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Identification of differentially expressed genes in two grape varieties cultivated in semi-arid and temperate regions from West-Bank, Palestine

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ABSTRACT

Plants respond to stress conditions by altering genetic pathways. In this study, we aimed to identify and analyze differentially expressed genes in leaves of two grape varieties (genotypes) that were grown in Palestine either in a semi-arid region with a prolonged drought and high temperature stress or in a temperate region with moderate stress levels. In total, twelve transcripts with altered expression patterns, either by stress or genotype, were identified with the differential display RT-PCR (DDRT-PCR) technique and validated via quantitative real-time PCR (qRT-PCR). Eight transcripts represent genes that are down-regulated by stress in the leaves of at least one variety, among of which are members of the *DEAD-box RNA helicase*, *Haloacid Dehalogenase* (*HAD*) *hydrolase*, *kinesin-like*, and *mitochondrial Adenine nucleotide transporter* (*ANT*) gene families. Two genes encoding for members of the *GDSL Lipase/Esterase* and *Multiprotein Bridging Factor* (*MBF*) gene families were found to be up-regulated in stressed leaves. Two transcripts coding for a NAC-domain containing protein and a WD-repeat containing protein, respectively, were found to be non-responsive to those abiotic stresses but are differentially expressed in a genotype-dependent manner.

1. Introduction

Grape (*Vitis vinifera* L.) is one of the worldwide popular crops with an estimation of about 77 million tons of grape production in 2013 (FAOSTAT, 2015). Their cultivation is commercially remunerative as their major products, mainly wine, berries, seeds, and leaves are widely consumed and used in several industries (Iriti and Faoro, 2006; Monagas et al., 2006; Aguilar et al., 2016). In the Mediterranean Basin, grapevines are cultivated in the temperate climate zones and thus experience seasonal periods of drought (Medrano et al., 2003; Chaves et al., 2010). Unlike other crop plants, grapevines are relatively tolerant to moderate levels of drought. Moreover, despite the negative impacts of drought on the total yield, it has positive and desired effects on fruit and wine qualities (Medrano et al., 2003; Deluc et al., 2009; Van Leeuwen et al., 2009).

In Palestine, grape cultivation goes back to ancient historical periods (Gorr, 1966), and it is currently a major contributor to the Palestinian agricultural sector with an estimated annual production of about 80.000 tons (Harb et al., 2015). Several old and local as well as new and introduced varieties (genotypes) are cultivated, mainly in the Hebron governorate at the south of the West Bank. In the northern districts of that governorate, summers are long, hot, and rainless, whereas winters are short, cold, and rainy. The annual average precipitations are between 400 and 800 mm. In contrast, the southern districts of the governorate are considered as semi-arid regions with a lower annual precipitation rate (200–300 mm) (Harb et al., 2015). It is worth mentioning that grapevine cultivation in Hebron-West Bank relies solely on rainfall with no supplementary irrigation. This imposes significant stress on the plants, especially during the growth period in spring/summer, when water becomes scarce causing drought stress which is typically combined with heat stress due to higher summer temperatures. Accordingly, especially in the southern parts of West

Abbreviations: ABA, abscisic acid; ANT, adenine nucleotide transporter; cDNA, DNA complementary to RNA; DDRT-PCR, differential display reverse transcription-polymerase chain reaction.; DNase, deoxyribonuclease; dNTP, deoxyribonucleoside triphosphate; DTT, dithiothreitol; DXS, 1-Deoxy-D Xylulose-5-Phosphate synthase; G6PDH, Glucose-6-Phosphate 1-Dehydrogenase; HAD, Haloacid Dehalogenase; HCF106, High Chlorophyll Fluorescence 106; KAC, Kinesin like protein for actin based chloroplast movement; MBF1a, Multiprotein Bridging Factor 1a; MEP, Methyl-Erythritol Phosphate; MLA *R*, mildew resistance (*R*) locus A; NAC, Petunia *NAM* (no apical meristem) and Arabidopsis *ATAF1*, 2 and *CUC2*; NPQ, non-photochemical quenching; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; SA, semi-arid; SDS, sodium dodecyl sulfate; SOG1, Suppressor of Gamma response 1; SOQ1, Suppressor of Quenching 1; SPPL, Signal Peptide Peptidase-Like; T, temperate; THF1, Thylakoid Formation 1; TOGR, Thermotolerant Growth Required 1

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Bank, the grape varieties must have developed adaptation mechanisms to cope with these harsh conditions. Studying such mechanisms was and still is a major topic in plant sciences.

Drought is considered as a major limiting factor for plant growth, performance, and productivity, causing serious agricultural yield losses worldwide. Plants' tolerance to abiotic stressors (i.e. drought) is known to be triggered by complex multicomponent signaling pathways, which restore cellular homeostasis and promote survival and adaptation. Stress-induced responses involve the differential expression of large sets of genes that are essential to drive such changes (Huang et al., 2008; Harb et al., 2010). Drought is known to trigger the production of the plant hormone abscisic acid (ABA) (Finkelstein et al., 2002) and numerous studies revealed that ABA and its corresponding signaling pathway forms a major part of the drought response regulatory network in plants (Zhu, 2002; Davies et al., 2005; Huang et al., 2008). Several ABA-related transcription factors (TFs) were reported to operate in drought signaling pathways (Tuteja, 2007; Golldack et al., 2014; Savoi et al., 2017). These TFs are identified to modulate the expression of downstream ABA-responsive genes and this modulation eventually leads to several cellular and physiological responses such as increasing levels of cytoplasmic organic osmolytes (Munns and Tester, 2008) and stomatal closure (Zhu, 2002; Davies et al., 2005). Moreover, organ and/or tissue specificity with regard to ABA signaling and responses is known in plants including grapes (Finkelstein, 2013; Rattanakon et al., 2016). Nevertheless, ABA-independent pathways, including gibberellic acid (GA)-, jasmonate (JA)-, reactive oxygen species (ROS)-, and lipid-dependent pathways, mediate drought-induced responses with growing evidences for cross-talks between them (reviewed in Kuromori et al., 2013; Golldack et al., 2014).

