

Suppression of 9-cis-Epoxy-carotenoid Dioxygenase, Which Encodes a Key Enzyme in Absciscic Acid Biosynthesis, Alters Fruit Texture in Transgenic Tomato^{1[W][OA]}

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Cell wall catabolism during fruit ripening is under complex control and is key for fruit quality and shelf life. To examine the role of absciscic acid (ABA) in tomato (*Solanum lycopersicum*) fruit ripening, we suppressed *SINCED1*, which encodes 9-cis-epoxycarotenoid dioxygenase (NCED), a key enzyme in the biosynthesis of ABA. To suppress *SINCED1* specifically in tomato fruits, and thus avoid the pleiotropic phenotypes associated with ABA deficiency, we used an RNA interference construct driven by the fruit-specific *E8* promoter. ABA accumulation and *SINCED1* transcript levels in the transgenic fruit were down-regulated to between 20% and 50% of the levels measured in the control fruit. This significant reduction in NCED activity led to a down-regulation in the transcription of genes encoding major cell wall catabolic enzymes, specifically polygalacturonase (*SIPG*), pectin methyl esterase (*SIPME*), β -galactosidase precursor mRNA (*SITBG*), xyloglucan endotransglycosylase (*SIXET*), endo-1,4- β -cellulose (*SICels*), and expansin (*SIExp*). This resulted in an increased accumulation of pectin during ripening. In turn, this led to a significant extension of the shelf life to 15 to 29 d compared with a shelf life of only 7 d for the control fruit and an enhancement of fruit firmness at the mature stage by 30% to 45%. In conclusion, ABA affects cell wall catabolism during tomato fruit ripening via down-regulation of the expression of major catabolic genes (*SIPG*, *SIPME*, *SITBG*, *SIXET*, *SICels*, and *SIExp*).

The plant hormone absciscic acid (ABA) not only regulates seed dormancy, plant growth, and responses to environmental stresses (Leung and Giraudat, 1998; Nambara and Marion-Poll, 2003; Finkelstein, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007) but also regulates fruit ripening (Kondo et al., 2002; Jiang and Joyce, 2003; Giovannoni, 2007; Galpaz et al., 2008). ABA in higher plants is formed from xanthoxin, via ABA aldehyde, by two oxidation reactions. The 9-cis-violaxanthin and 9-cis-neoxanthin are cleaved to xanthoxin (C_{15}) and C_{25} -epoxy-apo-aldehyde/ C_{25} -allenic-apo-aldehyde, in which reactions are catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED; Tan et al., 1997; Burbidge et al., 1999; Qin and Zeevaart, 1999;

Iuchi et al., 2001; Rodrigo et al., 2006). Since it was first isolated from the maize (*Zea mays*) *viviparous14* (*vp14*), mutant (Tan et al., 1997), NCED has been cloned and characterized in various plant species (Iuchi et al., 2000; Rodrigo et al., 2003; Wheeler et al., 2009). In higher plants, active ABA is either degraded to inactive compounds through an irreversible pathway starting with 8' hydroxylation and catalyzed by ABA 8'-hydroxylase (*CYP707As*) or is stored in bound form as ABA glucosylester, the formation of which is catalyzed by ABA glucosyltransferase (Zeevaart and Creelman, 1998; Zeevaart, 1999; Barthe et al., 2000). However, studies of ABA receptor proteins (Shen et al., 2006; Müller and Hansson, 2009; Pandey et al., 2009; Wu et al., 2009) and ABA signaling have defined the ABA signal transduction pathway (Ma et al., 2009; Melcher et al., 2009; Nishimura et al., 2009; Park et al., 2009), which includes three core components: the ABA receptors PYR/PYL/RCARs (hereafter referred to as "PYLs"); type 2C protein phosphatases (PP2Cs), which act as negative regulators; and subfamily 2 of SNF1-related kinases (SnRK2s), which act as positive regulators. Transcriptional regulation of these three core components has been investigated in tomato (*Solanum lycopersicum*) fruit (Sun et al., 2011). These experiments make a first step toward understanding ABA signal transduction in tomato by identifying candidate genes for signal transduction proteins.

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ABA can induce fruit ripening; for example, applying exogenous ABA to grape (*Vitis vinifera*) at the véraison stage enhances the production of several metabolites involved in fruit ripening (Chernys and Zeevaart, 2000; Ban et al., 2003; Cakir et al., 2003; Jeong et al., 2004; Deluc et al., 2007; Lacampagne et al., 2009; Wheeler et al., 2009; Giribaldi et al., 2010). Genome surveys of ABA-induced gene expression in vegetative tissues of *Arabidopsis* (*Arabidopsis thaliana*) demonstrate that ABA induces a complex reprogramming of plant metabolism and particularly affects genes involved in responses against stress and in stress-dependent signaling networks (Hoth et al., 2002; Suzuki et al., 2003; Seki et al., 2007).

Nevertheless, the role of ABA during fruit ripening remains obscure, and there have been few studies of ABA function in fruit tissues. Progression in ripening brings about the degradation of cell wall components, and this leads to softening of the fruit. The plant primary cell wall is a highly complex structure composed of diverse polysaccharides and structural proteins. The cell walls of fruit are generally composed of cellular microfibrils, tethered with xyloglycans embedded in pectin mesh, and glycoproteins (Carpita and Gibeaut, 1993). Cell wall changes associated with fruit softening are related to the expression of a number of hydrolase and transglycosylase genes (Huber, 1983). Among these genes, the role of polygalacturonase (PG) has been studied extensively, and increases in PG activity and mRNA levels have been observed during the ripening of several fruit varieties (Kramer et al., 1990; Fischer and Bennett, 1991; Hadfield and Bennett, 1998; Murayama et al., 2009). However, in tomato, PG activity alone is not sufficient to significantly impact fruit texture (Sheehy et al., 1988; Smith et al., 1988; Giovannoni et al., 1989; Smith and Gross, 2000); thus, it is likely to act in concert with additional factors to affect tomato fruit texture. Indeed, other enzymes involved in cell wall metabolism have also been identified in ripening fruit; these include pectin methylesterase (PME; Tieman et al., 1992), endo- β -1,4-glucanases (Cels; Lashbrook et al., 1998), and expansin (Exp1; Rose et al., 1997; Brummell et al., 1999b; Civello et al., 1999; Cosgrove, 2000a, 2000b). In addition, ethylene has variable effects on the activities of these hydrolases during ripening (Brummell and Harpster, 2001). However, it is unclear whether ABA plays a role in the above process, and direct observation of ripening phenotypes is lacking in transgenic lines.

ABA deficiency has been thoroughly studied using mutants of various plant species (Taylor et al., 2000; Galpaz et al., 2008). However, ABA-deficient mutants do not undergo normal fruiting, and the fruit has developmental defects such as small size and low quality; therefore, these mutants have not been useful for investigating the role of ABA in fruit ripening of tomatoes. To obtain direct evidence for whether ABA has a role in cell wall catabolism in tomato fruit, we designed a specific RNA interference (RNAi) vector driven by the fruit-specific promoter *E8* to down-

regulate the *SINCE1* gene. This allowed us to bypass the pleiotropic effects of ABA deficiency and investigate the role of ABA specifically during fruit ripening.

RESULTS

ABA Metabolism, Changes in ABA Accumulation, and Alteration in Gene Expression in Tomato Fruit

Although previous research has identified a role for ABA in fruit ripening (Zhang et al., 2009a; Sun et al., 2010), the exact mechanisms of ABA regulation remain unclear. To examine this, we first measured endogenous ABA levels, which peaked prior to ethylene production, at the turning stage of ripening (Fig. 1A). Next, we treated tomato fruit with exogenous ABA and found that fruit firmness decreased while sugar content increased. These changes occurred more rapidly than in the control fruit (Fig. 1, B and C). By contrast, treatment with the ABA synthesis inhibitor nordihydroguaiaretic acid (NDGA) suppressed and deferred the increase of sugar content and the decrease of firmness (Fig. 1, B and C). Next, we examined the expression levels of genes involved in ABA synthesis (*SINCE1/2*) and ABA catabolism (*SICYP707A1/2/3*). Expression levels of two *NCEDs* (*SINCE1* and *SINCE2*) and three *CYP707As* (*SICYP707A1*, *SICYP707A2*, and *SICYP707A3*) were measured by real-time quantitative reverse transcription (RT)-PCR using cDNAs from tomato fruits. The expression of these genes in the fruit displayed rather different patterns. The expression of *SINCE1* (Burbidge et al., 1999) increased rapidly to a peak at the turning stage (Fig. 1D), which correlated with ABA levels during fruit ripening. By contrast, the expression of *SINCE2* was high during the immature stage but then declined continually through the remaining stages of fruit ripening. As for *SICYP707A1/2/3*, the expression of *SICYP707A2* increased at the break and pink stages and reached a peak during the over-ripe stage, while the expression of *SICYP707A1* and *SICYP707A3* was very low throughout fruit development (Fig. 1E). Because *SINCE1* is the main gene involved in ABA biosynthesis in fruit, we targeted this gene for subsequent phylogenetic and reverse genetic analysis.

NCED Gene Cloning and Phylogenetic Analysis

To examine *NCED* regulation in fruits with different metabolic controls of ripening, we cloned *NCED* cDNAs from both climacteric (tomato, persimmon [*Diospyros kaki*], pear [*Pyrus pyrifolia*], and muskmelon [*Cucumis melo*]) and nonclimacteric (sweet cherry [*Prunus avium*], strawberry [*Fragaria ananassa*], cucumber [*Cucumis sativus*], and zucchini [*Cucurbita pepo*]) fruits. The *NCED* deduced amino acid sequences showed high levels of sequence identity within each species: 79.67% between *SINCE1* and -2, 64.09% between *CsNCED1* and -2, 70.47% between *DkNCED1* and -2, 75.41% between *FaNCED2* and -3, 64.08% between

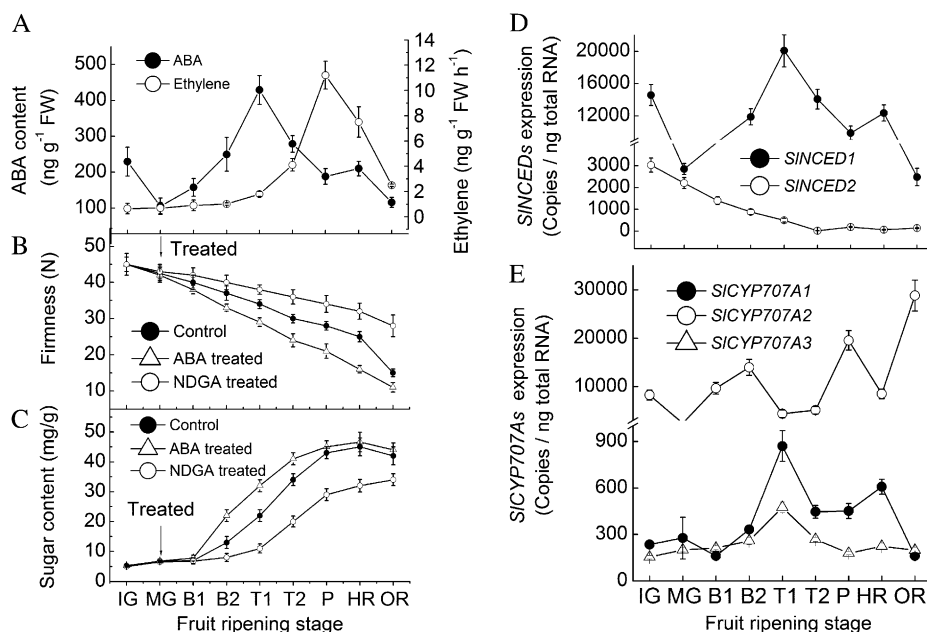


Figure 1. Changes in ABA, ethylene, fruit firmness, soluble sugar content, and expression of *SINCEDs* and *SICYP707As* in tomato fruits. A, Changes in endogenous ABA and ethylene production. FW, Fresh weight. B and C, Effect of exogenous ABA or NDGA treatment on fruit firmness and soluble sugar. The fruits on tomato plants were selected and tagged at 35 DAA. Fruits then were divided into three groups and were treated with ABA (100 μM), NDGA (100 μM), or distilled water. The fruits were each treated with 0.5 mL of solution by injection into the fruit from the pedicle with a medical injector. D and E, Absolute transcript level profiles for both *SINCEDs* and *SICYP707As*. During fruit development, tomato fruits were collected at the following times after anthesis (stages): 20 DAA (immature green [IG]), 35 DAA (mature green [MG]), 38 to 39 DAA (breaker [B1 and B2]), 40 to 41 DAA (turning [T1 and T2]), 42 DAA (pink [P]), 43 DAA (harvest red [HR]), and 45 DAA (over red [OR]). The transcript level of the *SAND* gene is shown as an internal loading control.

