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CANAN YÜKSEL ÖZMEN

FUNDA YILMAZ BAYDU

MOHAMMAD HASANZADEH

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GÜNSELİ BABAOĞLU

See next page for additional authors

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Assessment of drought stress responsive genes expression profiles and proline accumulation in a diverse set of grapevine rootstocks

Canan YÜKSEL ÖZMEN[1], Funda YILMAZ BAYDU[1], Mohammad HASANZADEH[2], Mert ÖKTEM[1], Günseli BABAOĞLU[1], Umut KİBAR[3], Saber DELPASAND KHABBAZİ[1], Kemal KAZAN[4], Ali ERGÜL[1,*]

[1]Biotechnology Institute, Ankara University, Ankara, Turkey

[2]Department of Plant Sciences, Faculty of Agriculture and Natural Resources-Moghan, University of Mohaghegh Ardabili, Ardabil, Iran

[3]Republic of Turkey, Ministry of Agriculture and Forestry, Agriculture and Rural Development Support Institution, Ankara, Turkey

[4]Commonwealth Scientific and Industrial Research Organization (CSIRO) Agriculture and Food, Queensland Bioscience Precinct, St. Lucia, Queensland, Australia

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Abstract: In order to study plant responses to drought stress, it is important to have markers for such responses. This issue has been a challenge for decades and numerous traits have been used for this purpose. In the current study, the influence of drought stress on proline biosynthesis, leaf water potential, and also gene expression levels of 10 genes probably involved in drought stress response regulation pathways were investigated. Also, all experiments were performed in both root and leaf tissues of studied rootstocks to investigate the response of plant tissues which is more suitable for the assessment of possible markers. As a result of the study, the highest percentage of proline increase in leaf and root tissues was mostly observed in susceptible rootstocks. The leaf water potential (-MPa) measurements showed the most decrease in leaf water potential values in highly tolerant rootstocks, so the maximum differences between control and drought stress treated plants were determined in SO4, 140Ru, 1045, and 44-53M rootstocks, respectively. Gene expression levels of *HAT5*, *RAP2-4, WRKY23, GRP*, *GDH, PRP2, GAS2, PD2, STPK*, and *GD1* genes were described in 18 different grapevine rootstocks and it was concluded that the root samples indicating more significant gene expression changes, could be more informative than leaf samples.

Key words: *Vitis vinifera* L., rootstocks, proline, drought, gene expression

1. Introduction

Abiotic stresses are among the most important factors limiting plant production in the world (Wang et al., 2004; Sahitya et al., 2018). Drought stress particularly has become one of the major problems preventing plant production in the majority of arable lands and according to some estimates, it will be increasingly important in the next 50 years (Dixit et al., 2018). Drought considerably reduces plant survival, growth, and development, as well as yield and quality through damage to important cellular components (Mizoi et al., 2012). During stress adaptation, plants undergo many molecular and physiological changes to alleviate the detrimental effects of stress (Vives-Peris et al., 2018). Under drought, plant leaves exhibit changes at molecular, biochemical, physiological, and morphological levels to improve water use efficiency. Some of the typical responses in leaf tissues may include adjustment of osmotic pressure, activation of Reactive Oxygen Species (ROS) mechanisms, and changes in cell wall elasticity, and metabolism (Prinsi et al., 2018). Molecular and genomic analyses have revealed many transcription factors (TFs) such as *DREB, WRKY, bHLH*, *bZIP* homeodomain transcription factors that regulate the expression of stress-inducible genes (Shinozaki et al., 2003; Chung et al., 2018; Mittal et al., 2018), providing a way to increase the capacity of a plant to tolerate drought stress (Bang et al., 2018).

The root is a plant organ that first encounters water deficiency and therefore, the morphological and physiological properties of roots may have a major impact on plant drought tolerance (Niu et al., 2018). It has been reported that, under certain conditions, a positive relationship between the size of the plant root system and

* Correspondence: ergul@ankara.edu.tr

tolerance to drought stress has been found (Ramireddy et al., 2018). However, compared to the above-ground parts of the plant, little is known about the underground responses to drought. Therefore, a better understanding of the physiological and molecular mechanisms associated with drought stress in plant roots is important to improve drought tolerance (Jiang et al., 2007; Molina et al., 2008).

Grapevine (*Vitis vinifera* L.) is an economically important fruit and a vast cultivation area has been assigned to viticulture over the world. Among them, the Mediterranean regions of Europe are located in areas affected by a seasonal drought during the ripening period of the grapevine, impacting the yield and fruit quality (Prinsi et al., 2018). Nevertheless, the grapevine is considered to be a relatively drought-tolerant species (Zhang et al., 2010; Aydemir and Ergül, 2021). Due to its extensive root system, it can survive even under extremely dry conditions, but drought may adversely affect the yield and quality (Marín et al., 2021). It is estimated that grapevine yield tends to decrease in drought conditions where leaf water potential falls below - 0.9 MPa (Mega pascal) (Grimes and Williams, 1990).

In viticulture, different American rootstocks are used to improve plant performance to overcome problems associated with biotic and abiotic stress factors such as drought, flooding, and salinity (Agaoglu et al., 2004; Gullo et al., 2018). Most of today's rootstocks have been developed in the 19th century to prevent the damages caused by the phylloxera beetle (*Phylloxera vastatrix radicicola*), which is one of the main pests in grapevine-growing countries (Serra et al., 2014). Cultivated grapevine (*Vitis vinifera* L.) cultivars and American rootstocks display different resistance to this pest. Cultivated grapevine is resistant to the leaf attack of this pest while American rootstocks are resistant to the root attack of Phylloxera (Buchanan and Amos, 1992; Ergül et al., 2010).

Apart from resistance to Phylloxera, the increasing possibility of water scarcity due to climate changes and water restriction makes studies on drought-tolerant rootstocks, an important aim of grapevine breeding programs (Tsegay et al., 2014; Bianchi et al., 2018). Resistance of grapevine rootstocks to drought stress varies significantly with genotype (Mullins et al., 1992). For instance, the rootstocks 110R, 140R, 44-53M, and 1103P are known to be highly drought tolerant while SO4, 99R, 420A, Fercal, 5BB, 161-49C, 41B, and Rupestris du Lot are known to have medium or low drought tolerance, respectively (Pavloušek, 2014). The genetic variability available for drought tolerance in these rootstocks enables the selection of material suitable for use in a given region.

Organic osmolyte accumulation such as sugar and amino acids (proline) contributes to drought tolerance in plants under drought stress conditions (Escalante - Magaña et al., 2019; Furlan et al., 2020). It has been shown that, during drought stress, proline content tends to increase with increasing water deficiency and the use of proline content as an indispensable and drought-sensitive marker has been reported in previous studies (Mohammadkhani and Heidari, 2008; Fulda et al., 2011). In this sense, proline accumulation can be used as a biochemical marker to predict increased stress tolerance in the breeding programs, and also in the development of drought-tolerant varieties through the hypothesis of increasing drought tolerance (Bayoumi et al., 2008).

