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Wound-Induced Ethylene Synthesis and Expression and Formation of 1-Aminocyclopropane-1-Carboxylate (ACC) Synthase, ACC Oxidase, Phenylalanine Ammonia-Lyase, and Peroxidase in Wounded Mesocarp Tissue of *Cucurbita maxima*

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1-Aminocyclopropane-1-carboxylate (ACC) synthase was rapidly induced in mesocarp tissue of *Cucurbita maxima* after wounding in the cut surface layer in 1 mm thickness (ca. 9 cells) (first layer) in both the enzyme activity and the levels of transcript. This led to a rapid accumulation of ACC and hence ethylene production. In the inside tissue (1–2 mm) (second layer), no significant induction of ACC synthase was observed, which resulted in a low level of ACC, although ethylene was evolved at a much lower rate than the first one. In contrast to ACC synthase, ACC oxidase was induced markedly in both the first and second layers and the development of its activity and the levels of mRNA remained high until later stages. It was considered that wound ethylene was closely associated with the development of ACC oxidase, since 2,5-norbornadiene (NBD), an inhibitor of ethylene action, substantially suppressed it. Phenylalanine ammonia-lyase (PAL) greatly increased in activity after wounding similarly to that of ACC synthase, in which increase in PAL activity occurred predominantly in the first layer. Induction of peroxidase activity after wounding had a close correlation in profile with that of ACC oxidase in that marked increases in the activity were observed in both the first and second layers and were strongly suppressed by NBD application. Four peroxidase isozymes were found by PAGE, among which a fraction was newly detected after wounding.

Key words: ACC oxidase — ACC synthase — *Cucurbita maxima* — Peroxidase — Phenylalanine ammonia-lyase — Wound-induced ethylene.

The plant hormone ethylene is produced in response to various kinds of environmental stress, 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, Yang and Hoffman 1984, Abeles et al. 1992). 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.

Mesocarp tissue of winter squash (*Cucurbita maxima*) fruit produces a large amount of ethylene in response to wounding (Hyodo et al. 1983), in which ACC synthase activity increased rapidly resulting in ACC accumulation to a greater extent. It was found that the induction of ACC synthase in the mesocarp tissue was suppressed by exogenous or endogenous ethylene (Hyodo et al. 1985, 1993). ACC synthase was purified to homogeneity from the wounded mesocarp tissue of winter squash fruit and its properties were characterized by Nakajima and Imaseki (1986). A cDNA for wound-induced ACC synthase of winter squash fruit was cloned and mRNA for ACC synthase was demonstrated to have accumulated after wound treatment (Nakajima et al. 1990). ACC oxidase activity was also induced in response to wounding, while its level was enhanced by ethylene in the wounded mesocarp tissue (Hyodo et al. 1993).

PAL is the first enzyme in the phenylpropanoids metabolism in plants. It 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 phenylpropanoids synthesis in lignification and flavanoids formation (Hanson and Havir 1981, Grisebach 1981). PAL transcripts increased in abundance in response to fruit ripening and wounding in melon (Diallinas and Kanellis 1994). In the mesocarp tissue of *Cucurbita maxima* PAL activity increased rapidly after wounding (Hyodo and Fujinami 1989).

Lignification is believed to play a role in defense response to wounding and disease by developing physical barriers in plants (Grisebach 1981). Peroxidase has been implicated in lignin formation at the step of polymerization of monolignols (Grisebach 1981). In the wounded meso-

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; DEPC, diethyl pyrocarbonate; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid); NBD, 2,5-norbornadiene; PAL, phenylalanine ammonia-lyase; SAM, *S*-adenosylmethionine.

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carp tissue of *Cucurbita maxima* peroxidase activity was significantly induced, which was suppressed by exposure to NBD (Hyodo et al. 1991), suggesting that ethylene is involved in the induction of peroxidase in this tissue.

In the present study, we investigated the mechanism of wound-induced ethylene production and increases in enzyme activity and gene expression of ACC synthase and ACC oxidase. We also investigated increases in enzyme activity of PAL and peroxidase in response to wounding in mesocarp tissue of *Cucurbita maxima*. We used two layers excised from the cut surface successively in 1-mm thickness for analyzing ethylene synthesis and expression and induction of the related enzymes. The objective of this study was to investigate the mechanism by which these enzymes related to ethylene synthesis and action were induced in response to wound stress.

Materials and Methods

Plant material—Winter squash fruit (*Cucurbita maxima* Duch. cv. Ebisu) were obtained at a local market. The fruit were cut in half and incubated at 25°C under humidified air. The mesocarp discs of 12 mm diameter and 1-mm thickness, each for the first layer (0–1 mm) and the second layer (1–2 mm), were excised from the fruit successively with a cylinder microtome (Ikemoto-Rika). Excised discs of each layer were immediately frozen with liquid nitrogen except for the discs for ethylene production assay and stored at −80°C until used.