PpNCED1 and -2, 79.44% among *VvNCED1*, -2, and -3, and 74.42% among *CmNCED1*, -2, and -3. Based on the alignment of these protein sequences, the four-domain structure, including conserved His residues, was conserved within the NCEDs (Supplemental Fig. S4, black triangles).

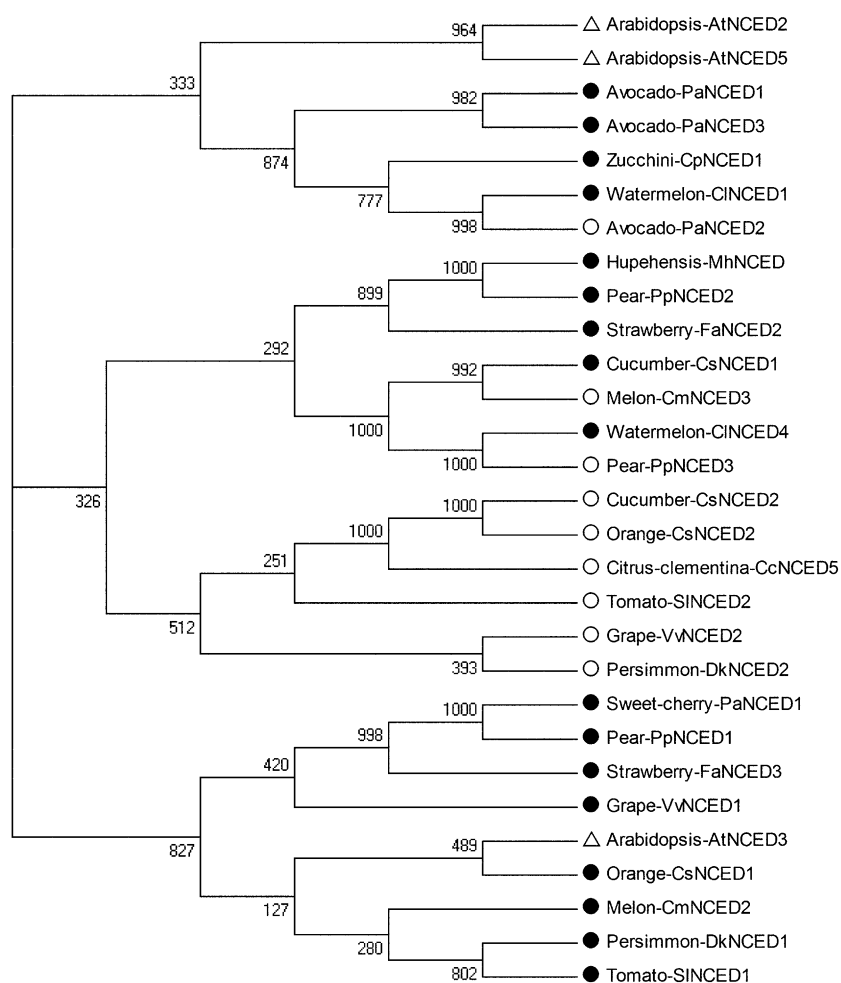
The expression of *NCED* genes was examined in fruits, and three main patterns were observed. The first pattern, which correlated well with ABA levels, was observed for genes such as *SINCED1*, *DkNCED1*, *PacNCED1* (Ren et al., 2011), and *CsNCED1*, which increased after the mature green stage, peaked at the turning stage, and then declined rapidly during the postharvest stage (Fig. 1D; Supplemental Fig. S6). The second expression pattern was observed for genes including *SINCED2* (Cui et al., 2010), *DkNCED2*, and *PpNCED3*, which were expressed at higher levels at the immature stage and then decreased continually during fruit development. A third gene expression pattern was observed for genes including *CINCED4* (Li et al., 2011), which were expressed at lower levels during early fruit development and then increased rapidly at later stages when the fruit became fully ripe. Taking together, interestingly, most genes with similar expression patterns clustered together in the phylogenetic tree (Fig. 2), but phylogenetic clustering showed no relationship with fruit type (climacteric or non-

climacteric). However, because we do not yet know the precise structure of these proteins, it is unclear how different amino acid sequences impact their functions (Supplemental Fig. S4). Further experiments are required to clarify the roles of these proteins during fruit ripening.

CYP707A Gene Cloning and Phylogenetic Analysis

To examine ABA catabolism in climacteric and non-climacteric fruits, we also cloned CYP707A cDNAs from the above-mentioned fruits. The genes identified were designated *SICYP707A1*, *SICYP707A2*, and *SICYP707A3* in tomato; *DkCYP707A1* in persimmon; *PpCYP707A1* and *PpCYP707A2* in pear; *CmCYP707A1* in muskmelon; *PacCYP707A1*, *PacCYP707A2*, *PacCYP707A3*, and *PacCYP707A4* in sweet cherry; *FaCYP707A1* in strawberry; *CsCYP707A1* and *CsCYP707A2* in cucumber; and *CiCYP707A1* in watermelon (*Citrullis lanatus*). The deduced amino acid sequences of these clones shared 70% sequence similarity with each other. Based on alignments of the protein sequences, most domains were well conserved in the different proteins (Supplemental Fig. S5). Phylogenetic analysis revealed that the CYP707A proteins in fruits were closely related to Arabidopsis (*AtCYP707As*) and other species (Fig. 3). Similar to *NCED* genes, *CYP707A* genes also showed

Figure 2. Phylogenetic tree of the *NCED* gene subfamily. A neighbor-joining phylogenetic tree was constructed using the ClustalX software and deduced protein sequences. Genes that showed increased expression increasingly as ABA accumulated in fruits are marked with black circles, and genes that displayed a gradual reduction in expression during fruit development are marked with white circles. White triangles indicate genes in *Arabidopsis*. Branch lengths are proportional to the number of amino acid substitutions. All sequence data mentioned in this article can be found in GenBank, and accession numbers are shown in Supplemental Table S3. Different species are represented as follows: tomato (Sl), *SINCE*D1 and *SINCE*D2; watermelon (Cl), *CINCE*D1, *CINCE*D2, and *CINCE*D3; muskmelon (Cm), *CmNCED*2 and *CmNCED*3; cucumber (Cs), *CsNCED*1 and *CsNCED*2; sweet cherry (Pac), *PacNCED*1; avocado (*Persea americana* [Pa]), *PaNCED*1, *PaNCED*2, and *PaNCED*3; grape (Vv), *VvNCED*1 and *VvNCED*2; *Arabidopsis* (At), *AtNCED*2, *AtNCED*3, and *AtNCED*5; zucchini (Cp), *CpNCED*1; persimmon (Dk), *DkNCED*1 and *DkNCED*2; pear (Pp), *PpNCED*1, *PpNCED*2, and *PpNCED*3; strawberry (Fa), *FaNCED*2 and *FaNCED*3; *Malus hupehensis* (Mh), *MhNCED*.



three distinct expression patterns in fruit (Ren et al., 2010; Li et al., 2011), and genes expressed according to these patterns clustered together in the phylogenetic analysis (Fig. 3; Supplemental Fig. S5). There is plainly a tendency in this system for genes showing similar expression patterns to cluster together in phylogenetic trees.

Suppression of *SINCE*D1 by RNAi Alters the Expression of Genes Involved in ABA Metabolism and Signaling

To examine the role of ABA during fruit ripening, we next inhibited ABA synthesis by using RNAi to reduce the levels of the key ABA biosynthetic enzyme *NCED*1. It is important for RNAi transgenic tomato plants and their fruit to be as normal and healthy as their control cognates, but inhibition of ABA synthesis has severe pleiotropic effects throughout the plant. To suppress *SINCE*D1 specifically in fruits and thereby avoid collateral negative effects on plant growth, we used the fruit-specific promoter *E8* to create plants that expressed a fruit-specific *SINCE*D1-RNAi construct. The RNAi hairpin structure was made up of an antisense/sense fragment of 427 bp from a conserved segment of *SINCE*D1. Because of the high protein

sequence identity between *SINCE*D1 and *SINCE*D2, we examined the effects of this construct on both *SINCE*D1 and *SINCE*D2. Four independent transgenic lines were generated, and all the RNAi lines showed normal development and fruiting, similar to the control fruit. Four *SINCE*D1-RNAi plants and their segregated lines were evaluated after cultivation in a greenhouse for three successive generations. First, we confirmed the efficacy of the RNAi method, finding that *SINCE*D1 levels were down-regulated throughout fruit development (Fig. 4A). Indeed, we also found *SINCE*D1 small interfering RNAs (siRNAs; Fig. 4K), indicating that silencing was effective in these plants. At the same time, we also found that *SINCE*D2 levels were down-regulated slightly at later stages of fruit development but not at early stages (Fig. 4B). In all transgenic lines, ABA levels in all fruits were down-regulated by 20% to 50% (Fig. 4C). To determine whether the altered ABA levels affected other components of the ABA signaling response, we examined *SICYP707A2* and found that its expression was also down-regulated in the transgenic tomato fruits (Fig. 4D). We also examined the expression levels of core components of the ABA signal transduction machinery, including *SIPYLs* (*SIPYL1* and *SIPYL2*), *SIPP2Cs*

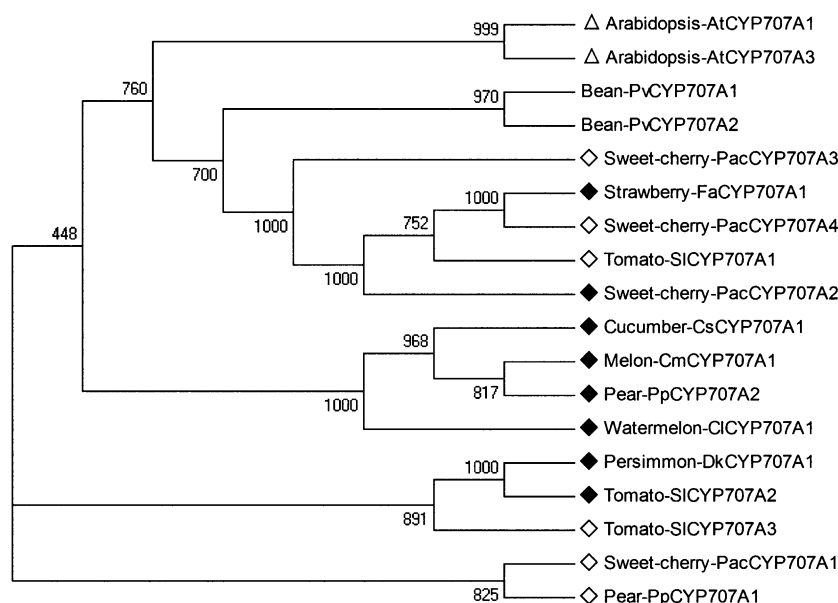


Figure 3. Phylogenetic tree of the *CYP707A* gene subfamily. A neighbor-joining phylogenetic tree was constructed using the ClustalX software and deduced protein sequences. Genes with high expression in fruits are marked with black diamonds, and genes showing low expression are marked with white diamonds. White triangles indicate genes in Arabidopsis. Branch lengths are proportional to the number of amino acid substitutions. All sequence data mentioned in this article can be found in GenBank, and accession numbers are shown in Supplemental Table S4. Different species are represented as follows: tomato (Sl), *SlCYP707A1*, *SlCYP707A2*, and *SlCYP707A3*; bean (*Phaseolus vulgaris* [Pv]), *PvCYP707A1* and *PvCYP707A2*; sweet cherry (Pac), *PacCYP707A1*, *PacCYP707A2*, *PacCYP707A3*, and *PacCYP707A4*; Arabidopsis (At), *AtCYP707A1* and *AtCYP707A3*; strawberry (Fa), *FaCYP707A1*; cucumber (Cs), *CsCYP707A1* and *CsCYP707A2*; persimmon (Dk), *DkCYP707A1*; watermelon (Cl), *ClCYP707A1* and *ClCYP707A2*; pear (Pp), *PpCYP707A1* and *PpCYP707A2*; muskmelon (Cm), *CmCYP707A1*.

(*SIPP2C* and *SIPP2C1*), and *SlSnRK2s* (*SlSnRK2C* and *SlSnRK2.3*). These genes generally have high expression in fruit (Sun et al., 2011), but in the transgenic fruits, we found that these genes were all up-regulated during fruit ripening (Fig. 4, E–J). This indicates that *SINCE1-RNAi* may influence ABA receptor activity and signal pathway sensibility, thereby affecting the physiological and biochemical processes involved in fruit ripening.

