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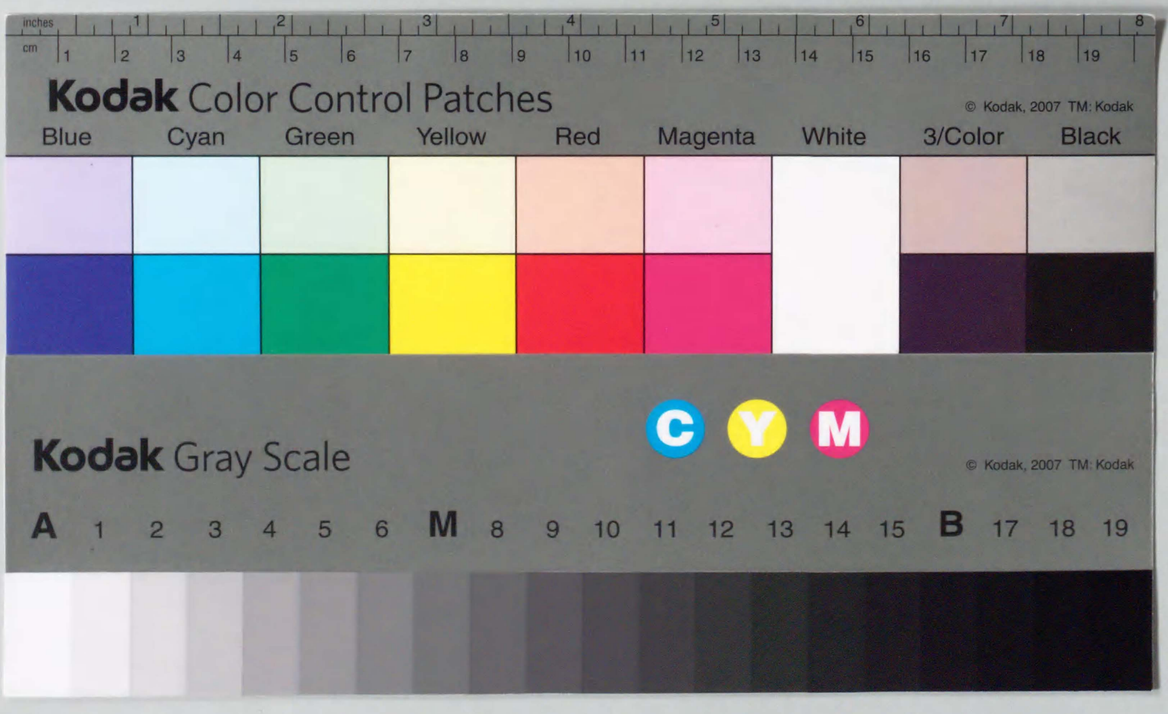

Regulatory Mechanism of the Expression of a Wound-inducible ACC Synthase Gene in Winter Squash (*Cucurbita maxima*)

Takahiro WATANABE

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Contents

Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
bp	base pairs
cDNA	complementary deoxyribonucleic acid
cv.	cultivar
dCTP	deoxy cytidine 5' triphosphate
DDC	diethyldithiocarbamic acid
DMSO	dimethyl sulfoxide
DPI	diphenylene iodonium
GC-MS	gas chromatography-mass spectrometry
JA	jasmonic acid
MeJA	methyljasmonate
Mes	2-morpholinoethanesulfonic acid, monohydrate
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PAL	phenylalanine ammonia-lyase
PR	pathogenesis-related
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
SAM	S-adenosyl-L-methionine
SOD	superoxide dismutase
v/v	volume/volume
w/v	weight/volume
X–XO	xanthine plus xanthine oxidase

Abstract

The production of ethylene by higher plants is enhanced by mechanical wounding and pathogen attack. The ethylene produced is referred to as "stress ethylene" and various lines of evidence support a role for ethylene as a mediator of the wound signal. Although the wound-inducible production of ethylene has been reported to be regulated by expression of genes for 1-aminocyclopropane-1-carboxylate (ACC) synthases in various plant species, the mechanism of such regulation is not sufficiently understood.

In recent years, many studies suggested that jasmonic acid (JA) and reactive oxygen species (ROS) also play important roles as mediators in early responses to wounding or attack by pathogens. However, the relationships between these three mediators of the wound signal, namely, JA, ROS and ethylene, have not yet been clarified. In this thesis, I examined the regulatory mechanisms of the expression of a gene for ACC synthase and showed that the harmonious production of three mediators was required for the responses of a plant to wounding.

The production of ethylene by mesocarp tissue of winter squash (*Cucurbita maxima* Duch.) is induced by mechanical wounding. I isolated a cDNA fragment (WSACS2) from the wounded mesocarp tissue of winter-squash fruit by the reverse transcriptase-polymerase chain reaction using primers that corresponded to two conserved amino acid sequences found in ACC synthases. The sequence of WSACS2 cDNA was almost identical to that of the cDNA for a wound-inducible ACC synthase (CM-ACS1) reported by Nakajima *et al.* (1990) with only one nucleotide difference. The expression of *CM-ACS1* in tissue disks was examined by Northern blotting analysis with the use of ^{32}P-labeled WSACS2 as the CM-ACS1 probe. The accumulation of the *CM-ACS1* transcript was induced within 1 h after wounding, and its amount

increased gradually for 6 h thereafter. The pattern of the expression of *CM-ACS1* was corresponded to that of the production of ethylene after wounding. These results suggest that the production of ethylene in tissue disks is mainly regulated by the level of the *CM-ACS1* transcript.

It has been shown that JA is accumulated in response to wounding and that the JA produced acts as a mediator of wound signal. O'Donnell *et al.* (1996) reported that the production of ethylene was stimulated by exogenously applied JA in tomato plants. To clarify the role of JA on the wound-induced production of ethylene, I measured levels of JA in tissue disks by gas chromatography-mass spectrometry. The JA content increased within 2 h after wounding and its level was about 5-folds at 3 h as compared to that of intact tissue disks. The ethylene production was stimulated by exogenously applied JA within 1 h and, at each time point during the incubation, it was approximately twice that in control disks. Furthermore, JA stimulated the accumulation of the *CM-ACS1* transcript within 30 min after the treatment, and the effects of JA continued for 7 h. The stimulatory effect was dependent on the concentration of JA over a range of concentrations from 10^{-7} to 10^{-3} M.

These results suggest that JA stimulated the production of ethylene by activating the expression of *CM-ACS1* in the disks of mesocarp tissue. However, the accumulation of the transcript increased prior to the production of JA. Therefore, I examined the effects of ROS on the expression of *CM-ACS1*, because ROS are produced immediately after wounding, prior to the production of JA.

The levels of ROS that were generated in the tissue disks were measured by using chemiluminescence reagents and it was found that the chemiluminescence signal became apparent within a few seconds after wounding. The increase in chemiluminescence was suppressed by the addition of diphenylene iodonium (DPI), which is known as an inhibitor of

superoxide-generating NAD(P)H oxidases. Ethylene production was also inhibited by the treatment with DPI. Furthermore, I showed that the accumulation of *CM-ACS1* mRNA in the disks was inhibited by treatment with DPI and the inhibitory effect was dependent on the concentration of DPI from 10^{-7} M to 10^{-4} M. The addition of xanthine and xanthine oxidase, which generate O_2^-, strongly induced the synthesis of the *CM-ACS1* transcript within 30 min. The addition of superoxide dismutase (SOD), which catalyzes the conversion of superoxide (O_2^-) to H_2O_2 and O_2, repressed the accumulation of the *CM-ACS1* transcript. However, the addition of H_2O_2 to the disks did not affect the induction. These results indicate that O_2^-, one type of ROS, might participate in the expression of the gene for the wound-inducible ACC synthase.

As mentioned above, the accumulation of the *CM-ACS1* transcript was stimulated by both JA and ROS in the disks. However, the relationship between ROS and JA remained unknown. To elucidate whether ROS regulates the expression of *CM-ACS1* through stimulation of JA production, the effects of ROS on the JA content of disks and the effects of inhibitors of the production of JA and ROS on the accumulation of the *CM-ACS1* transcript were examined. DPI had no effect on the increase in JA content but DPI strongly suppressed the accumulation of the *CM-ACS1* transcript both 1 and 2 h after the wounding. Acetylsalicylic acid, an inhibitor of the synthesis of JA, did not inhibit the accumulation of the transcript within 1 h but did inhibit accumulation within 2 and 3 h after wounding.

Based on the results obtained in this study, I proposed a working hypothesis for the regulatory mechanisms for the expression of a wound-inducible gene for ACC synthase. In wounded mesocarp tissue of winter-squash, both ROS and JA affect the production of ethylene by modulating the accumulation of the transcript of a wound-inducible gene for ACC synthase (*CM-ACS1*) but they act independently. The expression of *CM-ACS1* is

induced initially by ROS that are generated in disks upon wounding and then by JA, the level of which increases at a later time after wounding.

This is a first report showing the regulatory mechanisms of the expression of a wound-inducible gene for ACC synthase, and the relationships between the production of three mediators of wound signal, namely, JA, ROS and ethylene in the response of a plant to wounding.

General Introduction

Higher plants are continually challenged throughout their life cycle by a variety of adverse external stimuli, which they cannot move away from in the same manner as animals. The inability to move away from an adverse environment has led them to evolute adaptive mechanisms which enable to respond rapidly to a variety of stress conditions with the proper defense. One of the most severe stresses is the wounding that might result from the attacks of insects and microbes or mechanical injury (cutting, bruising). Plants respond to wounding by inducing a defense response characterized by the expression of a set of proteins, mainly aimed at wound healing and prevention of pathogen invasion. These responses include (a) reinforcement of the cell wall by deposition of callose, lignin, and hydroxyproline-rich glycoproteins, (b) synthesis of the antimicrobial compounds phytoalexin, and (c) production of proteinase inhibitors and lytic enzymes such as chitinase and glucanases (Collinge and Slusarenko 1987, Hahlbrock and Scheel 1987, Dean and Kuc 1987, Bowles 1990). Moreover, induction of defense-related proteins involves transcriptional activation of the corresponding genes and, as a consequence, correlates with a substantial alteration in the pattern of gene expression in the wounded plant (Lawton and Lamb 1987). A lot of mechanisms which allow the harmonious regulation of these responses remains unknown because the responses to wounding is very complex.

Ethylene is a plant hormone which regulates plant developmental processes from seed germination to senescence. It is also closely associated with the responses to a wide variety of environmental stresses such as pathogen infection, touch, drought and mechanical injury (Abeles *et al.* 1992, Kende 1993, Morgan and Drew 1997). In studies of plant responses to wounding, various evidences support a role of ethylene as a mediator of the wound signal, inducing the expression of defense genes that include genes for

proteinase inhibitors (Linthorst *et al.* 1993, Botella *et al.* 1996), phenylalanine ammonia-lyase (PAL) (Rickey *et al.* 1991), and pathogenesis-related proteins (Memelink *et al.* 1990, Ishige *et al.* 1993).

Ethylene is synthesized from methionine *via* S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylate (ACC), and ACC synthase which forms ACC from SAM is a rate-limiting enzyme (Yang and Hoffman 1984). This enzyme is encoded by a family of highly divergent genes (Rottmann *et al.* 1991) and each gene is independently regulated and differentially expressed in response to various signals. Therefore, this family of genes provides an ideal system for studies on complex signal transduction pathways and their relationships to stress responses. The genes for ACC synthases that are induced by wounding are distinguishable from other members of this family. Wound-inducible genes for ACC synthase have been cloned from many kinds of plant including zucchini (Huang *et al.* 1991), tomato (Liang *et al.* 1992, Lincoln *et al.* 1993), *Arabidopsis* (Oetiker *et al.* 1997) and winter squash (Nakajima *et al.* 1990). The inducibility and patterns of expression of these genes have been examined, but the mechanisms that regulate their expression have not yet been clarified.

The role of jasmonic acid (JA) and reactive oxygen species (ROS) as mediators in early responses to wounding or attack by pathogens has been well reviewed (Wasternack and Parthier 1997, Low and Merida 1996).

