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Enhancement of spermidine content and antioxidant capacity in transgenic pear shoots overexpressing apple *spermidine synthase* in response to salinity and hyperosmosis

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ABSTRACT

In our previous work, an apple *spermidine synthase* (*SPDS*)-overexpressing transgenic European pear (*Pyrus communis* L. 'Ballad'), line no. 32 (#32), demonstrated attenuated susceptibility to stress treatment. In the current paper, changes in enzymatic and non-enzymatic antioxidant capacity of the transgenic pear (line #32) were investigated in response to NaCl or mannitol stress. Under non-stressed conditions (before stress treatment), spermidine (Spd) contents and SPDS activity of line #32 were higher than those of the non-transformant (wild type). However, no significant differences were detected between line #32 and the wild type as regards contents of malondialdehyde (MDA) and H_2O_2, and activities of antioxidant enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR). When exposed to NaCl or mannitol stress, both the wild type and line #32 exhibited accumulation of Spd with the latter accumulating more. The transgenic line contained higher antioxidant enzyme activities, less MDA and H_2O_2 than the wild, implying it suffered from less injury. These results suggested that increase of Spd content in the transgenic line could, at least in part, lead to enhancing enzymatic and non-enzymatic antioxidant capacity.

1. Introduction

During the last decade, cultivated land in several regions of the world has been affected by environmental stresses like salt and drought, which hinders crop cultivation and yield (Wild, 2003; Rengasamy, 2006). It is predicted that these environmental stresses will become more intense and frequent with climate change, especially global warming. On the other hand, the world population is estimated to reach about 10 billion by 2050, which will witness serious food shortages, and such food shortages are already a daily occurrence in some areas of the world, especially in African countries. Therefore, it is proposed that some lands unsuitable for crop cultivation at present have to be exploited in order to maintain stable food supplies to satisfy the needs of growing population. In this context, crops that can tolerate these harsh environments should be developed so as to accelerate the use of the untapped lands. Unfortunately, the development of stress-tolerant crops using conventional breeding system met with slow progress due to its time-consuming and labor-intensive nature. As an alternative, gene-transfer method paves the way for accelerating the creation of crops with increased stress tolerance. To this end, it is necessary to select some potential genes that can efficiently confer the environmental stress tolerance to plants.

It has been demonstrated that environmental stresses including salt and hyperosmosis generate reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals, in plants (Park et al., 2000; Zhu, 2001; Leshem et al., 2007). Imbalance between production of ROS and the quenching activity of antioxidants resulted in oxidative stress that can cause harmful damage to plants (Hernández et al., 1999). Two types of antioxidants have been shown to be involved in scavenging of ROS. The first type is an array of antioxidant enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR), which have been reported to be implicated in stress tolerance (Gueta-Dahan et al., 1997; Sairam and Srivastava, 2002). SOD catalyzes the dismutation

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of superoxide to H_2O_2 (Bowler et al., 1992). APX, MDHAR, and GR, which are enzymes in the ascorbate (AsA)-glutathione cycle, are responsible for elimination of harmful H_2O_2 and thus can protect plants from ROS-derived damage (del Río et al., 1998). APX, functioning in the first step of AsA-glutathione cycle, is the most important plant peroxidase involved in H_2O_2 detoxification (Noctor and Foyer, 1998). The second type is non-enzymatic antioxidants, such as AsA, carotenoids, phenolics and proline, which also play a key role in scavenging free radicals in plants (Hernández et al., 2000; Blokhina et al., 2003; Verma and Mishra, 2005). Therefore, manipulation for enhancing enzymatic/non-enzymatic antioxidant levels could be an important strategy to create stress-tolerant plants.

Polyamines, including spermidine (Spd, a triamine), spermine (Spm, a tetramine), and their obligate precursor putrescine (Put, a diamine), are aliphatic amines widely present in living organisms. These molecules are involved in the regulation of many basic cellular processes, including DNA replication, transcription, translation, cell proliferation, modulation of enzyme activities, cellular cation–anion balance and membrane stability (Smith, 1985; Tabor and Tabor, 1984; Walden et al., 1997). It has been illustrated that polyamines also play pivotal roles in plant physiological and developmental processes, such as morphogenesis, pollen viability, senescence, fruit ripening, and responses to biotic and abiotic stresses (Evans and Malmberg, 1989; Galston and Sawhney, 1990; Bouchereau et al., 1999; Pandey et al., 2000; Takahashi et al., 2003; Ziosi et al., 2006). Recently, a large body of study shows that plant polyamines are involved in the acquisition of tolerance to such stresses as high and low temperatures, salinity, hyperosmosis, hypoxia and atmospheric pollutants (Liu et al., 2007). Furthermore, genetic transformation of several plant species with polyamine biosynthetic genes encoding arginine decarboxylase (ADC), ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (SAMDC) or Spd synthase (SPDS) led to improved environmental stress tolerance (Liu et al., 2007). It is of interest to note that transgenic plants overexpressing ADC (Prabhavathi and Rajam, 2007), SPDS (Kasukabe et al., 2004, 2006; Wen et al., 2008), or SAMDC (Wi et al., 2006) could tolerate multiple stresses including salinity, drought, low and high temperature, and paraquat toxicity. Such multiple abiotic stress tolerance is of practical importance since plants often suffer from several concurrent forms of environmental stress during their life cycle. In order to elucidate the molecular mechanism underlying the role of polyamines in stress tolerance, Kasukabe et al. (2004) compared transcriptional profiling between *Arabidopsis thaliana* transformants overexpressing SPDS and untransformed plants subjected to chilling stress based on a leaf cDNA microarray, which demonstrated that an array of genes involved in stress response was highly transcribed in the transgenic plants. However, the exact metabolic processes that result in stress tolerance after the introduction of polyamine biosynthetic genes are still largely unknown.