Under field conditions, however, plants are often concurrently exposed to several abiotic/biotic stress combinations (i.e. drought and heat stresses). Despite the fact that various components are shared in the signaling pathways for different stresses (reviewed in Pandey et al., 2015), recent studies indicated that plant responses to stress combinations varies significantly at molecular and physiological levels and cannot be deduced from the responses to specific stresses applied under controlled conditions (reviwed in Mittler, 2006; Suzuki et al., 2014). In addition, plant responses to a simultaneous occurrence of different stresses tend to be highly complex as it results from different, and sometimes opposing, signaling pathways that may interact and/or inhibit each other (Mittler, 2006; Suzuki et al., 2014). Thus, it is necessary to study plant tolerance and adaptation to stress combinations under conditions mimicking field environment or real field conditions.

In this study, we aimed to investigate and identify differentially expressed genes that potentially act in grapevine plants adapted to drought and high temperature stresses under field conditions. For this, two local grapevine varieties, namely “Beituni” and “Shami”, were selected that are widely cultivated in Palestine. This study is a further step toward not only a better characterization of local Palestinian grape varieties, in particular their adaptation to the local harsh environmental conditions, but also to better understand adaptation mechanism in grapes under combined stress conditions.

2. Material & Methods

2.1. Plant material

Grapevine leaves from “Beituni” and “Shami” varieties were collected from two different geographic regions of the West Bank-Palestine. The first location is Al-Dahria, which is 655 m above sea level and considered as a semi-arid region (average summer temperature = 25 °C; average annual rainfall rate = 255 mm; and potential monthly evapo-transpiration of 101.4 mm for the June–September period). The second location is Beit Ommar, which is 987 m above sea level and considered as a temperate region (average summer temperature = 21 °C; average annual rainfall rate = 500 mm, and potential monthly evapo-transpiration of 100.6 mm for the June–September period). The selection of these locations aimed to assess the influence of severe abiotic stresses, in particular drought and high temperature, in the semi-arid region compared to the moderate abiotic stresses in the temperate region. The collection time of leaves was during the main flush of vegetative growth (June 2013). Healthy leaves without any apparent infection symptoms were harvested and directly snap-frozen in liquid nitrogen and kept after that at − 80 °C until subsequent analyses.

2.2. RNA extraction and cDNA synthesis

Grape leaves were ground to fine powder under liquid nitrogen. 300 mg per sample were taken for total RNA extraction according to Chang et al. (1993) with slight modifications, namely the pellet was dissolved in SSTE buffer (1 M NaCl, 0.5% SDS, 10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)) before the final separation using chloroform:isoamylcohol (24:1) solution. RNA quality and quantity were assessed by gel-electrophoresis and NanoDrop spectrophotometer (Thermo Fischer Scientific), respectively. Genomic DNA contaminants were digested by DNaseI (NEB) for 1 h at 37 °C, and 6 µg of DNA-digested RNA were used further for cDNA synthesis. The first strand synthesis was performed in a total volume of 20 µL using Superscript III RT (Thermo Fischer Scientific). The reaction setup was as follows: 6 µg of RNA was mixed with 1 µL of 100 µM for one of the base-anchored primers (Table S1), and 1 µL of dNTPs (10 mM each). The mixture was incubated at 65 °C for 5 min and then transferred onto ice for 3 min. After that 4 µL of 5 × first strand buffer, 1 µL 0.1 M DTT, 1 µL of Superscript III RT (200 units), and water were added to a final volume of 20 µL. The reaction was incubated at 50 °C for 60 min followed by 15 min at 70 °C.

2.3. Differential display RT-PCR (DDRT-PCR)

Second strand synthesis and PCR amplification was performed in a 20 µL reaction mixture, using 2 µL of RT mix from the first strand cDNA. Each reaction mixture contains 2 µL of 10 × PCR buffer, 0.5 µL dNTPs (10 mM each), 2 µL each of one anchored primer (Table S1), one of the arbitrary primers (Table S1), and 0.25 µL Taq polymerase (5 U/µL, Genaxxon bioscience). The PCR reactions were as follows: 95 °C for 30 s followed by primer annealing at 40 °C for 2 min, extension at 72 °C for 30 s for 28 cycles followed by final extension at 72 °C for 5 min.

The DDRT-PCR products were loaded onto 2% agarose gels containing ethidium bromide (0.25 µg/mL) and separated by electrophoresis at 100 V. Amplification products were visualized by UV light and the product sizes were determined by comparison against the GeneRuler 100 bp plus DNA marker (Thermo Fisher Scientific). PCR products representing transcripts with differential expression patterns between either the two genotypes or the two geographical regions were excised from the gels and eluted using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The eluted bands were re-amplified and re-eluted from the agarose gel, cloned into pJET1.2/blunt end cloning vector (Thermo Fisher Scientific), and transformed into DH5α *E.coli* competent cells that were selected on LB agar plates supplemented with 100 µg/mL ampicillin. Bacterial colonies harboring insert-containing plasmids were picked for propagation and plasmid purification for sequencing using NucleoSpin® Plasmid EasyPure kit (Macherey-Nagel).

2.4. Analysis of obtained ESTs

The obtained cDNA nucleotide sequences were analyzed by homology searches using BLASTN and BLASTX (Altschul et al., 1990) against the *V. vinifera* genome sequences deposited in the “EnsemblPlant” (http://plants.ensembl.org/index.html) and the NCBI databases (Altschul et al., 1990). BLASTX against the *Arabidopsis thaliana* database (https://www.arabidopsis.org/) was performed to search for

Arabidopsis homologues. *Arabidopsis* genes/proteins were considered as homologues based on positive best reverse BLAST hit results, otherwise they were considered as best matches.

2.5. Validation of DDRT-PCR results via qRT-PCR

For the cDNA synthesis for qRT-PCR, RNA from four biological independent replicates per genotype (two per location) were used as described above with the exception that 2 µg DNA-digested RNA from each replicate was used and polyT_{23} oligonucleotides replaced the one base-anchored primers. Gene-specific primers for sequences/genes obtained via DDRT-PCR were designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) with amplicon sizes ranging between 75 and 200 bp (Table S1). SYBR Green chemistry was used to perform the qPCR analysis on a Bio-Rad iQ5 cycler. The PCR program was as follows: 95 °C for 10 min for Taq polymerase activation and DNA denaturation, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30s, and 72 °C 30 s. Melting curve analyses were performed to control the specificity of the primer pairs. The expression levels of the target genes were calculated relative to the transcript abundance of the reference gene *UBC* encoding for UBIQUITIN-CONJUGATING ENZYME E2 (NCBI: EE253706, EMBL: VIT_15s0046g01400) (Borges et al., 2014) employing relative quantification with efficiency correction (Livak and Schmittgen, 2001).

