Effect of Dropping on *Le-ACS2* Accumulation Around the Mechanically Stressed Site of the Tomato Fruit

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ABSTRACT. Ethylene production and the accumulation of the 1-aminocyclopropane-1-carboxylic acid synthase (ACS; EC 4.4.1.14) gene were determined in tomato (*Solanum lycopersicum* L.) fruit that were dropped from a height of 5 cm. Dropped fruit had higher ethylene production than nondropped controls, and this lasted for at least 10 h. Maximum accumulation of *Le-ACS2*, one of the members of the *Le-ACS* multigene family, was achieved 5 h after dropping, and changes in accumulation tracked closely with ethylene production. In comparison with control fruit, substantial accumulation of *Le-ACS1A*, *Le-ACS4*, or *Le-ACS6* in dropped fruit was not observed. These results indicated that the increased ethylene production following fruit dropping was most likely regulated by *Le-ACS2* transcripts. The transfer of dropping stimuli from directly stressed tissues was investigated by measuring *Le-ACS2* accumulation at various positions on the dropped fruit. *Le-ACS2* was mainly induced in the fruit pericarp, and there was low accumulation in the fruit interior. The *Le-ACS2* accumulation linearly decreased with increasing distance along the pericarp from the stressed site. This implied that accumulation of *Le-ACS2* was dependent on stress levels, while most ethylene that was derived from dropping was produced at the stressed site. Using levels of *Le-ACS2* accumulation, the ethylene production of tomato fruit at mechanically impacted sites was estimated to be about 50 times higher than that of nondropped controls.

Ethylene is important in plant growth, including germination through seedling, flowering, and fruit development to senescence (Abeles, 1973). Ethylene production is enhanced by stimuli such as infection, mechanical stress, pollination, ripening, high temperature, hypoxia, noxious chemicals, auxins, and leaf and organ senescence (Diaz et al., 2002; Peng et al., 2005; Yang and Hoffman, 1984). Ethylene is biosynthesized from methionine via *S*-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid. ACC synthase (ACS) catalyzes the conversion of *S*-adenosylmethionine to ACC, and ACC is then oxidized to ethylene, which is catalyzed by ACC oxidase (ACO; EC 1.4.3). ACS catalyzes the rate-limiting step for ethylene production in more complex plants (Yang and Hoffman, 1984). This enzyme was first identified in tomato tissue (Boller et al., 1979; Yu et al., 1979) and is encoded by a multigene family (Rottmann et al., 1991). Northern blot analysis indicated that ACS activity was closely related to the level of its mRNA accumulation (Dong et al., 1991; Olson et al., 1991). Individual members of the *ACS* family are regulated differently in

response to environmental and endogenous cues and developmental changes (Owino et al., 2002; Yip et al., 1992).

Wounding is a severe stress for plants and can be caused by physical stresses, pathogen infection, or insect attack (Cheong et al., 2002). Wounded plants show protective reactions that aid healing of damaged tissues and activate defense mechanisms to prevent further damage (Leon et al., 2001). Ethylene has an important role as a wound signal mediator and induces wound-response genes that encode a proteinase inhibitor (O'Donnell et al., 1996), phenylalanine ammonia-lyase, and chitinase (Marcos et al., 2005). Therefore, previous studies have focused on the mechanism through which mechanical stress induces ethylene biosynthesis.

The induction of *ACS* gene expression from wounding is well recorded (Kende, 1993; Nakajima et al., 1990). Lincoln et al. (1993) observed that two members of the *Le-ACS* gene family (*Le-ACS2* and *Le-ACS4*) were induced by wounding in ripening tomato fruit, and that *Le-ACS2* accumulated more rapidly and to a greater extent than *Le-ACS4*. There was accumulation of *Le-ACS1A*, *Le-ACS2*, and *Le-ACS6* in tissues after wounding, but *Le-ACS2* did not accumulate in tissue that had only been touched (Tatsuki and Mori, 1999). Thus, induced

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ACS genes depend on the type of stress and/or stress levels. In most previous studies, slicing and/or touching were used for mechanical stress; however, the identity of the particular *ACS* gene that plays a role in ethylene biosynthesis caused by dropping as a mechanical stress has not yet been clarified. In addition, the changes in ethylene production and *ACS* accumulation around the stressed site itself have not been well investigated.

