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Note

Improvement in the ozone tolerance of poplar plants with an antisense DNA for 1-aminocyclopropane-1-carboxylate synthase

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Abstract Genetically transformed lombardy poplar (*Populus nigra* L. var. *italica* Koehne) were regenerated after the co-cultivation of stem segments with *Agrobacterium tumefaciens* strain LBA4404, which harbored a binary vector that included an antisense DNA for ozone-inducible 1-aminocyclopropane-1-carboxylate (ACC) synthase from poplar leaves. Lower rates of ozone-induced ethylene production were observed in transgenic plants than in wild-type plants. Ozone-induced visible damage was attenuated in these lines, and the extent of damage was positively related to the level of ozone-induced ethylene production. In one of these ozone-tolerant lines, the levels of transcripts for ozone-inducible endogenous ACC synthases were suppressed compared with those in wild-type plants, demonstrating that ozone-inducible ACC synthases have a key role in the expression of leaf damage by ozone exposure. Thus, transgenic trees with air pollution tolerance were developed for the first time among woody plants.

Key words: Air pollution tolerance, 1-aminocyclopropane-1-carboxylate synthase, ethylene, lombardy poplar, ozone.

Tropospheric ozone (O_3) concentrations have increased globally by about 36% since pre-industrial times (Intergovernmental Panel on Climate Change, 2001, 2007), and are predicted to adversely affect forest ecosystems during the coming decades (Ashmore 2005; Fowler et al. 1999). O_3 is an air pollutant in many industrialized and developing countries. It is the main oxidant component of photochemical smog and causes leaf damage in many plant species (Kangasjärvi et al. 1994; Matyseek and Sandermann 2003), contributing substantially to crop loss and forest decline (Preston and Tingey 1988).

Before leaf damage appears, O_3 induces many biochemical reactions, such as ethylene production, reactions involved in the hypersensitive response found in pathogen infection, and reactions involved in flavonoid synthesis (Kangasjärvi et al. 1994). In the O_3-induced responses, the production of ethylene, a plant hormone, is one of the earliest detectable events (Craker 1971). A tight correlation between the rate of O_3-induced ethylene production and the extent of leaf damage has been reported (Tingey et al. 1976). It also has been shown that an inhibitor of hormonal action of ethylene reduced O_3-induced leaf damage (Bae et al. 1996; Mehlhorn and Wellburn 1987). An O_3-sensitive cultivar of tobacco showed higher O_3-induced ethylene production than that from a tolerant cultivar (Langebartels et al. 1991). These findings strongly suggest that a hormonal action of ethylene participates in the promotion of leaf damage by O_3 exposure.

Ethylene is synthesized from S-adenosyl-L-methionine via 1-aminocyclopropane-1-carboxylate (ACC) in higher plants, and ACC synthase (ACS; EC 4·4.1·14) often catalyses the rate-limiting step in ethylene biosynthesis (Yang and Hoffman 1984). The genes encoding ACS comprise a divergent multigene family and are differentially regulated in expression, depending on the developmental phase and environmental conditions (Kende 1993). In some solanaceous plants, two types of isogenes of ACS, one transiently induced within 2 h of O_3 exposure (early O_3-inducible ACSs), the other after a further 1 to 2 h delay in induction (late O_3-inducible ACSs), sequentially accumulate during O_3 exposure (Nakajima et al. 2001; Schlagnhaufer et al. 1997). In tomato plants, 1 h of O_3 exposure is sufficient to induce leaf damage, which suggests that the early O_3-inducible ACSs probably have a pivotal role in the appearance of leaf damage by O_3 treatment (Bae et al. 1996). The

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improvement in O_3 tolerance of tobacco plants with an antisense DNA for the early O_3-inducible ACSs has been successfully reported (Nakajima et al. 2002; Wi and Park 2002).

Poplar is a favorable model plant among forest trees because of its small genome size, short rotation cycle, rapid growth rate, and ease of vegetative propagation. Transgenic poplars variously improved in insect pest resistance, herbicide resistance, growth rate, woody quality, etc., and they have increased through the development of methods for genetic engineering in recent years (Confalonieri et al. 2003). We have also succeeded in controlling the morphological features in poplar by engineering the overexpression of the rice homeobox gene *OSH1* (Mohri et al. 1999). To our knowledge, there is no report of transgenic poplars with improved air pollutant resistance. In this study, we tried to suppress the expression of O_3-inducible ACSs in poplar by introducing an antisense DNA for a poplar early O_3-inducible ACS and analyzed the characterization of the transgenic poplar under O_3 exposure.