2.6. Statistical analysis

Student's *t*-test analysis was performed for the qRT-PCR data sets. Significance was set at $p \leq 0.05$. Standard error (S.E.) values were also calculated and included.

3. Results

3.1. Identification of differentially expressed genes

RNA isolated from leaves of the “Beituni” or “Shami” grape genotypes grown in either semi-arid (Al-Dahria) or temperate (Beit Ommar) regions in West-Bank, Palestine, were used for cDNA synthesis followed by several DDRT-PCRs. The use of various primer combinations for performing DDRT-PCRs led to the isolation, cloning, and sequencing of twenty PCR products from bands that appeared with different intensities in the gels. These products represent transcripts that are potentially differentially expressed in the two genotypes in response to the varied abiotic stress levels in the growth locations, or between the genotypes regardless the stress/location. However, from the twenty isolated and sequenced PCR products from the initial differential DDRT-PCR patterns we were able to confirm the differential expression of only twelve transcripts by qRT-PCR (Fig. 1, Table 1) and thus we focused on them.

3.1.1. Down-regulated genes

Out of twelve identified transcripts, eight were cloned from corresponding PCR products showing bands with a higher intensity derived from leaves collected from the temperate region, indicating a negative effect on their gene expression by abiotic stresses prevailed in the semi-arid region (Fig. 1, Table 1). The abiotic stress down-regulated genes are encoding for a putative MITOCHONDRIAL ADENINE NUCLEOTIDE TRANSPORTER (ANT) protein (VIT_09s0054g00790), a putative KINESIN-LIKE protein (VIT_00s0407g00080), a putative HALOACID DEHALOGENASE (HAD) HYDROLASE (VIT_14s0060g01030), a putative plastid-localized 1-DEOXY-D XYLULOSE-5-PHOSPHATE SYNTHASE (DXS, VIT_00s0218g00110), a putative DEAD-BOX ATP-DEPENDENT RNA HELICASE, a putative SIGNAL PEPTIDE PEPTIDASE-LIKE (SPPL, VIT_05s0062g01250), a cytoplasmic GLUCOSE-6-PHOSPHATE 1-DEHYDROGENASE (G6PDH, VIT_14s0171g00490), and a putative disease-resistant protein (VIT_14s0030g00960). The ANT protein is a transmembrane transporter protein that is composed of 356 amino acids belonging to the large mitochondrial carrier protein family (MCF). According to a Pfam analysis (http://pfam.xfam.org/), the protein harbors 3 Mito_carr functional regions (PF00153). For the KINESIN-LIKE protein the current gene model (VIT_00s0407g00080) seems to be incomplete with a protein composed of 297 amino acids and no clear functional domains. However, comparison of the protein sequence against the NCBI and TAIR databases indicated a resemblance to members of the kinesin-like motor protein family. The HAD HYDROLASE protein consists of 1078 amino acids with homology to the *Arabidopsis* SUPPRESSOR OF QUENCHING 1 (AtSOQ1). This grape protein contains several functional domains including a haloacid dehalogenase-like hydrolase (HAD_2, PF13419) domain at its N-terminus, a Thioredoxin-like (Thioredoxin_8, PF13905) domain, and a NHL-repeat (NHL, PF01436) at the C-terminus. The plastid-localized DXS consists of 718 amino acids and carries the DXP_synthase_N (PF13292) and Transketolase_C (PF02780) functional domains at its N- and C- terminus, respectively. The putative DEAD-BOX RNA HELICASE is a member of a huge gene family with about 40 members in *V. vinifera*. The protein consists of 732 amino acids, with a DEAD/DEAH box helicase (DEAD, PF00270) functional domain at its N-terminus and a conserved C-terminal helicase domain (Helicase_C, PF00271). The putative SPPL protein with 533 amino acids harbors a signal peptide peptidase (Peptidase_A22B, PF04258) functional domain at its C-terminus. The cytoplasmic G6PDH is a functionally well-characterized protein that catalyzes the first rate-limiting step of the oxidative pentose-phosphate pathway (Esposito, 2016). This protein is composed of 516 residues and the genome of *V. vinifera* encodes for at least five G6PDH proteins. The putative disease-resistant protein is composed of 1075 residues and harbors two functional domains namely a NB-ARC (PF00931) domain at the N-terminus and a leucine-rich repeat (LRR_8, PF13855) in the middle of the protein. This protein shows similarities to mildew resistance (*R*) locus A (MLA) proteins.

3.1.2. Up-regulated genes

Two transcripts were isolated and cloned from PCR products showing stronger intensities in the DDRT-PCR products derived from semi-arid grown plants (Fig. 1, Table 1). These transcripts represent genes encoding a putative GDSL (Esterase/Lipase, VIT_08s0007g02260) protein that is composed of 256 amino acids harboring a Lipase_GDSL_2 (PF13472) functional domain and putative MULTIPROTEIN BRIDGING FACTOR 1a (MBF1a, VIT_19s0014g01260) protein belonging to a class of small DNA binding proteins that act as transcription co-activators (Takemaru et al., 1997). The MBF1a protein consists of 142 amino acids and harbors two functional regions; MBF1 (PF08523) at the N-terminus and a helix-turn-helix (HTH_3, PF01381) motif at the C-terminus.

3.1.3. Genotype-dependent differential expression

Two additional transcripts were isolated and cloned from PCR products showing stronger intensities in the DDRT-PCR products derived from the “Shami” grape variety compared to the “Beituni” variety in both locations (Fig. 1E, Table 1). These transcripts represent genes coding for a putative SUPPRESSOR OF GAMMA response (SOG1, VIT_01s0011g02990) homologue and a putative WD-repeat containing protein (VIT_04s0023g02460). The SOG1 protein with 430 amino acids contains a NAC-domain known to function as a transcriptional regulator and DNA-damage response mediator in plants (Yoshiyama et al., 2009), while the WD protein with 492 amino acids harbors two WD40 (PF00400) functional domains and is predicted to be nucleus-localized and to act as a transcriptional regulator.