In our previous work, *in vitro* shoots of a transgenic European pear (*Pyrus communis* L. 'Ballad') line, no. 32 (line #32), overexpressing apple SPDS (*MdSPDS1*) showed attenuated susceptibility to NaCl, mannitol and $CuSO_4$ stresses compared with the untransformed one (Wen et al., 2008). In the present study, attempts were made to examine the metabolic relationships between polyamines and enzymatic/non-enzymatic antioxidant levels and to elucidate the mechanism that enhances the tolerance of multiple environmental stresses in this line. To this end, we investigated changes in the activities of polyamine biosynthetic (SPDS, SAMDC, ADC, ODC) and antioxidant enzymes (SOD, APX, MDHAR, and GR) in line #32 exposed to NaCl (150 mM) or mannitol (300 mM). In addition, the contents of free Put, Spd and Spm and some non-enzymatic antioxidants, such as AsA, dehydroascorbate (DHA) and proline were also assessed. Levels of malondialdehyde (MDA) and H_2O_2were measured as damage indicators. Based on the results, possible involvement of Spd in stress alleviation in *SPDS*-overexpressing transgenic European pear was discussed.

2. Results

2.1. Shoot growth under stress conditions

Increment (percentage) of the fresh weight and shoot height in line #32 and the wild type was followed for 15 d after the start of NaCl or mannitol treatment. Three days after the stress, no obvious differences in the growth were detected between line #32 and the wild type (data not shown). It was noted that fresh weight (FW) and shoot height (SH) in both line #32 and the wild type were reduced by NaCl, but to a lesser extent in the transgenic line on day 7 (Fig. 1a and b). The same tendencies were observed in mannitol treatment on day 7, although mannitol led to more serious growth impairment than NaCl did (Fig. 1a and b). The inhibitory effects of both stress treatments on growth of line #32 and the wild type were more noticeable on day 15 (Fig. 1a and b). Morphological abnormalities like chlorotic and necrotic damages were observed in the wild type leaf, which were less severe in line #32 (Fig. 1c). When the inhibition of fresh weight and shoot height were expressed as reduction percentage, line #32 showed less reduction than the wild type at both stages regardless of NaCl or mannitol stress (Fig. 1a and b), which suggested that line #32 showed better stress tolerance than the wild type, in line with Wen et al. (2008).

2.2. Changes in activities of SPDS, SAMDC, ADC and ODC

Our previous report (Wen et al., 2008) showed a high expression level of the transgene (*MdSPDS1*) in line #32, but activities of SPDS and other polyamine biosynthetic enzymes in this line were not examined. In the present work, before stress treatments, the SPDS activity in line #32 (7.9 nmol mg^{-1} protein h^{-1}) was confirmed to be nearly twice that in the wild type (4.2 nmol mg^{-1} protein h^{-1}) (Fig. 2a). NaCl stress enhanced the SPDS activity in line #32 by about 6.3- and 7.7-fold on days 3 and 7, respectively. The wild type increased its SPDS activity by approximately 4.9-fold on day 3, then declined to about 2.0-fold on day 7. As a result, SPDS activities in line #32 were 2.4- (day 3) and 7.0-fold (day 7) higher than those in the wild type. Similar responses were observed under mannitol stress, but to a less extent (Fig. 2a).

Almost same activity of SAMDC, which supplies decarboxylated *S*-adenosylmethionine to SPDS as a substrate, was present in line #32 (4.2 nmol $^{14}CO_2$ mg^{-1} protein h^{-1}) and the wild type (4.6 nmol $^{14}CO_2$ mg^{-1} protein h^{-1}) before stress treatment (Fig. 2b). NaCl stress strongly enhanced the SAMDC activity in line #32, with approximately 6.8- and 9.9-fold increases on days 3 and 7, respectively, in comparison to 5.2- and 2.3-fold increase in the wild type at the corresponding time points. Quantitatively, SAMDC activities in line #32 were 1.4-fold higher than the wild type on day 3 and 4.6-fold higher on day 7. Compared with NaCl, mannitol caused less increase in the SAMDC activity: 3.4- and 3.9-fold increases in line #32 and 1.6- and 2.8-fold increases in the wild type on days 3 and 7, respectively (Fig. 2b). However, under mannitol stress, SAMDC activities in line #32 were still 2.3- and 1.5-fold higher than those in wild type on days 3 and 7, respectively.

The ADC and ODC activities before stress treatment were about 1/2 and 1/5 of SAMDC in line #32 and the wild type (Fig. 2c and d). The ADC activity was enhanced by NaCl or mannitol, to a greater extent in line #32 than in the wild type (Fig. 2c). Similar results were obtained for the ODC activity upon NaCl treatment (Fig. 2d). Under mannitol stress, the ODC activity was induced on day 3, which declined on day 7 in both plants to the same extent.