Shoot cultures derived from peeled twigs of mature lombardy poplar (Mohri et al. 1996) were maintained on a solid medium that contained Murashige and Skoog's basal salts (Murashige and Skoog 1962), Gamborg's B5 vitamins (Gamborg et al. 1968), and 3% (w/v) sucrose (MSB5S medium) supplemented with 0.8% (w/v) agar and 0.5 mg/l 3-indolebutyric acid (IBA). Shoot cultures were incubated at 25°C under cool, white fluorescent light ($30\,\mu E \cdot m^{-2} s^{-1}$, 16 h photoperiod) and subcultured every two months.

Complementary DNAs for O_3-inducible ACSs from poplar leaves were cloned by reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA ($0.4\,\mu g$) from O_3-treated leaves was used for reverse transcription at 42°C for 30 min using RT-PCR beads (Ready-To-Go; Amersham Pharmacia Biotech). The cDNA was amplified by PCR with specific primers. The primers were made on the basis of conserved amino acid sequences of plant ACSs; their sequences were TT(TC)CA(AG)GA(TC) TA(TC)CA(TC)GGI(TC)TICC and GTICCIA(AG)IGG (AG)TTIGAIGG(AG)TT. The PCR conditions were 1 min at 94°C for denaturation, 2 min at 55°C for primer annealing, 3 min at 72°C for primer extension, for 50 cycles. The amplified cDNAs were subcloned into a pGEM-T Easy kit (Promega, Madison, WI, USA), and sequenced.

A blunt-ended cDNA for O_3-inducible poplar ACS (AcNO. AB033502: PO-ACS1) was ligated in an antisense orientation to the cauliflower mosaic virus 35S promoter, and replaced the β-glucuronidase coding region of the binary vector pBI35S-Hm (Figure 1). The disarmed strain *Agrobacterium tumefaciens* LBA4404 (Hoekema et al. 1983), harboring the binary vector, was

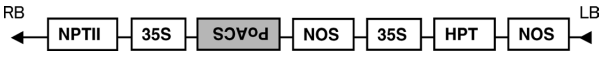

Figure 1. Structure of the construct used for transformation. NPT II, a gene encoding neomycin phosphotransferase II; 35S, cauliflower mosaic virus 35S promoter; NOS, nopalin synthase terminator; HPT, a gene encoding hygromaycin phosphotransferase; RB/LB, right/left border of T-DNA.

used in transformation experiments. *A. tumefaciens* was grown overnight at 28°C in liquid Luria-Bertani medium (Sambrook et al. 1989) in the presence of $25\,mg\,l^{-1}$ kanamycin and $300\,mg\,l^{-1}$ streptomycin. The overnight culture was diluted with liquid MSB5S medium to 5×10^8 cells ml^{-1} for the transformation of the poplar tissue.

Transformation and regeneration of lombardy poplar were performed as described in a previous study (Mohri et al. 1996). In brief, stem segments from cultures of poplar shoots were co-cultivated for 30 min with the above mentioned diluted culture of *A. tumefaciens* (Horsch et al. 1985). The stem segments were then blotted with sterile filter paper and incubated for two days on MSB5S medium (pH 5.8) supplemented with 0.3% (w/v) Gelrite (Scott Laboratories, Inc., Carson, CA) and $200\,\mu M$ acetosyringone (4-acetyl-2, 6-dimethoxyphenol; Aldrich, Milwaukee, WI). Each segment was washed three times with liquid MSB5S medium and then once with liquid MSB5S medium, which contained $500\,mg\,l^{-1}$ cefotaxime (Sigma, St. Louis, MO). After blotting with sterile filter paper, segments were planted on selective callus-induction medium [MSB5S medium containing $150\,mg\,l^{-1}$ kanamycin, $10\,mg\,l^{-1}$ hygromycin, $500\,mg\,l^{-1}$ cefotaxime, $500\,mg\,l^{-1}$ carbenicillin, $0.5\,mg\,l^{-1}$ 2,4-dichlorophenoxyacetic acid and 0.3% (w/v) Gelrite]. Calli were allowed to develop for four weeks, and they were then excised from stem segments and transferred to selective shoot-regeneration medium [MSB5S medium supplemented with $150\,mg\,l^{-1}$ kanamycin, $500\,mg\,l^{-1}$ carbenicillin, $2\,mg\,l^{-1}$ *trans*-zeatin, $0.2\,mg\,l^{-1}$ 6-benzyladenine and 0.3% (w/v) Gelrite]. Regenerated shoots were induced to produce roots by incubation on selective rooting medium [MSB5S medium that contained $75\,mg\,l^{-1}$ kanamycin, $2\,mg\,l^{-1}$ hygromycin, $500\,mg\,l^{-1}$ carbenicillin, $0.5\,mg\,l^{-1}$ IBA, 0.02 mg/l α-naphthaleneacetic acid and 0.8% (w/v) agar]. Kanamycin- and hygromycin-resistant, putative transgenic shoots were selected by determination of the insertion and expression of HPT gene (Igasaki et al. 2000).

