Changes in the Activity and Expression of 1-Aminocyclopropane-1-Carboxylate (ACC) Synthase, ACC Oxidase, and Phenylalanine Ammonia-lyase in Asparagus Spears in Response to Wound-induced Ethylene Synthesis

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Abstract. The molecular and enzymatic changes that follow harvesting of asparagus are important aspects for postharvest deterioration. To define the factors contributing to postharvest deterioration, the early changes in ethylene production and the activities and expression of 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACC oxidase, and phenylalanine ammonia-lyase (PAL) were studied in whole spears and in excised top and bottom portions. As a result of wounding, ethylene production was found higher in both top and bottom portions compared with whole spears but followed the same trend reaching the peak at 16 hours after harvest. ACC synthase was rapidly induced in excised top portions but no significant ACC synthase activity was observed in excised bottom portions. In top portions, ACC synthase reached a peak 8 hours after harvest and thereafter started to decline. In contrast to ACC synthase, ACC oxidase was induced markedly in both top and bottom portions and remained high until 16 hours after excision. On the other hand, PAL greatly increased in activity in bottom portions but not in excised tops. Northern blot analysis showed that increased mRNA levels coincided well with the excision-promoted increases in enzyme activity and ethylene production.

The plant hormone ethylene is produced in response to various kinds of environmental stresses, such as wounding, physical load, disease, exposure to low temperature and chemicals, and water stress (Hyodo, 1991). Ethylene is synthesized from methionine via S-adenosylmethionine (SAM) and ACC in higher plants (Adams and Yang, 1979). ACC synthase and ACC oxidase play essential roles in this pathway. ACC synthase catalyzes the conversion of SAM to ACC, whereas ACC oxidase catalyzes the oxidation of ACC to ethylene. The cloning and expression of ACC synthase and ACC oxidase genes has facilitated more biochemical and molecular studies of these enzymes. On the other hand, phenylalanine ammonia-lyase (PAL), the first enzyme in phenylpropanoid metabolism in

plants, catalyzes the elimination of ammonia from L-phenylalanine to form *trans*-cinnamic acid. PAL is induced by various kinds of stimuli including ethylene and wounding and regulates phenylpropanoid synthesis in lignification and flavonoid formation (Grisebach, 1981; Hanson and Havir, 1981).

As an initial step in elucidating the genetic response of asparagus spears to harvest, the changes in mRNA activity in tips of harvested spears were first investigated by King and Davies (1992). A harvest-induced ACC oxidase gene (pAS-ACO; AB083187) from tips of asparagus spears was reported recently, in which maximum pAS-ACO expression appeared after 3 d of harvesting and coincided well with ACC oxidase activity and ethylene production (Bhowmik et al., 2002). We have cloned and sequenced a cDNA for ACC synthase from asparagus spears. The 792-bp cDNA sequence for ACC synthase designated as pAS-ACS (AB111528) was highly homologous to other ACC synthase genes in the database. Recently we have also isolated an asparagus cDNA clone coding the enzyme PAL (pAS-PAL; AB102677) from a cDNA library prepared from asparagus spears (Bhowmik et al., 2003). The induction of PAL in both top and bottom portions of spears increased rapidly after harvest.

As the asparagus spears are not a large ethylene-producing commodity (Haard et

al., 1974; Hennion and Hartmann, 1990; Papadopoulou et al., 2001), very little research has been conducted on the rates of ethylene production and its deteriorative effects during storage. Haard et al. (1974) followed ethylene production by freshly harvested spears in relation to isoperoxidase changes. Hennion et al. (1992) indicated that postharvest lignification in etiolated asparagus spears is caused primarily by enhanced syringaldazine oxidase activity and ethylene is involved in the control of this activity, and Chang (1979) measured the ethylene accumulation by green and white asparagus during one day storage. Ethylene within the storage environment, whether produced by the stored product or other sources, is known to cause a significant stress to many harvested products. The hormone affects the rate of metabolism of many succulent plant products and is generally active at very low concentrations. In some recent studies on vegetables like broccoli, cabbage, carrots, and lettuce, a very small amount of ethylene has been shown to increase the rate of respiration, alter the activity of a number of enzymes, and increase membrane permeability (Wills et al., 1999). Cellular changes induced by ethylene result in acceleration of senescence and the deteriorative processes that accompany it.

