Expression analysis of three *h*-type thioredoxin isoforms in three Iranian grape (*Vitis vinifera* L.) cultivars, indicating differential expression in different tissues

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Abstract

Thioredoxins (Trxs) are small ubiquitous disulfide reductases that participate in dithiol-disulfide exchange reactions. In contrast to animals and prokaryotes that typically possess one or a few genes encoding Trxs, higher plants contain eight different Trx types: *f, m, x, y, z, o, s,* and *h*. Trx *h* with multiple forms is involved in different processes such as seed germination, cellular protection against oxidative stress, self-incompatibility etc. The expression analysis of the three *h*-type Trx genes, called VvTrx *h*1, VvTrx *h*2 and Vv*CxxS*2 was studied in different tissues of three grape cultivars (Askari, Red Seedless and White Seedless) at various develpmental stages by semiquantitative reverse transcription polymerase chain reaction (RT-PCR). The grape Trx *h* genes were expressed in berry, leaf, petiole, cluster, stem, root, and seed tissues at different developmental stages. The higher expression of the isoforms was observed in berry tissues as compared to those of the roots. In berry tissues, the expression of VvTrx *h*1, VvTrx *h*2 and Vv*CxxS*2 isoforms were analyzed at six growth stages, including 14, 28, 42, 56, 82, and 110 days post anthesis (dpa). The highest level of expression was observed at veraison stage (56 dpa). The expression of grape Trx *h* genes was also analyzed in leaf, petiole and cluster tissues at three developmental stages including young, mid and old stages with a imprecise pattern of expression in comparison to the berry tissues. In contrast to VvTrx *h*1 and Vv*CxxS*2 isoforms, VvTrx *h*2 showed the highest level of expression in different tissues, suggesting a major role for this isoform in grape.

Keywords: Thioredoxin *h*, Grape, Expression analysis, Semiquantitative RT-PCR.

Introduction

Thioredoxins (Trxs) are small ubiquitous disulfide reductases that are present in all organisms from prokaryotes to higher eukaryotes, and play a regulatory role in a variety of cellular processes (Gelhaye *et al*., 2004). Animals and prokaryotes typically possess one or a few genes encoding Trxs, whereas genome sequencing projects have revealed a wide range of genes encoding Trx in plants, specially in *Arabidopsis* with at least 48 Trx and Trx-like genes (Gelhaye *et al*., 2005; Meyer *et al*., 2008; Oliveira *et al*., 2010). Higher plants contain two Trx systems; one is ferredoxin-dependent whereas the other one is NADPH-dependent. The ferredoxin-dependent system, located in the chloroplast, is composed of nuclear-encoded Trxs, *f, m, x, y,* and *z* (Lemaire *et al*., 2007; Arsova *et al*., 2010; Hall *et al*., 2010). These Trxs are reduced by ferredoxin via ferredoxin-dependent thioredoxin reductase (FTR) (Buchanan *et al*., 2002;

Jacquot *et al*., 2002). The NADPH-dependent system is composed of Trxs *o*, *h*, and *s* which are reduced by NADPH via a flavin enzyme NADPH-dependent thioredoxin reductase (NTR) (Laloi *et al*., 2001; Rouhier *et al*., 2002; Alkhalfioui *et al*., 2008). The Trx *h* complex family has been most intensively studied in different plants and they have been showed to be involved in multiple processes such as seed germination (Maeda *et al*., 2003), cellular protection against oxidative stress (Serrato and Cejudo, 2003), self-incompatibility (Haffani *et al*., 2004), inactivation of toxic proteins (Joudrier *et al*., 2005) etc. They are divided in three different subgroups I, II and III. Members of subgroup I and II are reduced by NTR, while reduction of Trxs *h* subgroup III is dependent on the GSH (glutathione)/Grx (glutaredoxin) system (Gelhaye *et al*., 2003).

