Suppression of 9-cis-Epoxycarotenoid Dioxygenase, Which Encodes a Key Enzyme in Abscisic Acid Biosynthesis, Alters Fruit Texture in Transgenic Tomato¹[W][OA]

Liang Sun², Yufei Sun², Mei Zhang², Ling Wang, Jie Ren, Mengmeng Cui, Yanping Wang, Kai Ji, Ping Li, Qian Li, Pei Chen, Shengjie Dai, Chaorui Duan, Yan Wu, and Ping Leng*

College of Agriculture and Biotechnology, China Agricultural University, Beijing 100193, China (L.S., Y.S., L.W., J.R., M.C., Y.W., K.J., P.Li, Q.L., P.C., S.D., C.D., Y.W., P.Le.); and Department of Biochemistry and Molecular Biology, School of Life Sciences, Peking University, Beijing 100871, China (M.Z.)

Cell wall catabolism during fruit ripening is under complex control and is key for fruit quality and shelf life. To examine the role of abscisic acid (ABA) in tomato (Solanum lycopersicum) fruit ripening, we suppressed SNCED1, which encodes 9-cis-epoxycarotenoid dioxygenase (NCED), a key enzyme in the biosynthesis of ABA. To suppress SNCED1 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 SNCED1 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 (SlPG), pectin methyl esterase (SlPM), β-galactosidase precursor mRNA (SlTBG), xylanogucan endotransglycosylase (SixET), endo-1,4-beta-cellulose (SlCels), and expansin (SlExp). 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 (SlPG, SlPM, SixET, SlCels, and SlExp).

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² These authors contributed equally to the article.
* Corresponding author; e-mail pleng@cau.edu.cn

The plant hormone abscisic 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.
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 Zeexaat, 2000; Ban et al., 2003; Čakir 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 (Carpena 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-1,4-

regulate the SINCED1 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 (SINCED1/2) and ABA catabolism (SICYP707A1/2/3). Expression levels of two NCEDs (SINCED1 and SINCED2) and three CYP707AAs (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 SINCED1 (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 SINCED2 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 overred stage, while the expression of SICYP707A1 and SICYP707A3 was very low throughout fruit development (Fig. 1E). Because SINCED1 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 SINCED1 and -2, 64.09% between CsNCED1 and -2, 70.47% between DrNCED1 and -2, 75.41% between FaNCED2 and -3, 64.08% between

...the role of ABA specifically during fruit ripening.
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 SlNCED1, 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 SlNCED2 (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 ClNCED4 (Li et al., 2011), which were expressed at lower levels at the immature stage and then increased continually during fruit development. A third gene expression pattern was observed for genes including ClNCED4 (Li et al., 2011), which were expressed at lower levels at the immature stage and then increased continually during fruit development.

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 ClCYP707A1 in watermelon (Citrullus 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).
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 SiNCED1 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 SiNCED1. 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 SiNCED1 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 SiNCED1-RNAi construct. The RNAi hairpin structure was made up of an antisense/sense fragment of 427 bp from a conserved segment of SiNCED1. Because of the high protein sequence identity between SiNCED1 and SiNCED2, we examined the effects of this construct on both SiNCED1 and SiNCED2. Four independent transgenic lines were generated, and all the RNAi lines showed normal development and fruiting, similar to the control fruit. Four SiNCED1-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 SiNCED1 levels were down-regulated throughout fruit development (Fig. 4A). Indeed, we also found SiNCED1 small interfering RNAs (siRNAs; Fig. 4K), indicating that silencing was effective in these plants. At the same time, we also found that SiNCED2 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...
(SLPP2C and SLPP2C1), 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 SlNCED1-RNAi may influence ABA receptor activity and signal pathway sensitivity, thereby affecting the physiological and biochemical processes involved in fruit ripening.

**Phenotype of Fruit with Suppressed SINCED1 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. SINCED1-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 SINCED1 Expression**

The transgenic fruits with suppressed SINCED1 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 family, we measured the expression levels of genes involved in pectin catabolism in fruits with suppressed SlNCED1 expression.
family, polygalacturonase (SlPG), polygalacturonase isoenzyme 1 β-subunit (SlPG-β), pectin methylesterase (SlPE and SlPMEU1), tomato mRNA for pectin esterase clones 1 and 2 (SlPEC1 and SlPEC2), and tomato PG found in leaf (SlTPGL) 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 SlPG, SlPG-β, SlPMEU1, and SlPE was strongly reduced during ripening compared with controls, which showed high expression levels during ripening (Fig. 6). By contrast, in control fruits, SlPEC1, SlPEC2, and SlTPGL were expressed at low levels and changed little during fruit development. During ripening in the transgenic fruit, SlTPGL was strongly down-regulated, while SlPEC2 was slightly up-regulated (Fig. 6). Within the β-galactosidase precursor mRNA (SlTBG) gene family, the expression of SlTBG5 to -7 increased after the mature green stage and peaked during the pink stage, while the expression of SlTBG1 to SlTBG4 changed little during fruit development. SlTBG6 and SlTBG7 were strongly down-regulated in the transgenic lines, while there were no marked differences between control and RNAi lines for the other five SlTBG genes. Within the xyloglucan endotransglycosylase (XET) gene family, expression of SIXET16, SIXETm, the XET-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 hydro-lase (SIexTH3 and SIexTH9) 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 little during fruit development.
at high levels during ripening, but these were strongly down-regulated in transgenic fruits. Other genes, such as SlCel4 and SlCel5, were expressed consistently at extremely low levels during all stages of maturity. The expression of expansin (SlEXP1) increased from the mature green stage and peaked at the pink stage, but it was down-regulated in the transgenic lines.

