Rapid paper

Disruption of a Gene Encoding C₄-Dicarboxylate Transporter-Like Protein Increases Ozone Sensitivity Through Deregulation of the Stomatal Response in Arabidopsis thaliana

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To understand better the plant response to ozone, we isolated and characterized an ozone-sensitive (ozs1) mutant strain from a set of T-DNA-tagged Arabidopsis thaliana ecotype Columbia. The mutant plants show enhanced sensitivity to ozone, desiccation and sulfur dioxide, but have normal sensitivity to hydrogen peroxide, low temperature and high light levels. The T-DNA was inserted at a single locus which is linked to ozone sensitivity. Identification of the genomic sequences flanking the T-DNA insertion revealed disruption of a gene encoding a transporter-like protein of the tellurite resistance/C₄-dicarboxylate transporter family. Plants with either of two different T-DNA insertions in this gene were also sensitive to ozone, and these plants failed to complement ozs1. Transpiration levels, stomatal conductance levels and the size of stomatal apertures were greater in ozs1 mutant plants than in the wild type. The stomatal apertures of ozs1 mutant plants responded to light fluctuations but were always larger than those of the wild-type plants under the same conditions. The stomata of the mutant and wild-type plants responded similarly to stimuli such as light, abscisic acid, high concentrations of carbon dioxide and ozone. These results suggest that OZS1 helps to close stomata, being not involved in the responses to these signals.

Keywords: Arabidopsis thaliana — Mutant — Oxidative stress — Ozone — Stomata — Transporter.

Introduction

Ozone (O₃) is a gaseous air pollutant that causes damage to natural and cultivated plants, resulting in crop loss and forest decline (Bobbink 1998, Nouchi 2002a). Ozone enters leaves through the stomata, dissolves in the water on the cell surface, and produces reactive oxygen species (ROS) through its reaction with biomolecules and through disruption of normal cellular processes (Nouchi 2002b). Ozone and/or ROS then trigger a variety of responses at the molecular level, such as changes in signal transduction and gene expression (Kubo 2002, Tamaoki et al. 2003a, Mahalingam et al. 2005), which result in inhibition of photosynthesis and growth, visible leaf injury and acceleration of senescence. Molecular responses of plants to ozone resemble the hypersensitive response induced by infection with virulent pathogens (Kangasjarvi et al. 1994, Sharma and Davis 1997, Sandermann et al. 1998). Two mechanisms for ozone-induced damage have been proposed. One is based on ROS toxicity and the other is based on programmed cell death (PCD), wherein ROS acts as a signal together with other signaling molecules, for instance phytohormones such as ethylene and salicylic acid (SA) to induce PCD of leaf cells (Rao and Davis 1999, Rao et al. 2000, Rao and Davis 2001, Mano 2002, 2003).
Overmyer et al. 2003). On the other hand, another phytohormone, jasmonate (JA), is suggested to play defensive roles in the induction and/or expansion of PCD (Overmyer et al. 2000, Rao et al. 2002, Kanna et al. 2003).

To help elucidate the molecular mechanisms of plant responses to ozone, several Arabidopsis mutants with altered sensitivity to ozone have been isolated and characterized (Kangasjärvi et al. 2005). These mutants can be divided into three groups based on gene function. One group comprises those with defects in antioxidative systems. A mutant called vecl was the first mutant isolated for increased ozone sensitivity and has a defect in the ascorbate biosynthetic pathway (Conklin et al. 1999). Another mutant with a defect in cytosolic dehydroascorbate reductase (cytDHAR), which is a component of the ascorbate recycling system, was also found to be ozone sensitive (Yoshida et al. 2006).

The second group consists of mutations that affect signal transduction. The ozone-sensitive rcd1 mutant (Overmyer et al. 2000) produces a large amount of ethylene during ozone exposure and is less sensitive to JA than the wild type (Ahlfors et al. 2004). Moreover, an ethylene-overproducing mutant (eto1) also exhibits an ozone-sensitive phenotype (Rao et al. 2002, Tamaoki et al. 2003b). Rao et al. (2000) reported that ozone exposure increases JA accumulation and that mutants with defects in JA synthesis (fad3/7/8) or JA signaling (jar1) are highly sensitive to ozone. The ojil mutant strain, isolated on the basis of ozone sensitivity, showed lower sensitivity to methyl jasmonate than wild-type plants, along with higher levels of ethylene emission during ozone exposure (Kanna et al. 2003).