Jasmonic acid (JA) and its methyl ester, methyljasmonate (MeJA), are natural plant compounds that elicit a variety of responses in plants. These responses range from effects on growth and development to the induction of wound-responsive genes involved in plant defense. Although they have been studied for only a short time compared with other plant growth regulators including ethylene, many evidences suggest that jasmonate plays an important role in plant regulatory processes, acting in a manner characteristic of other plant hormones (Sembder and Parthier 1993). In particular, recent reports

show that JA has an important role as a mediator of the wound signal, inducing, for example, the expression of defense genes that include genes for proteinase inhibitors (Farmer and Ryan 1990), vegetative storage proteins (Mason and Mullet 1990), and pathogenesis-related proteins (Rickauer *et al.* 1997). Jasmonic acid is synthesized from α-linolenic acid *via* the lipoxygenase pathway, comprising oxidation of α-linolenic acid, cyclisation of the resulting hydroperoxide to 12-oxo-phytodienoic acid, and further conversion by reduction and ß-oxidation (Vick and Zimmerman 1983). This synthetic pathway of JA is referred to as octadecanoid signaling pathway. It has also been reported that mechanical wounding triggers an increase in the endogenous levels of JA in some kinds of plant materials including soybean hypocotyls (Creelman *et al.* 1992), *Avena* leaves (Albrecht *et al.* 1993), tobacco leaves (Seo *et al.* 1995) and *Arabidopsis* leaves (Laudert *et al.* 1996). O'Donnell *et al.* (1996) reported that JA induced the production of ethylene, and that ethylene and JA together regulated the wound-inducible expression of a gene for a proteinase inhibitor in tomato plants. However, whether JA induced the production of ethylene *via* activation of the expression of genes for wound-inducible ACC synthases have not yet been clarified.

Partially reduced forms of molecular oxygen which include superoxide (O_2^-) and hydrogen peroxide (H_2O_2) can react with many cellular components leading to the oxidative destruction of the cell (Halliwell and Gtteridge 1989). In contrast to molecular oxygen, these species are capable of unrestricted oxidation of various cellular components and are therefore collectively named "reactive oxygen species" (ROS). ROS are usually formed within cells as byproducts of certain metabolic reactions. This process occurs at a low rate during growth under normal environmental conditions, but stresses that disrupt the physiological homeostasis of the cell cause a dramatic increase in the production of ROS (Allen 1995, Asada 1992). Increased production of ROS also occurs when plants is injured and attacked by

pathogens (Apostol *et al.* 1989, Anderson *et al.* 1991, Doke *et al.* 1991, Yahraus *et al.* 1995). Moreover, ROS have been considered to contribute to the defense-responses of plants in several ways. Firstly, reactive oxygen species may lead to the cross-linking of cell-wall proteins rendering the cell wall more resistant to attack by fungal enzymes (Bradley *et al.* 1992); secondly, these ROS may act directly as toxins against pathogens (Mehdy 1994); and thirdly, ROS may act as second messengers for the activation of genes encoding defense related proteins (Lamb and Dixon 1997). Dai and An (1995) reported that methyljasmonate (MeJA) induced the expression of a gene for nopaline synthase and that the induction of the activity of the promoter of the gene for nopaline synthase was caused by H_2O_2, which is one type of ROS.

As mentioned above, it has been shown that each of three mediators of wound signal, namely, ethylene, JA and ROS have an important role in plant wound-response, respectively. However, the relationships between these three mediators have not yet been clarified. The clarification of the regulatory mechanisms for the expression of wound-inducible genes for ACC synthases are important to understand the wound-induced production of ethylene and the relationships between three mediators of wound signal. Therefore, I started a study for the regulatory mechanisms of the expression of a wound-inducible gene for ACC synthase in winter-squash mesocarp tissue. Fruits of winter squash (*Cucurbita maxima*) have been used for the research of wound-induced production of ethylene. Hyodo *et al.* (1985) reported that the rate of production of ethylene by winter-squash mesocarp tissue was strongly increased by mechanical wounding. Nakajima *et al.* (1990) identified a gene for wound-inducible ACC synthase in winter squash and designated as *CM-ACS1*. In this study, I revealed that the wound-induced expression of *CM-ACS1* was stimulated by JA. This is the first report showing that JA can regulate the expression of a wound-inducible gene for ACC synthase. This

finding supports the results described by O'Donnel *et al.* (1996), and indicates that the harmonious production of ethylene and JA is needed for the response of plants to wounding. On the other hand, the expression of *CM-ACS1* was also stimulated by ROS and this stimulatory effect of ROS was not caused by an increase in the synthesis of JA.

In this thesis, I described the effect of JA on the expression of *CM-ACS1* in wounded mesocarp tissue of winter-squash in Part I and the effect of ROS on it in Part II. The relationships between JA, ROS, and ethylene were described in Part III. Based on the results reported in this thesis, I have proposed a working hypothesis for the regulatory mechanisms of the expression of a wound-inducible gene for ACC synthase, *CM-ACS1*.

Part I. Effects of jasmonic acid on production of ethylene and expression of the gene for a wound-inducible 1-aminocyclopropane-1-carboxylate synthase in winter squash (*Cucurbita maxima*)

Introduction

Mechanical wounding of plant tissue stimulates the production of ethylene (Kende 1993) and the ethylene that is produced is an important mediator of wound signals, inducing the expression of various defense genes (Botella *et al.* 1996, Rickey and Belknap 1991).

Ethylene is synthesized from methionine *via* S-adenosyl-L-methionine and ACC (Adams and Yang 1979). The enzymes catalyzing the individual steps of this pathway are S-adenosyl-L-methionine synthase, ACC synthase and ACC oxidase, respectively. The biosynthesis of ethylene is primarily regulated at the level of ACC synthase (Yang and Hoffman 1984). This enzyme is encoded by a family of highly divergent genes (Rottmann *et al.* 1991), and genes for ACC synthase that are induced by wounding are distinguishable from other members of this family. The genomic DNA and cDNA for wound-inducible ACC synthase have been isolated from zucchini (Huang *et al.* 1991) and *Arabidopsis* (Liang *et al.* 1992), and winter squash (Nakajima *et al.* 1990) and tomato (Lincoln *et al.* 1993, Oetiker *et al.* 1997), respectively. The inducibility and patterns of expression of these genes have been examined, but the mechanisms that regulate their expression remain unknown.

Jasmonic acid (JA) plays important roles as a signal mediator in responses of plants to wounding (Farmer and Ryan 1992, Creelman and Mullet 1997), and mechanical wounding triggers increases in endogenous levels of JA (Creelman *et al.* 1992, Albrecht *et al.* 1993, Seo *et al.* 1995, Laudert *et al.* 1996). O'Donnell *et al.* (1996) reported that JA induced the production of ethylene, and that ethylene and JA together regulated the wound-inducible expression of a gene for a proteinase inhibitor in tomato plants. However, the effect of JA on the expression of the gene for wound-inducible ACC synthase have not yet been clarified. The clarification of the

effects of JA on the expression of wound-inducible genes for ACC synthases is important to understand the wound-induced production of ethylene and the concomitant effects of ethylene and JA on the expression of defense-related genes. In this part, I described the increase in endogenous JA content by mechanical wounding and the stimulatory effect of JA on the expression of a gene for a wound-inducible ACC synthase (*CM-ACS1*) in winter-squash mesocarp tissue.

Materials and Methods

Plant materials

Fruits of winter squash (*Cucurbita maxima* Duch cv. Ebisu) were purchased from a local market and stored at 11°C prior to use. Each fruit was kept at 25°C for 5 h before experiments, and the mesocarp was cut into disks 2 mm thick and 11 mm in diameter. The tissue disks were pooled and selected randomly for use in all experiments.

Production of ethylene

Five freshly prepared disks were placed in a 22.5-ml glass vial. The glass vial was sealed with a silicone stopper and incubated at 25°C for 2 h or 8 h. During the 2-h incubation, a 2-ml sample of gas was removed at 20-min intervals from the vial with a syringe that was inserted through the silicone stopper. During the 8-h incubation, a sample gas was removed at 1-h intervals from the vial. To examine the effect of JA on the production of ethylene, five disks treated with or without 1 mM JA, were placed in a 22.5-ml glass vial. During the incubation, a 2-ml sample of gas was removed at 1-h intervals from the vial as described above. The amount of ethylene in each sample was measured with a gas chromatograph (GL-380; GL Sciences Inc., Tokyo, Japan), which was equipped with a flame ionization detector and an alumina column. The instrument was calibrated with a standard sample of ethylene (212 μl/ l).

Treatment of disks

Four freshly prepared disks were put into 20 ml of test solution and

the solution was vacuum-infiltrated into the disks for 2.5 min. Jasmonic acid (JA) was dissolved in 200 μl of dimethyl sulfoxide (DMSO) and added to 200 ml of 10 mM Mes buffer (pH 5.7) to give a final concentrations of 1 mM. The solution of JA was diluted with 10 mM Mes buffer (pH 5.7) to various concentrations, as indicated. Ibuprofen, piroxicam, and esculetin were dissolved in 200 μl of DMSO and added to 200 ml of 10 mM Mes buffer (pH 5.7) at final concentrations of 100 μM (ibuprofen) and 1 mM (piroxicam and esculetin), respectively. Control disks were treated with 10 mM Mes buffer (pH 5.7) containing 0.1 % DMSO. The treated disks were blotted and incubated in a Petri dish at 25°C for various times, as indicated. After incubation, disks were immediately frozen in liquid nitrogen and stored at –80°C prior to extraction of nucleic acids and JA.

Quantitative analysis of JA in disks of mesocarp tissue

JA was extracted from 15 g of tissue disks as described by Lehmann *et al.* (1995) for each assay. To estimate the recovery of JA during extraction, 400 ng of [$^2H_2$](±)-JA was added as described by Nojiri *et al.* (1992) to the extract during the first extraction step. After extraction with ether, the organic phase was further purified by isocratic (55% methanol, 0.1% acetic acid) reverse-phase high-performance liquid chromatography (HPLC) on a column of Lichrospher 100 RP-18 (15 μm; Hewlett-Packard, Wilmington, DE) at a flow rate of 1 ml/min. Fractions with the same retention time as JA, namely 9.5 min, were collected, evaporated to dryness, and dissolved in 200 μl of ethyl acetate. Each sample was methylated with freshly prepared diazomethane and the resultant methyl jasmonate was quantitated by GC-MS with selected ion monitoring (Seo *et al.* 1999). All data were corrected by reference to the recovery of [$^2H_2$](±)-JA .

Isolation of RNA

Total RNA was extracted from the disks of winter squash that had been incubated at 25°C for 1 h after slicing, as described by Prescott and Martin (1987). RNA was purified by successive precipitation in lithium chloride and ethanol and used for the synthesis of cDNA. Total RNA was also prepared from the tissue disks that had been treated with various chemicals for various times for Northern blotting analysis.

Isolation of cDNA for ACC synthase

To isolate fragments of a gene for ACC synthase, I synthesized two degenerate oligonucleotide primers that were homologous to conserved regions of ACC synthases. One primer was a mixed-sense primer, 5'-ATICA[A/G]ATGGGI[T/C]TIGCIGA[A/G]AA[T/C]CA-3', that was based on the amino acid sequence IQMGLAENQ and the other was a mixed-antisense primer, 5'-GTICCIA[A/G]IGG[A/G]TTIGAIGG[A/G]TT-3', based on the sequence NPSNPLGT. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with 1 μg of total RNA and a GeneAmp RNA PCR Kit (Perkin-Elmer Japan Co. Urayasu, Japan). The parameters for PCR were 35 cycles of heating at 94°C for 1 min, at 55°C for 2 min and at 72°C for 3 min. The products of PCR were analyzed by electrophoresis on a 2% agarose gel and recovered with a Mermaid kit (BIO 101, Inc., La Jolla, Calif., USA). The recovered product was cloned into the pCR II vector (Invitrogen, San Diego, Calif., USA) as described in the TA Cloning Instruction Manual from Invitrogen.

Sequencing of DNA

The cloned DNA was sequenced with an automated DNA sequencer (model 377; Perkin-Elmer) with a Taq Dye Primer Cycle Sequencing kit (Perkin-Elmer). The sequenced cDNA was designated WSACS2.

Preparation of a cDNA probe

The isolated cDNA was cleaved by *Eco*RI from the pCR II vector that had been amplified in *Escherichia coli* (INVαF'). It was purified by electrophoresis on a 2% agarose gel and recovered. The insert was labeled with [α-^{32}P]dCTP by the random-priming method with a Multiprime DNA labeling system (Amersham International, Amersham, Bucks., UK).

