Phytochemistry 69 (2008) 2133–2141

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

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

Lixiong He [a,1], Yusuke Ban [b], Hiromichi Inoue [a], Narumi Matsuda [c], Jihong Liu [d], Takaya Moriguchi [a,b,*]

[a] National Institute of Fruit Tree Science, Tsukuba, Ibaraki 305-8605, Japan
[b] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan
[c] Yamagata General Agricultural Research Center, Horticultural Experiment Station, Sagae, Yamagata 991-0043, Japan
[d] National Key Laboratory of Crop Genetic Improvement, National Centre of Crop Molecular Breeding, Huazhong Agricultural University, Wuhan 43000, China

ARTICLE INFO

Article history:
Received 10 December 2007
Received in revised form 20 April 2008
Available online 27 June 2008

Keywords:
Pyrus communis
Rosaceae
Antioxidant enzymes
Polyamines
Stress tolerance
Transgenic plant

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.

© 2008 Elsevier Ltd. All rights reserved.

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

* Corresponding author. Address: National Institute of Fruit Tree Science, Tsukuba, Ibaraki 305-8605, Japan. Tel.: +81 29 838 6500; fax: +81 29 838 6437.
E-mail address: takaya@affrc.go.jp (T. Moriguchi).
[1] Present address: College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha, Hunan Province 410128, China.

0031-9422/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2008.05.015

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.

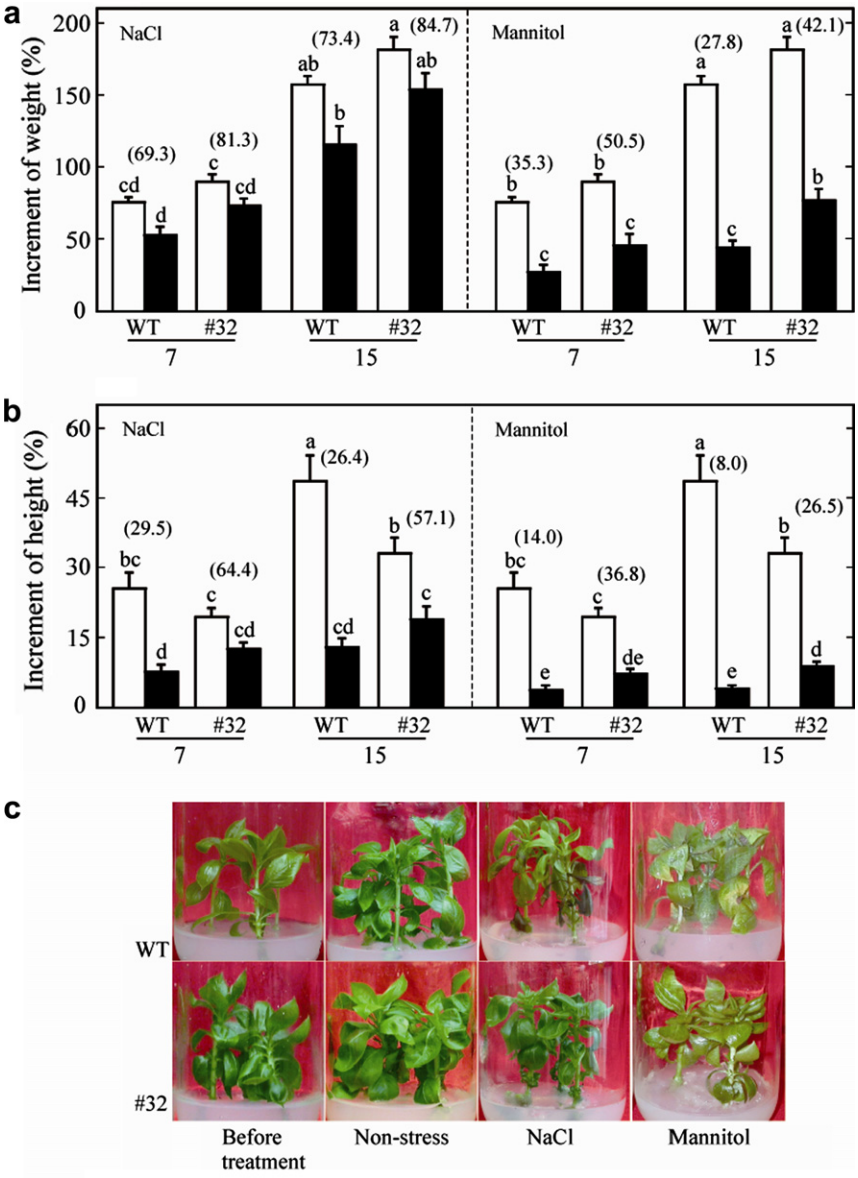

Fig. 1. Characteristics of transgenic pear line #32 and the wild type with (solid columns, stressed) or without (open columns, non-stressed) NaCl or mannitol treatment. The increment percentages of FW (a) and of SH elongation (b) 7 and 15 days after the stress treatments are shown, and the values in parentheses show the percentage of the stressed shoots compared with the non-stressed ones (set to 100%) in the same line. Values within the same stress treatment followed by different letter are significantly different at $p < 0.05$ (c). Morphological comparison between line #32 and the wild type under NaCl or mannitol treatment on day 15, together with these plants before treatment and under non-stressed conditions.

Thus, notable increases in the activities of SAMDC, ADC, and ODC occurred in both plants under stress treatments. Although the increases of these enzyme activities varied with the stress treatment, line #32 showed greater increases in the activities than the wild type with either NaCl or mannitol treatment.

2.3. Changes in polyamine contents

Before stress treatment, the Spd and Spm contents in line #32 were higher than in the wild type, and the Put contents in the two plants were not significantly different (Table 1). With NaCl treatment, the Put and Spd contents in both plants were increased on day 3 before a decline on day 7. The Spm content in the wild type showed the same fluctuation as Put and Spd, whereas line #32 showed an increase only on day 7. When subjected to mannitol stress, the Put content in line #32 increased steadily. In the wild type, the Put was at the same level as in line #32 by day 3, and it decreased on day 7. The Spd and Spm contents showed variations depending on the time in both plants. Although the changes in free polyamines did not follow rigid tendencies, Spd and Spm exhibited apparently larger increase in line #32 than in the wild type, resulting in a higher ratio of (Spd + Spm)/Put in the former (Table 1).

