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Molecular characterization of salt stress in grapevine cultivars (*Vitis vinifera* L.) and rootstocks

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Summary

Salt stress is an important factor inducing the expression of many genes; *e.g.* the osmotin gene is one of the very important genes responding to NaCl stress. After exposure to NaCl stress, the osmotin gene expression level was investigated in 6 grape cultivars grown in GAP (Southeast Anatolian region including the provinces Gaziantep, Şanliurfa, Diyarbakır, Mardin, Batman, Siirt and Şırnak) and in 4 grape cultivars and 7 rootstocks recommended to the GAP region. Expression levels were investigated by Northern blot analysis. The osmotin expression level was higher in scion cultivars than in rootstocks. Within grapevine cultivars, the highest osmotin gene expression level was observed in cv. Tahannebi, followed by Hönüsü, another GAP region cultivar. The osmotin gene expression levels of two grape cultivars, Ata sarisi and Alphonse Lavallée recommended to the GAP region, were lower than those of Hönüsü. On the other hand the GAP region cultivars Ağ Besni, Rumi, Kabarcık, Dımışkı and the recommended cultivars Razakı and Italia were significantly different in their expression levels.

Among rootstocks 1616 C displayed the highest expression level, followed by 99R; they were followed by 1613C. In 110R the expression level was slightly lower than in the above mentioned ones and 41B. In SO4 and 5C the level of expression remained at the control level.

K e y w o r d s : grapevine, rootstock, salt stress, gene expression, northern blot analysis, Turkey.

Introduction

Salt stress induces many metabolic changes with effects on growth and development. During adaptation to salt stress besides physiological changes (Watad *et al*. 1986; Binzel *et al.* 1987; Hirayama and Mihara 1987; La Rosa *et al.* 1987), changes in expression of many genes were shown in different biological systems (Singh *et al.* 1989; Holland *et al.* 1993; Garcia *et al.* 1998).

Osmotin and osmotin-like proteins constitute a major group of proteins responding to salt stress (Loulakakis 1997 a; Singh *et al*. 1989). The osmotin gene was originally isolated from tobacco plants cultured in a medium with low osmotic potential (Singh *et al.* 1989).

The expression patterns of the genes coding for osmotin and osmotin-like proteins are quite complicated (La Rosa *et al.* 1992; Zhu *et al.* 1995). These genes are not only controlled during development but also by factors like NaCl, ABA, salicylic acid, virus infection, low temperature, wounding or ethanol which have an impact on the control of gene expression (Loulakakis 1997 a; Singh *et al.* 1989; Zhu *et al.* 1995). In addition, these genes are also involved in the resistance to fungal disease (Zhu *et al.* 1995; Salzman *et al.* 1998; Yun *et al.* 1998).

In *Vitis* the full length pVVOSM 1 cDNA clone has been defined (Loulakakis 1997 b). After the expression patterns of the clone that displayed high homology to annual plant osmotin and osmotin-like proteins had been investigated, a progressive accumulation of steady-state levels of mRNA was observed (Loulakakis 1997 a).

In the current study osmotin gene expression levels were analysed by Northern Blot analysis in various grapevine cultivars and rootstocks in response to NaCl stress.

Material and Methods

P l a n t m a t e r i a l: Grapevine cultivars from the Southeastern part of Turkey and some other grapevine cultivars and rootstocks recommended to the Southeast Anatolian (GAP) region including Gaziantep, Şanlıurfa, Diyarbakır, Mardin, Batman, Siirt and Şırnak provinces, were collected from their original habitats. For this purpose cuttings from grapevines and rootstocks were grown in pots containing perlite + soil + washed torf (2:1:2) for two years. After NaCl treatment (Tab. 2), young leaves and shoots were used for RNA and DNA isolation. The grapevine cultivars and rootstocks used in the study are listed in Tab. 1.

To determine the time and the dosage of NaCl required to induce osmotin gene expression, a time course experiment with various doses was conducted (Tab. 2). For this part of the study two samples, cv. Razakı and the rootstock, cv. 1616 C were used.

