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'Superior Seedless' grapevine grafted on three rootstocks grown on calcareous soil under diluted brackish water irrigation. II. Expression of antioxidant genes

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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Abstract: Grapevine rootstocks that can absorb brackish water and maintain satisfactory growth of the grapevine scion might be a feasible management practice in areas suffering scarce water resources. The objective of this study was to evaluate the expression of antioxidant genes in 'Superior Seedless' leaves grafted on R110 (*Vitis berlandieri* x *V. rupestris*), 41B (*V. berlandieri* x *V. vinifera*) and P1103 (*V. berlandieri* x *V. rupestris*) in response to diluted brackish water irrigation at three levels: 1.5, 3.0 and 5.0 dS m^{-1} in addition to the 0.8 dS m^{-1} control. Results revealed that after salinity exposure for two weeks, the transcript levels of *APX*, *Mn-SOD* and *MDAR* increased in 'Superior Seedless' leaves grafted on the different rootstocks. However, their expression levels in response to salinity were noticeably higher in plants grafted on P1103 and R110 compared to 41B. The expression of *CAT* gene showed obvious enhanced level in plants grafted on P1103 in response to salt exposure. Meanwhile, the expression of *CAT* gene in 'Superior Seedless' scion grafted on 41B or R110 showed almost unchanged level in control and stressed conditions. Down-regulation of *CuZn-SOD* was recorded in leaves of 'Superior Seedless' grafted on P1103. Slight up-regulation of this gene in response to saline condition was recorded when scion was grafted on 41B or R110. The expression of *GPX* was enhanced in scion grafted on P1103 and 41B. On the other hand, scion grafted on R110 showed decreased expression of *GPX* in response to salt treatment. Grapevine rootstocks that have *V. rupestris and V. berlandieri* in their parentage are good candidates for salinity tolerance.

1. Introduction

Grapes (*Vitis* sp.) are considered as one of the world's major commercially grown fruit crops. In Jordan, grapes are ranked in second place after olives regarding the total area planted. The total area planted with grapes is 3806 ha (FAO, 2014). More than half of that area is under irrigation and Jordan is now ranked as the world's second water-poorest country.

Irrigation with low quality water during the whole growing season of the crops, even the tolerant ones, does not always produce high yield. Mixing low quality water; such as dam brackish water, with conventional quality irrigation water in ratios to keep the salinity of the irrigation water below the threshold of the target crop might be an acceptable management practice and was used by many researchers (Abdel Gawad and Ghaibeh, 2001). Alternating conventional quality water with brackish water is another management practice. Its application would be easier because it does not need containers for mixing two different sources of irrigation water. The conventional quality irrigation water can be used during the sensitive stages of plant growth and the brackish water during the non-sensitive or less sensitive stages.

Considerable yields were obtained using saline irrigation water (4-12 dS m^{-1}) in crops that had been previously defined as moderately sensitive to salt stress (Bustan *et al*., 2004). Furthermore, in some crops (e.g., tomato) the reduction in the fresh yield was compensated by an increase in fruit dry weight and other quality parameters (Mizrahi *et al.*, 1988). Bustan *et al.* (2005) reported that the combination of fresh (1.2 dS m^{-1}) and brackish (7 dS m^{-1}) irrigation water increased the yield level of melon to that of fresh water plants whereas it brought about the improvement of fruit quality typical to brackish water plants, thus providing an attractive approach to optimize late-summer melon production.

Plants subjected to saline environment use different mechanisms to overcome such abiotic stress. To avoid a disorder of ion homeostasis under saline conditions, plant cells have to maintain a low Na^+ concentration and keep a high H^+ concentration in the cytosol where enzymes for metabolism are located (Zörb *et al.*, 2005).

Usually, plants use different ways to maintain a low cytosolic sodium concentration, including restricting Na^+ influx, maintaining active Na^+ efflux, and compartmentalizing Na^+ into the vacuole, etc. (Rubio *et al.*, 1995). These mechanisms involve a number of Na^+/H^+ antiporter proteins that are localized in plant plasma and vacuolar membranes (Vasekina *et al.*, 2005). They catalyze the exchange of Na^+ for H^+ across membranes and the energy needed is generated by the H^+-adenosine triphosphatase and H^+-pyrophosphatase (Niu *et al.*, 1995; Shi and Zhu, 2002).

