Isolation of an Ozone-Sensitive and Jasmonate-Semi-Insensitive *Arabidopsis* Mutant (*oji1*)

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A novel ozone-sensitive mutant was isolated from *Arabidopsis* T-DNA tagging lines. This mutant revealed severe foliar injury and higher ethylene emission than the wild type under ozone exposure. The ozone-induced injury and ethylene emission were suppressed by pretreatment with aminoethoxyvinyl glycine, an inhibitor of ethylene biosynthesis, both in this mutant and wild-type plants. Pretreatment with methyl-jasmonate (MeJA) at 10 μM, however, suppressed the ozone-induced ethylene emission and foliar injury only in the wild-type plants. This mutant was less sensitive to jasmonate than the wild type, estimated by the MeJA-induced inhibition of root elongation and ozone-induced expression of *AtVSP1*, a jasmonate-inducible gene. Thus, this mutant was named *oji1* (ozone-sensitive and jasmonate-sensitive 1). These results suggest that the ozone sensitivity of *oji1* is caused by the increase in ozone-induced emission of ethylene as a result of low sensitivity to jasmonate, which plays defensive roles under stress conditions.

**Keywords**: *Arabidopsis* — Ethylene — Jasmonate — Mutant — Ozone.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; AVG, aminoethoxyvinyl glycine; ROS, reactive oxygen species; JA, jasmonate; MeJA, methyl jasmonate; SA, salicylic acid; PCD, programmed cell death; PPFD, photosynthetic photon flux density; PR protein, pathogenesis-related protein.

Introduction

With the increasing global population and industrial productivity, air pollution prevails as a serious environmental problem. Ozone, which is the major component of photochemical oxidant, damages plants severely. When plants are exposed to ozone at a high concentration, visible injury appears on leaves as a result of the death of mesophyll cell clusters (Treshow and Anderson 1989). Two hypotheses have been proposed for the mechanism of ozone-induced damages: one is based on the detrimental oxidation of biomaterials such as lipids, proteins and DNA by reactive oxygen species (ROS) generated during ozone exposure (Sakaki et al. 1983, Mudd 1996), the other is based on the ozone-induced activation of programmed cell death (PCD) which is similar to the pathogen-induced hypersensitive response (Kangasjärvi et al. 1994, Sharma and Davis 1997, Sandermann et al. 1998, Rao et al. 2000). Since both hypotheses have been supported by persuasive results as referred to above, it seems likely that such mechanisms underlie ozone-induced damages (Rao and Davis 1999, Koch et al. 2000). The latter hypothesis, i.e. PCD induced by ozone, however, is recently gathering more attention because of the interest in the molecular mechanisms of cell death as the defensive responses of plants to environmental stresses.

Ozone-induced ROS are thought to act as signal transduction molecules, which promote, for instance, the production of some phytohormones such as ethylene, salicylic acid (SA) and jasmonate (JA) in plants (Kangasjärvi et al. 1994). Among these phytohormones, ethylene and SA are supposed to act in the induction and/or expansion of the leaf damages, while JA is suggested to play defensive roles (Overmyer et al. 2000, Rao et al. 2002).

Suppression of ethylene production has been reported to effectively reduce ozone-induced damages. For example, a transgenic tobacco plant transformed with an antisense DNA encoding tomato 1-aminocyclopropane-1-carboxylate (ACC) synthase 6, an ozone-induced ethylene biosynthetic enzyme, showed less foliar injury than the wild type after ozone exposure (Nakajima et al. 2002). On the other hand, an ozone-sensitive *Arabidopsis* mutant, *rcd1* (Overmyer et al. 2000), produced a large amount of ethylene during ozone exposure. Furthermore, an ethylene-overproducing *Arabidopsis* mutant, *eto1*, exhibited phenotype with greater ozone sensitivity than
the wild type (Rao et al. 2002, Tamaoki et al. 2003). In this context, it can be thought that ethylene emission amplifies ozone-induced leaf injury: namely, the amount of ethylene production closely correlates with ozone-induced leaf damage.