3.2. Validation of DDRT-PCR results via qRT-PCR

Since we have excised and cloned DDRT-PCR products that have shown varying intensities in the samples derived from varying locations or different genotypes, we performed qRT-PCR analyses to confirm and

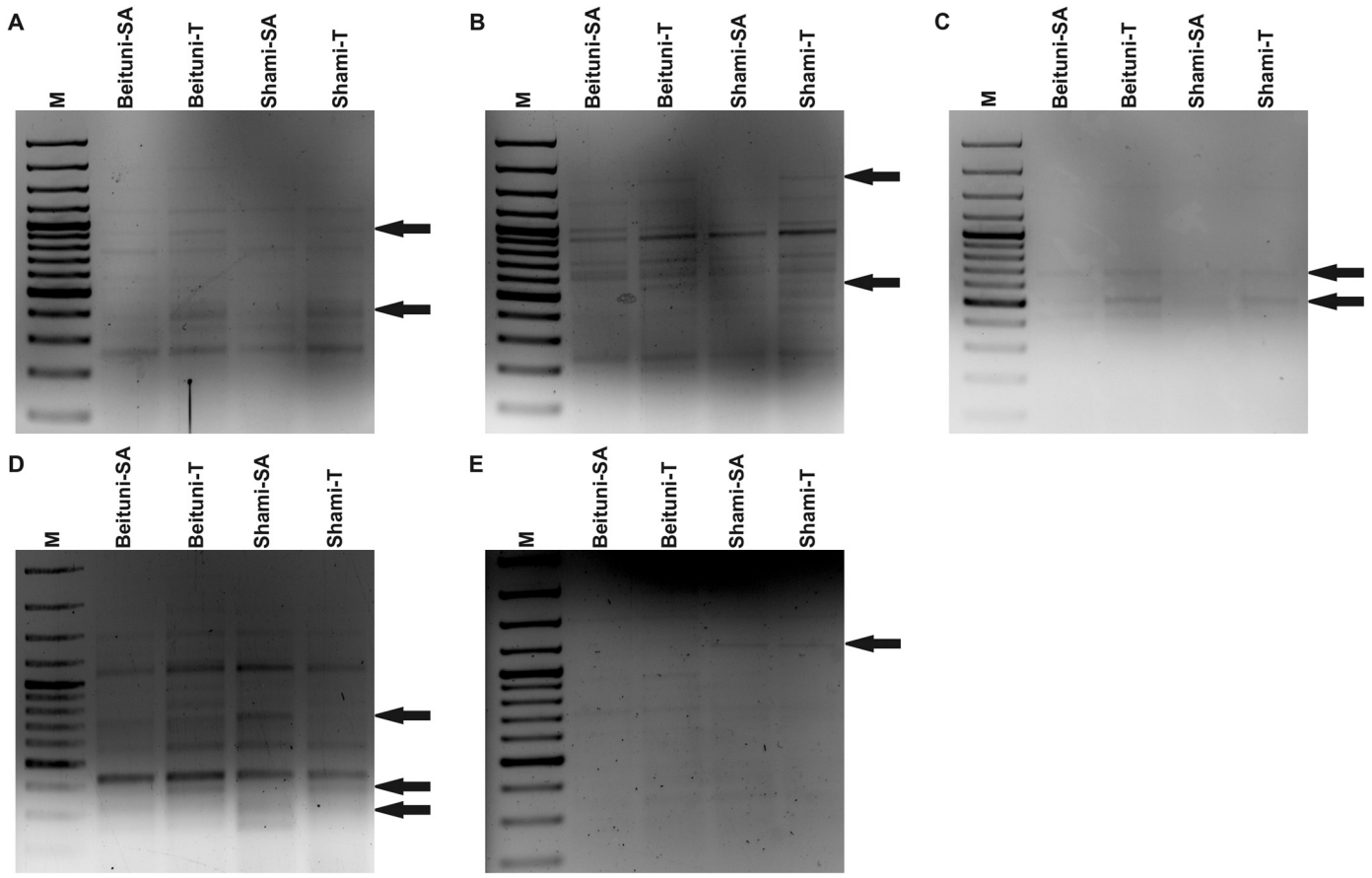

Fig. 1. Identification of differentially expressed transcripts in leaves of "Beituni" and "Shami" grapes grown in semi-arid (SA) or temperate (T) regions. 2% agarose gels showing separated amplified PCR products obtained by DDRT-PCRs using different primer combinations. A) HT11(A)-AP1, B) HT11(G)-AP8, C) HT11(A)-AP4, D) HT11(C)-AP1, E) HT11(G)-AP1. Arrows point to PCR products that were excised from the gel and further analyzed as described. Marker (M): GeneRuler 100 bp plus DNA ladder.

Table 1
Transcripts regulated by drought stress in the leaves of the two grape genotypes tested in this study.

