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Functional characterization of the apple *MdSAMDC2* gene by ectopic promoter analysis and over-expression in tobacco

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Abstract

The expression of *MdSAMDC2* gene, which encodes *S*-adenosylmethionine decarboxylase (SAMDC) in apple, was up-regulated by low temperature, salt and drought stresses. To identify its *in vivo* biological functions in the responses to stresses, the promoter region of *MdSAMDC2* was isolated and characterized by analyzing the *cis*-acting regulatory elements and GUS reporter gene by an *Agrobacterium*-mediated transformation. GUS activity was enhanced upon salt and cold stresses, indicating that *MdSAMDC2* promoter region controls gene transcription under stresses. In parallel, several lines of the transgenic tobacco plants over-expressing *MdSAMDC2* were obtained. The contents of three polyamines greatly increased in the transgenic lines compared with the non-transgenic WT control. Upon exposed to low temperature (4 °C), salt (150 and 250 mM NaCl) and osmotic (20 % polyethylene glycol) stresses, transgenic plants produced more free polyamines and more active antioxidative enzymes such as superoxide dismutase and catalase than the WT control. Meanwhile, malondialdehyde content, an indicator for membrane lipid peroxidation, decreased in transgenic plants relative to the WT control. Thus, over-expression of *MdSAMDC2* in tobacco conferred tolerance to stresses.

Additional key words: antioxidative enzymes, lipid peroxidation, low temperature, *Nicotiana tabacum*, osmotic stress, salinity.

Introduction

Apple trees are perennial woody plants and abiotic stresses such as drought, salinity, extreme temperatures have huge impact on their survival, geographic distribution, fruit productivity and quality. However, it is of great difficulty to recreate a fruit tree cultivar with improved stress resistance using conventional breeding programs, mainly due to the high heterozygosity in genome and self-incompatibility in sexual fertilization (Wen *et al*. 2008). Genetic engineering using molecular techniques provides an alternative approach. Present engineering strategies of achieving tolerance to adverse environmental challenges rely on the over-expression of one or several genes that regulate other genes from various pathways, or that encode enzymes crucial for the synthesis of functional and structural protectants, such as osmolytes and antioxidants, or that encode stress-tolerance conferring proteins (Vinocur and Altman 2005, Valliyodan and Nguyen 2006).

Polyamines putrescine (Put), spermidine (Spd) and spermine (Spm) play multiple roles in growth and development as well as in the responses to diverse stresses (Evans *et al*. 1989, Su and Bai 2008). Their biosynthetic pathways have been well-characterized. In plants, Put is either synthesized from ornithine *via* ornithine decarboxylase (ODC) or from arginine *via* arginine decarboxylase (ADC). The conversions of Spd from Put, as well as Spm from Spd, are catalyzed by *S*-adenosylmethionine decarboxylase (SAMDC) which is a rate-limiting enzyme in polyamine biosynthesis. Genes encoding SAMDCs have been cloned and characterized from yeast (Pajunen *et al*. 1988, Kashiwagi *et al*. 1990) and a variety of plant species such as potato (Mad Arif *et al*. 1994), spinach (Bolle *et al*. 1995), *Tritordeum*

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Abbreviations: CAT - catalase; GUS - β-glucurodinase, MDA - malondialdehyde; PCR - polymerase chain reaction; PEG - polyethylene glycol; SOD - superoxide dismutase; WT - wild type.
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(Dresselhaus *et al*. 1996), carnation (Lee *et al*. 1997), *Arabidopsis* (Franceschetti *et al*. 2001), pea (Marco and Carrasco 2002), soybean (Tian *et al*. 2004) and rice (Pillai and Akiyama 2004).

Because of the great impact of polyamines on plant development and in the plant responses to stresses, there have been increasing researches focused on the function of *SAMDC* genes in many plant species. Transgenic plants overexpressing *SAMDC* gene exhibited improved tolerance to salt and drought stresses in rice (Roy and Wu 2002), as well as to salt, drought and fungal wilts in tobacco (Waie and Rajam 2003). In addition to stress responses, transgenic potato with antisense *SAMDC* cDNA showed reduced *SAMDC* transcripts and modified phenotypes (Kumar *et al*. 1996). Moreover, tomato plants overexpressing *SAMDC* gene exhibited enhanced fruit nutrient content, fruit juice quality and vine life (Mehta *et al*. 2002).