Northern blotting analysis

Total RNA was isolated from the disks of winter squash after they had been treated with the various test solutions described above. Total RNA (15 μg per lane) was subjected to electrophoresis on a 1.17% agarose gel that contained 0.66 M formaldehyde and then bands of RNA were transferred to a Gene Screen Plus membrane (Du Pont, Boston, Mass. USA) by capillary action with 10x SSC (1x SSC = 16.7 mM NaCl, 16.7 mM sodium citrate, pH 7.0), as recommended by the manufacturer of the membrane. After baking at 80°C, the membrane was preincubated in 1 M NaCl, 1% SDS and 10% (w/v) dextran sulfate (sodium salt) at 60°C for 2 h. The denatured ^{32}P-labeled probe and denatured salmon-sperm DNA were then added to the prehybridization solution, and the membrane was incubated at 60°C for 18 h. Post-hybridization washes were performed twice successively for 5 min each with 2x SSC at room temperature and twice for 30 min with 2x SSC, 1% SDS at 60°C. The washed membrane was subjected to autoradiography with an

intensifying screen. Then it was washed with boiling 0.01x SSC, 0.01% SDS to dehybridize the probe and the blot was rehybridized using a 23 S rRNA gene to confirm that equal amounts of RNA had been loaded in each lane. All experiments were repeated at least three times and typical results were shown in figures.

Chemicals

JA, ibuprofen, piroxicam and esculetin are products of Sigma (Sigma chemical Co., St. Louis, Mo., USA).

Results

Wound-induced production of ethylene

As reported by Hyodo *et al.* (1985), the rate of production of ethylene by winter-squash mesocarp tissues was increased by mechanical wounding (Fig. I-1). The rate of production was elevated within 1 h after wounding and continued to increase dramatically for 7 h.

Isolation and characterization of a cDNA for a fragment of ACC synthase

Since the production of ethylene by winter squash mesocarp tissues increased within 1 h of mechanical wounding, total RNA was isolated from tissues that had been incubated for 1 h after wounding for preparation of a cDNA for ACC synthase. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with the mixed oligonucleotide primers described above and 1 μg of total RNA as template. A DNA fragment of about 500 bp was amplified, isolated and ligated into the plasmid vector.

The cDNA fragment was characterized by sequencing. The cDNA was 451 bp long and encoded 150 amino acids. A homology search revealed that the sequence of the cDNA was identical at positions 187-637 to that of the cDNA for a wound-inducible ACC synthase (CM-ACS1; accession D01032) reported by Nakajima *et al.* (1990), with only one nucleotide difference, at position 497 from cytosine to thymine (Fig. I-2). Therefore, the cDNA was designated WSACS2.

Wound-induced accumulation of the *CM-ACS1* transcript

The expression of *CM-ACS1* in tissue disks was examined by Northern blotting analysis with the use of ^{32}P-labeled WSACS2 as the CM-ACS1 probe. The accumulation of the *CM-ACS1* transcript was induced within 1h after wounding, and increased gradually thereafter (Fig. I-3). This inducibility and pattern of expression was correlated with the characters of *CM-ACS1* that have been reported by Nakajima *et al.* (1990).

Effect of mechanical wounding on the JA content of mesocarp tissue

As shown in Figure I-4, the concentration of JA in freshly prepared mesocarp tissues was about 7 ng/g FW. The JA content remained low at 1 h and then rapidly increased to 2.7-folds and 5.0-folds at 2 h and 3 h after wounding, respectively. This is the first report showing that the synthesis of JA is stimulated by wounding in mesocarp tissue. Similar time-course increase in wound-induced JA has been reported in previous studies with soybean hypocotyls (Creelman *et al.* 1992) and tobacco leaves (Seo *et al.* 1995).

Effects of ibuprofen and other inhibitors of lipoxygenases on the accumulation of the *CM-ACS1* transcript

It has been suggested that JA is synthesized *via* a lipoxygenase-mediated metabolic pathway (Farmer and Ryan 1992). Therefore, the effects of inhibitors of lipoxygenases on the accumulation of the *CM-ACS1* transcript were examined in wounded mesocarp tissues of winter squash. As shown in Figure I-5a, ibuprofen slightly suppressed the accumulation of the *CM-ACS1* transcript. Esculetin and piroxicam also slightly suppressed the accumulation of the *CM-ACS1* transcript (Fig. I-5b). These results indicated that

lipoxygenases might be involved in the synthesis of some factor(s) responsible for the accumulation of the *CM-ACS1* transcript.

Effects of JA on the production of ethylene after wounding

The amount of JA in the disks was elevated 2 h after the wounding (Fig. I-4) and the expression of *CM-ACS1* was inhibited by the treatment of inhibitors of lipoxygenases (Fig. I-5). Therefore, the effect of JA on the production of ethylene was examined in wounded mesocarp tissue. As shown in Figure I-6, ethylene production was stimulated by JA within 1 h and, at each time point during the incubation, it was approximately twice that in control disks.

Effect of JA on the accumulation of the *CM-ACS1* transcript

In order to clarify the role of JA, the effects of JA on the expression of *CM-ACS1* was examined. As shown in Figure I-7a, JA stimulated the accumulation of the transcript within 30 min after the treatment, and the stimulatory effect 1 h after the treatment was dependent on the concentration of JA over a range of concentrations from 10^{-7} to 10^{-3} M (Fig. I-7b). Moreover, the stimulatory effect of JA on the expression of *CM-ACS1* continued for 7 h (Fig. I-8).

Discussion

In higher plants, mechanical wounding induces the production of ethylene *via* the expression of genes that encode wound-inducible ACC synthases (Sato *et al.* 1989, Nakajima *et al.* 1990, Lincoln *et al.* 1993). However, the regulation of such gene expression is poorly understood. As shown in Figures I-1 and I-2, the expression of the *CM-ACS1* gene, which encodes ACC synthase was induced in response to mechanical wounding followed by production of ethylene in mesocarp of winter squash (*Cucurbita maxima*). These results suggest that the production of ethylene was mainly regulated by the level of *CM-ACS1* transcript in wounded mesocarp tissue.

There are many reports showing that JA is produced by wounding and that JA causes numerous physiological responses, which include the expression of various defense-related genes (Farmer and Ryan 1990, Mason and Mullet 1990, Rickauer *et al.* 1997, Wasternack and Parthier 1997). To clarify the role of JA on the production of ethylene, JA was quantified in wounded mesocarp tissue of winter squash fruit. The amount of JA in disks was elevated 2 h after wounding and continued to increase for another hour (Fig. I-4). Since it has been proposed that JA is synthesized *via* a lipoxygenase-mediated pathway (Farmer and Ryan 1992), the effects of three different inhibitors of lipoxygenases, namely, ibuprofen (Ellis *et al.* 1993), esculetin (Fourinier *et al.* 1993) and piroxicam (Peña-Cortés *et al.* 1993) on the expression of *CM-ACS1* were examined. Esculetin, a flavonoid compound, was reported to inhibit tobacco lipoxygenase (Fouriner *et al.* 1993). Ibuprofen and piroxicam, anti-inflammatory drugs, were reported to inhibit soybean lipoxygenase (Sircar *et al.* 1983). As shown in Figure I-5, three different inhibitors of lipoxygenases (ibuprofen, esculetin and piroxicam) partially suppressed the accumulation of the *CM-ACS1* transcript. There was a difference in the rank order of inhibitory potency and in no case

was the effect complete. However, these results reflect those of Fournier *et al.* (1993), who suggested that the effects of such inhibitors varied among plant materials.

As shown in Figure I-6, ethylene production was stimulated by treatment of disks with JA. Moreover, the expression of *CM-ACS1* was stimulated within 30 min after treatment with JA and the stimulatory effect was dependent on the concentration of JA (Fig. I-7a and Fig. I-7b). These results suggest that JA stimulated the production of ethylene by activating the expression of *CM-ACS1* in the disks of mesocarp tissue. The stimulatory effect of JA on the expression of *CM-ACS1* continued for 7 h (Fig. I-8). This result indicates that the production of ethylene in wounded mesocarp tissue is regulated by JA for a long time after wounding.

In this part, I described that the amount of JA in mesocarp tissues of winter squash was increased by mechanical wounding and that the JA produced increased the accumulation of the transcript of a wound-inducible gene for ACC synthase (*CM-ACS1*). Furthermore, it was revealed that the effect of JA on the expression of *CM-ACS1* continued for a long time after wounding. This is the first report showing that JA can induce the expression of a wound-inducible gene for ACC synthase. However, it is difficult to explain the accumulation of the *CM-ACS1* transcript at the early stage (within 1 h) after wounding because the JA content remained low at 1h.

Figure I-1. Changes with time after wounding in the rate of production of ethylene by wounded mesocarp tissues of winter squash. Data represent means ± SD of results from three independent experiments.

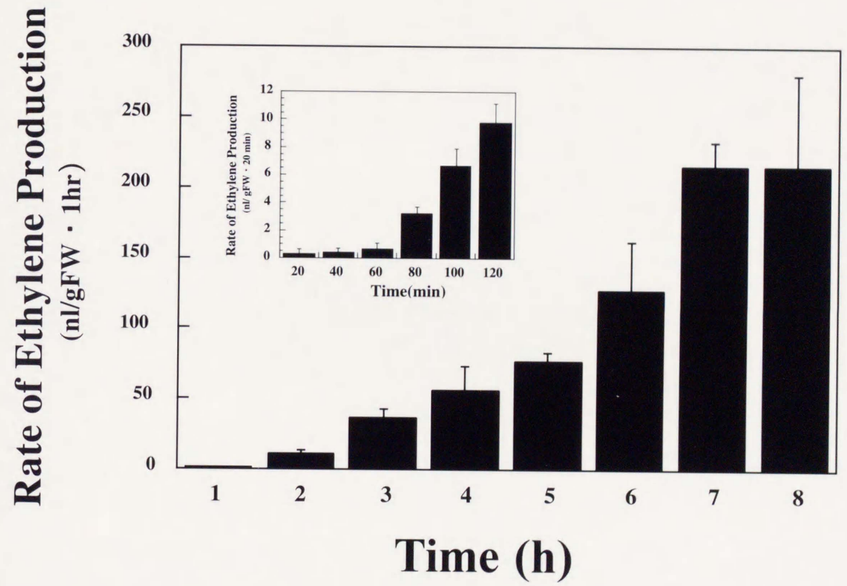


Figure I-2. Nucleotide sequence of the isolated cDNA fragment (WSACS2). The sequence of CM-ACS1 (cDNA for a wound-inducible ACC synthase) is shown below that of WSACS2. Asterisks show the same nucleotides.


```
WSACS2     1'        TCAGCTTTCCTTTGATATGATTGTTGACTGGATTAGAAAACACCCCGAAGCTTC
                     ************************************************************
CM-ACS1  181" AGAAAATCAGCTTTCCTTTGATATGATTGTTGACTGGATTAGAAAACACCCCGAAGCTTC

          55' GATTTGTACACCGAAAGGACTTGAGAGATTCAAAAGCATTGCCAACTTTCAAGATTACCA
              ************************************************************
         241" GATTTGTACACCGAAAGGACTTGAGAGATTCAAAAGCATTGCCAACTTTCAAGATTACCA

         115' TGGCTTACCAGAGTTTCGAAATGGGATTGCGAGTTTTATGGGGAAGGTAAGGGGTGGAAG
              ************************************************************
         301" TGGCTTACCAGAGTTTCGAAATGGGATTGCGAGTTTTATGGGGAAGGTAAGGGGTGGAAG

         175' GGTACAATTCGACCCGAGTCGGATTGTGATGGGTGGCGGTGCGACCGGAGCGAGCGAAAC
              ************************************************************
         361" GGTACAATTCGACCCGAGTCGGATTGTGATGGGTGGCGGTGCGACCGGAGCGAGCGAAAC

         235' CGTCATCTTTTGTTTGGCGGATCCGGGGGATGCTTTTTTGGTTCCTTCTCCATACTATGC
              ************************************************************
         421" CGTCATCTTTTGTTTGGCGGATCCGGGGGATGCTTTTTTGGTTCCTTCTCCATACTATGC

         295' TGCATTTGATCGAGATTTGAAATGGCGAACACGAGCACAAATAATTCGGGTCCATTGCAA
              ********** ***************************************************
         481" TGCATTTGATCGAGATCTGAAATGGCGAACACGAGCACAAATAATTCGGGTCCATTGCAA

         355' CAGCTCGAACAACTTCCAAGTCACAAAGGCAGCCTTAGAAATAGCCTACAAAAAGGCTCA
              ************************************************************
         541" CAGCTCGAACAACTTCCAAGTCACAAAGGCAGCCTTAGAAATAGCCTACAAAAAGGCTCA

         415' AGAGGCCAACATCAAAGTGAAGGGTGTTATAATCACC
              ************************************
         601" AGAGGCCAACATCAAAGTGAAGGGTGTTATAATCACCAATCCCTCAAATCCCTTAGGCAC
```

Figure I-3. Changes with time after wounding in the accumulation of the *CM-ACS1* transcript. The levels of transcript were analyzed by Northern blotting. The experiment was repeated at least 3 times and typical results are shown.