DDRT-PCR primer combination	PCR Sequence length	Regulation by drought	Transcript ID & accession number	Encoded protein	*Arabidopsis* homologue(s)
HT11(G)-AP8	545 bp	Down-regulated	EnsemblPlant: VIT_18s0001g14540 NCBI: XM_002273407.4	Putative DEAD-Box RNA helicase	AT4G16630
HT11(A)-AP4	718 bp	Down-regulated	EnsemblPlant: VIT_14s0060g01030 NCBI: XM_002277528.3	Putative HAD hydrolase (SOQ1)	AT1G56500
HT11(A)-AP1	407 bp	Down-regulated	EnsemblPlant: VIT_00s0407g00080 NCBI: XM_010648494.2	Putative kinesin-like protein	AT5G65460[a]
HT11(A)-AP4	511 bp	Down-regulated	EnsemblPlant: VIT_09s0054g00790 NCBI: XM_003632874.3	Putative ANT	AT3G20240
HT11(A)-AP1	934 bp	Down-regulated	EnsemblPlant: VIT_00s0218g00110 NCBI: XM_002266889.4	Putative DXS	AT4G15560[a]
HT11(A)-AP1	928 bp	Down-regulated	EnsemblPlant: VIT_05s0062g01250 NCBI: XM_002268539.3	Putative SPPL	AT2G43070
HT11(G)-AP8	1626 bp	Down-regulated	EnsemblPlant: VIT_14s0171g00490 NCBI: XM_002266491.4	Putative G6PDH	AT5G40760
HT11(C)-AP1	407 bp	Down-regulated	EnsemblPlant: VIT_14s0030g00960 NCBI: XM_010661676.1	Putative MLA10 disease resistant protein	AT4G27190[a]
HT11(C)-AP1	312 bp	Up-regulated	EnsemblPlant: VIT_08s0007g02260 NCBI: XM_002280260.4	Putative GDSL	AT3G11210 AT2G38180
HT11(C)-AP1	683 bp	Up-regulated	EnsemblPlant: VIT_19s0014g01260 NCBI: XM_003634619.2	Putative MBF1a	AT2G42680
HT11(G)-AP1	1180 bp	No regulation	EnsemblPlant: VIT_01s0011g02990 NCBI: XM_010649666.2	Putative SOG1 (NAC-domain containing protein)	AT1G25580
HT11(G)-AP1	1235 bp	No regulation	EnsemblPlant: VIT_04s0023g02460 NCBI: XM_002275683.4	Putative WD-repeat containing protein	AT4G18905

[a] *Arabidopsis* best match.

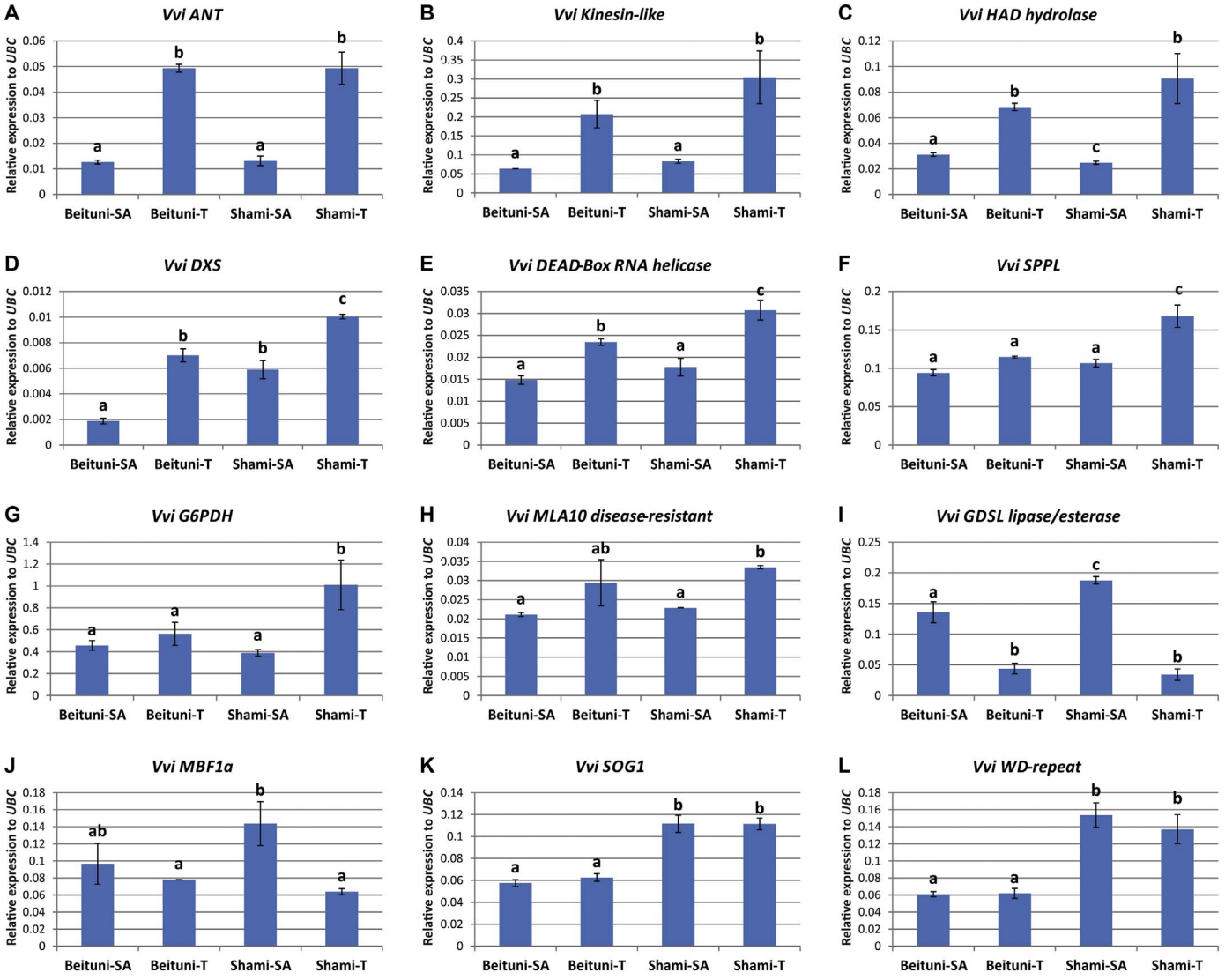

Fig. 2. Expression analysis of identified genes in leaves of "Beituni" and "Shami" grape varieties grown in semi-arid (SA) and temperate (T) regions by qRT-PCR using gene-specific primers. Relative expression values were obtained by normalization to the *VviUBC* control gene. Error bars indicate means ± SE (n = 2). Different letters indicate statistically significant differences between the samples as determined by the Student's *t*-test ($P < 0.05$).

validate the expected differential gene expression within the respective RNA samples. Fig. 2 shows the results obtained from qRT-PCRs using gene-specific primers for all transcripts listed above. For those mRNAs designated as down-regulated in both varieties by abiotic stresses, qRT-PCR revealed significant decreases in the expression of six genes (*ANT*, *kinesin-like*, *HAD hydrolase*, *DXS*, *DEAD-box RNA helicase*, and *SPPL*) (Fig. 2A–F). The expression of the remaining two genes (*G6PDH* and *MLA10 disease-resistant* protein) was found to be significantly inhibited only in the "Shami" variety in response to abiotic stress (Fig. 2G, H). In the "Beituni" variety, there was only a non-significant declining trend in their expression in response to abiotic stresses.

