, the amount of reducing sugars was colorimetrically determined at 540 nm. The reaction mixture for the SPS assay consisted of 100 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 20 mM NaF, 10 mM UDP-glucose, and 10 mM fructose-6-phosphate. The subsequent steps were the same as in the procedure described in the SUS assay in the synthesis direction. ACI and ALI activities were determined as described by Yu et al. (2017b) with slight modifications. The enzyme extract was incubated in a reaction mixture consisting of 100 mM Na-acetate (pH 4.8) and 100 mM sucrose at a ratio of 2:3 (v/v) at 37°C for 40 min. Subsequent steps were the same as described for the SUS assay in the hydrolysis direction. ALI assay followed the same procedure as

the ACI assay except that Na-phosphate (pH 7.5) was used instead of Na-acetate (pH 4.8).

Raffinose synthase (RAS, EC 2.4.1.82), stachyose synthase (STS, EC 2.4.1.67), acid α-galactosidase (ACG, EC 3.2.1.22), and alkaline α-galactosidase (ALG, EC 3.2.1.22) were assayed as the enzymes related to synthesis and hydrolysis of RFOs. RAS and STS activities were determined as described by Peterbauer et al. (2001) with slight modifications. The reaction mixture for the RAS assay consisted of 100 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 20 mM NaF, 10 mM UDPG, 5 mM galactinol, and 40 mM sucrose. Reaction mixture for the STS assay was the same as that for the RAS assay except that 20 mM raffinose was used instead of 40 mM sucrose. After the enzyme extracts were incubated in the reaction mixtures at a ratio of 2:3 (v/v) at 30°C for 2 h, the products were quantified by using an HPLC system with a Sugar-Pak column. ACG and ALG were assayed according to the method described by Bachmann et al. (1994) with slight modifications. The enzyme extract was incubated in a reaction mixture consisting of 200 mM citrate-phosphate buffer and 0.2 mM *p*-nitrophenyl-α-D-galactopyranoside at a ratio of 1:4 (v/v) at 37°C for 15 min. The pH for ACG and ALG assays were set at 5.5 and 7.5, respectively. The reaction was terminated by heating at 100°C for 5 min after the addition of 200 mM K_2HPO_4. After cooling at room temperature, the released *p*-nitrophenol was colorimetrically determined at 405 nm.

Total amylase (TAM) from α-amylase (EC 3.1.1.1) and β-amylase (EC 3.1.1.2), which are involved in starch degradation, was assayed as described by Cheng et al. (2009). The enzyme extract was incubated in a reaction mixture consisting of 100 mM Na-acetate (pH 6.5), 1.5 mM NaF, 5 mM $Ca(NO_3)_2$, and

0.5% starch at a ratio of 1:4 (v/v) at 30°C for 45 min. The subsequent steps were the same as in the procedures described for the ACI assay.

Determination of relative gene expressions

Total RNA was extracted from approximately 100 mg of shoot internode powders as described by Chang et al. (1993). The complementary DNA (cDNA) was synthesized using a PrimeScript™ First Strand cDNA Synthesis Kit (Takara Bio Inc., Otsu, Japan). Seventeen target genes of the seven enzymes assayed in the present study were selected and their primers were designed using the Primer3Plus (Untergasser et al., 2007) based on the DNA sequences from the Genome Database for Rosaceae (Table II-1). Relative expressions were determined by normalization against the expression of peach *RNA polymerase II* and *translation elongation factor 2* according to Tong et al. (2009). The quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed in a LightCycler® 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) using a program of 45 cycles at 95°C for 5 s and 60°C for 30 s with an initial incubation at 95°C for 30 s. The reaction mixture consisted of 200 nM each of forward and reverse primers, cDNA equivalent to the amount synthesized from 10 ng of the total RNA, and 1× TB Green™ Premix Ex Taq™ (Takara Bio Inc.) in a 10-μL reaction.

Statistical analysis

Statistically significant differences among the means were determined by the Student's *t*-test at $P < 0.05$, 0.01, or 0.001 using the SAS enterprise 4.3 (SAS Co., Cary, NC, USA). Pearson correlation was used to quantify relationships of cold

Table II-1. Primer sequences used for gene expression analysis by qRT-PCR.

Gene	Primer sequence	Product size (bp)
Acid invertase	F: AAGCTTTGCTCAAGGTGGAA	161
	R: ATGGGCGTATGAAGCAGAG	
Invertase 1	F: AGGTCAAGTTGCCAAGCAGT	176
	R: TCGGCTACACAAAAGCTGTG	
ppa004334m	F: AGCAGTTCAGGAATGCGAGT	197
	R: GCCGGTTATCCACACCTAGA	
ppa023176m	F: AAATCCAGGCGCAACATAAG	248
	R: TTCTTTGGCAAAACCCTCAC	
ppa025926m	F: CAAAGGTCACTCCCTGCATT	242
	R: ATAAGCCTTTGCAAGCTCCA	
Prupe.1G352200.1	F: TCCCCTTCTTCTTGGTTGTG	246
	R: GTGGGGATTCCAACATCATC	
Prupe.4G041000.1	F: ACCTGGGCAAGTATCACGTC	189
	R: TCGGACGTCACATCCAATAA	
Prupe.8G125500.1	F: GGTTCAATTGGGAATCATGG	224
	R: ACTATCGTGGAAGGCGCTTA	
Sucrose phosphate	F: TTGAGGCTACAGGAAAGGAAAG	184
synthase 1	R: GGACGCTCCTCTGAATGAATAG	
Sucrose phosphate	F: GTGTCTTACGGGGAAGTCCA	169
synthase 2	R: ATATGGCCAAGGCTGTTGAC	
Sucrose synthase 1	F: ATGAGGAGAAGGCTGAGATGAAG	226
	R: CAAGTAGCGAATGTTGGAAGTC	

Table II-1. Continued

Gene	Primer sequence	Product size (bp)
Sucrose synthase 3	F: ATGCGTTGATGTTGAATGA	197
	R: AGAGGGTGTCTCGGGTGGA	
Sucrose synthase 4	F: GAAATGGCGGAGATTGAAAA	150
	R: AGGCTGCACAAAGACACCTT	
Sucrose synthase 5	F: TTTGACATCTATCCCTACCTT	162
	R: AATAGTTGCCTGAGTAATCCC	
Sucrose synthase 6	F: GGGCTGACCAATCTGTCTACT	126
	R: CTGCCAAATATCCTATGTGCT	
RNA polymerase II	F: AAGCATACACCTATGATGATGAAG	128
	R: CTTTGACAGCACCAGTAGATTCC	
Translation	F: GGTGTGACGATGAAGAGTGATG	129
elongation factor 2	R: TGAAGGAGAGGGAAGGTGAAAG	

F, Forward; R, reverse.

hardiness with carbohydrate content, enzyme activity, and relative gene expression in the peach shoots. Graph plotting and Pearson correlation analysis were performed using the SigmaPlot 12.0 program (Systat Software, Inc., San Jose, CA, USA).

RESULTS AND DISCUSSION

Seasonal changes in cold hardiness

Cold hardiness, expressed as LT_{50} values, in the shoots of the two peach cultivars from August to April changed concurrently with changes in air temperature (Fig. II-1). The LT_{50} values in the shoots of both cultivars decreased rapidly from the beginning of October (Fig. II-1b), minimized in January, and then increased gradually. No significant differences were shown in LT_{50} values between the two cultivars until November. From December to March, however, the LT_{50} values were significantly lower in SM than in OD, indicating that SM is cold-hardier than OD.

Based on seasonal changes of LT_{50} values (Fig. II-1), CA and DA of the two peach cultivars might occur from October to January and from January to April, respectively. The rapid increase in cold hardiness starting in October and the peak in cold hardiness in January (Fig. II-1b) implied that the peach trees had completed the CA process and attained maximal cold hardiness prior to the coldest month, as reported in many woody plants including peach (Yu et al., 2017a), blueberry (Lee et al., 2012), and rhododendron (Lim et al., 1998).

Cold hardiness in the shoots of the two peach cultivars was rapidly strengthened during CA and weakened during DA, concurrently with changes in air temperature (Fig. II-1). Although cold hardiness in both cultivars changed similarly, SM shoots was significantly cold-hardier in mid-winter and maintained its cold hardiness longer during DA than OD shoots (Fig. II-1b). These observations suggest that OD is more vulnerable to freezing temperatures in mid-winter and to sudden temperature drops in spring than SM. These differential capacities of the

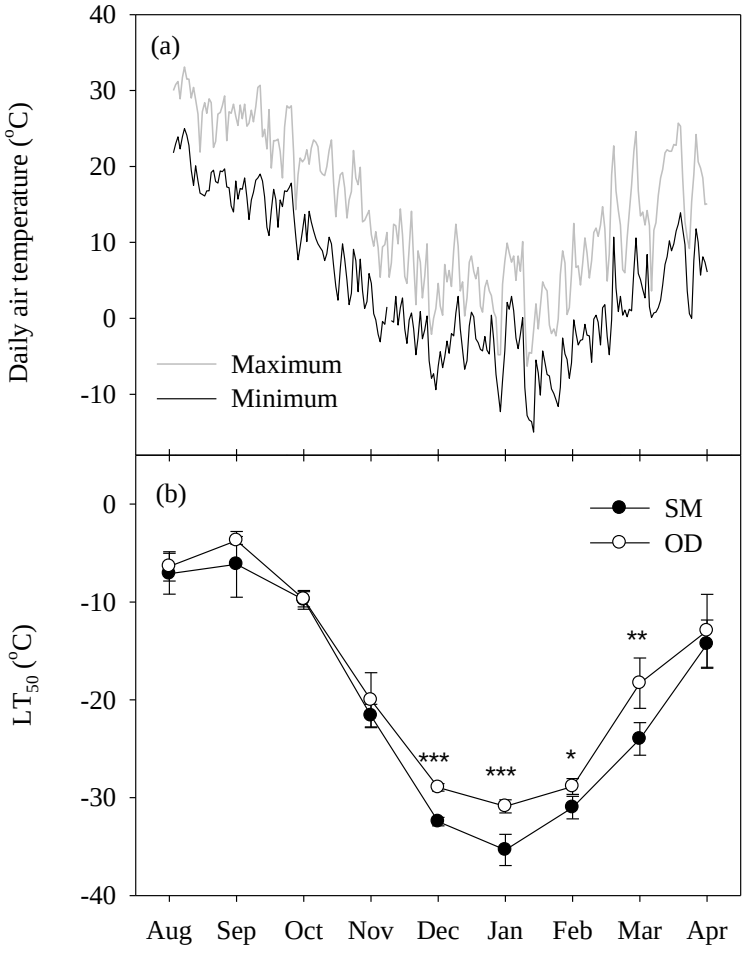


Fig. II-1. Seasonal changes in daily air temperature and cold hardiness from August, 2017 to April, 2018. (a) Daily maximum and minimum air temperature at the experimental site (35°8'N, 127°0'E) obtained from the Korea Meteorological Administration. (b) Cold hardiness, expressed as LT_{50} values, the temperature at which 50% injury occurred, in the shoot of 'Soomee' (SM) and 'Odoroki' (OD) peach trees. Values are means ± standard errors (n = 3). Asterisks ($^{*, **, ***}$) on the bars indicate significance between the cultivars within the same sampling dates using the Student's t-test at $P < 0.05$, 0.01, or 0.001, respectively.

two cultivars to withstand freezing stress might be a result of genetic adaptation for environmental conditions. SM and OD were bred in Suwon (37º2'N, 127º0'E), Republic of Korea and Nagano (36º4'N, 138º1'E), Japan, respectively, and the average minimum daily temperature of January is lower in Suwon (–7.9ºC) than in Nagano (–4.3ºC). These environmental differences, especially in the minimum temperature, during winter might be associated with their genetic background for cold hardiness.

Seasonal changes in soluble sugar and starch contents

Total soluble sugar contents in the shoots of the two cultivars increased rapidly from October, reached their highest levels in SM and OD in January and February, respectively, and then decreased steadily (Fig. II-2a). Total soluble sugar contents from December to March were significantly higher in SM than in OD, even though they were not significantly different between the two cultivars or significantly higher in OD than in SM during the rest course of the experiment. In contrast, starch contents increased from August and reached their highest levels in September and October in SM and OD, respectively (Fig. II-2b). Since then, they decreased, reached the lowest levels in January, and then increased until March (Fig. II-2b). Starch contents from October to February were significantly lower in SM than in OD.

Six soluble sugars (sucrose, sorbitol, fructose, glucose, raffinose, and stachyose) were detected in the shoots of both cultivars during the experimental period (Fig. II-3). Of the detected soluble sugars, sucrose was the predominant soluble sugar, comprising about half of the content of total soluble sugars (Figs. II-2a and 3a). Sucrose contents in both cultivars were maintained at constant and

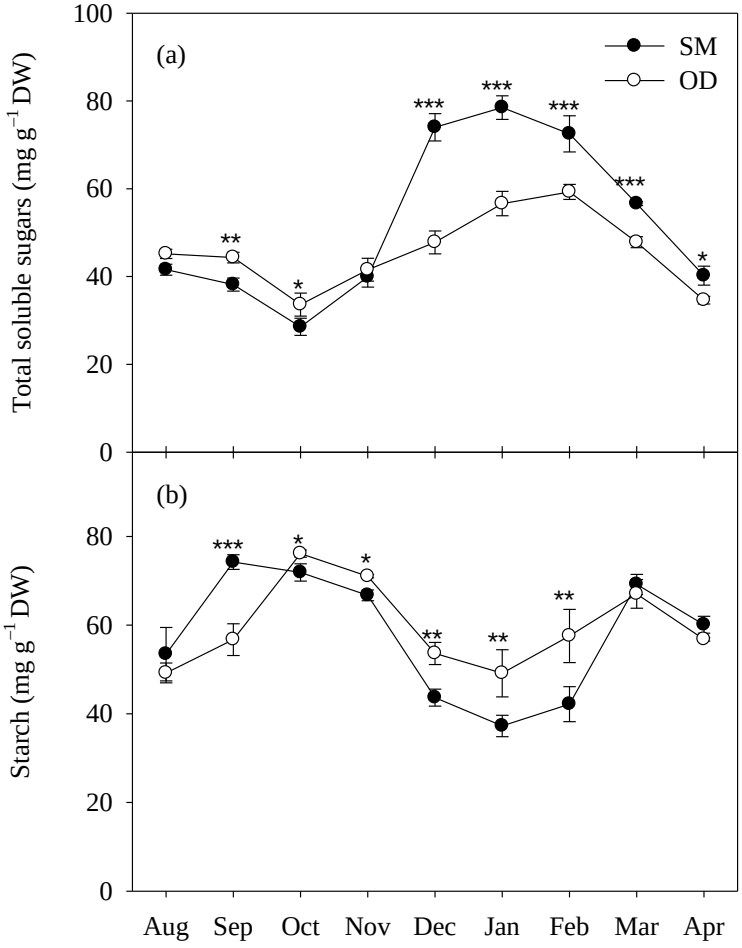


Fig. II-2. Seasonal changes in (a) total soluble sugar and (b) starch contents in the shoot of 'Soomee' (SM) and 'Odoroki' (OD) peach trees. Values are means ± standard errors (n = 3). Asterisks ($^{*, **, ***}$) on the bars indicate significance between the cultivars within the same sampling dates using the Student's t-test at $P < 0.05$, 0.01, or 0.001, respectively.

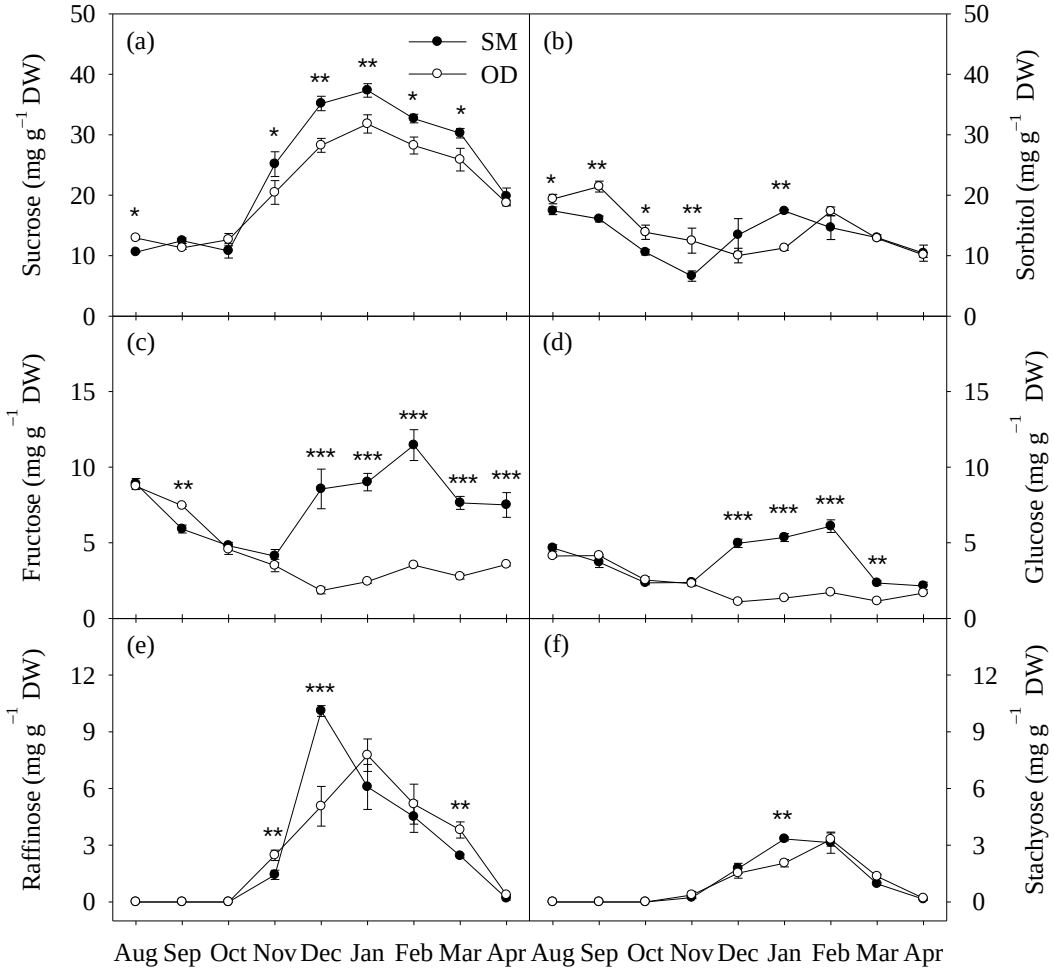


Fig. II-3. Seasonal changes in (a) sucrose, (b) sorbitol, (c) fructose, (d) glucose, (e) raffinose, and (f) stachyose contents in the shoots of 'Soomee' (SM) and 'Odoroki' (OD) peach trees. Values are means ± standard errors (n = 3). Asterisks ($^{*, **, ***}$) on the bars indicate significance between the cultivars within the same sampling dates using the Student's t-test at $P < 0.05$, 0.01, or 0.001, respectively.

relatively low levels until October. Since then, they increased rapidly, peaked in January, and decreased thereafter (Fig. II-3a). From November to March, sucrose contents were higher in SM than in OD. However, sorbitol contents in both cultivars fluctuated throughout the experimental period (Fig. II-3b).

