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ORIGINAL PAPER

Heat and water stress induce unique transcriptional signatures of heat-shock proteins and transcription factors in grapevine

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Abstract Grapevine is an extremely important crop worldwide. In southern Europe, post-flowering phases of the growth cycle can occur under high temperatures, excessive light, and drought conditions at soil and/or atmospheric level. In this study, we subjected greenhouse grown grapevine, variety Aragonez, to two individual abiotic stresses, water deficit stress (WDS), and heat stress (HS). The adaptation of plants to stress is a complex response triggered by cascades of molecular networks involved in stress perception, signal transduction, and the expression of specific stress-related genes and metabolites. Approaches such as array-based transcript profiling allow assessing the expression of thousands of genes in control and stress tissues. Using microarrays, we analyzed the leaf transcriptomic profile of the grapevine plants. Photosynthesis measurements verified that the plants were significantly affected by the stresses applied. Leaf gene expression was obtained using a high-throughput transcriptomic grapevine array, the 23K custom-made Affymetrix Vitis GeneChip. We identified 1,594 genes as differentially expressed between control and treatments and grouped them into ten major functional categories using MapMan software. The transcriptome of Aragonez was more significantly affected by HS when compared with WDS. The number of genes coding for heat-shock proteins and transcription factors expressed solely in response to HS suggesting their expression as unique signatures of HS. However, a cross-talk between the response pathways to both stresses was observed at the level of AP2/ERF transcription factors.

Keywords Heat stress · Microarrays · Transcriptomics · *Vitis vinifera* · Water deficit stress

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Introduction

As sessile organisms, plants are constantly exposed to changes in temperature and other environmental factors. Worldwide, extensive agricultural losses are attributed to temperatures above the normal optimum, sensed as heat stress (HS), that often occur in combination with drought, high light, or other forms of abiotic stresses (Mittler 2006). Photosynthesis is a physiological process particularly sensitive to HS either by a decrease in CO_2 fixation due to closing of stomata or to impairment of photochemical reactions (Wang et al. 2010). HS disturbs cellular homeostasis and can lead to severe retardation in growth and development and even to death. HS and water deficit stress (WDS) represent two abiotic stresses that often occur simultaneously in the field, namely in traditional grapevine-growing areas as is the case of southern Europe, a Mediterranean climate region. Furthermore, several available scenarios for climate change suggest an increase in aridity in the Mediterranean region in the near future (Jones et al. 2005). Drought effects and irrigation treatments in grapevine have been comprehensively studied at the physiological level (Chaves et al. 2007). The response of plants to drought, salt, and co-occurring stresses highlights photosynthesis and cell growth as among the primary processes affected by water or salt stress (Chaves et al. 2009; Cramer et al. 2007). In fact, abiotic stresses lead to a series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and productivity (Wang et al. 2003). Drought, salinity, extreme temperatures, and oxidative stress are often

interconnected and may induce similar cellular damage. For example, oxidative stress, which frequently accompanies high temperature and drought, may cause denaturation of functional and structural proteins (Wang et al. 2003). It is common that these diverse environmental stresses activate similar signaling pathways and cellular responses. For instance, drought can cause a variety of symptoms common to other primary abiotic stresses, such as heat, high concentrations of salt and other toxic solutes, and nutrient deficiency, and the symptoms can vary in location and time (Roy et al. 2011). Furthermore, there is a diversity of mechanisms and combinations of mechanisms which can be used by plants to tolerate each of these stresses (Roy et al. 2011). The major categories of genes activated upon the onset of abiotic stress include those involved in signaling cascades, those coding for proteins directly associated to the protection of membranes, those involved in water and ion uptake and transport such as aquaporins and ion transporters, and those coding for heat-shock proteins (Hsps) and chaperones, late embryogenesis abundant (LEA) proteins, osmoprotectants, and free radical scavengers (Wang et al. 2003). The adaptation of field plants to multiple stresses is a complex response that relies upon molecular networks that regulate stress perception, signal transduction, and expression of specific stress-related genes and metabolites (Vinocur and Altman 2005). Plants adapt to changing environmental conditions by responding to different simultaneous stress signals (Reusink and Buell 2005). One example is that under HS plants avoid raising leaf temperature by opening the stomata and increasing transpiration. However, when HS is combined with WDS, the control of leaf temperature is compromised because stomata do not open (Mittler 2006). The primary effects of individual stresses analyzed in grapevine under controlled conditions can elucidate important resistance and/or tolerance mechanisms (Cramer 2010). Aragonez (syn "Tempranillo") is an important red variety highly used in wine making which has been studied at the level of berry transcriptomics (Grimplet et al. 2009), but not of the leaves. This variety has been considered as isohydric ("drought avoider") (Chaves et al. 2010) both in the field as in greenhouse trials (Medrano et al. 2003; Sousa et al. 2006) because its stomatal guard cells react to chemical and hydraulic signals by closing the stomata and maintaining leaf water potential, despite the decrease of soil and root water potentials. The result is a relatively constant leaf water potential, but a declining stomatal conductance as the stomata are closed. Consequently, there is little initial relationship between soil water potential and leaf water potential.