The expression analysis of Trx *h* genes has been investigated in different plants. In *Arabidopsis*, the expression pattern of the eight Trx *h* genes was studied in different tissues and high level of expression was detected for most of the AtTrx *h* genes in calli and during the growth phase of cell suspension (Reichheld *et al*., 2002). The expression of poplar Trx *h*1 and *h*2 genes, and Trx *h*3 and *h*4 genes of pea were clearly detected in all compartments of the plants (Gelhaye *et al*., 2002; Montrichard *et al*., 2003). In contrast, the expression of the two tobacco Trxs *h* appears to be limited to the growing cells of both mature and non-dividing cells (Brugidou *et al*., 1993). Also, northern hybridization studies revealed that the grass Trxs *h* (*Lolium perenne*; LpTrx *h*, *Hordeum bulbosum*; HbTrx *h*, *Phalaris coerulescens*; PcTrx *h*, *Secale cereale*; ScTrx *h*) are most highly expressed in the mature pollen and stigma and at a much lower level in leaves and roots (Juttner *et al*., 2000).

In the present research project, we studied the level of gene expression of the three Trx *h* genes, called VvTrx *h*1, VvTrx *h*2 and Vv*CxxS*2, on different grape (*Vitis vinifera* L.) tissues of three Iranian cultivars at different developmental stages. These cultivars were selected based on their commercial importance and morphological features. Our results demonstrated that Trx *h* genes have different expression patterns in different tissues.

Materials and Methods

Plant Materials

Berries, leaves, petioles, clusters, stems, and roots were collected from three Iranian grape (*Vitis vinifera* L.) cultivars; Askari, Red Seedless and White Seedless, from the Grape Research Station, Takistan-Qazvin, Iran, during the 2008 field season. Berries were collected at six ripening stages including 14, 28, 42, 56, 82, and 110 days post anthesis (dpa). Leaves, petioles and clusters (harvested at young, mid, and old stages), and green stems and young roots were used for RNA extraction. In Askari cv., seeds were also removed from the berries at veraison stage by gently breaking open the berries in liquid nitrogen. All samples were immediately frozen in liquid nitrogen at the time of collection and then stored at –80 °C until extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from various grape tissues at different developmental stages as described by Reid *et al*. (2006) with slight modifications. First strand cDNA was synthesized from 5 µg of total RNA treated with RNase-free DNase I (Fermentas), using RevertAid^{TM} M-MuLV reverse transcriptase (Fermentas) and Oligo $(dT)_{18}$ (Qiagen) as the initiation primer (Table 1). The composition of each reaction was as follows; 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 10 mM dithiothreitol, 50 ng Olig

Table 1. Nucleotide sequence of the oligonucleotide primers used for RT-PCR.

Gene Name	Primer Name	Sequence (5'-3')	Melting Temperature (ºC)	Size of Amplicon (bp)
VvTrx *h*1	VTrx1F	TCTAAAGAGGCATCGAGAAA	68.0	368
	VTrx1R	TTAGGCAGTAGCCATGTGCT	67.0	
VvTrx *h*2	VTrx2F	ATGGCGGAAGAGGGACAA	71.1	345
	VTrx2R	TCAAGCAGTTGCATGCTTCT	67.0	
Vv*CxxS*2	VTrx3F	ATGGAAAATCAGGAGCCG	62.6	381
	VTrx3R	CTAGGCTACATACACGCGAAA	61.7	
AtAct2	AtAct2F	GTTAGCAACTGGGATGATATGG	66.8	530
	AtAct2R	AGCACCAATCGTGATGACTTG	69.6	

$(dT)_{18}$ primer, 500 µM of each dNTP and 200 units of Revert Aid^{TM} M-MuLV RT. Reverse transcription was carried out at 42 ºC for 1 h and terminated by heating to 70 ºC for 10 min. cDNA was recovered by ethanol precipitation and its concentration was determined spectrophotometrically