Phenotype of Fruit with Suppressed *SINCE1* Expression

To determine whether the changes in ABA levels affect fruit development, we next examined the phenotype of the transgenic fruit. The transgenic fruit was about 30% to 40% smaller by weight than the control fruit at harvest stage (Fig. 5A), but the total yields of the transgenic fruit and the control fruit were almost the same because the transgenic lines had more fruits per plant (Table I). The shelf life was measured mainly by fruit firmness, fruit weight loss, and fruit surface gloss. The firmness of the freshly picked fruit was about 50 Newton (N), and fruit with firmness of less than 8 N was considered to have lost its commercial value. Shelf life, therefore, was defined as the days required for the fruit firmness to gradually reduce from 50 to 8 N. When the transgenic fruits were harvested and placed in the laboratory, their shelf

life was 15 to 29 d, while the shelf life of the control fruit was only 5 to 7 d (Fig. 5B). Transgenic fruits were significantly firmer and more flexible than controls during the ripening stages after the mature green stage. *SINCE1-RNAi* fruits were firmer compared with control fruits, and the differences were statistically significant at the pink stage (Fig. 5C). Also, ethylene released by the transgenic fruit was higher than in the control fruit (Fig. 5D). The ripe transgenic fruits were deep red while the color of the typical control fruit was pink (Fig. 5, E–M), and the transgenic fruits had considerably fewer seeds. Seed number was approximately 140 to 180 in control fruit, but there were only four to 10 seeds in the transgenic fruits; however, these seeds could germinate normally and grow into normal tomato plants (Table I).

Transcriptional Regulation of Genes Involved in Cell Wall Catabolism in Fruits with Suppressed *SINCE1* Expression

The transgenic fruits with suppressed *SINCE1* showed substantial alterations in phenotype, including changes in firmness that likely result from changes in cell wall composition and structure. To examine the molecular causes of these alterations, we next measured the expression levels of genes involved in cell wall catabolism. Within the pectin catabolism gene

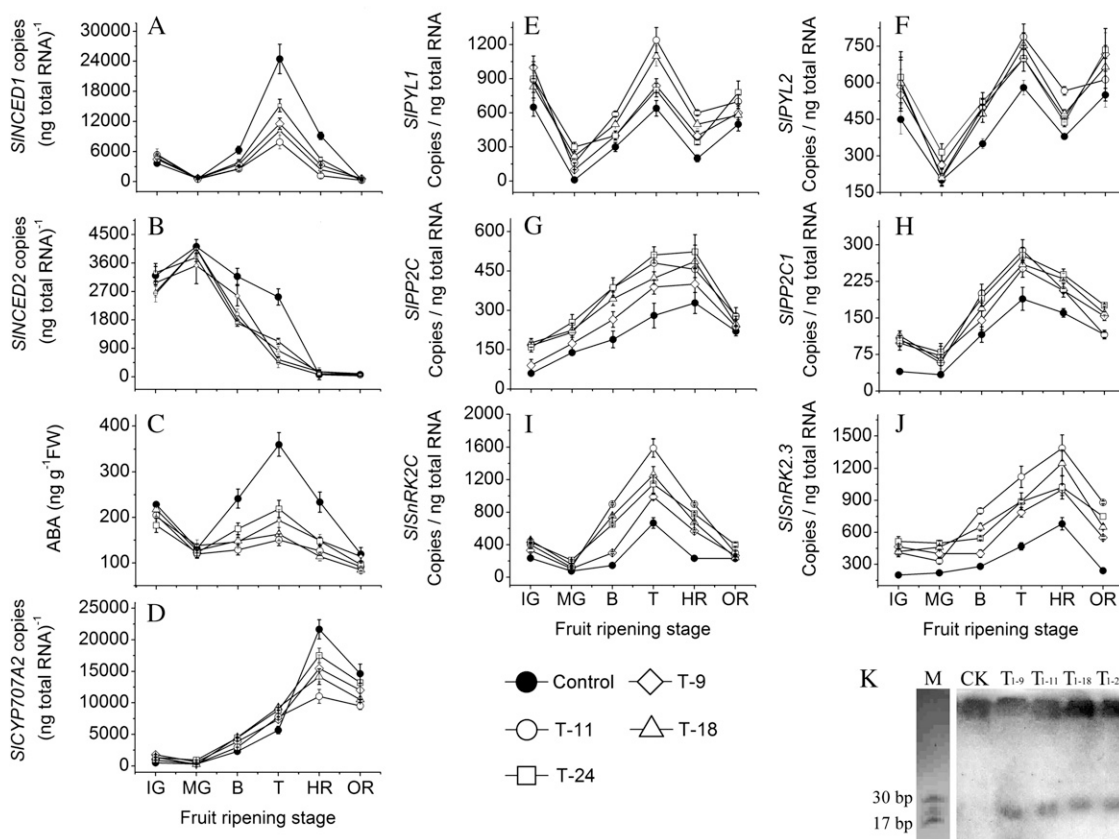


Figure 4. Suppression of *SINCED1* by RNAi alters the expression of genes. A, B, and D to J, Transcriptional regulation of nine genes involved in ABA metabolism and signaling was assessed in fruits from four independent transgenic plant lines (T1 generation) and control fruits using quantitative real-time PCR. During fruit development, tomato fruits were collected at the following times after anthesis (stages): 20 DAA (immature green [IG]), 35 DAA (mature green [MG]), 38 DAA (breaker [B]), 40 DAA (turning [T]), 42 DAA (pink [P]), 43 DAA (harvest red [HR]), and 45 DAA (over red [OR]). C, ABA content changes during transgenic and control fruit development. K, Detection of 25-bp siRNA that comes from degradation of the *SINCED1* gene. FW, Fresh weight; CK, control fruits.

family, polygalacturonase (*SIPG*), polygalacturonase isoenzyme 1 β -subunit (*SIPG- β*), pectin methylesterase (*SIPE* and *SIPMEU1*), tomato mRNA for pectin esterase clones 1 and 2 (*SIPEC1* and *SIPEC2*), and tomato *PG* found in leaf (*SITPGL*) are the major genes involved in the regulation of fruit cell wall catabolism (Smith et al., 1988; Giovannoni et al., 1989; Arrowsmith and de Silva, 1995; Brummell et al., 1999b, 2001; Cosgrove, 2000b; Powell et al., 2003; Flors et al., 2007). In the transgenic fruits, expression of *SIPG*, *SIPG- β* , *SIPMEU1*, and *SIPE* was strongly reduced during ripening compared with controls, which showed high expression levels during ripening (Fig. 6). By contrast, in control fruits, *SIPEC1*, *SIPEC2*, and *SITPGL* were expressed at low levels and changed little during fruit development. During ripening in the transgenic fruit, *SITPGL* was strongly down-regulated, while *SIPEC2* was slightly up-regulated (Fig. 6). Within the β -galactosidase precursor mRNA (*SITBG*) gene family, the expression of *SITBG5* to -7 increased after the mature green stage and peaked during the pink stage, while the expression of *SITBG1* to *SITBG4* changed

little during fruit development. *SITBG6* and *SITBG7* were strongly down-regulated in the transgenic lines, while there were no marked differences between control and RNAi lines for the other five *SITBG* genes. Within the xyloglucan endotransglycosylase (*XET*) gene family, expression of *SIXET16*, *SIXETm*, the tXET-B2 mRNA for xyloglucan endotransglycosylase (*SltXET-B1* and *SltXET-B2*), and the *XET* mRNA for endoxyloglucan transferase (*SIXET1*) was higher than for other members of the family, including *XET2*, *XET4*, and *XETB1*, during fruit ripening. Additionally, most *SIXETs* were down-regulated, while *XET2*, *XET4*, and *XETB1* changed little during fruit ripening, and there were no marked differences between control and transgenic fruits during fruit development. The expression of xyloglucan endotransglucosylase hydrolase (*SIXTH3* and *SIXTH9*) increased from the mature green stage and peaked during the pink stage, while these genes were strongly down-regulated in the transgenic fruits during ripening. Compared with other genes in the endo-1,4- β -glucanase precursor (*SICel4*) gene family, *SICel1* and *SICel2* were expressed

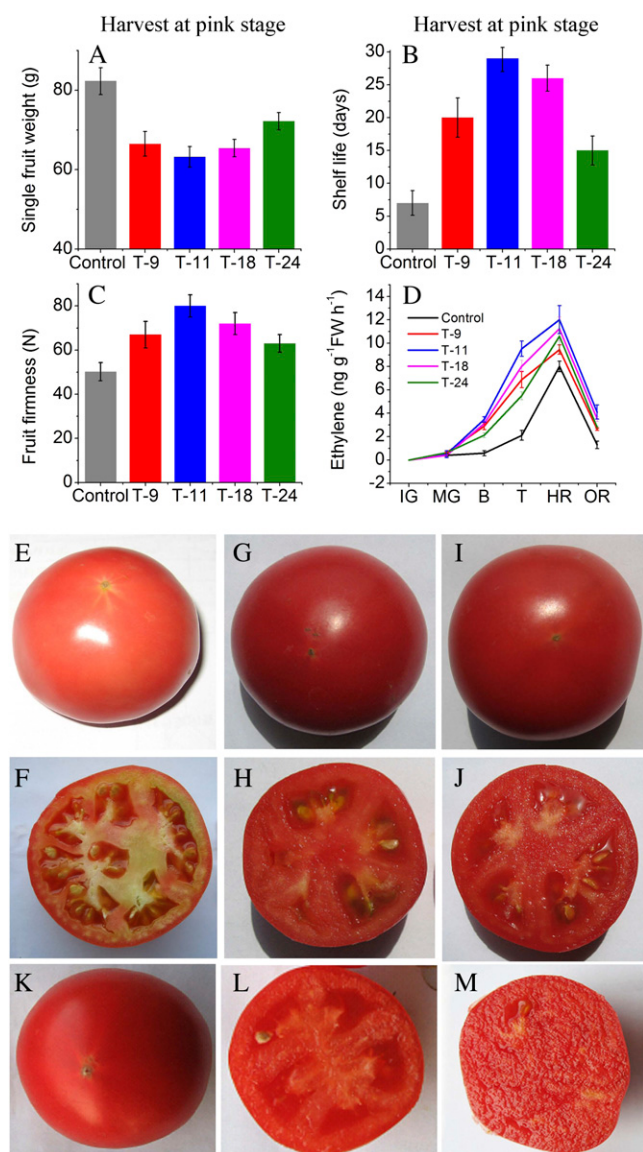


Figure 5. Ripening of transgenic tomato fruits with suppressed *SINCED1* gene expression. Four independent transgenic lines (T-9, T-11, T-18, and T-24) and control fruits were harvested at the pink stage (commercial maturity) in the greenhouse for three successive generations for the determination of fruit firmness, single fruit weight, and ethylene production. To study shelf life, fruit was stored for up to 4 weeks at 85% to 90% relative humidity and 20°C. Data shown are means of three replicates. Mean separation was tested by *t* test, with significance at the 5% level. A, Single fruit weight. B, Shelf life. C, Fruit firmness. D, Ethylene production. FW, Fresh weight. Fruit stages are as in Figure 4. E and F, Control fruits. G and H, Transgenic line 11. I and J, Transgenic line 18. K and L, Transgenic line 24. M, Transgenic line 9.

at high levels during ripening, but these were strongly down-regulated in transgenic fruits. Other genes, such as *SICel4* and *SICel5*, were expressed consistently at extremely low levels during all stages of maturity. The expression of expansin (*SIEXP1*) increased from the mature green stage and peaked at the pink stage, but it was down-regulated in the transgenic lines.

Effect of *SINCED1*-RNAi on the Physiology and Biochemistry of Tomato Fruit

To further examine the effect of alterations of ABA levels in developing tomato fruit, we next directly tested the effects on cell wall composition by measuring the levels of key cell wall components and the activities of key cell wall degradation enzymes in transgenic and control fruits. At the mature stage, the contents of water-soluble pectin, covalently bonded pectin, and ion-attached pectin in transgenic fruit were all significantly higher than in control fruit (Fig. 7A). However, the hemicellulose and cellulose contents of transgenic fruit were only slightly higher than control fruit at the mature stage (Fig. 7B). The viscosity of juice from the transgenic lines increased, as expected, as a function of ripening stage of the fruit at the breaker, turning, and pink stages, and this pattern was similar to that in control fruit (Fig. 7C). However, the viscosity of juices from the transgenic fruits was significantly greater than in the control fruit at the breaker, turning, and pink stages. The juice extraction rate in the control fruit was higher than that in the transgenic fruits at harvest stage (Fig. 7D). Moreover, during ripening, the activities of polygalacturonase (EC 3.2.1.15), pectin methylesterase (EC 3.1.1.11), and cellulase enzymes were reduced in the transgenic fruits (Fig. 7, E–G). The trait changes caused by RNAi in transgenic fruits were inherited stably in the next generation.