The third group of ozone-sensitive mutants includes those with a defect in stomatal regulation. Ahlfors et al. (2004) have shown that the above-mentioned mutant rcd1 has higher stomatal conductance than what is observed in the wild-type plant. Another mutant strain, rcd3, was isolated in the same screen as rcd1 and also has higher stomatal conductance than the wild type (Kangasjärvi et al. 2005). However, only a few mutants in the third group have thus far been isolated and characterized, despite the fact that the stomatal response is the first step of plant defense against ozone, and thus is likely to be important to plant ozone sensitivity.

Stomata provide the main pathway for gas exchange, and their aperture is regulated in response to environmental stimuli such as light, drought, the concentration of carbon dioxide (Schroeder et al. 2001) and ozone (Torsøenhaugen et al. 1999, Ahlfors et al. 2004), as well as endogenous stimuli such as ABA (MacRobbie 1998). Ion transport is required for volume changes of guard cells, which mediates the opening and closing of stomata, although the research is at too early a stage to construct a detailed description of the events of stomatal movement. In the present study, we have isolated and characterized a novel ozone-sensitive mutant. The results show that the responsible gene encodes a transporter-like protein and that stomatal aperture of the mutant is larger than that of the wild-type plant, showing normal responses to light, ABA, carbon dioxide and ozone.

Results and Discussion
Isolation of an ozone-sensitive mutant, ozs1, and identification of the OZS1 gene

Several ozone-sensitive mutants were isolated in a screen of T-DNA-tagged strains of Arabidopsis thaliana, ecotype Columbia (Col-6, gl1-1; Campisi et al. 1999) based on the degree of visible damage to the foliage of ozone-treated seedlings. The mutant strains were backcrossed with wild-type plants, and segregation of ozone sensitivity and kanamycin resistance were analyzed in the F2 populations. One mutant line, named ozn-sensitive1-1 (ozs1), showed severe foliar damage after exposure to 0.2 µl l⁻¹ ozone (Fig. 1A), and had lower chlorophyll content and higher levels of ion leakage than wild-type plants after ozone exposure (Fig. 1B, C). The ozs1 mutation segregated in a ratio of 3:1 in terms of both ozone and kanamycin resistance, suggesting that the line is recessive for the ozone-sensitive phenotype and has a T-DNA insertion at a single site in the genome. The results of Southern blot analysis supported this assumption (Fig. 2A). To investigate linkage between the ozone-sensitive phenotype and the T-DNA insertion, genomic DNA samples isolated from 44 individual F2 plants with the ozone-sensitive phenotype were subjected to PCR using primers that recognize the GUS (β-glucuronidase) gene, a component of the T-DNA construct. All 44 plants have T-DNA sequence, indicating a tight linkage between the ozone-sensitive phenotype and the T-DNA insertion (data not shown).

The gene disrupted by the insertion was identified by analyzing the sequence flanking the T-DNA insert, which was amplified by thermal asymmetric interlaced PCR (TAIL-PCR). PCR products of two different sizes were obtained, a 450 bp fragment [left border to the annealing position of the arbitrary degenerate (AD) primer 1, or AD1] and a 900 bp fragment (left border to AD2). Partial sequences of the At1g12480 gene were found by comparison of the DNA sequence of the amplified products with the Arabidopsis gene sequence (Fig. 2B). The results of a further analysis indicate that at least two T-DNA fragments are inserted at a single site but in opposite directions in exon 2 of At1g12480 (Fig. 2B). However, we were unable to detect right border sequences connected to the genome sequence. To investigate the At1g12480 transcript, RNA was extracted from both ozs1 and wild-type plants and
subjected to reverse transcription–PCR (RT–PCR) analysis. An RT–PCR product consistent with the expected size (377 bp) was amplified from wild-type RNA but was not obtained from RNA from ozs1 mutant plants (Fig. 2C). The sequence of the 377 bp product from the wild-type plants was analyzed and corresponds to a subregion of the *At1g12480* gene (Fig. 2B). Therefore, the transcript for this gene does not appear to exist in the mutant plants.