Treatment of the fruit with NBD—The fruit cut in half were placed in an 87.8-liter tight plastic container. Liquid NBD was applied onto a sheet of filter paper in a small petri dish in the container, which was sealed with a cover and vinyl tape in which NBD quickly volatilized. The amount of liquid NBD was calculated to give a desired gaseous concentration of 4,000 μl liter^{-1}.

Assay of ethylene production—Two sampled discs were placed in a 17-ml vial and sealed with a silicon rubber cap for 30 min at 20°C. A 1-ml gas was sampled from the internal atmosphere with a plastic hypodermic syringe and injected into a gas chromatograph (Hitachi 163) fitted with an alumina column at 70°C and a flame ionization detector to assay ethylene concentration. The rate of ethylene production was expressed as nmol ethylene per h per g FW.

Extraction of ACC synthase and PAL—Two frozen discs (ca. 0.2 g) were homogenized with a mortar and pestle in 3 ml of extraction buffer consisting of 0.1 M EPPS-KOH buffer, pH 8.5, 10 mM 2-mercaptoethanol, and 10 μM pyridoxal phosphate at 2°C. The homogenate was centrifuged at 14,000 × *g* for 20 min at 4°C. The supernatant was desalted by passing through PD-10 column (Amersham Pharmacia Biotech) with 10 mM EPPS-KOH buffer, pH 8.5, containing 10 mM 2-mercaptoethanol and 10 μM pyridoxal phosphate. The eluent was assayed for ACC synthase and PAL activities.

Assay of ACC synthase activity—ACC synthase activity was assayed in a reaction mixture that consisted of 50 mM EPPS-KOH buffer, pH 8.5, 50 μM SAM, and the enzyme preparation in a total volume of 1 ml. The reaction mixture was incubated for 30 min at 30°C and then the reaction was stopped by adding 0.1 ml of 40 mM $HgCl_2$. ACC formed in the reaction was assayed by the method of Lizada and Yang (1979). ACC synthase activity was expressed as nmol ACC formed per h per g FW.

Assay of PAL activity—PAL activity was determined spectrophotometrically by measuring the increase in A_{290} due to the formation of *trans*-cinnamic acid. The reaction mixture consisted of 50 mM EPPS-KOH buffer, pH 8.5, 10 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 sample was incubated for 1 h at 40°C. The activity was expressed as nmol *trans*-cinnamic acid formed per h per g FW of the mesocarp tissue. The calculation was based on a molar absorption coefficient for cinnamate of 9,500 M^{-1} cm^{-1}.

Extraction and assay of ACC and ACC oxidase—Two discs were homogenized in 2 ml of extraction buffer consisting of 0.1 M Tris-HCl, pH 7.2, 5 mM dithiothreitol, 30 mM Na-ascorbate, and 10% glycerol (v/w) at 2°C. The homogenate was centrifuged at 14,000 × *g* for 20 min at 4°C. The supernatant was used for assay of ACC and ACC oxidase. ACC content was assayed by the method of Lizada and Yang (1979). ACC oxidase activity was measured as described previously (Kato and Hyodo 1999). ACC oxidase activity was expressed as nmol ethylene formed per h per g FW.

Extraction and assay of peroxidase—Two discs were homogenized in 1 ml of extraction buffer consisting of 50 mM K-phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM Na-ascorbate, 0.2 M KCl, and 20% glycerol (w/v). The homogenate was centrifuged at 14,000 × *g* for 20 min at 4°C. The supernatant was passed through PD-10 column which had been equilibrated with 50 mM K-phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM Na-ascorbate, and 20% glycerol (w/v). The eluent was used for assay of peroxidase and electrophoresis on native PAGE.

Assay of peroxidase was carried out in the medium that consisted of 50 mM K-phosphate buffer, pH 7.0, 20 μM syringaldazine, and 0.1 mM H_2O_2 in a total volume of 3 ml. The reaction was started by the addition of H_2O_2 and the increase in A_{530} was monitored by spectrophotometry (Hitachi U-2000). Activity was expressed as an increase in the A_{530} per min per g FW of the tissue.

Protein assay—Protein was assayed by the method of Bradford (1976) using bovine serum albumin as a standard.

Native PAGE—Twenty μg protein in the extract of peroxidase was loaded on a 12.5% polyacrylamide gel (Atto Corp.) and separated by electrophoresis at 4°C. After the electrophoresis, the gel was stained for peroxidase in a medium consisting of 50 mM K-phosphate buffer, pH 7.0, 20 μM syringaldazine, and 0.1 mM H_2O_2 at 20°C.

