Wound-responsive cis-Element in the 5'-Upstream Region of Cucumber Ascorbate Oxidase Gene

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The cucumber (Cucumis sativas) AAO1 gene (former name, AsoI) encodes an ascorbate oxidase that catalyzes the oxidation by molecular oxygen of ascorbic acid to dehydroascorbate. CsAAO1 mRNA concentrations rose rapidly after mechanical wounding of cucumbers. To study the wound-responsive expression of CsAAO1 in detail, we examined transgenic tobacco plants harboring a CsAAO1 promoter-β-glucuronidase fusion gene. CsAAO1 promoter activity in leaves of the tobacco was induced by wounding. Analysis of the regulatory properties of 5'-deleted promoter fragments showed that a putative wound-responsive cis-element (WRE) was located −736 to −707 bp from the translation initiation site. DNA binding factors that bound specifically to the putative WRE sequence were identified in tobacco nuclear extracts by gel retardation assays.

Key words: ascorbate oxidase; cucumber; promoter; wound; cis-element

Ascorbate oxidase (AAO; EC 1.10.3.3) catalyzes the oxidation by molecular oxygen of ascorbic acid to dehydroascorbate, with the formation of water. AAO is expressed abundantly in the fruit of Cucurbitaceae plants such as cucumber, zucchini, and pumpkin.1) Plant AAO contains four copper atoms, with one type I (blue copper center), one type II (normal copper center), and two type III (binuclear center) copper atoms per subunit of the homodimeric enzyme.2) cDNAs encoding plant AAO have been cloned from cucumber (Cucumis sativas),3 zucchini (Cucurbita pepo medullosa),4 pumpkin (Cucurbita sp Ebisu Nankin),5 melon (Cucumis melo),6 and tobacco (Nicotiana tabacum).7) The cucumber gene, previously named AsoI, has been renamed CsAAO1 here.

AAO activity is high in the growing phase of cultured pumpkin cells.8) AAO production is induced by auxin in pumpkin fruit tissues.9) Lin and Varner studied the expression of the AAO gene in fruit and leaves of the zucchini plant, and found that concentrations of the AAO protein and corresponding mRNA are highest in actively growing tissues.10) Genomic DNA encoding AAO has been isolated from cucumber11) and pumpkin.12) CsAAO1 is expressed at high concentrations in cucumber fruit and stems.13) Kerk and Feldman have found that the AAO gene is specifically expressed in the quiescent center of maize roots and may be involved in the organization of root meristems.14) The expression of a pumpkin AAO promoter-β-glucuronidase (GUS) chimeric gene was induced by auxin in pumpkin fruit, indicating that an auxin-responsive cis-element in the 5'-upstream region acts in the transcriptional regulation of the AAO gene.12) AAO expression is under cell-cycle control in cultured tobacco cells.15) However, the relationship between the type of AAO and the mode of gene expression is unclear.

Here, we report as a novel regulation of the AAO gene that the expression of the CsAAO1 gene is induced by mechanical wounding. Wounding leads to dramatic physiological changes in plants. Several wound-induced genes have been studied by functional analysis of their promoter region in transgenic plants, including those encoding proteinase inhibitors,16–18) phenylalanine ammonia-lyase,19) chalcone synthase,20) pathogenesis-related proteins,21) nopaline synthase,22) soybean storage proteins,23) and peroxidases.24,25) Oligosaccharides, systemin, jasmonic acid, abscisic acid, ethylene, and electric signals seem to be components of signal transduction pathways leading to the expression of genes encoding wound-response proteins. Transcriptional regulation involves interactions between nuclear transcription factors and cis-elements in the 5'-upstream region or intron of the
target genes. Palm et al. found an interaction between a nuclear protein(s) from wounded tomato leaves and a 10-bp cis-element within the promoter of the potato proteinase inhibitor 1K gene. Wound-inducible nuclear factors bind to a cis-element in the 5′-upstream region of the mannopine synthase gene. Expression of the barley HVLox1 gene is regulated by an interaction between a trans-factor in the WRKY family and a cis-element containing a W-box sequence. G-box and PAL-box sequences are essential for expression of the prxC2 gene encoding a wound-inducible horseradish peroxidase. TFHP-1 and Ntlin1 proteins bind to the G-box and the PAL-box of the prxC2 promoter, respectively, and function as trans-factors for their expression. Despite these findings, however, the precise molecular mechanisms underlying wound-induced gene expression have not been examined thoroughly. Here, we describe the functional analysis of the CsAAO1 promoter, which helps to govern the extent of wound-induced expression of its gene, by 5′-deletion and DNA-protein binding assays in vitro.

