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BRIEF COMMUNICATION

Effects of salt and osmotic stresses on free polyamine content and expression of polyamine biosynthetic genes in *Vitis vinifera*

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Abstract

Grape (*Vitis vinifera* L.) seedlings grown *in vitro* were treated with either 200 mM NaCl or 350 mM mannitol for 7 d. Both salinity and osmotic stress caused significant increase in electrolyte leakage. From the three commonly occurring free polyamines (PA), only conspicuous accumulation of putrescine was found in the NaCl-treated seedlings. Four PA biosynthetic genes encoding arginine decarboxylase (*pVvADC*), *S*-adenosylmethionine decarboxylase (*pVvSAMDC*), spermidine synthase (*pVvSPDS*) and spermine synthase (*pVvSPMS*) were successfully isolated. While induction of *pVvADC* was observed from the 1st day of salt treatment, *pVvSAMDC* and *pVvSPMS* were induced only at late stage of stress. As for expression levels of genes in the mannitol-treated seedling, either temporary (*pVvADC* at day 1) or late (*pVvSPMS* at days 5 and 7) induction was observed.

Additional key words: abiotic stress, electrolyte leakage, grape, mannitol, NaCl, putrescine, spermine, spermidine.

Salinity and drought are two of the most important abiotic stresses that limit plant geographic distribution, affect plant growth, development and productivity (Flowers 2004). A variety of cellular or molecular changes take place in stressed plants like quick induction of specific genes (Shinozaki and Yamaguchi-Shinozaki 2007). In addition, accumulation of organic solutes, such as free proline and sugars, is an important physiological process for adaptation to the stresses (Aghaleh *et al*. 2009). Apart from these compounds, a large spectrum of studies have demonstrated that polyamines (PAs), mainly spermidine (Spd), spermine (Spm) and their diamine precursor, putrescine (Put), are involved in biotic and/or abiotic stress response (Liu *et al*. 2007, Kusano *et al*. 2008 and references therein).

Enhancement of stress tolerance in grape *via* modulating endogenous PAs through genetic engineering or exogenous application of PAs has been described (Verma and Mishra 2005, Liu *et al*. 2006b, Wi *et al*. 2006, Farooq *et al*. 2009). It is thus anticipated that tolerant grape germplasms can be obtained *via* manipulation of PA biosynthesis. Previously, isolation of genes encoding arginine decarboxylase (ADC) and *S*-adenosylmethionine decarboxylase (SAMDC) has been reported (Primikirios and Roubelakis-Angelakis 1999, Tassoni *et al*. 2007) and interrelationships between PAs and abscission or embryogenesis was identified (Aziz *et al*. 2001, Aziz 2003, Bertoldi *et al*. 2004). However, so far information is still scarce on PA accumulation and expression profiles of the biosynthetic genes under abiotic stresses in grape. Therefore, in the present study we used *in vitro* grown grape seedlings to investigate how PA biosynthesis responds to osmotic and salt stress.

Seedlings of grape (*Vitis vinifera* L. cv. Neo Muscat) were grown on Murashige and Skoog (1962; MS) medium with 1 μM *α*-naphthaleneacetic acid (NAA) and 2.5 μM N^6- benzylaminopurine (BA), 0.6 % agar and 3 % sucrose (pH 5.8). After rooting on MS medium with 0.1 μM NAA,