The aim of the current study is to comparatively analyze the physiological and molecular responses of different grapevine rootstocks to drought stress. We investigated the association between drought tolerance and proline accumulation in a diverse set of grapevine rootstocks that are known to differ in drought tolerance features. In addition, we examined the expression profiles of 3 transcription factors and 7 selected stress-responsive genes to determine if their expressions could be used as a marker to predict drought stress features.

2. Materials and methods

2.1. Plant material and drought stress treatment

In this study, 18 different rootstocks of *Vitis vinifera* L. including 110R, 140Ru, 1103P, 99R, SO4, 8B, 420A MGt, 161-49C, 41B MGt, Fercal, 44-53M, Rupestris du Lot, Dog Ridge, 1613C, 1616C, 1045, Ramsey, 5BB and also a medium drought tolerant cultivar "Cabernet Sauvignon" (CS) (Cochetel et al., 2020) were provided from National Collection Repository of Viticulture Research Institute- Tekirdağ, Turkey. The genetic origins and drought tolerance features of the analysed rootstocks are given in Table 1. During the plant cultivation period, scions with 4–5 buds belonging to the rootstocks and CS cultivar were taken into sterilized soil-filled pots (7 L) to produce healthy roots and shoots (approximately 80–100 cm) under growth chamber conditions (24 °C and 16 h light/8 h dark). In addition, prior to the stress application, it was ensured that the plants were not in contact with any pathogen and the plants were regularly checked for possible disease symptoms.

In the application of stress, the stress plants were not irrigated for 16 days (similar to Cramer et al. (2007)) and the control ones were irrigated daily at a rate of 1 L of 1 : 10 Hoagland nutrient solution per 7 L soil-filled pot (Hoagland and Arnon, 1950) (containing macronutrients: K_2SO_4, KH_2PO_4, $MgSO_4.7\ H_2O$, $Ca(NO_3)_2.4\ H_2O$, KCl and micronutrients: H_3BO_3, $MnSO_4$, $CuSO_4.5H_2O$, NH_4Mo, $ZnSO_4.7\ H_2O$) at the same time and rate. Three biological replications (pot) and three technical replications (plant per pot) for each stress and control condition were prepared. On the 16th day, the plants were deemed to be un-

Table 1. Genetic origins of the grape rootstocks and their drought tolerance phenotypes reported by previous studies (1: susceptible, 2: medium tolerant, 3: highly tolerant).

Rootstock	Hybrid combination (female × male)	Samson and Casteran, 1971	Fregoni, 1977	Carbonneau, 1985	Pavloušek, 2014	Padgett et al., 2003	Satisha et al., 2016	Wani et al., 2013	Hunter et al., 2014	Consolidated Drought Phenotype
110R	*V. rupestris* × *V. Berlandieri*	Good	Highly tolerant	Highly tolerant	-	-	-	-	-	3
140Ru	*V. rupestris* × *V. Berlandieri*	Average	Highly tolerant	Highly tolerant	-	-	-	-	-	3
1103P	*V. rupestris* × *V. Berlandieri*	Good	Highly tolerant	Tolerant	-	-	-	-	-	3
99R	*V. rupestris* × *V. Berlandieri*	Average	Average tolerant	Tolerant	-	-	-	-	-	2
SO4	*V. riparia* × *V. Berlandieri*	Weak	Weak tolerant	-	-	-	-	-	-	1
8B	*V. riparia* × *V. Berlandieri*	-	-	-	Medium	-	-	-	-	2
420A MGt	*V. riparia* × *V. Berlandieri*	Weak	Weakly tolerant	Sensitive	-	-	-	-	-	1
5BB	*V. riparia* × *V. Berlandieri*	Bad	Weak tolerant	Sensitive	-	-	-	-	-	1
161-49C	*V. riparia* × *V. Berlandieri*	Weak	Mid tolerant	Sensitive	-	-	-	-	-	1
41B MGt	*V. vinifera* × *V. berlandieri*	Average	Highly tolerant	Sensitive	-	-	-	-	-	1
Fercal	*(V. berlandieri* × *Colombard No. 1) x EM 333*	Average	-	Sensitive	-	-	-	-	-	1
44-53M	*(V. cordifolia* × *V. Rupestris)* × *V. riparia*	Good	Highly tolerant	Highly tolerant	-	-	-	-	-	3
Rupestris du Lot	*V. rupestris*	Bad	Weak tolerant	Sensitive	-	-	-	-	-	1
Dog Ridge	*V. labrusca* 'Canadice' (and perhaps *V. berlandieri*) × *V. rupestris*	-	-	-	-	-	Moderate	-	-	2
1613C	(*V. Labrusca* × *V. riparia* × *V. vinifera*) × (*V. riparia* × *V. rupestris* × V. *Labrusca* Canadice)	-	-	-	-	-	-	Weak	-	1
1616C	*V. riparia* × (*V. riparia* × *V. Rupestris* × Candicans)	-	-	-	-	-	-	Weak	-	1
1045	Ganzin 1 × *V. Berlandieri* Resseguier 2	-	-	-	-	-	-	-	Good	3
Ramsey	*V.* × champinii	-	-	-	-	Highly tolerant	-	-	-	3

der stress based on their MPa values, and the root and leaf tissue samples were harvested from these plants.

2.2. Leaf water potential (- MPa) measurements

Leaf water potential (-MPa) measurements were conducted using a Model 600 Pressure Chamber Instrument according to the manufacturer's protocol (PMS Instrument Company, Albany, USA). Leaf water potential measurements were made with three biological and three technical replicates. After measurements, leaves were frozen with liquid nitrogen and stored at –80 °C for RNA extraction. The measured MPa values were compared with the control values using the t-test at $p \leq 0.05$ significance level.

2.3. Determination of free proline levels

Free proline (µmol/g FW) levels of leaves and roots along with controls were measured with a spectrophotometer (Shimadzu, North America) as described by Bates et al. (1973). The analyses were performed in three biological replicates and three technical replicates and the mean free proline amounts and standard deviations ($p \leq 0.05$) were calculated. The percentage of proline increase (%) was calculated using Excel, Microsoft software.

2.4. Primer design and Real-Time quantitative PCR (qRT - PCR) analysis

In this study, the genes to be studied were selected based on the microarray data obtained from the unpublished Ph.D. thesis of Yüksel (2015). In the thesis of Yüksel (2015), the Cabernet Sauvignon (CS) grape cultivar and 5BB rootstock obtained through tissue culture were adapted to quartz sand in a growth chamber and drought stress was performed as unirrigation for 7 days on sufficiently growing plants. After drought stress, transcriptome analyses were made in root tissues with the microarray technique (GeneChip™ *Vitis vinifera* Genome Array) and stress-related genes were determined.