Approaches such as array-based transcription profiling allow the assessment of expression levels of thousands of genes in control and stress tissues. The huge amount of data that can be analyzed offers a unique opportunity to infer the principles that govern the regulation of gene expression in plants (Rizhsky et al. 2004). However, the most important advantage of microarray-based technology is that large data sets from different experiments can be combined together in a single database, which allows gene expression profiles from either different samples or samples obtained using different treatments to be compared with each other and analyzed together. As these lists can be long, it is hard to interpret the desired experimental treatment effect on the physiology of the analyzed organism, e.g., via selected metabolic or other pathways. For *Vitis vinifera*, gene ontologies and data visualization software have been implemented to overcome this problem (Pontin et al. 2010). Greenhouse plants obtained from cuttings pruned from field plants were subjected to individual HS and WDS, and leaf RNA was analyzed with the oligonucleotide array 23K Affymetrix GrapeGen GeneChip corresponding to circa 68 % of genes annotated in the grapevine genome (12X version) (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). Transcript profiling of grapevine leaf response to WDS and HS was obtained for the first time using the custom GrapeGen GeneChip, the 12X annotation of grapevine genome, and MapMan software for functional classification of differentially expressed genes. It is expected that the results obtained so far and presented in the current study will be instrumental for the molecular analysis of grapevine responses to environmental stress.

Materials and methods

Plant material and growth conditions

V. vinifera L. variety Aragonez shoots were collected in the field during the winter pruning season. Collected shoots were disinfected with 2 g L^{-1} fungicide (mixture of cyprodinil and fludioxonil), wrapped in absorbent paper, and placed at 4 °C for circa 2 months. Then, they were placed in distilled water under controlled conditions (25 °C, 50 μmol m^{-2} s^{-1}) for 2 weeks upon which they had developed roots and several new leaves. They were transplanted to pots filled with sterilized soil collected in the field and placed in the greenhouse under 200 μmol m^{-2} s^{-1} light intensity, 16 h light/8 h dark photoperiod, temperature of 25 °C at day/23 °C at night, and irrigation when necessary with nutrient solution (Knight and Knight 2001). When plants were circa 50 to 60 cm high, with more than ten expanded leaves, stresses were applied to groups of six plants, with the following experimental setup: HS −1 h at 42 °C; WDS, irrigation withdrawn until pre-dawn leaf Ψ_w=−0.9 MPa, a standard value for intense stress in greenhouse grown plants. It took circa 4 days to attain those Ψ_w values. To assess the physiological effects of stress treatments, light responses (A/I) curves were measured on the third fully expanded leaf from four plants per treatment and in the control, immediately after HS, and when Ψ_w was −0.9 MPa in WDS (Coito et al. 2012), using an open

gas exchange system (LI-6400XT Portable Photosynthesis System, LI-COR Biosciences, Lincoln NE, USA) connected to an individual leaf chamber 6400-02B LED Light Source 2.5×2.5 cm. Light curves were performed at ambient CO_2 (approximately 350 μmol mol^{-1} CO_2) at different irradiances (I) with measurements recorded every 380 s or less when photosynthesis rate had stabilized, at each irradiance, rising stepwise from 0 to 1,500 μmol m^{-2} s^{-1}. Statistically significant differences between the control and the stress condition were determined for each irradiance (Rhue et al. 1978) by a two-tailed t test ($p<0.05$; Supplementary Fig. 1).