RNA isolation—Total RNA was isolated according to the method of Ikoma et al. (1996). Fifteen frozen discs (ca. 3 g) were ground to fine powder in liquid nitrogen with a mortar and pestle. The powder was mixed with 12.5 ml of lysis buffer consisting of 150 mM Tris-borate buffer, pH 7.5, 50 mM EDTA, 2% SDS, and 1% 2-mercaptoethanol and 12.5 ml of phenol/chloroform/isoamyl alcohol (25 : 24 : 1, v/v/v) in a 50-ml polypropylene conical tube. After centrifugation at 5,000 × *g* for 20 min, the aqueous phase was transferred to a 50-ml conical tube. This extraction was repeated twice with phenol/chloroform/isoamyl alcohol. The aqueous phase in a 50-ml conical tube was mixed quickly with 0.25 volume of ethanol and 0.11 volume of 5 M K-acetate and then chloroform/isoamyl alcohol (49 : 1, v/v) was immediately added to the aqueous phase at 1 : 1 ratio. After re-centrifugation, the aqueous phase was transferred to 30-ml DEPC-treated Teflon tube. Total RNA was precipitated by the addition of LiCl (3 M final concentration) and incubated at −20°C overnight. The total RNA was pelleted by centrifugation at 20,000 × *g* for 30 min and resuspended in DEPC-treated water. Furthermore, this precipitation procedure was carried out twice and then the total RNA was

precipitated with 2.5 volumes of ethanol in the presence of K-acetate at −80°C.

Amplification of poly(A)$^+$ RNA by RT-PCR—The first strand cDNA was synthesized from 5 μg of the total RNA by reverse transcriptase with Oligo-(dT) primer according to the instruction of Ready-To-Go™ T-Primed First-Strand Kit (Amersham Pharmacia Biotech). PCR was performed in a total volume of 100 μl containing 1 μl of the first strand cDNA reaction products, 10 μl of 10 × PCR Buffer II, 2.5 mM $MgCl_2$, 200 μM deoxynucleotides, 100 pmol of primers, and 2.5 units of AmpliTaq Gold™ DNA polymerase (Perkin Elmer). The primers (AAATCCCTTAGGCACAACA as the upstream primer and TTTGCGAAACAAACTCGAAA as the downstream primer) designed on the basis of cDNA sequence of ACC synthase determined by Nakajima et al. (1990) were used to amplify ACC synthase cDNA. The primers (AA/[CT]/TGGGG/[CT]/TTCTTTGAG/[CT]/T as the upstream primer and TC/[AG]/TC/[CT]/TGGAA/[AG]/AG/[CG]/A/[AG]/GAT as the downstream primer) were designed by the common sequences of ACC oxidase that have been reported to amplify ACC oxidase cDNA. The PCR procedure for ACC synthase cDNA 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 Gene Amp PCR System 9600 (Perkin Elmer). The annealing temperature was changed to 52°C for ACC oxidase. The PCR products were confirmed by agarose gel electrophoresis.

Cloning and sequencing of cDNAs—The amplified cDNAs were ligated to the plasmid pCR II and cloned into *Escherichia coli* using TA Cloning Kit (Invitrogen). The sequences were determined using Taq Dye Primer Cycle Sequencing kit and Taq Dye Terminator Cycle Sequencing kit (Perkin Elmer) with 373S DNA Sequencing System (Perkin Elmer).

Northern blot analysis—Ten μg of total RNA was separated on a 1.2% agarose-denaturing formaldehyde gel containing 20 mM MOPS, pH 7.0, 0.5 mM Na-acetate, and 1 mM EDTA. After electrophoresis for 2 h, 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 (Boehringer Mannheim) by capillary action with 20 × SSC and then the membrane was baked for 3 h at 80°C.

cDNA encoding ACC synthase (*CMa-ACS*) was labeled with DIG RNA labeling kit using T7 RNA polymerase (Boehringer Mannheim) and cDNA encoding ACC oxidase (*CMa-ACO*) was labeled with DIG DNA labeling kit (Boehringer Mannheim). The membrane for *CMa-ACS* was prehybridized at 67°C with 5 × SSC, 50% formamide, 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 2% Blocking reagent (Boehringer Mannheim) for 4 h, while the membrane of *CMa-ACO* was prehybridized at 41°C with 7% SDS, 50% formamide, 5 × SSC, 50 mM sodium phosphate, pH 7.0, 0.1% *N*-lauroylsarcosine, and 2% Blocking reagent for 4 h. Hybridization was performed at 67°C for *CMa-ACS* and at 41°C for *CMa-ACO* in the same prehybridization buffers.