As a result of microarray analyses, 3 transcription factor genes with upregulation in CS and downregulation in 5BB rootstock encoding; Ethylene responsive transcription factor RAP 2-4 (RAP2-4, probe set ID: 1619927-S-at), Homebox-leucine zipper HAT5 (HAT5, probe set ID: 1615011_at) and WRKY transcription factor (C/TTGACC/T, W boxes) (WRKY23, probe set ID: 1622333_at) were selected. Other 7 probes were determined as Proline-rich protein 2 (PRP2, probe set ID: 1621384_at) and Glutamate decarboxylase 1 (GD1, probe set ID: 1607457_at) which did not show significant expression change in CS cultivar but were significantly downregulated in 5BB rootstock, and also the Glutamate dehydrogenase (GDH, probe set ID: 1612389_at), Glycine-rich protein (GRP, probe set ID: 1607606_at), Serine/ threonine-protein kinase (STPK, probe set ID: 1621120_at), Proline dehydrogenase 2 (PD2, probe set ID: 1617293_s_at), Galactinol synthase 2 (GAS 2, probe set ID: 1608229_s_at) probes which upregulated at least 3 folds in 5BB rootstock compared to CS cultivar based on Yüksel (2015) and Degenkolbe et al. (2013).

Primer pairs for these transcripts were designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and NCBI (National Center for Biotechnology Information) reference sequences (*Vitis vinifera* L.) of the genes (Table 2). Designed gene-specific primers were used to produce a PCR product of approximately 200–300bp in grapevine.

Total RNA extraction from leaf and root tissues was performed according to the protocol described previously by Tattersall et al. (2005). Agarose gel (1%, w/v) electrophoresis and Nanodrop Spectrophotometer (ND-1000) were employed to confirm the quality and concentration of the isolated RNAs. cDNAs were

Table 2. The list of primer sequences used in this study.

			Sequence (5' - > 3')	
Primer	**Microarray Probe set ID**	**NCBI Reference Sequence**	**Forward (F) Primer**	**Reverse (R) Primer**
EIF4α	-	XM_002277667.3	F: gatgtgatccaacaggcacaa	R: catgaaccctcacaccgaga
HAT5	1615011-at	XM_002271656.4	F: ctgaacaggtacatctgctgga	R: gcttagtcttccaccgtgct
RAP2 - 4	1619927-S-at	XM_003635401.3	F: gagacaacggcattggggaa	R: taagcctcgcgaagtcacc
WRKY23	1622333-at	XM_002277846.3	F: agcgaggttgatcatctgga	R: gccggatccttgaaacacct
GRP	1607606_at	XM_002276814.4	F: agctagctgaaacagccgaa	R: cgtcttccacatcctcaccg
GDH	1612389_at	NM_001281110.1	F: tgacatggaagaccgccgta	R: gtccggtgcaggtacatcag
PRP2	1621384_at	NM_001281239.1	F: cctgaacacaagcctccgat	R: attcttcggaagtcgcggtt
GAS2	1608229_s_at	XM_002279121.4	F: acagagcatacgtggccttc	R: tctggttcttgtaaaccggca
PD2	1617293_s_at	XM_002282733.3	F: ccacctccacaatcgacctc	R: aaatcgtcctcgcagtccag
STPK	1621120_at	XM_002283296.4	F: tgggaaagctgcagacacat	R: cagagaaggccttggagcag
GD1	1607457_at	XM_002285231.4	F: gccaggaaaatgctatggcg	R: tgtaggcaggcacaatccag

synthesized from total RNA using the First Strand cDNA synthesis kit (Roche, Cat No: 04897030001) following the manufacturer's protocol. qRT - PCR amplifications were conducted using a Light Cycler 480 Real-Time system (Roche). qRT - PCR reactions were performed in a 10 µL reaction mixture containing 0.4–0.8 µL forward and reverse primer (10 pmol), 2 µL cDNA (500 ng/µL), 5 µL LightCycler® 480 SYBR Green I Master (Roche) and ddH_2O. For each primer, the standard close were prepared (efficiency and slope values were closed to 2.2 and –3.2, respectively) from serial dilutions (i.e. 1/10 to 1/100,000) of a control cDNA. The amplification reaction was started with an initial denaturation at 95 °C for 2 min. It was followed by denaturation (15 s at 95 °C), annealing (1 min at 53–55–58 °C, according to the optimized annealing temperature (Tm) of the primer), and elongation (1 min at 72 °C) steps conducted in 40 cycles. The specificity of qRT-PCR amplification (presence of dimers) was checked by a melting curve analysis after the last cycle. The qRT-PCR conditions were optimized for high amplification efficiency (>95%) for all primer pairs used. The Ct (cycle threshold) values on the amplification curves were obtained between the 20th and 35th cycles. All Real-Time quantitative PCR analyses were performed with three biological replicates and three technical replicates according to Ibrahime et al. (2019).

2.5. Statistical analysis

The relative gene expression levels were calculated by using the REST 2009 online software according to the $2^{-\Delta\Delta CT}$ (the delta-delta-Ct or ddCt) algorithm and then were normalized by using the expression value of the *eIF4α* (*Vitis vinifera* eukaryotic initiation factor 4A - 8, Gene ID: LOC100261822, GenBank accession number: XM_002277667.3) housekeeping gene (Livak and Schmittgen, 2001). The reaction efficiency (RE) and the confidence interval (CI) values were considered as 1% and 95%, respectively.

3. Results

In this study, we examined drought tolerance in grapevine rootstocks through the investigation of drought-responsive gene expression profiles and their possible association with proline accumulation.