Expression Analysis

The expression of the VvTrx *h*1 (NCBI GenBank accession number HM370524), VvTrx *h*2 (HM370525) and Vv*CxxS*2 (HM370528) genes were analyzed in different tissues at different developmental stages by semi-quantitative RT-PCR. The RT-PCR reactions were performed using 1/20 of the reverse transcription reaction in a final volume of 20 µl containing of 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1 % Triton X-100, 0.1 mg/ml BSA, 200 µM of each of dNTPs, 20 pM of each primer (forward and reverse) and one unit of Taq DNA polymerase (Fermentas). Analysis of the cDNA sequences of VvTrx *h*1 and VvTrx *h*2 genes revealed a high degree of homology at the nucleotide level in the exonic sequences. However, the 5'-UTR was used for forward primer designation for VvTrx *h*1 gene (Table 1). The primers specific to *Arabidopsis* actin gene (*AtAct2;* NCBI GenBank accession number AF485783) were used as a control (Table 1). PCR reactions were set up out in a thermal cycler programmed (Techne-U.K) under the following conditions: 3 min at 94 ºC followed by 30 cycles of 30 s at 94 ºC, 1 min at 58 ºC, and 30 s at 72 ºC, with a final extension for 5 min at 72 ºC. Actin gene was amplified in the same PCR conditions as grape Trx *h* genes with the same amplification cycles (30 cycles) and was used as an internal control to normalize each sample for variations in the amounts of RNA. To control for possible genomic DNA contamination, parallel reactions were carried out where reverse transcriptase activity was inactivated by incubation at 95 ºC. A negative control lacking template was included in each set of RT-PCR amplification. Reactions were performed in triplicates. Five microliters of the amplified products were separated by 1.5 % (w/v) agarose gel electrophoresis and quantified using ImageJ software (W.S. Rasband; 1997-2007; National Institutes of Health; http://rsb.info.nih.gov/ij).

Results and Discussion

To date, numerous ESTs coding for grape Trx *h* genes have been recorded among the entire grape ESTs present in the NCBI GenBank database. Around 472 ESTs were

identified for the whole Trx *h* group. The highest numbers of records belong to VvTrx *h*2 with 140 identified ESTs. Also, 103 ESTs were found for VvTrx *h*1 and Vv*CxxS*2 isoforms. The identified ESTs for grape Trx *h* genes are present in all grape tissues, although no EST was found for VvTrx *h*2 and Vv*CxxS*2 for stem and seed tissues. Based on the number of identified ESTs in different tissues, grape Trx *h* genes appear to be transcribed at high levels in berries and bud tissues, and at lower levels in cluster tissues. Overall, the number of identified ESTs for VvTrx *h*2 gene for different tissues is more than VvTrx *h*1 and Vv*CxxS*2 isoforms with the exception of cluster and seed tissues.

Expression of VvTrx *h*1, VvTrx *h*2 and Vv*CxxS*2 isoforms was analyzed in different tissues of grape Askari, Red Seedless and White Seedless cultivars at different developmental stages by semiquantitative reverse transcription (RT)-PCR. It was shown that grape Trx *h* genes were expressed in all tissues studied at different developmental stages, although they appear to be expressed in berry tissues more than other tissues (Fig. 1). In *Arabidopsis*, the expression of five Trx *h* genes was shown to be in a range of different tissues. The five Trx *h* messages were abundant in the aerial plant organs such as young and mature leaves, siliques, flower buds, and flowers and less abundant in stems (Rivera-Madrid *et al*., 1995). The expression of the eight Trx *h* genes has also been analyzed in different *Arabidopsis* organs. In the whole plant, with the exception of AtTrx *h*8, all Trx *h* mRNAs were detected in almost all organs, however their levels of expression differed from one another (Reichheld *et al*., 2002). Similarly, the PtTrx *h*1 and *h*2 genes from poplar has been found in all compartments of the plant, but they were clearly more abundant in leaves (Gelhaye *et al*., 2002). Also, the pea Trx *h*3 and *h*4 genes are clearly expressed in all tissues, while Trx *h*1 and *h*2 genes are only detected in green leaves at a very low level (Montrichard *et al*., 2003). In contrast, the transcripts of NtTrx *h*1 and *h*2 isoforms from tobacco were observed to be abundant in growing tissues but undetectable in mature non-dividing cells (Brugidou *et al*., 1993).

In berry tissues, the expression of VvTrx *h*1, VvTrx *h*2 and Vv*CxxS*2 isoforms was analyzed at 14, 28, 42, 56, 82, and 110 days post anthesis (dpa). The isoforms showed an increase in the amount of transcripts from 14 dpa to veraison stage (56 dpa) and then there was a decrease to the ripening stage (110 dpa) (Fig. 1A-C).

One representative gel is shown from three independent replicates. Relative band intensities were normalized to the *AtAct2* band intensity (100%). Each histogram represents the mean ± SD obtained from three independent RT-PCR reactions.