2.4. Changes in activities of antioxidant enzymes

Line #32 and the wild type possessed similar activities of antioxidant enzymes before stress treatment (Fig. 3). The SOD activity in both plants was enhanced under NaCl stress on day 3. On day 7, its activity in the wild type decreased, but steadily increased in line #32 (Fig. 3a). Under mannitol stress, SOD activity increased on day 3 in both plants, line #32 being higher than the wild type (Fig. 3a) On day 7, the decrease or slight increase in SOD activity was

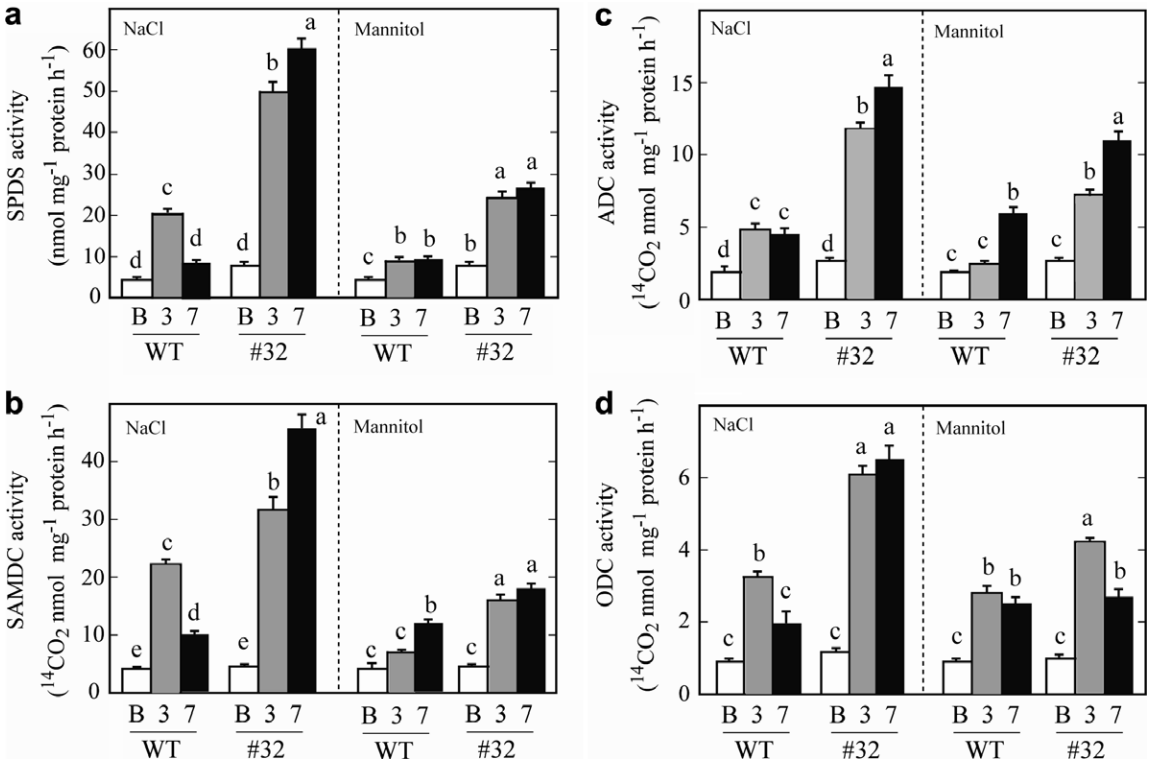

Fig. 2. Activities of SPDS (a), SAMDC (b), ADC (c), and ODC (d) in line #32 and the wild type under NaCl or mannitol stress at before treatment (B) or 3 and 7 days after treatment. Values within the same stress treatment followed by different letter are significantly different at $p < 0.05$.

Table 1
Polyamines and ratios of (Spd + Spm)/Put in line #32 and wild type with NaCl or mannitol treatment before treatment (B), 3 and 7 days after treatment

Polyamine	Day	NaCl		Mannitol	
		WT	#32	WT	#32
Put (nmol g^{-1} DW)	B	127.66 ± 19.02 d	127.35 ± 13.44 d	127.66 ± 19.02 c	127.35 ± 13.44 c
	3	331.17 ± 38.96 b	402.55 ± 51.97 a	197.99 ± 18.43 b	234.89 ± 28.47 ab
	7	236.41 ± 36.58 c	302.38 ± 31.45 b	128.67 ± 11.46 c	296.09 ± 32.15 a
Spd (nmol g^{-1} DW)	B	86.59 ± 12.34 d	258.26 ± 28.34 c	86.59 ± 12.34 c	258.26 ± 28.34 b
	3	213.55 ± 30.18 c	629.13 ± 55.54 a	129.63 ± 25.51 c	406.32 ± 28.21 a
	7	145.82 ± 24.72 d	415.65 ± 25.97 b	108.75 ± 15.26 c	290.13 ± 30.13 b
Spm (nmol g^{-1} DW)	B	24.23 ± 4.96 b	45.92 ± 9.08 b	24.23 ± 4.96 a	45.92 ± 9.08 a
	3	53.93 ± 7.86 b	36.93 ± 7.28 b	22.64 ± 7.56 a	27.85 ± 4.43 a
	7	31.93 ± 7.30 b	95.64 ± 14.28 a	16.52 ± 3.71 a	33.35 ± 4.25 a
(Spd + Spm)/Put	B	0.87	2.39	0.87	2.39
	3	0.81	1.65	0.77	1.85
	7	0.75	1.69	0.97	1.11

For each type of polyamine, values followed by different letters in the same stress treatment are significantly different at $p < 0.05$.

observed in both plants under NaCl or mannitol treatment, although this was not significant when compared with the activity in each plant on day 3. The APX activity was induced with NaCl or mannitol stress in both plants on day 3 with much high level in line #32 (Fig. 3b). On day 7, the tendency in APX activity in both plants was the same as that in SOD activity on day 7 under NaCl or mannitol. MDHAR activity increased upon NaCl treatment in both plants, but the wild type responded to a lesser degree than line #32 (Fig. 3c). Upon exposure to mannitol-induced osmotic stress, the MDHAR activity was induced on day 3, followed by a decrease on day 7, which was still significantly higher in line #32 than in the wild type (Fig. 3c). Change in GR activity followed the same trend as that of APX under NaCl, and was similar to MDHAR under mannitol stress (Fig. 3d). The MDHAR and GR activities seemed to be more sensitive to NaCl than to mannitol. Thus, it was apparent that these enzyme activities tended to be higher in line #32 than in the wild type, and induction of these enzyme activities was less pronounced with mannitol stress than with NaCl stress.