We have previously constructed cDNA libraries from asparagus spears and isolated cDNA clones for ACC oxidase (pAS-ACO; AB083187), ACC synthase (pAS-ACS; AB111528) and PAL (pAS-PAL; AB102677). We report here the changes in these enzyme activities and the mechanisms by which these enzymes are induced and related to ethylene synthesis in response to wound stress.

Materials and Methods

Plant material. Green asparagus (*Asparagus officinalis* L. 'Welcome') spears were harvested at ~9 AM from Kagawa Agricultural Experiment Station, Kagawa, Japan, in July, 2002. Spears were immediately brought to the laboratory and washed and weighed. At ~9:30 AM the top and bottom portions were excised and incubated at 20 °C for 0, 8, 16, 24, and 48 h under humid and dark conditions. About 1 h passed between harvest and the time 0. After harvest we divided spears into segments to check and compare the changes in ethylene production and enzyme activities due to excision and also due to the differences in maturity level in top and bottom tissues. The term top and bottom refer to the apical and basal 20 mm of 250-mm spears, respectively. Three replications of seven spears were used in the experiment. After storage treatment, samples were immediately stored at –80 °C until needed for enzyme assay and RNA extraction.

Extraction and assay of ACC oxidase. Enzyme extraction was performed according to the method described by Fernandez-Maculet and Yang (1992). Two grams of the frozen tissues was added in liquid nitrogen and homogenized in a cooled mortar and pestle with 4 mL extraction buffer consisting of 0.1 M Tris-HCl, pH 7.2, 5 mM dithiothreitol, 30 mM Na-ascorbate, 10% glycerol (v/w), and 5%

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PVPP. The resulting homogenate was filtered through four layers of Kimwipes and the filtrate was centrifuged at 15,000 g_n for 30 min at 4 °C. The supernatant was used for the assay of ACC oxidase. Enzyme activity was assayed at 30 °C in 2-mL reaction mixtures containing 1.7 mL extraction buffer, 50 μ M FeSO₄, 1 mM ACC, and 0.2 mL crude extract.

Ethylene measurement. Ethylene produced in the 13.2-mL vial sealed with silicon rubber cap for 1 h at 20 °C was determined by gas chromatography. A 1-mL gas was sampled from the internal atmosphere with a plastic hypodermic syringe and injected into the gas chromatograph. The rate of ethylene production was expressed as microliters of ethylene per hour per kilogram fresh weight.

Extraction and assay of PAL activity. Two g of spear tissues were homogenized at 2 °C with a mortar and pestle in 10 mL of 0.1 M borate buffer, pH 8.8, and 1.0 g PVPP. A further 10 mL of the buffer was added to the homogenate, which was then centrifuged at 14,000 g_n for 20 min. The supernatant was used for enzymatic assays. Protein concentration was measured by Lowry (Lowry et al., 1951) method. PAL activity was determined spectrophotometrically by measuring the increase in A₂₉₀ due to the formation of *trans*-cinnamic acid. The reaction mixture consisted of 50 mM borate buffer, pH 8.8, 20 mM L-phenylalanine, and the enzyme preparation in a total volume of 3 mL. A sample without L-phenylalanine was used as a blank. The activity was expressed as nanomoles of *trans*-cinnamic acid formed per milligram of protein.

Extraction and assay of ACC synthase. ACC synthase extraction was carried out with 2 g of spear tissues that were homogenized in a mortar and pestle with 4 mL of extraction buffer consisting of 400 mM K-phosphate buffer, pH 8.5, 10 μ M pyridoxal phosphate, 0.5% mercaptoethanol, and 20% glycerol at 2 °C. The resulting homogenate was filtered through four layers of Kimwipes and the filtrate was charged on a dry Sephadex G-25 column (1.5 cm i.d. $\times$ 7 cm) prepared by centrifuging at 3,000 g_n for 3 min at 4 °C just before use. The eluent was re-centrifuged at 3,000 g_n for 5 min and was used for the enzyme assay, according to the method described by Lizada and Yang (1979).