In Arabidopsis, JA is known to be involved in many physiological processes including pollen production (Feys et al. 1994), inhibition of root elongation (Staswick et al. 1992), fruit ripening, senescence (reviewed in Creelman and Mullet 1997), and responses to pathogens, pest, wound and elicitor (Turner et al. 2002). In some previous studies, roles of JA were investigated in relation to responses to ozone. Rao et al. (2000) reported that ozone exposure increased JA accumulation in Arabidopsis and that both JA-signaling-deficient (jar1) and JA synthesis (fad3/7/8) mutants were highly sensitive to ozone. In poplar, a JA-insensitive hybrid clone displayed more ozone sensitivity than a JA-sensitive one (Koch et al. 2000). Activation of the JA signaling pathway by pretreatment of methyl jasmonate (MeJA) or by wounding before ozone exposure decreased ozone injury in tobacco (Örvar et al. 1997). Additionally, exogenous supplement of MeJA abolished ozone-induced cell death in the Arabidopsis ecotype Cvi-0 (Rao et al. 2000). These observations suggest that ozone sensitivity is strongly correlated with JA insensitivity and that the JA-related signaling pathway plays defensive roles in ozone-induced damage.

Ethylene, SA and JA may also have cross-talk in ozone-induced signaling pathways. Rao et al. (2002) stated that SA and ethylene act cooperatively in the damaging mechanism induced by ozone. They found that SA content in eto1, which is ethylene overproducing and ozone-sensitive, was higher than the wild type during ozone exposure, and that a SA-deficient transgenic Arabidopsis (Col: NahG) and a SA-signaling mutant (npr1) revealed lower emission of ethylene than the wild type during ozone exposure.

It has been pointed out that plants respond to pathogen attack through a common pathway between ethylene and JA. For example, ERF1 is known as a transcription factor that is responsive to both ethylene and JA (Lorenzo et al. 2003). Additionally, JA and ethylene concomitantly activated defensive genes, such as plant defensin 1.2, pathogenesis-related protein (PR)-5 and PR-1 in Arabidopsis (Penninckx et al. 1998, Xu et al. 1994). On the other hand, downregulation of JA signaling by ethylene has been reported. In wound signaling, ethylene suppressed the expression of JA-inducible genes, such as jasmonate related (JR)1, JR2 and vegetative storage protein (VSP) in Arabidopsis (Rojo et al. 1999). Similarly, exogenous ethylene suppressed the gene expression of JA-inducible genes in tobacco (Shoji et al. 2000). Although ethylene and JA are thought to act antagonistically in ozone-induced responses (Rao et al. 2000), detailed molecular mechanisms in the interaction of ethylene and JA have not been described.

We report here an ozone-sensitive mutant, oji1, which shows enhanced ethylene emission during ozone exposure and has decreased sensitivity to JA. JA synthesis in this mutant seemed to be normal since JA content in oji1 was similar to that in the wild type, while JA signaling was likely to be depressed judging from root elongation under the supplement of MeJA and JA-inducible gene expression. This is the first report showing downregulation of ethylene emission by JA-signaling during ozone exposure. Analysis of this mutant may contribute to dissection of the mechanism in antagonistic interaction between ethylene and JA in ozone-induced signaling pathways.

Fig. 1  Leaf damage by ozone in wild type and oji1. (a) Seedling of in wild type and oji1 before and after ozone exposure. Sixteen-day-old seedlings (0 h) were exposed to 200 nl liter–1 ozone for 8 h, and then left in fresh air for 12 h (Ozone 8 h) Severe visible foliar injury can be seen in oji1. (b) Ion leakage from leaves during ozone exposure. Electroconductivity from each three leaves in 1 ml of DW was measured at indicated time. Relative values (%) to whole ion content after autoclaving are shown. Values of ozone-unexposed leaves at each time were subtracted from values of ozone exposed ones (n = 4 ± SE).
Ozone sensitive Arabidopsis mutant, *oji1*