2.5. Changes in AsA, proline, H_2O_2, and MDA contents

The contents of AsA and DHA varied in a manner depending on plant or stress (Table 2), but their contents in line #32 were generally higher than in the wild type. The AsA content in line #32 treated with NaCl or mannitol increased on day 3, followed by slight decrease on day 7. The AsA in the wild type under NaCl stress peaked on day 3, whereas mannitol caused an initial decrease in the AsA content, followed by an increase by day 7. The DHA content in line #32 was significantly higher than that of the wild type with NaCl treatment, but this tendency was less obvious with

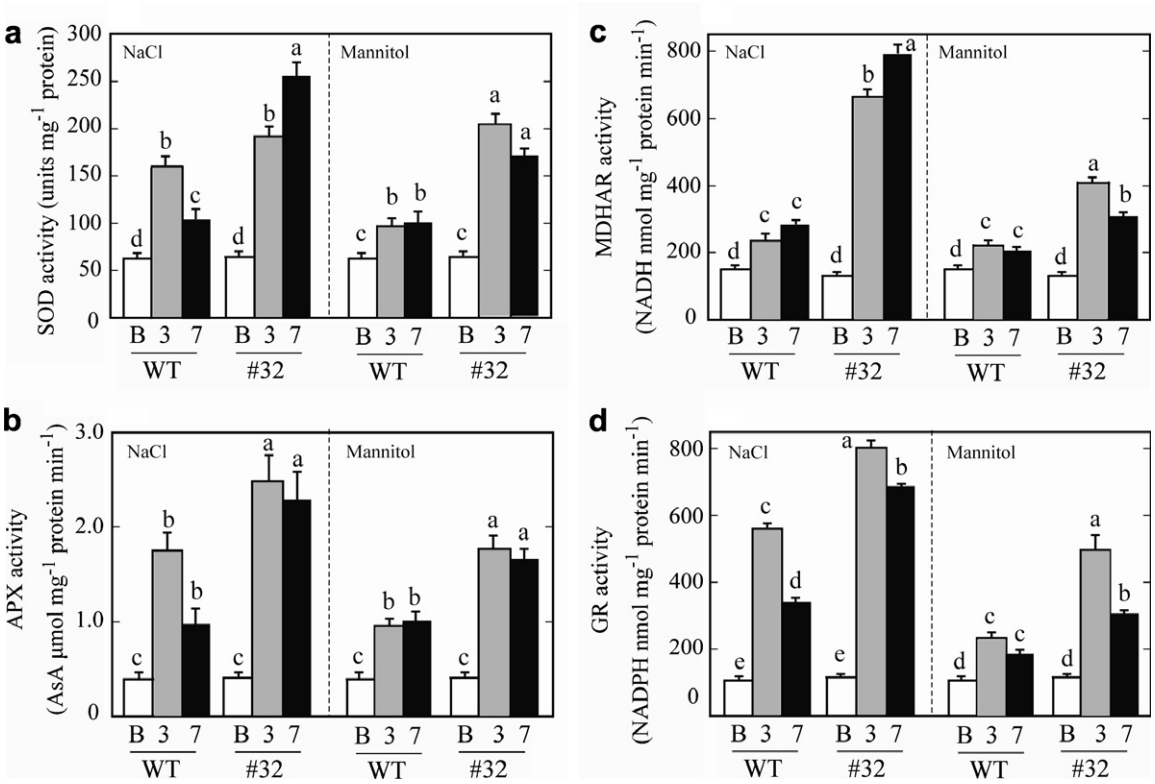

Fig. 3. Activities of SOD (a), APX (b), MDHAR (c), and GR (d) in line #32 and the wild type under NaCl or mannitol stress at before treatment (B) or 3 and 7 days after treatment. Values within the same stress treatment followed by different letter are significantly different at $p < 0.05$.

Table 2
AsA, DHA, ratios of AsA/DHA and proline in line #32 and wild type with NaCl or mannitol treatment before treatment (B), 3 and 7 days after treatment

	Day	NaCl		Mannitol	
		WT	#32	WT	#32
AsA (μmol g^{-1} DW)	B	16.41 ± 1.59 c	32.48 ± 2.32 b	16.41 ± 1.59 c	32.48 ± 2.32 b
	3	28.13 ± 3.65 b	72.49 ± 4.64 a	10.92 ± 0.97 c	50.84 ± 2.53 a
	7	19.60 ± 2.84 c	68.64 ± 4.40 a	19.97 ± 1.32 c	33.12 ± 1.61 b
DHA (μmol g^{-1} DW)	B	44.41 ± 2.05 c	63.34 ± 2.15 ab	44.41 ± 2.05 b	63.34 ± 2.15 a
	3	40.82 ± 1.74 c	55.30 ± 1.98 b	41.09 ± 3.74 b	48.44 ± 5.97 b
	7	41.52 ± 2.27 c	69.07 ± 2.28 a	34.42 ± 1.97 b	41.91 ± 6.18 b
AsA/DHA	B	0.37	0.51	0.37	0.51
	3	0.69	1.37	0.27	1.05
	7	0.47	0.99	0.58	0.79
Proline (μmol g^{-1} DW)	B	47.82 ± 3.25 d	45.94 ± 4.82 d	47.82 ± 3.25 d	45.94 ± 4.82 d
	3	112.90 ± 7.18 c	146.59 ± 14.24 bc	62.98 ± 4.90 cd	89.83 ± 2.95 b
	7	161.89 ± 8.59 b	222.92 ± 11.08 a	70.21 ± 3.63 bc	130.29 ± 4.77 a

For each parameter, values within the same stress treatment followed by different letters are significantly different at $p < 0.05$.

mannitol treatment. The AsA/DHA ratio in line #32 was higher than in the wild type under both stress conditions (Table 2), indicating a better redox status in line #32. The contents of proline, which has been shown to be important in regulating osmotic potential or scavenging a hydroxyl radical and singlet oxygen (Smirnoff and Cumbes, 1989; Rani, 2007), were increased following NaCl and mannitol stress in both plants, but line #32 possessed significantly higher content than the wild type on day 7 (Table 2).

Before stress treatment, nearly the same amount of H_2O_2 was observed in the two plants (Table 3). When exposed to NaCl or mannitol the wild-type exhibited a 2.5- to 3-fold increase of H_2O_2, which was, however, increased to less extent in line #32 under the same stressful condition. Consequently, the H_2O_2 content was lower in line #32 than in the wild type on both day 3 and 7. Contents of MDA, an indicator of lipid peroxidation, showed the same tendency as H_2O_2 (Table 3), indicating that line #32 had better membrane integrity than the wild type.