SAMDC enzymes are encoded by more than one gene in many, if not all, plants. A case is exemplified by *Arabidopsis* which genome contains four SAMDC (Franceschetti *et al*. 2001), and even more yet to be determined. In apple, two SAMDC genes were isolated and named as *MdSAMDC1* and *MdSAMDC2* (Hao *et al*. 2005). RNA gel blot analysis showed that *MdSAMDC1* is mainly involved in fruit development and cell growth, while *MdSAMDC2* in responses to chilling and salt stresses (Hao *et al*. 2005). These results suggested a potential contribution of *MdSAMDC2* to apple tree in polyamine accumulation and the tolerance to environmental stresses, although direct evidence is still lacking. Therefore, aim of this study was to elucidate physiological roles of *MdSAMDC2* gene and its involvement in polyamine accumulation and abiotic stress tolerance.

Materials and methods

Genomic DNA was extracted from leaf tissues of the 10-year-old apple (*Malus domestica* Borkh. cv. Orin) grown in orchard as described by Porebski *et al*. (1997). Promoter region cloning of *MdSAMDC2* gene was performed with polymerase chain reaction (PCR)-based genomic walking method as described by Wu and Liu (2006). Subsequently, the promoter sequence of *MdSAMDC2* was inserted into the *Hin*dIII/*Bam*HI digestion sites of vector pBI121 at the upstream of β-glucurodinase (*GUS*) gene, to replace the *CaMV 35S* promoter. The resulting construct was introduced into tobacco with *Agrobacterium*-mediated transformation as described by Luo *et al*. (2006). The independent transformants were screened by PCR and reverse transcriptase (RT)-PCR. Histochemical staining of plant tissue for GUS activity was performed according to Jefferson *et al.* (1987). Quantitative assay of GUS was conducted with the dye binding assay as described by Bradford (1976).

To over-express *MdSAMDC2* in tobacco (*Nicotiana tobaccum* L. cv. NC89), the cDNA was cloned from *pBluescript* and then ligated into donor vector *pDonor207* and entry vector *pMDC32* in turn by a entry cloning (BP) reaction and expression cloning (LR) reaction. The two recombine reactions were carried out according to the manufacture of *Gateway BP Clonase*™ enzyme mix and *Gateway LR Clonase*™ enzyme mix (*Invitrogen*, Carlsbad, USA).

Agrobacterium tumefaciens strain LBA4404 transformed with *pMDC32- 35S::MdSAMDC2* construct was used to transform tobacco leaf discs as described by Luo *et al*. (2006). To confirm transgene integration, total genomic DNA was isolated from the putative transgenic and control plants according to the method described by Porebski *et al*. (1997). PCR was conducted using *35S* promoter forward primer (5-GATGTGATATCTCCAC TGACGTAAG-3) and *MdSAMDC2* specific reverse primer (5-CTAGATCTTTGCCAT-3). The thermal conditions were 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 90 s at 72 °C.

Total RNA was isolated according to the manufacture of *TRIzol* reagent (*Invitrogen*) recommendation from the transgenic and non-transgenic (WT) tobacco plants. RNA gel blotting was performed with *NorthernMax* kit (*Ambion*, Austin, USA) as described by the manufacturer's instructions. About 10 μg of total RNAs for each sample were electrophoresed on 1.2 % formaldehyde denatured agarose gel, blotted onto a *Hybond*^{N} membrane (*Amersham Bioscience*, Piscataway, USA). The membrane was probed with a-^{32}P labeled *MdSAMDC2* probe. Hybridization was carried out at 68 °C in hybridization solution buffer containing 0.02 % SDS, 5× SSC, 2 % blocking reagent, 0.1 % *N*-lauroylsarcosine and 50 % formamide. After hybridization, the membranes were washed twice for 5 min each time by washing buffer containing 0.1× SSC and 0.1 % SDS at 68 °C. Hybridization was detected by autoradiography.

Free polyamines in the transgenic and WT tobacco plants were quantified as described previously (Hao *et al*. 2005).