Fructose and glucose contents in SM decreased from August to November, increased thereafter until February, and then decreased again (Fig. II-3c and d). However, no such changes were observed in OD. Both fructose and glucose contents in OD decreased until December and then were maintained until April (Fig. II-3c and d). The fructose contents in both cultivars were approximately 2-fold higher than the glucose contents throughout the experimental period. Both raffinose and stachyose were detected in the shoots of the two cultivars from November. From November, raffinose contents in both cultivars increased rapidly, reached the highest levels in December and January in SM and OD, respectively, and then decreased (Fig. II-3e). Similarly, stachyose contents changed during the experimental period (Fig. II-3f) about one month after the change in raffinose contents (Fig. II-3e). The raffinose and stachyose contents changed about one month earlier in SM than in OD. The raffinose contents were approximately 3-fold higher than the stachyose contents during the experimental period detected.

Seasonal changes in enzyme activities

SUS (in the synthesis direction) and SPS activities in both cultivars remained relatively low from August to November, increased rapidly until January, and then were sustained until April (Fig. II-4a and b). The SUS activities (in the synthesis direction) were similar in SM and OD throughout the experimental period (Fig. II-4a), while the SPS activities from January to April were significantly higher in

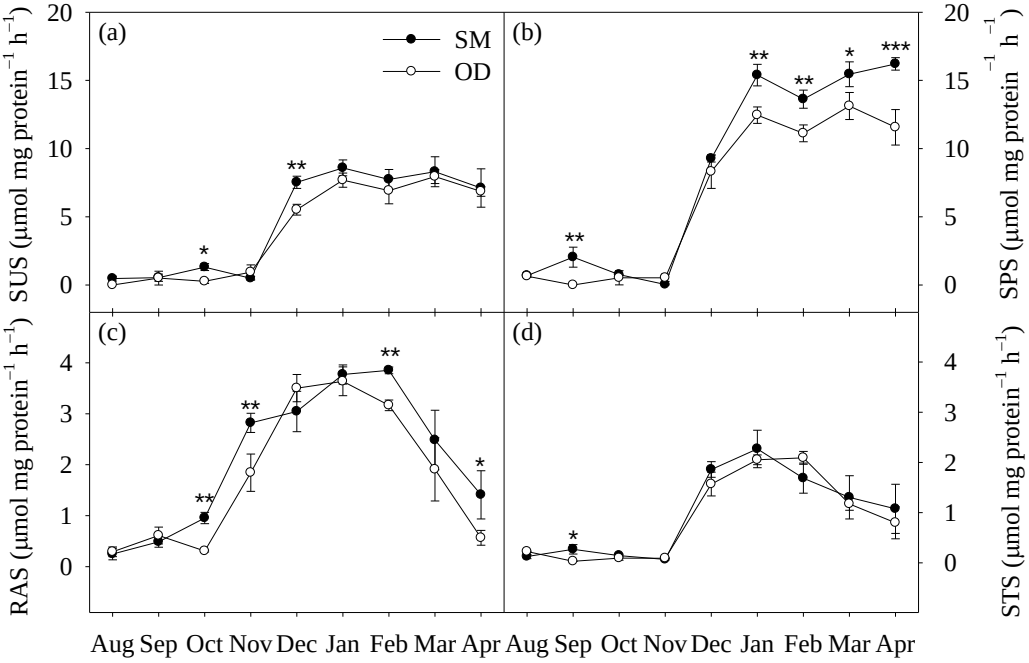


Fig. II-4. Seasonal changes in the activities of enzymes involved in the synthesis of sucrose and raffinose family oligosaccharides in the shoots of 'Soomee' (SM) and 'Odoroki' (OD) peach trees. (a) Sucrose synthase (SUS) (in the synthesis direction), (b) sucrose phosphate synthase (SPS), (c) raffinose synthase (RAS), and (d) stachyose synthase (STS) activities. Values are means ± standard errors (n = 3). Asterisks $^{(*, **, ***)}$ on the bars indicate significance between the cultivars within the same sampling dates using the Student's *t*-test at $P < 0.05$, 0.01, or 0.001, respectively.

SM than in OD (Fig. II-4b). RAS activities in SM and OD increased rapidly until February and January, respectively, and decreased thereafter (Fig. II-4c). Although STS activities in both cultivars remained low from August to November, the activities increased rapidly from November to January and February in SM and OD, respectively, and then decreased steadily (Fig. II-4d). RAS activities were mostly significantly higher in SM than in OD, while STS activities were not significantly different between the two cultivars throughout the experimental period. In addition, the magnitude of the increase was approximately 2-fold higher in RAS activity than in STS activity. RAS activity increased approximately one month earlier than SUS (in the synthesis direction), SPS, and STS activities (Fig. II-4).

SUS activities (in the hydrolysis direction) in both cultivars fluctuated from August to February and increased rapidly thereafter (Fig. II-5a). TAM activities increased abruptly from September to November with a higher magnitude in SM than in OD and then decreased rapidly in December. The decreased TAM activities were sustained until February and then restored slightly (Fig. II-5b). ACI activities increased rapidly from October, reached the highest levels in December, and then decreased steadily (Fig. II-5c). The increased ACI activities were significantly higher in SM than in OD. However, both cultivars displayed minor changes in ALI activities throughout the experimental period (Fig. II-5d). ACG and ALG activities in both cultivars remained low until February and then increased steadily (Fig. II-5e and f), similarly to SUS activity (in the hydrolysis direction) (Fig. II-5a). The ACG and ALG activities were not significantly different between the two cultivars.

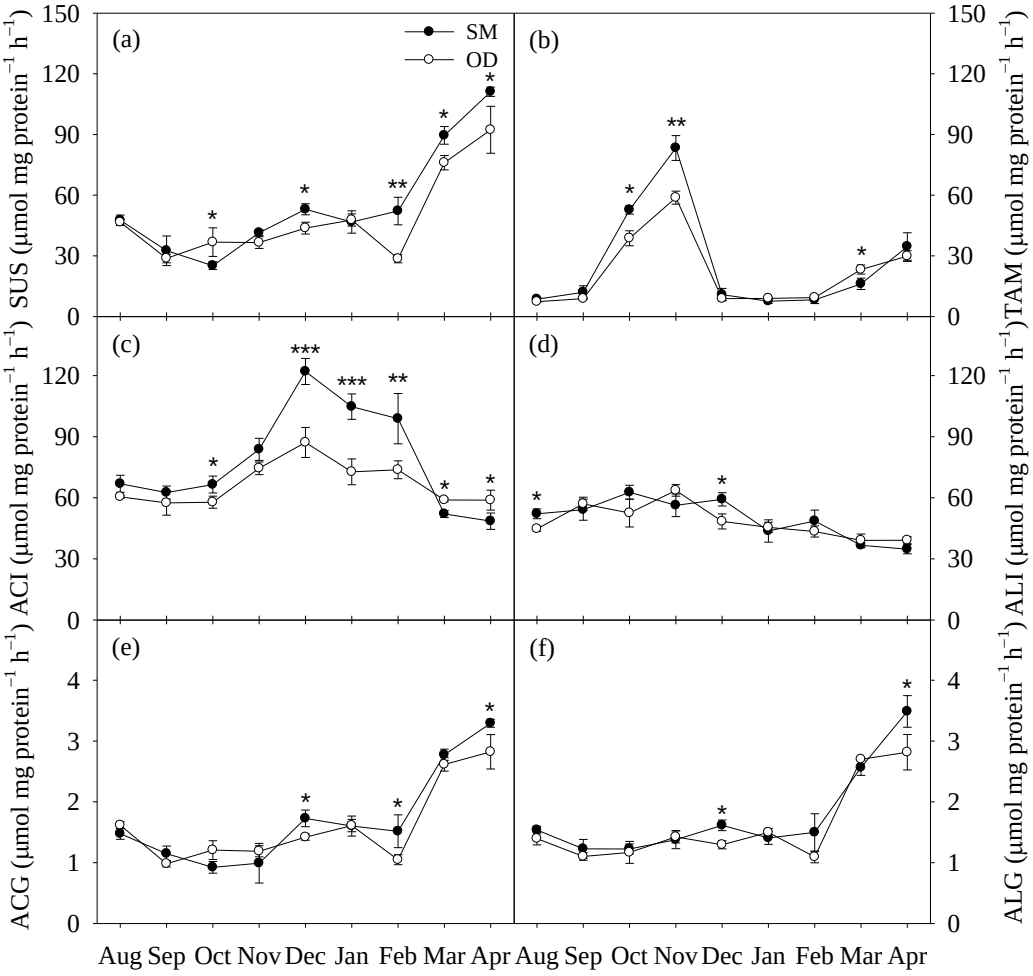


Fig. II-5. Seasonal changes in the activities of enzymes involved in the degradation of sucrose, raffinose family oligosaccharides, and starch in the shoots of 'Soomee' (SM) and 'Odoroki' (OD) peach trees. (a) Sucrose synthase (SUS) (in the hydrolysis direction), (b) total amylase (TAM), (c) acid invertase (ACI), (d) alkaline invertase (ALI), (e) acid α-galactosidase (ACG), and (f) alkaline α-galactosidase (ALG) activities. Values are means ± standard errors (n = 3). Asterisks (*,**,***) on the bars indicate significance between the cultivars within the same sampling dates using the Student's t-test at $P < 0.05$, 0.01, or 0.001, respectively.

Seasonal changes in relative gene expressions

SUS1 expressions in both cultivars increased gradually from August to February and decreased rapidly thereafter (Fig. II-6a). *SUS2* expressions remained low from August to October and then increased rapidly until December. Since then, they decreased rapidly to almost ground levels in February and then were maintained until April (Fig. II-6b). *SUS3* expressions increased gradually from August and were highest in October. Since then, they decreased rapidly until November and increased again to a smaller degree thereafter (Fig. II-6c). *SUS4*, *SUS5*, and *SUS6* expressions increased until November, February, and October, respectively, in both cultivars and then decreased (Fig. II-6d, e, and f). Among the six *SUS* genes, *SUS1*, *SUS2*, *SUS4*, and *SUS5* were more highly expressed in SM than in OD during the experimental period (Fig. II-6a, b, d, and e), while the *SUS*3 and *SUS6* expressions were not significantly different between the two cultivars (Fig. II-6c and f).

SPS1 slightly increased until November and decreased thereafter in both cultivars, while *SPS2* remained low until September and then increased rapidly (Fig. II-7). And they reached the highest levels in November and December in SM and OD, respectively, and then decreased slightly until March. However, the *SPS1* and *SPS2* expressions were not significantly different between the two cultivars throughout the experimental period.

Expressions of *ppa023176m* encoding RAS remained low from August to October, increased thereafter until February and December in SM and OD, respectively, and then decreased (Fig. II-8a). Expressions of *ppa025926m* encoding STS remained low from August to November, increased slightly until January, and decreased thereafter (Fig. II-8b). Compared to the *ppa023176m*

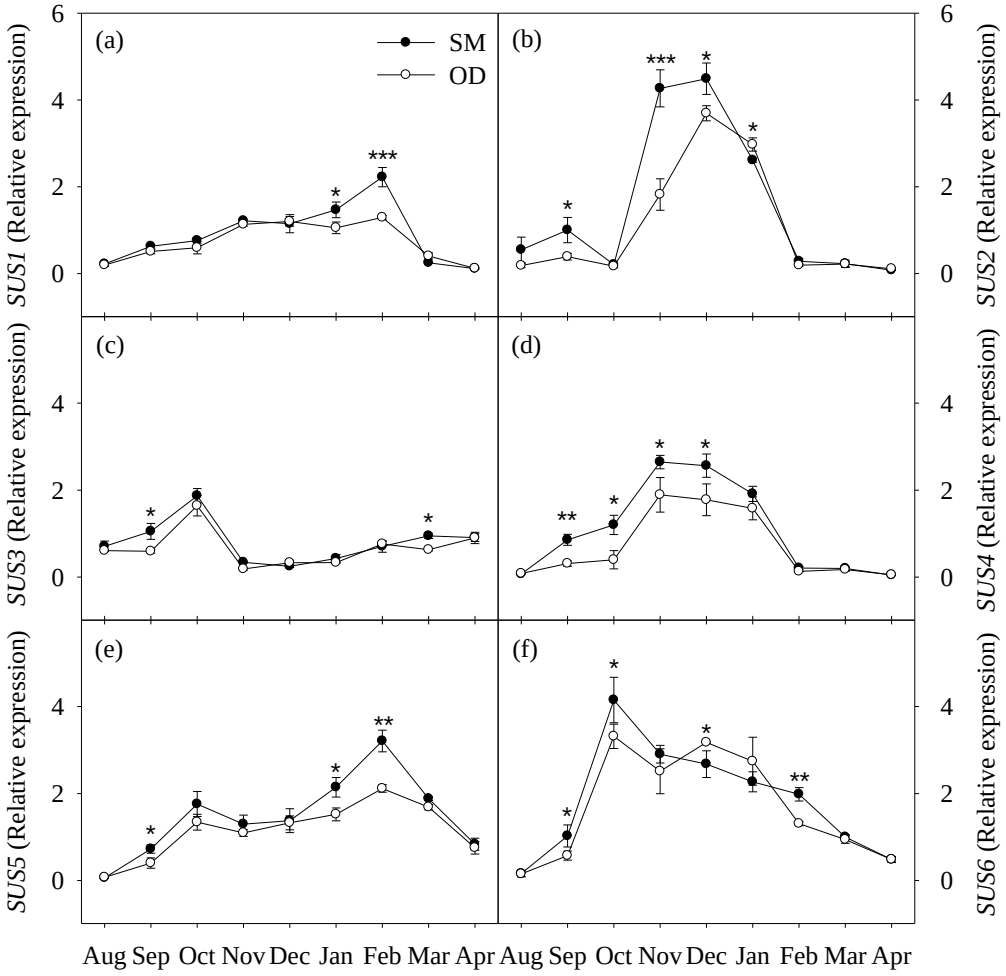


Fig. II-6. Seasonal changes in the relative expressions of genes encoding sucrose synthase (SUS) in the shoot of 'Soomee' (SM) and 'Odoroki' (OD) peach trees. (a) *SUS1*, (b) *SUS2*, (c) *SUS3*, (d) *SUS4*, (e) *SUS5*, and (f) *SUS6*. Values are means ± standard errors (n = 3). Asterisks ($^{*, **, ***}$) on the bars indicate significance between the cultivars within the same sampling dates using the Student's *t*-test at $P < 0.05$, 0.01, or 0.001, respectively.

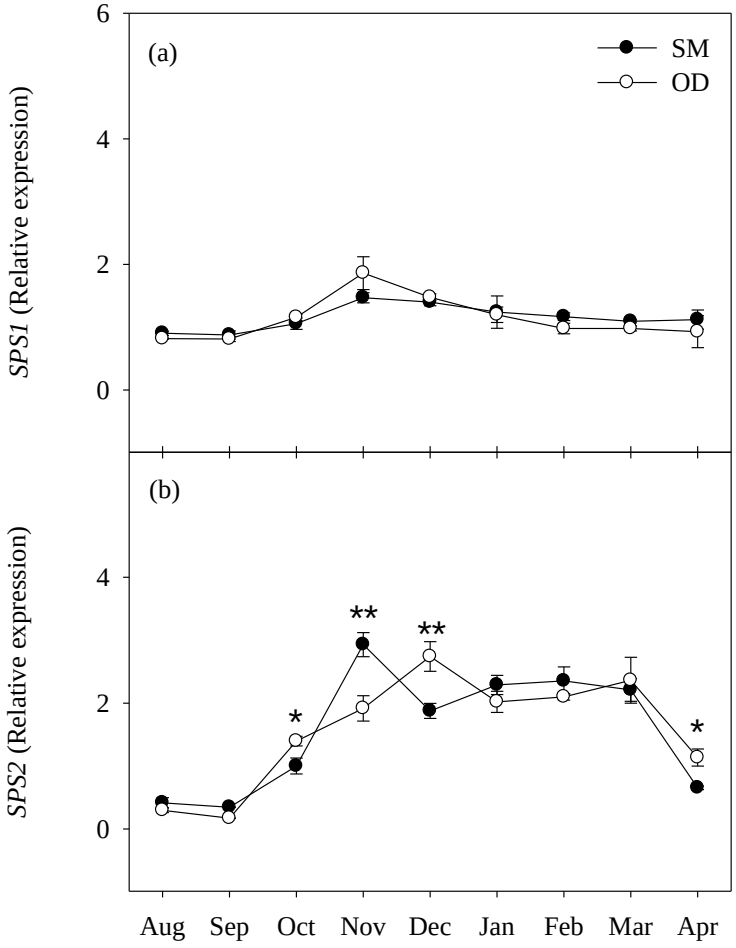


Fig. II-7. Seasonal changes in the relative expressions of genes encoding sucrose phosphate synthase (SPS) in the shoots of 'Soomee' (SM) and 'Odoroki' (OD) peach trees. (a) *SPS1* and (b) *SPS2*. Values are means ± standard errors (n = 3). Asterisks (*, **, ***) on the bars indicate significance between the cultivars within the same sampling dates using the Student's t-test at $P < 0.05$, 0.01, or 0.001, respectively.