**Results**

*Isolation and genetic mapping of a novel ozone-sensitive mutant, *oji1***

Visible foliar injury as a result of cell death is a simple indicator for detecting ozone sensitivity. Thus, we isolated ozone-sensitive mutants that showed severe leaf injury. Approximately 6,500 lines of T-DNA-transformed *Arabidopsis* plants (Feldmann and Marks 1987) were exposed to 200 nl liter⁻¹ ozone for 24 h and five lines displaying ozone-induced lesions on rosette leaves were identified. These mutant lines were then crossed with their wild type, Wassilewskija-2 (Ws-2), and three lines with the linkage between ozone-sensitivity at F₂ progeny and T-DNA insertions were selected. In the F₂ progeny of each
line, an ozone-sensitive phenotype segregated as a single recessive Mendelian trait. These three mutant lines were crossed with each other and ozone sensitivity was assayed in the F1 progeny. No ozone-sensitive plants were observed, indicating that these mutants represent independent loci of mutation in the genome. Further characterization of the most sensitive mutant, oji1, is presented here. It should be noted that the phenotype of oji1 was different from wild type (Fig. 1a). The whole plant size and leaf width of oji1 were slightly smaller and its leaf color was a little brighter (pale/less green) than those of wild-type plants, while the petiole of oji1 was relatively longer. This mutant was subjected to back-cross purification three times, after which only one copy of T-DNA was detected with the Southern blot analysis using the left border sequence of T-DNA (pAK1003, Feldmann and Marks 1987) as a probe (data not shown). The ozone-sensitive F2 progeny from the oji1 × Landsberg erecta (Ler) was used for mapping with simple sequence length polymorphisms (SSLP) markers. Using 21 ozone-sensitive oji1 individuals from F2 progeny, we were able to position the locus in chromosome 3 at 70±11.9 centimorgans from a SSLP marker ciw4. No mutants with similar phenotypes have previously been reported around this region.

Visible foliar damage on leaves of oji1 plants appeared earlier than in wild-type plants and it was markedly severe after an 8 h exposure to 200 nl liter⁻¹ ozone (Fig. 1a). The propagation of the lesion in oji1 was more extended than in the wild type at 24 h after the beginning of ozone exposure (data not shown). As a more quantitative indicator of cellular damage, ion leakage from leaves was measured during the ozone exposure. At 6 h after the beginning of ozone exposure, ion leakage in the wild type increased up to 10-fold of the initial level, while an increase in ion leakage in oji1 appeared earlier and more severe than in the wild type (Fig. 1b).

**Ethylene emission in oji1 was higher than in the wild type during ozone exposure**

Increased ethylene emission from ozone-treated plants is an early, consistent marker for ozone sensitivity (Tingey et al. 1997).
Ozone sensitive Arabidopsis mutant, oji1

Excessive ethylene emission during ozone exposure has been reported to enhance PCD in an ozone-sensitive Arabidopsis mutant, rcd1 (Overmyer et al. 2000). Fig. 2 reveals that oji1 had higher ethylene emission throughout the ozone exposure than the wild type. While ethylene emission from the wild type increased from 3 h after the beginning of the ozone exposure, it began to increase at 1 h in oji1 and kept increasing until 6 h (Fig. 2). At 3 h, ethylene emission from oji1 peaked at the level of approximately 1.6-fold of the wild type. The peak in the wild type was at 6 h and in a lower level than oji1.

Ozone-induced ethylene emission is known to be caused by the activation of enzymes for its biosynthesis, ACC synthase and ACC oxidase (Nakajima et al. 2002). Thus, we tested the effect of aminoethoxyvinyl glycine (AVG), a specific inhibitor of ACC synthase, on ethylene emission in oji1. Ethylene emission was almost completely inhibited by pretreatment with 100 μM AVG both in oji1 and wild-type plants (Fig. 3a). Ion leakage from leaves was also depressed by the same treatment in both oji1 and wild-type plants (Fig. 3b). These results suggest that enhanced ethylene emission propagates the damage in oji1 and that the ozone-induced ethylene emission is caused through activation of its biosynthetic pathway.

oji1 has reduced sensitivity to jasmonate

Since JA is supposed to play defensive roles under the stress caused by ozone (Rao et al. 2000, Overmyer et al. 2000), the effect of exogenously applied MeJA on ozone-induced injury was examined. The application of 1 μM MeJA did not affect ethylene emission or ion leakage both in Ws-2 and in oji1 during ozone exposure (data not shown). A high concentration (100 μM) of MeJA suppressed ethylene emission and ion leakage both in oji1 and in the wild type. However, a lower concentration (10 μM) of MeJA suppressed these symptoms only in the wild type but not in oji1 (Fig. 4a, b). Therefore, it seems that JA sensitivity is reduced in oji1. For further confirmation of this possibility, the inhibition of root elongation by MeJA was examined. Root elongation was inhibited by 0.1 μM MeJA in the wild type while no or very little inhibition of elongation was observed in oji1. On the other hand, 10 μM of MeJA showed stronger effect on inhibition of the root growth (Fig. 5). At 0 μM, root length of oji1 was shorter than wild type, probably, reflection of smaller plant size of oji1 than wild type as described above.