Therefore, it seems that the highest expression level of grape Trx *h* genes is related to veraison stage in berry tissues. In contrast to VvTrx *h*1 and Vv*CxxS*2 isoforms, VvTrx *h*2 showed the highest level of expression, whereas the lowest amount of transcripts was observed in Vv*CxxS*2 isoform. Similar to VvTrx *h*2, VvTrx *h*1 gene showed a considerable increase in amount, from 14 dpa to veraison stage (56 dpa), then remained approximately constant to 82 dpa, and then there was a relatively dramatic decrease to 110 dpa (Fig. 1A-C). Despite Red Seedless cv., the expression pattern of Vv*CxxS*2 was similar to Askari and White Seedless cultivars i.e. an imperceptible increase in amount of transcript from 14 dpa to 82 dpa and a gradual decrease to 110 dpa (Fig. 1A and C).

The expression of VvTrx *h*1, VvTrx *h*2 and Vv*CxxS*2 isoforms was also analyzed in leaf, petiole and cluster tissues at young, mid and old stages. Higher expression of these genes in leaf, petiole and cluster tissues was observed in mid, young and young stages, respectively (Fig. 2A-C). Unlike VvTrx *h*1 and Vv*CxxS*2 isoforms, VvTrx *h*2 showed the highest level of expression in leaf,