RNA isolation and hybridization to Affymetrix GeneChips

The third to fifth fully expanded leaves from four plants in control, WDS, and HS were collected, pooled, and frozen for RNA extraction. We used two biological replicates from the control and each stress. Total RNA was extracted through the method described by Reid et al. (2006) to give yield and purity necessary to perform microarray hybridizations. Samples were analyzed at the Genomics Unit of the Spanish National Centre for Biotechnology (CNB-CSIC, Madrid). RNA integrity analyses were done with an Agilent Bioanalyzer 2100 using the NanoChip protocol. Biotinylated RNA was prepared from 2 μg of total RNA according to the standard Affymetrix protocol. Briefly, RNA were reverse transcribed to produce first strand cDNA using an oligodeoxythymidylic acid 24 primer with a T7 RNA polymerase promoter site added to the 3′ end. After second strand synthesis, in vitro transcription was performed using T7 RNA polymerase and biotinylated nucleotides, to produce biotin-labeled cRNA. Labelled cRNA was fragmented to the 50–200-bp size range, and quality control was checked using the Bioanalyzer 2100 using the NanoChip protocol. If the quality control was correct, then 10 μg of fragmented cRNA was hybridized to the GrapeGena 520510F array (Affymetrix, Santa Clara, CA). Each sample was added to a hybridization solution containing 100 mM 2-(*N*-morpholino)ethanesulfonic acid, 1 M Na+, and 20 mM of EDTA in the presence of 0.01 % of Tween-20 to a final cRNA concentration of 0.05 μg/ml. Hybridization was performed for 16 h at 45 °C. Each GeneChip was washed and stained with streptavidin-phycoerythrin in a Fluidics Station 450 (Affymetrix) following the EukGE-WS2v5 script. Upon completion of the washing, the chips were scanned at 1.56 μm resolution in a GeneChip® Scanner 3000 7G System (Affymetrix). The software used was GeneChip Operating Software.

GeneChip data analysis

Scanned arrays were analyzed first with Affymetrix Expression Console software to obtain Absent/Present calls using the MAS 5.0 method. Subsequent analysis was carried out with DNA Chip Analyzer 2008. The six arrays were normalized to a baseline array with median CEL intensity by applying an Invariant Set Normalization Method (Li and Wong 2001b). Normalized CEL intensities of the arrays were used to obtain model-based gene expression indices based on a perfect match-only model (Li and Wong 2001a). Replicate data (duplicates) were weighted genewise by using inverse squared standard error as weights. All genes compared were considered to be differentially expressed if the 90 % lower confidence bound of the fold change between experiment and baseline was above 1.6 and 1.3, based on false discovery rate. The lower confidence bound criterion means that we can be 90 % confident that the fold change is a value between the lower confidence bound and a variable upper confidence bound. Li and Wong (2001a) have shown that the lower confidence bound is a conservative estimate of the fold change and therefore more reliable as a ranking statistic for changes in gene expression. For a second analysis, Partek Genomics Suite 6.5 was used. Here, the six arrays were normalized and modeled using Robust Multichip Averaging. Differential expression was determined using t test. Finally, a p value cutoff of 0.05 was used to select differentially expressed genes in drought and heat stress, respectively. A gene was declared to be differentially expressed in a given condition (WDS or HS) only when it had a presence call in both replicates. The advantages of using two statistical procedures was providing the necessary solidity to biological information and to make the selection of differential expressed genes robust, since only two replicates were used per sample. The subsequent validation of this approach was performed by quantitative real-time PCR (qRT-PCR).

Analysis of redundant probe sets

Redundant probe sets are probe sets that measure different regions of the same target gene. Previous work showed that some of the lack of agreement between the profiles from the redundant probe sets is likely associated with incorrect gene models and annotation problems (Cui and Loraine 2009). Thus, the potential ability of redundant probe sets to shed light on the regulation of mRNA variants is somewhat clouded by ambiguities in annotation, i.e., mapping probe sets onto their putative target genes. Some of these efforts have helped to expose problematic or potentially faulty probes, such as probes that map to multiple locations in the genome or, conversely, probes that do not appear to map to any location within the designated target locus.