RNA isolation. Total RNA was extracted according to the Hot Borate method of Wan and Wilkins (1994). Briefly, 3 g of frozen spear tips was ground to fine powder in liquid nitrogen with a prechilled mortar and pestle. The powder was mixed with 10.5 mL (at 3.5 mL g_n^{-1} tissue) of hot borate extraction (XT) buffer consisting of 0.2 M Na borate decahydrate, 30 mM EGTA, 1% (w/v) SDS, 10 mM dithiothreitol, 1% Nonidet P-40, and 2% (w/v) polyvinylpyrrolidone in a 50-mL conical tube. The homogenate was incubated with 315 μ L (at 105 μ L g_n^{-1} tissue) Proteinase K at 42 °C for 1.5 h and then with 840 μ L (at 280 μ L g_n^{-1} tissue) 2 M KCl on ice for 1 h. After centrifugation at 10,000 g_n for 20 min at 4 °C, 1/3 volume of 8 M LiCl was added to the supernatant to a final concentration of 2 M. The precipitate was collected by centrifugation at 10,000 g_n for 20 min at 4 °C and the pellets were dissolved in 2 mL of 10 mM Tris-HCl (pH

7.5) buffer and extracted with 2 M K-acetate (pH 5.5). The RNA in the supernatant was precipitated with 100% ethanol and collected by centrifugation at 9,000 g_n for 30 min. The RNA pellets were washed with 70% ethanol and resuspended in DEPC-treated water.

Amplification of poly (A)⁺ RNA for ACC synthase by reverse transcriptase-polymerase chain reaction (RT-PCR). The first strand cDNA was synthesized from 2 g of the total RNA by reverse transcriptase with Oligo-(dT) primer according to the instruction of SUPER SCRIPT preamplification system for first strand cDNA synthesis (GIBCOBRL, Tokyo, Japan). PCR was performed in a total volume of 25 μ L containing the first strand cDNA reaction products, 10 $\times$ PCR Buffer, MgCl₂, dNTP, First Start Taq DNA polymerase (Roche), and primers. The primers for PCR were synthesized to two amino acid domains conserved in various ACC synthase genes, IQMGLAE for the sense primer (5'-TYCARATGGGTCTHGC DGAA-3') and AGLFCWV for the antisense primer (5'-ACCCARCARAASARDCCNGC-3'), respectively. The *Sal* I and *Not* I restriction site sequences were also included at 5'-end of the sense and antisense primer to facilitate cloning of PCR product. The PCR procedure started with 10 min at 95 °C and was carried out 35 cycles of 30 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C, and 10 min at 72 °C with ASTEC program temperature control system PC-700. The PCR products were confirmed by agarose gel electrophoresis.

Cloning and sequencing of ACC synthase cDNA. The amplified cDNA was ligated to the plasmid pSPORT⁺ and cloned into *Escherichia coli* (DH-5 α) *Not* I-*Sal* I-cut (BRL, Tokyo, Japan). Sequencing was performed by the cycle sequencing method using GATC-Bio Cycle sequencing kit and a DNA sequencer GATC 1500 Long-Run system (GATC GmbH, Konstanz, Germany).

Sequence data analysis. Sequence analysis was performed using GENETYX-MAC Ver.7 computer software. Homology searches with the Genbank and the EMBL databases were

performed using the homology program in the software.

Preparation of the digoxigenin (DIG)-UTP-labeled RNA probe. The cloned RT-PCR product including the encoded region of ACC synthase gene was cleaved by *Not* I and *Sal* I from the pSPORT⁺ vector that had been amplified in *Escherichia coli* (DH-5 α) and it was purified and recovered by gel electrophoresis. Antisense DIG-labeled RNA probe was prepared according to the instructions of DIG RNA labeling kit (Boehringer Mannheim) using SP6 RNA polymerase.