For those genes that were expected to be up-regulated by abiotic stresses, qRT-PCR data confirmed the significant increase in the expression of the *GDSL* gene in both genotypes (Fig. 2I), while the expression of the *MBF1a* gene was significantly up-regulated only in the "Shami" variety grown in the semi-arid region with a non-significant increasing trend in the "Beituni" variety (Fig. 2J). Table 2 shows the fold changes in the gene expression for the analyzed genes in both grape varieties.

For both genotype-dependent genes, *SOG1* (NAC-domain containing) and the gene encoding the WD repeat-containing protein, the qRT-PCR analyses confirmed their genotype-specific expression with a

Table 2
Fold change in gene expression of stress-responsive genes in leaves of Beituni and Shami grape varieties grown in the semi-arid (SA) region relative to those grown in the temperate (T) region (SA/T). Values are calculated based on normalized qRT-PCR data.

Transcript ID	Encoded protein	Fold change (SA/T)	
		Beituni	Shami
VIT_18s0001g14540	Putative DEAD-Box RNA helicase	− 1.6	− 1.7
VIT_14s0060g01030	Putative HAD hydrolase (SOQ1)	− 2.2	− 3.7
VIT_00s0407g00080	Putative Kinesin-like protein	− 3.3	− 3.7
VIT_09s0054g00790	Putative ANT	− 3.9	− 3.8
VIT_00s0218g00110	Putative DXS	− 3.7	− 1.7
VIT_05s0062g01250	Putative SPPL	NSC	− 1.6
VIT_14s0171g00490	Cytosolic G6PDH	NSC	− 2.6
VIT_14s0030g00960	Putative MLA10 disease resistant protein	NSC	− 1.5
VIT_08s0007g02260	Putative GDSL	+ 3.1	+ 5.5
VIT_19s0014g01260	Putative MBF1a	NSC	+ 2.3

NSC: no significant change.

significant higher expression in the “Shami” variety compared to “Beituni” (Fig. 2K, L).

4. Discussion

Drought and heat are considered as major stressors affecting plant growth and productivity worldwide. In response to such stresses, plants developed mechanisms such as tolerance, resistance, and avoidance that help them to maintain performance under these limiting conditions. Modulating the expression of genes embedded in genetic and signaling programs underlying developmental and physiological processes is a well-known plant response to stressful conditions. Here, we report the differential expression of several genes in two grapevine varieties cultivated under different drought levels together with a probable influence of heat on their expression.

One of the down-regulated genes we found in both varieties is coding for a putative ANT protein (VIT_09s0054g00790). This *Vitis* ANT protein is a homologue to the uncharacterized *Arabidopsis* carrier protein (AT3G20240) and shows similarity to another well-characterized carrier, *Arabidopsis* BRITTLE 1 (AtBT1; AT4G32400) (Kirchberger et al., 2008; Bahaji et al., 2011a; Bahaji et al., 2011b). Interestingly, recent studies on grapes reported the down-regulation of this putative *ANT* gene in leaves, roots, and berries of various varieties in response to drought (Corso et al., 2015; Savoi et al., 2016) (Table S2), which goes in line with our findings.

The *Vitis* putative KINESIN-LIKE protein (VIT_00s0407g00080) is a member of the Kinesin-14B (KIN-14B) family within the eukaryotic highly conserved Kinesin superfamily. This superfamily is constituted of microtubule motor proteins acting in vesicle and organelle transportation, cytokinesis, morphogenesis, cell wall organization, and signal transduction (Li et al., 2012). Specifically, this *Vitis* protein shows high similarity to the *Arabidopsis* KINESIN LIKE PROTEIN FOR ACTIN BASED CHLOROPLAST MOVEMENT 2 (AtKAC2, AT5G65460). AtKAC2 together with AtKAC1 acts in chloroplast movement and their positioning in response to light (Suetsugu et al., 2010, 2012, 2016; Shen et al., 2015). Several reports indicated that dehydration and ABA trigger microtubule disruption in plant cells (i.e. guard cells) (Jiang et al., 1996; Pollock and Pickett-Heaps, 2005), pointing to a probable role of kinesin proteins in the stress response. In this regard, He et al. (2005) reported the down-regulation of *AtKAC2* representing the *Arabidopsis* closest homologue to this grape KINESIN-LIKE protein under salt-stress conditions. In addition, a nuclear localized kinesin protein was found to be differentially regulated by dehydration stress in chickpea (*Cicer arietinum*) (Pandey et al., 2008). In *Vitis*, this putative Kinesin-like gene was reported recently to be down-regulated by prolonged drought stress in leaves of two Italian grape varieties (Dal Santo et al., 2016), which is in agreement with our findings (Table S2).

RNA helicase motor proteins constitute one of the highly conserved families among living organisms that act in RNA metabolism and remodeling (Jankowsky, 2011). Previous studies revealed that several plant RNA helicases are involved in mediating responses and tolerance to various abiotic stresses such as members of the stress-related DEAD-box proteins (reviewed in Vashisht and Tuteja, 2006). In this sense, Zhu et al. (2015) characterized two tomato DEAD-box RNA helicase genes, *SlDEAD30* and *SlDEAD31*, and found that both are induced by salt, while *SlDEAD31* is induced also by heat, cold, and drought. Furthermore, transgenic tomato plants overexpressing *SlDEAD31* exhibited enhanced salt and drought tolerance. Another DEAD-box RNA helicase, the rice Thermotolerant Growth Required1 (OsTOGR1), was induced in expression and activity under high temperature and was suggested to have a role in rRNA biogenesis and homeostasis under high temperature conditions (Wang et al., 2016a). The grape putative DEAD-box RNA helicase gene (VIT_18s0001g14540) we identified in our analysis to be down-regulated by abiotic stresses is also homologous to an *Arabidopsis* RNA helicase (AT4G16630) that was reported to be induced by cold stress (Mikkelsen and Thomashow, 2009) as well as in response to salt and cytokinin treatments (Golan et al., 2016). In contrast to the detected decrease in the expression of the *Vitis* putative RNA helicase gene by drought/heat in this study, other recent studies reported that this grape gene is induced by drought in the berries (Savoi et al., 2016) and in the leaves by cold (Xin et al., 2013) indicating tissue specific variations in the gene expression in response to different stressors (Table S2).