Plants exposed to salt stress showed enhanced formation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and singlet oxygen (Mittler, 2002). The harmful effects of these molecules are referred to as oxidative stress (Halliwell, 2006). Plants have evolved an enzymatic antioxidant system to reduce the ROS levels. This enzymatic system includes superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidase (Apel and Hirt, 2004). The development of salt-tolerant crops is a practical solution to sustain agricultural productivity. Because of the complexity of the trait, traditional crop-breeding programs aimed at improving tolerance to salinity have limited success. Therefore, understanding the cellular and molecular bases of salinity-tolerance mechanisms is essential for marker-assisted selection and genetic engineering of salt tolerance in economic crops. Understanding the responses of plants to the major environmental stressor salinity is an important topic for the biotechnological application of functional mechanisms of stress adaptation. Plant engineering strategies for cellular and metabolic reprogramming to increase the efficiency of plant adaptive processes may either focus on (1) conferring stress tolerance by directly reprogramming ion transport processes and primary metabolism or (2) by modulating signaling and regulatory pathways of the adaptive mechanisms. The second approach seems to be more perspective because it is likely that signaling and regulatory factors orchestrate as key signaling components the transcriptional and translational control of group (1) adaptive mechanisms (Diédhiou *et al*., 2008; Popova *et al.*, 2008).

Most knowledge on molecular mechanisms involved in plant salt responses and adaptation has been derived from analyses of the glycophytic models *Arabidopsis thaliana* and rice. Such knowledge is lacking in *Vitis* species, therefore detecting expression changes of antioxidant defense genes is the objective of this study. Since the understanding of a plants response to a stress requires an evaluation of stress induced changes in gene expression, the expression of major defense genes (superoxide dis-

mutase, ascorbate peroxidase, catalase, glutathione peroxidase, monodehydroascorbate reductase) in 'Superior Seedless' grafted on different rootstocks has been examined by RT-PCR.

2. Materials and Methods

Plant material

Three grape rootstocks were evaluated in this study: R110 (*Vitis berlandieri* x *V. rupestris*), 41B (*V. berlandieri* x *V. vinifera*) and P1103 (*V. berlandieri* x *V. rupestris*). The rootstocks were purchased from Les Pépiniéristes du Comtat, Sarrians, France.

After being imported, 'Superior Seedless' bud cultivar was grafted on the rootstocks in a local nursery; Al-Bushra Nurseries, May, 2013. Grafted plant materials were planted in polyethylene bags filled with peatmoss. The one year old grafted grapevine rootstocks were grown for several months to allow for the formation of a well developed root system before applying treatments. Fertilizers and fungicides were applied as necessary.

Soil and water

The soil was brought from the southern Jordan Valley. Soil was crushed and sieved through 1 cm sieve and plastic pots (the working volume of the pots was 44 L) were filled with 50 kg each in order to roughly have a bulk density of 1.14 g cm^{-3}. The bulk density is within the typical range of bulk densities of agricultural soils. If bulk density was higher, infiltration would be very slower and hydraulic conductivity would be slower as well. Low hydraulic conductivity would exacerbate the osmotic effect and create anoxic conditions. The pots were placed in a controlled greenhouse. Grafted grapevines were transplanted in February and the growth was unified based on the number of buds and root length. The root system was cut back to 15 cm in length and the vegetative system was cut back to eight buds.

The water was brought from Al-Karameh dam located in the Jordan Valley and stored in a galvanized tank. Three levels of irrigation water salinity; in terms of electrical conductivity (EC), were applied: 1.5, 3.0 and 5.0 dS m^{-1} in addition to the 0.8 dS m^{-1} control. These three levels of diluted brackish water were used to find out the salt level that would result in a tolerable "adverse" effect to design alternate irrigation that would contribute to water saving. These concentrations were selected based upon the threshold EC of grapes (i.e. EC<2 dS m^{-1}). The treatments were prepared by mixing the dam water with tap water. A portable conductivity meter (Model Cond 3210, WTW, Germany) was used to measure the EC and to obtain the determined salinity levels. The twelve treatments were arranged in a randomized complete block design with three replicates. The grafted grapevines started to break the dormancy period during spring. Composite fertilizer (20:20:20), urea and ammonium sulphate were also applied to the grapevines and growth was again unified before applying the assigned treatments. Irrigation with the assigned treatments started in May. All pots received the same amount of water whenever irrigation was applied. Each pot received a total amount of irrigation water equal to 446 mm. Irrigation was scheduled according to evaporation readings from free water surface (in mm) taken every 48 hours and corrected using proper grapevine crop coefficient of 0.30 (according to Food and Agriculture Organization).