Northern blot analysis. Ten micrograms of total RNA was subjected to electrophoresis on a 1.0% agarose (Type II) gel containing 20 $\times$ MOPS and 37% formaldehyde. After electrophoresis for 30 min, RNA was visualized with ethidium bromide under UV light to confirm equal loading of RNA in each lane. RNA was transferred to a positively charged nylon membrane Hybond-N⁺ (Amersham Pharmacia Biotech) by capillary action with 20 $\times$ SSC and then after drying the membrane RNA was fixed under UV. The membrane was prehybridized at 50 °C with 5 $\times$ SSPE, 5 $\times$ Denhart's solution, formamide and 10% SDS for 3 h. Hybridization was performed at 50 °C using the gene specific antisense DIG-labeled RNA probe for 24 h using the same prehybridization buffer. After hybridization, the membrane was washed twice with 2 $\times$ SSPE containing 0.1% SDS for 10 min at room temperature, once with 1 $\times$ SSPE containing 0.05% SDS for 15 min at 65 °C and once with 0.2 $\times$ SSPE for 10 min containing 0.05% SDS at 65 °C. The membrane was also washed with Buffer A containing maleic acid and Tween 20 at room temperature and was blocked with 2% blocking reagent in maleic acid buffer for 30 min. Subsequently, the membrane was incubated with Anti-Digoxigenin-AP, Fab fragments (Boehringer Mannheim), in the blocking buffer for 30 min. Signals were detected by color reaction using 5-bromo-4-chloro-3-indolyl-phosphate and 4-Nitro blue tetrazolium chloride as the substrate.

Fig. 1. Changes in ethylene production in excised top, bottom and intact spears. Each point represents the mean of three replications and bars show SD about mean.

(A)

(B)

Fig. 2. Changes in ACC synthase activity and expression of pAS-ACS gene in the excised top and bottom portions of asparagus spears. (A) Changes in ACC synthase activity. Each point represents the mean of three replications and bars show SD about mean. (B) Northern blot analysis for ACC synthase. Equal loading of RNA was confirmed by staining a gel with ethidium bromide.

Cloning and sequencing of cDNA, sequence data analysis, probe synthesis, and northern blotting were performed as for ACC oxidase and PAL as described in our previous reports (Bhowmik et al., 2002, 2003).

Statistics. A randomized complete block design was adopted with three replications. The level of significance was calculated from the F value of ANOVA.

Results

Wound-induced ethylene synthesis. Ethylene production was found higher in both top and bottom portions compared with whole spears (Fig. 1). The production rate in the top portions was 2 to 6 times as much as that in the bottom portion and the rate in bottom portions was 1 to 1.5 times greater than intact spears. Ethylene production was rapidly induced in

both top and bottom portions of asparagus spears after excision. In the top, the production rate increased rapidly after excision, reaching a peak at 16 h and thereafter started to decline. On the other hand, the ethylene production rate in bottom increased slowly to a peak at 16 h after excision. The increase in production rate for intact spears was not significant except reaching a peak at 16 h.

Wound-induced ACC synthase. ACC synthase activity increased in the top portions after excision, reaching a peak at 8 h and then declined sharply (Fig. 2A). In the bottom portions, there was no specific inclining or declining pattern of ACC synthase activity throughout the experimental period. cDNA for ACC synthase was amplified by RT-PCR and the resultant cDNA designated as pAS-ACS was cloned. The sequence of pAS-ACS having 792 bp was found to be highly homologous to

other ACC synthase genes, showing 89.2% homology with bamboo (Matsui et al., 2003) and 86.3% homology with banana (Liu et al., 1999). Therefore, pAS-ACS clone was used as a probe for northern blot analysis. In northern blot analysis, the levels of transcripts for ACC synthase increased rapidly in the top portion immediately after excision (Fig. 2B). Expression of ACC synthase gene in top portions remained clearly detectable until 8 h but it was barely detectable in the bottom portions, which explains the lower activity of ACC synthase in that portion.