Stress tolerance of the transgenic and WT plants was tested on 15-d-old tobacco seedlings pre-incubated at 25 °C on Murashige and Skoog (MS) nutrient medium with 3 % sucrose and 1.2 % agar. Cold stress was imposed by growing seedlings on MS medium at temperature of 4 ± 1 °C under irradiance of 30 ± 2 μmol m^{-2} s^{-1}. Samples were taken after 0, 6 and 120 h. After 30 d, the seedlings were transferred back to 25 °C for recovery and their survival was scored visually after 4 d of recovery. To test salt tolerance, 15-d-old seedlings were transferred onto MS medium supplemented with 150 and 250 mM NaCl and grown at 25 °C and 30 ± 2 μmol m^{-2} s^{-1}. Samples were taken after 48 h. At the end of experiment (60 d), root length and leaf number and colour were determined. To test the tolerance to osmotic stress, 15-d-old seedlings were placed in MS medium containing 20 % PEG. The

samples were taken after 6 h. All experiments were repeated three times.

Oxidative damage to lipids was estimated by measuring the content of malondialdehyde (MDA) in leaf homogenates, prepared in 10 % trichloroacetic acid containing 0.65 % 2-thiobarbituric acid (TBA) and heated at 95 °C for 25 min, as described by Sofo *et al*. (2004). MDA content was calculated by correcting for compounds other than MDA, which absorb at 532 nm by subtracting the absorbance at 532 nm of a solution containing plant extract incubated without TBA from an identical solution containing TBA.

Frozen leaf segments (0.5 g) were crushed into fine powder in a mortar under liquid nitrogen. Soluble proteins were extracted by homogenizing the powder in 10 cm^3 of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 % polyvinylpyrrolidone. The homogenate was centrifuged at 15 000 *g* for 20 min at 4 °C and the supernatant was used for enzyme assays. Protein content was determined according to Bradford (1976) with bovine serum albumin as the standard.

Total superoxide dismutase (SOD, EC1.15.1.1) activity was assayed by measuring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Zhang and Kirkham (1994). A 3-cm^3 reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 0.1 cm^3 enzyme extract. The reaction mixtures were irradiated with two 15 W florescent lamps for 15 min. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of NBT as monitored at 560 nm.

Catalase (CAT, EC1.11.1.6) activity was determined by following the consumption of hydrogen peroxide (coefficient of absorbance 39.4 mM cm^{-1}) at 240 nm for 3 min (Zhang and Kirkham 1994). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 0.2 cm^3 of enzyme extract in a volume of 3 cm^3. One unit of CAT was defined as the amount decomposing 1 μmol of H_2O_2 in 1 min at 25 °C.

Free proline was measured according to the method reported by He *et al*. (2008).

The data were presented as the averages of three or four replicates. They were analyzed by *SPSS v12.0*. Statistically significant differences were tested at $P < 0.05$. Statistical analysis was performed using the Bonferroni-Dunn test.

Results

Cloning and sequence analysis of the promoter region of *MdSAMDC2* gene: A 643-bp promoter fragment in the upstream of MdSAMDC2 5'-UTR was amplified with TAIL-PCR. The nucleotide sequence of this genomic DNA was determined (Fig. 1). In this region, the *cis*-acting regulatory DNA elements were analyzed online using database *PLACE* (http://www.dna.affrc.go.jp/PLACE/index.html). It was found that TATA and CAAT boxes existed in this region as well as several stress associated *cis*-elements. TGTCA (BIHD10S) motif appeared two