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 SlPG, SlPME, SlXET16, SlCels, and SlExp1 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 SlNCED1-RNAi transgenic lines.
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

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 (n = 10) | T0 generation (2007) | 6 | 8 | 10 | 10 | 9 |
| Average seed number per fruit (n = 10) | T0 generation (2007) | 162 | 9 | 7 | 8 | 10 |
| Average fruit number per plant (n = 10) | T1 generation (2008) | 6 | 9 | 10 | 10 | 10 |
| Average seed number per fruit (n = 10) | T1 generation (2008) | 147 | 8 | 5 | 4 | 7 |
| Average fruit number per plant (n = 10) | T2 generation (2009) | 6 | 10 | 10 | 11 | 10 |
| Average seed number per fruit (n = 10) | T2 generation (2009) | 160 | 8 | 7 | 8 | 9 |

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.
DISCUSSION

SlNCED1-RNAi Affects Tomato Fruit Cell Wall Catabolism

The results of this study show that SlNCED1-RNAi driven by E8 effectively reduces the transcription of SlNCED1 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-amincyclopropane-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-
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-glucananase (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 expression of both SlPG and SlExp1 during ripening, compared with individual suppression of either SlPG or SlExp1 (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 (Deytieux 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 SlNCED1, indicating that the regulation of SIPEC1 and SIPEC2 expression is independent of ABA. Our data show that SINCED1-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 SlExp1 during transgenic fruit ripening (Figs. 5 and 6). The impact of SINCED1-RNAi on the consistency of the juice prepared from the transgenic

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 ± SD for at least three to four sets of observations.
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 SlPG, SlPME, SlXET16, SlCels, and SlExp1 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 (Grbić and Bleeker, 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, VvPME1 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; Deytieux-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 ± 2°C during the day and 18°C ± 2°C at night.
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), 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)20-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′-TGYAGGCGAAGGATGATGCA-3′; reverse, 5′-TCCACGCRCTCCAAHRTR- 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 NCEDs and SICYP70/As were aligned with their homologues from other species using ClustalX 2.012 software with the default settings. The phylogenetic trees were constructed by using the neighbor-joining method in MEGA 4.02 software with the bootstrap analysis of 1,000 replicates for evaluating the reliability of different phylogenetic groups. Tree files were viewed and edited 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 SlNCED1 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 then purified using a DNA Gel Recovery Kit, ligated into a pGEM-T Easy vector, and subsequently transformed into Escherichia coli DH5a. The positive clones were then sequenced by Invitrogen. The E8-antisense SINCEI-D:GUS-sense SINCEI-D: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-SINCEI 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 SINCEI-RNAi construct was introduced into tomato plants via A. tumefaciens LBA4404-mediated transformation (Supplemental Figs. S1 and S2). The GUS assay was modified from that of Jefferson (1987). (The complete method is provided in Supplemental Fig. S2.) Four independent SINCEI-RNAi transgenic plant lines (SINCEI-RNAi-9, SINCEI-RNAi-11, SINCEI-RNAi-18, and SINCEI-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), 3.5 μL of CDNA template, 10 μL of SYBR Premix Ex Taq (2×), 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 6.18 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 Espósito-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 SiNCED1-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% (v/v) polyacrylamide-urea gel and blotted onto a nylon membrane (Hybond N⁺; Amersham Biosciences). Then, the membrane was hybridized with the digoxigenin-labeled SiNCED1 DNA fragment probe (using primers shown in Table I) in high-salt buffer (7% [w/v] SDS, 5× SSC, 50 mM sodium phosphate [pH 7.0], 2% [v/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 Cellulose

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; Wegryn 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]. Extracts were centrifuged (10,000 g, 10 min). 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 the mixture was stirred for 10 min. The supernatant containing the water-soluble pectin was removed. Then, 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 (Matsushita and Hatanaka, 1992) using GalUA as a standard. Hemicellulose content was determined by the anthrone colorimetric assay with Suc as the standard (Doche, 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 washing buffer (the same was done with assay buffer, but there was no gelatin). Anti-mouse IgG coupled to alkaline phosphatase (100 µL of a 1:3,000 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 CH₃HO·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 antigens, 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 ethylene 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 SlPG, SlPME, Sicel5, SIXET16, and SlEXP1 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 ± se of all replicates.

To compare the effects between ABA, NDGA, and ethephon on fruit ripening, the fruits on tomato plants (SiNCED1-RNAi) 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 ± se (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 SiNCED1-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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