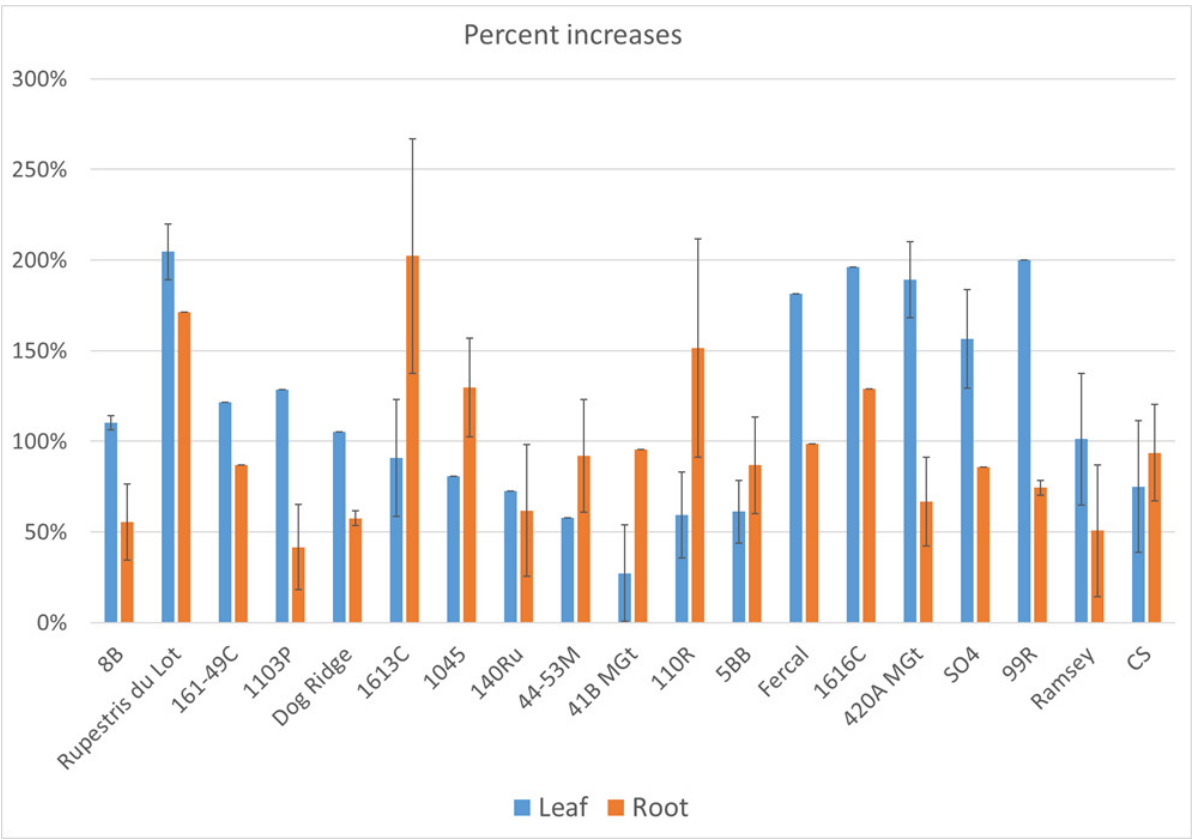

Figure 1. Percent increases (drought/control) at proline levels under drought conditions in leaf and root tissues of rootstocks. Standard error bars were drown according to the percent increase of the standard error of 3 replications of the control group and the standard error of 3 replications of stress group samples.

3.1. Proline accumulation

The percentage of proline increase in drought-exposed root and leaf tissues of 18 different rootstocks and CS are given in Figure 1. According to the results, proline increase percentage (%) was observed between 42% (1103P) and 202% (1613C) in root tissues, while this ratio was between 27% (41B MGt) and 205% (Rupestris du Lot) in leaf tissues. The highest percentage of proline increase (%) in both tissues was seen in Rupestris du Lot and 1613C rootstocks, which are known to be drought-sensitive ones. On the other hand, in the CS cultivar, no significant increase in proline percentage was found in both leaf (75%) and root (94%) tissues.

We observed strong increases in root proline levels in the drought-sensitive rootstocks Rupestris du Lot (171%) and 1613C (202%). A moderate increase was also observed in roots of the highly tolerant rootstock 110R (151%), while, the lower increase in root proline content of other highly tolerant and medium-tolerant rootstocks including 1103P (42%), Ramsey (51%), 8B (55%), and Dog Ridge (58%) were shown. The highest percentage of leaf proline increase was mostly observed in susceptible rootstocks such as Rupestris du Lot (205%), 1616C (196%), and Fercal (181%), 420A-MGt (189%), SO4 (156%), and also in medium-tolerant rootstocks such as 99R (200%), 8B (110%), and Dog Ridge (105%) (Figure 1).

3.2. Leaf water potential (- MPa) measurements

MPa values were determined in the leaves of drought-exposed rootstocks as well as in leaf samples of control plants of corresponding rootstocks. The differences in MPa values in control and stress-exposed samples of rootstocks and CS are indicated in Figure 2. Based on the results, MPa decrease values were found between –0.50 and –2.00, and a strong decrease in MPa values (>–1.50MPa) was observed especially in 140Ru, 1045, SO4, and 44-53M rootstocks. There was no clear correlation between the resistance of rootstocks to drought stress (Table 1) and MPa decrease rates. For example, the MPa decrease values were lower (between –0.50 and –1.00 MPa) in highly tolerant Ramsey and medium tolerant Dog Ridge compared to other rootstocks while, the MPa decrease values of highly tolerant rootstocks 140Ru, 1045, 44-53M, and 110R were determined to be >–1.50 MPa (Figure 2).

3.3. Expression analysis of transcription factors

The expression analysis of mentioned TF genes was evaluated by qRT-PCR to assess the gene expression levels in root and leaf tissues of 18 different rootstocks and CS cultivars when exposed to drought stress. The results indicated different expression patterns of increase or decrease in expression levels of genes under drought stress conditions.

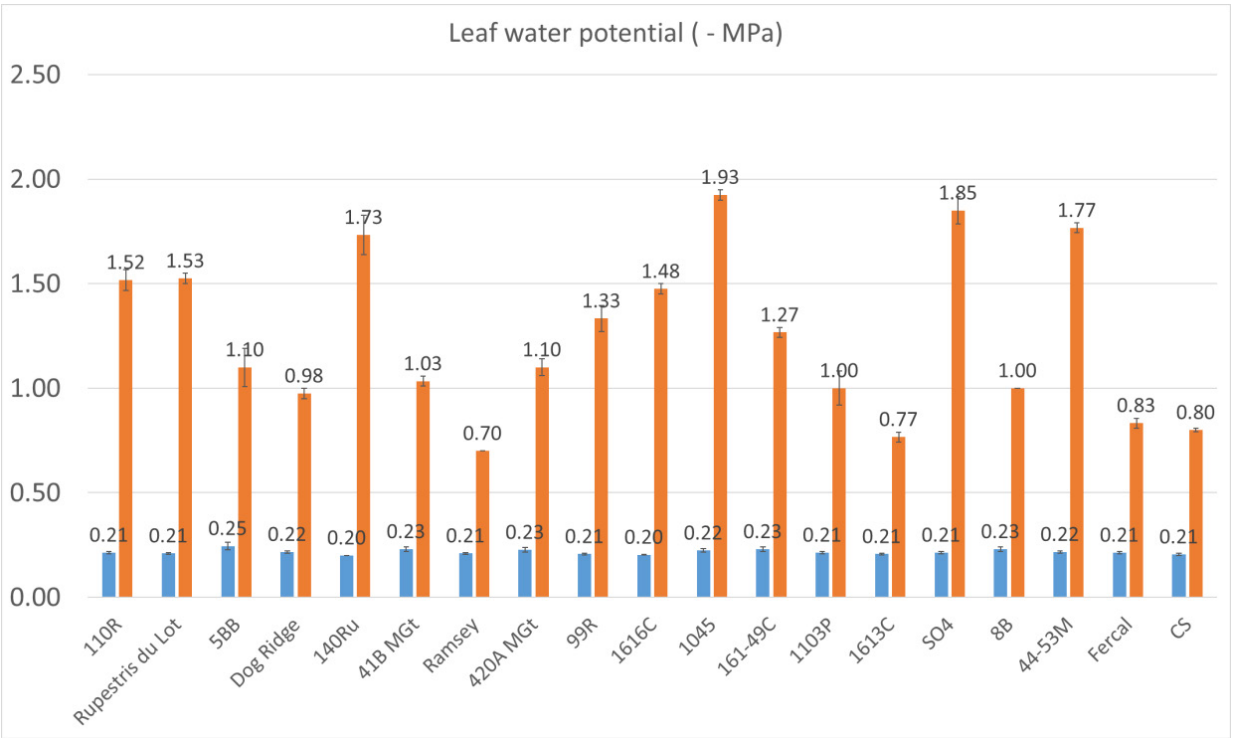

Figure 2. Leaf water potential (- MPa) measurements in leaf tissues of rootstocks (Blue column: control MPa mean and red column: stress MPa mean). Results were statistically analyzed using t-tests ($p \leq 0.05$).

