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Antisense inhibition of a *spermidine synthase* gene highlights the role of polyamines for stress alleviation in pear shoots subjected to salinity and cadmium

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ABSTRACT

Three transgenic European pear (*Pyrus communis* L.) lines with reduced *spermidine synthase* (*SPDS*) expression and spermidine (Spd) titers were developed using a construct containing an apple *SPDS* gene (*MdSPDS1*) in antisense orientation. After exposure to either salt or cadmium stress, growth inhibition was more severe in the antisense lines than in the wild-type (WT). The antioxidant system, as shown by glutathione (GSH) content, activity of glutathione reductase (GR) and superoxide dismutase (SOD), and proline accumulation, was not effectively induced under stress in the antisense lines as compared with the WT. The reduction in antioxidant system function in the antisense lines was accompanied by a greater accumulation of malondialdehyde (MDA), an indicator of lipid peroxidation. Growth inhibition, Spd level, and parameters indicative of the antioxidant system were significantly ameliorated by exogenous Spd application. Under either salt or cadmium stress, GSH content, GR and SOD activity, and proline accumulation were positively correlated with Spd, putrescine (Put), and total polyamine titers. Conversely, MDA level showed a significantly negative correlation with these polyamines under both stress conditions. Thus, the responses to stress treatments were first identified in the *SPDS* antisense European pears, and the results provide further evidence for the important role of polyamines in both salt and cadmium stress tolerance, in which the polyamines act, at least in part, by influencing the antioxidant system.

1. Introduction

The polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) are low-molecular-weight organic cations that are found in a wide range of organisms from bacteria to plants and animals. In plants, polyamines are involved in various physiological events such as development, senescence, and stress responses; in particular, the involvement of polyamines in environmental stress alleviation has been examined in detail (Bouchereau et al., 1999; Pang et al., 2007; Alcázar et al., 2010). During the past few years, various approaches had been employed to unravel key functions of different polyamines in the regulation of abiotic stress tolerance. Exogenous applications of polyamines and polyamine biosynthetic inhibitors have been tested in a variety of plant species (e.g., Songstad et al., 1990; Shen et al., 2000; Tang et al., 2004; Zhao and Qin, 2004; Verma and Mishra, 2005; Liu et al., 2006). These methods, however, suffer from some drawbacks: (i) absorption and catabolic flow of exogenously applied compounds cannot be precisely regulated, and (ii) application of inhibitors causes non-specific and pleiotropic effects on plant metabolism (Noh and Minocha, 1994; Bhatnagar et al., 2001; Duan et al., 2007). Alternatively, the use of transgenic plants is a powerful means to investigate the correlation between modulation of a single gene and its specific role. The response to environmental stress has

Abbreviations: ADC, arginine decarboxylase; CaMV, cauliflower mosaic virus; DIG, digoxigenin-dUTP; FWI, fresh weight increment (%); GR, glutathione reductase; GSH, glutathione; MDA, malondialdehyde; Put, putrescine; ROS, reactive oxidative species; SAMDC, *S*-adenosylmethionine decarboxylase; SHI, shoot height increment (%); SOD, superoxide dismutase; SPDS, spermidine synthase; Spd, spermidine; Spm, spermine; TBA, thiobarbituric acid.

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been examined using transgenic plants overexpressing various polyamine biosynthesis genes. For example, it has been reported that plants show high stress tolerance with overexpression of *arginine decarboxylase* (*ADC*) (Capell et al., 2004; Prabhavathi and Rajam, 2007), *spermidine synthase* (*SPDS*) (Kasukabe et al., 2004), or *S-adenosylmethionine decarboxylase* (*SAMDC*) (Wi et al., 2006). Nevertheless, the precise molecular mechanism by which polyamines control plant responses to stress stimuli, are largely unknown. Previously, we have generated transgenic European pear lines overexpressing apple *SPDS* (*MdSPDS1*), which has enhanced tolerance to multiple environmental stresses such as salt, hyperosmosis, and heavy metals, by inducing an antioxidant effect (He et al., 2008; Wen et al., 2008, 2010), which suggests that polyamines likely participate in the amelioration of oxidative status under abiotic stress.

The combination of gain- and loss-of-function analyses is a comprehensive and circumspect strategy to reveal the function of a target gene. Thus, in addition to overexpression of a particular polyamine biosynthetic gene, introduction of the corresponding antisense transgene provides an excellent way to address the contribution of the polyamine to abiotic stress alleviation and to confirm the amelioration of oxidative status by comparison with the results from the sense transgene. Toward this end, we further developed transgenic European pear with reduced *SPDS* expression and Spd titer via antisense technology, and subjected these transgenic antisense lines and the wild-type (WT) to environmental stresses. To enable us to build on the results previously obtained in the *MdSPDS1*-overexpressing European pear (Wen et al., 2008, 2010), we followed methods described in those previous reports by exposing the antisense lines to either salt or cadmium stress and by measuring the same parameters as those in the previous study (Wen et al., 2008, 2010), with the addition of proline content. We also investigated the effects of exogenous application of Spd on the restoration of the antioxidant parameters in the antisense lines which were subjected to either salt or cadmium stress. To our best knowledge, this is a first report for the investigation in *SPDS* antisense plants. The results presented herein further demonstrate a role for polyamines in stress tolerance in European pear, by increasing expression and function of the antioxidant system.