To determine whether JA synthesis is deficient in oji1, we measured JA contents in leaf during ozone exposure. JA contents in oji1 and wild type were almost similar before ozone exposure, and they started to increase at 3 h after ozone treatment, reaching to high level at 4 h. Interestingly, JA contents in oji1 were significantly enhanced; 2.6-fold higher than the wild type (see Fig. 7). Taken together, these results suggest that oji1 is less sensitive to JA than the wild type.

To investigate the JA-induced gene expression in oji1, RNA gel blot analysis was performed using AtVSP1, a JA inducible gene (Berger et al. 1996) as a probe. Expression of

Fig. 5  Jasmonate sensitivity estimated by root elongation. Seeds were sown on 1/2MS plates with or without 0.1 μM and 10 μM MeJA. The plates were then placed vertically for 7 d at 25°C under continuous light (100 μmol PPFD m² s⁻¹). SE: oji1 0 μM (n = 17), Ws-2 0, 10 μM, oji1 10 μM (n = 16) (*P <0.01). Absolute values at 0 μM are 12.3 mm (oji1), 18.1 mm (Ws-2), respectively.

Fig. 6  Changes in gene expression by ozone exposure. Total RNA (10 μg) were gel electrophoresed and blotted as described in Material and Methods. cDNA fragments of AtVSP1, PR-1 and tubulin4 were used as probes. Ethidium bromide staining of rRNAs is shown in the lower most panels as loading control. Histogram shows relative intensity of mRNA level to 0 h of ozone exposure. Experiment was repeated three times with similar results, and a representative northern blot image is presented in this figure.
Endogenous JA level of wild type (open square) and *oji1* (filled square). Data shown are from duplicated experiments.

**Discussion**

*oji1* is a novel ozone-sensitive mutant

*oji1* was more sensitive to ozone than the wild type, *Ws-2*, in the extent of visible foliar injury and in the level of damage on the cell membrane (Fig. 1a, b). We also showed that this mutant had reduced sensitivity to MeJA in inhibition of root elongation (Fig. 5) and expression of the *AtVSP1* gene (Table 1). As shown in Table 1, several ozone-sensitive mutants have been reported. *oji1* is thought to be a novel ozone-sensitive mutant judging from its mutation locus and characteristics.

Some ozone-sensitive mutants were *JA*-related mutants as well. Independent of ozone sensitivity, *JA*-related mutants have been classified into two groups; *JA*-synthesis mutants which are affected in *JA* biosynthesis such as *fad* mutants and *dde2* (McConn and Browse 1996, Malek et al. 2002), and *JA*-signaling mutants which are supposed to be disrupted in *JA* signal transduction such as *jar1* (Staswick et al. 1992), *coil* (Feyes et al. 1994), and *jin* mutants (Berger et al. 1996). Among *JA*-synthesis mutants, a triple mutant of fatty acid desaturase (*fad3/7/8*, McConn et al. 1997) which is deficient in *JA* synthesis has been reported as ozone sensitive (Rao et al. 2000). On the other hand, *JA*-signaling mutants previously investigated for their ozone sensitivity were all sensitive to ozone (Rao et al. 2000). *oji1* can be categorized as a *JA*-signaling mutant since its *JA* contents were not deficient (Fig. 7). Moreover, *JA* contents in *oji1* were higher than that of wild type after ozone treatment (Fig. 7). This result also suggests that *JA* biosynthesis in *oji1* functions normally. From the data it seems likely that *JA* accumulates in *oji1*. One possible reason for this may be that, *JA* in *oji1* is not consumed via its normal perception mechanisms for activating the defensive and/or morphological responses.