DXS catalyzes the first step in the Methyl-Erythritol Phosphate (MEP) pathway, which is one of the two known pathways for terpenoid/isoprenoid biosynthesis in living organisms (Estevez et al., 2001; Wright et al., 2014). The grape genome encodes several DXS members and we found VIT_00s0218g00110 to be down-regulated in the leaves collected from the semi-arid region. Interestingly, Pateraki and Kanellis (2010) showed an initial up-regulation and subsequent down-regulation of a *DXS* gene in the leaves of *Cistus creticus* upon prolonged drought or heat stress. Furthermore, since the emission of some volatiles is considered as a mean to measure fluxes within the MEP pathway, several studies indicated that stressful conditions can affect the MEP pathway, including a varying expression of the *DXS* genes, depending on the stress type, its severity, and persistence (Niinemets, 2010 and therein references). Mild to moderate drought stress, for example, does not affect isoprene and monoterpene emissions, while mild to moderate heat stress enhances such emissions probably to play a thermo-protective role in the leaves. However, prolonged and acute stresses, known to inhibit photosynthesis, would inhibit isoprene and monoterpene emissions (Niinemets, 2010 and therein references). Accordingly, this might explain the varying regulation patterns reported in recent studies for this grape *DXS* gene under drought conditions. For instance, Corso et al. (2015) found this gene to be down-regulated upon drought stress in the leaves and roots of two different grape hybrid varieties (Table S2) which is in line with our results. In contrast, Dal Santo et al. (2016) reported the up-regulation of this gene in the leaves of “Sangiovese” and “Montepulciano” grapes in response to drought (Table S2). One additional explanation might be that this gene demonstrates a genotype-dependent expression in response to drought which was not observed in the two varieties we analyzed herein. This gene is also differentially regulated under various other stress conditions (Table S2).

The putative HAD hydrolase encoding gene (VIT_14s0060g01030) was found to be down-regulated in the leaves collected from the semi-arid region. Its closest *Arabidopsis* homologue *AtSOQ1*, encoding a chloroplast thylakoid membrane protein, was recently shown to be implicated in non-photochemical quenching (NPQ) with a proposed function in maintaining the light harvesting efficiency under unfavorable conditions (Brooks et al., 2013). Moreover, further indications suggest a role of AtSOQ1 in acclimation to cold and high light stress (Malnoë et al., 2016 and personal communication). Similar to our results on the grape homologue, Pandey et al. (2013) and Bhaskara et al. (2012) reported the down-regulation of *AtSOQ1* during drought. Wang et al. (2016b) identified AtSOQ1 as an interacting protein in a complex containing the *Arabidopsis* proteins HIGH CHLOROPHYLL FLUORESCENCE 106 (AtHCF106) and THYLAKOID FORMATION 1 (AtTHF1). Both of these chloroplast-localized proteins are essential for thylakoid formation and seem to act as negative regulators of drought resistance in *Arabidopsis*. The single and double mutants of these proteins exhibited elevated levels of ROS in guard cells, improved stomatal closure, and consequently reduced water loss under drought conditions (Wang et al., 2016b). Thus, we assume that plants, including *V. vinifera,* that harbor homologues of these genes have developed a mechanism including the regulation of these genes/proteins expression and availability to mediate drought resistance and to reduce water loss. Besides our findings on the responsiveness of the *VviSOQ1* gene to abiotic stresses, several other studies on grapes showed that it is also regulated under other developmental conditions (Table S2).

The signal peptide peptidases (SPP) and the homologous SPP-like (SPPL) proteases are multi-transmembrane aspartic proteases that

hydrolyze their substrates within the plane of the cellular membranes. They belong to the conserved eukaryotic protein family of intramembrane-cleaving proteinases (I-CliP) (Weihofen et al., 2002; Voss et al., 2013). The *Vitis* gene VIT_05s0062g01250 that is down-regulated by drought codes for a putative SPPL protein with high sequence similarity to the *Arabidopsis* SPPL3. Both of the highly conserved aspartic active sites (YD and G*X*GD) are present in this grape putative SPPL protein. Regarding the involvement of plant SPP/SPPL proteases in abiotic stress responses, one study reported the up-regulation of a rice putative *SPPL2B* gene in leaves and roots in response to drought (Kohli et al., 2012). Together with our findings on the responsiveness of this *Vitis* putative *SPPL* member to combined drought and heat stress in "Shami" grapes, these results point to a yet unexplored role of these genes in plant stress adaptation.

VIT_14s0030g00960, another gene that is down-regulated by drought only in "Shami" grapes, encodes a putative disease-resistance protein that shows similarities to MLA10 *R* protein which belongs to the mildew resistance (*R*) locus A protein family. In barley, the MLA10 protein is known to confer resistance against powdery mildew fungus and to act in cell death signaling (Bai et al., 2012). Interestingly, this grape gene was not reported to be regulated after infection with *Erysiphe necator*, a powdery mildew causing fungus (Fung et al., 2008; Weng et al., 2014), but rather was found to be down-regulated in the berries of two different varieties that are infected with *Botrytis cinerea* causing noble-rot (Blanco-Ulate et al., 2015) (Table S2). However, supporting our results, this gene is down-regulated in the leaves of the "Montepulciano" grape variety in response to drought (Dal Santo et al., 2016) (Table S2).