Thus, apart from AsA content, DHA, proline, H_2O_2, and MDA were similar in line #32 and the wild type before treatment, but their values including AsA changed upon stress toward a more favorable direction for survival in line #32 than in the wild type.

3. Discussion

3.1. Morphological and biochemical features of line #32 under stress conditions

The transgnenic pear line #32 has been shown to suffer the least growth damage caused by NaCl, mannitol, and $CuSO_4$ among a batch of *MdSPDS1*-transgenic plants (Wen et al., 2008). Line #32

Table 3
H_2O_2 and MDA in line #32 and wild type with NaCl or mannitol treatment before treatment (B), 3 and 7 days after treatment

	Day	NaCl		Mannitol	
		WT	#32	WT	#32
H_2O_2 (nmol g^{-1} DW)	B	1019.56 ± 78.69 d	1105.05 ± 63.73 cd	1019.56 ± 78.69 d	1105.05 ± 63.73 d
	3	3041.79 ± 100.21 a	1448.84 ± 78.36 bc	2570.89 ± 77.76 a	1479.01 ± 70.43 c
	7	2868.23 ± 121.50 a	1592.45 ± 86.18 b	1890.30 ± 60.08 b	1528.60 ± 73.90 c
MDA (nmol g^{-1} DW)	B	234.25 ± 23.33 d	303.04 ± 17.21 cd	234.25 ± 23.33 c	303.04 ± 17.21 bc
	3	368.25 ± 29.19 c	316.76 ± 23.28 cd	266.51 ± 18.45 bc	272.09 ± 20.44 bc
	7	763.94 ± 47.67 a	482.58 ± 37.30 b	365.00 ± 32.68 a	325.81 ± 13.83 b

For each parameter, values in the same stress treatment followed by different letters are significantly different at $p < 0.05$.

and the wild type are favorable materials for the comparative study of stress tolerance and related physiological reactions in plants. Therefore, this transgenic line was employed in the current study with the intention of examining the metabolic relationships between polyamines and enzymatic/non-enzymatic antioxidant levels. In agreement with the previous report (Wen et al., 2008), under stress conditions line #32 showed less growth inhibition than the wild type, further supporting the reliability of this line's attenuated susceptibility to stress. However, free polyamine contents under non-stressed conditions in this study were not consistent with those of Wen et al. (2008), which might be possibly ascribed to different phytohormones used for maintenance of the shoots *in vitro* and for the stress treatments, indolebutyric acid (IBA) and zeatin in this study rather than N^6-benzylaminopurine in the previous one. Alteration of cellular polyamine content by phytohormones has been reported previously. For instance, Davis (1997) reported that free polyamine contents were different in the hypocotyls segments of leafy spurge (*Euphorbia esula* L.) when they were cultured on medium added with indoleacetic acid (IAA) or 2,4-dichlorophenoxy acetic acid. In another work, free polyamine contents of tobacco suspension cultures were also affected by IAA (Park and Lee, 1994).

It has been demonstrated that overexpression of Put biosynthetic genes, *ADC* or *ODC*, generally causes the production of high levels of Put (DeScenzo and Minocha, 1993; Bastola and Minocha, 1995; Burtin and Michael, 1997; Bhatnagar et al., 2001). By contrast, overexpression of *SPDS* led to a small increase of Spd despite the use of the constitutive promoter, *CaMV*35S (Franceschetti et al., 2004; Kasukabe et al., 2004; Wen et al., 2008). Indeed, Spd in line #32 was only about 2.4- to 2.9-fold higher than that in the wild type upon exposure to NaCl or mannitol, despite their distinct variation in stress tolerance. In our work, no transformants with extremely high Spd have been recovered. It is possible that transgenic individuals with excessively high Spd level could not regenerate successfully due to toxic nature of Spd beyond the lethal threshold content, as has been reported by Wen et al. (2008). It has to be pointed out that the increased magnitude in our work is comparable to those that have been reported earlier (Franceschetti et al., 2004; Kasukabe et al., 2004; Wen et al., 2008). Moderate increase of endogenous Spd even in case of over-expression of *MdSPDS1* implied that cellular polyamine contents underwent homeostatic regulation, as has been reported by others (Bhatnagar et al., 2002; Pang et al., 2006). This homeostatic regulation of Spd might explain, at least in part, why Spd content did not perfectly correspond to the enzymatic activity of SPDS on day 7, when its activity increased in line #32 and Spd content fell under stress. Therefore, other regulatory systems such as post translational regulation could exist to maintain polyamine homeostasis in cells.

3.2. Direct effect of the increased Spd and Spm on stress tolerance

In the current study, line #32 predictably showed higher level of free Spd than the wild type, which may result from high SPDS activity due to abundant transcriptional expression of the transgene, *MdSPDS1*. The expression of gene encoding polyamine oxidase, an enzyme responsible for degradation of Spd and Spm, was not obviously different between line #32 and the wild type (data not shown). On the other hand, H_2O_2, which is a product of polyamine catabolism, existed at low levels in line #32 compared with the wild type. It is plausible to suggest that the increased Spd biosynthesis is not accompanied by its increased catabolism. In addition, Spm content was also higher in line #32 than the wild type, leading to higher ratio of (Spd + Spm)/Put in the former. In our previous study, we found that the ratio of (Spd + Spm)/Put was always higher in line #32 than in the wild type exposed to NaCl, mannitol or $CuSO_4$ stress (Wen et al., 2008). Therefore, high ratio of (Spd + Spm)/Put in this line might have been correlated with the alleviation of stress-derived damage. Our work corroborates several lines of work reported previously, which demonstrated the importance of Spd and/or Spm for alleviating stress-derived cell injury. For instance, salt-tolerant rice and tomato genotypes increased their Spd and Spm levels in response to salt stress, which was not observed in the sensitive strains (Krishanamurthy and Bhagwat, 1989; Santa-Cruz et al., 1997). Increase of cellular Spd or Spm through exogenous addition of Spd or Spm has been demonstrated to confer tolerance to drought in *Arabidopsis* (Yamaguchi et al., 2007) and to copper in *Nymphoides peltatum* (Wang et al., 2007). The promotion of stress tolerance by free and congugated forms of Spd or Spm could be ascribed to direct role in ROS scavenging and membrane stabilization due to their polycationic properties, as has been shown elsewhere (Besford et al., 1993; Borrell et al., 1997; Kubiś, 2005).