After hybridization, the membrane was washed twice with 2 × SSC for 5 min at room temperature and then washed with 0.1 × SSC for 15 min at 67°C for *CMa-ACS* and 65°C for *CMa-ACO*. After the equilibration in Buffer A (0.1 M maleic acid and 0.15 mM NaCl, pH 7.5) for 5 min at room temperature, the membrane was blocked with 2% Blocking reagent in Buffer A for 30 min. Subsequently, the membrane was incubated with Anti-Digoxygenin-AP, Fab fragments (Boehringer Mannheim) in the blocking buffer for 30 min. The membranes were washed four times for 8 min each in Buffer A containing 0.3% Tween 20 and equilibrated with 0.1 M Tris-HCl buffer, pH 9.5 containing 0.1 M NaCl and 50 mM $MgCl_2$ for 5 min. Chemiluminescent reaction was carried out with CDP-Star™ (Boehringer Mannheim), chemiluminescent substrate, and the membrane was exposed to Hyper film™ ECL™ (Amersham).

Results

Wound-induced ethylene synthesis—Ethylene production was rapidly induced in the first layer including the cut surface of mesocarp tissue of *Cucurbita maxima* after wounding (Fig. 1). The production rate rose rapidly, reaching a peak 16 h after wounding, then declining to a basal level. In the second layer, inward mesocarp tissue, the ethylene production rate increased slowly to a peak at 16 h. The peak rate in the first layer was approximately five times as much as that in the second layer.

Wound-induced ACC synthase—ACC synthase activity increased rapidly in the first layer after wounding, reaching a peak at 8 h, then declining sharply (Fig. 2A). In the second layer, the increase in activity was not significant throughout the experiment period. cDNA for ACC synthase amplified by RT-PCR which corresponded to about 600 bp was cloned into the pCR II and sequenced. The 621-bp cDNA for ACC synthase designated as *CMa-ACS* contained pyridoxal phosphate-binding site of ACC synthase and was identical to the same region of the cDNA that encoded ACC synthase of wounded mesocarp tissue of *Cucurbita maxima* (Nakajima et al. 1990). Therefore, *CMa-ACS* clone was used as a probe for northern blot analysis. The levels of transcripts for ACC synthase increased quickly in the first layer immediately after wounding (Fig. 2B). Expression of ACC synthase gene in the first layer was clearly observed 4 h after wounding, which remained clearly detectable until 16 h. The expression of *CMa-ACS* mRNA was barely detectable in the second layer that may reflect in the much lower activity of ACC synthase in the second layer.

ACC levels increased markedly in the first layer,

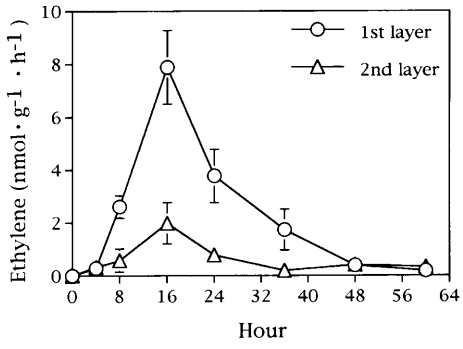

Fig. 1 Time course of ethylene production in the first layer and the second layer, respectively, of wounded mesocarp tissue of *Cucurbita maxima*. Data are the mean of three replicates. Bars represent S.E.s when larger than symbols.

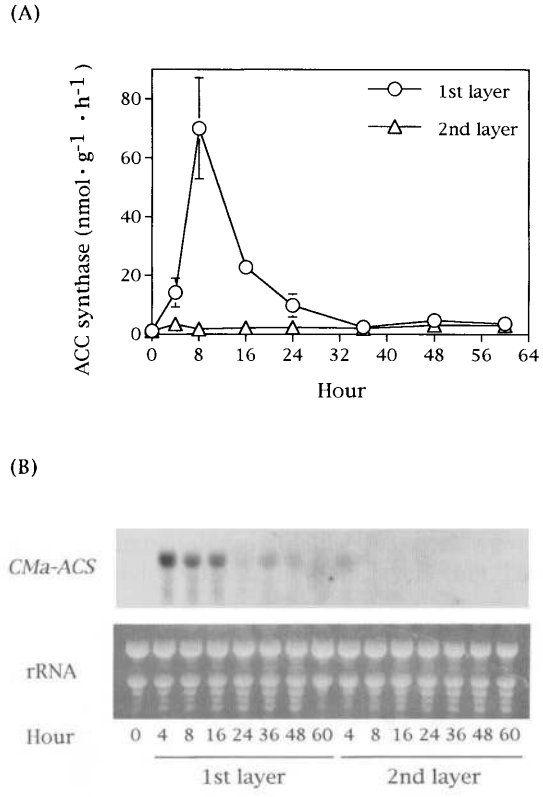

Fig. 2 Time courses of ACC synthase activity and expression of *CMa-ACS* gene in the first layer and the second layer, respectively, of wounded mesocarp tissue of *Cucurbita maxima*. (A) Changes in ACC synthase activity. Data are the mean of three replicates and bars represent S.E.s when larger than symbols. (B) Northern blot analysis for ACC synthase. Equal loading of RNA was confirmed by staining a gel with ethidium bromide.