The objective of the present study was to clarify the relationship between *Le-ACS* (*Le-ACS1A*, *Le-ACS2*, *Le-ACS4*, and *Le-ACS6*) accumulation and ethylene biosynthesis caused by the dropping of a whole tomato fruit. To this end, *Le-ACS* accumulation levels were quantified by real-time polymerase chain reaction (PCR) analysis according to the distance from each impact site, and the transfer of mechanical stress from impact sites to other sites was investigated.

Materials and Methods

PLANT MATERIALS. Greenhouse-grown ‘Momotaro’ tomato fruit were obtained from a commercial farm in Tsukuba city (Ibaraki Prefecture, Japan). Fruit were harvested at the mature green stage and were immediately transferred to a constant temperature of 25 °C, where they were held overnight to equilibrate temperature.

APPLICATION OF MECHANICAL STRESS. Individual fruit were dropped 10 times from a height of 5 cm onto concrete, which caused no visible damage. The fruit-concrete impacts were conducted by the following two methods. In the first method, the impacts were evenly distributed (ED) over the fruit equatorial surface (on each fruit, a different part of the equatorial surface was struck with each of the 10 impacts) to investigate the relationship between *Le-ACS* accumulation and ethylene biosynthesis caused by dropping. The second method investigated changes in accumulation of *Le-ACS* at various distances from the impact sites. Mechanical impact (10 times as previously described) was locally applied to only one arbitrary site on the equatorial surface of the fruit; this method was denoted “locally distributed” (LD).

Immediately after ED or LD treatment, four sets of tomato fruit (each set contained three replicates; one fruit for one replicate) were separately placed into 2-L acrylic jars and were kept at 25 °C. Four sets of control fruit that had not been dropped were stored under the same conditions.

MEASUREMENT OF ETHYLENE PRODUCTION RATE. Before the ethylene production was measured, acrylic jars were tightly sealed from the start of storage until the measurements. Ethylene production rates were calculated from the increases in ethylene concentration inside the jars. Ethylene production rates of ED-treated fruit were determined at 0, 2, 5, and 10 h, and one set of tomato fruit was removed at each sampling time and was used for mRNA analysis. Ethylene production rates of LD-treated fruit were measured hourly for 3 h and were then used for mRNA analysis. The ethylene production rate was measured one time.

GAS ANALYSIS. Gas analysis was conducted with a gas chromatograph (model GC-8A; Shimadzu, Kyoto, Japan) fitted with a flame-ionization detector and a Porapak Q column (Nihon Waters, Tokyo). Column and detector temperatures were set at 140 °C and 160 °C, respectively. Helium was used as the carrier gas.

PREPARATION OF TISSUES USED FOR mRNA ANALYSIS. After each ethylene production rate measurement at 0, 2, 5, and 10 h, one set of fruit from the ED treatment and the control was removed, and tissues from the equatorial pericarp and the fruit center were sampled. The samples were immediately frozen with liquid nitrogen and stored at –80 °C until mRNA analysis.

The tissues for mRNA analysis of the LD treatment were prepared after the 3-h ethylene production measurement. The tissues were sampled at different distances from the impact site (Fig. 1); the impact site of pericarp (Pa), the pericarp 2 and 4 cm from the Pa (Pb and Pc, respectively), tissues from the fruit center (C), and from just below Pa (Ip). Pericarp tissues from the ED-treated and tissues from pericarp and fruit center of control fruit were prepared similarly for mRNA analysis.