To confirm that disruption of the *At1g12480* gene is responsible for the mutant phenotype, additional mutant lines with insertions in this gene were searched from the insertion sequence database of The Arabidopsis Information Resource (TAIR: http://www.arabidopsis.org/portals/mutants/worldwide.jsp). Two strains with T-DNA insertions in *At1g12480*, SALK_137265 and SALK_099139, were identified (Fig. 2B) and were also found to be ozone sensitive based on the extent of visible leaf injury after exposure to ozone (data not shown). A complementation test with ozs1 was then performed. Transheterozygotes for SALK_137265 or SALK_099139 and ozs1 were ozone sensitive (Fig. 1B, C), strongly suggesting that disruption of the same gene is responsible for the ozone-sensitive phenotype in all three mutant strains.

*At1g12480*, hereafter referred to as *OZS1*, is located 4.26 Mbp from the short-arm end of chromosome 1 and encodes a putative membrane-bound transporter-like protein composed of 556 amino acids. The OZS1 protein is presumed to have 10 transmembrane domains (Fig. 3A), and has significant similarity to proteins in the tellurite resistance/C4-dicarboxylate-transporter (TDT) family (Fig. 3B, Saier et al. 1999). To date, three members of the TDT family have been functionally characterized. The *Escherichia coli* TehA protein functions to confer tellurite resistance on the bacteria (Taylor et al. 1994). The *Schizosaccharomyces pombe* Mae1 protein functions in uptake of malate and other dicarboxylates via a proton symport mechanism (Grobler et al. 1995). The final characterized family member is a *Saccharomyces cerevisiae* sulfite efflux pump (Park and Bakalinsky 2000). Four *OZS1*-related genes can be found in the Arabidopsis genome (Fig. 3B). The intracellular localization of OZS1 protein was predicted by using the WoLF PSORT (http://wolfpsort.org/) program that suggested the OZS1 protein is most probably localized at the plasma membrane. Based on previously reported results of microarray analysis, the level of *OZS1* transcript is high in shoots and sepals but is low in cultured cells, seeds and roots (Arabidopsis Membrane Protein Library, http://wardlab.cbs.umn.edu/arabidopsis/; ATTED-II, http://www.atted.bio.titech.ac.jp/). Though expression of the gene does not appear to be induced by ozone, it is induced by aging, nematode infection, heat shock, osmotic stress and genotoxic stress (Arabidopsis Membrane Protein Library, ATTED-II). This suggests that in addition to involvement in the response to ozone, OZS1 may also participate in responses to other environmental stresses.

**Analysis of the stress response in ozs1 mutant plants**

The growth and morphology of ozs1 mutant plants were similar to those of the wild type under normal
conditions [25°C, 50–60% relative humidity and a light intensity of 100 μmol photosynthetic photon flux density (PPFD) m⁻² s⁻¹], but severe chlorosis was observed after exposure to ozone, which had little effect on wild-type plants (Fig. 1A). The severity of ozone-induced injury to ozs1 plants was evaluated by measuring chlorophyll content (Fig. 1B) and ion leakage from leaves, which reflects membrane damage (Fig. 1C). We also compared ozone sensitivity in the wild type, Col-0 (wild type for SALK_099139), ozs1, SALK_099139 and the F1 progeny obtained after the cross between ozs1 and SALK_099139.

Plants homozygous for ozs1 or SALK_099139 and transheterozygotes showed similar degrees of damage in terms of both reduction of chlorophyll levels and the amount of ion leakage under conditions in which wild-type and Col-0 plants showed little sign of damage (Fig. 1B, C). The ozs1 plants had normal sensitivity to hydrogen peroxide (Fig. 4), suggesting that sensitivity to oxidative stress is not altered by disruption of OZS1. Sensitivity to low temperature (5°C) and high light levels (500 μmol PPFD m⁻² s⁻¹, 5-fold intensity of the growth condition) was also similar to that of wild-type plants (data not shown). However, ozs1 showed enhanced sensitivity to desiccation and sulfur dioxide, both in terms of appearance (Figs. 5A, 6A) and when evaluated quantitatively such as by measuring fresh weight (Fig. 5B) and ion leakage (Fig. 6B). These results suggest that ozs1 mutant plants do not have a defect in general resistance mechanisms, such as responses to oxidative stress, but may have a defect in gas absorption and transpiration.