Materials and Methods

Plant materials. The cucumber plant (Cucumis sativus cv. Shin-hokusei 1) was the source of the CsAAO1 gene and ascorbate oxidase. Nicotiana tabacum cv. Petit Havana SR-1 was used as the host plant in all gene transfer experiments. The plants were grown in a greenhouse and the leaves were used in the wound-responsive expression experiment.

Wound treatment of cucumber and tobacco leaves. Leaves of cucumber or transgenic tobacco plants grown on the Murashige and Skoog medium were cut into small pieces (1 cm), washed three times with sterilized water, and incubated in sterile 10 mM sodium phosphate buffer (pH 7.0) at 25°C. Twenty micrograms of RNA was separated on a 1% agarose gel containing 16% formaldehyde. After transfer to a ZetaProbeGT membrane (Bio-Rad Laboratories, Richmond, CA), hybridization was done with cDNAs labeled with [α-32P]dCTP with a BcaBEST labelling kit (Takara Shuzo Co., Ltd., Kyoto, Japan). The membrane was hybridized in a solution of 0.25 M Church buffer, 0.25 M NaCl, 7% SDS, 1% bovine serum albumin, and 1 mM EDTA at 65°C for 16 h. Next, the membrane was washed in 2× SSC and 0.1% SDS, then in 1× SSC and 0.1% SDS, and finally in 0.5× SSC and 0.1% SDS. All washing was at room temperature for 15 min.

Western blotting. Soluble protein was extracted from the leaves of cucumber plants at 0, 2, 4, and 8 h after wounding. The protein was separated on a 12.5% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories). Membranes were treated with a solution containing 3% skim milk suspended in TBST (25 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween-20), 1 h at room temperature. After incubation with rabbit anti-cucumber AAO polyclonal anti-serum at 1:1000 dilution, membranes were washed and incubated with a 1:2,500 dilution of goat anti-rabbit IgG conjugated with alkaline phosphatase. Immunodetection was done with ProtoBlot Western Blot AP Systems (Promega, Madison, WI).

Results

Construction of chimeric genes of CsAAO1 promoter and GUS gene. We constructed chimeric genes in which 1071, 736, 494, 368, or 117 bp of the 5′-flanking region of CsAAO1 was fused to the coding region of the GUS reporter gene. These chimeric genes were inserted into the binary vector plasmid pBI101Hm and amplified in Escherichia coli HB101. The modified binary vectors in E. coli cells were transferred to Agrobacterium tumefaciens EHA105 cells by triparental mating with the helper plasmid pRK2013.

Plant transformation. Tobacco was transformed with A. tumefaciens EHA105 that carried a modified form of pBI101 by the leaf-disk-infection method. Transformants were selected in the presence of kanamycin (100 μg/ml) and regenerated plants were grown in a greenhouse at 25°C.

Gel retardation assays. Nuclear protein was prepared as described by Staiger et al. from tobacco leaves. The binding reaction mixture contained 2 μg of nuclear proteins, 2 μg of poly(dt-dC), 5 ng of the radiolabelled probe DNA, and a 10- or 50-fold molar excess of the unlabelled competitor in DNA binding buffer (10 mM Tris-HCl, pH 8.0, 80 mM KCl, 1 mM dithiothreitol, 0.1% NP-40, and 7.5% glycerol). The reaction mixture was incubated for 30 min at 23°C and then put on a 4% polyacrylamide gel in TBE (89 mM Tris-HCl pH 8.0, 89 mM boric acid, and 2 mM EDTA) for electrophoresis. Labeled bands were detected by autoradiography.