Expression patterns of three TF genes (*HAT5, RAP2-4,* and *WRKY23*) were examined in roots and leaves under control and drought stress conditions. All three genes were induced in most of the rootstocks (Figure 3). Considering the expression level fold changes of these 3 genes in root tissue, it was revealed that the upregulation rates were greater than that of downregulation. While the highest significant upregulation was determined in 8B rootstock in *RAP2-4* (56.54 fold change) and *WRKY23* (33.66 fold change) genes, the lowest significant upregulation was observed in 1103P rootstock in the *HAT5* gene (2 fold change). The highest downregulation was found in the *WRKY23* gene (–28.50 fold change) in SO4 rootstock ($p \leq 0.05$) (Figure 3).

Interestingly, in leaf tissue, the highest significant gene upregulation and downregulation were observed in the *WRKY23* gene, in Fercal (6.45 fold change) and 1616C (–33.66 fold change) rootstocks, which are known to be drought sensitive. In addition, *WRKY23* was also strongly induced in root tissue of medium-tolerant rootstock 8B (33.56 fold change), while it was significantly repressed in roots of sensitive rootstock SO4 (–28.50 fold change) ($p \leq 0.05$) (Figure 3).

However, some interesting data were obtained in gene expression results of leaf and root tissues. For instance, the expression analyses indicated that *Homebox-leucine zipper* gene (*HAT5*) had the highest downregulation in the roots of drought-sensitive 5BB (–4.73 fold change) while revealed the highest upregulation in another drought-sensitive 1613C roots (29.39 fold change) and also leaf (3.99 fold change) tissues. This gene also had significant downregulation in 1616C leaf (–15.41 fold change) ($p \leq 0.05$). Interestingly, *RAP2-4* was strongly downregulated (statistically significant) in the leaf tissues of medium-tolerant rootstock 8B (–4.59 fold change) while strongly induced in the root tissues of the same rootstock (56.54 fold change). Similarly, *RAP 2-4* had a remarkable downregulation (–9.27 fold change) in the roots of the CS cultivar, while was upregulated strongly (3.81 fold change) in the same cultivar leaves ($p \leq 0.05$). *RAP2- 4* has also shown a greater upregulation in leaf samples than that of the root samples in different rootstocks (Figure 3).

Overall, according to the results, it is inferred that the reduction in gene expressions has mostly occurred in roots and leaf samples of susceptible rootstocks at a significant level whereas, the expression decrease was recorded in a small number of tolerant rootstocks. Among these 3 TFs genes studied, especially the *WRKY23* showing less significant gene expression (except 1103P, 1045, Fercal, and1616C rootstocks) appears to be the least informative gene.

3.4. Expression analysis of other selected stress-responsive genes

Considering the expression levels of selected (*GRP, GDH, PRP2, GAS2, PD2, STPK*, and *GD1*) genes in rootstocks, expression profiles were analysed in highly-tolerant, medium-tolerant, and sensitive rootstocks (Table 1, Figure 4). Although no correlation was found between the rootstocks studied and gene expression levels, expression profiles were separately specified in the root and leaf of rootstocks.

According to the expression analyses of 7 drought-responsive genes evaluated in root tissues, the highest gene upregulation was obtained in *GRP* gene (119.42 fold change) in Ramsey rootstock while, the highest gene downregulation was determined again in *GRP* gene (–43.88 fold change) in SO4 rootstock, which is known to be drought sensitive (Table 1). Interestingly, drought-sensitive rootstock SO4 showed downregulation in all genes (except *PRP2* and *PD2* genes) between 2.80 and 43.88 fold change, while rootstock 8B was upregulated in all genes between 15.35 and 182.38 fold change ($p \leq 0.05$). In addition, 5BB known as drought-sensitive rootstock also showed downregulation in all genes (except *GDH* and *PRP2* genes) between 2.36 and 11.99 fold change ($p \leq 0.05$) (Figure 4).

Generally, high levels of downregulation were observed in leaf tissues of different rootstocks. Particularly, in drought-sensitive 1616C, downregulation was observed in all genes, and the highest downregulations were recorded in the *GD1* (–266.65 fold change), and *GDH* (–85.07 fold change) genes, respectively. Although it was observed that the gene upregulation fold changes in leaf tissues were at low rates, the highest upregulation was detected in the *STPK* gene (26.97 fold change) in Fercal rootstock and also in the *GRP* gene (21.68 fold change) in 1613C rootstock ($p \leq 0.05$) (Figure 4).

Considering the results examined in all genes and tissues, the drought tolerance/sensitivity status of rootstocks (Table 1) was significantly revealed in both root and leaf tissues. For instance, it was determined that in the *GRP* gene, most strong downregulations were seen in susceptible rootstocks while most of the tolerant ones showed upregulated expression. This upregulation of *GRP* in the roots and leaves of Ramsey was significantly remarkable. In the *GAS* gene, most strong downregulations were also seen in susceptible rootstocks whereas, the medium-tolerant 8B showed a significantly upregulated profile in both root and leaf tissues. However, in leaf tissue, 44-53M rootstock known as highly tolerant, did not show any gene expression changes in all genes, while a similar situation was not encountered in root tissue samples of this rootstock. Similarly, the CS cultivar did not show expression changes in any gene in leaf tissue except *GDH* (–5.13 fold change). The CS cultivar showed significant downregulation in root tissue especially in *GRP* (–80.83 fold change) and *GDH* (–24.15 fold change) genes ($p \leq 0.05$) (Figure 4).