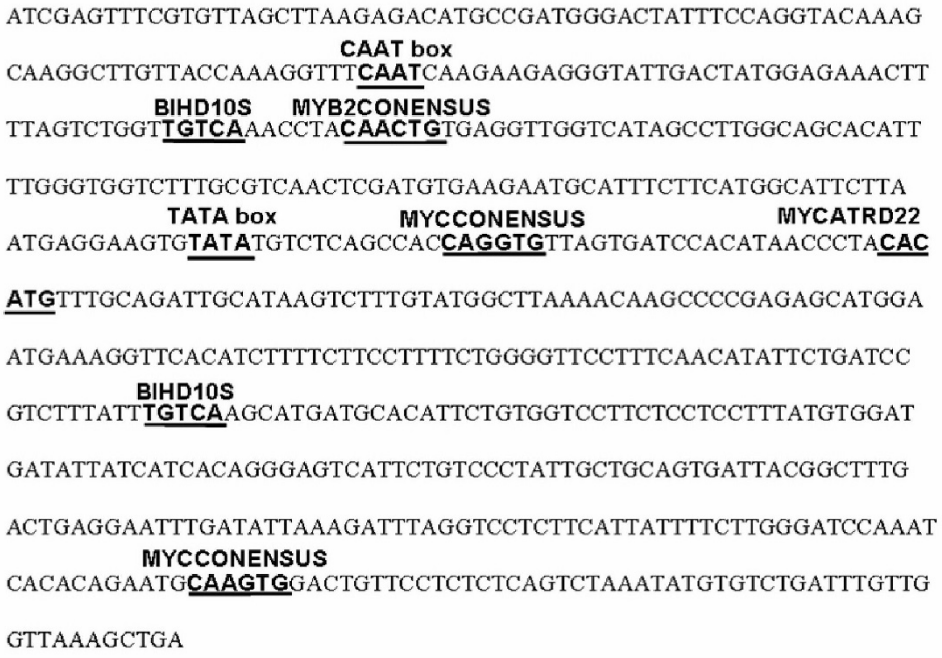

Fig. 1. Nucleotide sequence of the promoter region of the *MdSAMDC2*. The underlined sequences are homologous to BIHD10S (ACGTG), MYB (CAACTG) and MYC (CAGGTG and CACATG) motifs as well as the TATA and CAAT boxes.

times, which was involved in defensive reaction of plant to diseases, for example, as a binding site for OsBIHD1 in rice (Fig. 1). In addition, in the promoter region, there were several MYB (MYB2CONSENSUS as YAACKG) and MYC (MYCCONSENSUSAT as CANNTG and MYCATRD22 as CACATG) motifs which were also found in the promoters of the dehydration-responsive gene *RD22* as well as cold-responsive genes *CBF3* and *ICE1* (*Inducer of CBF Expression 1*) in *Arabidopsis* (Fig. 1). The findings suggest that the promoter region of MdSAMDC2 may be attributable to the stress-responsive expression of *MdSAMDC2*.

Expression analysis of *GUS* reporter gene driven by the *MdSAMDC2* promoter in transgenic tobacco plants: To examine whether the promoter region was involved in the stress-responsive expression of the *MdSAMDC2* gene, a chimeric gene construct with the *MdSAMDC2* promoter region fused to the *GUS* reporter gene was integrated into tobacco genome with *Agrobacterium*-mediated transformation. As a result, stable transgenic lines were obtained. GUS staining demonstrated the promoter activity of *MdSAMDC2* promoter region in T_0 transgenic tobacco (Fig. 2*A*,*B*). According to the GUS staining, three T2 transgenic lines PT1, PT4 and PT5 were chosen for further analysis of stress-responsive expression of the GUS reporter gene. A 2.07- to 2.84-fold increase (average 2.50-fold) in GUS activity was observed in the leaves of salt-treated tobacco plants relative to the control (Table 1). In parallel, we also examined the induction of GUS activity in T2 transgenic tobacco treated with low temperature. It was found that the GUS activities were 2.27 to 3.13 times (average 2.62 times) higher in cold-treated tobacco than the control (Table 1). Those data indicated that the *MdSAMDC2* promoter region was involved in the cold- and salt-responsive expression in transgenic tobacco.

Table 1. GUS activity [nmol(4-MU) mg^{-1}(f.m.) min^{-1}] in 30-d-old transgenic seedlings treated with salt (250 mM NaCl) or cold (4 °C) stresses. PT1, PT2 and PT5 are three independent transgenic lines. Means ± SE, $n = 3$. Values followed by different letters are significantly different at $P < 0.05$.

Line	Control	Salt stress	Cold stress
PT1	4.1 ± 0.53f	8.5 ± 0.94cd	9.3 ± 0.88ab
PT4	3.2 ± 0.50g	9.1 ± 1.08bc	7.9 ± 0.93e
PT5	3.1 ± 0.61g	8.0 ± 0.87de	9.7 ± 1.25a

Generation and identification of transgenic tobacco plants: To examine the *in vivo* function of *MdSAMDC2* in polyamine accumulation and stress tolerance, a construct containing *MdSAMDC2* gene driven by *CaMV 35S* promoter was introduced into tobacco with *Agrobacterium*-mediated transformation. Thirty-six independent, stable transgenic lines were confirmed with kanamycin-resistance, genomic DNA PCR and cDNA RT-PCR (data not shown). Then, RNA gel blot analysis was conducted to quantify the ectopic expression level of *MdSAMDC2* in transgenic tobacco. It was found that the expression level of *MdSAMDC2* was line dependent (Fig. 2C). Finally, the T2 transgenic lines T12, T13 and T20, which produced *MdSAMDC2* transcripts at different levels from low to high, were chosen for polyamine accumulation assay.