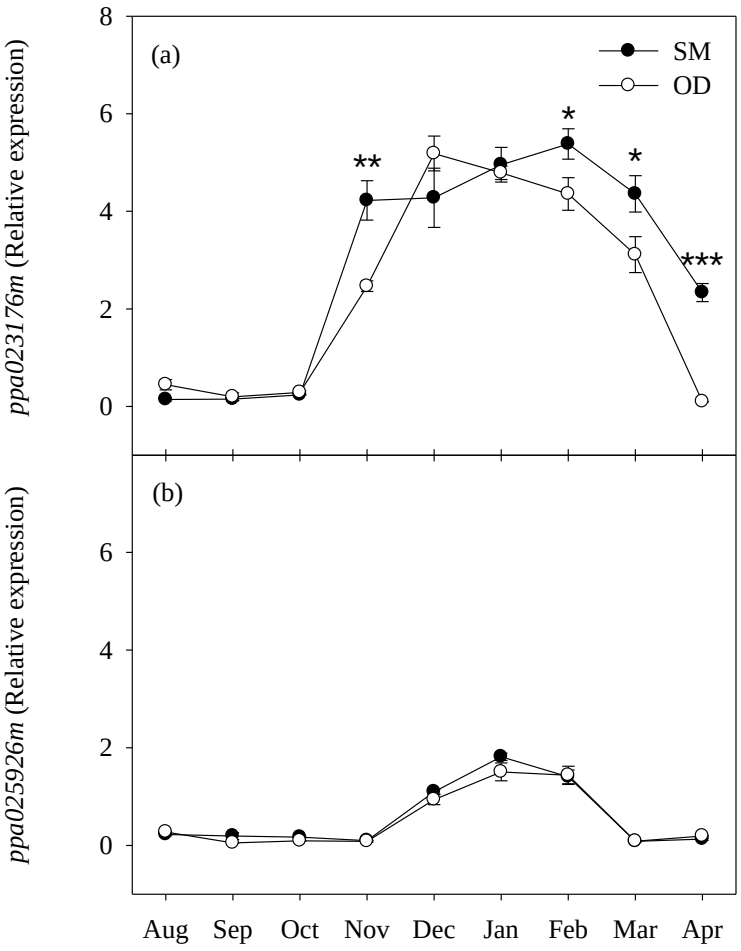


Fig. II-8. Seasonal changes in the relative expressions of genes involved in the synthesis of raffinose family oligosaccharides in the shoot of 'Soomee' (SM) and 'Odoroki' (OD) peach trees. (a) *ppa023176m* encoding raffinose synthase and (b) *ppa025926m* encoding stachyose synthase. Values are means ± standard errors (n = 3). Asterisks (*, **, ***) on the bars indicate significance between the cultivars within the same sampling dates using the Student's t-test at $P < 0.05$, 0.01, or 0.001, respectively.

expression, the *ppa025926m* expression up-regulated later and then down-regulated earlier during the experimental period. The expression levels in *ppa023176m* from February to April were significantly higher in SM than in OD (Fig. II-8a), while those in *ppa025926m* were similar in both cultivars throughout the experimental period (Fig. II-8b).

Expression of *AI* encoding ACI remained low from August to October and then increased rapidly until December (Fig. II-9a). Since then, they decreased until February, increased abruptly in March, and then decreased. From November to January, the expression levels of *AI* were significantly higher in SM than in OD (Fig. II-9a). Expressions of *INV1* encoding ALI increased steadily from August to January and then decreased rapidly, without significant differences between the two cultivars (Fig. II-9b). *Prupe.1G352200.1* encoding α-galactosidase sustained its low expression levels until February and then increased abruptly (Fig. II-9c). Expressions of *Prupe.4G041000.1*, another α-galactosidase encoding gene, did not change significantly throughout the experimental period (Fig. II-9d). Expressions of *Prupe.8G125500* encoding α-amylase significantly increased until October, decreased thereafter to the ground levels within one and two months in OD and SM, respectively, and were maintained until April (Fig. II-9e). Expression of *ppa004334m* encoding β-amylase remained low from August to September and then increased rapidly until November (Fig. II-9f). Since then, they decreased to be the lowest levels in February, increased abruptly in March, and then decreased. In both cultivars, the up-regulations were later but higher in *ppa004334m* expression than in *Prupe.8G125500* expression (Fig. II-9e and f). From October to November, the up-regulations in both gene expressions were higher in SM than in OD.

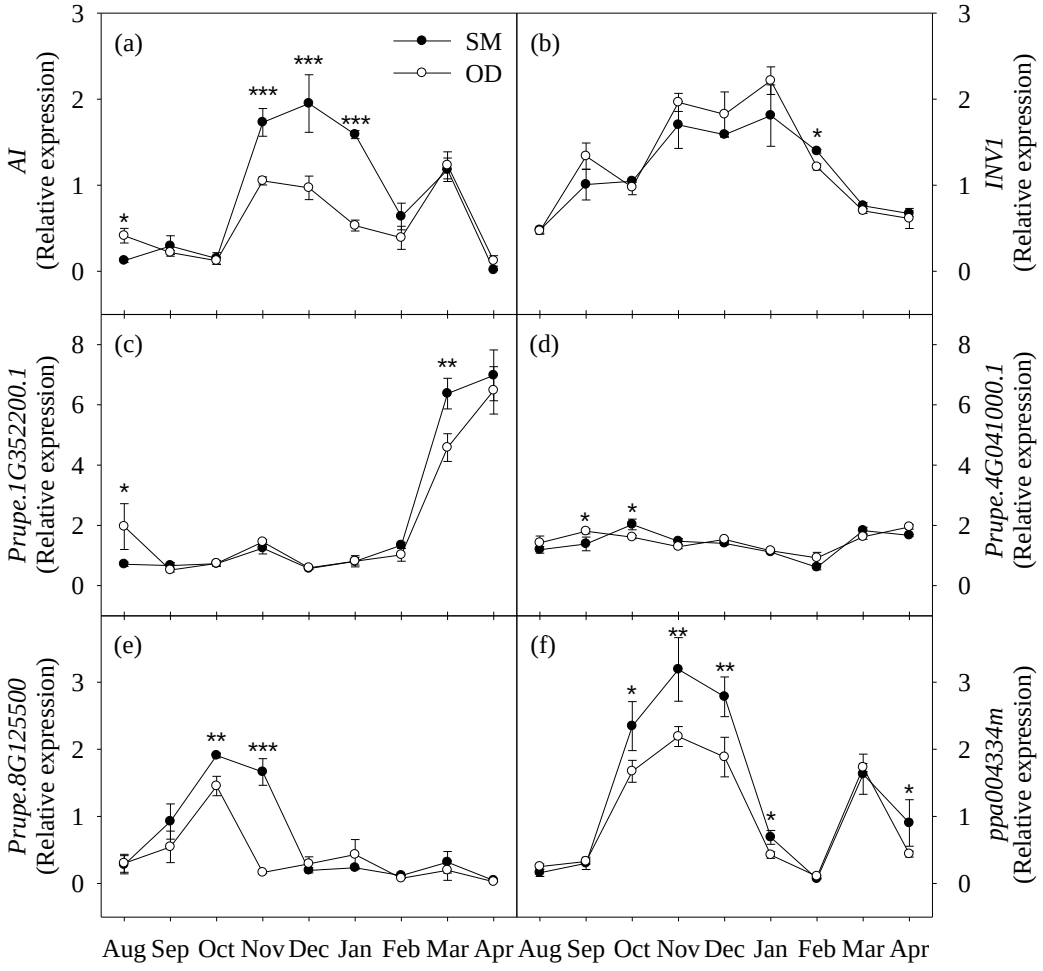


Fig. II-9. Seasonal changes in the relative expressions of genes involved in the degradation of sucrose, raffinose family oligosaccharides, and starch in the shoot of 'Soomee' (SM) and 'Odoroki' (OD) peach trees. (a) *AI* encoding acid invertase, (b) *INV1* encoding alkaline invertase, (c) *Prupe.1G352200.1* encoding α-galactosidase, (d) *Prupe.4G041000.1* encoding α-galactosidase, (e) *Prupe.8G125500* encoding α-amylase, and (f) *ppa004334m* encoding β-amylase. Values are means ± standard errors (n = 3). Asterisks ($^{*, **, ***}$) on the bars indicate significance between the cultivars within the same sampling dates using the Student's t-test at $P < 0.05$, 0.01, or 0.001, respectively.

Interconversion of soluble sugars and starch

Interconversion between soluble sugars and starch is common in woody plants during CA and DA (Kalberer et al., 2006; Pagter et al., 2008; Welling and Palva, 2008). Total soluble sugar content increased during CA, but decreased during DA (Fig. II-2a). Conversely, starch content decreased and increased during CA and DA, respectively (Fig. II-2b). The increase in total soluble sugar content during CA might be due to starch hydrolysis, since TAM activity increased (Fig. II-5b) and its related genes, *Prupe.8G125500* and *ppa004334m*, which encode α- and β-amylase, respectively, were up-regulated (Fig. II-9e and f). Starch hydrolysis into soluble sugars is triggered by low temperatures during CA (Kaplan et al., 2006; Thalmann and Santelia, 2017). SM showed a larger change in total soluble sugar and starch contents than OD, but their contents in both cultivars were similar from August to November (Fig. II-2a and b). Higher TAM activity and its related gene expression in SM during CA may account for the higher magnitude of starch degradation and higher total soluble sugar content, resulting in higher cold hardiness when compared to OD.

In both cultivars, starch accumulated until October (Fig. II-2b), resulting presumably from the translocation of photosynthates before leaf fall (Charrier and Améglio, 2011). The decrease in total soluble sugar content along with the increase in starch content during DA (Fig. II-2) may be due to starch synthesis or translocation from various reserves in preparation for growth resumption (Lee et al., 2012; Pagter et al., 2011). The decreases in both total soluble sugar and starch contents in March (Fig. II-2) could also be explained by growth resumption, such as bud burst and flowering (Morin et al., 2007). The decrease in starch content during this period might also be due to the increased activities of SUS (in the

hydrolysis direction) (Fig. II-5a), ACG (Fig. II-5e), and ALG (Fig. II-5f), and due to the increased expressions of *Prupe.1G352200.1* (Fig. II-9c) and *ppa004334m* (Fig. II-9f), which encode α-galactosidase and β-amylase, respectively.

Two types of amylases are responsible for starch hydrolysis. While α-amylase is involved in various developmental processes, such as seed germination (Chen et al., 2006), bud dormancy (Rubio et al., 2014), and fruit ripening (Fan et al., 2018), β-amylase is associated with stress responses (Kaplan et al., 2006; Yue et al., 2019) and cold hardiness (Lee et al., 2012; Peng et al., 2014; Rowland et al., 2008). Previous studies in blueberry shoots (Lee et al., 2012) and floral buds (Rowland et al., 2008) have drawn attention to the differential expressions of β-amylase gene to account for differences in cold hardiness between cultivars. The changes in relative expressions of *Prupe.8G125500* and *ppa004334m* during CA and DA (Fig. II-9e and f) demonstrate that the genes affect cold hardiness by changing starch metabolism, and at least partly account for cultivar differences in cold hardiness.

Correlation of cold hardiness with carbohydrate content, enzyme activity, and gene expression

LT_{50} values were negatively correlated with total soluble sugar contents in both cultivars ($r = –0.89^{***}$ and $–0.64^{***}$ for SM and OD, respectively; Table II-2). Of the detected soluble sugars, sucrose, raffinose, and stachyose contents were negatively correlated with LT_{50} values in both cultivars. Fructose and glucose contents were negatively correlated with LT_{50} value in SM, but positively with LT_{50} value in OD. The positive correlation between LT_{50} value and starch content was observed only in SM, but not in OD. The reverse was true for sorbitol content.

Table II-2. Correlations of cold hardiness (LT_{50}), with carbohydrate content, enzyme activity, and relative gene expression in the shoot of 'Soomee' (SM) and 'Odoroki' (OD) peach trees.

		Correlation coefficient (r)	
Variable		SM	OD
Carbohydrate	Starch	0.686^{***}	0.164^{NS}
	Total soluble sugars	-0.887^{***}	-0.639^{***}
	Sucrose	-0.961^{***}	-0.913^{***}
	Sorbitol	-0.054^{NS}	0.514^{**}
	Fructose	-0.453^{*}	0.796^{***}
	Glucose	-0.476^{*}	0.792^{***}
	Raffinose	-0.839^{***}	-0.889^{***}
	Stachyose	-0.855^{***}	-0.832^{***}
Enzyme	SUS (in the synthesis direction)	-0.749^{***}	-0.691^{***}
	SPS	-0.616^{***}	-0.641^{***}
	RAS	-0.918^{***}	-0.907^{***}
	STS	-0.774^{***}	-0.833^{***}
	SUS (in the hydrolysis direction)	-0.131^{NS}	0.048^{NS}
	TAM	0.209^{NS}	0.124^{NS}
	ACI	-0.695^{***}	-0.687^{***}
	ALI	0.162^{NS}	0.163^{NS}
	ACG	-0.131^{NS}	0.037^{NS}
	ALG	0.029^{NS}	0.074^{NS}

Table II-2. Continued

Variable		Correlation coefficient (*r*)	
		SM	OD
mRNA	*SUS1*	–0.653***	–0.755***
	SUS2	–0.487**	–0.666***
	SUS3	0.580**	0.404*
	SUS4	–0.416*	–0.545**
	SUS5	–0.664***	–0.742***
	SUS6	–0.281^{NS}	–0.474*
	SPS1	–0.509**	–0.407*
	SPS2	–0.792***	–0.841***
	ppa023176m	–0.912***	–0.935***
	ppa025926m	–0.783***	–0.796***
	AI	–0.734***	–0.427*
	INV1	–0.634***	–0.595**
	Prupe.1G352200.1	–0.170^{NS}	–0.138^{NS}
	Prupe.4G041000.1	0.379^{NS}	0.311^{NS}
	Prupe.8G125500	0.371^{NS}	0.585**
	ppa004334m	0.043^{NS}	0.156^{NS}

$^{NS, *, **, ***}$ Not significant or significant at $P < 0.05$, 0.01, or 0.001, respectively.

Among the enzymes assayed, SUS (in the synthesis direction), SPS, RAS, STS, and ACI activities were negatively correlated with LT_{50} values in both cultivars. However, such correlations were not observed with the activities of the other enzymes. Expressions of the two *SPS* genes and all *SUS* genes barring *SUS3* were negatively correlated with LT_{50} values in both cultivars. Among the genes analyzed, expressions of *ppa023176m* and *ppa025926m* involved in RFO synthesis were most significantly correlated with LT_{50} values.

Changes in soluble sugar contents and related metabolism

The highest sucrose content in January (Fig. II-3a) may be due to the increased activities of SPS and SUS (in the synthesis direction) in December and January (Fig. II-4a and b). Although SUS has both sucrose synthesis and hydrolysis activity, SUS activity in the synthesis direction, but not in the hydrolysis direction, was correlated with cold hardiness (Table II-2), and possibly contributed to cold hardiness enhancement. Increased SUS activity during CA was also reported in rhododendron (Christiaens et al., 2015) and *CsSUS* genes were up-regulated during CA in tea plants (Yue et al., 2015). In contrast, SUS activity was low and its related genes were expressed to low levels during CA in sweet cherry (Turhan and Ergin, 2012) and garden rose (Ouyang et al., 2019), indicating differences among plant species. The different expression patterns of the *SUS* family genes (Fig. II-6) may be due to the bidirectional role of SUS in converting sucrose to glucose and fructose, which is necessary for maintaining sucrose balance in the plant (Ruan, 2014).

SPS, a sucrose synthesizing enzyme, exhibited higher activity than SUS (Fig. II-4a and b). SPS activity (Fig. II-4b), and *SPS1* and *SPS2* expressions (Fig. II-7)

were significantly correlated with cold hardiness (Table II-2). These results corroborate the studies in poplar (Schrader and Sauter, 2002) and spruce needles (Loewe et al., 1996), showing a change in SPS activity during CA.

The gradual decrease in sucrose content beginning in February (Fig. II-3a) appeared to be facilitated by the increased activity of SUS (in the hydrolysis direction) in March (Fig. II-5a), and that of ACI between December and February (Fig. II-5c). Since SUS (in the hydrolysis direction) and invertase are both sucrose-hydrolyzing enzymes, they compete for sucrose as a substrate. SUS (in the hydrolysis direction) converts sucrose to D-fructose and UDP-glucose, while invertase splits sucrose into glucose and fructose. The reduced invertase activity might increase the likelihood of SUS using sucrose to prepare for growth resumption.

ACI activity was correlated with cold hardiness (Table II-2), corroborating the reports in acacia (Hauch and Magel, 1998) and sweet cherry (Turhan and Ergin, 2012). In particular, ACI activity and its related gene (*AI*) expression were higher in SM than in OD in mid-winter (Figs. II-5c and 9a), resulting in higher glucose and fructose contents in SM (Fig. II-3c and d). In OD, however, glucose and fructose contents did not increase during CA. Since glucose and fructose potentially double the osmolarity (Yue et al., 2015), sucrose degradation by ACI might contribute to cold hardiness enhancement in SM. The distinct differences of glucose and fructose contents between cold-hardy and cold-sensitive cultivars during mid-winter was also observed in other studies (Shin et al., 2015; Yu et al., 2017a). The glucose and fructose contents of cold-hardy cultivar were significantly higher than those of cold-sensitive cultivar in accordance with the results in the present study.

Sucrose affects cold hardiness development in various ways, including acting as an osmoprotectant in preventing intracellular ice formation (Thomashow, 1999). Sucrose and other soluble sugars facilitate deep supercooling in plant cells by maintaining intracellular water in the liquid phase at subzero temperatures (Kasuga et al., 2007). Sucrose also mediates signaling functions, such as the induction of expression of cold-responsive promoter (*COR78*) which directly facilitates CA in *Arabidopsis* (Rekarte-Cowei et al., 2008). In addition, sucrose triggers ACI activity to regulate sugar content in response to environmental stresses (Xiang et al., 2011). Therefore, the higher sucrose content in SM (Fig. II-3a) may account for its higher ACI activity during CA and DA (Fig. II-5c).