Western-induced expression of the CsAAO1 gene in cucumber

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RNA blotting. Total RNA was isolated from leaves by the aaurintricarbocinolyx acid method. Twenty micrograms of RNA was separated on a 1% agarose gel containing 16% formaldehyde. After transfer to a ZetaProbeGT membrane (Bio-Rad Laboratories, Richmond, CA), hybridization was done with cDNAs labeled with [α-32P]dCTP with a BcaBEST labelling kit (Takara Shuzo Co., Ltd., Kyoto, Japan). The membrane was hybridized in a solution of 0.25 M Church buffer, 0.25 M NaCl, 7% SDS, 1% bovine serum albumin, and 1 mM EDTA at 65°C for 16 h. Next, the membrane was washed in 2× SSC and 0.1% SDS, then in 1× SSC and 0.1% SDS, and finally in 0.5× SSC and 0.1% SDS. All washing was at room temperature for 15 min.

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A course of CsAAO1 Gene Expression and AAO Protein Production after Wounding in Cucumber.

A. Wound-induced expression of CsAAO1. Total RNA (20 μg/well) from unwounded or wounded (0.5–16 h) cucumber leaves was hybridized with a radiolabeled cDNA fragment of the CsAAO1 gene. B. Western blotting of AAO protein in cucumber leaves. Twenty micrograms of crude protein extracted from wounded leaves or 0.5 μg of purified AAO protein (lane C) were put in the lanes.

Fig. 2. 5′-Deletion Analysis of the CsAAO1 Promoter.

A deletion series of the 5′-upstream region of the CsAAO1 was constructed, generating the following sequences relative to the ATG initiation codon: −1071, −736, −707, −494, −368, and −117 bp. The GUS reporter gene was put under the control of a promoter fragment and the resulting promoter-GUS chimeric constructs were used to transform tobacco plants.

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wounded leaves of cucumber (Fig. 1B).

Cis-element in the 5′-upstream region of CsAAO1 for wound-responsive expression

A chimeric gene comprising the 1071-bp full-length CsAAO1 promoter and GUS was constructed (Fig. 2) and introduced into tobacco. The presence of the expected chimeric genes in the transgenic tobacco plants was confirmed by PCR (not shown). Leaves of five transgenic T1 plants were wounded in the same way as was done for the cucumber plants. GUS activity was increased by 1 or 2 days after wounding (Fig. 3A). This result indicates that CsAAO1 promoter activity increased by mechanical wounding in tobacco plants. GUS activity increased in tobacco containing CsAAO1 promoter fragments 736 bp or longer (Fig. 3AB), but not in those 707 bp or shorter (Fig. 3C-F). Therefore, the position of the wound-responsive cis-element (WRE) of the CsAAO1 gene was restricted to the 30 bp between −736 and −707 bp from the ATG initiation codon. The DNA sequence of the putative WRE contained a tandemly repeated 10-bp 5′-AA(A/T)GTATC(C/G)A-3′ motif (Fig. 4).

GUS activity in transgenic tobacco leaves harboring a −494- or −368-bp CsAAO1 promoter-GUS chimeric gene was about five times that in leaves expressing the −707-, −736-, or −1071-bp CsAAO1 promoter-GUS chimera (Fig. 3). These results suggest that another cis-element for the repression of the CsAAO1 promoter in healthy plants exists in the region between −494 and −707 bp in the 5′-upstream region of the CsAAO1 gene. A −117-bp DNA fragment without the CAAT or TATA boxes
had no promoter activity (Fig. 3F).

**Identification of DNA binding factors interacting with** *CsAAO1* **WRE**

These results suggested the presence of a transcription factor(s) in tobacco interacting with the putative WRE of *CsAAO1* to regulate wound-responsive transcription. Nuclear proteins prepared from unwounded or wounded tobacco leaves were examined by gel retardation with the WRE sequence as the probe (p1: Fig. 5A). A band shifted when the 30-bp sequence of the WRE and the nuclear extract were combined, but the shift did not occur when there was incubation with an excess of the unlabelled 30-bp DNA fragment (Fig. 5B). The binding factors of the WRE were found in both unwounded and wounded leaves, but the shift was different with wounding and without wounding. The results suggested the presence of nuclear factors that bind constitutively to the putative WRE in a DNA-sequence-specific way.