Table 2. Polyamine contents [nmol g^{-1}(f.m.)] in 15-d-old seedlings of three independent transgenic lines. Means ± SE, $n = 3$. Values followed by different letters are significantly different at $P < 0.05$.

Line	Put	Spd	Spm	Total PAs
WT	19.9 ± 1.13c	14.3 ± 1.56b	10.5 ± 0.85b	44.7 ± 0.42c
T12	21.5 ± 0.57bc	17.0 ± 0.14b	18.0 ± 1.27a	55.5 ± 1.06b
T13	24.5 ± 0.42b	22.8 ± 1.70a	22.0 ± 1.13a	70.3 ± 3.25a
T20	30.1 ± 0.99a	18.4 ± 0.99ab	22.6 ± 2.69a	71.1 ± 0.70a

HPLC analysis revealed that the free polyamine contents were greatly enhanced in the three transgenic lines (Table 2). Lines T12, T13 and T20 produced 1.08-, 1.23- and 1.51-fold more Put; 1.19-, 1.59- and 1.29-fold Spd; 1.71-, 2.10- and 2.15-fold Spm; respectively, relative to the WT control. The contents of total and each polyamine were consistent with the corresponding transcript levels of *MdSAMDC2* gene in three lines (Table 2; Fig. 2*C*). Those results support that *MdSAMDC2* is crucially involved in polyamine synthesis. Meanwhile, there was no significant growth and developmental abnormality found in three transgenic lines (data not shown), despite of their higher polyamine accumulation than in the WT.

Tolerance of transgenic tobacco plants to cold stress: The result showed that the survival rates of all three transgenic lines T12, T13 and T20 were higher (23.3 ± 6.40, 31.6 ± 8.35 and 72.0 ± 6.28, respectively) than the WT (only 12.0 ± 5.32 % of WT seedlings survived). Since T20 showed the highest cold tolerance, we examined if it accumulates more polyamines under cold condition than the WT control. The result demonstrated that low temperature led to a similar fluctuation in polyamine contents with time both in the line T20 and WT seedlings. However, line T20 seedlings always produced more Put, Spd, Spm and total free polyamines at all time points, in consistence with its higher survival rate when recovered from cold stress, than the WT (Table 3). Therefore, *MdSAMDC2* over-expression enhanced the polyamine contents, which then conferred cold tolerance to transgenic seedlings. To examine the proposed contribution of polyamines to maintain the stability of proteins and membranes, the lipid peroxidation and activities of antioxidative enzymes were

analyzed. MDA content was much lower in T20 than in WT, suggesting a less lipid peroxidation. By contrast, the activities of both SOD and CAT were higher at all time points in line T20 than in WT, indicating that the transgenic seedlings had greater antioxidative capability than the WT control. In addition, proline accumulated more in T20 than in WT (Table 4).