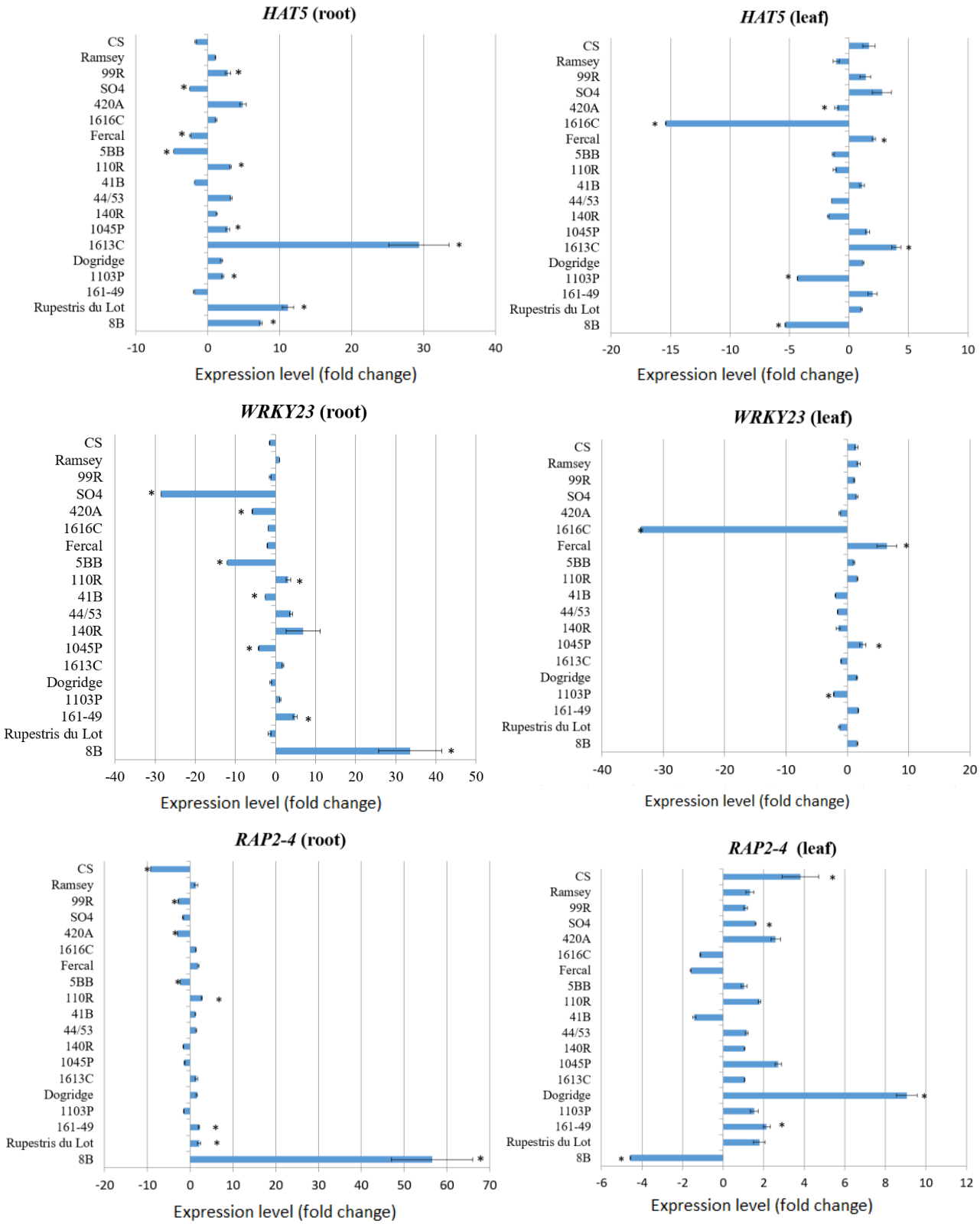

Figure 3. The relative gene expression of transcription factor genes (*: values were found to be significant (t-test ; $p \leq 0.05$) in statistical analysis).

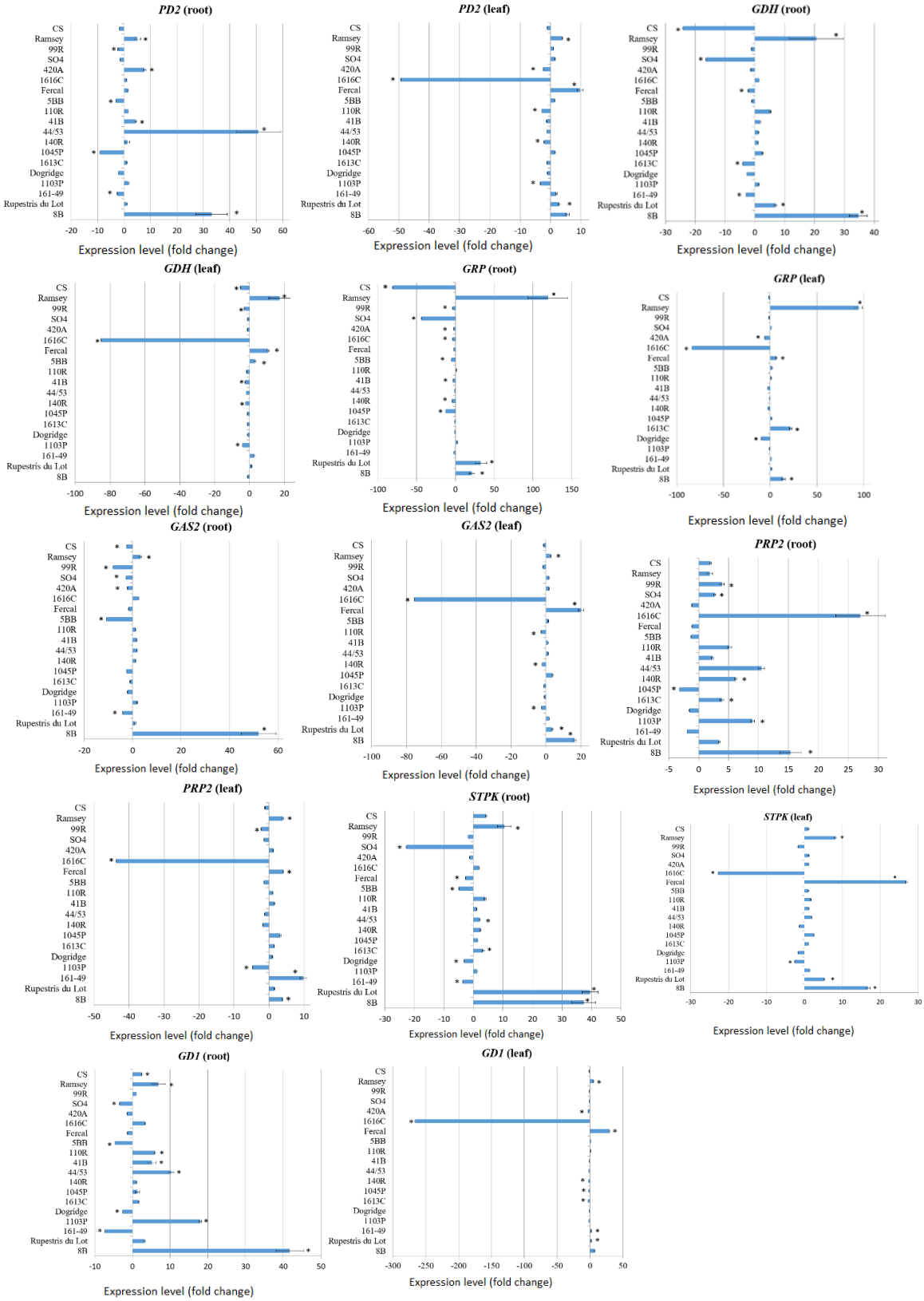

Figure 4. The relative gene expression of other selected genes (*: values were found to be significant (t-test; $p \leq 0.05$) in statistical analysis).