DNA and RNA extraction

Leaves were sampled starting from February until November, 2014 (every two weeks) and frozen in liquid nitrogen for analyzing DNA and RNA using kits (iNtRON, Korea). Total RNA was extracted from frozen leaf samples with the IQeasy™ Plus Plant RNA Extraction Mini Kit (iNtRON Biotechnology, Korea) according to user's manual. RNA concentration and purity were estimated based on absorbance at 260 and 280 nm. Two microgram of RNA was used to reverse transcribe the first strand cDNA using Power cDNA Synthesis System (iNtRON Biotechnology, Korea) according to the manufacturer's protocols with oligo $(dT)_{15}$ as a primer in a reaction volume of 20 µl.

The first strand cDNAs generated for all the samples were used for semi-quantitative RT-PCR to monitor the transcript levels. The gene-specific primers for RT-PCR were designed based on the basis of the sequences published in GenBank using the software Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The *EF-1α* gene was selected as a reference gene. The primer sequences are listed in Table (1). The PCR reaction was performed using iNtRON *i*-MAXTM II system (iNtRON, Korea). The same thermal profile was used for all PCR reactions; PCR was initiated with enzyme activation at 95°C for 2 min followed by 32 cycles of 40s at 95°C, 40s at 56 °C and 1 min at 72°C. Different amplification cycles were tried and the product of the 32 cycles was selected to be presented. All RT-PCR products were loaded in ethidium bromide-stained 1.5 % (w/v) agarose gel.

Table 1 - Primer pairs used in gene expression analysis

Gene	GeneBank ID	Primer pairs (5'→3')	Amplicon size (bp)
Ascorbate peroxidase (APX)	EU280159	F: GACAATGAAGCACCCAGAGGAG R: AATGGGCTTCAGCATAGTCAGC	542
CuZn-Superoxide Dismutase (CuZn-SOD)	AF056622	F: CTGCTCCATCTCGTGTCTTTCT R: ATCCACAATTGTTGCTTCAGCC	452
Mn-Superoxide Dismutase (Mn-SOD)	NP_001268135	F: AGAAAATCGCTAGGGTTAGGGC R: TACCCAGCAATGGAACCAAGTT	538
Catalase (CAT)	AF236127	F: AGGCCCAGTTCTTCTTGAGGAT R: AGGCAAGCATCTCATTCTCAGC	860
Glutathione Peroxidase (GPX)	XM_002272900	F: ATGTCGAAGCAAATACAGCAGG R: TGAGAGGGGAAGTTGTTGGGTA	472
Monodehydroascorbate reductase (MDAR)	NP_001267971	F: TCATGTTTGTGTTGGAAGCGGA R: GGACAGATCAAAGGCACGAGAG	868
Elongation factor 1α (EF-1α)	XP_002277159	F: ATTGTGGTCATTGGCCATGTTG R: CCTTCGAAACCAGAGATGGGAA	566

3. Results and Discussion

As an abiotic stress, salinity induces oxidative damage in plant cells through the increased generation of Reactive Oxygen Species (ROS) in different cell compartments (Mittler, 2002). The activation of antioxidant defense system reduces the level of ROS and minimizes the impact of oxidative stress and its associated damage. Plant cells possess antioxidant enzymes which have the ability to detoxify toxic ROS (Apel and Hirt, 2004).

Rootstock choice should be taken with careful consideration since the scion is dependent on the rootstock (Creasy and Creasy, 2009). Novel rootstocks are frequently used to confer resistance to environmental adversities in horticultural crops (Albacete *et al.*, 2015). A promising approach to improve salt tolerance of horticultural species is the use of grafting on salt-tolerant rootstocks (Colla *et al.*, 2010; Giuffrida *et al.*, 2014; Simpson *et al.*, 2015; Zrig *et al.*, 2016). Grapevine rootstock selection is a key factor and could be considered an important strategy to mitigate salinity stress. Grape rootstocks do influence the scion cultivar in many growth and physiological aspects (Gu, 2003). However, some contradictions can be found in the literature in terms of the salt tolerance of grapevine rootstocks implying that various factors are involved, which eventually determine grapevine response to salt stress. For example, Southey and Jooste (1991) found that American hybrids performed poorly in response to salinity when used as rootstocks for the cultivar 'Colombard'. In addition, Cavagnaro *et al.* (2006) concluded that Argentinean cultivars performed better than European cultivars in an *in vitro* salinity evaluation study. Regarding differences in ranking rootstocks, Dardeniz *et al.* (2006) indicated that 41B was the most salt resistant rootstock, followed by 140Ru and P1103, and the least resistant was 5 BB. On the other hand, Walker *et al.* (2002) showed that the highest salt resistance was obtained when P1003 was used as a rootstock.