ISOLATION OF TOTAL RNA AND REAL-TIME PCR. Total RNA was extracted from frozen tissues using the RNeasy Plant Mini Kit (Qiagen, Tokyo). The RNA samples were treated with RNase-free DNase (Qiagen) through column purification according to the manufacturer’s instructions. RNA content was quantified spectrophotometrically by measuring the A_{260} nm/ A_{280} nm ratio. The integrity of extracted RNA was electrophoretically analyzed on a 1.2% agarose gel with ethidium bromide staining. Following adjustment, 200 µg of RNA was reverse transcribed to synthesize cDNA using the ExScript RT reagent kit (TaKaRa Bio, Shiga, Japan). The 50-µL PCR reaction mixture consisted of 2 µL of cDNA, 2 µM primer, 13 µL of dH₂O, and 25 µL of Power Syber® Green PCR Master Mix (Applied Biosystems, Tokyo). *Le-ACS* mRNA was quantified three times, and the means and standard deviations were determined. To minimize the influence of differences in amplification efficiency, the amount of *Le-ACS* mRNA was normalized to 18S rRNA abundance. The real-time PCR primers were as follows:

Le-ACS1A (forward):
5’-TCATTGTTCTGAACCTGGTTGG-3’
Le-ACS1A (reverse):
5’-CCTTTGGCTGAAGCACAAAGT-3’
Le-ACS2 (forward):
5’-GAAGAATAATTTGAGACTTAGTTTTTCGAA-3’

Fig. 1. Cross-sectional diagram showing the location of samples taken from the tomato fruit for mRNA analysis. Pa = pericarp at stressed site; Pb and Pc = pericarp 2 and 4 cm away from Pa, respectively; C = tissues located at the center of the tomato fruit; Ip = location just below the pericarp.

Le-ACS2 (reverse):
 5'-AATTAAGTCTTAACGAACCTAATGGTGAGG-3'
Le-ACS4 (forward):
 5'-GGATTCGGATGTTTATGGATGC-3'
Le-ACS4 (reverse):
 5'-CATCGTACTCCCCATTTGAGGA-3'
Le-ACS6 (forward):
 5'-AGCTTAATGTTTCACCTGGTTGTTTC-3'
Le-ACS6 (reverse):
 5'-CTTCATTGTAGCATCATCCATATTAGC-3'
18S rRNA (forward):
 5'-TCCTAGTAAGCGCGAGTCATCA-3'
18S rRNA (reverse):
 5'-TCATTCAATCGGTAGGAGCGA-3'

DATA ANALYSIS. Statistical analysis of the accumulation of *Le-ACS2* after dropping the fruit was done using Tukey's test at 5% levels to test for significant differences.

Results

EFFECT OF DROPPING ON THE ETHYLENE PRODUCTION RATE AND *Le-ACS* ACCUMULATION. There was no immediate effect of ED treatment on ethylene production; however, the production rate had increased by 2 h and reached a peak after 5 h (Fig. 2). There was a subsequent decrease in ethylene production, but it did not return to control levels within 10 h. Ethylene production in control fruit remained constant.

Le-ACS1A expression changed little in the pericarp of ED fruit, while it was increased in control fruit at 5 h, peaking at 5 h (Fig. 3). In comparison with the pericarp, remarkably lower levels of *Le-ACS1A* accumulation were detected in the centers of control and ED fruit, and dropping did not affect *Le-ACS1A* accumulation. Enhanced *Le-ACS2* accumulation was observed in the pericarp of ED fruit within 2 h (Fig. 3), and a peak occurred after 5 h. The level of *Le-ACS2* subsequently decreased and reached the approximate basal level by 10 h. In controls, *Le-ACS2* expression levels were constant. In contrast to pericarp, dropping did not affect accumulation of *Le-ACS2* in the fruit center, where there was a consistently low level of accumulation. Accumulation of *Le-ACS4* was maintained at low levels in the pericarp and the center of dropped and control fruit. A slight increase in accumulation of *Le-ACS6* within 2 h and a subsequent return to basal levels was observed in the pericarp of ED-treated and control fruit (Fig. 3). In pericarp and the center of the fruit, no differences were observed in the pattern of *Le-ACS6* accumulation

Fig. 2. Ethylene production rate of dropped tomato fruit. Dropping impacts were evenly distributed over the equatorial surface of the fruit (▲). Data from untreated fruit were also indicated (□).