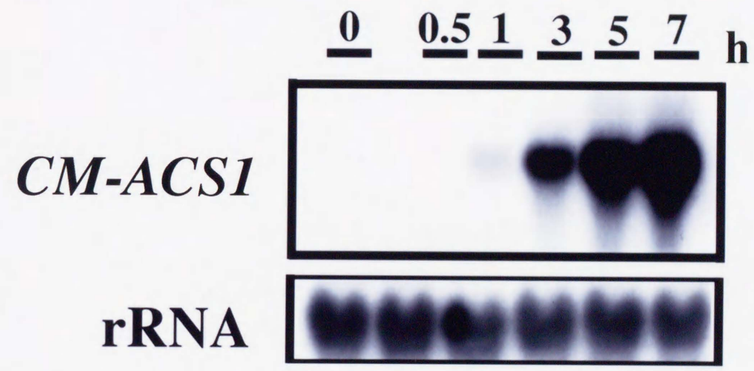


Figure I-4. Time course of changes in the JA content of wounded mesocarp tissues of winter squash. Tissue disks were incubated for various times as indicated prior to quantitation of JA by gas chromatography-mass spectrometry. Data represent means ± SD of results from three independent experiments.

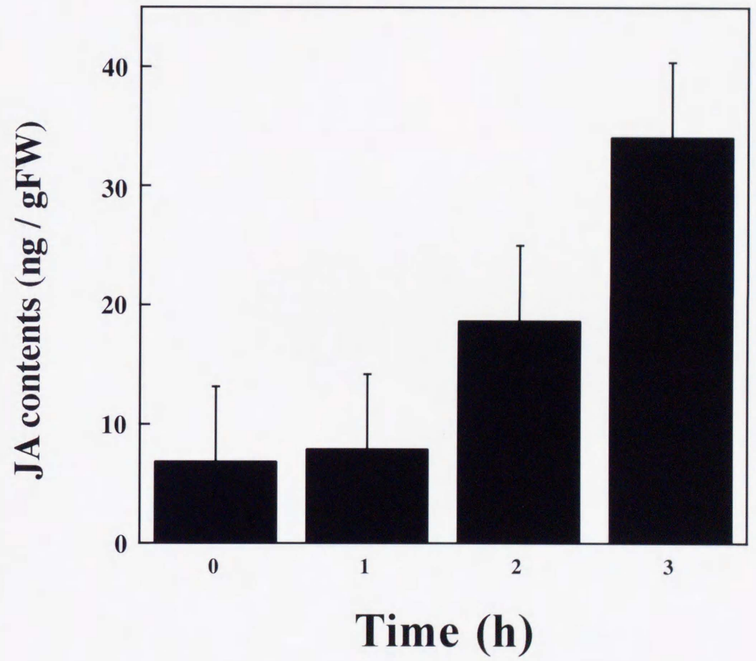


Figure I-5. The effects of three inhibitors of lipoxygenases on the expression of *CM-ACS1*. Disks were treated for the indicated times (min) with 100 μM ibuprofen (**a**), with 1 mM esculetin or with 1 mM piroxicam (**b**) to inhibit endogenous lipoxygenases. Control disks were treated with 10 mM Mes buffer (pH 5.7) containing 0.1 % (v/v) DMSO. The levels of transcript were analyzed by Northern blotting. The experiment was repeated at least 3 times and typical results are shown.

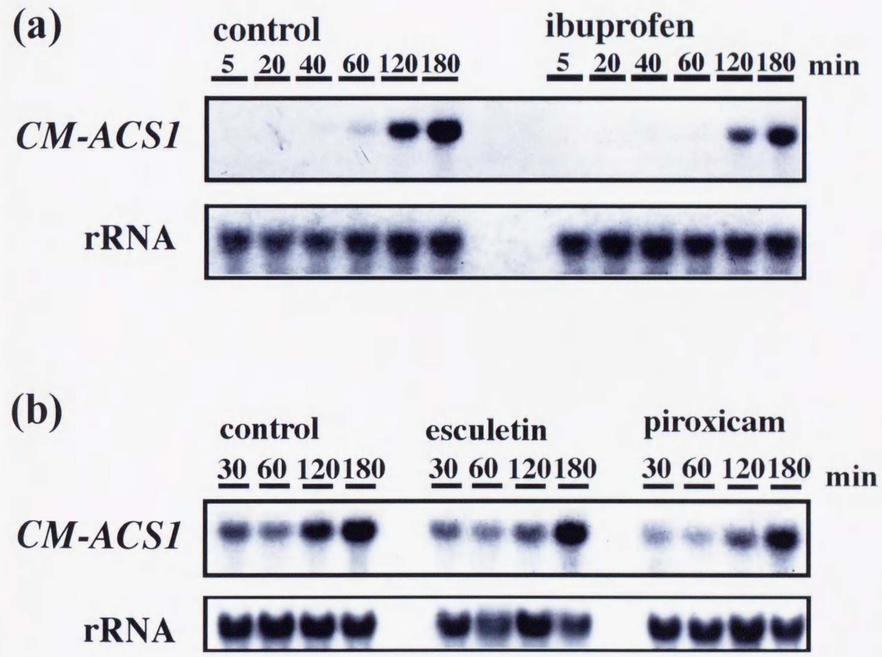


Figure I-6. The effects of JA on the production of ethylene by disks of mesocarp tissue. Disks were treated with (closed bar) and without (open bar) 1 mM JA and the production of ethylene was measured at the indicated times. Data represent means ± SD of results from three independent experiments.

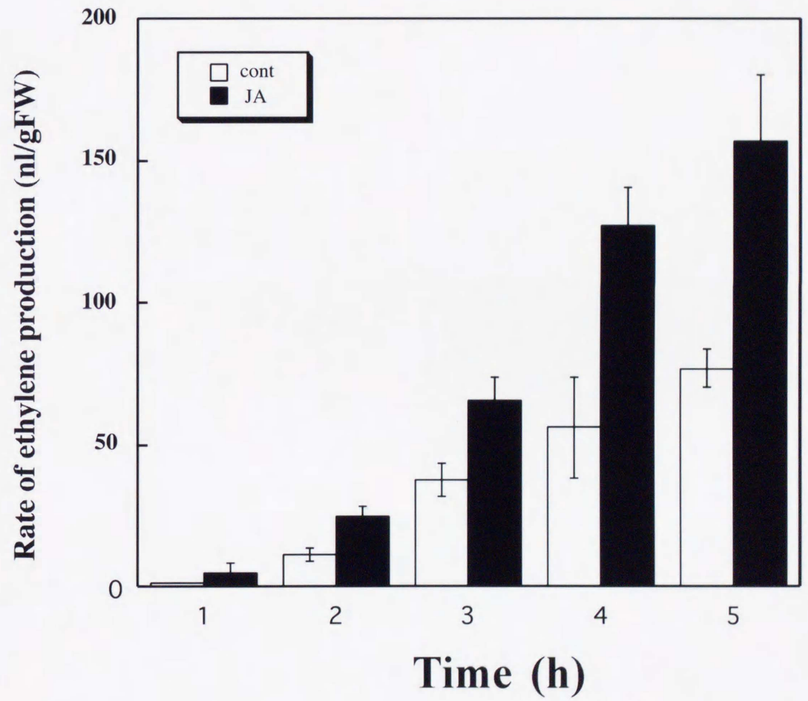


Figure I-7. The effects of JA on the accumulation of the *CM-ACS1* transcript. **(a)** Time course of accumulation of the transcript (during 3-h incubation after wounding). Disks were treated with and without 1 mM JA for the indicated times prior to analysis of transcript by Northern blotting. **(b)** Effects of the concentration of JA. Disks were incubated for 1 h with JA at various concentrations prior to analysis of transcripts by Northern blotting. The results obtained from disks incubated for 0 h and for 1 h with buffer only are indicated by c1 and c2, respectively. The experiment was repeated at least three times and typical results are shown.

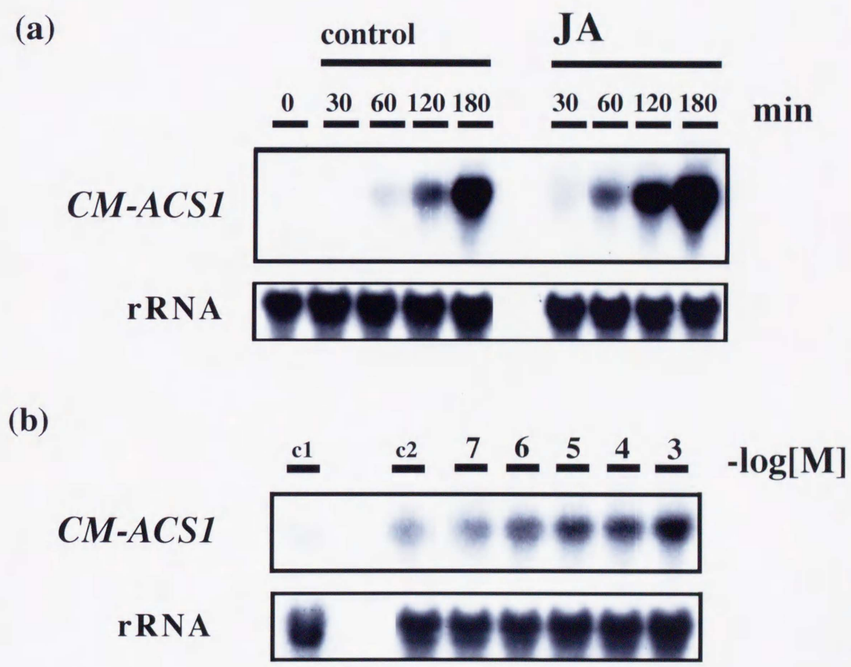
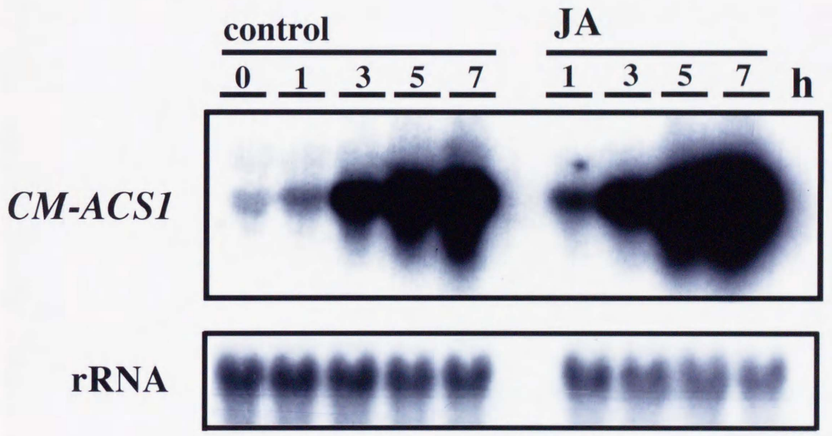


Figure I-8. The effects of JA on the accumulation of the *CM-ACS1* transcript. Time course of accumulation of the transcript (during 7-h incubation after wounding). Disks were treated with and without 1 mM JA for the indicated times prior to analysis of transcript by Northern blotting. The experiment was repeated at least three times and typical results are shown.