2. Materials and methods

2.1. Generation and confirmation of the antisense transgenes

MdSPDS1 (AB072915) was excised with *Bam*HI/*Kpn*I from pBluescript-*MdSPDS1* (Zhang et al., 2003) and then ligated into binary vector pBI121 in the antisense direction under the control of the *cauliflower mosaic virus* (*CaMV*) 35S promoter. *Agrobacterium tumefaciens* strain LBA4404 was transformed with the binary vector construct and then used to transform European pear (*Pyrus communis* L. 'Ballad'). The preparation of explant material of in vitro pear shoots, *Agrobacterium* inoculation, and selection procedures followed the method described by Matsuda et al. (2005).

RNA gel blotting was used to detect mRNA abundance in the antisense lines, with the WT included as a control. The sense- and antisense-strand RNA probes were labeled using the full-length sequence of *MdSPDS1* as a template and according to procedures supplied by the manufacturer of the digoxigenin-dUTP (DIG) RNA labeling kit (Roche Diagnostics, Mannheim, Germany). Other procedures for RNA gel blotting were performed according to the method of Ban et al. (2007).

Based on the RNA gel blotting result, lines representing different reduced levels of *MdSPDS1* expression were selected and analyzed with PCR and Southern blotting, so as to further confirm integration of the *MdSPDS1* transgene. Total genomic DNA was isolated from three selected antisense lines (AS21, AS22, and AS33) and the WT according to the method described by Porebski et al. (1997). PCR was conducted using a *CaMV* 35S-F (5′-TGT GAT AAC ATG GTG GAG CA-3′) and *MdSPDS1* C-F (5′-TGA TTG ACG CAA AAG CAA AG-3′) primer set, which produces an expected band of 824 bp. The thermal conditions were 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 90 s at 72 °C. For Southern blotting, 10 μg of genomic DNA was digested with *Dra*I, which has no restriction sites within *MdSPDS1*. After digestion, the DNA was separated on 1% agarose gels overnight, then neutralized and denatured, followed by transfer to nylon Hybond N membrane (Amersham Biosciences, Piscataway, NJ, USA) via capillary transfer. After the membrane was UV-crosslinked with a crosslinker (UV StrataLinker 2400, Stratagene Japan, Tokyo, Japan), it was subjected to prehybridization at 42 °C for 3 h in high-SDS hybridization buffer. The probe was the full-length sequence of *MdSPDS1* labeled with DIG (Roche Diagnostics) by PCR. Hybridization and subsequent procedures were performed according to the method of Ban et al. (2007). Membranes were used to expose X-ray film (Fuji Photo Film, Tokyo, Japan) to detect hybridization.

2.2. Stress treatment

In vitro cultured shoots of the selected antisense lines (AS21, AS22, and AS33) and the WT were maintained under 16 h/8 h light (photon flux density 55.6 $\mu mol\,s^{-1}\,m^{-2}$)/dark conditions at 25 °C in MS medium (Murashige and Skoog, 1962) containing B_5 organic components (Gamborg et al., 1968), 3% sucrose, 1.0 μM indole butyric acid, 4.0 μM zeatin, 4.0 μM N^6-benzylaminopurine, and 0.8% agar. The cultures were transferred to new medium at 4-week intervals. Age-matched shoots 5–6 cm in height from antisense lines and the WT were planted in bottles and cultured with or without salt (150 mM NaCl) or cadmium (150 μM $CdCl_2$) according to the results of previous reports (Wen et al., 2008, 2010). For confirmation of exogenous Spd effects, antisense lines and the WT were also subjected to salt + Spd (150 mM NaCl + 1.0 mM Spd) or cadmium + Spd (150 μM $CdCl_2$ + 0.5 mM Spd).

2.3. Growth of the antisense lines

To elucidate the response of the transgenic lines to long-term stress, growth parameters were investigated after 10 days for salt (salt + Spd) stress or 21 days for cadmium (cadmium + Spd) stress because plants are often subjected to such stresses for a long time. The shoot height and fresh weight were measured at the start and end of the experiment. The shoot height increment (%) (SHI) during the treatment period was calculated using the following equation: $SHI = ([SH_{at\ the\ end} - SH_{at\ the\ start}]/SH_{at\ the\ start}) \times 100$. The fresh weight increment (%) (FWI) was calculated by substituting FW for SH in the equation. After measurement of the net increment, shoots from the same bottle were sampled, the bottom ends of the shoots in contact with the medium were removed, and the shoots were either used for malondialdehyde (MDA) quantification or immediately frozen in liquid nitrogen and stored at −80 °C for further analysis. Meanwhile, another set of shoots was subjected to 30-day stress treatments for morphological observations.

2.4. Measurement of polyamines by HPLC

Free polyamines were quantified according to the method described by Song et al. (2002). Briefly, polyamines were extracted by homogenizing the shoots with 5% (w/v) perchloric acid. After centrifugation, the supernatant was preserved and the pellet was resuspended in 5% perchloric acid after several washes with the same solution. After dansylation, polyamines in the supernatant were quantified via HPLC. 1,6-Hexanediamine was used as an inter-