These transgenic plants were grown weekly with Hyponex [0.1% (v/v); Hyponex Japan Licensee, Tokyo, Japan], and pots were watered daily. Seedlings that were five to six weeks old were exposed to 0.6 ppm (Nakajima et al. 2002). O_3 in a growth chamber

(230 cm×190 cm×170 cm) at 25°C and 70% relative humidity, under light from metal halide lamps with a photosynthetic photon flux density (PPFD) of 300 μE m^{-2} s^{-1}. Ozone was generated from an O_3 generator (Sumitomo Seika Chemicals Co., Tokyo, Japan).

The rate of ethylene production was measured as described by Bae et al. (1996). Poplar plants at seven to eight weeks old were exposed to 0.6 ppm. O_3. Leaf discs were excised and incubated in sealed flasks under light for 1 h. Then, 1 mL of gas was withdrawn from each flask, and ethylene was analyzed by gas chromatograph equipped with a flame ionization detector (GC-7 A; Shimadzu, Tokyo, Japan).

RT-PCR was performed by using 1 μg of RNA from O_3-exposed leaves. Primers were designed to amplify the internal ACS (PO-ACS2) specifically. Their nucleotide sequences were AGGACATGGGACTCCCTGGCT and ACAAAGAGACCGGCTTTGCTTT. Amplified DNA was analyzed by agarose electrophresis.

Transgenic poplar plants were generated with an antisense DNA for an early O_3-inducible poplar PO-ACS2 gene. Twenty transgenic lines that showed kanamycin and hygromycin resistance (markers of successful transformation) were obtained. All transgenic plants contained the introduced HPT gene and showed constitutive expression of the antisense DNA for a poplar ACS gene, but the constitutive expression was not found in the wild-type plants (data not shown). The morphological features of the transgenic plants did not differ noticeably from nontransformants (Figure 2). A difference in growth rates was not found under strong light and sufficient nutrient conditions; however, a difference was sometimes observed when they were cultivated under weak light and insufficient nutrient conditions.

We examined the physiological characteristics of transgenic poplar plants to know their potential for O_3 tolerance. For the first time, we selected the best conditions for O_3 exposure. When the control poplar plants were exposed to 0.2 ppm O_3, which is fatal in tobacco plants (Nakajima et al. 2002), we could not find any leaf damages. When O_3 concentration increased to 0.6 ppm, their leaves withered and necrosis appeared on the surface within 24 h after the end of exposure. The injury tended to be more extensive in older leaves than in younger leaves (Figure 3). The result might indicate that lombardy poplar have higher O_3 tolerance than tobacco plants do. By contrast, some transgenic lines showed less visible damage and line 24 showed the highest resistance to the O_3 treatment (Figure 3).

We compared the level of O_3-induced ethylene production between the transgenic and wild-type plants during O_3 exposure to study the effectiveness of the transgenic plants. In wild-type plants, ethylene synthesis

Figure 2. Growth with transgenic and wild-type plants under conditions where nutrition was scare (Plants were grown weekly with Hyponex [0.001% (v/v)]);. Left: wild type, Right: antisense transgenic plant. Bar=10 cm.

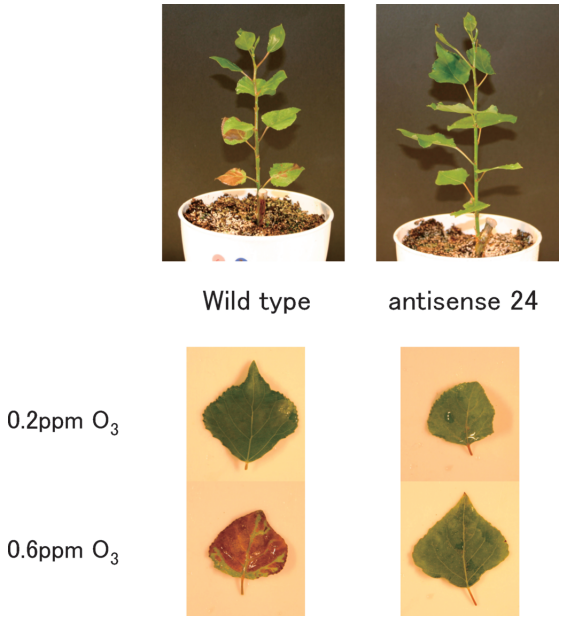

Figure 3. Leaf damage caused by 6 h O_3 exposure. Four- to five-week-old wild type (WT) and transgenic (antisense 24) poplar plants were exposed to 0.6 ppm. After the O_3 treatment, they were left in the light for 24 h.