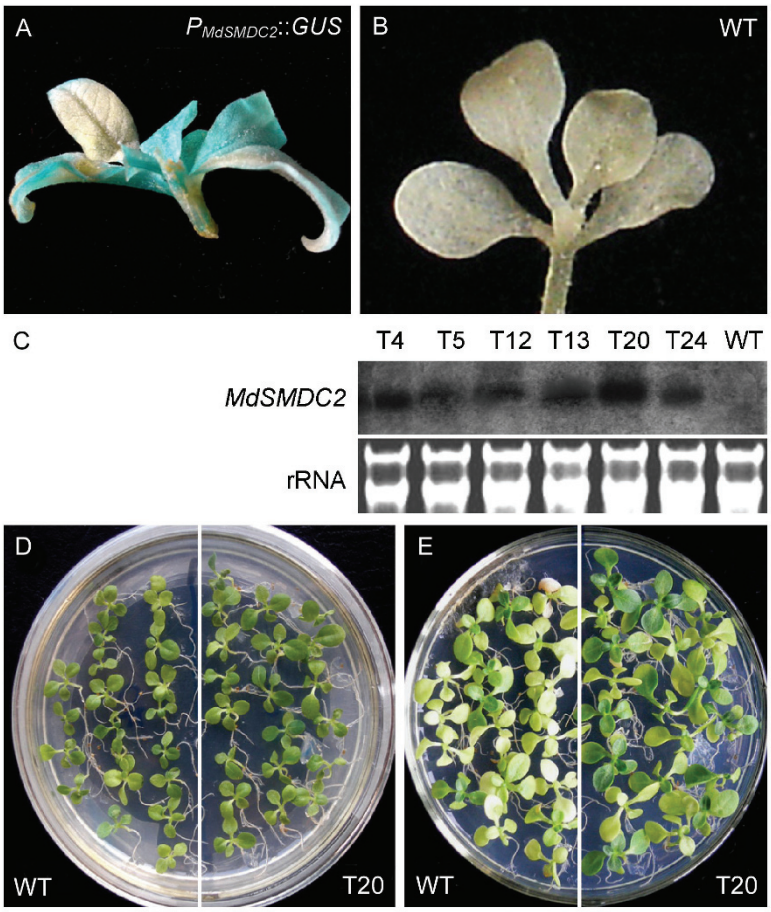

Fig. 2. GUS activity, transcript level and salt tolerance. *A*,*B* - GUS activity in a transgenic tobacco with the construct $P_{MdSAMDC2}$::*GUS* (*A*) and the non-transgenic control (WT) (*B*). *C* - The expression levels of *MdSAMDC2* gene in dependent transgenic lines T4, T5, T13, T20, T24 and the non-transgenic control (WT). The rRNA was used as loading control. *D* - 15-d-old seedlings of the transgenic line T20 and WT growing in the separate halves of the same agar plate under normal conditions. *E* - The same seedlings were exposed to 250 mM NaCl for another 60 d.

Table 3. Polyamine (PA) contents [nmol g^{-1}(f.m.)] in T20 and WT seedlings treated with cold (4 °C for 0, 6 and 120 h), NaCl (0, 150 and 250 mM for 48 h) and PEG 20 % for 0, 2 and 6 h). Means ± SE, $n = 3$. Values followed by different letters are significantly different at $P < 0.05$.

PAs	Line	Cold			NaCl			PEG		
		0 h	6 h	120 h	0 mM	150 mM	250 mM	0 h	2 h	6 h
Put	WT	19.9±1.13d	27.7±1.13cd	33.8±1.84c	19.9±1.13c	30.4±1.84b	22.9±1.70c	19.9±1.13e	81.1±2.12b	63.3±1.27c
	T20	30.1±0.99c	79.3±4.24b	97.1±2.97a	30.1±0.99b	37.8±2.26a	25.4±0.99bc	30.1±0.99d	111.4±2.26a	80.8±0.57b
Spd	WT	14.3±1.56c	60.8±5.66b	21.9±1.56c	14.3±1.56cd	18.7±1.13c	13.2±0.42d	14.3±1.56d	50.5±1.27c	60.8±2.12b
	T20	18.4±0.99c	86.0±2.69a	67.9±2.40b	18.4±0.99c	56.4±1.70a	35.0±1.27b	18.4±0.99d	78.4±1.27a	63.3±1.13b
Spm	WT	10.5±0.85d	12.8±2.26d	10.0±1.27d	10.5±0.85e	29.1±0.99b	18.6±0.94d	10.5±0.85c	20.8±1.27b	20.5±1.13b
	T20	22.6±2.69c	59.0±3.81a	34.4±1.69b	22.6±2.69cd	36.1±1.27a	24.7±1.56bc	22.6±2.69b	38.6±2.12a	31.8±2.97a
Total PAs	WT	44.7±1.70c	126.6±11.60c	65.7±5.09c	44.7±1.70c	78.2±5.66b	54.7±2.55c	44.7±1.70e	152.3±2.12c	144.6±0.28c
	T20	71.1±3.11c	199.1±12.59a	199.3±10.75a	71.1±3.11b	130.3±6.93a	85.0±1.98b	71.1±3.11d	228.4±1.41a	175.9±2.40b