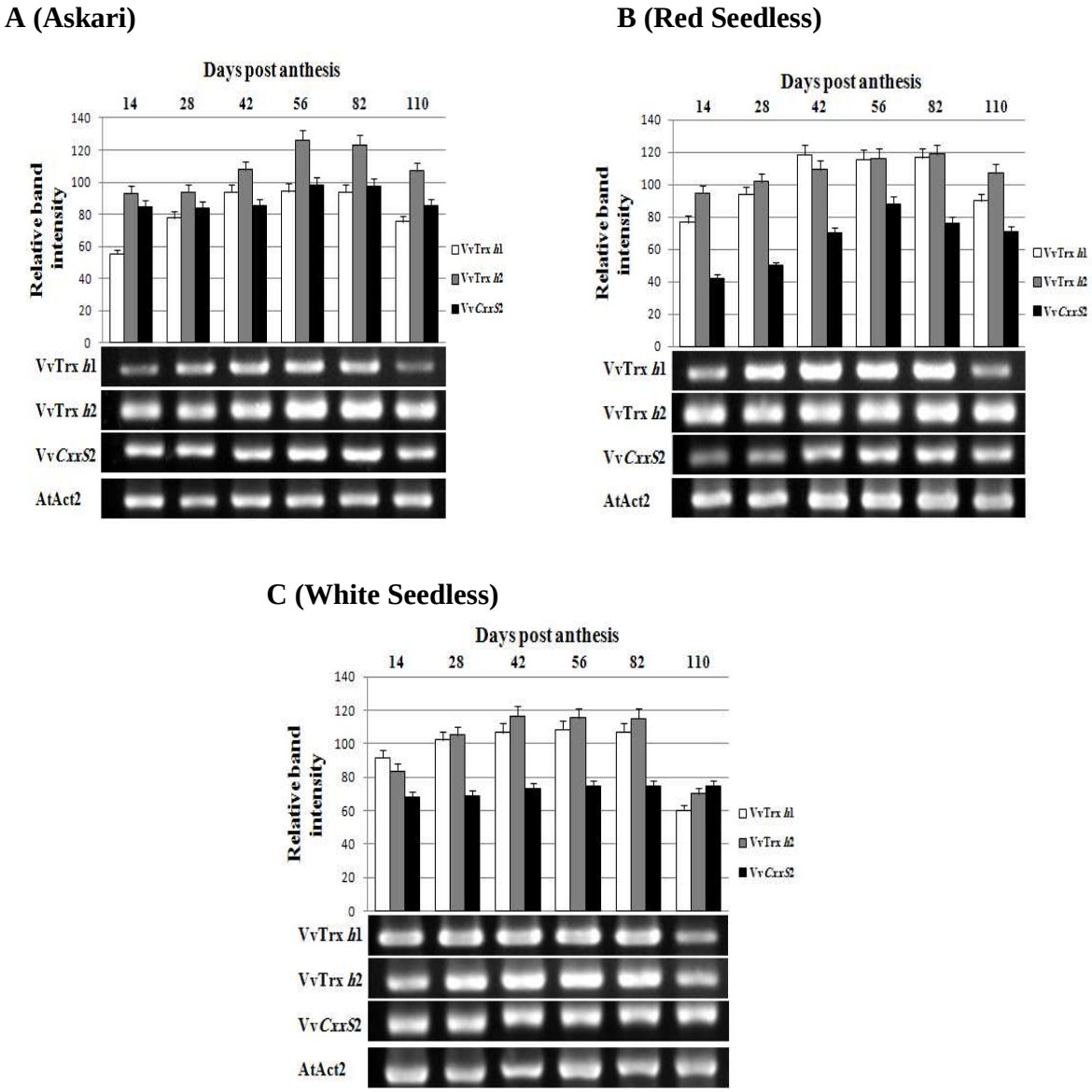

Fig. 1 Expression analysis of VvTrx *h*1, VvTrx *h*2 and Vv*CxxS*2 isoforms in berry tissue of A) Askari, B) Red Seedless and C) White Seedless cultivars at six times of sampling (14-110 dpa).

petiole and cluster tissues and the highest amount of transcripts was observed in seed tissues of Askari cv. (Fig. 2A) and petiole tissues of Red Seedless and White Seedless cultivars (Fig. 2A and C). VvTrx *h*1 and Vv*CxxS*2 isoforms also displayed the highest amount of transcripts in petiole tissues of the three cultivars (Fig. 2A-C). The expression of grape Trx *h* genes did not reveal a distinct pattern in leaf, petiole and cluster tissues of the three cultivars. The expression of VvTrx *h*1, VvTrx *h*2 and Vv*CxxS*2 isoforms was also confirmed in green stems, young roots and seeds of the cultivars. The lowest intensity of the transcripts was detected in the young roots of Trx *h* genes (Fig. 2A-C).