The enzyme G6PDH catalyzes the first step in the oxidative pentose phosphate pathway and thus regulates NADPH and pentose levels in plant cells (Esposito, 2016 and therein references). NADPH is required for the protection against oxidative damage that can be caused by environmental conditions that provoke oxidative stress (Esposito, 2016). Several studies reported the induction of *G6PDH* genes and/or protein activity in tobacco, soybean, and tomato plants in response to environmental stresses such as drought and heat (Scharte et al., 2009; Gong et al., 2012; Liu et al., 2013; Landi et al., 2016). It is assumed that this induction is needed to meet the high demand for NADPH as a reductant to reset redox homeostasis. In the leaves of *Morus alba*, drought did not significantly induce the expression of *G6PDH,* but it promoted an increase in G6PDH enzymatic activity (Yao and Wu, 2016). Interestingly the expression level of the grape gene we identified in this study (VIT_14s0171g00490), encoding a cytosolic *G6PDH* isoform, was found to be significantly reduced by drought in the leaves of the "Shami" variety but not in the "Beituni" variety. These results are in contrast with the above-mentioned findings from other plant systems. Moreover, Savoi et al. (2016) reported the up-regulation of this gene in grape berries of drought-stressed plants (Table S2). Additionally, the *Arabidopsis* closest homologue of this grape gene, AT5G40760, was reported to be induced by drought but inhibited by ABA treatment (Noctor et al., 2014). This might indicate a genotype- and/or tissue-specific regulation of this gene in response to drought. Nevertheless, we cannot exclude that other grape genes coding for cytosolic *G6PDH* are up-regulated in the leaves of the assessed varieties in response to stress.

One of the genes shown to be induced in both assessed varieties encodes for a putative GDSL lipase/esterase protein (VIT_08s0007g02260). GDSL proteins are involved in plant development and morphogenesis (Akoh et al., 2004). Moreover, several studies demonstrated the importance of GDSL and GDSL-like proteins in mediating the response to various types of biotic and abiotic stresses in plants (Oh et al., 2005; Naranjo et al., 2006; Kim et al., 2008; Kwon et al., 2009; Lee et al., 2009; Agee et al., 2010). In grapes, several recent publications indicated the differential expression of the identified putative *VviGDSL* gene under various developmental and stress conditions (Table S2). For instance, Dal Santo et al. (2016) reported the up-regulation of this gene in the leaves of "Sangiovese" grapes exposed to drought stress which is in accordance with our results. Moreover, this gene was also induced in berries of other varieties exposed to elevated temperatures (Carbonell-Bejerano et al., 2013; Rienth et al., 2014, 2016). However, other studies reported the down-regulation of this gene expression in leaves and berries of several grapevine varieties upon fungal infections (Table S2).

Another induced gene analyzed in our study codes for a putative MBF1a, a transcription co-activator that belongs to a small and highly conserved eukaryotic protein family acting in the control of gene expression (Takemaru et al., 1997). In *Arabidopsis*, AtMBF1 proteins (AtMBF1a-c) are reported to mediate several biotic and abiotic stress responses as well as to participate in phytohormone signaling pathways (Tsuda et al., 2004; Tsuda and Yamazaki, 2004; Arce et al., 2010). The *AtMBF1a* gene is induced by dehydration, and the constitutive expression of *MBF1a* in *Arabidopsis* led to elevated salt and glucose tolerance as well as enhanced resistance to fungal infection by *B. cinerea* (Kim et al., 2007). Moreover, genetic analysis of *Arabidopsis* gain- and loss-of-function mutants demonstrated that AtMBF1c acts upstream of salicylic acid and ethylene signaling during heat stress (Suzuki et al., 2008). *AtMBF1c* overexpression resulted in enhanced tolerance to heat, osmotic stress, and bacterial infection (Suzuki et al., 2005, 2008). The *V. vinifera* genome also encodes three members of the MBF1 family and regarding our gene of interest, VIT_19s0014g01260, recent studies indicated the induction of this putative *VviMBF1a* gene upon exposure to heat stress (Carbonell-Bejerano et al., 2013; Rienth et al., 2016) (Table S2). It is probable that the detected transcriptional induction of this gene in the leaves of the "Shami" variety, and to lesser extent in the "Beituni" variety, resulted from the combined drought and heat stresses. Indeed, members of the *MBF1* gene family, identified as ethylene-responsive transcriptional co-activators, were reported to be highly induced in *Arabidopsis*, *Nicotiana tabacum* (Rizhsky et al., 2002, 2004), and the desert legume *Retama raetam* (Pnueli et al., 2002) upon exposure to either drought or heat and especially when both stressors concur.

We found one gene (VIT_01s0011g02990) to be expressed in a genotype-dependent manner showing an approximately two-fold increased transcript level in the "Shami" variety compared to the "Beituni" variety. This gene encodes a putative NAC-domain containing protein and presents a member of the NAC domain family [Petunia *NAM* (no apical meristem) and Arabidopsis *ATAF1*, 2 and *CUC2*] (Yoshiyama et al., 2009). It has high similarity to *Arabidopsis* SOG1, a transcription factor that functions in the regulation of DNA damage response (Yoshiyama et al., 2009; Yoshiyama, 2015). We could not detect any differential regulation of this putative *VviSOG1* gene in response to abiotic stresses and until now recent studies indicated the regulation of this gene only in berries upon infection with *B. cinerea* and during development (Table S2).

Another gene with a genotype-dependent expression pattern encodes a putative WD-repeat containing protein (VIT_04s0023g02460). WD-repeat containing proteins comprise a diverse superfamily of regulatory proteins that play major roles in various mechanisms such as signal transduction, cytoskeletal dynamics, protein trafficking, nuclear export, RNA processing, chromatin modification, and transcriptional mechanisms (Stirnimann et al., 2010). Regarding the genotype-specific expression of the *Vitis* WD-repeat gene, recent large-scale studies indicated that this gene does not seem to be differentially regulated under biotic or abiotic stresses. However, the expression level of this gene was reported to be higher in leaves of the grape variety "Sangiovese" compared to the leaves of the "Montepulciano" variety with no clear effect during drought stress (Dal Santo et al., 2016) (Table S2), thus resembling our findings in this study.

In conclusion, we identified several differentially expressed genes in the leaves of two local Palestinian grape varieties upon exposure to abiotic stresses such as drought and heat. Comparison with the literature of other grape varieties and other plant species indicate that most of the genes we identified in our analysis are generally involved in

response to such stressors. However, we were also able to identify novel genes involved in stress responses such as the *SSPL* and the *MLA10* disease resistant, which were not shown yet to be involved in stress responses. Otherwise, also genes with potential genotype- and/or tissue-specific differential expression in stress responses were identified in our analysis (*DXS* and *G6PDH*). These specificities and the fact that field studies with combined stressors as in our study are still scarce indicate that more investigations are required to identify stress mechanisms in grapes – and other plants – in response to heat and drought stress.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aggene.2017.11.001.

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