Part II. Effects of reactive oxygen species on production of ethylene and expression of *CM-ACS1*

Introduction

As described in part I, the accumulation of the transcript of a wound-inducible gene for ACC synthase (*CM-ACS1*) increased gradually after wounding in mesocarp tissues of winter squash, and this pattern of expression was correlated with that of production of ethylene after wounding. Moreover, the expression of *CM-ACS1* was stimulated by JA, and the effect of JA continued for a long time after the treatment. However, the accumulation of the transcript increased within 1 h after wounding, prior to the increase of endogenous JA level (within 2 h). This result might suggest that the expression of *CM-ACS1* in the early stage (within 1 h) was regulated by another mediator of the wound signal, except for JA.

Reactive oxygen species (ROS) are produced immediately and transiently, and then their levels decrease after wounding and attack by pathogens. Doke *et al.* (1991) reported the transient (duration, 30 min) activation of O_2^- -generating systems in potato tuber tissues within 2-3 min after slicing. Iodonium compounds inhibit the activity of a variety of flavoproteins and at least one hemoprotein by inhibiting electron-transport system (O'Donnell *et al.* 1993). O'Donnell *et al.* (1993) reported that diphenylene iodonium (DPI), one kind of iodonium compound, inhibited the activity of plasma-membrane NAD(P)H oxidase, which is a flavoprotein and the principal enzyme involved in production of ROS in phagocytes. It has also been reported that this enzyme participates in the production of ROS in plants and that DPI inhibits the production of ROS in plants and in suspension-cultured plant cells upon infection by a pathogen (oxidative burst; Levine *et al.* 1994, Jabs *et al.* 1996).

Moreover, it has been suggested that ROS induced by wounding or attack by pathogens might be involved directly in defense-related responses, such as the generation of antimicrobial agents in tobacco (Peng and Kuc

1993), lignification in cucumber (Dean and Kuc 1987), production of phytoalexin in suspension-cultured soybean cells (Apostol *et al.* 1989), peroxidation of lipid in suspension cultured bean cells (Rogers *et al.* 1988), and in oxidative cross-linking of structural proteins in cell walls of suspension-cultured soybean and bean cells (Bradley *et al.* 1992). The possibility has also been discussed that ROS might regulate the gene expression of one kind of pathogenesis-related (PR) protein in tobacco leaves (Chen *et al.* 1993). However, in studies of the responses of higher plants to wounding, the relationships between the expression of the gene for wound-inducible ACC synthase and the roles of ROS have not yet been clarified.

In this part, I examined the effect of ROS on the expression of *CM-ACS1* in wounded mesocarp tissues.

Materials and Methods

Plant materials

Fruits of winter squash (*Cucurbita maxima* Duch cv. Ebisu) were purchased from a local market and stored at 11°C prior to use. Each fruit was kept at 25°C for 5 h before experiments, and the mesocarp was cut into disks 2 mm thick and 11 mm in diameter as described in Materials and Methods of Part I. The tissue disks were pooled and selected randomly for use in all experiments.

Treatment of disks

Four disks were put into 20 ml of test solution and the solution was vacuum-infiltrated into the disks for 2.5 min. Diphenylene iodonium (DPI) and H_2O_2 were dissolved in 10 mM Mes buffer (pH 7.5) at final concentrations of 100 μM and 10 mM, respectively. The solution of DPI was diluted with 10 mM Mes buffer (pH 7.5) to the various concentrations indicated. Xanthine oxidase and superoxide dismutase (SOD) were dissolved in 10 mM Mes buffer (pH 7.5) at 0.5 unit/ml and 50 units/ml, respectively. The solution of xanthine oxidase was mixed with a solution of xanthine [10 mM Mes buffer (pH 7.5) that contained 100 μM xanthine] just before vacuum-infiltration. Control disks were treated with 10 mM Mes buffer (pH 7.5). The treated disks were incubated at 25°C for various times, as indicated. After incubation, disks were immediately frozen in liquid nitrogen and stored at –80°C prior to extraction of nucleic acids.

Chemiluminescence assay of H_2O_2

The production of H_2O_2 by the disks was measured by a chemiluminescence assay with luminol as the active reagent and a scintillation counter (5000 AT; Beckman, Fullerton, Calif., USA) in the single-photon mode, as described by Auh *et al.* (1995) with slight modification. In this assay, chemiluminescence depends on the presence of both H_2O_2 and peroxidase (Seitz 1978). A freshly prepared disk of winter squash was placed in a scintillation vial that contained 2 ml of peroxidase solution (1 unit of peroxidase from horseradish per ml of 20 mM potassium phosphate buffer, pH 7.4) within 30 s after preparation of the disk. The reaction was started by the addition of 200 μl of 20 mM potassium phosphate buffer (pH 7.4) that contained 1 mM luminol. Chemiluminescence was recorded over 10-s intervals for 170 s. In some experiments, 1 mM diethyldithiocarbamic acid (DDC) was added to the reaction mixture as an inhibitor of SOD in the disks to block the conversion of O_2^- to H_2O_2.

Chemiluminescence assay of O_2^-

The generation of O_2^- by the disks was monitored by a chemiluminescence assay with lucigenin as the active reagent. This assay is specific for O_2^- and progressed nonenzymatically (Corbisier *et al.* 1987). A freshly prepared disk of winter squash was placed in a scintillation vial that contained 2 ml of the reaction buffer (Gly-NaOH buffer, pH 9.0, containing 1 mM EDTA, 1 mM sodium salicylate and 1 mM DDC) as described above. The DDC was added to the reaction mixture to inhibit the conversion of O_2^- to H_2O_2 and O_2 by endogenous SOD. The reaction was started by the addition of 200 μl of the above buffer that contained 1 mM lucigenin. Data were recorded as described above for the quantitation of H_2O_2. In experiments in which an inhibitor was added, DPI was first dissolved in DMSO and then diluted with the reaction buffer to 1, 5, and 10 mM. The concentration of

DMSO was kept at 0.1 % in all solution including the control.

Production of ethylene

Five disks which were treated with or without DPI solution were placed in a 22.5-ml glass vial. The glass vial was sealed with a silicone stopper and incubated at 25°C for various times, as indicated. During the incubation, a 2-ml sample of gas was removed at 1-h intervals from the vial with a syringe that was inserted through the silicone stopper. The amount of ethylene in each sample was measured with the same methods as described in Materials and Methods of Part I.

Isolation of RNA

The conditions for the isolation of RNA were the same as described in Materials and Methods of Part I.

Preparation of a cDNA probe

Because the sequence of WSACS2 obtained in this study (Part I) is identical to that of the CM-ACS1 (Nakajima *et al.* 1990) with only one nucleotide difference, the insert of WSACS2 was used as the CM-ACS1 probe. The insert of WSACS2 were isolated from the plasmid and labeled as described in Materials and Methods of Part I.

Northern blotting analysis

Total RNA (15 μg per lane) was subjected to electrophoresis on a formaldehyde gel and transferred to a Gene Screen Plus membrane (Du Pont,

Boston, Mass. USA) as described in Materials and Methods of Part I. The membrane was hybridized with ^{32}P-labeled CM-ACS1 probe, and conditions for the hybridization and wash were the same as described in Materials and Methods of Part I. The washed membrane was subjected to autoradiography with an intensifying screen. Then it was washed with boiling 0.01x SSC, 0.01% SDS to dehybridize the probe and the blot was rehybridized using a 23 S rRNA gene to confirm that equal amounts of RNA had been loaded in each lane. All experiments were repeated at least three times and typical results were shown in figures.

Chemicals

Diphenylene iodonium (DPI), DDC, luminol, lucigenin, xanthine, xanthine oxidase (from buttermilk), SOD (from horseradish), and peroxidase (from horseradish) are products of Sigma (Sigma chemical Co., St. Louis, Mo., USA).

Results

Quantitation of the wound-induced generation of ROS using a chemiluminescence assay

It has been reported that ROS are produced very rapidly and then their levels decrease transiently when plants are subjected to mechanical wounding (Doke *et al*. 1991). Therefore, levels of ROS were measured, in wounded mesocarp tissues of winter squash, using two different chemiluminescence reagents. In the experiment with luminol, the rate of production of the chemiluminescent signal reached a maximum level 130 s after the start of measurements and decreased slightly thereafter (Fig. II-1a). The increase in chemiluminescence was suppressed by the addition of DDC, to inhibit the activity of SOD which catalyzes the conversion of O_2^- to H_2O_2 and O_2 (Fig. II-1a). These results suggested that H_2O_2 was produced by SOD from O_2^- that had been generated by wounding. In the experiment with lucigenin, by contrast, the rate of production of the chemiluminescent signal reached a maximum 170 s after the start of measurements. Moreover, the increase in chemiluminescence was suppressed by the addition of DPI, an inhibitor of NAD(P)H-oxidase, in a dose-dependent manner (Fig. II-1b). These results indicated that the chemiluminescent signal was derived from ROS that had been produced by NAD(P)H oxidase. The rate of generation of O_2^- by the wounded tissue disks began to increase 60 s after wounding, remained high between 60 and 140 s, and decreased thereafter (Fig. II-1b). The time course of generation of H_2O_2, measured without DDC (Fig. II-1a), reflected the changes in the rate of generation of O_2^-, measured in the presence of DDC (Fig. II-1b).

Effect of DPI on the production of ethylene

As shown in Figure II-1, ROS were generated rapidly by mechanical wounding of mesocarp tissue and the generation was inhibited by DPI. To examine whether ROS might affect the production of ethylene, the production of ethylene was measured using the disks that had been treated with and without DPI. As shown in Figure II-2, ethylene production was considerably inhibited especially at the early period after DPI treatment.

Effects of ROS on the accumulation of the *CM-ACS1* transcript

The wound-induced production of ethylene in mesocarp tissues was inhibited by the treatment with DPI (Fig. II-2). Therefore, to examine the effects of DPI on the expression of *CM-ACS1*, the accumulation of *CM-ACS1* mRNA in tissue disks that had been treated with and without DPI was measured by Northern blotting analysis. As shown in Figure II-3a, DPI strongly inhibited the accumulation of the transcript and the inhibitory effect was dependent on the concentration of DPI from 10^{-7} M to 10^{-4} M (Fig. II-3b). These results suggested that ROS, that had been generated by wounding, played an important role in induction of the synthesis of the *CM-ACS1* transcript.

To examine the role of ROS in the expression in more detail, the experiments were done to apply ROS exogenously to the disks and to eliminate ROS enzymatically from the disks. As shown in Figure II-4, the addition of xanthine and xanthine oxidase (X-XO), which form a superoxide-generating system, to the disks strongly induced the accumulation of the *CM-ACS1* transcript within 30 min and the level of the transcript decreased thereafter. The addition of SOD repressed the accumulation of the *CM-ACS1* transcript (Fig. II-4). The addition of H_2O_2 to the disks did not affect the induction (Fig. II-4). These results indicated that O_2^-, one type of

ROS, might participate in the expression of the gene for the wound-inducible ACC synthase.

Discussion

As shown in Figures I-7 and I-8, the expression of a gene for wound-inducible ACC synthase (*CM-ACS1*) was stimulated by JA, and the stimulatory effect of JA continued for a long time after the treatment. However, the accumulation of the transcript increased within 1 h after wounding (Fig. I-3), prior to the increase of endogenous JA level (within 2 h; Fig. I-4). This result might suggest that the expression of *CM-ACS1* in the early stage (within 1 h) after wounding was regulated by another mediator of the wound signal, except for JA. Reactive oxygen species (ROS) are generated by mechanical wounding (Doke *et al.* 1991) and the generation of ROS occurs prior to the production of ethylene and JA. The roles of ROS in the control of gene expression have been discussed for some plant defense genes (Chen *et al.* 1993, Levine *et al.* 1994) but not for wound-inducible ACC synthases.