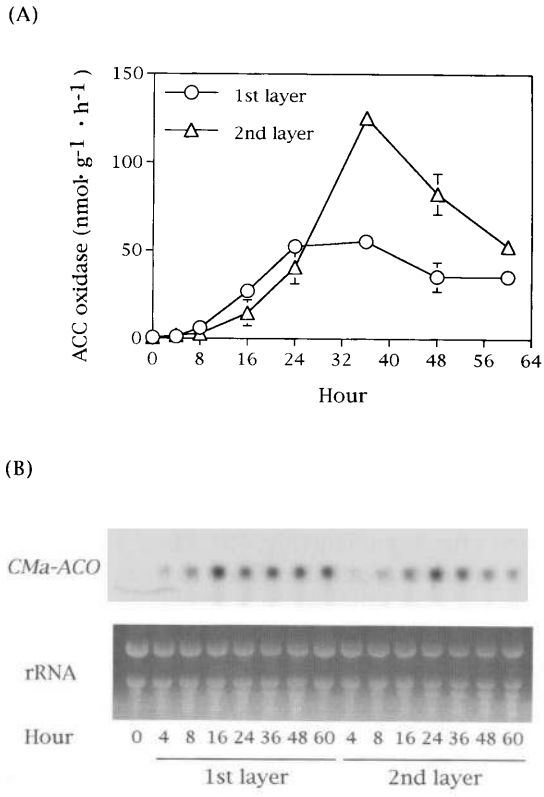

Fig. 4 Time courses of ACC oxidase activity and expression of *CMa-ACO* gene in the first layer and the second layer, respectively, of wounded mesocarp tissue of *Cucurbita maxima*. (A) Changes in ACC oxidase activity. Data are the mean of three replicates. Bars represent S.E.s and are contained within the symbols when not shown. (B) Northern blot analysis for ACC oxidase. Equal loading of RNA was confirmed by staining a gel with ethidium bromide.

peaking 16 h after excision, then declining sharply (Fig. 3). The marked increase in ACC content in the first layer may have resulted from the rapid rise in ACC synthase activity.

In the adjacent (2nd) layer ACC formation increased only marginally owing to a much lower level of ACC synthase activity.

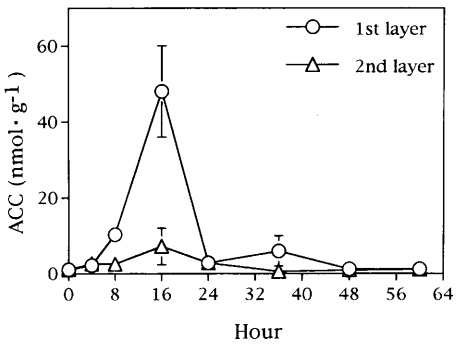

Fig. 3 Changes in ACC levels in the first layer and the second layer, respectively, of wounded mesocarp tissue of *Cucurbita maxima*. Data are the mean of three replicates. Bars represent S.E.s and are contained within the symbols when not shown.

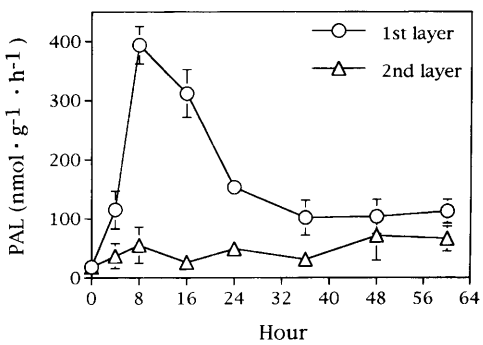

Fig. 5 Time course of the PAL activity in the first layer and the second layer, respectively, of wounded mesocarp tissue of *Cucurbita maxima*. The data are the mean of three replicates with bars representing S.E.s when larger than symbols.

Induction of ACC oxidase—In contrast to ACC synthase, ACC oxidase activity increased progressively in both layers and the high activity was maintained until the later stage after wounding (Fig. 4A). The activity of the inner layer (2nd) was found to be even much higher than the surface layer (1st). The activity of ACC oxidase in the first layer reached a maximum at 24 h and remained almost at the same level up to 60 h, whereas the activity in the second layer increased to a peak at 36 h. cDNA for ACC oxidase was amplified by the procedures of RT-PCR. The resultant cDNA designated as *CMa-ACO* was cloned. The sequence of *CMa-ACO* having 482 bp was found to be highly homologous to ACC oxidase genes isolated from other plant sources, showing 86.2% identical with melon (Balagué et al. 1993, Yamamoto et al. 1995) and 84.8% identical with cucumber (Perl-Treves et al. 1998, Shiomi et al. 1998), respectively. Expression of ACC oxidase gene was evident in both layers after wounding (Fig. 4B). The amounts of ACC oxidase transcripts greatly increased 16 h for the first layer and 24 h for the second layer after wounding in correlation with the increase in ACC oxidase activity.