After the application of the statistical treatment previously described, the redundancy was reduced from 54 % (chip redundancy) to 22.5 % in our data. To eliminate the presence of still problematic probe sets, we checked manually for a lack of consistency between these 22.5 % redundant probe sets through genome-based screening using Genoscope (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). If

the redundant probe sets for a given gene indeed measure the same target transcripts, then they should yield consistent results in the same experiment with the allowance of some variation. We did not find probe sets assigned to the same gene and showing differentially inconsistent (up versus down) expression changes. When two or more probe set sequences belong to the same group, these probe sets are considered redundant because they measure the same gene region or transcriptional unit.

Validation of the microarray results through qRT-PCR

Total RNA was extracted as described above. RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI). cDNA was synthesized from 2 μg of total RNA using oligo(dT)$_{20}$ in a 20-μL reaction volume using Revert Aid Reverse Transcriptase (Fermentas Life Science, Helsingborg, Sweden) according to the manufacturer's recommendations. Detailed description of the methodology used for qRT-PCR can be found in Supplementary Fig. 2.

Data availability

Microarray data analyzed in this study have been submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) under the number GSE36849.

Results

Analysis of differential gene expression

The statistical analyses of the microarray data resulted in 596 and 1,461 differentially expressed transcripts identified for water (WDS) and heat stress (HS), respectively (Fig. 1a). After redundancy removal, the final number of genes was reduced to 469 and 1,125, respectively, for WDS and HS (Fig. 1a, b; Supplementary Figs. 3 and 4). These numbers of differentially expressed genes represent 2.9 and 7.1 %, respectively, of the total number of nonredundant probe sets (15,800) present in the GrapeGen chip (Pontin et al. 2010). All further analyses focused on these two core sets. A number of genes encoding proteins of interest were found to be up- or down-regulated under stress conditions in the present study. The Venn diagram (Fig. 1b) shows 108 up-regulated and 62 down-regulated common genes after both stress treatments. Only one gene was found to be up-regulated in WDS and down-regulated in HS, while five genes down-regulated in WDS change the regulation tendency in HS (Fig. 1b). Additionally, promoter analysis of common genes to both stresses revealed similar GCC box-binding domains for APETALA2 (AP2)/ethylene-responsive factor (ERF) transcription factors as shown for five transcripts (Supplementary Fig. 5).

a

	LCB (WS >1.6) and (HS>1.3)&p-value<0.05		
	Up	Down	Total
HS	922	539	1461
WS	324	272	596
	Without redundancy		
HS	659	466	1125
WS	254	215	469

b

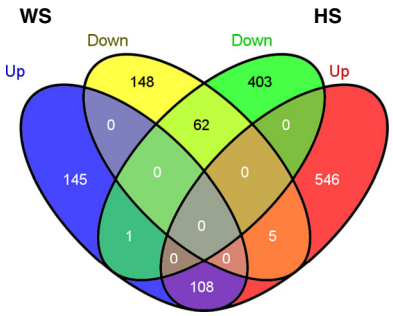

Fig. 1 Global expression data in grapevine, variety Aragonez, obtained from microarray analysis. **a** Number of probe sets on the Aragonez microarray showing significant expression changes (up- or down-regulation) in response to water and heat stresses in comparison to the control. *HS* heat stress, *WDS* water stress, *LCB* lower confidence bound of fold change. **b** Venn diagrams showing the number of probe sets that were common and distinct in each abiotic condition (Supplementary Figs. 3 and 4)

We showed that the application of principal components analysis to the expression data based on all genes present on the array allowed to summarize gene responses under different conditions (Fig. 2). The three-dimensional plot shows that the replicates for each experimental condition are well grouped with HS showing a much stronger effect than WDS. In fact, when compared to WDS, HS caused a more than twofold higher number of responsive genes. Principal component #1 explains 30.2 % of the variability and splits water and the control from heat stress. Principal component #2 explains 25.1 % and splits water from control. All the principal components together explained 75.6 % of the total variance (Fig. 2) and the remaining variability is mainly explained by experimental noise.