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Abbreviations: ADC - arginine decarboxylase; EL - electrolyte leakage; MS - Murashige and Skoog; ODC - ornithine decarboxylase; Put - putrescine; SAMDC - *S*-adenosylmethionine decarboxylase; Spd - spermidine; SPDS - spermidine synthase; Spm - spermine; SPMS - spermine synthase.
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0.8 % agar and 3 % sucrose (pH 5.8), the 20-d-old seedlings were transferred to new medium supplemented with or without either 200 mM NaCl or 350 mM mannitol (Lutts *et al*. 2004). All samples taken after 0, 1, 3, 5 and 7 d were immediately frozen in liquid nitrogen and stored at -80 ºC until use for RNA extraction and measurement of free PAs. Electrolyte leakage (EL) was measured at the onset of experiment (d 0) and 7 d after treatment as described previously (Liu *et al*. 2006b). RNA was isolated using hot borate (Wan and Wilkins 1994), and first-strand cDNA was synthesized with a cDNA synthesis kit (*Amersham Bioscience,* Piscataway, NJ, USA). Partial cDNA fragments of *ADC* (arginine decarboxylase), *SAMDC* (*S*-adenosylmethionine decarboxylase) and *SPDS* (spermidine synthase) were obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) according to Liu *et al*. (2006b) using degenerate primers (Table 1), while partial cDNA of *SPMS* (spermine synthase) was amplified as mentioned by Kitashiba *et al*. (2005). DNA sequences were analyzed using the *GCG* software package (*Accelrys*, San Diego, CA, USA) and *Genetyx-Mac 10.1* (*Software Development*, Tokyo, Japan).. The homologous fragments were labeled with digoxigenin-dUTP (DIG) by PCR (*Roche Diagnostics*, Tokyo, Japan) according to the manufacturer's instructions and used as probes for RNA gel blotting with the same procedure as Liu and Moriguchi (2007). Expression of grape ornithine decarboxylase (*ODC*) gene was assayed using a probe of peach *ODC* since the former was not obtained in this study (Liu *et al*. 2006a). Free PAs were extracted and measured by high performance liquid chromatography (HPLC, *Shimadzu*, Kyoto, Japan) according to Liu and Moriguchi (2007). The experiments were repeated twice with 3 replicates in each, and data of mean ± SD from a typical experiment are presented. The statistical analysis was performed by one-way *ANOVA*, taking $P < 0.05$ as significant.

RT-PCR gave rise to bands of expected size for *ADC* (1208 bp), *SAMDC* (584 bp), *SPDS* (335 bp) and *SPMS* (310 bp). Sequence analysis *via* homology search in the database showed that all of them were truly partial cDNAs of the PA biosynthetic genes. They were thus designated as *pVvADC* (*partial Vitis vinifera ADC*), *pVvSAMDC*, *pVvSPDS* and *pVvSPMS*, which have been deposited in DDBJ (DNA Data Bank of Japan) database under the accession numbers of AB240536, AB240537, AB240538 and AB240539, respectively (Table 2). Multiple sequence alignments demonstrated that their nucleotide sequences presented high identity to corresponding genes isolated elsewhere. cDNAs of *pVvADC* and *pVvSAMDC* herein were most identical (97 and 92 %) to their counterparts, X96791 and AJ567368, respectively, isolated from grape. In addition, *pVvSPDS* and *pVvSPMS* sequences shared the highest identity with those from peach (99 %, AB194105) and apple (84 %, AB204520), respectively.

Grape seedlings cultured in the control medium had normal leaves, whereas those treated with either salt or mannitol for 7 d exhibited wilting (data not shown). The

Table 1. Degenerate primers used to isolate partial fragments. For each pair of primers the upper sequence is the forward primer and the lower one is the reverse primer.

Target ESTs	Primer sequences (5'-3')
pVvADC	TACCARGGDGTHTAYCCDGTGAA
	CCRTCRCTRTCRCANGTYAARTC
pVvODC	TTYTAYGCYGTYAAATGYAACCC
	GTATGWGCRCCCATRTTMKSRAA
pVvSAMDC	GAYTCNTATGTNCTNTCNGAGTCNAG
	CTNGCRTARCTGAANCCRTCYTCNGG
pVvSPDS	AARGTIYTIGTIATHGGIGGIGG
	TGIGTISWIACIACICCICCIGG
pVvSPMS-a	GRGARGCWCAYTCHYTGAA
	CKCTNGGRTAWGTDGGRA
pVvSPMS-b	AYGARTGTGCNTAYCARGA
	GGRTCWGAWGARTCMACWAT

Table 2. Isolation of the four partial cDNA fragments and homology search in database.

ESTs	Size [bp]	Accession No.	The first hit in *BLASTN*
ADC	1208	AB240536	X96791 (grape)
SAMDC	584	AB240537	AJ567368 (grape)
SPDS	335	AB240538	AB194105 (peach)
SPMS	310	AB240539	AB204520 (apple)