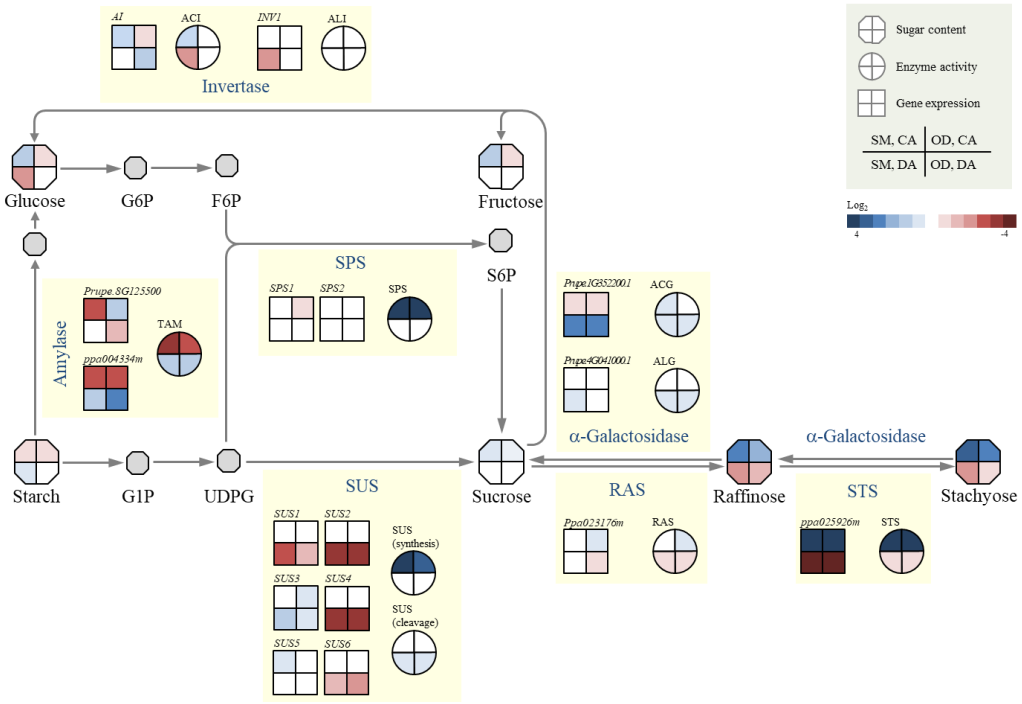
Raffinose and stachyose contents were significantly correlated with cold hardiness (Table II-2), even though they were detected in minor quantities between November and March (Fig. II-3e and f). RAS activity and its related gene (*ppa023176m*) expression increased in November (Figs. II-4c and 8a), while the raffinose content significantly increased in December (Fig. II-3e). Furthermore, raffinose contents peaked in December and January in SM and OD, respectively (Fig. II-3e), while stachyose contents reached at the highest level one month later (Fig. II-3f). RAS activity in November increased to a greater extent in SM than in OD (Fig. II-4c), presumably leading to higher raffinose content in SM during CA (Fig. II-3e). Since STS can utilize raffinose as a substrate, the earlier raffinose accumulation in SM may also facilitate earlier stachyose synthesis. This view is supported by the decrease in raffinose content in SM starting in January, with a concomitant increase in stachyose content (Fig. II-3e and f), even though the STS activities were similar in both cultivars (Fig. II-4d).

The decreases in raffinose and stachyose contents starting in February (Fig. II-

3e and f) appeared to be due to the reduced RAS and STS activities (Fig. II-4c and d), and their related gene (*ppa023176m* and *ppa025926m*) expressions (Fig. II-8a and b), along with the increased ACG and ALG activities (Fig. II-5e and f) and their related gene (*Prupe.1G352200.1*) expression (Fig. II-9c). Although little or no information is available regarding the changes in α-galactosidase activity in woody plants in association with cold hardiness, α-galactosidase activity and its related gene (*PhGAL*) expression were reported to increase in petunia during DA (Pennycooke et al., 2004).

RFOs act as compatible solutes to decrease the osmotic potential in the cell, thereby alleviating dehydration stress and stabilizing the membrane. RFOs separate lipid molecules and prevent membrane disorder by replacing water molecules (Hincha et al., 2003). According to the report of Carpita et al. (1979), a single raffinose molecule can replace about 30 water molecules, while sucrose can replace about 18 water molecules. Considering that starch synthesis is relatively slow at low temperatures, RFOs may have dual functions not only as osmoprotectants during CA, but also as alternative carbohydrate reserves for regrowth the following spring. The storage function of RFOs has been reported elsewhere (Bachmann et al., 1994; Sengupta et al., 2015).

The results of the present study demonstrate that the differential cold hardiness of peach cultivars is associated with differences in soluble sugar accumulation. Changes of sugar contents, enzyme activity, and gene expression were integrated in schematic pathway of sugar metabolism (Supplementary Fig. II-1). Glucose and fructose contents were significantly increased in SM than in OD during CA. This accumulation could be accounted for by sucrose degradation due to higher ACI activity and its related gene expression in SM. SM induced the



Supplementary Fig. II-1. Schematic pathway of sugar metabolism integrated with changes of sugar content, enzyme activity, and gene expression in the shoots of 'Soomee' (SM) and 'Odoroki' (OD) peach trees. Fold changes of sugar content, enzyme activity, and gene expression were evaluated during cold acclimation (CA, from October to January) and deacclimation (DA, from January and March). Grey octagons are undetected individual sugars. F6P, fructose-6-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; S6P, sucrose-6-phosphate.

synthesis of sucrose and RFOs more efficiently than OD. Distinct accumulation patterns of RFOs were also observed during winter with a time lag between raffinose and stachyose accumulation. Since sucrose can be used as a substrate to produce fructose, glucose, and RFOs, sucrose metabolism might play a pivotal role in seasonal changes in soluble sugar accumulation during CA and DA. These results indicate that systematic alterations in the levels of soluble sugars and starch are crucial for determining cold hardiness in peach trees.

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CHAPTER 3

Changes of cold hardiness and related transcriptomes in the shoot of a cold-hardy and a cold-sensitive peach (*Prunus persica*) cultivar during accelerated and delayed deacclimation

ABSTRACT

Cold acclimation and deacclimation (DA) are crucial to winter survival of deciduous fruit trees. In the aspects of global warming, premature DA increases the risk of subsequent freezing injuries on the trees. In this study, the DA resistance and changes in soluble sugars and related transcriptomes were compared in the shoots of a cold-hardy 'Soomee' (SM) and a cold-sensitive 'Kanoiwa Hakuto' (KH) peach (*Prunus persica*) cultivar during accelerated DA at 15ºC and delayed DA at −5ºC in February. KH shoots lost cold hardiness more rapidly during the accelerated DA than SM shoots, showing stronger DA resistance of SM shoots. Of 18,130 assembled transcripts, 2,303 and 1,814 were differentially expressed in SM and KH shoots, respectively, during the accelerated DA. The metabolic pathways best represented in both cultivars were 'carbohydrate metabolism', 'amino acid metabolism', and 'energy metabolism'. Among the transcripts annotated to the carbohydrate metabolism, 43 were annotated to encode 20 enzymes related to soluble sugar metabolism. During the accelerated DA, transcripts encoding catabolic enzymes, including β-galactosidase (LOC18769018 and LOC18783479) and β-glucosidase

(LOC18770931, LOC18779132, and LOC18779303) were up-regulated in SM relative to KH, while those encoding anabolic enzymes such as galactinol synthase (LOC18789982) and raffinose synthase (LOC18770520) were down-regulated. The differentially expressed genes were validated through quantitative reverse-transcription polymerase chain reaction analysis. These results suggest that cold hardiness in peach tree shoots is determined by the interconversion of soluble sugars and starch.

Key words: cold hardiness, deacclimation resistance, peach, *Prunus persica*, soluble sugars, transcriptome analysis

INTRODUCTION

Freezing can cause significant losses in the productivity and yield of deciduous fruit trees and limit their geographical distribution (Yu and Lee, 2020). To survive through the harsh winter, the trees adjust their cold hardiness via cold acclimation (CA) and deacclimation (DA) (Pagter et al., 2008; Vyse et al., 2019). Compared to other Rosaceae fruit trees, peach trees are more frequently injured by freezing temperatures (Jun et al., 2016) due to their relatively lower cold hardiness and earlier blooming (Hanke et al., 2012). Moreover, premature DA induced by unseasonably warm spells makes the peach tress vulnerable to low temperatures, and the sudden temperature drop can cause severe damage on the trees (Pagter et al., 2011). The characteristics of cold hardiness loss under warm temperature, DA resistance, is also regarded as an important factor to determine the cold hardiness of genotypes along with mid-winter cold hardiness (Espevig et al., 2014).

After chilling requirement has been met, the endodormancy of trees is usually broken and cold hardiness is decreasing with substantial increases in temperature (Kalberer et al., 2006). Among environmental factors, temperature is a key factor for the cold hardiness loss during DA, and DA is affected by both the temperature itself and the duration (Hoffman et al., 2014). According to the scenarios for climate change, mild spells may occur more frequently and for longer periods of time, and trees may be more exposed to temperature fluctuations (IPCC, 2014). According to a modeling study in spruce (Jönsson et al., 2004), global warming would lead to a temperature increase in the fall, thus, to an incomplete CA in which trees are also readily deacclimated. Paradoxically,

the risk of freezing injuries is increasing due to the global warming.

Physiological and biochemical changes during CA and DA are induced by reprogramming of transcriptomes (Sakuma et al., 2002; Wu et al., 2016). During CA, low temperatures activate C-repeat binding factors (CBFs) and then the CBFs alter the expression of about 170 cold regulated genes (*COR*s) (Fowler and Thomashow, 2002; Liu et al., 2019; Zhao et al., 2015). Coordinated responses of the *CORs* regulate metabolism of carbohydrates, lipids, and proteins enhancing cold hardiness (Liu et al., 2019; Zhao et al., 2015). Transcriptome analysis has been attempted to identify genes involved in the responses during CA in woody plants including eucalyptus (Gaete-Loyola et al., 2017), olive (Guerra et al., 2015), and peach (Yu et al., 2020). Studies have focused on the changes during CA, but not during DA, which are assumed as a reversal of CA.

The present study was conducted to compare DA resistance of a cold-hardy 'Soomee' (SM) and a cold-sensitive 'Kanoiwa Hakuto' (KH) peach cultivar. The trees were subjected to accelerated and delayed DA and their cold hardiness changes were compared along with the changes of soluble sugar and starch contents. The transcriptomic changes were also compared to select differentially expressed genes (DEGs) between the two cultivars. This study will provide a framework dataset for metabolic changes of peach trees during DA.

MATERIALS AND METHODS

Plant materials and treatments

One-year-old SM and KH peach trees were grown in experimental field of the National Institute of Horticultural and Herbal Science (NIHHS), Wanju (35°8'N, 127°0'E), Republic of Korea. The trees were transferred to walk-in chambers on February 2, 2018 being planted in 20-L pots for accelerated and delayed DA treatments. For the accelerated and delayed DA treatments, the trees were subjected to 15ºC and –5ºC, respectively, in February with a 11 h photoperiod corresponding to natural daylength. The temperatures for the accelerated and delayed DA treatments were chosen to maximize the temperature effect during DA, because the temperatures are the maximum and minimum in February when the DA usually occurs in Republic of Korea (Kwon et al., 2020).

The treatments were employed with three replications consisting of three trees each. Three shoots longer than 30 cm were randomly collected from each tree weekly up to 4 weeks of the treatments. The shoots for analyzing cold hardiness were wrapped in moist paper and placed in a plastic bag to prevent dehydration, while the shoot internodes for determining sugar and starch contents, and for analyzing transcriptomes were immediately frozen in liquid nitrogen, ground using a bead mill (TissueLyser II, Qiagen, Düsseldorf, Germany), and stored at –80°C until use.

Cold hardiness determination

Cold hardiness was estimated by electrolyte leakage analysis as described by Yu et al. (2017). To avoid the damage caused by bud separation from the shoots,

bud-attached shoots having 7-9 mm in diameter were cut into 5-cm pieces, rinsed under cold distilled running water for 15 s, and then placed in a 50-mL conical test tube containing 1 mL of distilled water. The tubes were incubated in a circulating water bath (VS-1203P4S-3C, Vision Scientific, Daejeon, Republic of Korea) equipped with a temperature controller (UP351E, Yokogawa Electric Korea Co., Seoul, Republic of Korea). The tubes were cooled at a rate of 5°C h^{-1} until the target temperatures were reached, maintained for 2 h at each target temperature, and thawed at a rate of 5°C h^{-1} to 4°C. The temperatures were monitored with a copper-constantan thermocouple and recorded using a data logger (CR-1000, Campbell Scientific Inc., Logan, UT, USA). After freezing treatment, the internodes from the bud-attached shoot segments were cut into 1-cm long pieces, placed in a 15-mL tube containing 8 mL deionized water, and then vacuum infiltrated for 2 min. The tubes were incubated at room temperature for 20 h on an orbital shaker (VS-8480, Vision Scientific) at 120 rpm. Electrical conductivity of the aliquots was measured using an electrical conductivity meter (COND 6+, Thermo Fisher Scientific, Waltham, MA, USA). After autoclaving at 120°C for 30 min, electrical conductivity was measured again. Using the percent-adjusted injury data, the temperature at which 50% injury occurred (LT_{50}) was calculated as described by Lim et al. (1998).

Cold hardiness was also evaluated by observing tissue browning, the shoots of SM and KH were exposed at –25ºC for 2 h after the accelerated DA and delayed DA for 4 weeks. Shoot samples were prepared and temperature change were employed as described above. After thawed to 4ºC, the shoots were incubated at 4ºC for additional 1 day and then cross sections of the shoots were observed under a stereo microscope (S8 APO, Leica, Wetzlar, Germany).

Determination of soluble sugar and starch contents

Soluble sugar contents were determined as described by Yu et al. (2017). Briefly, 100 mg of the shoot internode powders were incubated in 1 mL of 80% ethanol at 85°C for 15 min. After centrifugation at 6,300 *g* for 5 min, the supernatant was collected and the pellet re-extracted twice as described above. The supernatants from each extraction were combined and evaporated using an evaporator (N-EVAP^{TM}, Organomation Associates, Inc., West Berlin, MA, USA) with N_2 at 60°C, and the pellets were saved for further starch analysis. The evaporated extracts were dissolved in 3 mL of distilled water, and passed through a nylon syringe filter (0.45 μm pore size, Whatman 6789-1304, GE Healthcare, Little Chalfont, UK) and a C18 Sep-Pak cartridge (Waters Corp., Milford, MA, USA). The soluble sugars were analyzed using an HPLC (Dionex UltiMate 3000, Thermo Fisher Scientific) equipped with a Sugar-Pak 300 × 6.5 mm column (Waters Corp.) and a Shodex RI-101 detector (Showa Denko K.K., Kawasaki, Japan). Ten microliters of the filtered extract were injected into a Sugar-Pak column stored at 75°C. Distilled water was used as a solvent at a flow rate of 0.5 mL min^{-1}. Starch in the remaining pellets was determined using a Total Starch Assay Kit (Megazyme, Wicklow, Ireland) according to the manufacturer's instructions. Briefly, starches in the pellets were degraded with 3,000 U mL^{-1} thermostable α-amylase (Sigma-Aldrich, St. Louis, MO, USA) and the amount of released glucose was determined at 510 nm using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific).

Sample shoots were prepared to microscopically observe starch accumulation. Shoot internode tissues subjected to the accelerated DA and delayed DA for 4

weeks in February were incubated in a fixing agent consisting of 0.1 M phosphate (KH_2PO_4/K_2HPO_4) buffer (pH 7.2), 2.5% (v/v) glutaraldehyde, and 4% (w/v) sucrose for 24 h. Following the fixing, the tissues were rinsed with the phosphate buffer three times for 15 min each, and then postfixed with 1% (w/v) OsO_4 in the same buffer with 4% (w/v) sucrose for 4 h. The tissues were then rinsed again in the same manners as above, dehydrated in the alcohol series, transferred to propylene oxide, and embedded in epoxy resin. The samples were sliced into 2.5 μm in thickness using an ultramicrotome (Powertome X, RMC Boeckeler, Tucson, AZ, USA), stained using the periodic acid Schiff reaction (Clement et al., 1994), and observed under a light microscope (Axioscop 2, Carl Zeiss, Oberkochen, Germany).

Total RNA extraction

Total RNA was extracted from shoot internodes as described by Gambino et al. (2008) using cetyltrimethylammonium bromide buffer. A 900-μL aliquot of the extraction buffer was preheated to 65ºC and added to a 2-mL microcentrifuge tube containing 100 mg of the shoot internode powder, mixed thoroughly, and incubated at 65ºC for 30 min. An equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added, vortexed for 5 s, and centrifuged at 11,000 × g at 4ºC for 10 min. The 750-μL supernatant was recovered and again mixed with an equal volume of the chloroform:isoamyl alcohol. The 600-μL supernatant was transferred to a new 2-mL tube and an equal volume of 6 M LiCl was added. The mixture was incubated on ice for 30 min and centrifuged at 21,000 × g at 4ºC for 20 min to precipitate the RNA. The pellet was resuspended in 500 μL of preheated (65ºC) SSTE buffer consisting of 0.5% sodium lauryl sulfate, 1 M

NaCl, 1 M Tris-HCl (pH 8.0), and 10 mM EDTA with gentle shaking. An equal volume of the chloroform:isoamyl alcohol was added, and the mixture was centrifuged at 11,000 × g at 4ºC for 10 min. The 400-μL supernatant was mixed with 280 μL cold isopropanol and centrifuged at 21,000 × g at 4ºC for 15 min. The pellet was washed with 1 mL of 70% ethanol, dried, and resuspended in 20 μL of diethyl pyrocarbonate-treated water. Finally, the solution was heated at 65ºC for 5 min to dissolve the RNA. The quality of the extracted RNA samples was assessed by determining their A260/A280 ratios using a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific).

cDNA library construction, sequencing, and assembling

A total of 12 cDNA libraries were constructed from the RNA using a TruSeq RNA Sample Prep Kit 2 (Illumina, San Diego, CA, USA). They were sequenced using a HiSeq 2500 system (Illumina) to generate 101-bp paired-end reads. The quality of the resulting data was confirmed using the FastQC (version 0.11.5). Unwanted artifacts, including the adaptor sequences, low-quality reads, and short length reads (< 36 bp), were removed from the raw data using the Trimmomatic (version 0.32). The trimmed reads were mapped to the reference peach genome (GCA-00346465.2) using the Bowtie2 aligner (version 2.3.5) and the HISAT2 (version 2.1.0) after which the aligned reads were assembled into transcripts or genes using the StringTie (version 1.3.6).