Tolerance of transgenic line T20 to salt and osmotic stress: Salinity greatly affected the growth of T20 and WT seedlings. All leaves turned to be bleached to different degree, especially in WT seedlings (Fig. 2*D*,*E*). The root length and leaf number of T20 were 2.16 ± 0.36 cm and 6.8 ± 1.09, respectively, while those of WT control were 1.6 ± 0.35 cm and 4.8 ± 0.84, respectively. Therefore, the transgenic seedlings were bigger than the WT seedlings. Similar with the changes in cold treatment, the transgenic seedlings of line T20 always produced more individual and total free polyamines than the WT after treatment with 150 and 250 mM NaCl for 48 h (Table 3). Further, MDA content was lower in line T20 than in WT and the SOD and CAT activities were mostly higher in T20 than WT seedlings. In addition, there was more proline produced in T20 than in WT (Table 4). These results suggested that transgenic line displayed greater antioxidative capability and got less membrane damage under salt stress than WT.

When line T20 and WT were exposed to osmotic stress induced by 20 % PEG, similar results, *i.e*. more polyamines and proline, less MDA content as well as higher SOD and CAT activities in transgenic line than in the control, were observed (Tables 3,4).

Table 4. MDA and proline contents as well as SOD and CAT activities in T20 and WT seedlings treated with cold (4 °C for 0 - 120 h), NaCl (0, 150 and 250 mM for 48 h) and PEG (20 % for 0 - 6 h). Means ± SE, $n = 3$. Values followed by different letters are significantly different at $P < 0.05$.

		MDA [nmol·g^{-1}(f.m.)]		SOD [U·g^{-1}(f.m.)]		CAT [ΔA_{240}·g^{-1}(f.m.)·min^{-1}]		Proline [μg·g^{-1}(f.m.)]	
		WT	T20	WT	T20	WT	T20	WT	T20
Cold	0	6.6±0.40bc	4.8±0.50c	104.8±1.24e	168.6±8.37d	1.4±0.05de	2.3±0.07bc	10.1±1.15f	13.2±0.51ef
	6	8.2±1.31bc	5.0±1.01c	193.4±7.44bc	214.5±5.58a	2.3±0.35bc	3.3±0.35a	15.0±0.24e	26.0±0.25c
	24	12.6±3.12ab	6.7±1.50bc	189.3±0.30bc	200.0±4.96b	2.0±0.04cd	2.8±0.02ab	26.6±3.31c	24.5±1.40cd
	72	10.7±2.50abc	6.1±2.61bc	180.7±0.32c	185.5±6.82c	1.4±0.09de	2.2±0.06bc	33.7±0.41b	39.1±0.92a
	120	15.4±0.81a	11.5±3.30abc	181.4±0.48c	192.5±2.05bc	1.3±0.32e	1.2±0.09e	10.6±1.15ef	20.7±1.33cd
NaCl	0	6.6±0.40e	4.8±0.50f	104.8±1.24d	168.6±8.37b	1.4±0.05d	2.3±0.07b	10.1±1.15d	13.2±0.51c
	150	15.4±0.41c	11.4±0.63d	178.3±5.91b	209.6±0.59a	2.2±0.19bc	5.1±0.09a	19.4±1.11c	25.9±1.15a
	250	26.0±0.62a	20.5±0.51b	155.9±7.13c	156.4±6.27c	1.2±0.13d	1.8±0.09c	10.4±1.14cd	13.2±0.12c
PEG	0	6.6±0.40c	4.8±0.50c	104.8±1.24f	168.6±8.37e	1.4±0.05h	2.3±0.07e	10.1±1.15f	13.2±0.51de
	1.5	4.1±0.92c	5.2±1.42c	159.5±1.53e	200.7±7.78cd	3.8±0.02a	2.5±0.07cd	18.2±1.50c	51.0±2.75b
	3.0	15.7±1.64b	8.0±0.74c	170.4±8.38e	216.5±4.46c	1.9±0.02g	3.1±0.04b	21.8±0.25c	60.6±0.64a
	4.5	25.2±1.11a	23.5±1.72a	194.7±3.72d	327.0±2.79a	2.0±0.05f	3.8±0.04a	12.1±0.51def	12.5±0.89de
	6.0	23.8±0.33a	21.6±2.23ab	173.7±3.73e	258.6±6.86b	2.7±0.04c	3.1±0.03b	17.0±0.51cd	20.3±2.42c