was induced within 2 h after O_3 exposure (Figure 3). Although synthesis in the transgenic lines was also induced after 2 h after O_3 exposure, the levels were markedly lower than that of wild-type plants (data not shown). Most remarkable was line 24, whose ethylene production was about two-thirds that of the wild-type plant (Figure 4). We generated 20 transgenic lines that showed constitutive expression of the antisense DNA,

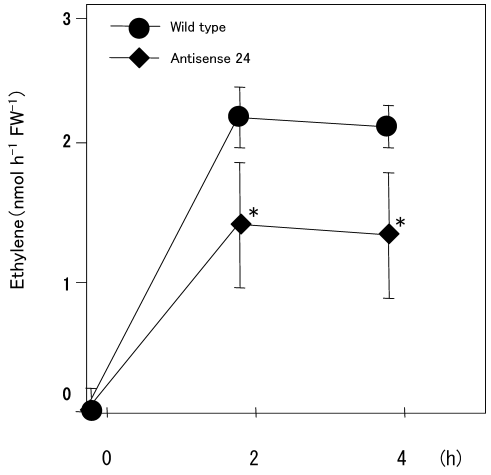

Figure 4. Ethylene production by transgenic (antisens 24) and wild-type plants during O_3 exposure. Four- to five-week-old plants were exposed to 0.6 ppm O_3 for 6 h. Ethylene production was measured as described under Materials and methods. Vertical bars represent standard errors from three replicates. * indicates a significant difference between transgenic (antisens 24) and wild-type plants at $P<0.05$ (*t*-test). △, antisense 24; ○,wild type.

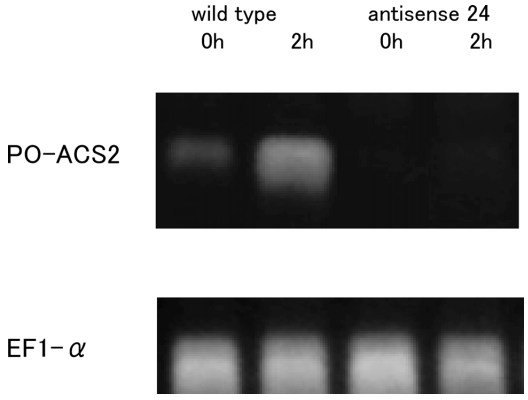

Figure 5. Change in level of transcript for PO-ACS2. RT-PCR was performed by using the specific primers for PO-ACS2 and EF1α (Control) as described in Materials and methods. Lane 1–4: 1 μg total RNA from leaves exposed to ozone for 0 or 2 h and were analyzed.

but only some lines showed O_3 tolerance and lower O_3-induced ethylene production. The variations in O_3 tolerance and the level of O_3-induced ethylene synthesis were probably caused by the putatively random integration of T-DNA into the poplar genome, and they probably also reflected the influence of the surrounding genomic sequences on the expression of the antisense ACS gene.

Figure 5 shows the pattern of expression of the PO-ACS2 gene in the control and transgenic poplar plants after O_3 exposure. The level of transcript for the PO-ACS2 gene in the control plants increased within 2 h of O_3 exposure. By contrast, expression of the PO-ACS2 gene in the transgenic plants was suppressed markedly even after 2 h of O_3 exposure.

Thus, we succeeded in the generation of O_3-tolerant transgenic poplar through the introduction of an antisense DNA for a poplar ACS gene. The transgenic poplar decreased the extent of leaf damage and the level of O_3-induced ethylene production (Figures 3, 4). The extent of leaf damage to transgenic polar was positively related to the level of O_3-induced ethylene production. This finding supports the hypothesis that ethylene is an important factor that determines plant sensitivity to O_3 (Mehlhorn and Wellburn 1987). In addition, we have also succeeded in the generation of O_3-sensitive transgenic poplar plants by over-expression of a poplar ACS gene (Mohri, unpublished results). The transgenic plants showed a susceptibility to O_3 and their leaves were severely damaged even after 0.2 ppm O_3 exposure.

In conclusion, the present study shows that the constitutive expression of an antisense DNA for an O_3-inducible ACS gene could improve the O_3 tolerance of poplar plants through suppressing the expression of early O_3-inducible ACS gene, indicating that these ACSs have key roles in the appearance of leaf damage by O_3 exposure. The result is almost consistent with that of tobacco (Nakajima et al. 2002). More recently, we also found that the O_3-tolerant transgenic poplar showed drought- and high salinity-tolerance (Kogawara et al. in preparation). This technology could be used for the improvement of O_3 tolerance in woody plants and may also be effective in conferring resistance to other environmental stresses.

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