Identification and functional annotation of the DEGs

The abundances of the assembled transcripts in each sample were expressed by normalized fragments per kilobase of transcript per million mapped reads

(FPKM) values. To effectively identify the DEGs, the assembled transcripts or genes showing zero FPKM in any sample were removed from the analysis. The filtered data were adjusted using the quantile normalization method after taking the $\log_2$(FPKM + 1) value to reduce the range of the data and evenly distribute the data. Statistical analysis was performed on the fold change values using the Student's *t*-test at $P < 0.05$. The functional annotations and gene set enrichment analysis were also performed using the DAVID tool (http://david.abcc.ncifcrf.gov) based on their associated gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) database categories (http://www.genome.jp/kegg).

Determination of relative gene expressions

A total of 16 DEGs analyzed in the present study were selected based on their fold changes and their primers were designed using Primer3Plus (Untergasser et al., 2007) based on the DNA sequences from the KEGG database (Table III-1). Relative expressions were determined by normalization against the expression of peach *RNA polymerase II* and *translation elongation factor 2* according to Tong et al. (2009). The quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed in a LightCycler® 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) using a program of 45 cycles at 95°C for 5 s and 60°C for 30 s with an initial incubation at 95°C for 30 s. The reaction mixture consisted of 200 nM each of forward and reverse primers, cDNA equivalent to the amount synthesized from 10 ng of the total RNA, and 1× TB Green™ Premix Ex Taq™ (Takara Bio Inc., Otsu, Japan) in a 10-μL reaction.

Table III-1. Primer sequences used for qRT-PCR of the selected differentially expressed genes (DEGs) in the shoots of 'Soomee' and 'Kanoiwa Hakuto' peach trees during accelerated deacclimation at 15ºC relative to delayed deacclimation at –5ºC in February.

Gene (LOC No.)	Enzyme code	Primer sequence
Up-regulated DEG		
18770798	3.2.1.21	F: GATATAACCACCGCCACCAC
		R: TTGGGAGACCAGGATCTGAC
18770812	2.4.1.82	F: GCCTGGGAAGCACAACTTTG
		R: GAAGAGTAGGCGCCAAACCT
18770931	3.2.1.21	F: GGGCCTTTGTGACGTTTTTA
		R: GGCCATTGCAAACTTGATTT
18776343	2.4.1.15	F: TGGAGCAATCAGGGTGAACC
		R: TTCCCAACTGGACGTGCTAC
18779132	3.2.1.21	F: AAAGGGCACGTAGCAGAGAA
		R: TTGCCTTGCTAGGCTTTTGT
18779303	3.2.1.21	F: GGCAACTGTCACTGCTTCAA
		R: GAGCTCGTCCACTGTGTTCA
18782349	2.4.1.21	F: TGGATACGCCTGGGAGGTTA
		R: GGTTCAGTCCACATGGCTCA
18783479	3.2.1.23	F: GCGACGACTTACCTCCAGAC
		R: TAAGGGGAGCCCTCTTCATT
Down-regulated DEG		
18767692	3.2.1.4	F: GGAGGGCTATCAGCGTGTTT
		R: GCGGTTCCACCACAATTGAC
18768098	2.4.1.14	F: GGGACTCTATGACGGCTTCG
		R: TCAGGCACATCCGATTGCTT

Table III-1. Continued.

Gene (LOC No.)	Enzyme code	Primer sequence
18773544	3.2.1.21	F: CCCAAAGAGCCCTCGACTTT
		R: GCTCGATTCATAGCCGTCCA
18774021	3.2.1.21	F: TTGGAGATGCTGTGCTGAAC
		R: GCATGAAAACACTCCCCACT
18774195	3.2.1.21	F: CAGCCAGAATGTTCAGCAAA
		R: GCATTCTCTCCTGCAAGGTC
18775030	2.4.1.1	F: GAAAGGGAGCACAGCCTCAT
		R: AGCCACCTCCGAGGAGTAAT
18780300	3.2.1.23	F: CGCACTTCAGGAGGACCATT
		R: TGATGTGATCCACCAAGCCC
18789982	2.4.1.123	F: GCTCAAGCTCCTCTCCAATG
		R: TGGTCTTGATTTTGCTGCTG

F, Forward; R, reverse.

RESULTS AND DISCUSSION

Cold hardiness: LT_{50} values and tissue browning

During the accelerated DA, cold hardiness, as expressed in LT_{50}, decreased in the shoots of SM and KH peach trees (Fig. III-1). From 2 weeks in the accelerated DA, the LT_{50} increased more rapidly in KH than in SM, showing that KH had low DA resistance. In contrast, the cold hardiness during the delayed DA remained almost unchanged. The two cultivars showed significant difference in cold hardiness during the accelerated DA, but not during the delayed DA.

Damaged tissues especially at cambium turned to brown during the accelerated DA (white arrows in Fig. III-2). Browning was more severe in phloem tissues of KH than in those of SM during both the accelerated DA and the delayed DA. Cambium tissues of KH also turned to dark brown during the accelerated DA, showing more severe damages than in those of SM (Fig. III-2b).

Along with the maximal mid-winter cold hardiness, DA resistance also attributes to the overall cold hardiness of perennial woody plants. However, DA resistance is not always correlated with the degree of maximal cold hardiness, implying that it is inherited independently (Kalberer et al., 2006). For example, ‘Concord’ grapevines are cold-hardier, but more rapidly deacclimated than ‘Cabernet Sauvignon’ grapevines (Wolf and Cook, 1992). As the characteristics of DA cannot simply be predicted from its mid-winter cold hardiness, both the maximal cold hardiness and DA resistance should be monitored to evaluate the cold hardiness. In this study, the cold hardiness more rapidly decreased during the accelerated DA than during the delayed DA (Fig. III-1). The rate and magnitude of cold hardiness changes depends on the ambient temperature and exposure

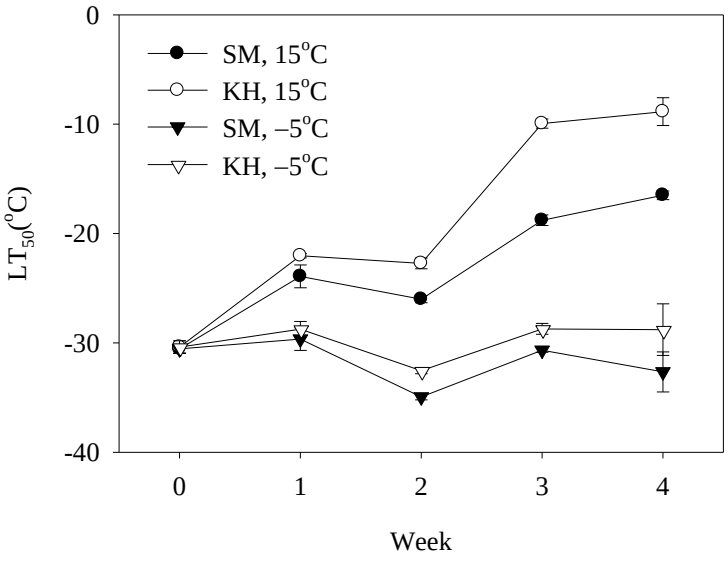

Fig. III-1. Changes in LT_{50} of the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation at 15ºC and delayed deacclimation at –5ºC in February. Values are means ± standard errors (n = 3).

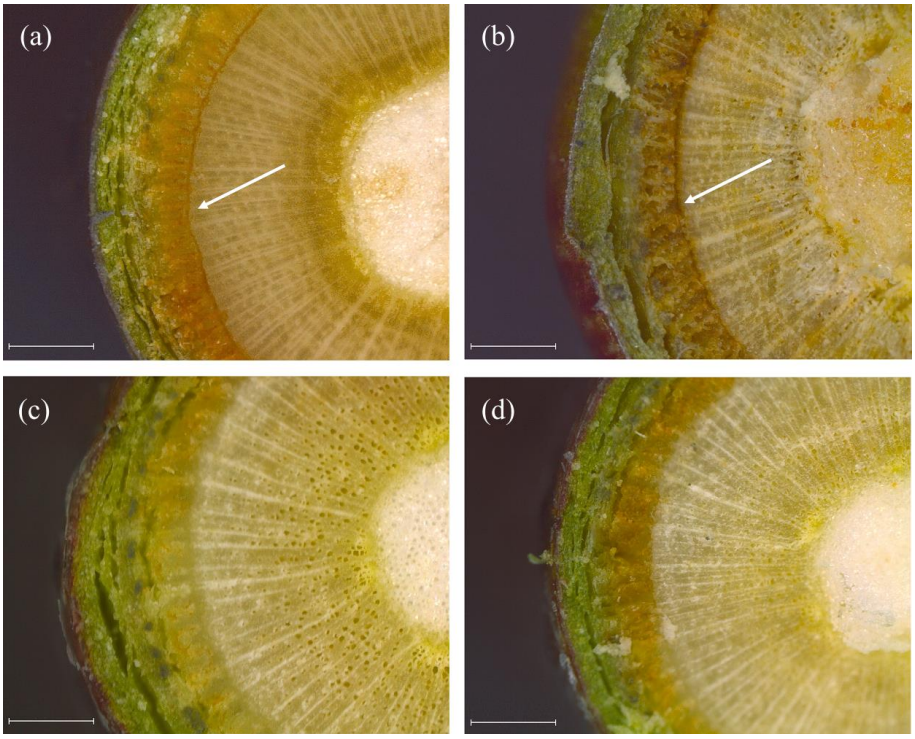

Fig. III-2. Cross sections of the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees with freezing injuries. The trees were subjected to accelerated deacclimation at 15ºC or delayed deacclimation at –5ºC in February and then freezing injuries were induced at –25ºC for 2 h. (a) SM at 15ºC, (b) KH at 15ºC, (c) SM at –5ºC, and (d) KH at –5ºC. White arrows indicate damaged area with brown discoloration. Scale bar = 0.5 mm.

duration. Considering that the energy for structural and functional changes is usually less required during DA than during CA (Browse and Lange, 2004), DA in which cold hardiness decrease rapidly is at high risk for freezing damages on deciduous fruit trees like peach.

Changes in soluble sugar contents

The contents of total soluble sugars increased during the delayed DA, but did not change during the accelerated DA (Fig. III-3a). From 3 weeks of the DA treatments, the total soluble sugar contents became significantly different between the two DA treatments. Starch contents gradually decreased during the accelerated DA, but did not change during the delayed DA (Fig. III-3b). During the accelerated DA and the delayed DA, the changes in total soluble sugar and starch contents were not significantly different between the two cultivars, except that starch contents after 4 weeks of the accelerated DA and the delayed DA were lower in SM than in KH (Figs. III-3b and 4), indicating more soluble sugars accumulated by starch degradation in SM.

The decrease in the total soluble sugar contents during the accelerated DA might be due to the growth resumption (Morin et al., 2007) as observed in other woody plants (Stushnoff et al., 1993). The total soluble sugars might be consumed for regrowth the following spring with increased respiration which were not offset by photosynthesis (Ögren, 1997). Since the flower buds of peach trees germinate earlier than leaf buds, the flower bud germination is dependent on the stored sugars in the shoots.

Various amounts of fructose, glucose, sorbitol, sucrose, raffinose, and stachyose were detected in the shoot of SM and KH trees subjected to the

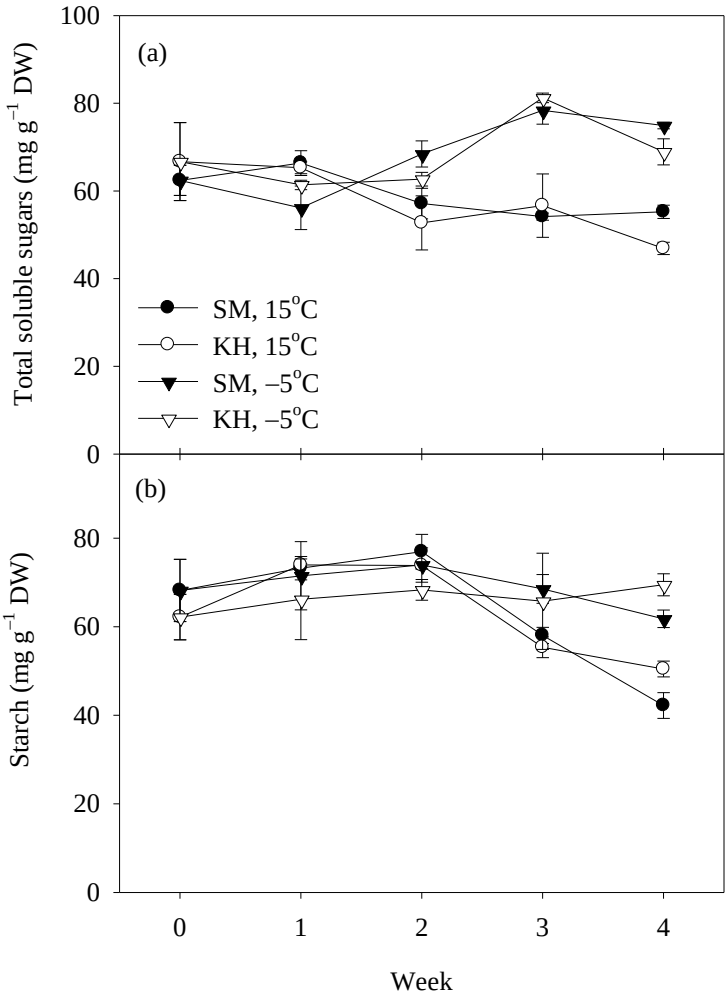

Fig. III-3. Changes in (a) total soluble sugar and (b) starch contents in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation at 15°C and delayed deacclimation at –5°C in February. Values are means ± standard errors (n = 3).

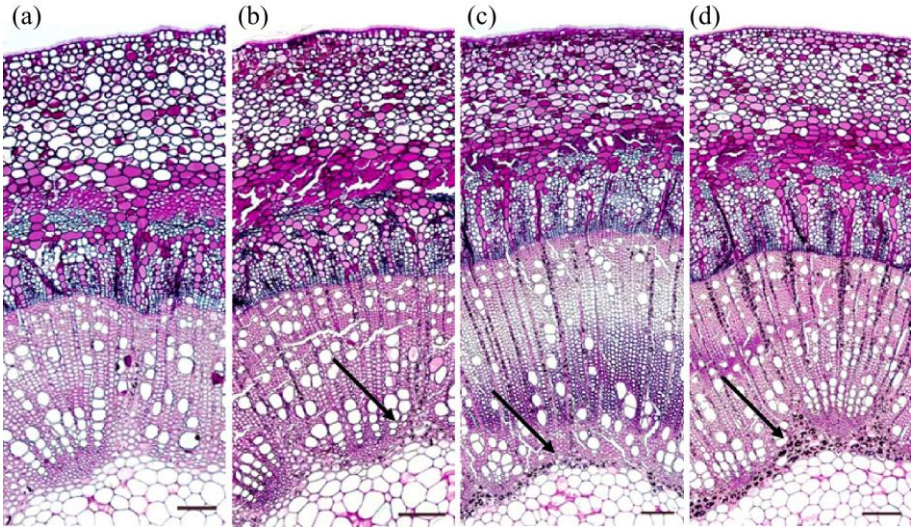

Fig. III-4. Accumulation of starch granules in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation at 15°C and delayed deacclimation at –5°C for 4 weeks in February. (a) SM at 15°C, (b) KH at 15°C, (c) SM at –5°C, and (d) KH at –5°C. Starch granules inside the parenchyma cells were dyed black and indicated with black arrows. Scale bar = 100 μm.

accelerated DA and the delayed DA (Fig. III-5). Sucrose was predominant, comprising about half contents of the total soluble sugars (Fig. III-5a). Sucrose content increased and slightly decreased during the accelerated DA and the delayed DA, respectively. However, sorbitol contents in both cultivars did not change significantly during the accelerated DA and the delayed DA (Fig. III-5b). At 4 weeks after the accelerated DA, fructose and glucose contents decreased in both cultivars, but they did not change during the delayed DA (Fig. III-5c and d). Raffinose and stachyose contents also decreased significantly during the accelerated DA, but they slightly increased during the delayed DA in both cultivars (Fig. III-5e and f). During the accelerated DA, raffinose and stachyose contents steadily decreased in both cultivars.

During the accelerated DA, starch might be hydrolyzed to soluble sugars and support metabolic process as an energy source for bud development and growth resumption (Morin et al., 2007; Ouyang et al., 2019). During the delayed DA, on the other hand, starch degradation and soluble sugar accumulation might be associated with maintaining the cold hardiness as the increases of sucrose, raffinose, and stachyose were observed (Fig. III-5a, e, and f). The fluctuation of starch, a major source of soluble sugars in woody plants, might be a consequence of net carbon gain or loss (Thalmann and Santelia, 2017).