Induction of PAL and POX activity in response to wounding—PAL activity increased rapidly in the first layer to a peak at 8 h after wounding (Fig. 5). The profile of increasing pattern was similar to the induction of ACC synthase. In the second layer, however, significant increases in the activity were not evident.

Peroxidase activity began to increase 16 h after wounding in both the first and second layers (Fig. 6A). The development of peroxidase activity progressed throughout the experimental period. The increasing patterns of peroxidase activity were much different from those of PAL, but similar to a manner of increase in ACC oxidase activity. Several isozymes of peroxidase in the extract from wounded mesocarp tissue were separated by polyacrylamide gel electrophoresis (Fig. 6B). At least four peroxidase isoenzymes were detected, which were designated as POX 1,

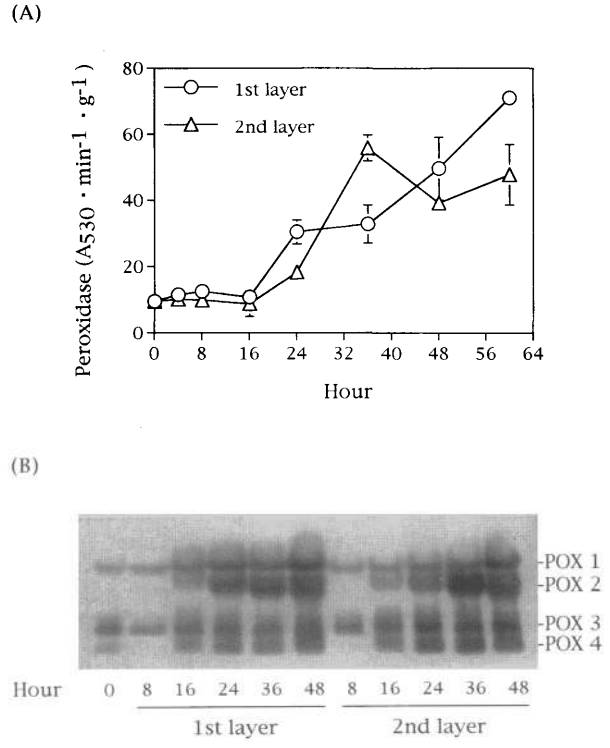

Fig. 6 Increasing patterns of peroxidase activity in the first layer and the second layer, respectively, of wounded mesocarp tissue of *Cucurbita maxima*. (A) Changes in peroxidase activity. Data are the mean of three replicates. Bars representing S.E.s are included in the symbols when not shown. (B) Polyacrylamide gel electrophoresis of peroxidase isozymes. The sample proteins were obtained from the same tissue as in (A). Each ten μg protein extracted from the both layers was loaded in individual lanes. After electrophoresis, gel was stained with a solution containing 20 μM syringaldazine and 0.1 mM H_2O_2.

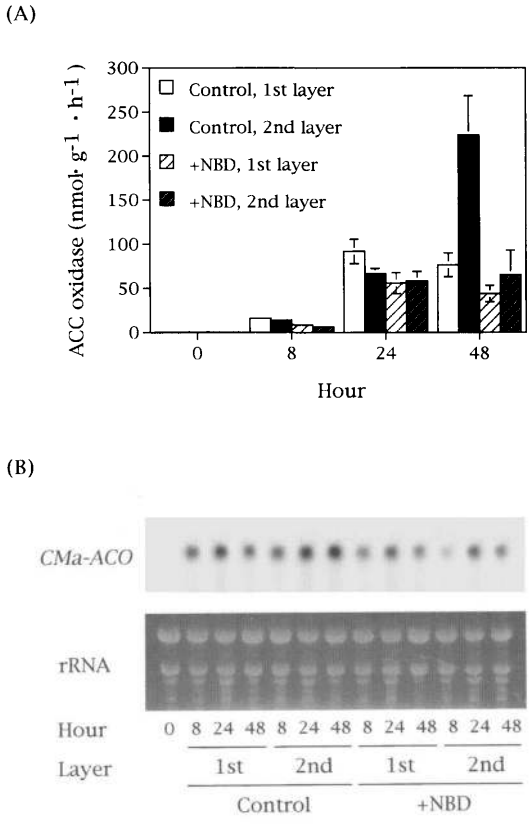

Fig. 7 Effect of NBD on induction of ACC oxidase activity and expression of *CMa-ACO* gene mRNA in the first layer and the second layer, respectively, of wounded mesocarp tissue of *Cucurbita maxima*. (A) ACC oxidase activity was extracted from the respective tissues that were incubated in the presence or absence of NBD. Data are the mean of three replicates. Bars represent S.E.s when larger than symbols. (B) Northern blot analysis for ACC oxidase. Equal loading of RNA was confirmed by staining a gel with ethidium bromide.