Effects of ABA, Ethephon, and 1-Methylcyclopropene on the Transcriptional Regulation of Cell Wall Catabolism Genes

To examine the interaction of ABA and ethylene in the regulation of genes encoding cell wall catabolic enzymes, we next treated control fruit with ethephon or 1-methylcyclopropene (1-MCP) in combination with ABA, to increase ethylene levels, and then examined gene expression. Exogenous ABA treatment markedly inhibited the expression of *SIPG*, *SIPME*, *SIXET16*, *SICels*, and *SIExp1* during the immature stages of fruit development, but it effectively promoted the expression of these genes in the turning stage; however, no significant differences were observed between control and treated fruit during the mature green stage (Fig. 8). By contrast, ethephon treatment promoted the expression of these genes during almost all of the developmental stages of the fruit. The differential effect of ABA on fruit ripening at different stages was further confirmed by measuring fruit firmness and sugar. These results confirmed that, among the diverse roles of ABA, ABA-induced fruit ripening occurs preferentially during the turning stage.

Effects of Exogenous ABA, Ethephon, and NDGA Treatment on Transgenic Fruit Ripening

We next examined the changes in fruit coloration after treatment with exogenous ABA, ethephon, or NDGA at 35 d after anthesis (DAA) on *SINCED1*-

Table 1. Seed numbers in control and *SiNCED1*-RNAi tomato fruits

The control fruit was from a nontransgenic plant. T-9, T-11, T-18, and T-24 were independent transgenic lines.

Fruit and Seed	Generation	Control	T-9	T-11	T-18	T-24
Average fruit number per plant (<i>n</i> = 10)	T0 generation (2007)	6	8	10	10	9
Average seed number per fruit (<i>n</i> = 10)		162	9	7	8	10
Average fruit number per plant (<i>n</i> = 10)	T1 generation (2008)	6	9	10	10	10
Average seed number per fruit (<i>n</i> = 10)		147	8	5	4	7
Average fruit number per plant (<i>n</i> = 10)	T2 generation (2009)	6	10	10	11	10
Average seed number per fruit (<i>n</i> = 10)		160	8	7	8	9

RNAi-24 plants (Fig. 9A). When transgenic fruits on tomato plants were treated with exogenous ABA, NDGA, or ethephon at the same time, the changes in fruit coloration induced by ABA or ethephon occurred at 38 DAA (Fig. 9B) or 40 DAA (Fig. 9C), and the changes in fruit coloration induced by distilled water

(control) and NDGA were delayed by 2 to 3 d at 42 and 43 DAA, respectively. Pectin content in transgenic fruits at 45 DAA was higher than in control fruit, but pectin content in the fruit treated with ABA or ethephon was higher than in the control but lower than in untreated RNAi fruit (Fig. 9G). The pectin content of

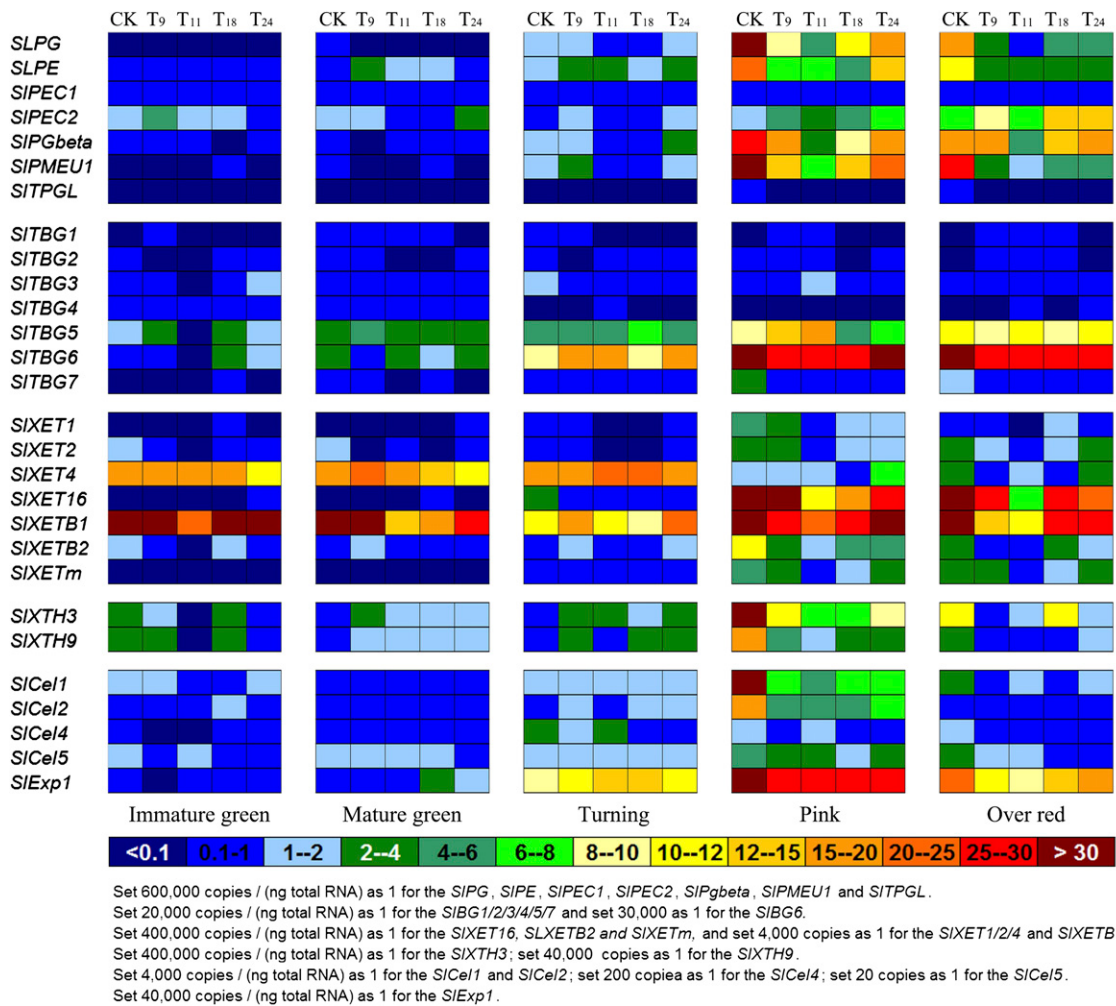


Figure 6. Transcriptional regulation of genes related to cell wall catabolism in control and transgenic fruits. During fruit development, tomato fruits were collected at the following times after anthesis (stages): 20 DAA (immature green), 35 DAA (mature green), 40 DAA (turning), 42 DAA (pink), and 45 DAA (over red). Data are from four independent transgenic lines (T1 generation) and control fruit. Expression of cell wall-degrading enzyme genes was detected by quantitative real-time PCR. All data are results of absolute quantitative expression analysis. The transcript level of the *SAND* gene was used as an internal loading control. CK, Control fruits.

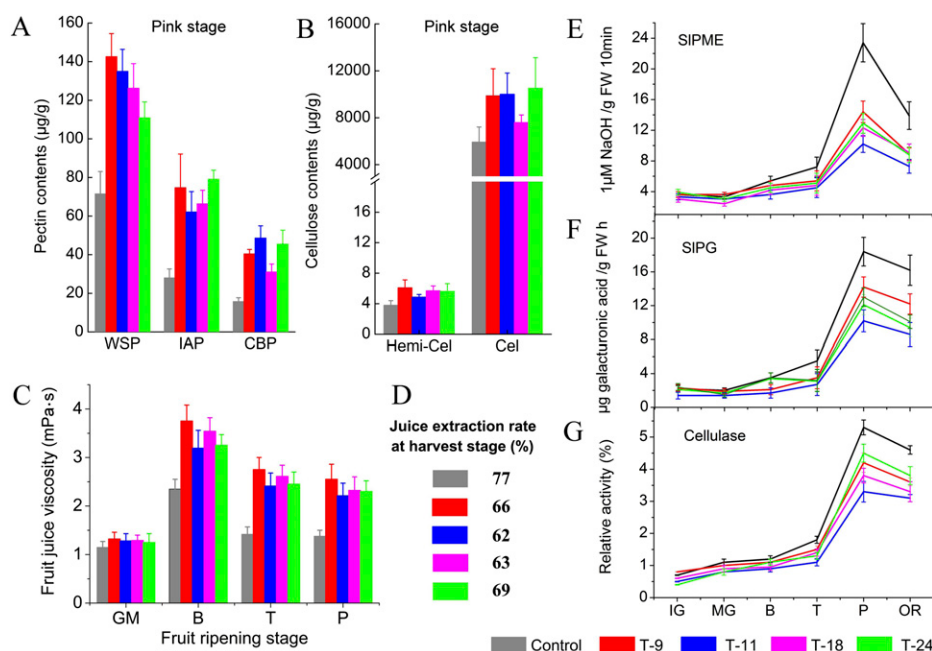


Figure 7. *SINCED1*-RNAi induced physiological and biochemical changes in tomato fruit. The contents of water-soluble pectin (WSP), ion-attached pectin (IAP), and covalently bonded pectin (CBP) in transgenic fruit are shown. A, Change in pectin contents, including WSP, IAP, and CBP. B, Changes in cellulose and hemicellulose contents. These data are from four independent transgenic plant lines of the T1 generation and control fruit. Data shown are means of three replicates. Mean separation was tested by *t* test, with significance at the 5% level. C and D, Juice viscosity (C) and percentage juice extraction (D). Juice was prepared using a winepress from fruit at the mature green (GM), breaker (B), turning (T), and pink (P) stages. A Bostwick consistometer was used to measure the flow properties of the juices. E to G, Activity of PG, PME, and cellulase enzymes in control and transgenic fruits during fruit development and ripening. FW, Fresh weight.

fruit treated with NDGA shows no significant change comparing with the *SINCED1*-RNAi fruit.

DISCUSSION

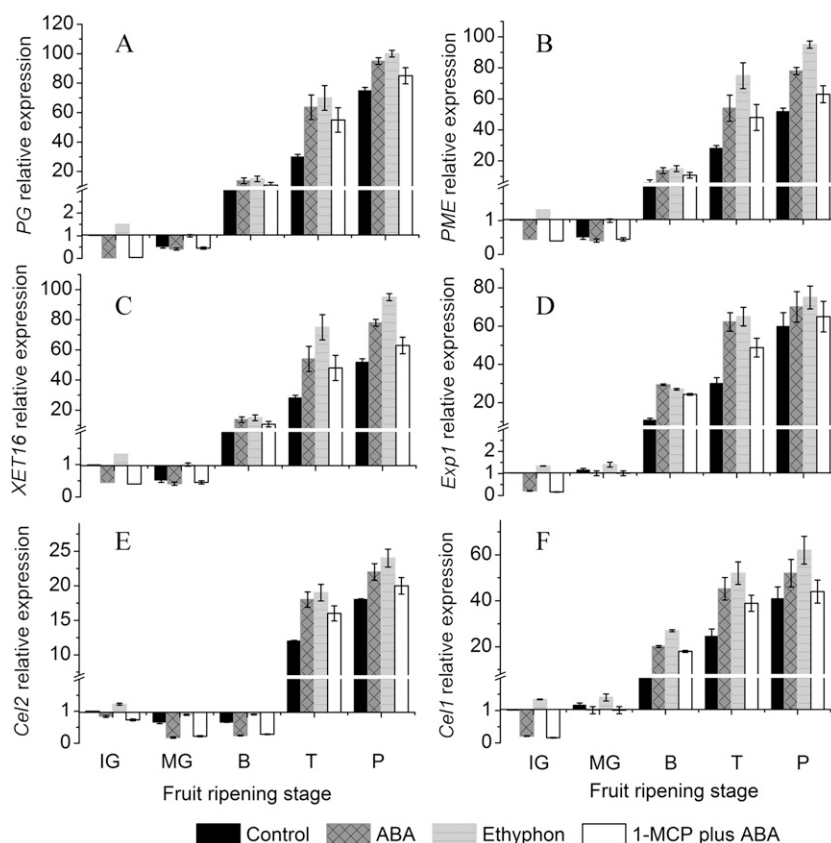
SINCED1-RNAi Affects Tomato Fruit Cell Wall Catabolism