Transcriptome sequencing data statistics

The transcriptomes of the shoots of SM and KH peach trees subjected to the accelerated DA and the delayed DA were sequenced. Generated reads were 34,523,499 to 39,739,876 with GC content of 45.8-46.4% and a Q30 of 94.3-94.8 (Table III-2), indicating that the trimmed data were significant. The Q30 means

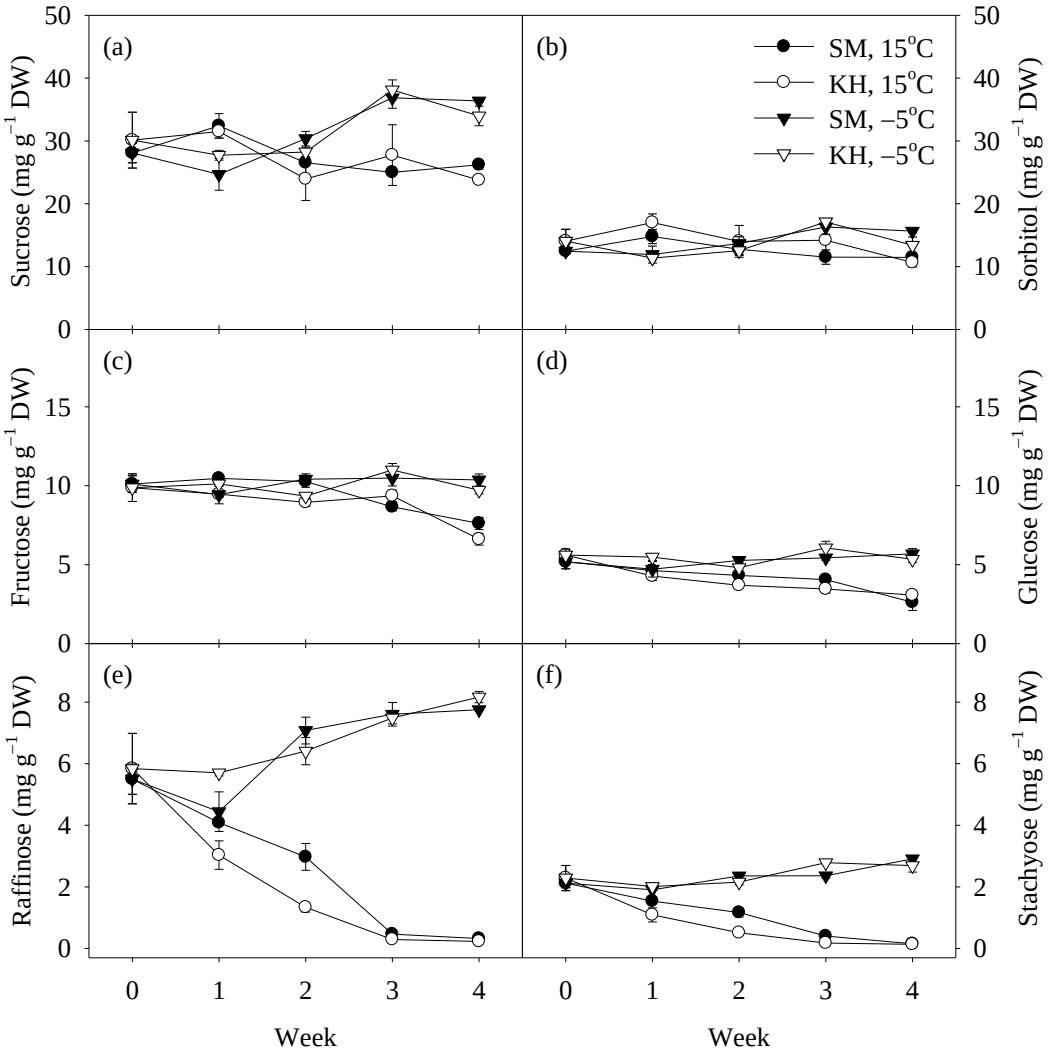

Fig. III-5. Changes of (a) sucrose, (b) sorbitol, (c) fructose, (d) glucose, (e) raffinose, and (f) stachyose contents in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation at 15°C and delayed deacclimation at –5°C in February. Values are means ± standard errors (n = 3).

Table III-2. Transcriptome sequencing data statistics from the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation (DA) at 15°C and delayed DA at –5°C in February.

Cultivar	DA at	Total read	Total base	GC content (%)	Q30 (%)	Mapping ratio (%)
SM	15 °C	39,739,876	4,013,727,476	46.0	94.7	96.5
	–5 °C	34,523,499	3,486,873,365	46.4	94.8	96.7
KH	15 °C	39,227,297	3,961,957,031	45.8	94.7	96.6
	–5 °C	36,692,761	3,705,968,827	46.4	94.3	96.9

the probability of an incorrect base call 1 in 1,000 times. The total bases of average of 3.48-4.01 × 10^9 were obtained for each sample, and almost all of the total reads were mapped to the reference peach genome (GCA_00346465.2), with a mapping ratio of 96.5-96.9% (Table III-2).

Functional annotation and GO term enrichment of the DEGs

Of the 26,411 assembled transcripts, 18,130 transcripts were identified in all the samples examined. During the accelerated DA, a total of 2,302 and 1,814 transcripts were differentially expressed in SM and KH, respectively, with a |fold change| > 2 ($P < 0.05$) (Fig. III-6). Among them, 1,174 and 784 transcripts were up-regulated, while 1,128 and 1,030 transcripts were down-regulated in SM and KH, respectively, during the accelerated DA as compared to those during the delayed DA. A total of 363 and 505 DEGs were commonly up- and down-regulated, respectively, in SM and KH during the accelerated DA relative to the delayed DA (Fig. III-6).

The up-regulated DEGs in SM and KH were classified into 35 functional terms within three main GO categories: 10 biological process terms, 7 cellular component terms, and 18 molecular function terms were significantly enriched (Fig. III-7). DEGs were associated with various biological process terms: 122 DEGs (14.2%) and 74 DEGs (16.6%) in SM and KH, respectively. DEGs were also associated with the cellular component terms (369 DEGs; 43.0%) and molecular function terms (367 DEGs; 42.8%) in SM. In KH, 163 DEGs (36.5%) and 209 DEGs (46.9%) were associated with cellular component and molecular function terms, respectively. The ‘integral component of membrane’ term was significantly enriched with 249 and 74 up-regulated DEGs in SM and KH,

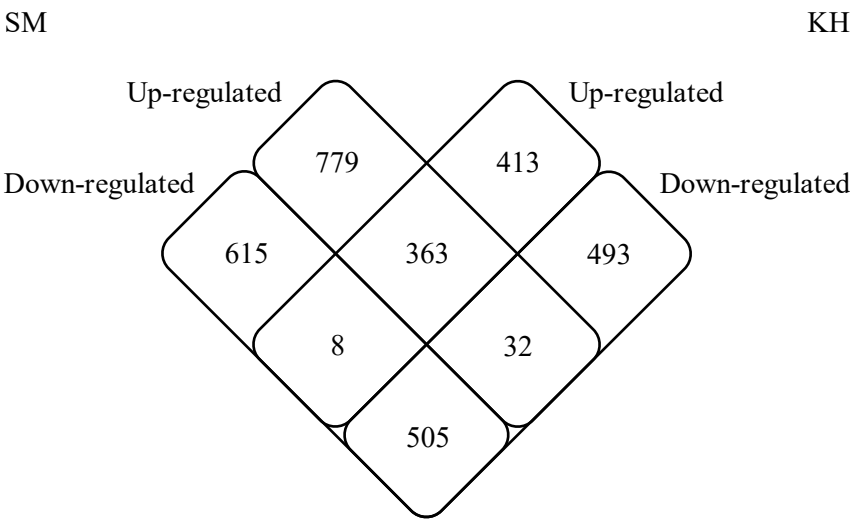

Fig. III-6. Venn diagram of differentially expressed genes (DEGs) in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation (DA) at 15ºC and delayed DA at –5ºC for 4 weeks in February. The DEGs with a |fold change| > 2 ($P < 0.05$) were selected based on the FPKM values during the accelerated DA as compared to those during the delayed DA.

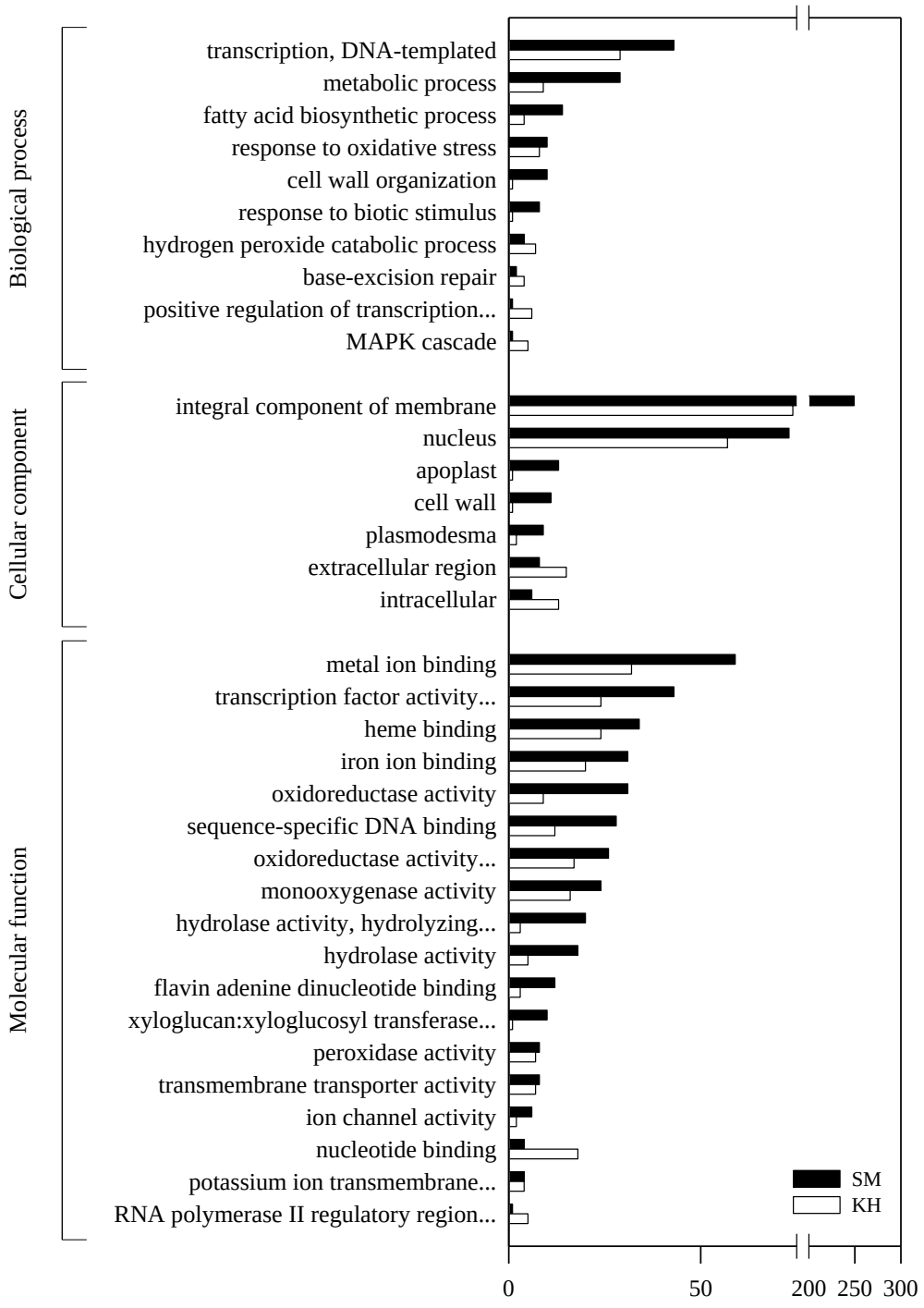
Biological process
Cellular component
Molecular function
transcription, DNA-templated
metabolic process
fatty acid biosynthetic process
response to oxidative stress
cell wall organization
response to biotic stimulus
hydrogen peroxide catabolic process
base-excision repair
positive regulation of transcription...
MAPK cascade
integral component of membrane
nucleus
apoplast
cell wall
plasmodesma
extracellular region
intracellular
metal ion binding
transcription factor activity...
heme binding
iron ion binding
oxidoreductase activity
sequence-specific DNA binding
oxidoreductase activity...
monooxygenase activity
hydrolase activity, hydrolyzing...
hydrolase activity
flavin adenine dinucleotide binding
xyloglucan:xyloglucosyl transferase...
peroxidase activity
transmembrane transporter activity
ion channel activity
nucleotide binding
potassium ion transmembrane...
RNA polymerase II regulatory region...
SM
KH
0
50
200
250
300

Fig. III-7. Gene ontology terms enriched up-regulated differentially expressed genes (DEGs) in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation (DA) at 15°C and delayed DA at –5°C for 4 weeks in February. The DEGs with a |fold change| > 2 ($P < 0.05$) were selected based on the FPKM values during the accelerated DA as compared to those during the delayed DA.

respectively. The down-regulated DEGs in SM and KH were classified into 35 functional terms within three main GO categories: 9 biological process terms, 12 cellular component terms, and 14 molecular function terms were significantly enriched (Fig. III-8). DEGs were associated with various biological process terms: 138 DEGs (22.2%) and 129 DEGs (21.0%) in SM and KH, respectively. DEGs were also associated with the cellular component terms (202 DEGs; 32.5%) and molecular function terms (282 DEGs; 45.3%) in SM. In KH, 202 DEGs (32.8%) and 284 DEGs (46.2%) were associated with cellular component and molecular function terms, respectively. The 'oxidoreductase activity' was the most highly enriched term (49 DEGs) in SM, while 'metal ion binding' was highly enriched in KH (39 DEGs).

Transcription factor (TF) identification and classification

A total of 219 DEGs were predicted as TFs and 39 TF families were identified by using the TF plant database. Major TF families enriched more than five DEGs were shown in Fig. III-9. Among the up-regulated TFs, the highest number of TFs were found in WRKY family followed by myeloblastosis TF (MYB) and no apical meristem, *Arabidopsis* transcription activation factor, and cup-shaped cotyledon (NAC) families in SM. In KH, MYB was the largest family in up-regulation. Among the down-regulated TFs, the largest family was ethylene responsive factor (ERF) in both cultivars.

In peach, more than 1,500 TFs have been identified and they are classified into 57 families and only four families, basic helix-loop-helix (bHLH), ERF, MYB, and NAC, have more than 100 identified TFs (Bianchi et al., 2015). In the present study, the ERF, WRKY, and MYB were the largest TF families with 28,

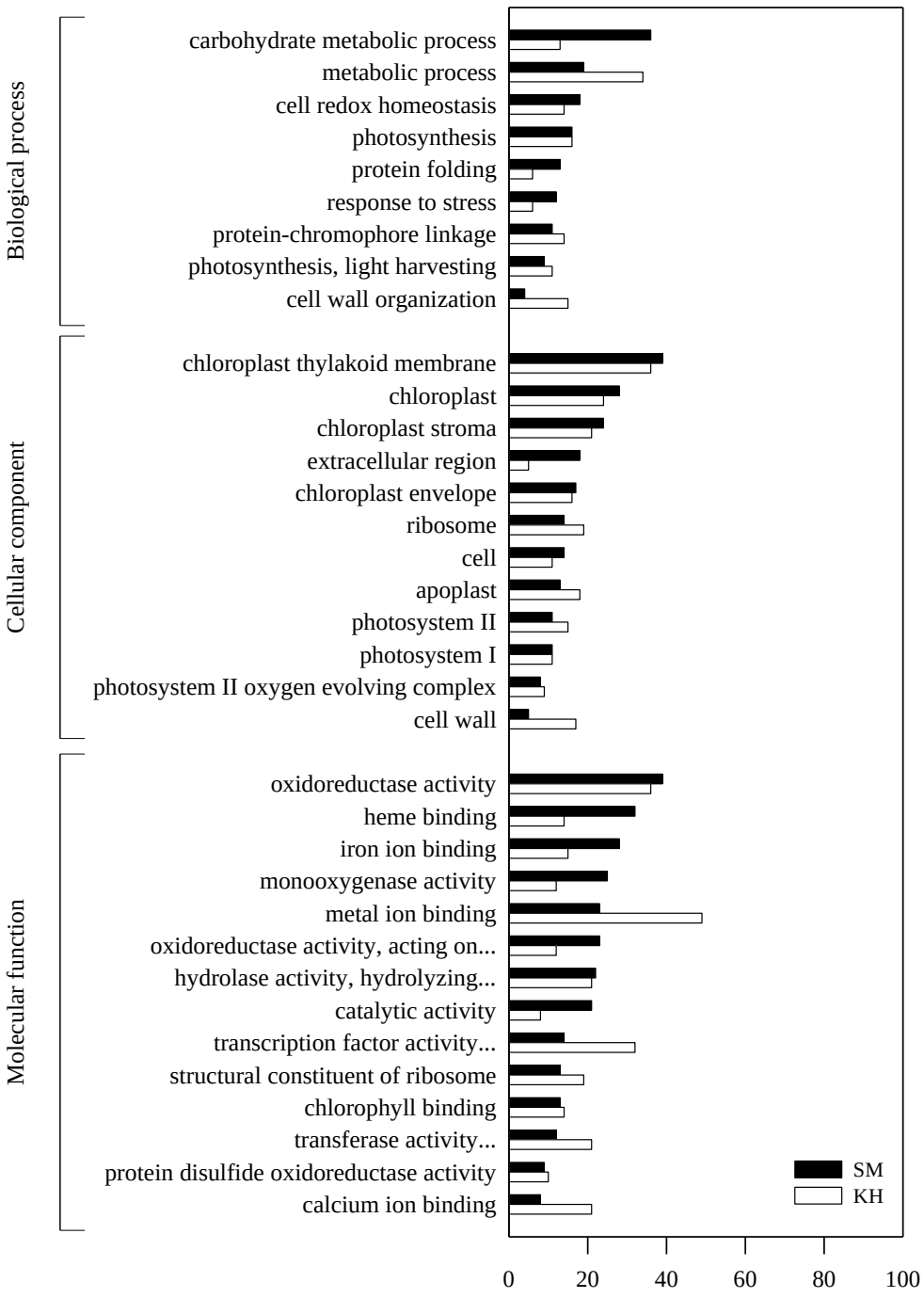
Biological process
Cellular component
Molecular function
carbohydrate metabolic process
metabolic process
cell redox homeostasis
photosynthesis
protein folding
response to stress
protein-chromophore linkage
photosynthesis, light harvesting
cell wall organization
chloroplast thylakoid membrane
chloroplast
chloroplast stroma
extracellular region
chloroplast envelope
ribosome
cell
apoplast
photosystem II
photosystem I
photosystem II oxygen evolving complex
cell wall
oxidoreductase activity
heme binding
iron ion binding
monooxygenase activity
metal ion binding
oxidoreductase activity, acting on...
hydrolase activity, hydrolyzing...
catalytic activity
transcription factor activity...
structural constituent of ribosome
chlorophyll binding
transferase activity...
protein disulfide oxidoreductase activity
calcium ion binding
SM
KH
0
20
40
60
80
100

Fig. III-8. Gene ontology terms enriched down-regulated differentially expressed genes (DEGs) in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation (DA) at 15°C and delayed DA at –5°C for 4 weeks in February. The DEGs with a |fold change| > 2 ($P < 0.05$) were selected based on the FPKM values during the accelerated DA as compared to those during the delayed DA.