Fig. 3. Time courses of relative *Le-ACS* accumulation levels. Data were standardized with maximum accumulation as 100% and are represented as the means of three analyses with SD.

between ED fruit and controls. Thus, compared with control fruit, enhanced accumulation of *Le-ACS* family members in the pericarp by dropping was clearly observed only for *Le-ACS2*. Based on these data, only *Le-ACS2* was analyzed in the following experiment to investigate the transfer of mechanical stress from the points of impact to other sites.

RELATIONSHIP BETWEEN DISTANCE FROM IMPACT SITE AND *LE-ACS2* ACCUMULATION. Ethylene production rates of dropped fruit were markedly higher than that of controls 3 h after dropping (Fig. 4). There was no difference in ethylene production rate between ED fruit and controls within 1 h; however, a subsequent rise in ethylene production was observed in ED fruit, while most control fruit maintained constant values. No significant difference in ethylene production rate between ED fruit and LD fruit was detected after 2 h.

There were very low levels of *Le-ACS2* accumulation in the pericarp and the center of control fruit, and these values were not substantially different from each other (Fig. 5). However, *Le-ACS2* accumulation was enhanced in dropped fruit. In ED fruit, there was enhanced accumulation in the pericarp that reached about 20 times the control values. The highest level of *Le-ACS2* was at the local impact site, Pa (Fig. 1), which was about 28 times higher than controls. Accumulation levels tended to be lower with increasing distance along the pericarp from the impact site (Pb and Pc). Dropping did not affect accumulation of *Le-ACS2* in the fruit center. There was a relatively high accumulation just below the pericarp of LD fruit (Ip).

The accumulation of *Le-ACS2*, expressed as values relative to controls, linearly decreased with distance from the impact site, and the following regression equation was obtained with a very high correlation ($R^2 = 0.99$).

$$y = -7.20x + 31.6 \quad [1]$$

where x is the distance (centimeters) from impact site, and y (–) is the accumulation of *Le-ACS2* relative to controls.

Fig. 4. Changes in the ethylene production rate of tomato fruit after dropping. The impacts were evenly distributed over the equatorial surface of the fruit (■) or locally applied to an arbitrary point on the surface of the tomato fruit (▲). The ethylene production rate of untreated fruit is also indicated (□).

Fig. 5. Accumulation levels of *Le-ACS2* in untreated fruit (P = pericarp tissues, C = tissues located at the center of fruit), fruit after the “evenly distributed” (ED) treatment, and fruit after the “locally distributed” (LD) treatment (Pa = pericarp at stressed site; Pb and Pc = pericarp 2 and 4 cm away from Pa, respectively) 3 h after dropping. Data were standardized with maximum accumulation level as 100%, and means of three analyses are represented with SD. Columns with the same letter are not significantly different ($P > 0.05$).

Discussion

Dropping fruit increases the rate of ethylene production (Fig. 2), consistent with reports of increased ethylene production induced by mechanical stress (Kende and Boller, 1981; Watanabe and Sakai, 1998).

Enhanced *ACS* accumulation caused by mechanical stress has also been previously reported (Bekman et al., 2000; Botella et al., 1995). Oetiker et al. (1997) found that the *Le-ACS2* transcript level paralleled induction of ethylene synthesis, and Lincoln et al. (1993) found that *Le-ACS2* accumulated in wounded tissue, indicating that *Le-ACS2* is wound inducible. In the present study, a rapid and significant increase in *Le-ACS2* was detected in the pericarp of fruit dropped from only 5 cm (Fig. 3). While several *ACS* genes (*CMe-ACS2*, *VR-ACS1*, and *AT-ACS6*) have been reported to be touch induced (Arteca and Arteca, 1999; Botella et al., 1995; Ishiki et al., 2000; Yoon et al., 1997), Tatsuki and Mori (1999) did not detect accumulation of *Le-ACS2* when mature green tomato fruit were touched. However, *Le-ACS2* was induced in wounded fruit, suggesting that *Le-ACS2* accumulation required severe cell damage. In the present study, no visible damage in tomato fruit was observed after dropping, but there was probably sufficient damage to induce *Le-ACS2* accumulation.