The results of this study show that *SINCED1*-RNAi driven by *E8* effectively reduces the transcription of *SINCED1* and the accumulation of ABA in fruit, which affects several metabolic pathways of fruit ripening in a pleiotropic manner. Fruit ripening consists of a series of biochemical processes under the control of a complex combination of hormonal pathways. Cell wall catabolism is one of the major fruit ripening-related changes, and this involves the dismantling of multiple polysaccharide networks by diverse families of wall-modifying proteins, including enzymes for pectin and cellulose catabolism. The degradation of pectin and cellulose depends on ethylene during the softening of climacteric fruits (Huber, 1983; Hiwasa et al., 2003; Ergun et al., 2005; Nishiyama et al., 2007). Flores et al. (2001) observed that in 1-aminocyclopropane-1-carboxylic acid oxidase-suppressed transgenic melon fruit, softening was accelerated by the application of exogenous ethylene, but this continued, albeit at a

reduced rate, after the removal of the ethylene, suggesting that softening was only partially ethylene regulated. Recently, functional interaction and synergism between ABA and ethylene during the early stages of fruit ripening have been reported (Zhang et al., 2009a; Sun et al., 2010). Moreover, the application of exogenous ABA or NDGA stimulated or inhibited the disassembly of cell wall polymer networks (Fig. 9), indicating that these processes are ABA regulated and that the corresponding wall-modifying genes might be similarly regulated. In this study, we examined the expression patterns of major cell wall catabolism-associated genes in both transgenic and control fruits. As might be expected, most cell wall catabolism-associated genes, especially the major genes, were down-regulated in transgenic fruits during fruit development and ripening; thus, the products of these genes may affect the depolymerization or modification of cell wall polysaccharides. As a result, the transgenic fruit had a firmer texture and longer shelf life, but a higher ethylene content, which can cause normal ripening (Fig. 5, A–D).

Among cell wall catabolism enzymes, PG is an important pectin-modifying enzyme (Ortega-Regules et al., 2008; Koyama et al., 2010). The expression of *SIPG* in ripening tomato fruits (Brady, 1987; Dellapenna et al., 1989; Hadfield and Bennett, 1998) is accompa-

Figure 8. Effects of exogenous treatment with ABA, ethephon, and 1-MCP with ABA on the expression of genes encoding PG, PME, cellulase (Cel1 and Cel2), Exp1, and XET16. During fruit development, tomato fruits were collected at the following times after anthesis (stages): 20 DAA (immature green [IG]), 35 DAA (mature green [MG]), 38 DAA (breaker [B]), 40 DAA (turning [T]), and 42 DAA (pink [P]). Fruits were then divided into four groups at each sampling time and treated with ABA (0.1 mM for 30 min by soaking; group 1), ethephon (2 mM for 1 h by soaking; group 2), 1-MCP for 24 h followed by ABA (0.1 mM for 30 min by soaking; group 3), or untreated (control; group 4). Data are means $\pm$ SD for at least three to four sets of observations.



nied by increases in the expression of other proteins whose functions are to depolymerize or modify polysaccharides in the cell wall, such as PME (Harriman et al., 1991; Willats et al., 2001; Phan et al., 2007), endo-1,4- β -glucanase (Lashbrook et al., 1994; Brummell et al., 1997), XET (Arrowsmith and de Silva, 1995), β -galactosidases (Carey et al., 2001), β -Gal (Ranwala et al., 1992; Smith and Gross, 2000), and pectate lyases (Brummell and Harpster, 2001). Generally, the major wall pectin, homogalacturonan, is cleaved more readily by PG and pectate lyase after deesterification (Vicente et al., 2007) by PME (Tieman, and Handa, 1994). Expansins are cell wall proteins associated with numerous tissues and stages of development and changes in fruit size and shape (McQueen-Mason and Cosgrove, 1994; Brummell et al., 1999b; Cosgrove, 2000a, 2000b). The role of *Exp1* in fruit ripening has been investigated in tomato and strawberry (Rose et al., 1997; Brummell et al., 1999b; Civello et al., 1999; Cosgrove, 2000a, 2000b). Tomato fruit softness is reduced and firmness is increased dramatically by the simultaneous suppression of expression of both *SIPG* and *SIExp1* during ripening, compared with individual suppression of either *SIPG* or *SIExp1* (Powell et al., 2003; Cantu, et al., 2008a, 2008b). XET, an enzyme found originally in berry skin, is also involved in cell wall restructuring during fruit ripening (Deytieu et al., 2007; Negri et al., 2008). Similar results were also reported in transcriptomic studies (Glissant et al.,

2008) and transcript analysis of grape berries (Nunan et al., 2001; Ishimaru and Kobayashi, 2002). Our results support the correlation between solubilization of the xyloglucan fraction and fruit softening and further suggest that this aspect of wall disassembly is ABA regulated (Fig. 6). Meanwhile, β -1,4-endoglucanases and XTHs have been suggested to play a synergistic role in the restructuring of the cellulose-xyloglucan network (Rose and Bennett, 1999; Flors et al., 2007). In this study, each gene of the pectin-degrading family showed a distinct pattern of ABA regulation. These observations indicate that the expression of major genes involved in cell wall catabolism is dependent on ABA and suggest that they are primary candidates responsible for ABA-dependent pectin disassembly. By contrast, the expression of genes such as *SIPEC1* and *SIPEC2* was only slightly affected by transgenic suppression of *SINCE1*, indicating that the regulation of *SIPEC1* and *SIPEC2* expression is independent of ABA. Our data show that *SINCE1*-RNAi apparently increases pectin content via down-regulation of the expression of these genes (Fig. 6), meaning that ABA might directly regulate gene expression, thereby influencing fruit firmness and shelf life. The transgenic fruit was found to be smaller in size than control fruit, and the smaller fruit size may be related to the down-regulation of *SIExp1* during transgenic fruit ripening (Figs. 5 and 6). The impact of *SINCE1*-RNAi on the consistency of the juice prepared from the transgenic

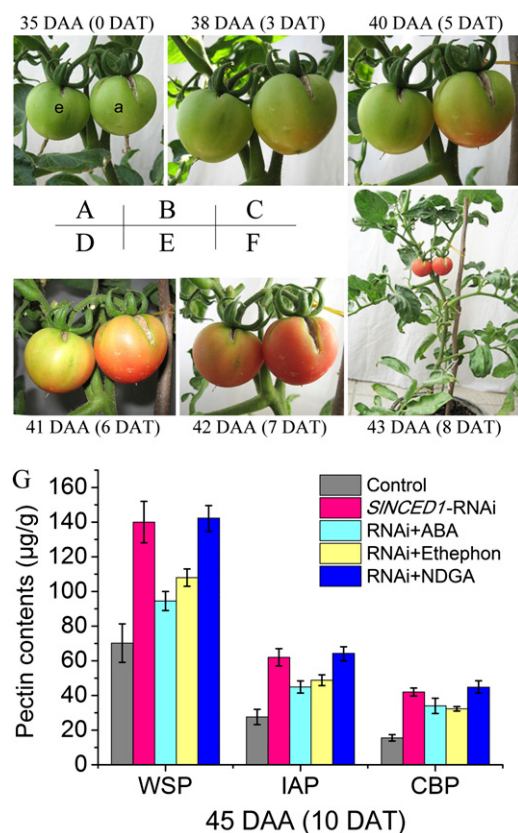


Figure 9. Effects of exogenous ABA, NDGA, and ethephon treatments on transgenic fruit ripening. The transgenic fruits (*SINCE1*-RNAi-24) were selected and tagged at 35 DAA. The fruits were each treated with 0.5 mL of ABA (100 μ M), ethephon (1 mM), or NDGA (100 μ M) solution by injection into the fruit from the pedicle with a medical injector. The control fruit was injected in 0.5 mL of distilled water. A, Zero days after treatment (DAT) with ABA or ethephon (a, ABA treatment; e, ethephon treatment). B to F, Three, 5, 6, 7, and 8 DAT with ABA (100 μ M) or ethephon (1 mM). G, Pectin contents of 45 DAA (10 DAT with ABA, ethephon, or NDGA). WSP, Water-soluble pectin; IAP, ion-attached pectin; CBP, covalently bonded pectin. Data are means $\pm$ SD ($n = 3$).

tomato lines agreed with earlier reports (Kalamaki et al., 2003; Powell et al., 2003).

The results of this study indicate that cell wall catabolism is a systematic process involving numerous enzymes and proteins that function with reciprocal and interdependent actions to complete this complex developmental procedure. Therefore, changing one or a few factors does not necessarily impact the whole system. However, it was possible to change this system of cell wall catabolism when multiple factors were changed simultaneously. Our results indicate that ABA is able to control the gene expression of the entire system of cell wall catabolism genes, meaning that the fine-tuning of a single factor can have a considerable impact on fruit texture. These findings provide an important framework for understanding the complex effects of ABA on fruit ripening.

Effect of Exogenous ABA and Ethylene on Tomato Fruit Softening

Exogenous ABA may affect the expression of *SIPG*, *SIPME*, *SIXET16*, *SICels*, and *SExp1* during the entire development and ripening process (Fig. 8), and the role of ABA varies with the stage of fruit development but not the ripening pattern. ABA and ethylene treatment markedly enhanced the expression levels of these six genes during the turning stage (Fig. 8), suggesting that these genes were not solely regulated by ethylene, since ABA was also able to enhance their expression. Thus, at least in part, ABA can induce cell wall catabolism in tomato fruit. However, ABA-induced ripening also requires other development-related factors, since the induction of *NCED* expression is not sufficient to lead to ripening in immature young fruit. Age-dependent functional specification has also been reported for ethylene-induced senescence (Grbi and Bleecker, 1995). Thus, age-dependent functional specification is a common method of functional diversification of these multifunctional hormones. It will be interesting to pinpoint the precise mechanisms that link the actions of these hormones to the different developmental stages of fruit.

Accumulating evidence also indicates that ABA indirectly participates in cell wall degradation by affecting the biosynthesis of ethylene (Fig. 5D). For example, *VpPME1* was up-regulated by ABA exposure in berries cultured in vitro, indicating a possible modification of pectin (Barnavon et al., 2001; Nunan et al., 2001; Deytieu-Belleau et al., 2008). One transcript up-regulated by ABA treatment encodes the grape-ripening-induced protein, GRIP13 (Davies and Robinson, 2000). These structural cell wall proteins are thought to be involved in providing additional support to the polysaccharide network in the cell walls by forming intermolecular cross-links. Thus, the differential transcript abundances of cell wall proteins, as well as pectin-modifying enzymes, may reflect the modification of cell walls occurring under ABA treatment.

In conclusion, our data provide direct evidence at the molecular level for a physiological role of ABA in cell wall catabolism related to fruit ripening. ABA directly participates in the cell wall catabolism involved in fruit ripening via the regulation of the expression of a suite of important genes during this process in tomato.

MATERIALS AND METHODS

Plant Materials and Gene Cloning

The *NCED* genes and the *CYP707A* genes were cloned by RT-PCR from cDNAs of fruit in tomato (*Solanum lycopersicum*), muskmelon (*Cucumis melo*), persimmon (*Diospyros kaki*), and pear (*Pyrus pyrifolia*) for climacteric fruit and in sweet cherry (*Prunus avium*), grape (*Vitis vinifera*), strawberry (*Fragaria ananassa*), and cucumber (*Cucumis sativus*) for nonclimacteric fruit.

Tomato plants from a selfed line (cv Jia Bao) were grown in natural light in a climate-controlled greenhouse at 25°C $\pm$ 2°C during the day and 18°C $\pm$ 2°C

during the night. During fruit development, fruits were sampled at the following times after anthesis (stages): 20 DAA (immature green), 35 DAA (mature green), 38 DAA (breaker), 40 DAA (turning), 42 DAA (pink), 43 DAA (harvest red), and 45 DAA (over red).

RNA Extraction, RT-PCR, and Sequencing

Total RNA was extracted from 1-g fruits using the hot borate method (Wan and Wilkins, 1994). Poly(A⁺) RNA was purified using oligo(dT)₃₀-latex (Takara) following the manufacturer's protocol. The synthesis of first-strand cDNA from the purified poly(A⁺) RNA was conducted using a Marathon cDNA Amplification Kit (Clontech). The cDNA of tomato flesh was used as a template for amplifying *NCEDs* with degenerate primers (forward, 5'-TTYGAYGGIGAYGGIATGGTICA-3'; reverse, 5'-TCCCAIGCRTTCCAIAARRT-GRAA-3') designed from the conserved sequences of plant *NCEDs*. To obtain the 3' nucleotide sequences, RACE-PCR was performed using the 3'-RACE System for RACE Kit (Invitrogen) according to the manufacturer's instructions. PCR was performed using the following conditions: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and with the final reaction being terminated at 72°C for an additional 10 min. The PCR products were sequenced by Invitrogen.