Molecular and physiological characters of *oji1*

By comparison with other ozone-sensitive accessions (Table 1), distinctive features of *oji1* may be clarified. In spite of drastically increased contents of *JA* in *oji1* during ozone exposure (Fig. 7), *AtVSP1* expression in *oji1* was the similar or even lower than that in wild type as shown in Fig. 6. *AtVSP1* expression in the other *JA*-related mutants and an ozone-sensitive ecotype *Cvi* was reduced by treatment with exogenous MeJA at approximately 50 μM (Berger et al. 1996, McConn et al. 1997, Rao et al. 2000, Ellis and Turner 2002). Endogenous *JA* concentration in *oji1* and the wild type was in the order of 10 μM (Rao et al. 2000) and exogenously supplied *JA* concentrations. *AtVSP1* expression induced by ozone has not been investigated in the other mutants listed in Table 1 and the difference in *JA*-sensitivity among ecotypes has not been described enough.

Sensitivity to *JA* was also estimated by inhibition of root elongation, a well-known physiological role of *JA* as a phyto-
Table 1  Molecular and physiological characters of ozone-sensitive accessions

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>JA signaling mutants (ecotype)</th>
<th>JA synthesis mutant (ecotype)</th>
<th>Ozone-sensitive ecotype</th>
<th>Ozone-sensitive mutants (ecotype)</th>
<th>Reference</th>
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<tbody>
<tr>
<td></td>
<td>oji1 (Ws-2)</td>
<td>coi1 (Col)</td>
<td>jin4 (Ws-2)</td>
<td>jar1 (Col)</td>
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<tr>
<td>Ascorbate content</td>
<td>WT</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Ethylene emission by ozone</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Chromosome (locus)</td>
<td>3 (70±11.9 cM)</td>
<td>2 (F-box protein)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
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WT, similar to wild-type phenotype; ND, not determined.

1 A gene expression induced by endogenous-JA during ozone exposure.
2 Exogenous JA-induced gene expression.
phormone, in oji1 and other accessions (Table 1). Four JA-signaling mutants including oji1 that were used to determine the effect of JA to root elongation displayed insensitivity to JA. Therefore, these four mutants are suggested to be defective in the perception of JA. Cvi showed normal JA-sensitivity in the inhibition of root elongation, unlike in the AtVSP1 expression, this observation implies the branching of JA-signaling pathways to morphogenesis and to defensive responses after the JA perception.

Since ascorbate content in oji1 was similar in level to the wild type (Table 1; data not shown), the reason for ozone sensitivity of oji1 may not be the reduced activity of the antioxidative system proposed for an ascorbate-deficient ozone-sensitive mutant, vtc1 (Conklin et al. 1996). This supports the adequacy of the application of the hypothesis of PCD induced by ozone to ozone sensitivity of oji1.

Among the ozone-sensitive mutants, only oji1 and rcd1 revealed enhanced ethylene emission by ozone. Propagation of leaf injury by a high amount of ethylene is clearly the reason for ozone sensitivity of these two mutants, while enhanced ethylene emission in rcd1 is not because of a defect in the pathway mediated by JA, since this mutant showed normal sensitivity to JA both in the inhibition of root elongation and in depression of ozone-induced injury (Fig. 5; Overmyer et al. 2000).

Ethylene emission in oji1 is through ethylene biosynthesis by ACC synthase

As mentioned above, ethylene is known as a trigger to propagate ozone-induced cell death (Overmyer et al. 2000, Nakajima et al. 2002). Thus, ozone sensitivity of oji1 is thought to be a result of its earlier and higher ethylene emission during ozone exposure than in the wild type (Fig. 2). Ethylene production, consequently, is an important key of ozone damage. Nakajima et al. (2002) have shown that the gene expression of ACC synthase, a key enzyme in ethylene biosynthesis, was increased by ozone exposure accompanied with an increase in ethylene emission in tomato, and the treatment by AVG, a specific inhibitor of ACC synthase, decreased ethylene emission and ozone-induced leaf damage (Bae et al. 1996, Tamaoki et al. 2003). Similarly, in oji1, AVG treatment completely inhibited ethylene emission and leaf injury during ozone exposure (Fig. 3a, b). Depression in ethylene emission both in wild type and in oji1 shown in Fig. 3a at 0 h is most probably thought to be because of 3 h pretreatment of AVG before ozone exposure. In Fig. 3b at 0 h, AVG-treated oji1 plants showed similar ion leakage to non-treated plants, whereas wild-type plants showed clear suppression by AVG. AVG solution (0 μM and 100 μM) which was supplied by spraying 3 h before ozone exposure included 0.05% Tween 20, for absorption of AVG inside of the leaf cells. oji1 might be affected by the detergent, and probably by spraying, more severe than the wild type. In all, these data suggest that ozone damage in oji1 is subsequent to an increase of ethylene production as a result of the activation of ACC synthase.