**Analysis of stomatal responses in ozs1 mutant plants**

As suggested indirectly by the phenotypes of ozs1 mutant plants in response to various stresses, stomatal conductance was higher in ozs1 plants than in the wild type (Fig. 7A). The higher stomatal conductance of ozs1 plants was supported by the results of measurement of leaf temperature using an infrared thermal imager. Thermal imaging clearly showed a lower leaf temperature in mutant leaves at two different growth stages, probably due to a higher transpiration rate than in the wild type (Fig. 7B). Whereas the size and density of stomata on the abaxial leaf epidermis were similar between the mutant and wild-type plants (Fig. 7C, quantitative data not shown), the stomatal aperture in ozs1 mutant plants was larger than in the wild type (Fig. 7C), which seemed sufficient to explain the higher stomatal conductance we observed. Moreover, these results are consistent with the sensitivity of ozs1 mutant plants to gaseous stress factors, ozone and sulfur dioxide, and the
faster loss of water in dry conditions observed in mutant plants.

Because *ozs1* appears to have a defect in regulation of stomatal aperture size, we next assayed stomatal responses to various stimuli known to affect stomatal aperture; namely, light, ABA, high concentrations of carbon dioxide (Schroeder et al. 2001) and ozone (Torsethaugen et al. 1999), and compared these between *ozs1* and wild-type plants. The stomatal apertures of both *ozs1* and wild-type plants showed similar fluctuations under daily light/dark cycles, but the aperture in *ozs1* mutant plants was always larger than that in the wild type (Fig. 8A). The stomatal aperture of both *ozs1* and wild-type plants decreased during prolonged dark periods, and began to increase after the start of the following light period, further suggesting that the mutant plants retain light responsiveness (Fig. 8B). The mutant plants also retained the ability to close stomata in response to ABA (Fig. 8C) and high concentrations of CO₂ (approximately 3-fold those of ambient air) or ozone (Fig. 8D). Therefore, OZS1 protein does not appear to be involved in regulation of stomatal aperture in response to the above-examined stimuli but may always help to close stomata. An ATP-binding cassette transporter with such a function has been reported to be present in the guard cell plasma membrane of *A. thaliana* (Klein et al. 2004). Moreover, maintenance of the stomatal state is known to be regulated in concert with water potential of the leaf (Raschke 1975). Stomatal osmotic pressure is regulated via the presence of solutes in guard cells, such as K⁺, Cl⁻, malate and sucrose (MacRobbie 1981). As reported previously, malate accumulated light-dependently and

Fig. 3 Predicted features of the OZS1 protein (At1g12480). (A) Hydrophobicity profile analyzed using an algorithm developed by Kyte and Doolittle (1982; GENETYX Corp., Japan). The 10 putative transmembrane domains are indicated with arrows and vertical light gray bars. (B) Phylogenetic tree of TDT family proteins and related proteins encoded by the *Arabidopsis* genome. Comparisons are based on the unweighted pair group method with arithmetic mean (UPGMA; GENETYX Corp., Japan). The number of mismatches divided by the length of the shorter sequence is shown on horizontal lines. At1g62262, At1g62280, At4g27970 and At5g24030 have significant similarity to OZS1 and are also found in the *Arabidopsis* genome. TehA, *Escherichia coli* tellurite resistance determinant; Mae1, a *Schizosaccharomyces pombe* protein that functions in uptake of malate and other dicarboxylates via a proton symport mechanism; SSU1, a *Saccharomyces cerevisiae* sulfite efflux pump.

Fig. 4 Sensitivity of *ozs1* and wild-type leaves to hydrogen peroxide. (A) Images of leaves after treatment with various concentrations of hydrogen peroxide. (B) Ion leakage from leaves after incubation in various concentrations of hydrogen peroxide for 20 h. The average and SD are shown (n = 3). wt, wild-type.
Sucrose accumulated light-independently in guard cells under osmotic stress (Asai et al. 1999). Thus, it is possible that OZS1 protein may act as a malate and/or sucrose transporter to regulate stomatal aperture size.