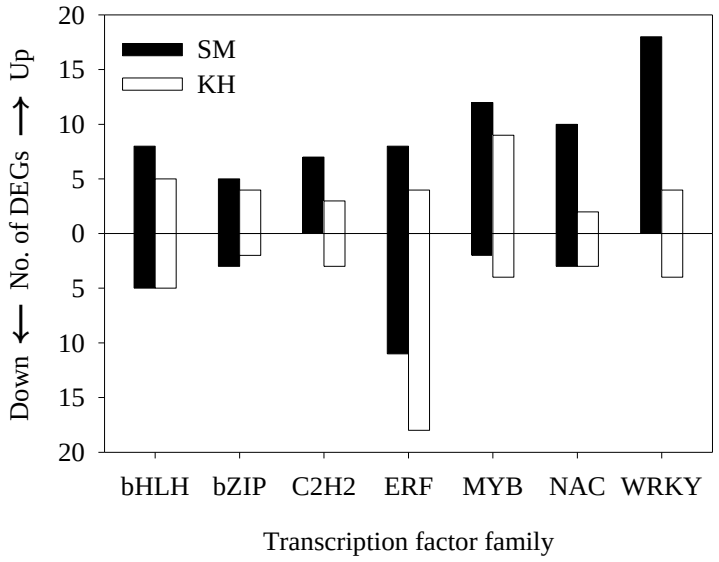

Fig. III-9. Differentially expressed transcription factor families enriched in the differentially expressed genes (DEGs) in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation (DA) at 15°C and delayed DA at –5°C for 4 weeks in February. The DEGs with a |fold change| > 2 (P < 0.05) were selected based on the FPKM values during the accelerated DA as compared to those during the delayed DA.

22, and 21 DEGs, respectively (Fig. III-9). The TF families are commonly involved in various biological and physiological responses, since the families are classified based on their characteristic DNA-binding domains (Hong, 2016).

Eighteen WRKY TFs were up-regulated in SM, while four WRKY TFs were up-regulated in KH (Fig. III-9). There were no down-regulated TFs in WRKY family in SM, but four TFs were down-regulated in KH. While the majority of functional analyses of WRKY TFs were restricted to abiotic stresses such as drought, high salinity, cold, and osmotic stresses (Chen et al., 2012), a few WRKY TFs have been described on regulating plant development. Overexpression of *WRKY13* and *WRKY15* promoted the stem elongation and root development (Yu et al., 2012; Zhou et al., 2008) and knockdown of *WRKY78* induced semi-dwarf phenotype in rice (Zhang et al., 2011). These growth enhancements suggest the activation of downstream genes which are involved in hormone signaling such as auxin and gibberellin (GA) (Zhou et al., 2008). Besides, an WRKY TF increased starch synthesis by binding to the sugar-responsive element of *iso1* promoter as an activator (Sun et al., 2005). Therefore, the up-regulation of WRKY TFs during DA in the present study probably contributes to the regrowth of plant and carbohydrate anabolism in the following spring through the sequential signal transduction and responses.

A total of 29 MYB TFs were identified, out of which 21 and 6 TFs were up- and down-regulated during DA (Fig. III-9). The most up-regulated genes were *MYB62* and *MYB6* in SM and KH, respectively. MYB TFs, the largest family in plants, also regulate diverse target genes and they are involved in various processes including cell differentiation and development, and defense and stress responses (Li et al., 2015). More than 20 MYB TFs have been reported to be up-

regulated by low temperature or during CA (Liu et al., 2019). Overexpression of *OsMYB4*, which is induced by cold stress, enhanced tolerance to cold and freezing stresses (Pasquali et al., 2008). On the other hand, overexpression of *AtMYB15* reduced freezing tolerance by suppressing the expression of *CBF* gene which confer freezing tolerance to plants (Agarwal et al., 2006). In the present study, more up-regulated MYB TFs were found than down-regulated TFs in both cultivars (Fig. III-9). Among the up-regulated MYB TFs, *MYB6*, *MYB62*, and *MYB108* were most significantly up-regulated in both cultivars. In *Arabidopsis*, similarly, *MYB6* was up-regulated under high temperature condition (30°C), down-regulating the anthocyanin biosynthesis (Rowan et al., 2009). The overexpression of *MYB62* in *Arabidopsis* suppressed flowering regulators such as *SOC1* and *SUPERMAN,* resulting in a GA-deficient phenotype (Devaiah et al., 2009). As peach trees flower after DA, the up-regulated MYB TFs during the accelerated DA in the present study might be involved in flowering by interfering with GA metabolism.

The ERF family which play crucial roles in biotic and abiotic stresses can be classified into ethylene-responsive element binding protein and dehydration-responsive element-binding protein (DREB) subfamilies (Sakuma et al., 2002). In the present study, 11 and 18 ERF TFs were down-regulated in SM and KH, respectively, only eight and four ERF TFs were up-regulated (Fig. III-9). Among the down-regulated ERF TFs, four *DREB1* and one *DREB2* genes were significantly down-regulated. DREB1 subgroup is known to be cold inducible and a major regulator of cold stress responses, while DREB2 plays important roles in dehydration and heat stress responses (Mizoi et al., 2012). The significant down-regulation of *DREB1* during DA might be related to the increased gene expression

during CA or under low temperature condition.

Functional annotation of the transcripts during accelerated DA

To understand the deeper insights into the biological process during DA, the DEGs were matched with KEGG pathway classification. Among the total of 2,303 and 1,814 DEGs of SM and KH, respectively, 667 and 515 DEGs were assigned to 124 pathways based on KEGG database. The DEGs were primarily linked to metabolism category followed by genetic information processing, environmental information processing, and organismal systems (Fig. III-10). Among the metabolism category, the most enriched pathway was carbohydrate metabolism followed by amino acid and lipid metabolism (Fig. III-11). The DEGs belong to carbohydrate metabolism were classified into 16 individual pathway maps (Fig. III-12) and starch and sucrose was the most enriched pathway map with 25 and 18 DEGs in SM and KH, respectively. A total of 20 enzymes involved in sugar metabolism were annotated and β-galactosidase (EC 3.2.1.23; LOC18769018 and LOC18783479) and β-glucosidase (EC 3.2.1.21; LOC18770931, LOC18779132, and LOC18779303) were significantly up-regulated in SM (Fig. III-13). Considering that β-galactosidase and β-glucosidase are involved in RFOs and starch degradation, respectively, their higher gene expressions might be associated with the higher sugar contents.

Transcripts involved in galactinol synthase (EC 2.4.1.123; LOC18789982) and raffinose synthase (EC 2.4.1.82; LOC18770520) were significantly down-regulated in SM relative to KH (Fig. III-13). A key role of galactinol synthase in RFO synthesis and cold hardiness has been reported in woody plants (Unda et al., 2012). In accordance with other studies, the transcript encoding galactinol

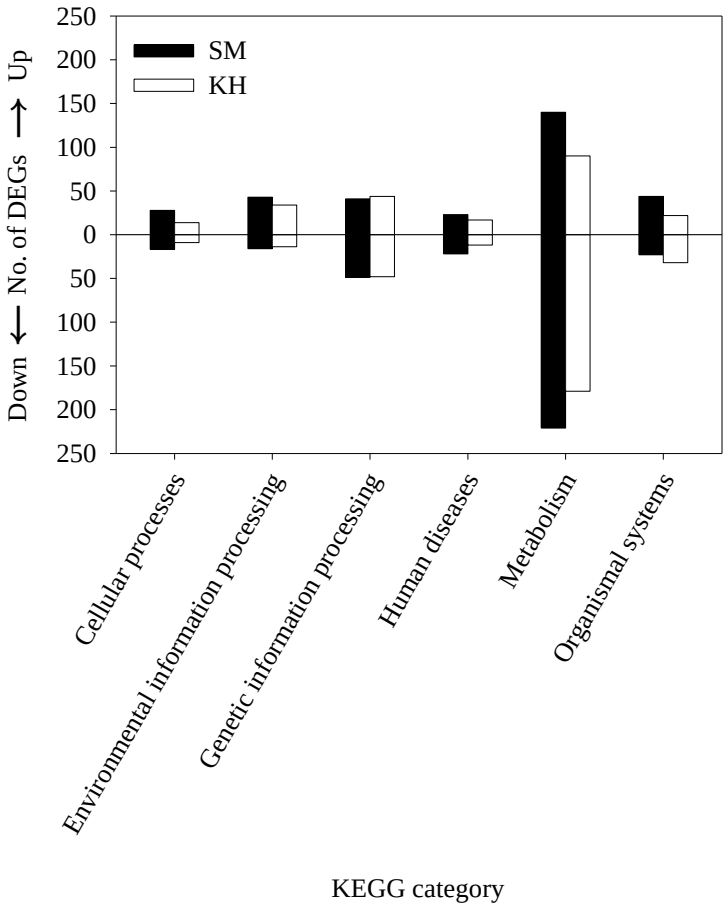

Fig. III-10. Kyoto Encyclopedia of Genes and Genomes (KEGG) categories enriched in the differentially expressed genes (DEGs) in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation (DA) at 15°C and delayed DA at –5°C for 4 weeks in February. The DEGs with a |fold change| > 2 ($P < 0.05$) were selected based on the FPKM values during the accelerated DA as compared to those during the delayed DA.

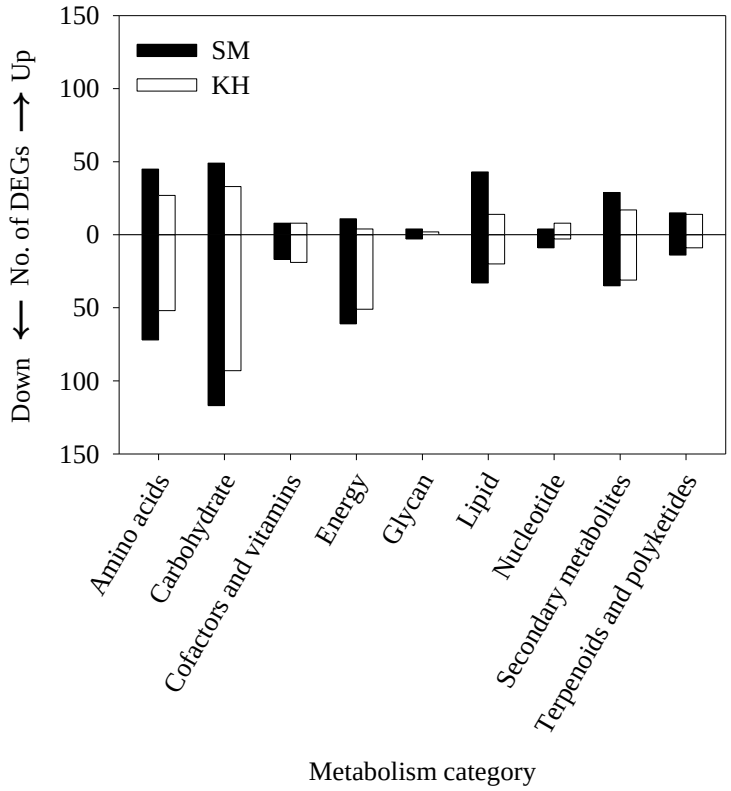

Fig. III-11. Metabolism categories, based on Kyoto Encyclopedia of Genes and Genomes database, enriched in the differentially expressed genes (DEGs) in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation (DA) at 15°C and delayed DA at –5°C for 4 weeks in February. The DEGs with a |fold change| > 2 ($P < 0.05$) were selected based on the FPKM values during the accelerated DA as compared to those during the delayed DA.

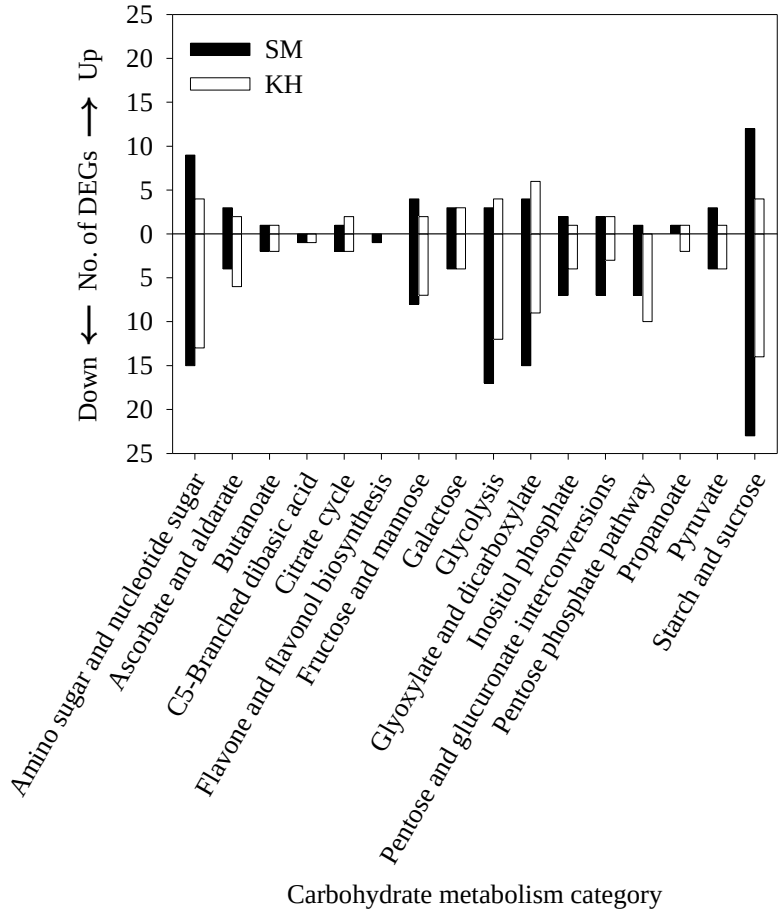

Fig. III-12. Carbohydrate metabolism categories, based on Kyoto Encyclopedia of Genes and Genomes database, enriched in the differentially expressed genes (DEGs) in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation (DA) at 15°C and delayed DA at –5°C for 4 weeks in February. The DEGs with a |fold change| > 2 ($P < 0.05$) were selected based on the FPKM values during the accelerated DA as compared to those during the delayed DA.

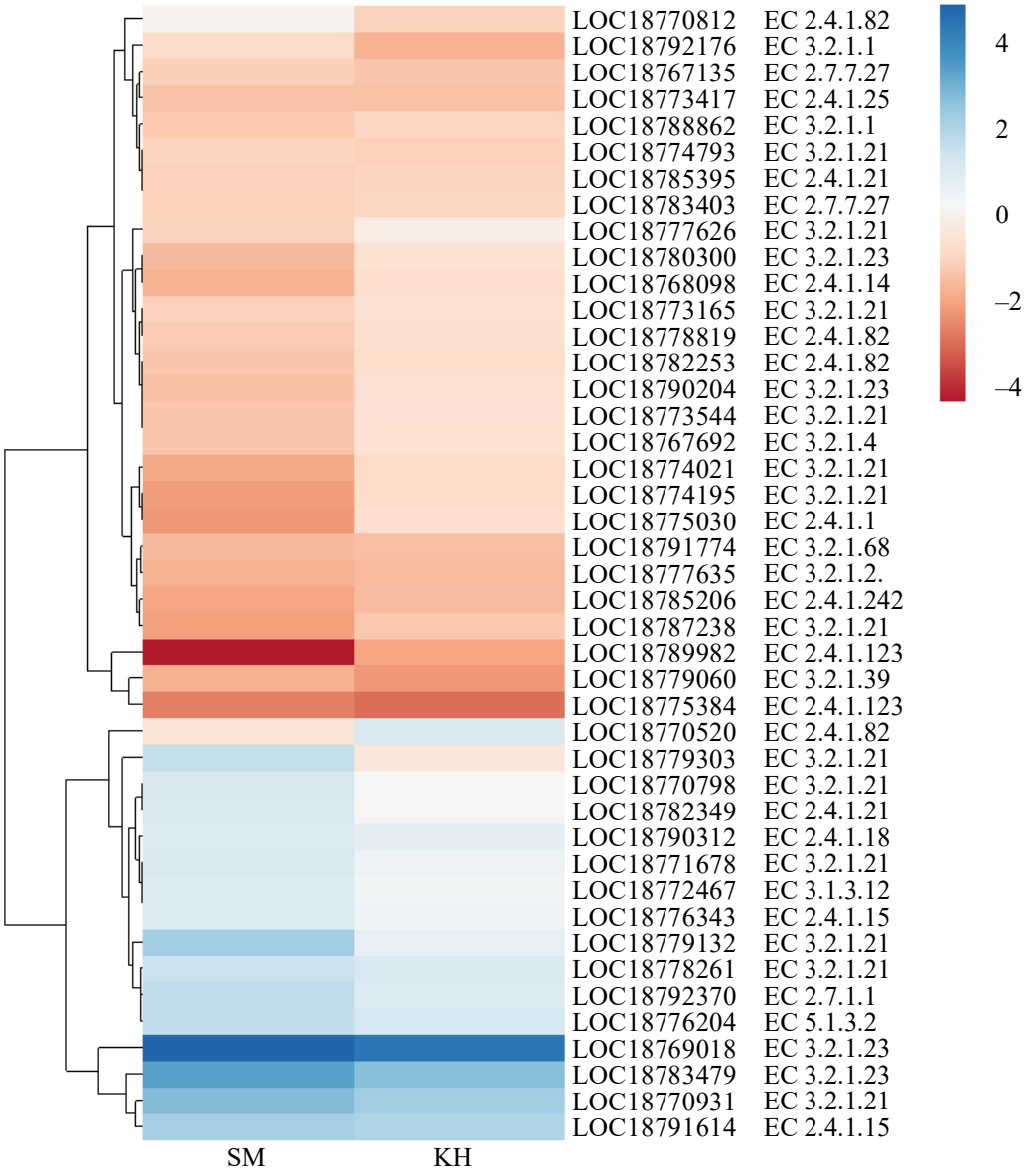

Fig. III-13. Heatmap of the $\log_2$ (fold change) values of 43 differentially expressed genes involved in starch and sucrose metabolism in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation at 15°C relative to delayed deacclimation –5°C for 4 weeks in February.

synthase was also significantly down-regulated during the accelerated DA.