N u c l e i c a c i d i s o l a t i o n a n d N o r t h e r n b l o t a n a l y s i s : DNA was isolated for probe preparation following the protocol by Lodhi *et al.* (1994) RNA isolation was performed from young leaves and shoot apices according to the method developed by J. T. Burger (pers. comm.): 2.5 - 3 g tissue was ground in liquid nitrogen and 10 volumes

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T a b l e 1

Grapevine varieties and rootstocks

Varieties	Rootstocks
Tahannebi	1616C (*Solonis* x *Riparia*)
Ağ Besni	1613C (*Solonis* x Othello)
Dımışkı	99R (Berlandieri x *Rupestris*)
Hönüsü	110R (*Berlandieri* x *Rupestris*)
Kabarcık	41B (Chasselas x *Berlandieri*)
Rumi	SO4 (*Berlandieri* x *Riparia*)
Ata sarısı	5C (*Berlandieri* x *Riparia*)
Razakı	
Italia	
Alphonse Lavallée	

T a b l e 2

NaCl dosage and time intervals of application to cvs Razaki and 1616 C

Treatment	Dosage NaCl, $mg \cdot l^{-1}$	Time intervals (d)
1	150	1
2	300	1
3	450	1
4	800	1
5	150	3.5
6	300	3.5
7	450	3.5
8	800	3.5
9	150	8
10	300	8
11	450	8
12	800	8
Control	-	-

of extraction buffer [3 M $NaClO_4$, 0.2 M Tris-HCl, pH 8.3, 5 % SDS (w/v), 8.5 % PVPP (w/v), 2 % PEG 6000 (w/v), just before using 1 % β-mercapto-ethanol (v/v)] was added and the solution was stirred for 30 min. After filtering by miracloth, 2.5 volumes of 100 % ethanol was added to the supernatant and they were kept at -20 °C for at least two h. The samples were centrifuged at 8,300 rpm for 15 min and pellets were washed with 70 % ethanol and re-suspended in 2 ml TE (10 mM Tris-HCl-pH 8.0, 1 mM EDTA). For cleaning, phenol: chloroform: isoamylalcohol (25:24:1) extraction was repeated 6 times. Nucleic acid precipitation was performed by adding 2.5 volumes 100 % ethanol, 0.1 volume 3M NaOAc and storing samples at -20 °C for at least two h. After centrifugation at 14,000 rpm for 20 min (4 °C) pellets were washed with 70 % ethanol, air-dried pellets were re-suspended in 100 µl DEPC treated-H_2O. To remove DNA from RNA 0.3 volumes 8M LiCl was added and the samples were stored at 4 °C for 16 h. In a last step samples were centrifuged at 14,000 rpm for 25 min, washed with 70 % ethanol and re-suspended in 50 µl DEPC treated-H_2O.

Total RNAs (20 µg per lane) were loaded to 1-2 % formaldehyde containing agarose gels. The equal loading of RNA to the wells was determined by visualizing and photographing the denaturing gel on UV light before transferring to nylon membranes (Fig. 4 b.). PCR amplified fragment of osmotin with the primers

VvO1 5'-AACTCAACAATGGGCCTCTG-3' and
VvO2 5'-TGCAACCACCGGTAGTCTTT-3'

were used in hybridization of the filters. The primers were designed by the program from http://www-genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi. The probe was labeled with the ($\alpha^{32}P$) dCTP using standard Mega-prime DNA labeling kit (Amersham). Hybridization and washing of the filters were performed as reported by KAZAN *et al*. (2002). Filters were exposed to X-omat (Kodak) films at -70 ºC for 4 d. The expression levels on the membranes were estimated by visual observation.

Osmotin gene expression was analyzed at the mRNA accumulation level by Northern Blot Analysis. Total RNA isolation was performed from leaf material collected from potted plants and the amount of RNA was determined by spectrophotometer (260/280 nm).

Results and Discussion

PCR analysis with the primers obtained from *Nicotiana tabacum* L. osmotin gene sequence, yielded a 500 bp sequence in grapevine which was used as probe in the study (Fig. 1). The successful amplification of the DNA sequence with the primers from *Nicotiana tabacum* osmotin gene, might indicate the existence of the same sequences in grapevine.

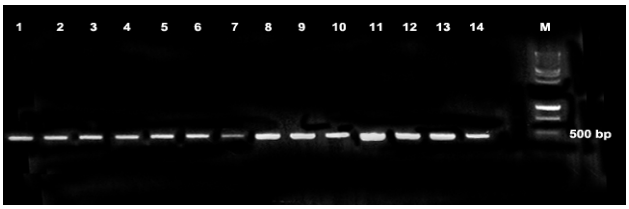

Fig. 1: The 500 bp DNA probe generated by the amplification of DNA from grapevine varieties and rootstocks. Lane 1; Ağ Besni, 2. Rumi, 3. Kabarcık, 4. Dımışkı, 5. Hönüsü, 6. Tahannebi, 7. Alphonse Lavallée, 8. Ata sarısı, 9. Razakı, 10. Italia, 11. 99R, 12.1616C, 13.1613C, 14.110R, M: molecular weight marker.