As for conjugated and bound forms of polyamnes, recently, the involvement of these forms in stress alleviation has been suggested (Dondini et al., 1999; Waie and Rajam, 2003; Roussos and Pontikis, 2007). Bouchereau et al. (1999) also showed the potential ability of conjugated polyamines for acting as free radical scavengers. In this study, the titers of conjugated polyamines also tended to be higher in line #32 than in the wild type (data not shown), which might indicate that the higher conjugated polyamine titers in line #32 than the wild type could also contribute to the better stress tolerance upon NaCl and mannitol stresses in this line.

3.3. Relationships between the increased Spd/Spm and antioxidant system

In addition to the participation of polyamine *per se* in stress tolerance, the enhanced Spd, along with Spm, may exert its effect by affecting the antioxidant system under stress. Recently, exogenous application of polyamines was found to reduce salt-induced oxidative damage by activating antioxidant enzymes, including APX, GR, and SOD, in Virginia pine (Tang et al., 2004; Tang and Newton, 2005). Similarly, Verma and Mishra (2005) reported that addition of polyamines could enhance activities of several antioxidant enzymes and content of non-enzymatic antioxidants, leading to less stress damage in *Brassica juncea* seedlings. In this study, before

stress treatment, line #32 showed higher SPDS activity and Spd contents than the wild type. However, with the exception of AsA, the enzymatic/non-enzymatic antioxidant levels and the H_2O_2 and MDA contents were similar between the transgenic and wild type plants, indicating that the transgene did not greatly affect the antioxidant system under normal conditions. Upon NaCl or mannitol stress, activities of four antioxidant enzymes, SOD, APX, MDHAR and GR, were induced to a higher extent in line #32 than in the wild type, coincident with the increase of Spd content in the former. Therefore, production of more Spd/Spm in line #32 may be involved in activation of the antioxidant system of the cells in response to adverse environment. The higher SOD activity in line #32 indicated its sufficient dismutation of the superoxide anion into H_2O_2 caused by stress. However, in line #32 possessed less H_2O_2 than the wild type, suggesting that enzymes in the AsA-glutathione cycle with high activities in line #32 functioned efficiently to remove the H_2O_2. In addition, content of non-enzymatic antioxidants like AsA and proline and the ratio of AsA/DHA in line #32 were also higher than those in the wild type, further supporting that the transgenic line was more potent in eliminating the ROS than the wild type. Activation of antioxidant systems, along with the direct participation in ROS scavenging and membrane stabilization, works together or separately to protect the cells from stress-derived damage, leading to better growth under stresses. It is noted that growth of line #32 was better under salt stress than under mannitol stress, which might be also explained by higher antioxidant activities in the salty condition. However, the underlying mechanism for such difference needs to be further elucidated in the future.

4. Conclusions

At this point, though it is still difficult to draw a clear-cut picture for the mechanism underlying the induced antioxidant capacity by introduction of *MdSPDS*, it might be partially possible to suggest that the enhanced Spd levels upon stresses in line #32 correlated with the defense against the oxidative stress. By contrast, those in the wild type might be insufficient to initiate an efficient oxidative defense machinery, although Spd levels increased in the wild type as well upon stress treatments when compared with those prior to stress treatment. Similar results have been reported before based on investigation of transgenic plants and exogenous application of polyamines (Capell et al., 2004). Transgenic rice plants expressing *Datura* ADC produced more Put that could be converted to Spd and Spm synthesis, leading to improved drought tolerance, while the wild type could not synthesize sufficient Put (Capell et al., 2004). Our work, in conjunction with earlier ones, provides accumulating convince that a slight but significant increase in Spd can give rise to desirable effect on stress tolerance, although physiological nature of stress tolerance remains to be characterized.

5. Experimental

5.1. Chemicals

All chemicals used in this study were purchased from Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or Sigma (Sigma–Aldrich Corp., St. Louis, MO, USA) unless otherwise stated.

5.2. Plant materials and stress treatments

Untransformed European pear (*P. communis* L. 'Ballad', wild type) and the transgenic line #32 overexpressing apple SPDS (*MdSPDS1*) under the control of the *CaMV*35S promoter were used in this study (Wen et al., 2008). *In vitro* shoot cultures of line #32 and the wild type were maintained on MS medium (Murashige and Skoog, 1962) containing B_5 organic components (Gamborg et al., 1968), 3% sucrose, 1.0 μM IBA, 5.0 μM zeatin, and 0.8% agar under a 16-h photoperiod at 25 °C. The cultures were transferred to fresh medium at four-week intervals. For stress treatments, *in vitro* shoots of line #32 and the wild type were incubated on MS medium containing 150 mM NaCl or 300 mM mannitol. The SH and FW were measured at the start of the experiment and compared with those of the wild type on the third and seventh day following NaCl or mannitol treatment. The net increments (percentage) in fresh weight (FWI_n) and shoot height (SHI_n) during a given period were calculated using the equation $FWI_n(SHI_n) = [FW\ (SH)_{at\ the\ end} - FW\ (SH)_{at\ the\ start}]/FW\ (SH)_{at\ the\ start} \times 100$ according to Wen et al. (2008). After measurement of the net increment, the shoots were sampled and immediately frozen in liquid nitrogen before storage at −80 °C until further analysis.

5.3. Quantification of polyamines by HPLC

Free polyamines were quantified according to the method described by Song et al. (2002) and expressed as dry weight (DW) basis.

5.4. Measurement of enzyme activities

The enzymes of SPDS, SAMDC, ADC, and ODC were extracted as described by Kasukabe et al. (2004). The ratio of the shoot weight to the extraction buffer volume was 1:3. The homogenate was centrifuged at 25,000*g* for 20 min, and the enzyme activities in the supernatant were measured. SPDS activity, expressed as nmol mg^{-1} protein h^{-1}, was assayed based on Kasukabe et al. (2004), and those of SAMDC, ADC, and ODC, expressed as nmol $^{14}CO_2$ mg^{-1} protein h^{-1}, were measured according to previous reports (Song et al., 2001; He et al., 2002) using a radioisotope. Protein was measured according to Bradford (1976).