As shown in Figure II-1, the generation of O_2^- was detectable immediately after wounding and the rate of generation of O_2^- reached a maximum within a few minutes. The addition of DPI, to inhibit the generation of O_2^- (Fig. II-1), inhibited the wound-induced production of ethylene and accumulation of the *CM-ACS1* transcript (Fig. II-2 and Fig. II-3). To inhibit the generation of O_2^-, DPI was needed at much higher concentrations than to inhibit both the production of ethylene and the expression of *CM-ACS1*. This might be due to a difference in the DPI treatment of the disks. As the generation of ROS was very rapid response to wounding, it was difficult to treat the disks with DPI solution using vacuum-infiltration in the experiment of O_2^- generation. Therefore, the disks were put in a solution of higher concentration of DPI. As shown in Figure II-4, exposure of the disks to an extracellular O_2^--generating system, X-XO, strongly induced the expression of *CM-ACS1* 30 min after the treatment and

the level of the transcript fell thereafter. The decrease in expression might be attributable to feedback inhibition by excess O_2^- (Legendre *et al.* 1993). The addition of SOD, to convert O_2^- to H_2O_2 and O_2, inhibited the accumulation of the *CM-ACS1* transcript (Fig. II-4). The limited extent of the inhibitory effect might have been due to the inability of SOD in the cells to convert O_2^- to H_2O_2 and O_2. In fact, the addition of H_2O_2 did not stimulate the expression of *CM-ACS1* (Fig. II-4).

In this part, I described that the expression of the *CM-ACS1* could be induced by O_2^-, one type of ROS, that was generated after wounding by NAD(P)H-oxidase in the plasma membrane.

Figure II-1. The rate of generation of O_2^- in mesocarp tissues of winter squash after wounding. **(a)** Effects of diethyldithiocarbamic acid (DDC) on the rate of generation of O_2^- after wounding. H_2O_2 was produced by endogenous SOD from the O_2^- generated and was quantitated with luminol as the chemiluminescence reagent. The chemiluminescent signals produced during the 10 s prior to each indicated time were plotted at the representative times indicated. ○ , Control ; ● , without a disk ; ◇ , + 1 mM DDC. **(b)** Effects of DPI on the generation of O_2^-. The O_2^- generated was quantitated with lucigenin as the chemiluminescence reagent. The chemiluminescent signals produced during the 10 s prior to each indicated time were plotted at the representative times indicated. ○ , Control ; ● , without a disk ; 1 mM (◇), 5 mM (△), and 10 mM (□) DPI. Data represent means ± SD of results obtained from three independent experiments.

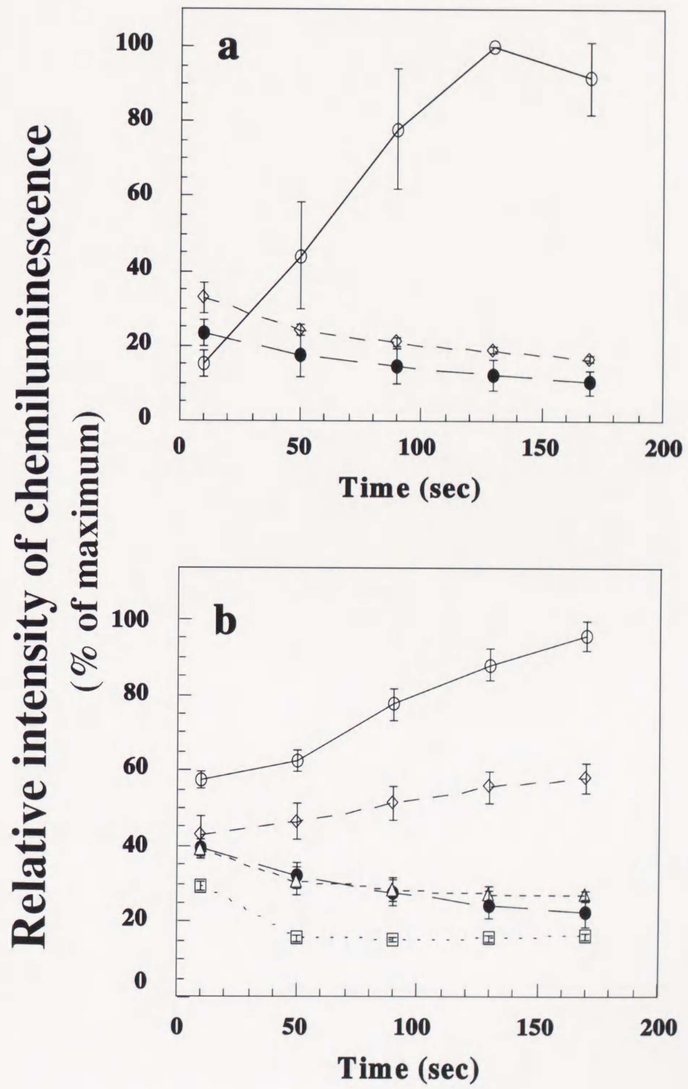


Figure II-2. The effects of an inhibitor of ROS-generating systems (diphenylene iodonium, DPI) on the production of ethylene. Disks treated with (closed bar) and without (open bar) DPI were incubated for various times as indicated and ethylene was quantitated at 1-h intervals. Data represent means ± SD of results from three independent experiments.

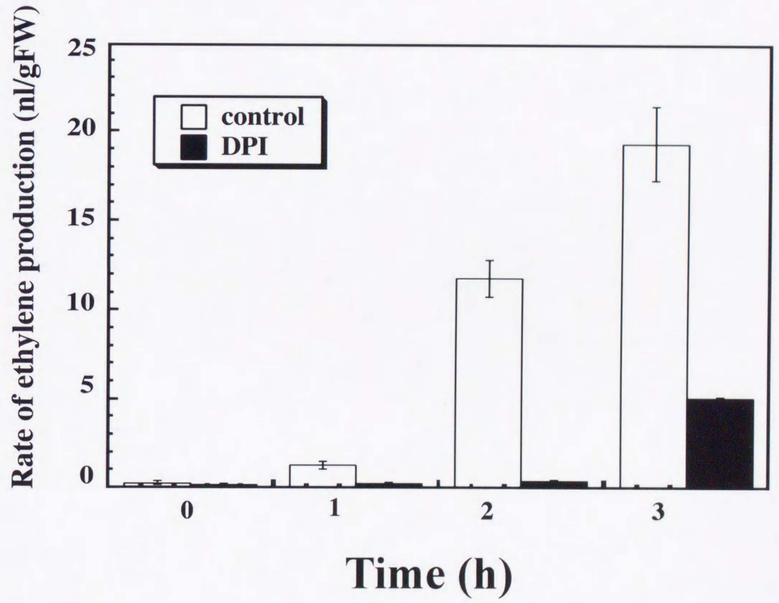


Figure II-3. The effects of DPI on the expression of *CM-ACS1*. **(a)** Time course. Winter-squash disks were treated with or without 100 μM DPI and incubated for the indicated times (h) prior to analysis by Northern blotting. **(b)** Effect of DPI concentration. The disks were incubated for 1 h with various concentrations of DPI prior to analysis by Northern blotting.

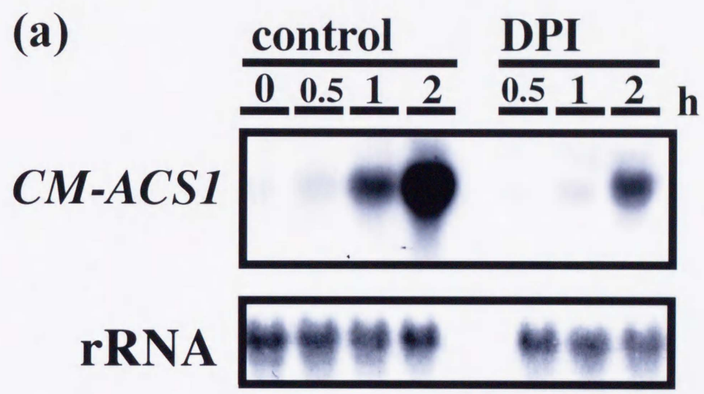
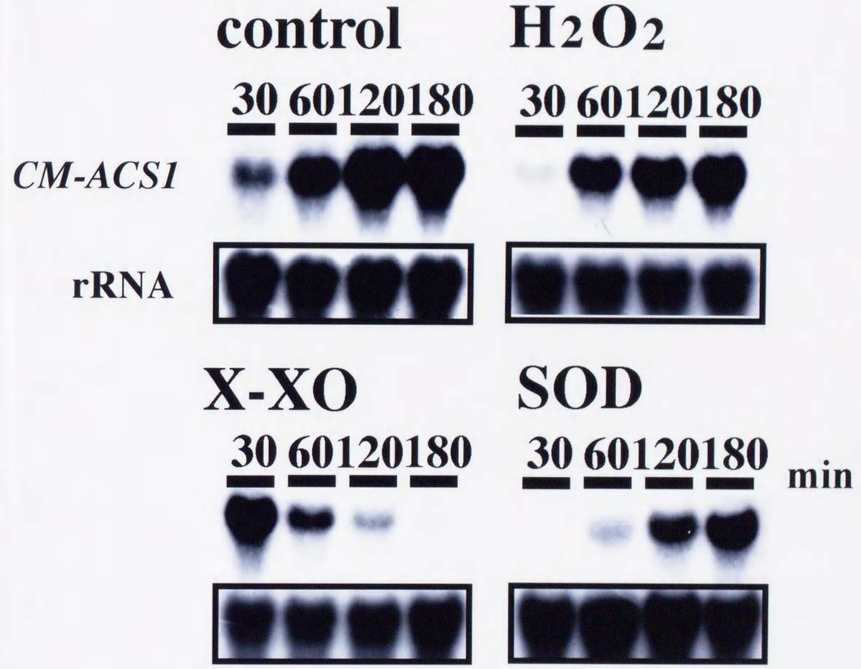


Figure II-4. Effects of the exogenous application of ROS and of the enzymatic elimination of ROS on the expression of *CM-ACS1*. Winter squash disks were treated for the times indicated (min) with 10 mM H_2O_2, with a mixture of 100 μM xanthine and 0.5 unit/ml xanthine oxidase (X-XO) to generate O_2^-, and with 50 units/ml SOD to eliminate O_2^-, respectively. Then samples were analyzed by Northern blotting. The boxed bands represent 23S rRNA. The experiment was repeated at least 3 times and typical results are shown.



Part III. Separate regulation of the expression of *CM-ACS1* by JA and ROS

Introduction

The expression of genes related to plant defenses is regulated by both ethylene and JA, and the simultaneous activation of signaling pathways triggered by ethylene and by jasmonate is needed to induce the expression of defense genes, as revealed by studies of mutants of *Arabidopsis* that are unable to respond either to ethylene or to JA (Phenninckx *et al.* 1999). These observations suggest that cross-talk occurs between the ethylene and jasmonate signaling pathways. Yu *et al.* (1998) reported that methyljasmonate (MeJA) and ROS enhanced nopaline synthase promoter expression *via* separate pathways. However, the relationships between the generation of ROS, the production of JA and the induction of gene expression after wounding have not been examined. As described in part I and part II, the wound-induced expression of *CM-ACS1* was stimulated by both JA and ROS, but the relationship between the effects of JA and ROS on the expression of *CM-ACS1* was not clear. However, two possibilities were considered. (i) O_2^- and JA might independently affect the expression of the *CM-ACS1*, and, indeed, there was a difference in the extent of gene expression 30 min after wounding between O_2^- and JA (Fig. I-6a and Fig. II-4). (ii) The accumulation of linolenic acid might be stimulated by O_2^- that had been generated immediately after wounding. Conconi *et al.* (1996) reported that linolenic acid, a starting material in the biosynthetic pathway of JA, was accumulated upon wounding of tomato leaves. Linolenic acid is converted to JA *via* a lipoxygenase-mediated pathway (Farmer and Ryan 1992) and JA induced the expression of the *CM-ACS1*.

In order to clarify the relationship between ROS and JA in the expression of the *CM-ACS1* gene in wounded mesocarp tissues of winter squash fruit, the effects of ROS on the JA content of disks and the effects of inhibitors of the production of JA and ROS on the accumulation of the *CM-*

ACS1 transcript were examined.

Materials and Methods

Plant materials

Fruits of winter squash (*Cucurbita maxima* Duch cv. Ebisu) were purchased from a local market and stored at 11°C prior to use. Each fruit was kept at 25°C for 5 h before experiments, and the mesocarp was cut into disks 2 mm thick and 11 mm in diameter as described in Materials and Methods of Part I. The tissue disks were pooled and selected randomly for use in all experiments.