Phylogenetic Analysis

Deduced amino acid sequences of *SINCEdS* and *SICYP707As* were aligned with their homologs from other species using ClustalX 2.0.12 software with the default settings. The phylogenetic trees were constructed by using the neighbor-joining method in MEGA 4.0.2 software with the bootstrap analysis of 1,000 replicates for evaluating the reliability of different phylogenetic groups. Tree files were viewed and edited using MEGA 4.0.2. The alignment results were edited and marked using BOXSHADE 3.21 software (http://www.ch.embnet.org/software/BOX_form.html).

Cloning of the RNAi Expression Construct

PCR primers containing restriction enzyme sites at their 5' and 3' ends were designed based on sequences published in GenBank (Supplemental Table S1). Antisense and sense *SINCEd1* fragments were amplified from tomato fruit cDNA. The *E8* promoter was amplified from tomato genomic DNA. *GUS* and the Nos terminator were amplified from a plasmid including *GUS* and Nos terminator sequences. The PCR products were electrophoresed and inspected on a 1.0% agarose gel and were then purified using a DNA Gel Recovery Kit, ligated into a pGEM-T Easy vector, and subsequently transformed into *Escherichia coli* DH5α. The positive clones were then sequenced by Invitrogen. The *E8::antisense SINCEd1::GUS::sense SINCEd1::Nos-terminator* were cloned into pBluescript SK+ (Invitrogen) in that order to form a construct called pBlue-RNAi. Following identification of the correct clone, the RNAi expression cassette was excised from pBlue-RNAi and then inserted into *SacI*/*SalI*-digested pCambia1305.1 (Invitrogen). Sequencing of the construct verified that the pCambia1305.1-*SINCEd1* RNAi vector (pCAM-RNAi) was correctly constructed.

Transformation and Histochemical GUS Assay for Transgenic Tomato Plants

The recombinant plasmid pCAM-RNAi was introduced into *Agrobacterium tumefaciens* LBA4404, and the *SINCEd1*-RNAi construct was introduced into tomato plants via *A. tumefaciens* LBA4404-mediated transformation (Supplemental Figs. S1 and S3). The GUS assay was modified from that of Jefferson (1987). (The complete method is provided in Supplemental Fig. S2.)

Four independent *SINCEd1*-RNAi transgenic plant lines (*SINCEd1*-RNAi-9, *SINCEd1*-RNAi-11, *SINCEd1*-RNAi-18, and *SINCEd1*-RNAi-24) were obtained and evaluated after cultivation in the greenhouse for three successive generations. Each independent transgenic line included 15 plants. Thirty non-RNAi azygotes were used as controls. We carried out the research in a closed greenhouse to avoid genetic contamination; also, control and transgenic plants were segregated for the duration of flowering.

Real-Time Quantitative RT-PCR Analysis

Total RNA isolated from samples was assessed by agarose gel electrophoresis, and RNA samples that were found to contain genomic DNA were

treated with an RNase Free DNase I kit (Takara) according to the manufacturer's recommendations to eliminate the genomic DNA. First-strand cDNA synthesis was conducted using the PrimeScript RT reagent kit (Takara) from 1.0 μg of total RNA, and then the cDNA samples were diluted 3-fold. The sequences of the primer pairs used for each gene are shown in Supplemental Table S2. Real-time PCR was performed using the SYBR Premix Ex Taq kit (Takara). Reactions contained 0.8 μL of primer mix (containing 4 μM of each forward and reverse primer), 1.5 μL of cDNA template, 10 μL of SYBR Premix Ex Taq (2×) mix, and 7.7 μL of water for a total volume of 20 μL. Reactions were carried out under the following conditions: 95°C for 30 s (one cycle); 95°C for 15 s, 60°C for 20 s, and 72°C for 15 s (40 cycles), using a Rotor-Gene 3000 system (Corbett Research). The PCR product of each gene was confirmed by agarose gel electrophoresis and sequencing. The amplified fragment of each gene was subcloned and used to generate efficiency curves. Relative fold expression for each gene was calculated by Rotor-Gene 6.1.81 software using the "two standard curves method," in which the value of the young fruit stage (or lower value sample) was set at 1. The *SAND* gene (SGN-U316474), encoding a *SAND* family protein, was selected as an internal control gene according to Expósito-Rodríguez et al. (2008) and Sun et al. (2011).

Determination of Fruit Firmness

Fruits were harvested from all of the plants in each of three replicate plantings at the different ripening stages mentioned above. Fruits were harvested in multiple lots, and each lot contained at least 20 fruits of each genotype for comparison. Fruits harvested at the different ripening stages were stored at 24°C in trays lightly covered with plastic wrap for 24 h before fruit firmness was measured. Flesh firmness was measured after the removal of fruit skin on three sides of each fruit using a KM-model fruit hardness tester (Fujihara). The strength of flesh firmness was recorded in N. Compression of each fruit was measured three times, and the average of the maximum force was used.

Determination of Soluble Sugar Content

Ten grams of sample was ground into powder in a mortar and pestle with liquid nitrogen. A total of 0.5 g of powder and 10 mL of 80% (v/v) ethanol were mixed and incubated for 3 min at 80°C, centrifuged at 10,000g for 10 min, and the supernatant was collected into a 100-mL triangular flask. Ten milliliters of 80% (v/v) ethanol was added to the residues, and the extraction was repeated twice. The supernatants were pooled, and the remaining residue was washed and filtered with 1 mL of 80% (v/v) ethanol. The filtrate was moved into a 10-mL test tube, and two drops of 5% *N*-naphthol was added and mixed. Then, concentrated sulfuric acid was added slowly along the wall of the tube, until there was no purple ring in the layer, indicating that the sugar was completely extracted from the sample. The combined supernatants were evaporated in a boiling-water bath and washed twice with 20 mL of ultrapure water, made up to a volume of 50 mL, and 2 mL was removed for LC-18 solid-phase extraction. The soluble sugar content was determined by the anthrone colorimetric method (Laurentin and Edwards, 2003) using a SP-1900UV spectrophotometer (Ledon Technologies). Standard D-(+)-Glc (G8270-100G) was purchased from Sigma. The assays were repeated three times.

Shelf Life of the Transgenic Fruits

The transgenic and control fruits were harvested during the pink stage (commercial maturity), and these were then stored for up to 4 weeks at 85% to 90% relative humidity and 20°C. During storage, batches of 10 fruits per replicate sample (including three replicates per transgenic line) were used to determine shelf life mainly by fruit firmness, but also by weight loss, decay incidence, ethylene production, internal fruit quality, and ABA content. Shelf life was defined as the days required for the firmness of the fruit to decrease from 50 to 8 N. Each of the repeat samples was stored individually in plastic boxes, and all plastic boxes were maintained in the same location of the storage room to ensure exposure to identical conditions.

Juice Preparation and Consistency Determination

Control and transgenic fruits were collected at the mature green, breaker, pink, and red ripe stages, weighed, and chopped into quarters. Juice was extracted twice using a benchtop finisher (R30) with a screen size of 0.84 mm. Juice consistency was determined using a Brookfield viscometer (DV-II+PRO) at 20°C. Triplicate measurements were performed for each sample.

Northern-Blot Analysis of siRNAs

To clarify whether the observed changes were caused by *SINCE1*-RNAi, we performed a siRNA test. siRNA northern-blot analysis was performed according to the method of Hamilton and Baulcombe (1999). siRNA was extracted from 10 g of flesh using the miRcute miRNA isolation kit (Tiangen), and then this was fractionated in a 15% (w/v) polyacrylamide-urea gel and blotted onto a nylon membrane (Hybond N⁺; Amersham Biosciences). Then, the membrane was hybridized with the digoxigenin-labeled *SINCE1* cDNA fragment probe (using primers shown in Table I) in high-SDS buffer (7% [w/v] SDS, 5× SSC, 50 mM sodium phosphate [pH 7.0], 2% [w/v] blocking reagent, and 0.1% *N*-lauroylsarcosine) containing 50% (v/v) formamide overnight at 42°C. After hybridization, membranes were washed twice at 37°C in 2× SSC and 0.1% (w/v) SDS for 15 min and then twice at 55°C in 0.1× SSC and 0.1% (w/v) SDS for 30 min. The membranes were subjected to immunological detection according to the manufacturer's instructions using CDP-Star as the chemiluminescent substrate for alkaline phosphatase (Roche Diagnostics).

Enzyme Assays for PG, PME, and Cellulase

Assays for PG (EC 3.2.1.15), PME (EC 3.1.1.11), and cellulase were conducted according to previously published methods (Gross, 1982; Jen and Robinson, 1984; Wegrzyn and MacRae, 1992; Andrews and Li, 1995). (The complete protocol is provided in the Supplemental Data section of enzyme assay of PG, PME, and cellulase.)

Cell Wall Material Extraction, Separation, and Determination

Cell wall material was extracted according to the method described by Huber (1983). Separation was performed according to the method described by Siddiqui et al. (1996). Two grams of pulp was ground with liquid nitrogen, and the powders were put into 50-mL centrifuge tubes containing 20 mL of 80% methanol (v/v) and then boiled for 30 min in a water bath. Then, the extract was centrifuged at 8,000 rpm min⁻¹ for 10 min, and the supernatant was discarded. Absolute ethyl alcohol (20 mL) and 20 mL of anhydrous acetone were added separately to the residue. This was stirred and centrifuged, and thus rough cell wall materials were obtained. After this, 15 mL of 90% dimethyl sulfoxide was added for 15 h before centrifugation. The supernatant was discarded, while the residue (cell wall materials) was dried to constant weight.

Then, 10 mL of 50 mmol L⁻¹ sodium acetate (pH 6.5) was added to the cell wall materials, and this was stirred for 10 min and centrifuged. The resulting supernatant contained the water-soluble pectin. After this, 10 mL of 50 mmol L⁻¹ sodium acetate containing 50 mmol L⁻¹ EDTA was added to the residue, resuspended for 10 min, and centrifuged. The resulting supernatant contained the ion-associated pectin. Next, 10 mL of 50 mmol L⁻¹ Na₂CO₃ was added to the residue, and this was again resuspended for 10 min completely and centrifuged. This time, the supernatant contained the covalently bonded pectin. Finally, 10 mL of 0.4 M KOH (containing 1% [w/v] sodium borohydride) was added to the residue, and this was resuspended for 10 min and centrifuged. The supernatant contained hemicellulose. The remaining substances were washed twice (once for 10 min) with distilled water and dried to constant weight; in this way, the cellulose was obtained.

The water-soluble pectin, ion-associated pectin, and covalently bonded pectin were determined by carbazole colorimetry assay (Matsushashi and Hatanaka, 1992) using GalUA as a standard. Hemicellulose content was determined by the anthrone colorimetric assay with Suc as the standard (Dische, 1962). The content of cell wall material was determined using a spectrophotometer (SP-1900UV; Ledon Technologies).

ABA Analysis

For ABA extraction, 1.0 g of pulp was ground in a mortar and homogenized in extraction solution (80% methanol [v/v]). Extracts were centrifuged at 10,000g for 20 min. The supernatant was eluted through a Sep-Pak C₁₈ cartridge (Waters) to remove polar compounds and then stored at -20°C for ELISA. The stepwise procedure for the indirect ELISA of ABA was as follows. Each well of a microtiter plate had been precoated with ABA-bovine serum albumin conjugate diluted in coating buffer by the manufacturer (China Agricultural University). To each well was added 50 μL of standard or sample

in assay buffer (8.0 g of NaCl, 0.2 g of KH₂PO₄, 2.96 g of Na₂HPO₄·12H₂O, 1.0 mL of Tween 20, and 1.0 g of gelatin, added to 1,000 mL of water) followed by 50 μL of ABA antibody (Invitrogen) diluted 1:2,000 in assay buffer. The plates were incubated for 0.5 h at 37°C and then washed four times with scrubbing buffer (the same was done with assay buffer, but there was no gelatin). Anti-mouse IgG coupled to alkaline phosphatase (100 μL of a 1:1,000 dilution) was added to each well, and the plates were incubated for 0.5 h at 37°C. The plates were washed as above, and then 100 μL of a 1 to 2 mg mL⁻¹ solution of *o*-phenylenediamine substrate and 0.04% (v/v) of 30% (v/v) hydrogen peroxide in substrate buffer (5.10 g of C₆H₈O₇·H₂O, 18.43 g of Na₂HPO₄·12H₂O, and 1.0 mL of Tween 20, added to 1,000 mL of water) were added to each well. After several minutes, 50 μL of 2.0 mol L⁻¹ H₂SO₄ was added to each well to terminate the reaction. The absorbance was read at 490 nm with a Thermo Electron (Labsystems) Multiskan MK3 (Pioneer). The concentration of ABA in a sample was calculated from Logit B/B₀-transformed standard curve data, where B and B₀ are the absorbance values in the presence and absence of the competing antigen, respectively.