The antioxidant enzymes, SOD, APX, MDHAR, and GR, were extracted according to Venisse et al. (2001) with some modifications. Shoots were homogenized in two fold volume of ice-cold 50 mM potassium phosphate buffer (pH 7.5) containing 2% (w/v) polyethylene glycol, 1 mM phenylmethylsulfonyl fluoride, 8% (w/v) polyvinylpolypyrrolidone and 0.01% (v/v) Triton X-100, followed by centrifugation for 20 min at 16,000*g* (4 °C). The enzyme activities in the supernatants were immediately assayed. SOD activity, expressed as unit mg^{-1} protein, was determined according to a method modified from Beyer and Fridovich (1987) with SOD Assay Kit-WST (Dojindo Molecular Technologies Inc., Kumamoto, Japan) following the manufacturer's instructions. One unit of SOD was defined as the amount of enzyme that causes a 50% decrease in the SOD-inhibitable nitroblue tetrazolium reduction. APX activity (AsA μmol mg^{-1} protein min^{-1}) was assayed by following the oxidation of AsA at 290 nm according to Nakano and Asada (1981) with minor modifications. In this method, 50 μl of extraction supernatant were added to 1 ml of a reaction mixture consisting of 80 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.2 mM H_2O_2, and 0.2 mM diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid dianhydride (DTPA). MDHAR activity (expressed as NADH nmol mg^{-1} protein min^{-1}) was measured by the oxidation of NADH at 340 nm according to Hossain et al. (1984) and Jahnke et al. (1991) with some modifications. For this measurement, 100 μl of extraction supernatant were added to 1 ml of reaction mixture consisting of 80 mM potassium phosphate buffer (pH 7.8), 0.5 mM AsA, 0.2 mM DTPA, 0.15 mM NADH, and 0.2 unit of AsA oxidase. For measurement of GR activity, expressed as NADPH nmol mg^{-1}protein min^{-1}, 100 μl of extraction supernatant were added to 1 ml of a reaction mixture consisting of 80 mM potassium phosphate buffer (pH 7.8), 0.2 mM DTPA, 0.15 mM NADPH, and

0.5 mM oxidized glutathione (Jahnke et al., 1991), followed by oxidation of NADPH at 340 nm.

5.5. Measurement of AsA, DHA, proline, H_2O_2, and MDA

For analysis of AsA, a method slightly modified from Sakaki et al. (1983) was used, in which pear shoots (1 g FW) were homogenized in 4 ml of 5% (w/v) metaphosphoric acid and centrifuged at 10,000*g* for 10 min at 4 °C. To convert AsA to DHA, 1 mM 2,6-dichlorophenolindophenol (DCIP) was added to the supernatant and mixed with 2.9 mM 2,4-dinitrophenylhydrazine, 0.3% w/v thiourea, and 0.66 M sulfuric acid. The mixture was incubated at 50 °C for 60 min before cooling in an ice bath to stop the reaction. Equal volume of 15.4 M sulfuric acid was then added, followed by determination of DHA content in the sample at absorbance of 520 nm. The content of AsA depends on difference between the contents of DHA in the sample with and without DCIP.

Proline was measured according to Chandler and Thorpe (1987) and Wren and Wiggall (1965). Shoots (50–80 mg FW) were homogenized in 4 ml of methanol:chloroform:water (12:5:1, v/v/v). After addition of H_2O (1.5 ml) and $CHCl_3$ (1 ml), the solution was mixed and centrifuged at 10,000*g* for 5 min at 4 °C. An aliquot (0.2 ml) of the upper phase was diluted with H_2O (0.8 ml), 2.5 ml of a 3:2 (v/v) mixture of 4 μmol ml^{-1} glycine in CH_3CO_2H and 6 M phosphoric acid and 2.5 ml of 40 mg ml^{-1} ninhydrin. The solution (6.0 ml) was incubated in a 95 °C water bath for 40 min. After cooling down at room temperature, toluene (5 ml) was added, which was mixed and then centrifuged at 10,000*g* for 5 min at 4 °C, before measurement of absorbance at 513 nm. The proline content was calculated using a calibration curve.

Measurement of H_2O_2 was performed using peroxidase enzyme according to Lee and Lee (2000). Pear shoots (1 g FW) were homogenized in 3 ml of 100 mM sodium phosphate buffer (pH 6.8). The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA, USA) and centrifuged at 18,000*g* for 20 min at 4 °C. To initiate the enzyme reaction, a 0.6-ml aliquot of the supernatant was mixed with 2.5 ml of peroxide reagent (83 mM sodium phosphate buffer (pH 7.5), 0.005% (w/v) *o*-dianisidine, and 40 μg ml^{-1} peroxidase) and incubated for 10 min at 30 °C in a water bath, followed by addition of 0.5 ml of 1 N perchloric acid to stop the reaction. The solution was centrifuged at 5000*g* for 5 min at 4 °C before absorbance of the supernatant at 436 nm was measured. The H_2O_2 contents were calculated using the calibration curve.

MDA was measured according to the method described by Sofo et al. (2004). Pear shoots (0.5 g FW) were homogenized in 5 ml of 10% (w/v) CCl_3CO_2H and centrifuged at 10,000*g* for 10 min at room temperature. Four ml of 0.5% (w/v) thiobarbituric acid in 20% (w/v) CCl_3CO_2H were added to a 1-ml aliquot of the supernatant. The mixture was boiled at 100 °C for 30 min and quickly cooled in ice. After centrifugation at 10,000*g* for 10 min at room temperature, the absorbance of the supernatant at 532 nm was measured and the non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using an extinction coefficient of 155 $mmol^{-1}$ cm^{-1}. All of these parameters were expressed as DW basis.

5.6. Statistical analysis

All of the presented data are mean values of at least three replicates with nine shoots and are shown as the mean ± SE. Statistical analysis was performed using the Bonferroni-Dunn test.

Acknowledgements

This research was supported in part by a grant from the Japan Society for the Promotion of Science (JSPS). The authors wish to thank Dr. Y. Kasukabe (Toyobo Co. Ltd., Shiga, Japan) for kindly providing decarboxylated *S*-adenosylmethionine and Prof. Wen (Guizhou University, China) for his critique of this manuscript. This work was undertaken when Dr. L. He was a visiting scientist at NIFTS.

References

Bastola, D.R., Minocha, S.C., 1995. Increased putrescine biosynthesis through transfer of mouse ornithine decarboxylase cDNA in carrot promotes somatic embryogenesis. Plant Physiol. 109, 63–71.

