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 cocultivation 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.

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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 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 obtained from O3-inducible poplar ACS and \(O_3\)-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 obtained from O3-inducible poplar ACS.

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 \(\beta\)-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 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 \(\times\) 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\(^{-1}\) \(\alpha\)-napthaleneacetic 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 O₃ 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 O₃. 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 O₃-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 electrophoresis.

Transgenic poplar plants were generated with an antisense DNA for an early O₃-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 O₃ tolerance. For the first time, we selected the best conditions for O₃ exposure. When the control poplar plants were exposed to 0.2 ppm O₃, which is fatal in tobacco plants (Nakajima et al. 2002), we could not find any leaf damages. When O₃ 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 O₃ tolerance than tobacco plants do. By contrast, some transgenic lines showed less visible damage and line 24 showed the highest resistance to the O₃ treatment (Figure 3).

We compared the level of O₃-induced ethylene production between the transgenic and wild-type plants during O₃ exposure to study the effectiveness of the transgenic plants. In wild-type plants, ethylene synthesis was induced within 2 h after O₃ exposure (Figure 3). Although synthesis in the transgenic lines was also induced after 2 h after O₃ 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,
but only some lines showed O₃ tolerance and lower O₃-induced ethylene production. The variations in O₃ tolerance and the level of O₃-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 O₃ exposure. The level of transcript for the PO-ACS2 gene in the control plants increased within 2 h of O₃ exposure. By contrast, expression of the PO-ACS2 gene in the transgenic plants was suppressed markedly even after 2 h of O₃ exposure.

Thus, we succeeded in the generation of O₃-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 O₃-induced ethylene production (Figures 3, 4). The extent of leaf damage to transgenic polar was positively related to the level of O₃-induced ethylene production. This finding supports the hypothesis that ethylene is an important factor that determines plant sensitivity to O₃ (Mehlhorn and Wellburn 1987). In addition, we have also succeeded in the generation of O₃-sensitive transgenic poplar plants by over-expression of a poplar ACS gene (Mohri, unpublished results). The transgenic plants showed a susceptibility to O₃ and their leaves were severely damaged even after 0.2 ppm O₃ exposure.

In conclusion, the present study shows that the constitutive expression of an antisense DNA for an O₃-inducible ACS gene could improve the O₃ tolerance of poplar plants through suppressing the expression of early O₃-inducible ACS gene, indicating that these ACSs have key roles in the appearance of leaf damage by O₃ exposure. The result is almost consistent with that of tobacco (Nakajima et al. 2002). More recently, we also found that the O₃-tolerant transgenic poplar showed drought- and high salinity-tolerance (Kogawara et al. in preparation). This technology could be used for the improvement of O₃ tolerance in woody plants and may also be effective in conferring resistance to other environmental stresses.

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