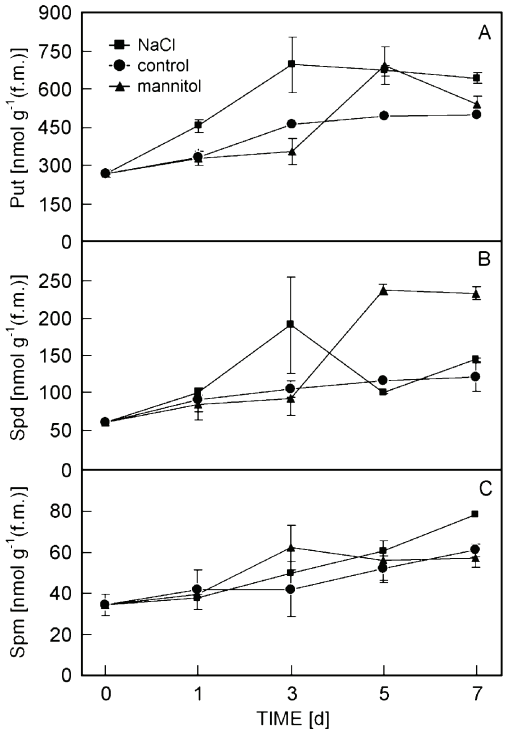

Fig. 1. Changes in free putrescine (Put, *A*), spermidine (Spd, *B*) and spermine (Spm, *C*), of *in vitro* grape seedlings sampled 0, 1, 3, 5 and 7 d after they were cultured on root-induction medium supplemented with mannitol, NaCl or without stress (control).

control seedlings did not show obvious change in EL during growth. However, treatment with both NaCl and mannitol led to significant increase in EL (41.9 and 47.8 %, respectively) relative to the control, indicating stress-induced damage of the seedlings.

When the seedlings were treated with NaCl Put content increased at day 1 and reached the peak at day 3, followed by a negligible decrease at the last day. At any given day Put in the salt-treated seedlings was higher than that in the control. During the whole course Put in the mannitol-treated seedlings exhibited no significant alteration compared with the control, with the exception of an increase at day 5 (Fig. 1*A*). The salt-treated seedlings showed higher Spd content only at day 3. Spd in the mannitol-treated seedlings were significantly higher than in the control at the last two days (Fig. 1*B*). Spm in the salt-treated seedlings showed increase from day 3 until the end of treatment, while that in the mannitol-treated seedlings was only higher at day 3 (Fig. 1*C*). It implied that under stress discrete increase of free PAs was observed, in agreement with what has been depicted before. Santa-Cruz *et al*. (1997) reported that free PAs were only accumulated at the start of stress treatment, while they began to decrease when the culture duration increased. In another work, Tonon *et al*. (2004) also showed that treatment of *Fraxinus angustifolia* callus with salt led to early and temporary increase in PAs, while prolonged treatment did not induce drastic accumulation. All of these showed that the PAs did not infinitely increase when the stresses progressed, suggesting that stress-induced accumulation of PAs may be limited to a certain period and/or is stress-type dependent (Botella *et al*. 2000).

The mRNA steady-state level of *pVvADC* in the control seedlings showed no remarkable change during 7 d culture. Osmotic stress led to slight induction of *pVvADC* 1 d after treatment, which, however, decreased to level close to the control in the following days. On the contrary, the expression of *pVvADC* was notably up-regulated by salt stress during the whole course (Fig. 2). Expression level of *ODC* was not obviously influenced by either of the stresses. As far as *pVvSAMDC* was concerned, its transcription level was unexpectedly high on the first day in the control and mannitol-treated seedlings. In the salt-treated seedlings its mRNA accumulation was observed at the last two sampling days, particularly at day 7. Expression of *pVvSPDS* was the weakest among the five genes and did not exhibit appreciable change between the control and the treated seedlings. Without stress treatment, transcriptional level of *pVvSPMS* was weak and did not show obvious change during the whole course. Under mannitol treatment the expression pattern of this gene was similar to that of the control, with the exception of an induction at day 7. The *pVvSPMS* began to accumulate at day 3 after NaCl treatment and continued to increase upto the last day (Fig. 2). Induction of the genes under stress implied that, at least in part, they possibly took part in or were important components for stress response. However, it is noted that the five genes were induced to different degrees. Although both ADC and