Effect of Exogenous ABA, NDGA, and Ethephon Application on the Expression of Genes Related to Cell Wall Catabolism and Fruit Ripening

The fruits harvested at different stages were divided into four groups. The first and second groups were directly soaked in 100 μM ABA or 500 μM ethephon solution for 10 min, respectively. The third group was treated with 1-MCP plus ABA; for this, 1-MCP (5 μL L⁻¹) was applied by injecting a measured volume of stock gas into sealed glass jars with the fruits inside, and then after 24 h the fruits were treated with ABA. The remaining fruits were used as a control and were soaked in distilled water for 10 min. The expression of *SIPG*, *SIPME*, *SICels*, *SIXET16*, and *SIExp1* was detected by real-time PCR. Samples were taken only at 3 d after drug treatments. Pulp tissue was separated, frozen in liquid nitrogen, and stored at -80°C until use. All biochemical measurements were replicated using samples from separate experiments. Data are expressed as means ± SD of all replicates.

To compare the effects between ABA, NDGA, and ethephon on fruit ripening, the fruits on tomato plants (*SINCE1*-RNAi-24) were selected and tagged at 35 DAA. The fruits were each treated with 0.5 mL of ABA (100 μM), ethephon (1 mM), or NDGA (100 μM) solution by injection into the fruit from the pedicle with a medical injector. The control fruit was injected with 0.5 mL of distilled water. Data are means ± SD (*n* = 3).

Sequence data in this article can be found in the GenBank/EMBL data libraries according to the accession numbers mentioned in the article.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Construction of hairpin construct of the *SINCE1*-RNAi and genetic transformation of tomato.

Supplemental Figure S2. Histochemical GUS assay for transgenic tomato plants.

Supplemental Figure S3. Detection for transformed tomato plants.

Supplemental Figure S4. Similarity of NCEDs based on deduced amino acid sequences.

Supplemental Figure S5. Similarity of CYP707As based on deduced amino acid sequences.

Supplemental Figure S6. Expression of *NCED* genes in other species.

Supplemental Table S1. Primers used for amplification of genes.

Supplemental Table S2. Specific primers used for real-time PCR analysis.

Supplemental Table S3. NCED sequences used for phylogenetic tree analysis.

Supplemental Table S4. CYP707A sequences used for phylogenetic tree analysis.

Supplemental Table S5. Specific primers used for real-time PCR analysis of *NCED* genes in other species.

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LITERATURE CITED

- Andrews PK, Li S (1995) Cell wall hydrolytic enzyme activity during development of nonclimacteric sweet cherry (*Prunus avium* L.) fruit. *J Hortic Sci* **70**: 561–567
- Arrowsmith DA, de Silva J (1995) Characterisation of two tomato fruit-expressed cDNAs encoding xyloglucan endo-transglycosylase. *Plant Mol Biol* **28**: 391–403
- Ban T, Ishimaru M, Kobayashi S, Goto-Yamamoto SN, Horiuchi S (2003) Absciscic acid and 2,4-dichlorophenoxyacetic acid affect the expression of anthocyanin biosynthetic pathway genes in 'Kyoho' grape berries. *J Hortic Sci Biotechnol* **78**: 586–589
- Barnavon L, Doco T, Terrier N, Ageorges A, Romieu C, Pellerin P (2001) Involvement of pectin methyl-esterase during the ripening of grape berries: partial cDNA isolation, transcript expression and changes in the degree of methyl-esterification of cell wall pectins. *Phytochemistry* **58**: 693–701
- Barthe P, Garello G, Bianco-Trinchant J, le Page-Degivry MT (2000) Oxygen availability and ABA metabolism in *Fagus sylvatica* seeds. *Plant Growth Regul* **30**: 185–191
- Brady CJ (1987) Fruit ripening. *Annu Rev Plant Physiol* **38**: 155–178
- Brummell DA, Bird CR, Schuch W, Bennett AB (1997) An endo-1,4-beta-glucanase expressed at high levels in rapidly expanding tissues. *Plant Mol Biol* **33**: 87–95
- Brummell DA, Hall BD, Bennett AB (1999a) Antisense suppression of tomato endo-1,4-beta-glucanase Cel2 mRNA accumulation increases the force required to break fruit abscission zones but does not affect fruit softening. *Plant Mol Biol* **40**: 615–622
- Brummell DA, Harpster MH (2001) Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Mol Biol* **47**: 311–340
- Brummell DA, Harpster MH, Civello PM, Palys JM, Bennett AB, Dunsmuir P (1999b) Modification of expansin protein abundance in tomato fruit alters softening and cell wall polymer metabolism during ripening. *Plant Cell* **11**: 2203–2216
- Burbidge A, Grieve TM, Jackson A, Thompson A, McCarty DR, Taylor IB (1999) Characterization of the ABA-deficient tomato mutant *notabilis* and its relationship with maize *Vp14*. *Plant J* **17**: 427–431
- Cakir B, Agasse A, Gaillard C, Saumonneau A, Delrot S, Atanassova R (2003) A grape ASR protein involved in sugar and abscisic acid signaling. *Plant Cell* **15**: 2165–2180
- Cantu D, Vicente AR, Greve LC, Dewey FM, Bennett AB, Labavitch JM, Powell ALT (2008a) The intersection between cell wall disassembly, ripening, and fruit susceptibility to *Botrytis cinerea*. *Proc Natl Acad Sci USA* **105**: 859–864
- Cantu D, Vicente AR, Labavitch JM, Bennett AB, Powell ALT (2008b) Strangers in the matrix: plant cell walls and pathogen susceptibility. *Trends Plant Sci* **13**: 610–617
- Carey AT, Smith DL, Harrison E, Bird CR, Gross KC, Seymour GB, Tucker GA (2001) Down-regulation of a ripening-related β -galactosidase gene (TBG1) in transgenic tomato fruits. *J Exp Bot* **52**: 663–668
- Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J* **3**: 1–30
- Chernys JT, Zeevaert JAD (2000) Characterization of the 9-cis-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in avocado. *Plant Physiol* **124**: 343–353
- Civello PM, Powell ALT, Sabehat A, Bennett AB (1999) An expansin gene expressed in ripening strawberry fruit. *Plant Physiol* **121**: 1273–1280
- Cosgrove DJ (2000a) New genes and new biological roles for expansins. *Curr Opin Plant Biol* **3**: 73–78
- Cosgrove DJ (2000b) Loosening of plant cell walls by expansins. *Nature* **407**: 321–326
- Cui MM, Sun L, Wang CL, Zhao SL, Liu ST, Xu XQ, Wang YP, Ji K, Li P, Leng P (2010) Cloning and tissue expression of a new 9-cis-epoxycarotenoid dioxygenase gene *LeNCED2* from tomato fruit. *J China Agri Univ* **15**: 20–26
- Davies C, Robinson SP (2000) Differential screening indicates a dramatic change in mRNA profiles during grape berry ripening: cloning and characterization of cDNAs encoding putative cell wall and stress response proteins. *Plant Physiol* **122**: 803–812
- Dellapenna D, Lincoln JE, Fischer RL, Bennett AB (1989) Transcriptional analysis of polygalacturonase and other ripening associated genes in Rutgers, *rin*, *nor*, and *Nr* tomato fruit. *Plant Physiol* **90**: 1372–1377
- Deluc LG, Grimplet J, Wheatley MD, Tillett RL, Quilici DR, Osborne C, Schooley DA, Schlauch KA, Cushman JC, Cramer GR (2007) Transcriptomic and metabolite analyses of Cabernet Sauvignon grape berry development. *BMC Genomics* **8**: 429–471
- Deytieu C, Geny L, Lapailleurie D, Claverol S, Bonneau M, Donèche B (2007) Proteome analysis of grape skins during ripening. *J Exp Bot* **58**: 1851–1862
- Deytieu-Belleau C, Vallet A, Donèche B, Geny L (2008) Pectin methyl-esterase and polygalacturonase in the developing grape skin. *Plant Physiol Biochem* **46**: 638–646
- Dische Z (1962) Measurement of cell wall neutral sugars. In RL Whistler, ML Wolfrom, eds, *Methods in Carbohydrate Chemistry*. Academic Press, New York, pp 475–481
- Ergun M, Jeong JW, Huber DJ, Cantliffe DJ (2005) Suppression of ripening and softening of 'Galia' melons by 1-methylcyclopropene applied at pre-ripe or ripe stages of development. *HortScience* **40**: 170–175
- Expósito-Rodríguez M, Borges AA, Borges-Pérez A, Pérez JA (2008) Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. *BMC Plant Biol* **8**: 131–143
- Finkelstein RR (2006) Studies of abscisic acid perception finally flower. *Plant Cell* **18**: 786–791
- Fischer RL, Bennett AB (1991) Role of cell wall hydrolases in fruit ripening. *Annu Rev Plant Physiol Plant Mol Biol* **42**: 675–703
- Flores F, Romojaro F, Ben Amor M, Pech JC, Latche A (2001) Differential sensitivity to ethylene of the various ripening pathways of ethylene-suppressed cantaloupe melons. *Acta Hort* **553**: 151–153
- Flors V, Leyva MdeL, Vicedo B, Finiti I, Real MD, García-Agustín P, Bennett AB, González-Bosch C (2007) Absence of the endo- β -1,4-glucanases Cel1 and Cel2 reduces susceptibility to *Botrytis cinerea* in tomato. *Plant J* **52**: 1027–1040
- Galpaz N, Wang Q, Menda N, Zamir D, Hirschberg JP (2008) Absciscic acid deficiency in the tomato mutant *high-pigment 3* leading to increased plastid number and higher fruit lycopene content. *Plant J* **53**: 717–730
- Giovannoni JJ (2007) Fruit ripening mutants yield insights into ripening control. *Curr Opin Plant Biol* **10**: 283–289
- Giovannoni JJ, DellaPenna D, Bennett AB, Fischer RL (1989) Expression of a chimeric polygalacturonase gene in transgenic *rin* (ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening. *Plant Cell* **1**: 53–63
- Giribaldi M, Gény L, Delrot S, Schubert A (2010) Proteomic analysis of the effects of ABA treatments on ripening *Vitis vinifera* berries. *J Exp Bot* **61**: 2447–2458
- Glissant D, Dedaldechamp F, Delrot S (2008) Transcriptomic analysis of grape berry softening during ripening. *J Int Sci Vigne Vin* **42**: 1–13
- Grbi V, Bleecker AB (1995) Ethylene regulates the timing of leaf senescence in *Arabidopsis*. *Plant J* **8**: 595–602
- Gross KC (1982) A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide. *HortScience* **17**: 933–934
- Hadfield KA, Bennett AB (1998) Polygalacturonases: many genes in search of a function. *Plant Physiol* **117**: 337–343
- Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**: 950–952
- Harriman RW, Tieman DM, Handa AK (1991) Molecular cloning of tomato pectin methylesterase gene and its expression in Rutgers, ripening inhibitor, nonripening, and never ripe tomato fruits. *Plant Physiol* **97**: 80–87
- Hiwasa K, Kinugasa Y, Amano S, Hashimoto A, Nakano R, Inaba A, Kubo Y (2003) Ethylene is required for both the initiation and progression of softening in pear (*Pyrus communis* L.) fruit. *J Exp Bot* **54**: 771–779
- Hoth S, Morgante M, Sanchez JP, Hanafey MK, Tingey SV, Chua NH (2002) Genome-wide gene expression profiling in *Arabidopsis thaliana*