Wound-induced ACC oxidase and PAL. In contrast to ACC synthase, ACC oxidase activity increased in both top and bottom portions and the increased activity was maintained until 16 h after excision (Fig. 3A). Expression of ACC oxidase gene was evident in both top and bottom portions (Fig. 3B). The ACC oxidase transcripts showed maximum expression until 16 h after excision and coincided well with the peak of ACC oxidase enzyme activity. Like ACC oxidase, the increase in PAL activity in both top and bottom portions also continued until 16 h after excision; thereafter the level of activity started to decline (Fig. 4A). Maximum pAS-PAL expression appeared 16 h after excision in the bottom portions and coincided with the peak of enzyme activity (Fig. 4B). In the top portions, however, significant pAS-PAL expression was not evident.

Discussion

Although asparagus is considered to be a low to medium ethylene-producing commodity (Haard et al., 1974; Hennion and Hartmann, 1990; Papadopoulou et al., 2001), the excised top and bottom portions of asparagus spears produced a significant amount of ethylene in response to excision. With a marked rise in ethylene production, ACC synthase activity was induced in the top portions and followed by a significant increase in the expression of ACC synthase gene (Fig. 1, 2A and B). In contrast to the top, the activity of ACC synthase in the bottom was too low to induce ethylene production. Thus, induction of ACC synthase is considered to be primarily important for wound-induced ethylene synthesis. This type of finding was also reported in other vegetables like bamboo shoot (Matsui et al., 2003), tomato (Tatsuki and Mori, 1999), and cucumber (Shiomi et al., 1998).

In contrast to ACC synthase, there was a marked increase in ACC oxidase mRNA and ACC oxidase activity of asparagus spears, suggesting well that the transcription of pAS-ACO led to the increase in wound-induced ACC oxidase activity. In both top and bottom portions, a marked increase in ACC oxidase suggests that ethylene production is not limited by ACC oxidase activity. ACC oxidase can be induced by various factors such as fruit ripening (Ross et al., 1992), auxin (Shiomi et al., 1998), wounding (Matsui et al., 2001; Kato et al., 2002), disease (Ohtsubo et al., 1999), and senescence of flower (Wang and Woodson, 1992) and vegetative tissues (Peck et al., 1993; Kim and Yang, 1994). Our result

(A)

(B)

Fig. 3 Changes in ACC oxidase activity and expression of pAS-ACO gene in the excised top and bottom portion of asparagus spears. (A) Changes in ACC oxidase activity. Each point represents the mean of three replications and bars show SD about mean. (B) Northern blot analysis for ACC oxidase. Equal loading of RNA was confirmed by staining a gel with ethidium bromide.

was consistent with that observed in wound-induced bamboo (Matsui et al., 2001), cucumber (Shimoi et al., 1998), pea (Peck et al., 1993), and broccoli (Kato et al., 2002). In those studies, results have been shown that suggest wound-induced ethylene participated in the regulation of ACC oxidase. However, in our previous experiment (Bhowmik et al., 2002), the time lag between wounding and ACC oxidase gene expression appeared to be longer than that of present study.

The development of PAL activity in asparagus spears was similar in a number of respects to other wound-induced tissue systems (Kato et al., 2000; Rubery and Fosket, 1969). There was an increase in PAL activity in both top and bottom portions of the spears that continued

for ~16 h. Thereafter PAL activity started to decline. Although PAL activity in both top and bottom portions followed almost the same pattern, the activity was higher in bottom than top portions. High PAL activity associated with the bottom portions, or tissue near to cut surfaces, such as we observed, has been noted previously (Engelsma, 1968; Goldstein et al., 1972). In spite of a higher rate of ethylene production in the top portions lower activity of PAL in the top suggests that the endogenous ethylene evolved is not directly related to wound-induced PAL in asparagus. Besides ethylene, some other factor(s) may also be involved for PAL activity. Further research is necessary to investigate the factor(s) controlling PAL activity in top portions of asparagus spears.

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(A)

(B)

Fig. 4 Changes in PAL activity and expression of pAS-PAL gene in the excised top and bottom portion of asparagus spears. (A) Changes in PAL activity. Each point represents the mean of three replications and bars show sd about mean. (B) Northern blot analysis for PAL. Equal loading of RNA was confirmed by staining a gel with ethidium bromide.

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