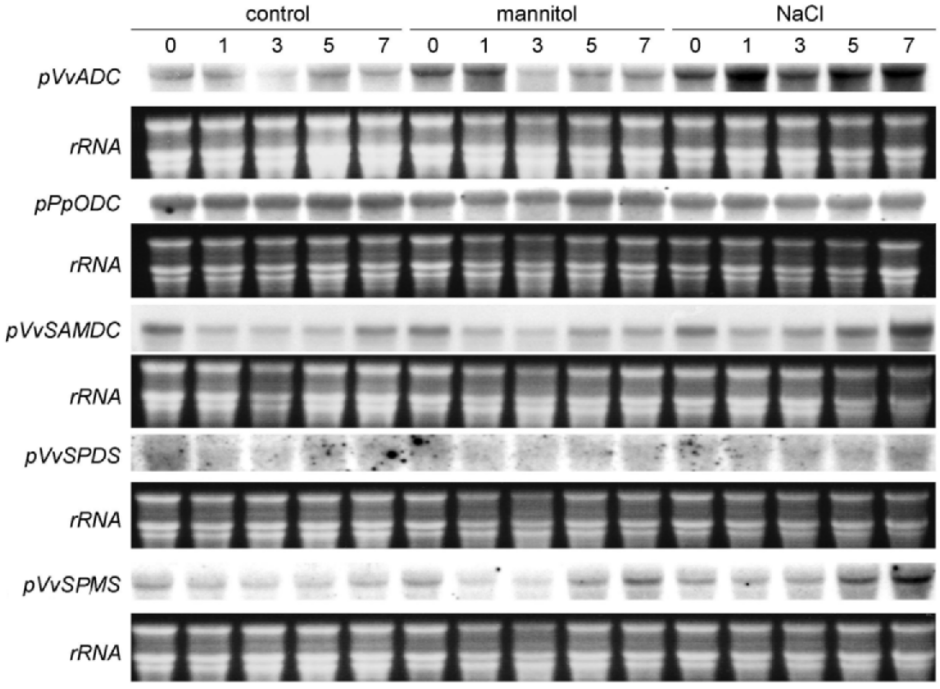

Fig. 2. Expressions of grape PA biosynthetic genes, based on RNA gel blotting with probes from grape (*pVvADC*, *pVvSAMDC*, *pVvSPDS*, *pVvSPMS*) and peach (*pPpODC*), of *in vitro* seedlings that were sampled 0, 1, 3, 5 and 7 after they were cultured on root-induction medium supplemented with mannitol, NaCl or without stress (control). Equal loading of RNA is shown as rRNA stained by ethidium bromide for each gene.

ODC are responsible for putrescine biosynthesis, ODC encoding gene showed minor change when the seedlings were exposed to both stressors, whereas ADC encoding gene was upregulated by either mannitol or NaCl, implying that ADC might be more intimately related to stress response than ODC, consistent with previous concept that ADC plays key role in stress response (Urano *et al*. 2004, Liu *et al*. 2009). SAMDC encoding gene has also been shown to be induced by stresses in previous reports (Urano *et al*. 2003), but the induction in grape was only observed in the salt-treated seedlings at the last two days, suggesting that stress response of this gene may be plant-specific or limited to certain stressor. Nevertheless, the possibility that there are other SAMDC encoding genes in grape that may be more closely linked to stress adaptation could not be ruled out. Interestingly, abundance of *pVvSPMS* was enhanced by either mannitol (day 7) or NaCl (days 5 and 7), implying this gene may be implicated in stress response. Previously, SPMS encoding gene was shown in connection with cell expansion and ripening of apple fruit (Kitashiba *et al*. 2005). It needs further work to illustrate the physiological significance of the induction of this gene by stress, which has been delineated elsewhere (Liu and Moriguchi 2008).

Herein the *in vitro* grape seedlings were subjected to two types of abiotic stresses, salinity and high osmoticum, that had equivalent osmotic potential (Lutts *et al*. 2004). However, their effects on PA accumulation and gene expression were distinct, suggesting that they had different influences on PA biosynthesis. Treatment with NaCl led to obvious and persistent accumulation of Put, coupled with higher and/or earlier induction of the PA biosynthetic genes than osmotic stress. The underlying reason for this disparity may be that NaCl causes not only osmotic stress, but also nutrient imbalance and ion toxicity (Zhu 2001). In addition, salinity also results in oxidative stress due to accumulation of reactive oxygen species, indicating that NaCl has resulted in more profound influences or more severe damage than osmotic stress (Tonon *et al*. 2004). In this regard, plants under salt stress may respond in a more active manner, through accumulation of more PAs, stronger and/or quicker gene induction, which functions to mitigate or repair the stress-derived damage.

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