Putative role of OJI1: low-JA-sensitivity may cause enhanced ethylene emission in oji1

Activities of phospholipase and non-specific acyl hydrolases have been reported to be stimulated by ozone, and as a result, oxidized fatty acids for JA biosynthesis were released (Creelman and Mullet 1997). These released oxidized fatty acids are supposed to cause an increase of JA contents during ozone exposure, which has been reported in Arabidopsis (Rao et al. 2000). On the other hand, a decrease in ozone damage by exogenously added MeJA has been reported (Overmyer et al. 2000). These results suggest that JA synthesized by ozone plays defensive roles by suppression on progressing of the damage. In other words, JA signaling may act antagonistically to the signaling for induction and/or propagation of ozone-induced leaf injury. As shown in Fig. 4, supplement of exogenous MeJA markedly suppressed ozone-induced ethylene emission, as well as ozone-induced leaf damage, both in oji1 and the wild type. This result indicates that one of the roles of JA in suppression of ozone-induced leaf injury through its signaling pathway(s) may be prevention of ethylene production.

As shown in Fig. 8, signaling pathways of JA and ethylene are stimulated in the process of ozone-induced cell death and the JA signaling pathway is supposed to be obstructed in oji1. Accordingly, OJI1, which is thought to be disrupted in the oji1 mutant, may conduct JA sensitivity at some point in the pathway from JA perception to the point of suppression of ethylene production, presumably suppression of the expression of ethylene biosynthetic enzyme such as ACC synthase.

While OJI1 is supposed to regulate ethylene biosynthesis by activating ACC synthase or up stream in the JA signaling, JAR1 is not thought to interact with the process of ethylene biosynthesis (Overmyer et al. 2000). In jar1, ozone-induced lesion was not rescued by supplement of exogenous MeJA (Rao et al. 2000), jar1 showed higher expression of PR-1 than wild type at 6 h after the beginning of ozone exposure (Rao et al. 2000), whereas PR-1 expression in oji1 was similar to wild type at the same time (data not shown). At 24 h, however, PR-1 expression in oji1 was higher than that in the wild type (Fig. 6). These results suggest that oji1 may respond to SA in a different way from jar1 during ozone exposure. Two hypotheses are possible: one is that JAR1 and OJI1 are involved in diverged signaling pathways leading to ozone-induced cell death, the other is that the difference in the background ecotype caused different responses in oji1 and jar1. Tamaoki et al. (2003) proposed that a difference in ethylene sensitivity and/or production may cause the difference in ozone sensitivity between Ws-2 and Col-0: Ws-2 displayed higher ozone-induced ethylene emission and higher sensitivity to ozone than Col-0 (Tamaoki et al. 2003).

The target gene of oji1 was preliminarily detected by thermal asymmetric interlaced (TAIL)-PCR, in chromosome 3 between At3g61810 (putative beta-1, 3, glucanase: PR-2) and At3g61820 (putative cnd41 like protein), in accord with the
result from mapping. Expression of these genes during ozone exposure and function in JA signaling are under investigation.

In addition to dissecting the JA-signaling pathway in detail, further investigation of oji1 is required, such as the expressions of JA-responsive and biosynthetic genes, ethylene biosynthetic genes, and changes in contents of JA precursors during ozone exposure. Additionally, it would also be very interesting to examine SA relationships in oji1 in regard to ozone sensitivity. The SA content in the leaf has also been reported to be increased by ozone in Arabidopsis (Overmyer et al. 2000, Rao et al. 2000). An Arabidopsis ecotype, Cvi-0, has been known for its extreme ozone-sensitivity with high accumulation of SA during ozone exposure, while expression of exogenously introduced salicylate hydroxylase (NahG) in transgenic Cvi-0-reduced ozone-induced cell death (Rao et al. 2000).