According to the preliminary NaCl treatment assays, 800 $mg \cdot l^{-1}$ was accepted as maximum amounts for further experiments. In order to estimate the maximum expression of the osmotin gene, different amounts of salt were applied in various time intervals to cv. Razakı chosen as a model. After application of 150, 300, 450, 800 $mg \cdot l^{-1}$ NaCl (Fig. 2, lanes 1,2,3,4) for one day and 150 $mg \cdot l^{-1}$ for 3.5 d (lane 5) transcript accumulation levels remained close to the accumulation observed in the control. On the other hand after the applica-

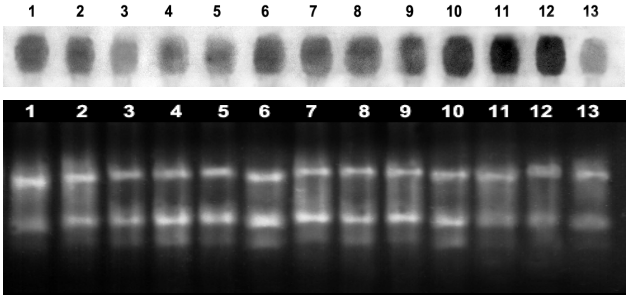

Fig. 2: Determination of stress inducing NaCl dosages and time intervals for grapevine varieties. 1 d NaCl treatment: 1. lane: 150 mg·l^{-1}; 2. lane: 300 mg·l^{-1}. 3. lane: 450 mg·l^{-1}; 4. lane: 800 mg·l^{-1}. 3.5 d NaCl treatment: 5. lane: 150 mg·l^{-1}; 6. lane: 300 mg·l^{-1}; 7. lane: 450 mg·l^{-1} ; 8. lane: 800 mg·l^{-1}. 8 d NaCl treatment: 9. lane: 150 mg·l^{-1}; 10. lane: 300 mg·l^{-1}; 11. lane: 450 mg·l^{-1}; 12. lane: 800 mg·l^{-1}; 13. lane: control.

tion of 300, 450, 800 mg·l^{-1} of NaCl for 3.5 d (lanes 6, 7, 8) an increase in the level of mRNA accumulation was observed. After 8 d the accumulation had reached a maximum. In cv. Razakı, salt treatment raised gradually the steady-state mRNA level depending both on the dosage and duration of the treatment.

Parallel experiments conducted with rootstocks displayed quite different results (Fig. 3). When expression of the control sample was taken into consideration (lane 13), salt treatment for a 1-d-period (lanes 1,2,3,4) induced an increase in the mRNA level. The increase, starting at the 800 mg·l^{-1} treatment for one day (lane 4), reached a maximum after application of 150, 300, 450 mg·l^{-1} NaCl for 3.5 d (lanes 5, 6 and 7). Beginning at 800 mg·l^{-1} NaCl for 3.5 d a decrease of the mRNA level was observed (lanes 8, 9, 10, 11, 12). After 8 d the level of mRNA was lowest in all treatments. Whether the decrease observed in the transcript level is associated with a refractory period in the gene may be revealed by further studies.

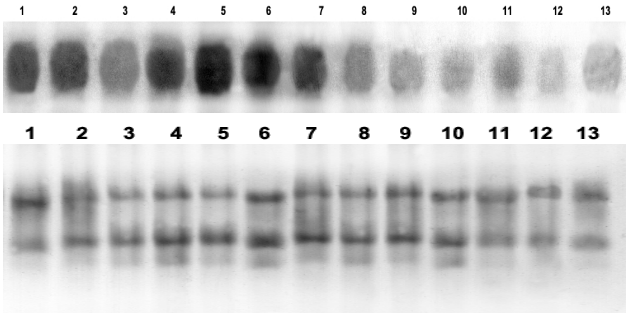

Fig. 3: Determination of the stress inducing NaCl dosages and time intervals for rootstocks. 1 d NaCl treatment: 1. lane: 100 mg·l^{-1}; 2. lane: 300 mg·l^{-1}; 3. lane: 450 mg·l^{-1}; 4. lane: 800 mg·l^{-1}. 3.5 d NaCl treatment: 5. lane: 100 mg·l^{-1}; 6. lane: 300 mg·l^{-1}; 7. lane: 450 mg·l^{-1}; 8. lane: 800 mg·l^{-1}. 8 d NaCl treatment: 9. lane: 100 mg·l^{-1}; 10. lane: 300 mg·l^{-1}; 11. lane: 450 mg·l^{-1}; 12. lane: 800 mg·l^{-1}; 13. lane: control.

In order to identify the alteration in mRNA accumulation levels in different grapevine varieties and rootstocks, the values obtained from preliminary experiments were used. To analyze the expression differences, grapevine cultivars were treated with 450 mg·l^{-1} NaCl for 8 d. On the other hand rootstocks were treated with 300 mg·l^{-1} NaCl for 3.5 d.