Besford, R.T., Richardson, C.M., Campos, J.L., Tiburcio, A.F., 1993. Effect of polyamines on stabilization of molecular complexes in thylakoid membranes of osmotically stressed oat leaves. Planta 189, 201–206.

Beyer Jr., W.F., Fridovich, I., 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal. Biochem. 161, 559–566.

Bhatnagar, P., Glasheen, B.M., Bains, S.K., Long, S.L., Minocha, R., Walter, C., Minocha, S.C., 2001. Transgenic manipulation of the metabolism of polyamines in poplar cells. Plant Physiol. 125, 2139–2153.

Bhatnagar, P., Minocha, R., Minocha, S.C., 2002. Genetic manipulation of the metabolism of polyamines in poplar cell. The regulation of putrescine catabolism. Plant Physiol. 128, 1455–1469.

Blokhina, O., Virolainen, E., Fagerstedt, K.V., 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Anal. Bot. 91, 179–194.

Borrell, A., Carbonell, L., Farràs, R., Puig-Parellada, P., Tiburcio, A.F., 1997. Polyamines inhibit lipid peroxidation in senescing oat leaves. Physiol. Plant. 99, 385–390.

Bouchereau, A., Aziz, A., Larher, F., Martin-Tanguy, J., 1999. Polyamines and environmental challenges: recent development. Plant Sci. 140, 103–125.

Bowler, C., Van Montagu, M., Inzé, D., 1992. Superoxide dismutase and stress tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 83–116.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burtin, D., Michael, A.J., 1997. Overexpression of arginine decarboxylase in transgenic plants. Biochem. J. 325, 331–337.

Capell, T., Bassie, L., Christou, P., 2004. Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. Proc. Natl. Acad. Sci. USA 101, 9909–9914.

Chandler, S.F., Thorpe, T.A., 1987. Characterization of growth, water relations, and proline accumulation in sodium sulfate tolerant callus of *Brassica napus* L. cv Westar (canola). Plant Physiol. 84, 106–111.

Davis, D.G., 1997. 2,4-Dichlorophenoxy acetic acid and indoleacetic acid partially counteract inhibition of organogenesis by difluoromethylornithine. Physiol. Plant. 101, 425–433.

del Río, L.A., Pastori, G.M., Palma, J.M., Sandalio, L.M., Sevilla, F., Corpas, F.J., Jiménez, A., López-Huertas, E., Hernández, A., 1998. The activated oxygen role of peroxisomes in senescence. Plant Physiol. 116, 1195–1200.

DeScenzo, R.A., Minocha, S.C., 1993. Modulation of cellular polyamines in tobacco by transfer and expression of mouse ornithine decarboxylase cDNA. Plant Mol. Biol. 22, 113–127.

Dondini, L., Bonazzi, S., Del Duca, S., Bregoli, A.M., Serafini-Fracassini, D., 1999. Acclimation of chloroplast transglutaminase to high NaCl concentration in a polyamine-deficient variant strain in *Dunaliella salina* and in its wild type. J. Plant Physiol. 158, 185–197.

Evans, P.T., Malmberg, R.L., 1989. Do polyamines have roles in plant development? Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 235–269.

Franceschetti, M., Fornale, S., Tassoni, A., Zuccherelli, K., Mayer, M.J., Bagni, N., 2004. Effect of *spermidine synthase* overexpression on polyamine biosynthetic pathway in tobacco plants. J. Plant Physiol. 161, 989–1001.

Galston, A.W., Sawhney, R.K., 1990. Polyamines in plant physiology. Plant Physiol. 94, 406–410.

Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151–158.

Gueta-Dahan, Y., Yaniv, Z., Zilinskas, B.A., Ben-Hayyim, G., 1997. Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in *Citrus*. Planta 203, 460–469.

He, L., Nada, K., Kasukabe, Y., Tachibana, S., 2002. Enhanced susceptibility of photosynthesis to low-temperature photoinhibition due to interruption of chill-induced increase of S-adenosylmethionine decarboxylase activity in leaves of spinach (*Spinacia oleracea* L.). Plant Cell Physiol. 43, 196–206.

Hernández, J.A., Campillo, A., Jiménez, A., Alarcón, J.J., Sevilla, F., 1999. Response of antioxidant systems and leaf water relations to NaCl stress in pea plants. New Phytol. 141, 241–251.

Hernández, J.A., Jiménez, A., Mullineaux, P., Sevilla, F., 2000. Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defences. Plant Cell Environ. 23, 853–862.

Hossain, M.A., Nakano, Y., Asada, K., 1984. Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. Plant Cell Physiol. 25, 385–395.

Jahnke, L.S., Hull, M.R., Long, S.P., 1991. Chilling stress and oxygen metabolizing enzymes in *Zea mays* and *Zea diploperennis*. Plant Cell Environ. 14, 97–104.

Kasukabe, Y., He, L., Nada, K., Misawa, S., Ihara, I., Tachibana, S., 2004. Overexpression of *spermidine synthase* enhances tolerance to multiple

environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis thaliana*. Plant Cell Physiol. 45, 712–722.

Kasukabe, Y., He, L., Watakabe, Y., Otani, M., Shimada, T., Tachibana, S., 2006. Improvement of environmental stress tolerance of sweet potato by introduction of genes for *spermidine synthase*. Plant Biotechnol. 23, 75–83.

Krishanamurthy, R., Bhagwat, K.A., 1989. Polyamines as modulators of salt tolerance in rice cultivars. Plant Physiol. 91, 500–504.

Kubiś, J., 2005. The effect of exogenous spermidine on superoxide dismutase activity, H_2O_2 and superoxide radical level in barley leaves under water deficit conditions. Acta Physiol. Plant. 27, 289–295.

Lee, D.H., Lee, C.B., 2000. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. Plant Sci. 159, 75–85.

Leshem, Y., Seri, L., Levine, A., 2007. Induction of phospatidylinositol 3-kinase-mediated endocytosis by salt stress leads to intracellular production of reactive oxygen species and salt tolerance. Plant J. 51, 185–197.

Liu, J.-H., Kitashiba, H., Wang, J., Ban, Y., Moriguchi, T., 2007. Polyamines and their ability to provide environmental stress tolerance to plants. Plant Biotechnol. 24, 117–126.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880.

Noctor, G., Foyer, C., 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 249–279.

Pandey, S., Ranade, S.A., Nagar, P.K., Kumar, N., 2000. Role of polyamines and ethylene as modulators of plant senescence. J. Biosci. 25, 291–299.