Treatment of disks

To examine the effects of chemicals on the accumulation of the *CM-ACS1* transcript, four freshly prepared disks were put into 20 ml of test solution and the solution was vacuum-infiltrated into the disks for 2.5 min, as described in Part I. The treated disks were blotted and incubated in a Petri-dish at 25°C for various times, as indicated. Acetylsalicylic acid and DPI were dissolved in 10 mM Mes buffer (pH 5.7) at final concentrations of 5 mM and 100 μM, respectively. In the control experiment, disks were treated with 10 mM Mes buffer (pH 5.7). To examine the effects of acetylsalicylic acid on JA content and ethylene production, disks were incubated in a Petri-dish for 1 h before the treatment with the solution of acetylsalicylic acid. After incubation, the disks were immediately frozen in liquid nitrogen and stored at –80°C prior to extraction of nucleic acids and JA.

Quantitative analysis of JA in disks of mesocarp tissue

JA was extracted from 15 g of tissue disks treated with or without test

solutions and quantitated as described in Materials and Methods of Part I.

Production of ethylene

Five disks which were treated with or without test solution were placed in a 22.5-ml glass vial. The glass vial was sealed with a silicone stopper and incubated at 25°C for various times, as indicated. During the incubation, a 2-ml sample of gas was removed at 1-h intervals from the vial with a syringe that was inserted through the silicone stopper. The amount of ethylene in each sample was measured with the same methods as described in Materials and Methods of Part I.

Isolation of RNA

The conditions for the isolation of RNA were the same as described in Materials and Methods of Part I.

Preparation of a cDNA probe

The insert of WSACS2 were isolated from the plasmid and labeled as described in Materials and Methods of Part I.

Northern blotting analysis

Total RNA (15 μg per lane) was subjected to electrophoresis on a formaldehyde gel and transferred to a Gene Screen Plus membrane (Du Pont, Boston, Mass. USA) as described in Materials and Methods of Part I. The membrane was hybridized with ^{32}P-labeled CM-ACS1 probe, and conditions for the hybridization and wash were the same as described in Materials and

Methods of Part I. The washed membrane was subjected to autoradiography with an intensifying screen. Then it was washed with boiling 0.01x SSC, 0.01% SDS to dehybridize the probe and the blot was rehybridized using a 23 S rRNA gene to confirm that equal amounts of RNA had been loaded in each lane. All experiments were repeated at least three times and typical results were shown in figures.

Chemicals

Acetylsalicylic acid and diphenylene iodonium (DPI) are products of Sigma (Sigma chemical Co., St. Louis, Mo., USA).

Results

Effects of acetylsalicylic acid on the production of JA and ethylene

It has been reported that acetylsalicylic acid inhibits the synthesis of JA and suppresses the expression of a gene for a proteinase inhibitor in tomato plant (Peña-Cortés *et al.* 1993, Doares *et al.* 1995). As shown in Figure III-1a, acetylsalicylic acid inhibited the accumulation of JA in disks of winter squash mesocarp tissue, and it also inhibited the production of ethylene (Fig. III-1b). However, the inhibitory effect of acetylsalicylic acid on ethylene production was low 1 h after wounding and then increased gradually.

Effects of DPI on the production of JA and ethylene

As described in part II, ROS were generated rapidly by mechanical wounding of mesocarp tissue and the accumulation of *CM-ACS1* transcript was suppressed by treatment of the tissue with DPI, an inhibitor of the generation of ROS (Levine *et al.* 1994, Jabs *et al.* 1996, Tenhaken *et al.* 1998). To examine whether ROS might affect the production of JA, the contents of JA were quantified in disks that had been treated with and without DPI. As shown in Figure III-2a, the production of JA was unaffected by the treatment with DPI, indicating that ROS were not involved in the enhanced synthesis of JA. However, ethylene production was considerably inhibited especially at the early period after the treatment with DPI.

Effects of acetylsalicylic acid and DPI on the accumulation of the *CM-ACS1* transcript

DPI suppressed the accumulation of the *CM-ACS1* transcript both 1

and 2 h after the treatment, but renewed accumulation of the transcript was evident at 3 h (Fig. III-3). In contrast to the results of treatment with DPI, acetylsalicylic acid did not inhibit the accumulation of the transcript 1 h after the treatment. However, the clear inhibitory effects of acetylsalicylic acid were observed 2 and 3 h after the treatment. In particular, at 3 h after treatment, the accumulation of the transcript was strongly inhibited by acetylsalicylic acid, rather than by DPI (Fig. III-3). These results suggested that the accumulation of the *CM-ACS1* transcript at the early stage was mainly induced by ROS and at the later stage of the 3-h incubation by JA, respectively.

Discussion

As described in part I and part II of this thesis, the wound-induced accumulation of the *CM-ACS1* transcript was stimulated by both ROS and JA. In this part, I examined the effects of ROS on the JA content of disks and the effects of inhibitors of the production of JA and ROS on the accumulation of the *CM-ACS1* transcript to clarify the relationships between ROS and JA in the expression of *CM-ACS1*.

In part II of this thesis, it was demonstrated that the generation of ROS in mesocarp tissue of winter squash began immediately after wounding and that the ROS generated induced the accumulation of the *CM-ACS1* transcript. Therefore, JA contents were quantified using the disks treated with and without DPI, an inhibitor of the generation of ROS (Levine *et al*. 1994, Jabs *et al*. 1996, Tenhaken *et al*. 1998). In contrast to the inhibitory effects of acetylsalicylic acid which is an inhibitor of JA synthesis (Fig. III-1), DPI had no effect on the synthesis of JA in tissue disks (Fig. III-2). These results suggest that the effects of ROS on the accumulation of the *CM-ACS1* transcript were not caused by an increase in the synthesis of JA in the disks.

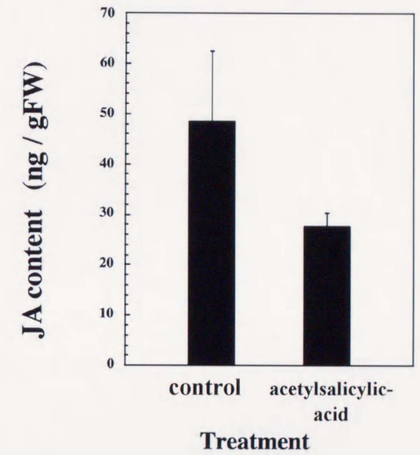
To clarify the roles of ROS and JA in the accumulation of the *CM-ACS1* transcript, the effects of acetylsalicylic acid and DPI on levels of the transcript were examined. As shown in Figure III-3, the accumulation of the *CM-ACS1* transcript was inhibited both by acetylsalicylic acid and by DPI but the inhibitory effects of the two inhibitors were different. The expression of the transcript after 1 and 2 h was strongly suppressed when disks were treated with DPI immediately after wounding, but the inhibitory effect of DPI observed after 1 and 2 h was partially reversed at 3 h (Fig. III-3). DPI, an iodonium compound, inhibits the activity of NAD(P)H oxidase in the plasma membrane. This enzyme is the most important enzyme in the intracellular generation of ROS (O'Donnell *et al*. 1993) and it is transiently activated

immediately after wounding (Doke *et al.* 1991). Therefore, these results suggest that ROS, generated immediately after wounding, was needed for induction of the expression of *CM-ACS1* at the early stages after wounding (within 2 h). The fact that the clear inhibitory effect of DPI continued for 2 h might suggest that ROS affected on the expression of *CM-ACS1* indirectly *via* the induction of another signal, except for JA. In contrast to the effects of DPI, acetylsalicylic acid had no detectable effect on the expression of the *CM-ACS1* transcript at 1 h. However, an inhibitory effect of acetylsalicylic acid could be observed 2 h after treatment and an even greater inhibitory effect was observed at 3 h (Fig. III-3). The timing of the action of acetylsalicylic acid on the accumulation of the *CM-ACS1* transcript coincided with the time course of the increase in the level of JA (Fig. I-4). These results suggest that acetylsalicylic acid suppressed the expression of *CM-ACS1* by inhibiting the synthesis of JA at the later stages of the incubation (after 2 h) and thereby inhibited the production of ethylene.

In this part, I showed that the expression of *CM-ACS1* was stimulated by ROS at the early stage (within 2 h) and by JA at the later stage (after 2 h) during 3 h-incubation after wounding. Moreover, I demonstrated that the ROS were not involved in the enhanced synthesis of JA. These results indicate that the expression of *CM-ACS1* is separately regulated by each of ROS and JA, which are independently generated at different period after wounding.

Figure III-1. Effects of acetylsalicylic acid on the changes in levels of JA and the production of ethylene. (**a**) The effect of acetylsalicylic acid on JA content. Freshly prepared disks were incubated in a Petri dish for 1 h and then treated with and without 5 mM acetylsalicylic acid as described in Materials and Methods. The treated disks were incubated for 2 h prior to the extraction of JA. (**b**) The effects of acetylsalicylic acid on ethylene production. Disks treated with (closed bar) and without (open bar) 5 mM acetylsalicylic acid were incubated for various times as indicated and ethylene was quantitated at 1-h intervals by gas chromatography. Data represent means ± SD of results from three independent experiments.

(a)



(b)

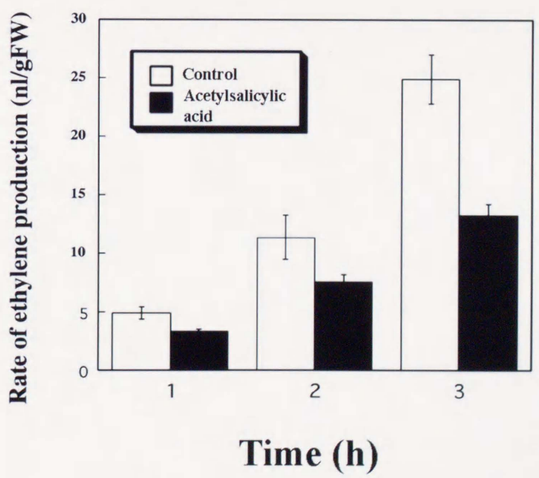
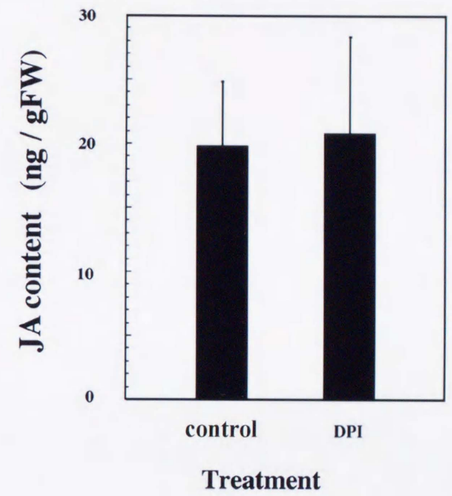


Figure III-2. Effects of DPI on the changes in levels of JA and the production of ethylene. **(a)** The effect of DPI on JA content. Disks were treated with and without 100 μM DPI immediately after wounding and then incubated for 3 h prior to quantitation of JA. **(b)** The effects of DPI on ethylene production. Disks treated with (closed bars) and without (open bars) DPI were incubated for various times as indicated and ethylene was quantitated at 1-h intervals. Data represent means $\pm$ SD of results from three independent experiments.