The *GD1* gene was induced significantly in root tissues of tolerant rootstocks such as 1103 P, 44-53M, 110R, and Ramsey, while all downregulation cases in roots and leaves were related to sensitive rootstocks. Similarly, in the *STPK* gene, all of the downregulations were seen in susceptible rootstocks whereas, the highly-tolerant Ramsey and medium-tolerant 8B and Rupestris du Lot rootstocks recorded the highest levels of significant induction in roots and leaves. However, in leaf samples, the susceptible rootstock 1616C showed significant downregulation in the *STPK* gene, while a significant upregulation of this gene was observed in another susceptible rootstock (Fercal). Although different genes studied in various rootstocks showed various variations in expression profiles, they displayed similarities in leaf sample diagrams to each other, as well as in root sample diagrams. For example, the *GD1, STPK, PRP, PD2, GDH, GAS,* and *GRP* genes in leaf tissues of 1616C, Fercal, 8B, Ramsey, 1103P, 5BB, 44-53M, and 161-49C rootstocks showed a similar pattern of changes ($p \leq 0.05$) (Figure 4).

4. Discussion

Many rootstocks are used to minimize the adverse effects of potentially deleterious biotic and abiotic stress factors such as drought, excess water, or salinity. It is known that rootstock plays an important role in drought stress tolerance and appropriate rootstock selection can improve transpiration efficiency and water usage (Gullo et al., 2018). Therefore, a better understanding of potential mechanisms associated with drought stress tolerance of rootstocks is of great importance.

In this study, we investigated drought tolerance and proline accumulation in a diverse set of grapevine rootstocks. In addition, we examined the expression profiles of a number of stress associated genes to determine if their expressions could be used as a marker to predict drought stress specification.

4.1. Proline accumulation

In many studies, it has been reported that the proline amount in the roots of different plants is increased against drought stress and helps to tolerate stress, and reduces oxidative damages which are associated with stress (Manivannan et al., 2007; Ashraf and Foolad, 2007; Mohammadkhani and Heidari, 2008; Ahmad et al., 2017; Goñi et al., 2018). Also, Irani et al. (2021) reported that the proline content of grapevine leaves increased significantly with drought stress. In the current study, a significant increase observed in proline accumulation in studied grapevine rootstocks supports the view of proline contribution to drought tolerance response in grapevine rootstocks. In particular, proline accumulation observed in both leaf and root samples of Rupestris du Lot, 1616C, 1613C, and Fercal rootstocks, which are known to be drought sensitive, could be a part of the drought response mechanisms in these rootstocks.

In our study, although no significant correlation could be established between proline accumulation and drought tolerance (data not shown), it could be noted that drought-sensitive rootstocks showed a stronger increase in proline content relative to highly tolerant rootstocks in response to drought in leaf and root tissues.

Although in various studies investigating the effects of stress on different genotypes, an increase in proline levels has been measured sometimes in tolerant genotypes and sometimes in susceptible genotypes (Schafleitner et al., 2007; Al Hassan et al., 2016; Morosan et al., 2017), but only a broader analysis based on a large number of cultivars grown under the same experimental conditions and the same stress treatments could determine whether stress responses based on proline aggregation may be a good criterion for inferencing the stress tolerance of different genotypes or not (Arteaga et al., 2020). Accordingly, based on our results obtained under controlled greenhouse conditions on a high number of grapevine rootstocks, it could be noted that proline increase could not be used as a reliable nonbiological stress biomarker.

Although proline is often considered a trait for measuring the ability of plants to tolerate drought stress conditions (Ahmad et al., 2017), it is not possible to describe genotypes with higher proline concentrations as being more tolerant merely for this reason (Szabados and Savouré, 2010). Our result is in accordance with Morosan et al. (2017) indicating the proline increase at higher concentrations in a more sensitive cultivar of *Phaseolus vulgaris* than a tolerant one after 3 weeks of drought stress. So, the authors concluded that the proline could be considered as a marker showing the stress extent the plant is affected.

4.2. Leaf water potential measurements of rootstocks after drought stress application

It is known that drought can negatively affect the photosynthesis rate by decreasing the leaf water potential (Siddique et al., 2000; Baccari et al., 2020). However, in some studies, in irrigated control grapevine genotypes, leaf water potential threshold value has been given at an average value of –0.8 MPa and lower, while it is assumed that grapevines are exposed to drought stress at an average value of –1.2 MPa and are exposed to extreme drought stress at a value of –1.5 MPa (Vincent et al., 2007).

In our study, MPa decreased values in 1045, Rupestris du Lot, 44-53M, S04, 140Ru, and 110R rootstocks were found to be similar to the finding of Vincent et al. (2007) and it was identified that these rootstocks were exposed to drought stress. However, among these rootstocks, 1045, 44 -53M, 140Ru, and 110R are known as drought-tolerant rootstocks (Fregoni, 1977; Carbonneau, 1985), and the hypothesis that these rootstocks could tolerate drought by preserving the water status could not be proven in this measurement. However, in another study evaluating the

drought tolerance of grapevine rootstocks, the Rupestris du Lot and 5BB were also reported as drought-sensitive rootstocks (Serra et al., 2014). In our study, it was observed that these rootstocks had lower decreases in MPa values compared to the tolerant 1045, 44-53M, 140Ru, and 110R rootstocks. Accordingly, although there were significant differences in leaf water potential values among rootstocks, no significant correlation was found between MPa values and drought tolerance.

4.3. Expression analysis of transcription factors and other selected stress-responsive genes

At the molecular level, gene expression plays a key role in drought tolerance response. The availability of gene expression data could be contributed to the identification of many transcription factor (TF) genes involved in the regulation of abiotic and biotic stress in plants (Zhang et al., 2012; Zhao et al., 2018). TFs could act in a specific manner as activators or suppressors of gene expression (Broun, 2004). Previous studies have reported that some TF gene families such as *HD - ZIP, WRKY,* and *RAP2* react to abiotic stress conditions such as drought (Lin et al., 2008; Zhang et al., 2014; Wei et al., 2016). The *HAT-5* and *RAP 2-4*, TF gene families have been induced during drought stress through an ABA-dependent signaling pathway (Lin et al., 2008; Hu et al., 2012). Therefore, it is useful to examine the expression level of these genes in genotypes that differ in drought tolerance. In the current study, the strong upregulation in roots of medium-tolerant 8B in *HAT-5* (7.35 fold change), *WRKY23* (33.56 fold change), and *RAP 2-4* (56.54 fold change) transcription factors was remarkable during drought stress. Among these genes, the *WRKY23* gene was reported as a candidate gene that could be used in drought stress tolerance studies (Kiranmai et al., 2018). However, it was not possible to find a relationship between their expressions and other physiological measurements. Also, considering all the TF genes, the root samples indicating more significant gene expression changes, could be more informative than leaf samples (Figure 3).