POX 2, POX 3, and POX 4, respectively, as a result of staining with syringaldazine. Those isozymes, POX 1, POX 3, and POX 4 were also detectable in fresh tissue (0 h), but their amounts increased greatly in both layers after wounding. POX 2, however, was newly detected in response to wounding and its abundance increased 16 h after wounding. The same results were observed when guaiacol was used as a substrate in place of syringaldazine.

Effect of NBD on the induction of ACC oxidase and peroxidase—To investigate the role of endogenous ethylene in the induction of ACC oxidase and peroxidase, winter squash fruit cut in half were treated with 4,000 μl liter^{-1} NBD, a competitive inhibitor of ethylene action (Sisler and Yang 1984). The induction of ACC oxidase activity was substantially suppressed by the application of NBD in both layers (Fig. 7A), especially a strong reduction in the activity by NBD was observed in the second layer at 48 h. Expression of *CMa-ACO* mRNA was also prevented by the exposure to NBD in the both layers (Fig. 7B). The effect of NBD on the accumulation of the transcripts in the second layer seemed much greater than that in the first layer. These results suggest that induction of ACC oxidase is largely attributable to endogenous ethylene evolved in response to wounding.

The induction of peroxidase activity was also inhibited by the application of NBD in both the first and second layers (Fig. 8A). Similarly to ACC oxidase, the peroxidase activity in the second layer at 48 h was greatly suppressed. It also appeared that the enzyme activity of peroxidase isozymes stained on the gel after electrophoresis was suppressed by the NBD treatment (Fig. 8B). From these results it is considered that ethylene is closely associated at least in part with the induction of peroxidase in the wounded tissue.

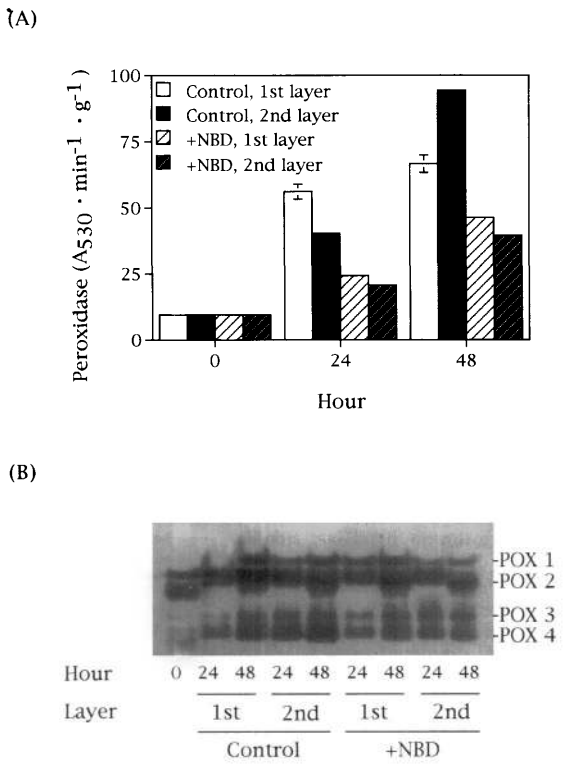

Fig. 8 Effect of NBD on increase in activity and development of isozymes of peroxidase in the first layer and the second layer, respectively, of wounded mesocarp tissue of *Cucurbita maxima*. (A) Peroxidase activity was extracted from the respective tissues that were incubated in the presence or absence of NBD. The data are the mean of three replicates and bars representing S.E.s are included in the symbols when not shown. (B) Polyacrylamide gel electrophoresis of peroxidase isozymes. The sample proteins were obtained from the same tissue as in (A). The amount of protein loaded and staining were as described in Figure 6.

Discussion

Mesocarp tissue of *Cucurbita maxima* produced a large amount of ethylene in response to wounding. We prepared two layers from the cut surface successively in 1 mm thickness consisting of approximately 9 cells that were sliced at specified times during incubation and used for the subsequent analyses. ACC synthase activity was rapidly induced in the first layer followed by a marked rise in ACC level and then ethylene production (Fig. 1, 2A, 3). The rapid rise in ACC synthase activity occurred concurrently with the significant increase in the expression of ACC synthase gene (Fig. 2B). In contrast to the first layer, the activity of ACC synthase in the second layer was too low to induce a marked rise in ACC formation and thereby ethylene production. Thus induction of ACC synthase is considered to be primarily important for wound-induced ethylene synthesis. It is possible that wound ethylene detected in the second layer may have in part resulted from translocatable ethylene moved from the first layer (Woltering et al. 1995).