(a)



(b)

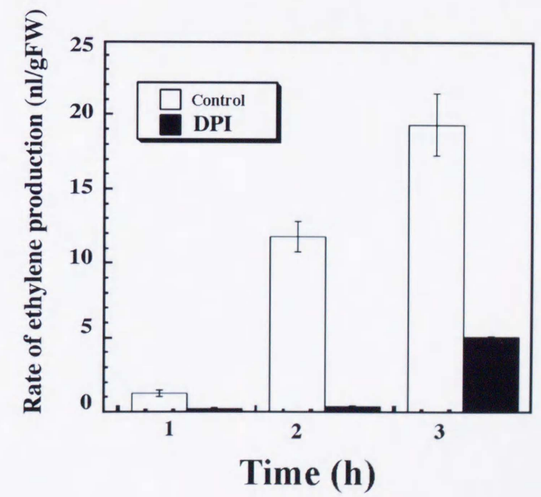
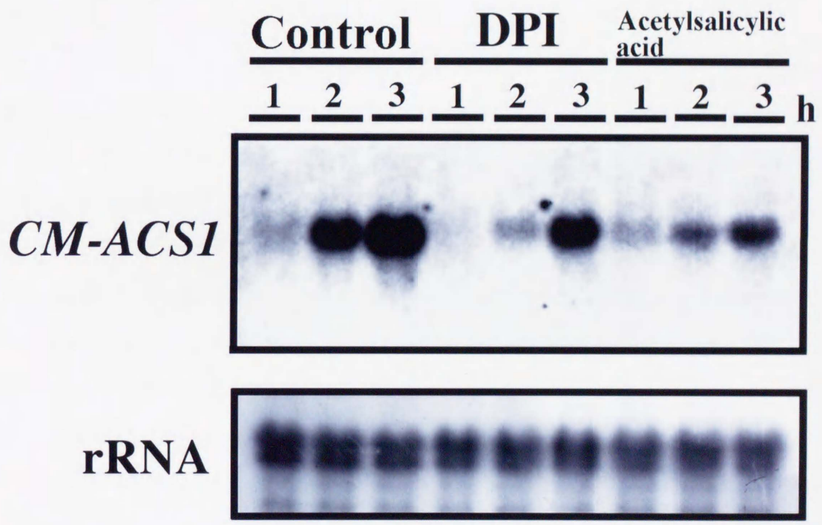


Figure III-3. Effects of acetylsalicylic acid and DPI on the expression of the *CM-ACS1* transcript. Disks were treated with 5 mM acetylsalicylic acid or with 100 μM DPI immediately after wounding and incubated for the indicated times prior to analysis of transcripts by Northern blotting. The control experiment was performed with buffer only. The experiment was repeated at least three times and typical results are shown.



General Discussion

One of the most severe environmental stresses that plants encounter during their life cycle is wounding. Plants respond to wound stress by activating a set of genes that encode proteins involved in healing of damaged tissue and in protection against pathogen infection. In recent years, much effort has been directed to the elucidation of the signal transduction pathways that mediate wound stress on the expression of defense-related genes. JA, ROS and the phytohormone ethylene have been considered to be mediators of wound signal.

In higher plants, mechanical wounding induces the production of ethylene *via* stimulation of the expression of genes that encode wound-inducible ACC synthases. However, the regulation of such gene expression is poorly understood. I showed the expression of the *CM-ACS1* gene, which encodes ACC synthase was induced in response to mechanical wounding followed by production of ethylene in mesocarp of winter squash (*Cucurbita maxima*). These results suggest that the production of ethylene was mainly regulated by the level of *CM-ACS1* transcript in wounded mesocarp tissue. In this study, I examined the roles of JA and ROS on the expression of *CM-ACS1* to clarify the regulation of the wound-induced production of ethylene.

In Part I, I showed that the production of ethylene was mainly regulated by the level of *CM-ACS1* transcript in wounded mesocarp tissue, and examined the effects of JA on the wound-induced production of ethylene. The amount of JA in the disks of mesocarp tissue was elevated 2 h after wounding and continued to increase for another hour. Furthermore, the production of ethylene and the accumulation of the *CM-ACS1* transcript were stimulated by exogenously applied JA in wounded mesocarp tissues. These results indicated that the production of JA was activated by wounding and that the JA produced stimulated the production of ethylene *via* activating the

expression of *CM-ACS1*. In fact, the addition of inhibitors of lipoxygenase to inhibit the wound-induced increase of JA suppressed the expression of *CM-ACS1*. From these results, JA is considered to be one of the factors that regulate the production of ethylene in wounded mesocarp tissue of winter squash.

In part II, I examined that effects of ROS on the wound-induced production of ethylene in tissue disks. The generation of superoxide (O_2^-), one type of ROS, was detectable immediately after wounding and the rate of generation of O_2^- reached maximum within a few minutes. This generation was inhibited by the addition of diphenylene iodonium (DPI), an inhibitor of the generation of O_2^-, in a dose-dependent manner. Furthermore, the production of ethylene and accumulation of *CM-ACS1* transcript at early stage after wounding (within 2 h) was strongly inhibited by treatment with DPI. Moreover, exposure of the disks to an extracellular O_2^--generating system, X-XO, strongly induced the expression of *CM-ACS1* 30 min after wounding. On the other hand, the addition of H_2O_2, another type of ROS, did not stimulate the expression of *CM-ACS1*. These results suggest that O_2^- generated by wounding causes the increase in the production of ethylene by activating the expression of *CM-ACS1* at the early stage after wounding. From these results, O_2^- is considered to contribute in the regulation of the ethylene production in wounded mesocarp tissue of winter squash.

In Part III, I showed the separate effects of JA and ROS on the expression of *CM-ACS1*. Acetylsalicylic acid, an inhibitor of synthesis of JA, inhibited the increase of JA, but DPI had no effect on the synthesis of JA in tissue disks. Moreover, the expression of *CM-ACS1* was inhibited by both DPI and acetylsalicylic acid but the inhibitory effects of the two inhibitors were different. DPI strongly inhibited the accumulation of the *CM-ACS1* transcript both 1 and 2 h after wounding, but renewed accumulation of the transcript was evident at 3 h. In contrast to the results of treatment with DPI,

acetylsalicylic acid did not inhibit the wound-induced accumulation of the transcript 1 h after the treatment. However, clear inhibitory effects of acetylsalicylic acid were observed 2 and 3 h after the treatment. In particular, at 3 h after treatment, the accumulation of the transcript was strongly inhibited by acetylsalicylic acid, rather than by DPI.

From the results reported in the thesis, I propose a working hypothesis concerning the regulatory mechanisms of the expression of *CM-ACS1* (Fig. IV-1a). In this study, I showed that both ROS and JA affect the production of ethylene by modulating the accumulation of the transcript of a wound-inducible gene for ACC synthase (*CM-ACS1*) but that they act independently (Fig. IV-1a). Moreover, I showed that ROS and JA induced the accumulation of the *CM-ACS1* transcript at different periods during 3 h-incubation after wounding. Namely, the expression of *CM-ACS1* at the early stage (within 2 h) is regulated by ROS that are generated immediately after wounding and at the later stage (after 2 h) by JA, the level of which increases at 2 h after wounding. At 2 h after wounding, the first inducer (ROS), which regulate the production of the ethylene in the early stages, might be taken the place by the second inducer (JA) which regulate that in the later stages (Fig. IV-1b). Ethylene has an important role as a mediator of wound signal which regulate the expression of various defense-related genes both at the early stage and at the later stage after wounding (Rickey *et al.* 1991, Ishige *et al.* 1993). The results described above suggest that the two inducers, which are generated at different time periods, are needed to regulate the rapid induction and continuous increase of the ethylene production.

As shown in Figure IV-1a, O_2^- is generated *via* activating NAD(P)H oxidase and the O_2^- generated regulates the expression of *CM-ACS1*. However, the molecular mechanism for the induction of *CM-ACS1* by O_2^- is unknown. Since O_2^- cannot permeate cell membranes, Jabs *et al.* (1996) proposed that O_2^- might be perceived by O_2^--monitoring molecules in the

plasma membrane and that the converted cytosolic signal might lead to gene activation. The fact that the inhibitory effect of DPI continued for 2 h may suggest that ROS affected on the expression of *CM-ACS1* indirectly *via* the induction of another signal as proposed by Jabs *et al.* (1996). In addition to ROS, I showed that the contents of JA is increased in wounded mesocarp tissue and that the JA regulates the expression of *CM-ACS1*. It has been shown that JA accumulates in response to wounding *via* activating octadecanoid signaling pathway in which lipoxygenase acts as a key enzyme (Wasternack and Parthiner 1997). In mesocarp tissue of winter-squash, the wound-induced expression of *CM-ACS1* is regulated by JA that is synthesized *via* activating octadecanoid signaling pathway (Part I).

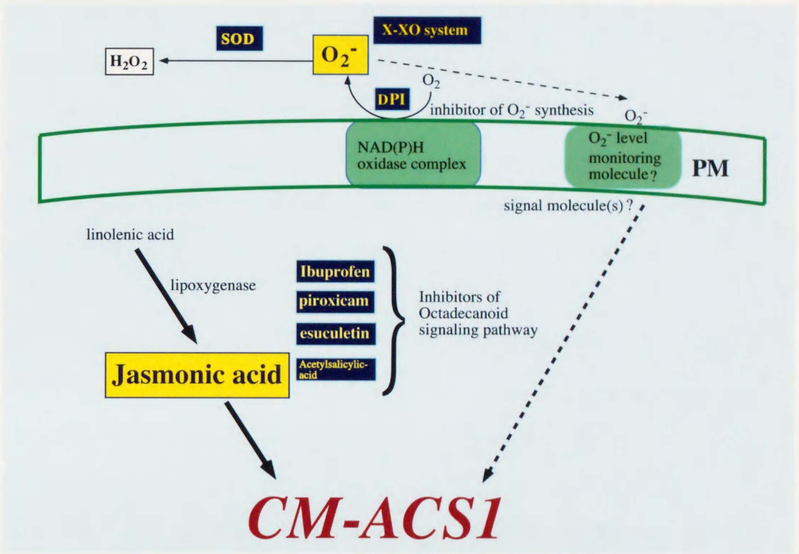
As described above, the production of three mediators of wound signal, namely, JA, ROS and ethylene, is harmoniously regulated with different time-course after wounding (Fig. IV-1b). In recent years, many studies suggest that ROS has also important roles in responses of plants to wounding and attack by pathogens, but the relationships between the production of ethylene and that of ROS has not yet been clarified. This is a first report showing that ROS stimulates the production of ethylene. On the other hand, it has also been shown that JA has important roles as a key signal molecule in wound signal transduction pathways. However, there is few reports showing the stimulatory effect of wound-induced JA on the expression of wound-inducible ACC synthase gene. It was reported that exogenously applied JA enhanced the production of ethylene in potato tuber and in tomato leaves, respectively (Greulich *et al.* 1995, O'Donnell *et al.* 1996). These observations support the results obtained in this study. A common regulatory mechanism that regulates the expression of genes for wound-inducible ACC synthases may be conserved in many of plants.

In higher plants, mechanical wounding induces the complex defense responses including the expression of various defense-related genes and

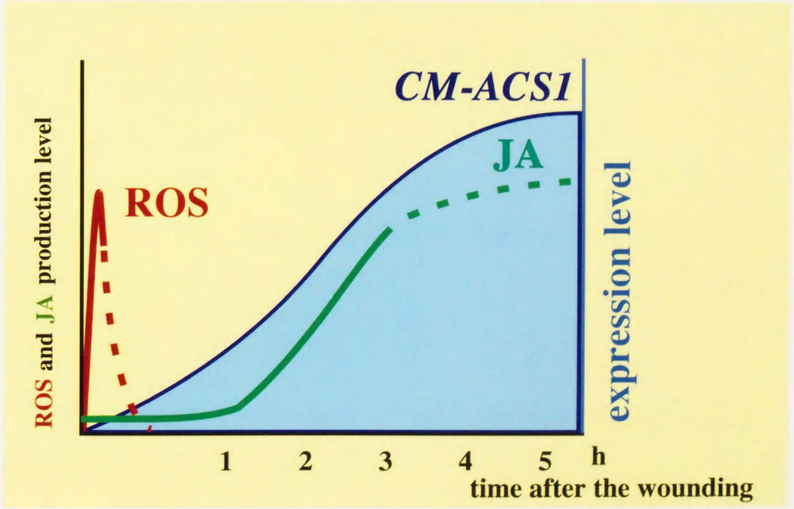
which are regulated by various mediators of wound signal. Recently, it has been reported that the expression of *PDF1.2* gene, a defense-related gene, was regulated by JA, ROS and ethylene (Mackrness *et al*. 1999). It appears that a regulatory mechanism that allows the harmonious production of various mediators of the wound signal, namely, JA, ROS and ethylene, is also needed for the harmonious regulation of the complex response of a plant to wounding.

Figure IV-1. **(a)** A working hypothesis for the mechanisms that control expression of the *CM-ACS1* gene. *O_2^- level monitoring molecule*, a hypothetical molecule that monitors levels of O_2^-; *PM*, plasma membrane. **(b)** Time course analyses of JA and ROS production and of the expression of *CM-ACS1*. The red and green line show the production level of ROS and JA, respectively. The blue line shows the level of the *CM-ACS1* transcript.

(a)



(b)



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Gene 14 Winter Squash (*Cucurbita maxima*)