To investigate the importance of the 10-bp motif in the sequence of the WRE for binding to nuclear proteins, we prepared three DNA fragments, p2, p3, and p4, as probes for the gel retardation assay. p2 was a 26-bp fragment lacking a 4-bp spacer sequence between the two 10-bp motifs, p3 was the 19 bp at the 5'-end of the WRE, and p4 was the 14 bp at the 3'-end of the WRE (Fig. 5A). Results of gel retardation assays showed retarded bands in lanes containing p2, but not in those containing p3 or p4 (Fig. 5C). These results suggested that the tandemly repeated 10-bp motif, but not the 4-bp spacer, was essential for recognition of the binding region by nuclear proteins.

**Discussion**

We present here the first report showing that the *AAO* gene is inducible by wounding. To understand the induction of *CsAAO1* transcription by wounding, it is necessary to identify the cis-acting elements and trans-acting factors involved in the wound response. To obtain information about the functional organization of the *CsAAO1* promoter, we constructed *CsAAO1* promoter-GUS fused genes. GUS activity by wounding was induced in transgenic tobacco leaves. Deletion analysis of the *CsAAO1* promoter region showed that the 30 bp from –736 to –707 bp upstream of the ATG codon were necessary for wound-inducible expression in transgenic tobacco. The DNA sequence of this putative WRE contained a tandemly repeated 10-bp 5'-AA(A/
TGTATC(C/G)A-3’ motif. This sequence motif has not been found in the 5'-upstream region of other AAO genes. A database search showed that this 10-bp motif is in the 5'-upstream region of a pea seed lipoxygenase gene (AATGTATCGA at −536 to −527 bp)\(^{39}\) and a soybean proteinase inhibitor gene (AATGTATCCA at −274 to −265 bp)\(^{40}\) but those 10-bp motifs are not tandemly repeated. Lipoxygenase activity increased in response to mechanical wounding of rye seedlings.\(^{41}\) Lipoxygenase is involved in the synthetic pathway of a regulatory molecule, jasmonic acid, produced via the octadecanoid pathway from linolenic acid. Methyl jasmonic acid induces the expression of defense-related proteins (the proteinase inhibitor PI-I of tomato and PI-II of potato) in response to wounding.\(^{42-44}\) Therefore, the 10-bp motif may be a consensus wound-responsive sequence. The next question is whether the tandemly repeated 10-bp motif is sufficient to increase the level of promoter activity after wounding. The G-box and the PAL-box motif are needed for the expression of the prxC2 gene in response to wounding.\(^{29,32}\) The G-box motif is needed for some responses, such as those to abscisic acid, light, anaerobiosis, and ethylene.\(^{45}\) The G-box and the PAL-box, however, were not found in the 5'-upstream region of the CsAAO1 gene.

In the gel retardation assay, a nuclear factor(s) in tobacco leaves bound to the 30-bp putative WRE. The tandemly repeated 10-bp motif was essential for this binding. The wound inducibility of gene expression implies the existence of a signal transduction pathway in response to the environmental stimuli. In the final step of this pathway, a wound-specific transcription factor(s) interacts with WRE, trans-activating CsAAO1 gene transcription. Differences in the shift of the band and in band intensity between the wounded and non-wounded conditions may depend on the mode of modification of the DNA binding factor. Isolation of cDNA(s) encoding the DNA binding factor(s) will provide useful information regarding the molecular mechanisms of transcriptional regulation in wound-response formation.

The AAO gene is expressed at high levels during elongation of cultured tobacco cells (N. tabacum, BY2).\(^{15}\) Transgenic BY2 cells harboring a pumpkin AAO gene under the control of the cauliflower mosaic virus 35S promoter elongate more rapidly than wild-type cells when hormones are applied.\(^{46}\) These results suggest that AAO is important in plant cell elongation. The maize AAO gene is expressed specifically in the quiescent center of roots,\(^{14}\) and AAO expression in cultured tobacco cells is under cell-cycle control.\(^{15}\) Accumulation of AAO mRNA is repressed within 6 h when melon fruits are wounded; the significance of wound-responsive AAO expression is not known.

In plants, a variety of biochemical changes occur in tissues affected by pathogens, pests, or mechanical trauma. Molecular breeding of disease- and insect-resistant crops has been done with so-called resistance genes such as the gene encoding chitinase.\(^{47,48}\) The combination of resistance genes and promoters such as CsAAO1 that respond to wounding stress would increase resistance against pathogens and pests.

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