ACC synthase has been shown to be induced by various factors such as fruit ripening (Dong et al. 1991, Nakatsuka et al. 1998), auxin (Kim et al. 1992), senescence (Woodson et al. 1992), and various kinds of stresses including mechanical stimuli and wounding (Nakajima et al. 1990, Tatsuki and Mori 1999) and disease (Ohtsubo et al. 1999). In the present study we investigated the wound-induced expression of ACC synthase and found that the wound effect was exhibited exclusively in the surface layer of 1 mm thickness in both accumulation of ACC synthase mRNA and ACC synthase activity. Watanabe and Sakai (1998) demonstrated that in the wounded tissue of *Cucurbita maxima* active oxygen species and methyl jasmonate were involved in the induction of ACC synthase gene. Felix and Boller (1995) suggested the involvement of systemin in the induction of ACC synthase activity. The increase in the activity and the transcription of ACC synthase mRNA

were suppressed by the exogenous or endogenous ethylene (Hyodo et al. 1985, Nakajima et al. 1990, Nakatsuka et al. 1998, Peck and Kende 1998). The wound ethylene produced may have related to the sharp decline of ACC synthase mRNA and its enzyme activity.

In contrast to ACC synthase, there was a marked increase in ACC oxidase mRNA and ACC oxidase activity in the second layer after wounding (Fig. 4) as well as in the first layer. This promotion of ACC oxidase induction is explained to be mediated at least in part by endogenous ethylene because the treatment with NBD significantly suppressed this induction at both levels of ACC oxidase mRNA and its enzyme activity (Fig. 7). It has been demonstrated that ACC oxidase is induced by various factors such as fruit ripening (Ververidis and John 1991, Dong et al. 1992, Balagué et al. 1993, Nakatsuka et al. 1998), senescence (Woodson et al. 1992, Pogson et al. 1995, Kasai et al. 1996), wounding (Hyodo et al. 1993, Kim and Yang 1994, Bouquin et al. 1997) and infection by pathogens (Kim et al. 1998, Ohtsubo et al. 1999). In these studies results have been shown that endogenous or exogenous ethylene participated in the regulation of ACC oxidase (Woodson et al. 1992, Hyodo et al. 1993, Kim and Yang 1994, Kim et al. 1997, Kwak and Lee 1997). Kim et al. (1997) and Kwak and Lee (1997) further suggested that protein phosphorylation and dephosphorylation were responsible for the signal transduction in ethylene mediated expression of ACC oxidase gene.

PAL plays an essential role in biosynthesis of phenylpropanoids including flavonoids, chlorogenic acid and lignin in plants (Hanson and Havir 1981, Hahlbrock and Scheel 1989, Lewis and Yamamoto 1990). The rapid rise in PAL activity observed in the first layer of wounded tissue of *Cucurbita maxima* (Fig. 5) may contribute to the progress in lignification observed in the surface layer of the wounded tissue (Hyodo et al. 1991). The increasing behavior of PAL activity resembled that of ACC synthase developed in the first layer. NBD applied to the cut fruit during incubation failed to yield a significant inhibition to the development of PAL in the first layer (data not shown). This, together with the results that there were no significant increases in PAL activity in the second layer (Fig. 5) may indicate that the endogenous ethylene evolved is not directly related to wound-induced PAL in *Cucurbita maxima*.

Peroxidase activity in mesocarp tissue of *Cucurbita maxima* greatly increased after wounding both in the first and second layers in a similar manner (Fig. 6A). In addition, a peroxidase isozyme, POX 2, was newly formed and increased in the amount after wounding (Fig. 6B). Other three isozymes also increased in the amount by wounding (Fig. 6B). That ethylene was involved in the enhancement of peroxidase activity was demonstrated by the results that NBD treatment significantly suppressed the increase in peroxidase activity (Fig. 8). The promotion of peroxidase activity and its transcripts level by the exogenous ethylene was shown (Imaseki 1970, Ishige et al. 1993). In a previous paper we reported that the administration of aminoethoxyvinylglycine, an inhibitor of ACC synthase, strongly reduced the subsequent development of peroxidase in the wounded mesocarp tissue (Hyodo et al. 1991). These results indicate that endogenous ethylene produced in response to wounding may participate in the induction of peroxidase. A physiological role played by peroxidases induced by wounding is considered to be related to lignin synthesis (Grisebach 1981, Lewis and Yamamoto 1990).

In both cases of ACC oxidase and peroxidase, the enzyme induction still continued even when the ethylene production fell off and stayed at a low level. Furthermore, the effect of NBD seemed incomplete on reducing the development of both enzymes. These findings imply that some factor(s) may also be involved besides ethylene in the elicitation of ACC oxidase and peroxidase in wounded mesocarp tissue of *Cucurbita maxima*.

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