Comparison of DEGs between SM and KH

Transcriptomes were also compared between the two cultivars and the top ten up- and ten down-regulated DEGs were selected based on their fold changes and expression volume. Top ten up- and ten down-regulated DEGs in SM shoots relative to KH shoots were shown in Tables III-3 and 4, respectively, and the fold change values of the DEGs were significantly correlated with the cold hardiness, suggesting that the DEGs influence cold hardiness in peach tree shoots.

The significantly regulated DEGs were involved in metabolic process such as carbohydrate and lipid metabolism. For example, probable xyloglucan endotransglucosylase protein 23 (LOC18792321), acidic endochitinase (LOC18785306), uridine disphosphate (UDP)-glucuronate 4-epimerase 6 (LOC18793895), galactinol synthase 1 (LOC18789982), 2,3-bisphosphoglycerate -independent phosphoglycerate mutase (LOC18772584), α-glucan phosphorylase (LOC18775030), and β-glucosidase 11 (LOC18774195) were related to carbohydrate metabolism, while linoleate 13S-lipoxygenase 3-1, chloroplastic (LOC18783171), putative lipid-transfer protein DIR1 (LOC18785612), and Δ^{12}-fatty-acid desaturase FAD2 (LOC18769718) were related to lipid metabolism.

Dehydration-responsive genes were reported to be up-regulated during CA and involved in membrane stabilization during desiccation stress. They included low-temperature-induced 65 kDa protein (Visconti et al., 2019), late embryogenesis abundant protein 29 (Olvera-Carrillo et al., 2011), dehydrin Xero 2 (Hanin et al., 2011), and desiccation-related protein PCC13-62 (Rodriguez et al., 2010). In the present study, DREB 1A (LOC18787317) and dehydrin Xero 2

Table III-3. Top ten up-regulated differentially expressed genes in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation (DA) at 15°C relative to delayed DA at –5°C for 4 weeks in February.

Gene (LOC No.)	Description	Fold change (expression volume) SM	KH	SM/KH	Correlation coefficient (r)
18792321	Probable xyloglucan endotransglucosylase/hydrolase protein 23	1.11 (8.85) *	–17.85 (6.79) *	19.80 (7.75) ***	–0.91 ***
18787317	Dehydration-responsive element-binding protein 1A	3.32 (6.67) ***	–3.09 (4.79) *	10.24 (5.65) *	–0.87 ***
18767063	Classical arabinogalactan protein 1	1.77 (7.32) ***	–5.57 (5.62) NS	9.84 (6.42) **	–0.91 ***
18776420	Probable calcium-binding protein CML27	–1.24 (7.70) ***	–10.29 (5.90) *	8.29 (6.74) **	–0.91 ***
18767807	Pathogenesis-related protein 1	34.1 (3.80) ***	5.89 (2.54) **	5.79 (3.10) **	–0.83 ***
18768170	(RS)-Norcoclaurine 6-O-methyltransferase	–2.33 (7.90) **	–10.52 (5.93) *	4.51 (6.84) ***	–0.91 ***
18785306	Acidic endochitinase	16.36 (6.23) ***	2.24 (5.25) NS	7.32 (5.72) **	–0.85 **

18792372	Zinc finger protein ZAT10	2.09 (8.15) ***	-3.05 (6.82) NS	6.36 (7.46) **	–0.91 ***
18783171	Linoleate 13S-lipoxygenase 3-1, chloroplastic	3.46 (5.52) ***	–1.99 (4.36) NS	6.89 (4.9) **	–0.91 ***
18793895	UDP-glucuronate 4-epimerase 6	–1.11 (7.08) **	–6.69 (5.69) *	6.02 (6.35) **	–0.91 ***

Fold changes were determined based on the FPKM values during the accelerated DA as compared to those during the delayed DA.

Correlation coefficients were determined between cold hardiness (LT_{50}) and log_2(fold change) of the selected DEGs.

NS, *, **, ***Not significant or significant at $P < 0.05$, 0.01, or 0.001, respectively.

Table III-4. Top ten down-regulated differentially expressed genes in the shoots of 'Soomee' (SM) and 'Kanoiwa Hakuto' (KH) peach trees subjected to accelerated deacclimation (DA) at 15°C relative to delayed DA at –5°C for 4 weeks in February.

Gene (LOC No.)	Description	Fold change (expression volume) SM	KH	SM	Correlation coefficient (r)
18766910	Non-symbiotic hemoglobin	–9.34 (5.00) **	1.49 (6.88) NS	–13.87 (5.86) ***	0.91 ***
18768928	Bidirectional sugar transporter SWEET1	–7.96 (3.18) ***	–1.11 (5.27) NS	–7.16 (4.09) **	0.86 ***
18785612	Putative lipid-transfer protein DIR1	–10.01 (5.79) ***	–1.50 (7.26) *	–6.65 (6.49) ***	0.90 ***
18769991	Dehydrin Xero 2	–14.53 (7.09) **	–3.05 (8.24) ***	–4.77 (7.64) ***	0.92 ***
18789982	Galactinol synthase 1	–19.46 (6.24) ***	–3.96 (7.33) **	–4.91 (6.76) ***	0.91 ***
18775351	Osmotin–like protein	–3.90 (5.31) **	1.62 (5.89) NS	–6.32 (5.60) *	0.85 ***
18769718	Δ^{12}-Fatty-acid desaturase FAD2	–8.98 (7.16) ***	–2.53 (8.08) *	–3.54 (7.61) **	0.88 ***

18772584	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase	–3.81 (7.09) **	–1.33 (8.02) NS	–2.86 (7.54) *	0.85 ***
18775030	α-Glucan phosphorylase, H isozyme	–4.61 (6.56) **	–1.60 (7.50) **	–2.89 (7.02) **	0.88 ***
18774195	β-Glucosidase 11	–4.32 (5.84) **	–1.67 (6.77) **	–2.60 (6.29) **	0.90 ***

Fold changes were determined based on the FPKM values during the accelerated DA as compared to those during the delayed DA.

Correlation coefficients were determined between cold hardiness (LT_{50}) and $\log_2$(fold change) of the selected DEGs.

NS, *, **, ***Not significant or significant at $P < 0.05$, 0.01, or 0.001, respectively.

(LOC18769991) were significantly up- and down-regulated, respectively, in SM relative to KH (Tables III-3 and 4).

Validation of DEG data by qRT-PCR

During the accelerated DA, eight and eight DEGs involved in starch and sucrose metabolism were up- and down-regulated, respectively, in SM shoots relative to KH shoots. The 16 DEGs with a |fold change| > 2 ($P < 0.05$) were selected for validation and their expression patterns were analyzed by using qRT-PCR (Figs. III-14 and 15). Ten DEGs showed higher relative expression levels during the accelerated DA than during the delayed DA (Fig. III-14). SM had especially higher relative expression levels in LOC18770798, LOC18779132, and LOC18779303, which all encode β-glucosidase. These results demonstrated that the in silico data were useful for elucidating the sugar metabolism involved in cold hardiness of peach trees.

In conclusion, peach tree shoots lose their cold hardiness during the accelerated DA along with the reduction of soluble sugar and starch contents. During the accelerated DA, starch might be hydrolyzed to soluble sugars and support metabolic process as an energy source for bud development and growth resumption. Transcripts encoding catabolic enzymes, including β-amylase (EC 3.2.1.2) and β-glucosidase (EC 3.2.1.21), were up-regulated during the accelerated DA, while anabolic enzymes such as galactinol synthase (EC 2.4.1.123) and raffinose synthase (EC 2.4.1.82) were down-regulated, presumably facilitating RFO degradation and loss of cold hardiness. Validation of DEG through qRT-PCR analysis confirmed that interconversion of soluble sugars and starch are the key to determining cold hardiness in peach trees.

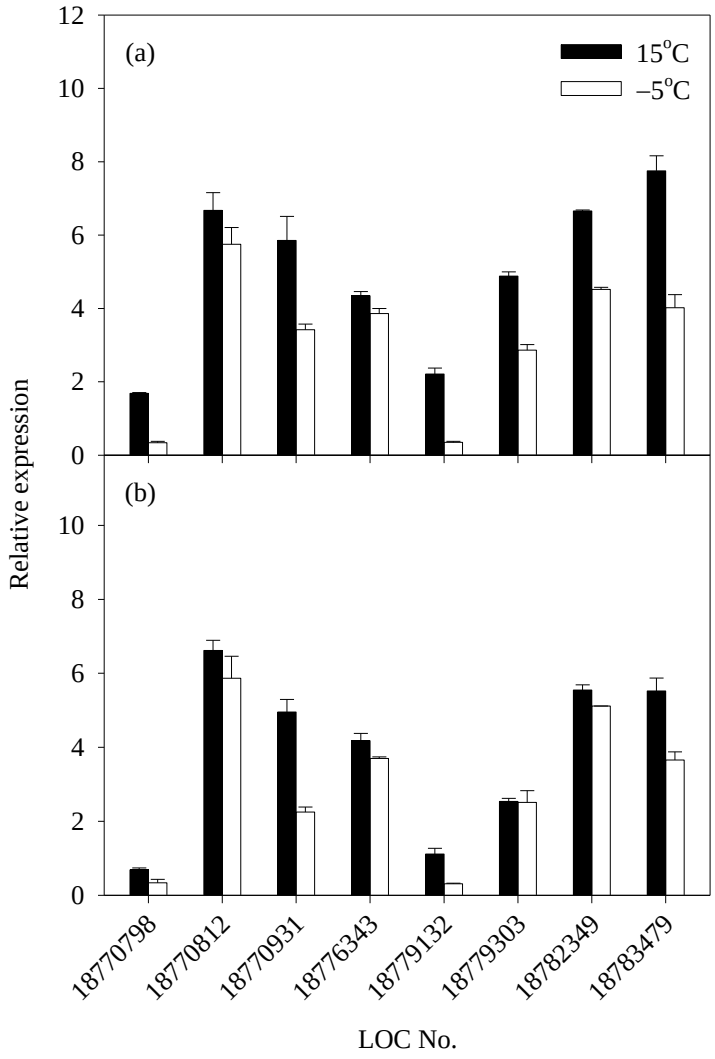

Fig. III-14. Relative expression for the validation of up-regulated differentially expressed genes in the shoots of (a) 'Soomee' and (b) 'Kanoiwa Hakuto' peach trees subjected to accelerated deacclimation (DA) at 15°C relative to the delayed DA at –5°C for 4 weeks in February. Expression levels were normalized against the expression of the peach *RNA polymerase II* and *translation elongation factor 2*. Values are means ± standard errors (n = 3).

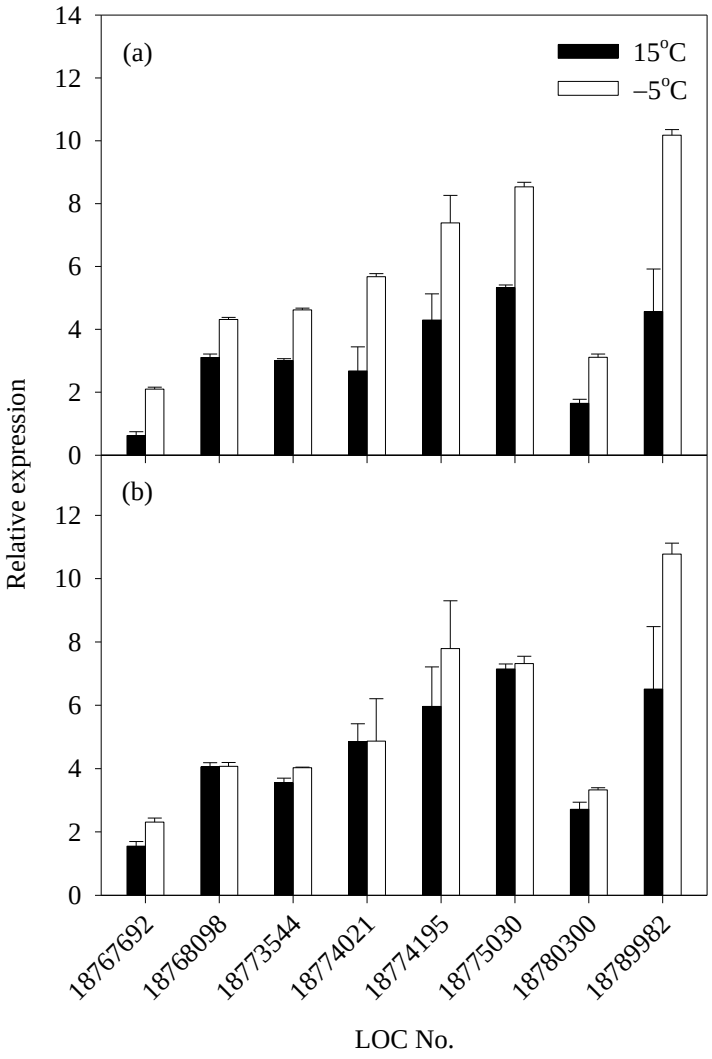

Fig. III-15. Relative expression for the validation of down-regulated differentially expressed genes in the shoots of (a) 'Soomee' and (b) 'Kanoiwa Hakuto' peach trees subjected to accelerated deacclimation (DA) at 15°C relative to the delayed DA at –5°C for 4 weeks in February. Expression levels were normalized against the expression of the peach *RNA polymerase II* and *translation elongation factor 2*. Values are means ± standard errors (n = 3).

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CONCLUSIONS

In this study, the cold hardiness of peach trees was dependent on endogenous factors such as dormancy status and sugar metabolism. Dormancy break of peach cultivars were around February with decreasing cold hardiness. Chilling requirement of peach cultivars were estimated and the cultivars with low chilling requirement might be readily deacclimated when exposed to warm spells. The changes in sugar metabolism which was different between a cold-hardy and a cold-sensitive peach cultivar also affected cold hardiness. Total soluble sugar contents from December to March were significantly higher in the cold-hardy cultivar than in the cold-sensitive cultivar. The increase in total soluble sugar content during cold acclimation might be due to hydrolysis, since total amylase and acid invertase activity increased. Transcriptome analysis also revealed transcriptional regulation associated with soluble sugar metabolism with alteration of cold hardiness. Transcripts encoding catabolic enzymes were up-regulated during the accelerated DA at 15ºC, while those encoding anabolic enzymes were down-regulated. The information of chilling requirement and cold hardiness of peach cultivars can be used for selecting suitable cultivation area. The identification of genes encoding soluble sugars which significantly accumulated during winter in cold hardy cultivars might be useful in peach breeding programs.

ABSTRACT IN KOREAN

온대 과수는 휴면을 통해 생장을 일시적으로 멈추고 저온 순화를 통해 내한성을 증가시켜 겨울철 저온에 적응하기 한다. 본 논문에서는 복숭아 품종별로 동해가 다르게 나타나는 원인을 구명하기 위해 휴면과 저온 순화 과정 중의 생리적 변화를 비교하였다. 복숭아 품종의 휴면은 1월과 2월 사이에 타파되었으며, Chill Hours, Utah, Dynamic 모델 등을 적용한 결과, 263-2123 chill hour, 377-1134 chill unit, 21.3-74.8 chilling portion의 저온 요구도를 나타내었다. 저온 요구도가 낮은 품종은 휴면 타파 시기가 빠르기 때문에 쉽게 탈순화되어 늦겨울과 초봄의 저온에 동해를 입을 가능성이 높은 것으로 판단되었다. 내한성이 강한 '수미' 품종과 약한 '오도로끼' 품종의 신초를 대상으로 하여 시기별로 유리당 함량의 변화를 조사하고 주요 당과 관련된 효소 활성의 변화, 관련 유전자의 발현양을 조사하였다. 두 품종은 시기별로 내한성이 유사하게 변화하였으나 12월부터 3월까지는 '수미'가 더 강한 내한성을 나타내었다. 이 시기의 총 유리당 함량은 '수미'에서 더 높았으며 관련 유전자 발현양도 더 높았다. 복숭아 신초에서는 유리당 중에서 sucrose의 함량이 가장 높은 것으로 조사되었으며 내한성과의 상관관계가 가장 높았다. 또한 fructose와 glucose는 순화 기간 동안 '수미'에서만 유의하게 증가하였으며 '오도로끼'에서는 증가하지 않았다. 이는 '수미'에서 활성이 높았던 acid invertase에 의해 sucrose가 fructose와 glucose로 분해되었기 때문인 것으로 보인다. Raffinose와 stachyose는 11월부터 4월 사이에서만 검출되었으며 이외의 시기에서는 검출되지 않아 내한성과 유의한 상관관계를 보였다. 품종별 탈순화 반응에 따른 대사 변화를 분석하기 위해 '수미'와 '가납암백도'

품종을 2월에 촉진 탈순화(15°C)와 지연 탈순화(–5°C) 처리하고 내한성, 전사체, 유리당과 전분 함량 변화를 분석하였다. 4주간의 촉진 탈순화 과정에서 내한성의 소실이 '수미'에서보다 '가납암백도'에서 더 빠르게 이루어졌으며 전분 함량의 감소는 '수미'에서 더 크게 나타났다. 전사체 분석 결과 'carbohydrate metabolism'에 연관된 차별 발현 유전자가 가장 많이 나타났으며, 그 중에서 'starch and sucrose metabolism'에 관련된 것이 가장 많았다. 43개의 차별 발현 유전자가 'starch and sucrose metabolism'에 연관되어 있었고, 이들은 20개의 효소를 암호화하였다. 촉진 탈순화 과정에서 β-galactosidase(LOC18769018, LOC18783479), β-glucosidase(LOC18770931, LOC18779132, LOC18779303)와 같은 분해 효소 유전자의 발현이 '가납암백도'에서보다 '수미'에서 더 증가하였으며, galactinol synthase(LOC18789982)나 raffinose synthase (LOC18770520)와 같은 합성 효소 유전자의 발현은 '수미'에서보다 '가납암백도'에서 더 증가하였다. 본 실험을 통해 유리당 대사에 관련된 유전자 발현과 효소 활성의 변화가 품종별로 차이가 있다는 것을 확인하였으며 이는 품종 간에 내한성이 다르게 나타나는 데 영향을 끼침을 알 수 있었다.

핵심어: 내한성, 복숭아, 온대 과수, 저온 순화, 저온 요구도, 지구 온난화, 탈순화, 휴면