- reveals new targets of abscisic acid and largely impaired gene regulation in the *abi1-1* mutant. *J Cell Sci* **115**: 4891–4900
- Huber DJ (1983) Polyuronide degradation and hemicellulose modification on ripening tomato fruit. *J Am Soc Hortic Sci* **108**: 405–409
- Ishimaru M, Kobayashi S (2002) Expression of a xyloglucan endo-transglycosylase gene is closely related to grape berry softening. *Plant Sci* **162**: 621–628
- Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (2001) Regulation of drought tolerance by gene manipulation of 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. *Plant J* **27**: 325–333
- Iuchi S, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2000) A stress-inducible gene for 9-*cis*-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. *Plant Physiol* **123**: 553–562
- Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol Biol Rep* **5**: 387–405
- Jen JJ, Robinson ML (1984) Pectolytic enzymes in sweet bell peppers (*Capsicum annuum* L.). *J Food Sci* **49**: 1085–1087
- Jeong ST, Goto-Yamamoto N, Kobayashi S, Esaka M (2004) Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. *Plant Sci* **167**: 247–252
- Jiang Y, Joyce DC (2003) ABA effects on ethylene production, PAL activity, anthocyanin and phenolic contents of strawberry fruit. *J Plant Growth Regul* **39**: 171–174
- Kalamaki MS, Powell AL, Struijs K, Labavitch JM, Reid DS, Bennett AB (2003) Transgenic overexpression of expansin influences particle size distribution and improves viscosity of tomato juice and paste. *J Agric Food Chem* **51**: 7465–7471
- Kondo S, Ponrod W, Kanlayanarat S, Hirai N (2002) Abscisic acid metabolism during fruit development and maturation of mangosteens. *J Am Soc Hortic Sci* **127**: 737–741
- Koyama K, Sadamatsu K, Goto-Yamamoto N (2010) Abscisic acid stimulated ripening and gene expression in berry skins of the Cabernet Sauvignon grape. *Funct Integr Genomics* **10**: 367–381
- Kramer M, Sanders R, Sheehy R, Melis M, Kuehn M, Hiatt W (1990) Field evaluation of tomatoes with reduced polygalacturonase by antisense RNA. In A Bennett, S O'Neill, eds, *Horticultural Biotechnology*. Liss, New York, pp 347–355
- Lacampagne S, Gagne S, Geny L (2009) Involvement of abscisic acid in controlling the proanthocyanidin biosynthesis pathway in grape skin: new elements regarding the regulation of tanning composition and leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) activities and expression. *J Plant Growth Regul* **29**: 81–90
- Lashbrook CC, Giovannoni JJ, Hall BD, Fischer RL, Bennett AB (1998) Transgenic analysis of tomato endo-beta-1,4-glucanase gene function: role of cell in floral abscission. *Plant J* **13**: 303–310
- Lashbrook CC, Gonzalez-Bosch C, Bennett AB (1994) Two divergent endo-beta-1,4-glucanase genes exhibit overlapping expression in ripening fruit and abscising flowers. *Plant Cell* **6**: 1485–1493
- Laurentin A, Edwards CA (2003) A microtiter modification of the anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates. *Anal Biochem* **315**: 143–145
- Leung J, Giraudat J (1998) Abscisic acid signal transduction. *Annu Rev Plant Physiol Plant Mol Biol* **49**: 199–222
- Li Q, Li P, Sun L, Wang YP, Ji K, Sun YF, Dai SJ, Chen P, Duan CR, Leng P (2011) Expression analysis of β -glucosidase genes that regulate abscisic acid homeostasis during watermelon (*Citrullus lanatus*) development and under stress conditions. *J Plant Physiol* **169**: 78–85
- Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**: 1064–1068
- Matsushashi S, Hatanaka C (1992) Difference between the free and conjugated galacturonate residues in their color reaction with carbazole or m-hydroxybiphenyl reagents. *Biosci Biotechnol Biochem* **56**: 1142–1143
- McQueen-Mason S, Cosgrove DJ (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. *Proc Natl Acad Sci USA* **91**: 6574–6578
- Melcher K, Ng LM, Zhou XE, Soon FF, Xu Y, Suino-Powell KM, Park SY, Weiner JJ, Fujii H, Chinnusamy V, et al (2009) A gate-latch-lock mechanism for hormone signalling by abscisic acid receptors. *Nature* **462**: 602–608
- Müller AH, Hansson M (2009) The barley magnesium chelatase 150-kD subunit is not an abscisic acid receptor. *Plant Physiol* **150**: 157–166
- Murayama H, Arikawa M, Sasaki Y, Dal Cin V, Mitauhashi W, Toyomasu T (2009) Effect of ethylene treatment on expression of polyuronide-modifying genes and solubilization of polyuronides during ripening in two peach cultivars having different softening characteristics. *Postharvest Biol Technol* **52**: 196–201
- Nambara E, Marion-Poll A (2003) ABA action and interactions in seeds. *Trends Plant Sci* **8**: 213–217
- Negri AS, Prinsi B, Rossoni M, Failla O, Scienza A, Cocucci M, Espen L (2008) Proteome changes in the skin of the grape cultivar Barbera among different stages of ripening. *BMC Genomics* **9**: 378–397
- Nishimura N, Hitomi K, Arvai AS, Rambo RP, Hitomi C, Cutler SR, Schroeder JI, Getzoff ED (2009) Structural mechanism of abscisic acid binding and signaling by dimeric PYR1. *Science* **326**: 1373–1379
- Nishiyama K, Guis M, Rose JKC, Kubo Y, Bennett KA, Wangjin L, Kato K, Ushijima K, Nakano R, Inaba A, et al (2007) Ethylene regulation of fruit softening and cell wall disassembly in Charentais melon. *J Exp Bot* **58**: 1281–1290
- Nunan KJ, Davies C, Robinson SP, Fincher GB (2001) Expression patterns of cell wall-modifying enzymes during grape berry development. *Planta* **214**: 257–264
- Ortega-Regules A, Ros-García JM, Bautista-Ortín AB, López-Roca JM, Gómez-Plaza E (2008) Changes in skin cell wall composition during the maturation of four premium wine grape varieties. *J Sci Food Agric* **88**: 420–428
- Pandey S, Nelson DC, Assmann SM (2009) Two novel GPCR-type G proteins are abscisic acid receptors in *Arabidopsis*. *Cell* **136**: 136–148
- Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TFF, et al (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**: 1068–1071
- Phan TD, Bo W, West G, Lycett GW, Tucker GA (2007) Silencing of the major salt-dependent isoform of pectinesterase in tomato alters fruit softening. *Plant Physiol* **144**: 1960–1967
- Powell ALT, Kalamaki MS, Kurien PA, Gurrieri S, Bennett AB (2003) Simultaneous transgenic suppression of LePG and LeExp1 influences fruit texture and juice viscosity in a fresh market tomato variety. *J Agric Food Chem* **51**: 7450–7455
- Qin XQ, Zeevaert JAD (1999) The 9-*cis*-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proc Natl Acad Sci USA* **96**: 15354–15361
- Ranwala AP, Suematsu C, Masuda H (1992) The role of β -galactosidases in the modification of cell wall components during muskmelon fruit ripening. *Plant Physiol* **100**: 1318–1325
- Ren J, Chen P, Dai SJ, Li P, Li Q, Ji K, Wang YP, Leng P (2011) Role of abscisic acid and ethylene in sweet cherry fruit maturation: molecular aspects. *NZ J Crop Hort* **39**: 1–14
- Ren J, Sun L, Wu JF, Zhao SL, Wang CL, Wang YP, Ji K, Leng P (2010) Cloning and expression analysis of cDNAs for ABA 8'-hydroxylase during sweet cherry fruit maturation and under stress conditions. *J Plant Physiol* **167**: 1486–1493
- Rodrigo MJ, Alquezar B, Zacarías L (2006) Cloning and characterization of two 9-*cis*-epoxycarotenoid dioxygenase genes, differentially regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis* L. Osbeck). *J Exp Bot* **57**: 633–643
- Rodrigo MJ, Marcos JF, Alférez F, Mallent MD, Zacarías L (2003) Characterization of Pinalate, a novel *Citrus sinensis* mutant with a fruit-specific alteration that results in yellow pigmentation and decreased ABA content. *J Exp Bot* **54**: 727–738
- Rose JKC, Bennett AB (1999) Cooperative disassembly of the cellulose-xyloglucan network of plant cell walls: parallels between cell expansion and fruit ripening. *Trends Plant Sci* **4**: 176–183
- Rose JKC, Lee HH, Bennett AB (1997) Expression of a divergent expansin gene is fruit-specific and ripening-regulated. *Proc Natl Acad Sci USA* **94**: 5955–5960
- Seki M, Umezawa T, Urano K, Shinozaki K (2007) Regulatory metabolic networks in drought stress responses. *Curr Opin Plant Biol* **10**: 296–302
- Sheehy RE, Kramer M, Hiatt WR (1988) Reduction of polygalacturonase activity in tomato fruit by antisense RNA. *Proc Natl Acad Sci USA* **85**: 8805–8809

- Shen YY, Wang XF, Wu FQ, Du SY, Cao Z, Shang Y, Wang XL, Peng CC, Yu XC, Zhu SY, et al (2006) The Mg-chelatase H subunit is an abscisic acid receptor. *Nature* **443**: 823–826
- Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. *J Exp Bot* **58**: 221–227
- Siddiqui S, Brackmann A, Streif J, Bangerth F (1996) Controlled atmosphere storage of apples: cell wall composition and fruit softening. *J Hortic Sci* **71**: 613–620
- Smith CJS, Watson CF, Ray J, Bird CR, Morris PC, Schuch W, Grierson D (1988) Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. *Nature* **334**: 724–726
- Smith DL, Gross KC (2000) A family of at least seven β -galactosidase genes is expressed during tomato fruit development. *Plant Physiol* **123**: 1173–1183
- Sun L, Wang YP, Chen P, Ren J, Ji K, Li Q, Li P, Dai SJ, Leng P (2011) Transcriptional regulation of *SIPYL*, *SIPP2C*, and *SISnRK2* gene families encoding ABA signal core components during tomato fruit development and drought stress. *J Exp Bot* **62**: 5659–5669
- Sun L, Zhang M, Ren J, Qi J, Zhang G, Leng P (2010) Reciprocity between abscisic acid and ethylene at the onset of berry ripening and after harvest. *BMC Plant Biol* **10**: 257–268
- Suzuki M, Ketterling MG, Li QB, McCarty DR (2003) Viviparous1 alters global gene expression patterns through regulation of abscisic acid signaling. *Plant Physiol* **132**: 1664–1677
- Tan BC, Schwartz SH, Zeevaart JAD, McCarty DR (1997) Genetic control of abscisic acid biosynthesis in maize. *Proc Natl Acad Sci USA* **94**: 12235–12240
- Taylor IB, Burbidge A, Thompson AJ (2000) Control of abscisic acid synthesis. *J Exp Bot* **51**: 1563–1574
- Tieman DM, Handa AK (1994) Reduction in pectin methylesterase activity modifies tissue integrity and cation levels in ripening tomato (*Lycopersicon esculentum* Mill.) fruits. *Plant Physiol* **106**: 429–436
- Tieman DM, Harriman RW, Ramamohan G, Handa AK (1992) An anti-sense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit. *Plant Cell* **4**: 667–679
- Vicente AR, Saladie M, Rose JKC, Labavitch JM (2007) The linkage between cell wall metabolism and fruit softening: looking to the future. *J Sci Food Agric* **87**: 1435–1448
- Wan CY, Wilkins TA (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal Biochem* **223**: 7–12
- Wegrzyn TF, MacRae EA (1992) Pectinesterase, polygalacturonase, and β -galactosidase during softening of ethylene-treated kiwifruit. *Hort-Science* **27**: 900–902
- Wheeler S, Loveys B, Ford C, Davies C (2009) The relationship between the expression of abscisic acid biosynthesis genes, accumulation of abscisic acid and the promotion of *Vitis vinifera* L. berry ripening by abscisic acid. *Aust J Grape Wine Res* **15**: 195–204
- Willats WGT, McCartney L, Mackie W, Knox JP (2001) Pectin: cell biology and prospects for functional analysis. *Plant Mol Biol* **47**: 9–27
- Wu FQ, Xin Q, Cao Z, Liu ZQ, Du SY, Mei C, Zhao CX, Wang XF, Shang Y, Jiang T, et al (2009) The magnesium-chelatase H subunit binds abscisic acid and functions in abscisic acid signaling: new evidence in Arabidopsis. *Plant Physiol* **150**: 1940–1954
- Zeevaart JAD (1999) Abscisic acid metabolism and its regulation. In G Bernardi, ed, *Biochemistry and Molecular Biology of Plant Hormones*. Elsevier Science, Amsterdam, pp 189–207
- Zeevaart JAD, Creelman RA (1998) Metabolism and physiology of abscisic acid. *Annu Rev Plant Physiol Plant Mol Biol* **39**: 439–473
- Zhang M, Yuan B, Leng P (2009a) The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *J Exp Bot* **60**: 1579–1588