The ozs1 mutant can be an appropriate material to dissect the mechanism of stomatal movement, although it was isolated as an ozone-sensitive mutant in this study. Further analyses of ozs1 mutant plants are currently underway, as well as characterization of the OZS1 protein, such as its tissue-dependent expression and its intracellular localization. Furthermore, we are measuring the malate content of epidermis and guard cells in ozs1 and wild-type plants. The transporting activity of OZS1 for malate and other solutes will also be investigated.

**Materials and Methods**

**Screen for ozone-sensitive mutant strains**

T-DNA-transformed *A. thaliana* L. ecotype Col-6, gl1-1 seeds were obtained from the Arabidopsis Biological Research Center, Ohio State University, Columbus, OH, USA. Seeds were sown on blocks of rock wool (Minipot, Nittobo, Tokyo, Japan) and chilled at 4°C for 1 week, and the resulting seedlings were grown in a growth chamber at 25°C under 14 h of light per day at 100 μmol PPFD m⁻² s⁻¹ from white fluorescent lamps and 50–60% relative humidity. Fourteen-day-old seedlings were exposed to 0.2 μl l⁻¹ ozone in a growth chamber at 25°C and a relative humidity of 70% under continuous light from fluorescent lamps with 100 μmol PPFD m⁻² s⁻¹. Then, plants with severe visible damage were isolated and those that reproducibly showed ozone sensitivity were back-crossed twice with the wild type (Col-6, gl1-1) in an attempt to eliminate other mutations from the mutant genome. To test for kanamycin resistance, seedlings were grown on Murashige and Skoog medium–agar plates with kanamycin at 25 mg l⁻¹ for 10 d in a growth chamber, and kanamycin-sensitive plants were identified by the characteristic bleached cotyledon and lack of roots. The test of kanamycin resistance was also carried out in F₁ progeny obtained from crossing ozs1 to SALK_137265 or SALK_099139.

**Identification of the OZS1 gene**

In order to prepare DNA for genomic PCR, approximately 3.0 mg of leaf tissue was macerated in a microfuge tube using a plastic mini-pestle, followed by addition of 200 μl of extraction buffer (200 mM Tris–HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS) and homogenization. After ethanol precipitation, the pellet was dissolved in 200 μl of TE buffer (10 mM Tris–HCl;
T-DNA probes were prepared from the pD991 plasmid (kindly provided by Roche, Basel, Switzerland) according to the manufacturer’s instructions. Genomic DNA was extracted from 14-day-old plants using Nucleon PhytoPure (GE Healthcare, Uppsala, Sweden) and purified using the QIAquick Gel Extraction Kit (Qiagen, Germantown, MD, USA). DNA probes were labeled using a digoxigenin system following the manufacturer’s instructions (Roche, Basel, Switzerland). DNA fragments were isolated and purified from an agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Germantown, MD, USA). DNA probes were labeled using a digoxigenin system (Roche, Basel, Switzerland). For TAIL-PCR (Liu et al. 1995), genomic DNA was extracted from ozs1 plants and then the T-DNA-flanking sequence was amplified using the following T-DNA-specific and AD primers.

Left border primers: Oligo 154, 5-TGATCCATGTAAGTTTCCCG-3; Oligo 155, 5-ATAAGCTCGGAGATCTAC-3; Oligo 156, 5-CCTATAAAATACGAGCGGATG-3. Right border primers: Oligo 86, 5-TGGGGCCTAAACTTTTGTTG-3; Oligo 123, 5-GCATGCAAGCTTTGGCAGCCTGG-3; Oligo 124, 5-TGACGACCTCAATTGGAGC-3. AD primers: AD1, 5-NCTCAAG(T/A)TGTGTT-3; AD2, 5-NGTCGAAG(C/A)TGANA(T)GAA-3; AD3, 5-(A/T)GTGNAG(A/T)ANCAAGA-3; AD5, 5-(A/T)TGNTAG(C/G)GTNCTTCG-3; AD1a, 5-TGA(A/T)GNAG(G/C)ANCAAG(A/C)AGA-3; AD2a, 5-AAG(A/T)GNAG(A/T)ANCA-3; AD5a, 5-GATGCAGCTTCTCTCTGCT-3.