Comparing grapevine cultivars, the expression levels for cvs Ağ Besni, Rumi, Kabarcık, Dımışkı were almost identical (Fig. 4, lanes 1, 2, 3, 4 respectively) but were higher than the expression level observed under basal conditions. Cv. Hönüsü showed a higher transcript accumulation (lane 5) compared with the above mentioned 4 cultivars. On the other hand, the highest gene expression level was observed in cv. Tahannebi (lane 6). Although cv. Ata sarısı displayed a higher signal than cv. Alphonse Lavallée, both signals remained lower than the signal obtained for cv. Hönüsü.

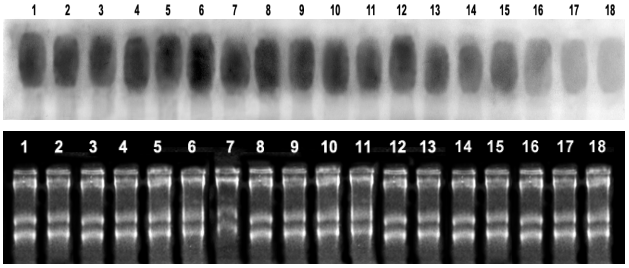

Fig. 4: (**a**) Expression levels of grapevine varieties and rootstocks. Lanes 1-10 show results of grapevine varieties (300 mgl^{-1}, 8 d). Lanes 11-17 show results obtained from rootstocks. 1. lane: Ağ Besni, 2. lane: Rumi, 3. lane: Kabarcık, 4. lane: Dımışkı, 5. lane: Hönüsü, 6. lane: Tahannebi, 7. lane: Alphonse Lavallée, 8. lane: Ata sarısı, 9. Razakı, 10. lane: Italia, 11. lane: 99-R, 12. lane: 16-16C, 13. lane: 16-13C, 14. lane: 110R, 15. lane: 41 B, 16. lane: SO 4, 17. lane: 5 C, 18. lane: Control. (**b**) The respective total RNAs on formaldehyde containing denaturing gels**.**

The expression levels obtained for cvs. Razakı and Italia (lanes 9 and 10) were lower than those for cv. Hönüsü but similar to cvs. Ağ Besni, Rumi, Kabarcık, Dımışkı (lanes 1, 2, 3, 4). In conclusion, if cultivars are put into sequence according to their expression levels, cv. Tahannebi obtained the highest level. This is followed by cv. Hönüsü, a GAP region variety which is followed by recommended cultivars of the GAP region, Ata sarısı and Alphonse Lavallée. They are followed by GAP region cvs. Ağ Besni, Rumi, Kabarcık, Dımışkı and the recommended cvs. Razakı and Italia. The latter two show expression levels with no significant differences.

Results obtained from rootstocks are also displayed in Fig. 4 (lanes 11,12, 13, 14, 15, 16 and 17). Transcript accumulation levels decreased from 99R, 1616C, 1613C, 110R, 41B, SO4 to 5C (Fig. 4, lanes 11, 12, 13, 14, 15, 16 and 17). 1616C (lane 12) displayed the highest mRNA accumulation level and followed by 99 R (lane 11) and 1613 C (lane 13). 110R (lane 14) showed a lower level of transcript accumulation. The signal displayed by 41B (lane 15) was very close to the signal observed under basal conditions. On the other hand the expression levels of SO4 and 5C (lanes 16 and 17) were quite low compared to the others.

According to previous data on salt resistance of rootstocks and cultivars, reported by Howell (1987) an approximated level of tolerance was 3 g·l^{-1} NaCl for *V. vinifera* cultivars and 1.5 g·l^{-1} for American rootstocks, *i.e.* lower values were reported for rootstocks. Our data indicate that

the approximate level of tolerance was at 0.8 $g \cdot l^{-1}$ NaCl for 1616C and 0.4 $g \cdot l^{-1}$ NaCl for 41B which was also reported as very sensitive.

According to our data higher expression levels were recorded for grapevine cultivars than rootstocks (Fig. 4), which confirms previous results.

Among rootstocks the maximum osmotin expression level was observed for cv. 1616C which was previously found to belong to the most resistant rootstocks (Howell 1987). Cv. 41B had an expression level close to the control, which was also reported by Howell (1987).

To our knowledge there are no published data on the salt tolerance of 99R, 1613C, 110R , 41B, SO4 and 5C. According to the current data, it can be suggested that the maximum resistance for 99R and 1613C (Fig. 4, lanes 11 and 13) was close to 0.8 $g \cdot l^{-1}$ as indicated for 1616C. On the other hand, from expression levels of 110R being close to 41B (lanes 14 and 15), while levels of SO4 and 5C (lanes 16 and 17) being lower than 41B (lane 15), it can be suggested that the resistance of 110R may be close to 41B and the resistance of SO4 and 5C may be lower than 41B.

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