Discussion

In the previous study, RNA gel blot analysis showed that *MdSAMDC2* was regulated by cold and salt stresses (Hao *et al*. 2005). To further examine how *MdSAMDC2* responds to those stresses, its promoter region was isolated and analyzed. It was found that several typical *cis*-acting regulatory elements existed in the promoter region. Of those, MYB and MYC motifs are generally involved in multiple responses of plants to stresses (Simpson *et al*. 2003). MYC motifs in *CBF3* and *ICE1* promoter regulate gene transcription in cold, salt or dehydration conditions (Meshi and Iwabuchi 1995, Chinnusamy *et al*. 2003). Furthermore, GUS activity assay in tobacco ectopically expressing *GUS* gene driven by *MdSAMDC2* promoter demonstrated the involvement of *MdSAMDC2* gene in stress responses. However, it is yet to be determined how the *MdSAMDC2* promoter responds to stresses. A more detailed analysis of the *cis*-elements that control expression of the *MdSAMDC2* gene should help us to understand the molecular mechanisms of the regulation of gene expression by multiple stresses.

To verify the function of *MdSAMDC2 in planta*, transgenic tobacco plants over-expressing *MdSAMDC2* were obtained and examined. With enhanced polyamine contents, transgenic seedlings were more tolerant to cold, salt and drought stresses, especially line T20. It is likely to be true that the over-expression of *MdSAMDC2* in tobacco results in an increased activity of SAMDC enzyme, which then produce more aminopropyl to promote the biosynthesis of Spd and Spm, as reported in other plant species (Li and Chan 2000, Wi *et al*. 2006). With regard to the increase of Put in the transgenic tobacco lines, it may comes from the feedback regulation by the transformation of Put into Spd or the revertant transformation of Put from Spd through the acetylation mechanism (De Agazio *et al*. 1995). This is also consistent with the results reported in cucumber (Shen *et al*. 2000) and *Limonium sinuatum* (Duhaze *et al*. 2002). Furthermore, transgenic lines over-expressing *MdSAMDC2* produced more polyamines than the WT control. It is deemed that polyamine accumulation resulting from *SAMDC* over-expression can increase the activity of ADC and ODC enzymes which are directly involved in Put synthesis (Mo and Pua 2002;

Thu-Hang *et al*. 2002). In addition, the total polyamine contents drop down in WT seedlings at 120 h compared with 6 h in cold treatment, as well as at 6 h relative to 2 h in PEG treatment, suggesting a possible down-regulation or crosstalk with other metabolic pathways.

It has been well documented that reactive oxygen species (ROS) production and antioxidative enzyme (including SOD and CAT) activities change in plants upon exposure to abiotic stresses. In higher plants, polyamines are able to affect membrane fluidity by binding to phospholipids (Tiburcio *et al*. 1993) and mediate biotic and abiotic stress responses such as pathogen infection, osmotic stress, potassium deficiency and wounding (Watson *et al*. 1996, Cowley *et al*. 2002, Pérez-Amador *et al*. 2002, Capell *et al*. 2004, Tonon *et al*. 2004, He *et al*. 2008). In parallel, it has been reported that increased polyamine content might activate gene expression of antioxidative enzymes in response to abiotic stress (Wi *et al*. 2006). In this study, it was also found that the polyamines might be involved in keeping defense enzyme more stable and active, which is helpful to eliminate stress-induced ROS. This is consistent with results reported in watermelon where the increased antioxidative enzyme activities under the chilling temperature treatment were associated with increased polyamine contents (Kwon *et al*. 2003).

RNA gel blot analysis showed that *MdSAMDC2* gene was positively induced by cold and salt stresses as previously reported (Hao *et al*. 2005). In this study, ectopically transgenic analysis of promoter region further proved the constitutive expression of *MdSAMDC2* in different tissues or organs. In addition, it was also found that ectopic over-expression of *MdSAMDC2* gene in tobacco improved the tolerance of transgenic seedlings to stresses. It indicates that *MdSAMDC2* has a great potential as target gene in genetic engineering for the improvement of fruit tree stress tolerance. However, all data of this study come from ectopic over-expression *MdSAMDC2* in tobacco. The exact roles of *MdSAMDC2* in apple tree *per se* are yet to be determined.

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