Total RNA for RT-PCR was extracted from 14-day-old plants of ozs1 and the wild type using the RNeasy Plant Mini Kit (Qiagen). Subsequently, 350 ng of mRNA was obtained from 75 μg of total RNA, and 300 ng of this was used for RT-PCR, which was carried out with Sensiscript following the manufacturer’s instructions (Qiagen) and with the following primer sets derived from exon 2 (OZ31028-1F4) and exon 3 (OZ31028-1R3): 377 bp product primers: OZ31028-1F4, 5-TCGGGCCTAACTTTTGGTG-3; OZ31028-1R3, 5-TCGCGATCAATTTCTTCA-3; OZ31028-1R3, 5-GATGCAGCTTCTCTCTGCT-3.

Analysis of stress responses

Fourteen-day-old mutant and wild-type plants were exposed to ozone as described above and ozone sensitivity was measured according to the following three parameters: visual injury, chlorophyll content and ion leakage from leaves. To determine total chlorophyll content, the first and second leaves of two plants were detached by cutting at the petiole, combined and used for measurement according to the method of Arnon (1949). To measure ion leakage levels, the first and second leaves of three plants were detached by cutting at the petiole, combined and shaken in 1 ml of distilled water for 1 h at 100 r.p.m. Then, the electroconductivity of the bathing water was measured with an ion conductivity meter (B-173; Horiba, Tokyo, Japan). The leaves were then autoclaved together with the remaining water, and the conductivity of the autoclaved solution was also measured. Relative ion leakage was obtained by dividing the conductivity of the pre-autoclaved solution by that of the autoclaved solution.

To test sensitivity to hydrogen peroxide, three of the first or second leaves from 14-day-old plants were dipped into 1 ml of various concentrations of hydrogen peroxide, vacuum-infiltrated for 2 min, then incubated under continuous light of 100 μmol m⁻² s⁻¹ at 25°C for 20 h, and the electroconductivity of the water was measured as described above.

To analyze drought sensitivity, twenty 14-day-old ozs1 or wild-type plants were carefully drawn out from rock wool, left on a plastic case at 25°C and 70% humidity, and their fresh weight was measured at 10-min intervals for 1 h. The relative weight was obtained by dividing the fresh weight obtained at each time point by that at the starting time. The experiment was repeated five times, and the average and SD were obtained.

Sensitivity to sulfur dioxide was analyzed similarly to the test for ozone sensitivity. That is, 14-day-old ozs1 and wild-type plants...
were exposed to sulfur dioxide at 1 μl l⁻¹ under a light intensity of 100 μmol PPFD m⁻² s⁻¹ for 2 h and then moved into fresh air for 1 d. Chlorophyll content and ion leakage levels from leaves of these plants were then measured as described above.

**Analysis of stomatal responses**

Most of the following experiments were conducted using the first or the second leaf of plants cultivated for 2 weeks in a growth chamber under 14 h of light per day at 100 μmol PPFD m⁻² s⁻¹.
25°C and 60% humidity. Leaf stomatal conductance was measured using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE, USA) equipped with a 6400-15 Arabidopsis chamber (Li-Cor). The gas exchange rate was also checked by thermal imaging analysis using a thermo-tracer TH7102MX (NEC, Tokyo, Japan). Stomatal apertures were measured on epidermal peels prepared from leaves, using an IMT-2 inverted microscope system (Olympus, Tokyo, Japan) equipped with a micrometer. For most of the analyses, the average and SD of 20 samples were calculated.

Acknowledgments

We thank Dr. T. Jack for providing the T-DNA tagging lines and pD991; the SALK Institute Genomic Analysis Laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutants; and the Arabidopsis Biological Research Center for distributing the T-DNA tagged lines. We also thank Dr. H. Oguma for allowing us to use a thermo-tracer. S.B. was a JSPS fellow.

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