Materials and Methods

Mutant screening, crossing and genetic mapping

T-DNA transformed Arabidopsis thaliana L. ecotype Ws-2 seeds (Arabidopsis Biological Research Center; Ohio State University, Columbus, U.S.A.; Feldmann and Marks 1987) were grown on glass wool at a density of 5,000 plants m⁻² for 2 weeks in a growth chamber (100 μmol PPFDM² s⁻¹, 14 h light, 25°C, 60% relative humidity) and exposed to 200 nl liter⁻¹ ozone for 24 h. The ozone-treated plants were screened visually for individuals with enhanced damage after 24 h. Candidates of ozone-sensitive mutants were selected and allowed to self-pollinate to obtain T₅ progeny for further screening. Selected mutants were crossed Ws-2 twice or three times to determine the link between ozone sensitivity and T-DNA insertion, and the inheritance of the phenotype. The T-DNA-inserted mutants were then crossed with each other to perform allelism test. oji1 was crossed with Landsberg electa (Ler) for mapping. The ozone-sensitive F₂ progeny of oji1 × Ler were used for linkage analysis by using SSLP markers. Southern blotting was performed following the standard method using 2 μg of genomic DNA from oji1. A 1.4 kb EcoRI fragment of the left border sequence of pAK1003 (Feldmann and Marks 1987) was used as a probe.

Plant materials and growth conditions

Seeds of Ws-2 and oji1 were sown on 2×2.3 cm² blocks of glass wool (Minipot, Nittobo, Tokyo, Japan). Then, they were kept at 4°C for 2 d for vernalization treatment. Seedlings were grown in a growth chamber at 22°C, under 14 h-light at 100 μmol photosynthetic photon flux density (PPFD) m² s⁻¹ from white fluorescent lamps, at 50–60% relative humidity. Sixteen-day-old seedlings were used for all experiments. These seedlings were exposed to ozone at 200 nl liter⁻¹ in a growth chamber at 25°C, 70% relative humidity, under continuous light from metal halide lamps with 300 μmol PPFD m² s⁻¹.

Measurement of ethylene emission

The ethylene production from seedlings was measured as described by Tamaoki et al. (2003). Three sets of 16-day-old seedlings except roots were collected into a sealed glass vial of 2 ml volume (GL Science, Tokyo, Japan) at each sampling time. After incubation for 1 h at 100 μmol PPFDM² s⁻¹ at 24°C, 1 ml of gases was taken out of the vial by a plastic syringe and the amount of ethylene was determined with a gas chromatograph with a flame ionization detector (GC-7A, Shimadzu, Tokyo, Japan).

MeJA and AVG treatment

Sixteen-day old seedlings were sprayed with each concentration of MeJA in a sealed plastic case by a sprayer 0.5 h before ozone exposure. AVG treatment was performed as described previously (Tamaoki et al. 2003). Sixteen-day-old seedlings were sprayed with a solution of AVG containing 0.05% Tween 20 in a sealed plastic case 3 h before ozone exposure. Then, the seedlings were exposed to 200 nl liter⁻¹ ozone. As a control, seedlings were sprayed with a solution containing 0.05% Tween 20.

Inhibition of root elongation by MeJA

Surface-sterilized seeds were germinated on a 1/2MS (Murashige and Skoog 1962) plate containing each concentration of MeJA. Plates were placed at 4°C for 4 d, then laid vertically under continuous light of 100 μmol PPFD m² s⁻¹ at 22°C for 7 d.

RNA isolation and northern gel blot analysis

Total RNA was extracted from seedlings using an RNeasy plant mini kit (Qiagen, MD, U.S.A.). Northern gel blot analysis was carried out essentially as described previously (Tamaoki et al. 1995). cDNA clones used as probes (AtVSP1, PR-1, tubulin4) here were isolated as described previously (Matsuyama et al. 2002).

Quantification of endogenous JA

Approximately 200 mg Arabidopsis plants were used for JA extraction. Endogenous JA levels were determined by LC-MS/MS (HPLC – Agilent 1100 HPLC system, Hewlett-Packard, Waldbronn, Germany; Column – Capcell PaK C18 column (150×4.6 mm, Shiseido, Tokyo, Japan) eluted with 85% aqueous methanol, 0.3 ml min⁻¹ at 25°C, MS – API-2000, LC/MS/MS, PE-SCIEX, Concord, Ontario, Canada) as described previously (Rakwal et al. 2002) with some modifications. Briefly, a chloroform extraction step was added prior to filtration on a C-18 cartridge (a method to be published elsewhere).

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