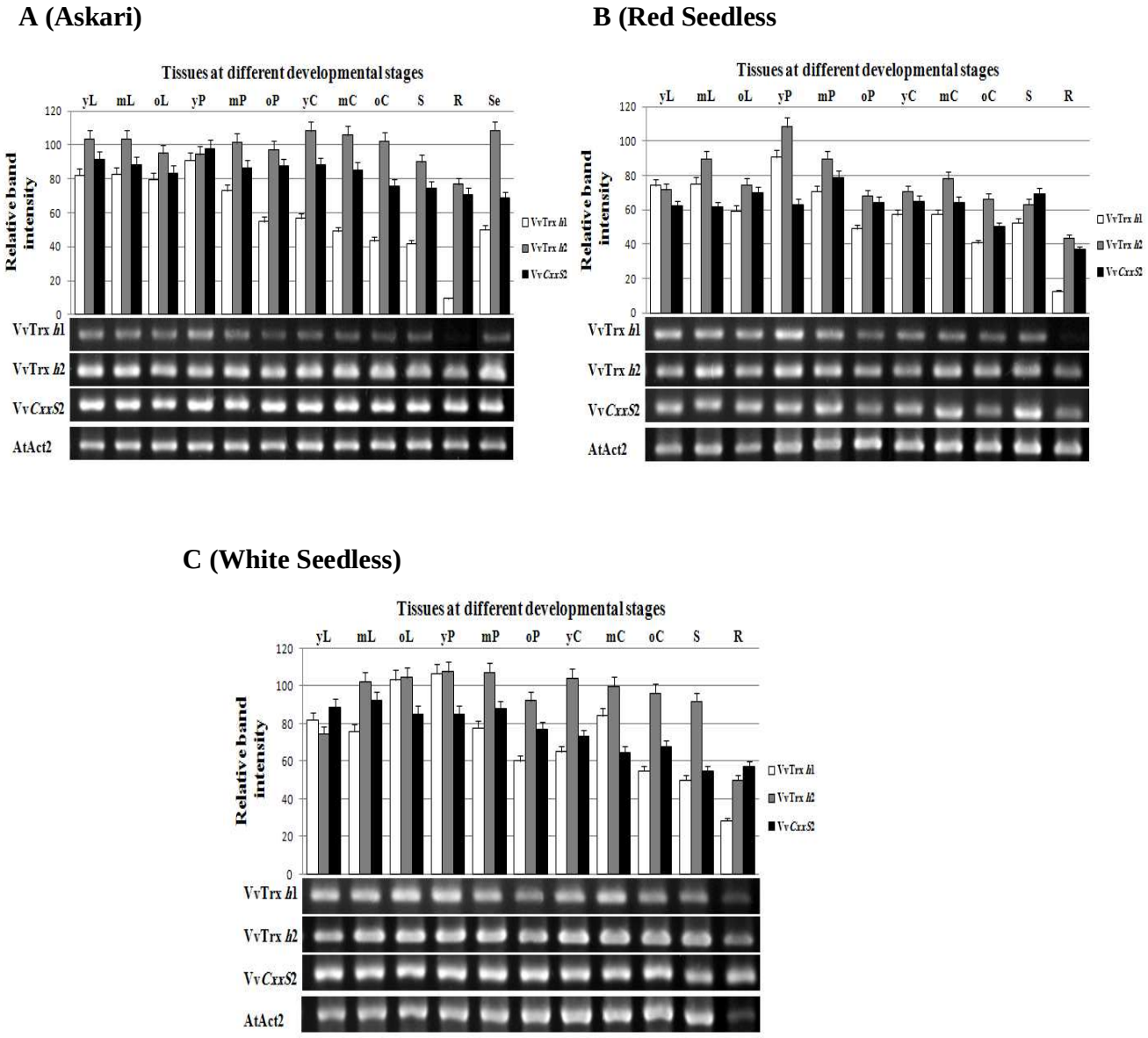

Fig. 2. Expression analysis of VvTrx *h*1, VvTrx *h*2 and Vv*CxxS*2 isoforms in leaf, petiole, cluster, stem, root, and seed tissues of A) Askari, B) Red Seedless and C) White Seedless cultivars (One representative gel is shown from three independent replicates. Relative band intensities were normalized to the *AtAct2* band intensity (100%). Each histogram represents the mean ± SD obtained from three independent RT-PCR reactions. yL, young leaf; mL, mid leaf; oL, old leaf; yP, young petiole; mP, mid petiole; oP, old petiole; yC, young cluster; mC, mid cluster; oC, old cluster, S, stem; R, root; Se, seed).

In stems, VvTrx *h*2 was expressed at the highest level in Askari and White Seedless cultivars (Fig. 2A and C).

Also, in contrast to White Seedless cv., the highest intensity of the transcripts was detected in VvTrx *h*2 in roots (Fig. 2A and B). The lowest intensity of the transcripts was also related to VvTrx *h*1 in stems, roots and seed tissues. (Fig. 2A-C). Similarly, the mRNAs corresponding to AtTrx *h*2, *h*4, and *h*5 from *Arabidopsis* were detected at very low levels in roots, whereas *h*1 and *h*3

mRNAs were undetectable (Rivera-Madrid *et al*., 1995). The grass Trx *h* that are highly expressed in the mature pollen and stigma, were also found at a much lower level in leaves and roots (Juttner *et al*., 2000). Moreover, the expression analysis of *Arabidopsis* AtTrx *h*9 gene showed a low level of expression in root and leaf tissues in amount (Meng *et al*., 2010).

Conclusion

In this study, the expression of the three Trx *h* genes, VvTrx *h*1, VvTrx *h*2 and Vv*CxxS*2, was analyzed in different tissues of grape Askari, Red Seedless and White Seedless cultivars at different developmental stages. The obtained results revealed that grape Trx *h* genes are expressed in all tissues at different developmental stages with a higher expression in berry tissues compared to those of roots. In contrast to VvTrx *h*1 and Vv*CxxS*2 isoforms, VvTrx *h*2 exhibited the highest intensity of the transcripts in different tissues, suggesting a probable important role in grape. The observed differences in the expression patterns of grape Trx *h* genes proposes that the corresponding proteins may have different functions in grape and may indicate that the number of Trx *h* genes present in grape to be even greater, which might be accounted as the future work.

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