In the pericarp of control fruit, *Le-ACS1A* accumulated, while levels in the dropped fruit changed very little (Fig. 3). It is difficult to explain this result; however, *Le-ACS1A* might have accumulated in response to touch to the fruit at the start of experiment. In combination with the remarkably low *Le-ACS1A* accumulation in the center of the fruit, it seemed that the *Le-ACS1A* accumulation did not correlate with the change in ethylene production caused by dropping. The accumulation of *Le-ACS4* found in this study was unaffected by dropping. This was similar to the reports of Tatsuki and Mori (1999) and Lincoln et al. (1993), which indicated that no accumulation of *Le-ACS4*

in mature green tomato fruit was detected after wounding. Enhancement in *Le-ACS6* accumulation was observed in pericarp of dropped fruit (Fig. 3). However, the increased accumulation was also detected in the pericarp of control fruit. Additionally, in the center of the fruit, no differences in *Le-ACS6* accumulation patterns between control and dropped fruit were observed. *Le-ACS6* was considered to be unrelated to the ethylene production caused by dropping. Thus, gene accumulation (except for *Le-ACS2*) was considered to be only slightly related to ethylene biosynthesis caused by dropping.

The dropping-induced change in *Le-ACS2* correlated well with ethylene production at 5 h (Figs. 2 and 3). This result indicated that enhanced ethylene production of dropped tomato fruit was at least partly regulated by *Le-ACS2* transcription. High *ACS* accumulation rapidly enhances the ACS level (Kato et al., 2000), which acts to increase ethylene production via ACC accumulation. However, there was a large discrepancy between *Le-ACS2* accumulation and ethylene production at 10 h; ethylene production was still high at 10 h, whereas *Le-ACS2* accumulation had reached about basal levels. This may have been due to a delay in ethylene release as it travels from the tomato tissue to the exterior. Additionally, there could be a large lag time between the accumulation of *Le-ACS2* in response to mechanical stress and the actual biosynthesis of ethylene.

When dropping impacts were applied to only one surface (LD), there was marked accumulation of *Le-ACS2* at the Pa site (Fig. 3). The tissue at this one site was more severely stressed than the others. In the pericarp, accumulation of *Le-ACS2* decreased with increasing distance from the impact site. Enhanced *Le-ACS2* expression at sites removed from the impact site (Pb and Pc) was probably caused by physical transmission of the dropping impact. In addition to the pericarp, a centrally directed transmission of mechanical stress is suggested because *Le-ACS2* was induced just below the pericarp (Ip). These results indicated that the accumulation level of *Le-ACS2* depended upon stress levels, and ACC accumulation and ethylene biosynthesis were mainly induced close to the impact site.

A linear relationship [Eq. 1] was obtained between accumulated levels of *Le-ACS2* and distance from the local impact site. In this equation, the *y*-value was the gene accumulation level relative to the control (namely ratio of gene accumulation level to that of the control), and the accumulation level equivalent for the control fruit was calculated by substituting 1 for the *y*-value. This calculation produced an *x*-value of 4.25 cm, indicating that the impact applied to one site could enhance *Le-ACS2* accumulation up to 4.25 cm away along the pericarp, although the estimation is extrapolated outside the range of actual data points. If the ethylene production rate was assumed to be proportional to the gene accumulation level, then ethylene production at the impact site would be 50 times higher than that of control fruit. Although LD-treated tomato fruit produced more ethylene, most of it was probably emitted directly from the local impact site.

In the present study, we investigated the *Le-ACS* genes that play an important role for ethylene biosynthesis caused by dropping. Compared with nondropped fruit, dropped fruit produced a higher accumulation level of *Le-ACS2* only. The results indicate that *Le-ACS2* accumulation potentially regulates the burst of ethylene production observed after dropping. In addition, the high accumulation level of *Le-ACS2* in locally impacted sites indicated that expression of *Le-ACS2* is regulated by tissue stress levels.

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