Analysis of the expression level of other selected genes associated with drought revealed that the gene expression levels were independent of their drought tolerance phenotypes. Similarly, in earlier studies, the transcriptomic data obtained from different grapevine genotypes were reported to be independent of genotype specification (Catacchio, 2019; Khadka, 2019). It has been elucidated that Glycine-rich protein (*GRP*) may be involved in the ABA signaling pathway (Sachetto - Martins et al., 2000). In the present study, *GRP* gene expression profiles indicating a significant increase in the root tissues of most tolerant genotypes and a significant decrease in most susceptible ones could be used as a root drought marker gene in drought stress of grapevine genotype.

Glutamate decarboxylase (*GD1*) is known to take part in the development of plants through the Ɣ-aminobutyric acid (GABA) pathway and both are known to be involved in defense against abiotic stress (Mousavi and Hotta, 2005; Yu et al., 2014). Considering the significant upregulation of *GD1* in the root tissues of medium tolerant rootstock 8B (41.76 fold change) and highly tolerant rootstocks 1103P (18.01 fold change), 44-53M (10.33 fold change), and Ramsey (6.94 fold change), it could be noted that the expression of this gene is induced in response to drought stress resulting in increased drought tolerance of mentioned genotypes. Therefore, *GD1* could be a useful root drought marker gene used in drought stress selection studies.

Galactinol synthase (*GAS2*) synthesizes regulatory compounds against plant stress. It is believed that the raffinose family of oligosaccharides (*RFO*s) plays a critical role in drought tolerance. Galactinol synthase (*GOLS*) and Raffinose synthase (*RAFS*) are two key enzymes responsible for raffinose biosynthesis (Peterbauer et al., 2002; Gangl et al., 2015). So, *GOLS* uses *UDP*-galactose and *myo-inositol* to synthesize galactinol (Karner et al., 2004), while *RAFS* uses galactinol and sucrose to catalyze the raffinose production (Peterbauer et al., 2002; Li et al., 2020). Therefore, a study of *GOLS* genes can help us extend our understanding of how plants respond to adverse conditions (Chu et al., 2018). George et al. (2018) reported that overexpression of *AtGolS2* in transgenic *Arabidopsis* caused an increase in endogenous galactinol and raffinose amount and showed reduced transpiration from leaves to improve drought tolerance. In our study, the significant upregulation expression of *GAS2* in the root (52 fold change) and leaf (16.45 fold change) tissues of 8B rootstock was also supported by MPa values, which indicated a decrease in transpiration in leaf tissues for drought tolerance.

Proline dehydrogenase (*PD2*) as a key enzyme in controlling cellular homeostasis, responds to stress according to the intensity and persistence of stress through the decrease in proline dehydrogenase activity or proline catabolism (Ramanjulu and Sudhakar, 2000). In recent studies, it was elucidated that the accumulation of proline is mostly observed under drought- stress conditions (Yamada et al., 2005). Sezgin et al. (2018) reported that induction and increase in the expression levels of the Proline dehydrogenase gene were observed in maize under drought-stress conditions. Upregulation of *PD2* in roots of 44-53M (50.81 fold change) which is stated to be highly tolerant in root and also in medium tolerant rootstock 8B could indicate the increased amount of proline in these tolerant rootstocks to protect against drought effects. In addition, it is consistent with the increased level of proline against drought as a result of proline measurements synthesized in the root.

It has been reported that the proline-rich protein (*PRP2*) gene also is induced by abiotic adversities and creates a response against drought stress (Thomas et al., 2003; Abou-Elwafa 2018). Accordingly, considering the expression levels of *PD2* and *PRP2*, primarily in 8B (33.17 and 15.35 fold changes, respectively) and 44-53M (50.81 and 10.48 fold changes, respectively) rootstocks, and also in general, it could be cited that at the molecular level, the reaction against drought occurs first in the roots. In addition, according to the results and significantly substantial increase of this gene in roots of tolerant 8B, 1103P, 44-53M, 140RU, 110R, Ramsey, 99R, and Dog ridge rootstocks, the *PRP2* could be used as a root drought marker gene in drought stress studies on the grapevine.

Glutamate dehydrogenase (*GDH*) enzyme appears to be important in the assimilation of ammonia under various stress conditions such as high temperature, salinity, water stress, starvation, environmental pollution, aging, and other abnormalities (Srivastava and Singh, 1987). An increase in *GDH* activity during aging seems to be a common feature in plants. A significant increase in NAD-GDH activity during aging indicates that the enzyme also functions in the energy production pathway (Cammaerts and Jacobs, 1985). Statistically significant upregulation of *GDH* in roots and leaves of tolerant rootstocks such as medium tolerant 8B (34.71 fold change in root) and highly tolerant Ramsey (20.63 fold change in root and 17.17 fold change in leaf) suggests that these rootstocks need more ammonia assimilation. Besides, a remarkable increase in *GDH* transcripts in most tolerant genotypes indicates that this gene can be used as a root drought marker gene in stress studies.

Serine/threonine-protein kinases (*STPK*) regulate various processes through interacting with proteins and also are involved in developmental regulation (Afzal et al., 2008). Serine/ threonine protein kinases include a subgroup of calcium-dependent protein kinases (*CDPCs*), CDPK-associated kinases (*CRKs*), calmodulin-dependent protein kinases (*CaMKs*), and *SnRKs* that are stress-inducible and have an essential role in plant defense response (Diédhiou et al., 2008). In this study, *STPK* is strongly upregulated in roots of the drought-sensitive rootstock Rupestris du Lot (39.58 fold change) and the medium tolerant 8B (37.40 fold change) and also in highly tolerant Ramsey (10.47 fold change) rootstocks. In leaf tissues, significantly upregulated expression of this gene in drought-sensitive rootstock Fercal (26.97 fold change) and medium tolerant rootstock 8B (16.74 fold change) suggests that the *STPK* gene might participate in stress response.

Based on the results, no significant correlation was found between the expression profiles of 10 selected genes studied in this research and drought stress phenotypes of different rootstocks (data not shown). However, among the stress-responsive genes studied, those showing significant upregulation mostly in tolerant rootstocks and/or remarkable downregulation mostly in tolerant ones could be applied as a useful molecular marker in future studies on drought stress assessment. It has been observed that genes used in the present study, were induced more in root than in leaf tissues. In this sense, due to the mentioned reasons and gene upregulation mostly in tolerant rootstocks and/or downregulation in susceptible ones, the *GRP*, *GD1*, *GDH,* and *PRP2* genes were determined as drought stress-related root marker genes in grapevine.

5. Conclusion

The present study shows the complex regulation of stress response through various genetic, biochemical, and physiological pathways. Although the expression of selected genes in some cases was independent of the drought specificity of rootstock, these genes represent general stress genes that respond to drought conditions via different pathways. Genes that show significantly strong expression increase or decrease may be candidates for more comprehensive physiological and molecular research on drought tolerance. The current study presents useful data that could provide new insights into future transcriptional and posttranslational modification studies and also could improve understanding of drought stress regulation in different grapevine genotypes.

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