To determine whether NaCl-induced stress and rootstock type were able to regulate the expression levels of genes responsible for antioxidant defense response, a semi-quantitative RT-PCR assay was performed for the analysis of six major antioxidant enzyme genes (*APX*, *CAT*, *CuZn-SOD*, *Mn-SOD*, *GPX*, and *MDAR*) (Fig. 1).

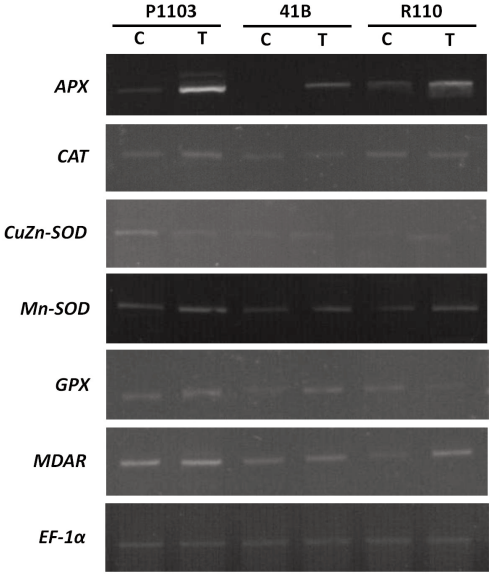

Fig. 1 - Semi-quantitative RT-PCR expression analysis of major antioxidant enzyme genes in 'Superior Seedless' grafted on different rootstocks and exposed to salinity for two weeks. The *EF-1α* gene was used as the internal control for normalization of loading.

Plants utilize antioxidant enzymes, such as: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDAR), as defense mechanisms against salinity and do have the ability to detoxify toxic ROS (Apel and Hirt, 2004).

Their expression has been studied in many crops as previously mentioned in the introduction part. However, such knowledge is lacking in *Vitis* species, specifically in *Vitis berlandieri* and *rupestris*. Meanwhile, their expression is extensively studied in both *vinifera* rootstocks and cultivars (Carvalho *et al.*, 2015).

After salinity exposure for two weeks, the transcript levels of *APX*, *Mn-SOD* and *MDAR* were increased in leaves of 'Superior Seedless' grafted on the different rootstocks. However, their expression levels in response to salinity were noticeably higher in plants grafted on P1103 and R110 compared to 41B. The expression of *CAT* gene showed obvious enhanced level in plants grafted on P1103 in response to salt exposure. However, the expression of *CAT* gene in 'Superior Seedless' scion grafted on 41B or R110 showed almost unchanged level in control and stressed conditions.

Down-regulation of *CuZn-SOD* was recorded in leaves of 'Superior Seedless' grafted on P1103, while, slight up-regulation of this gene in response to saline condition was recorded when scion was grafted on 41B or R110. In response to salinity stress, the expression of *GPX* was enhanced in scion grafted on P1103 and 41B. On the other hand, scion grafted on R110 showed decreased expression of *GPX* in response to salt treatment.

The expression of these genes was evaluated every two weeks, and only the results after the first two weeks of the stress treatment were presented since no differences in expression were noticed at the other time points between different treatments. This indicates early differential responses to salt stress at the level of antioxidant defense genes. Such responses were previously reported in other plant species (Ellouzi *et al.*, 2014; Ranjit *et al.*, 2016).

4. Conclusions

Rootstock choice is critical in determining grapevine performance and productivity under saline conditions. Expression levels of *APX*, *Mn-SOD* and *MDAR* were noticeably higher in plants grafted on P1103 and R110 compared to 41B. The expression of *CAT* and *GPX* genes showed obvious enhanced level in plants grafted only on P1103 in response to salt exposure. *CuZn-SOD* was down-regulated in leaves of 'Superior Seedless' grafted on P1103 and up-regulated when scion was grafted on 41B or R110. Rootstocks with enhanced expression levels of antioxidant defense genes, such as superoxide dismutase (SOD) and catalase (CAT) can possibly eliminate or reduce detrimental effects of ROS. Thus, grapevine rootstocks that possess this defense line are more preferable to be utilized for soils subjected to salinity or grapevines irrigated with diluted brackish water. Moreover, additional studies are needed to investigate the salt-stress responses of enzymatic and non-enzymatic antioxidant components in grapevine grafted on contrasting rootstocks.

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