Pang, X.-M., Nada, K., Liu, J.-H., Kitashiba, H., Honda, C., Yamashita, H., Tatsuki, M., Moriguchi, T., 2006. Interrelationship between polyamine and ethylene in 1-methylcyclopropene treated apple fruits after harvest. Physiol. Plant. 128, 351–359.

Park, J., Choi, H.-J., Lee, S., Lee, T., Yang, Z., Lee, Y., 2000. Rac-related GTP-binding protein in elicitor-induced reactive oxygen generation by suspension-cultured soybean cells. Plant Physiol. 124, 725–732.

Park, K.Y., Lee, S.H., 1994. Effects of ethylene and auxin on polyamine levels in suspension cultured tobacco cells. Physiol. Plant. 90, 382–390.

Prabhavathi, V.R., Rajam, M.V., 2007. Polyamine accumulation in transgenic eggplant enhances tolerance to multiple abiotic stresses and fungal resistance. Plant Biotechnol. 24, 273–282.

Rani, G., 2007. Changes in protein profile and amino acids in *Cladophora vagabunda* (Chlorophyceae) in response to salinity stress. J. Appl. Phycol. 19, 803–807.

Rengasamy, P., 2006. World salinization with emphasis on Australia. J. Exp. Bot. 57, 1017–1023.

Roussos, P.A., Pontikis, C.A., 2007. Changes of free, soluble conjugated and bound polyamine titers of jojoba explants under sodium chloride salinity in vitro. J. Plant Physiol. 164, 895–903.

Sakaki, T., Kondo, N., Sugahara, K., 1983. Breakdown of photosynthetic pigments and lipids in spinach leaves with ozone fumigation: Role of active oxygens. Physiol. Plant. 59, 28–34.

Santa-Cruz, A., Acosta, M., Perez-Alfocea, F., Bolarin, M.C., 1997. Changes in free polyamine levels induced by salt stress in leaves of cultivated and wild tomato species. Physiol. Plant. 101, 341–346.

Sairam, R.K., Srivastava, G.C., 2002. Changes in antioxidant activity in sub-cellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. Plant Sci. 162, 897–904.

Smirnoff, N., Cumbes, Q.J., 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28, 1057–1060.

Smith, T.A., 1985. Polyamines. Annu. Rev. Plant Physiol. 36, 117–143.

Sofo, A., Dichio, B., Xiloyannis, C., Masia, A., 2004. Effects of different irradiance levels on some antioxidant enzymes and on malondialdehyde content during rewatering in olive tree. Plant Sci. 166, 293–302.

Song, J., Nada, K., Tachibana, S., 2001. The early increase of *S*-adenosylmethionine decarboxylase activity is essential for the normal germination and tube growth in tomato (*Lycopersicon esculentum* Mill.) pollen. Plant Sci. 161, 507–515.

Song, J., Nada, K., Tachibana, S., 2002. Suppression of S-adenosylmethionine decarboxylase activity is a major cause for high-temperature inhibition of pollen germination and tube growth in tomato (*Lycopersicon esculentum* Mill.). Plant Cell Physiol. 43, 619–627.

Tabor, C.W., Tabor, H., 1984. Polyamines. Annu. Rev. Biochem. 53, 749–790.

Takahashi, Y., Berberich, T., Miyazaki, A., Seo, S., Ohashi, Y., Kusano, T., 2003. Spermine signaling in tobacco: activation of mitogen-activated protein kinase by spermine is mediated through mitochondrial dysfunction. Plant J. 36, 820–829.

Tang, W., Newton, R.J., 2005. Polyamines reduce salt-induced oxidative damage by increasing the activities of antioxidant enzymes and decreasing lipid peroxidation in Virginia pine. Plant Growth Regul. 46, 31–43.

Tang, W., Newton, R.J., Outhavong, V., 2004. Exogenously added polyamines recover browning tissues into normal callus cultures and improve plant regeneration in pine. Physiol. Plant. 122, 386–395.

Venisse, J-S., Gullner, G., Brisset, M.-N., 2001. Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora*. Plant Physiol. 125, 2164–2172.

Verma, S., Mishra, S.N., 2005. Putrescine alleviation of growth in salt stressed *Brassica juncea* by inducing antioxidative defense system. J. Plant Physiol. 162, 669–677.

Waie, B., Rajam, M.V., 2003. Effect of increased polyamine biosynthesis on stress response in transgenic tobacco by introduction of human *S*-adenosylmethionine gene. Plant Sci. 164, 727–734.

Walden, R., Cordeiro, A., Tiburcio, A.F., 1997. Polyamines: small molecules triggering pathways in plant growth and development. Plant Physiol. 113, 1009–1013.

Wang, X., Shi, G., Xu, Q., Hu, J., 2007. Exogenous polyamines enhance copper tolerance of *Nymphoides peltatum*. J. Plant Physiol. 164, 1062–1070.

Wen, X.-P., Pang, X.-M., Matsuda, N., Kita, M., Inoue, H., Hao, Y.-J., Honda, C., Moriguchi, T., 2008. Over-expression of the apple *spermidine synthase* gene in pear confers multiple abiotic stress tolerance by altering polyamine titers. Transgenic Res. 17, 251–263.

Wi, S.J., Kim, W.T., Park, K.Y., 2006. Overexpression of carnation *S*-adenosylmethionine decarboxylase gene generates a broad-spectrum tolerance to abiotic stresses in transgenic tobacco plants. Plant Cell Rep. 25, 1111–1121.

Wild, A., 2003. Soils, Land and Food: Managing the Land During the Twenty-First Century. Cambridge University Press, Cambridge.

Wren, J.J., Wiggall, P.H., 1965. An improved colorimetric method for the determination of proline in the presence of other ninhydrin-positive compounds. Biochem. J. 94, 216–220.

Yamaguchi, K., Takahashi, T., Berberich, T., Imai, A., Takahashi, T., Michael, A.J., Kusano, T., 2007. A protective role for the polyamine spermine against drought stress in *Arabidopsis*. Biochem. Biophys. Res. Commun. 352, 486–490.

Zhu, J.-K., 2001. Plant salt tolerance. Trends Plant Sci. 6, 66–71.

Ziosi, V., Bregoli, A.M., Bonghi, C., Fossati, T., Biondi, S., Costa, G., Torrigiani, P., 2006. Transcription of ethylene perception and biosynthesis genes is altered by putrescine, spermidine and aminoethoxyvinylglycine (AVG) during ripening in peach fruit (*Prunus persica*). New Phytol. 172, 229–238.