Functional clustering analysis

To analyze the expression profiles of genes in different biological functional groups, the 1,594 selected genes were annotated for biological processes using Grapegene ontology (http://bioinfogp.cnb.csic.es/tools/grapegendb/). The annotated genes were then categorized into ten functional groups and analyzed based on the gene expression levels (Table 1). The functional

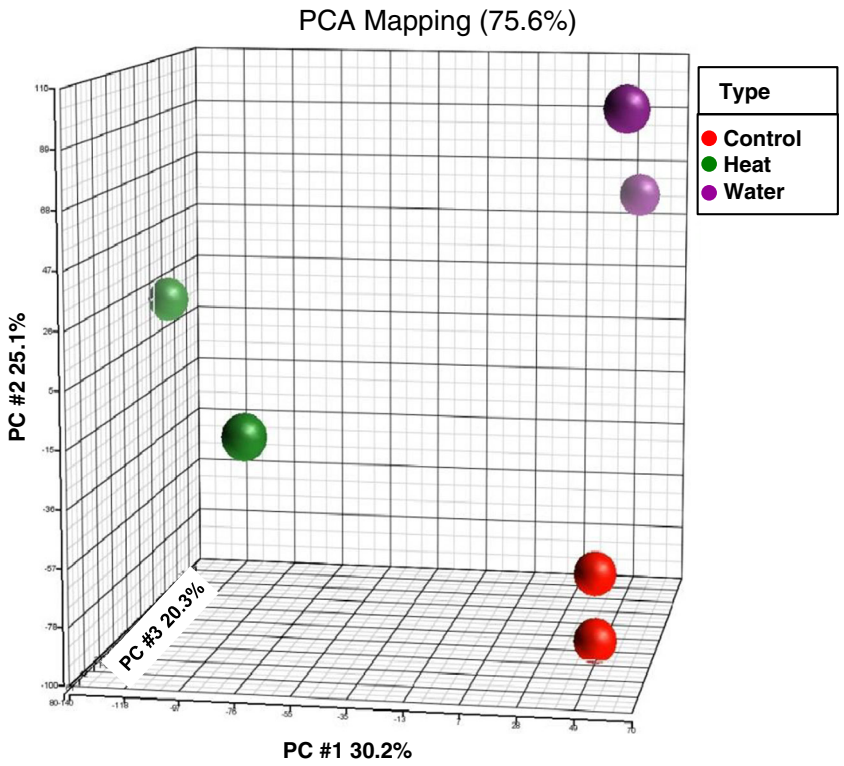

Fig. 2 Principal component analysis of array data. The expression values of all samples from the arrays were analyzed by principal components analysis

classification of the differentially expressed genes and the pictorial representation of the significantly modulated classes were accomplished using MapMan onthology (Thimm et al. 2004) with files specifically designed for the GrapeGen GeneChip (Pontin et al. 2010), followed by the 12Xv0 version of the GrapeGen GeneChip functional annotations (http://bioinfogp.cnb.csic.es/tools/grapegendb/) supported by Genoscope annotation 12X. Considering the number of genes assigned to each functional category and their expression level, in the present study, the most relevant category was unknown which includes genes not yet annotated (Fig. 3 and Table 1), as already reported for this species (Cramer et al. 2007). Nevertheless, a meaningful number of functional categories and subcategories (protein metabolism, transcription factors, signaling, and abiotic stress) including a number of genes expressed at significantly up- or down-regulated levels are presented in Figs. 4, 5, 6, and 7. Gene expression is presented as log2 fold change (Supplementary Figs. 6 and 9) where each

Table 1 Functional categories present in GrapeGen Chip. Number of probe sets showing differential expression in water and heat stresses after removing the redundancy

BIN (MapMan)	Functional category	No. of Bin probe sets (GrapeGen Chip)	No. of differentially expressed probe sets		Total
			Water stress	Heat stress	
1	Cellular process	1,140	29	64	93
2	Development	156	4	8	12
3	Diverse functions	305	9	12	21
4	Metabolism	5,865	127	323	450
5	Regulation	1,395	50	140	190
6	Response to stimulus	705	18	32	50
7	Signaling	1,487	56	125	181
8	Transport	1,104	24	40	64
9	Unknown	7,986	148	377	525
10	Xenoprotein	388	4	4	8
Total		20,532	469	1,125	1,594

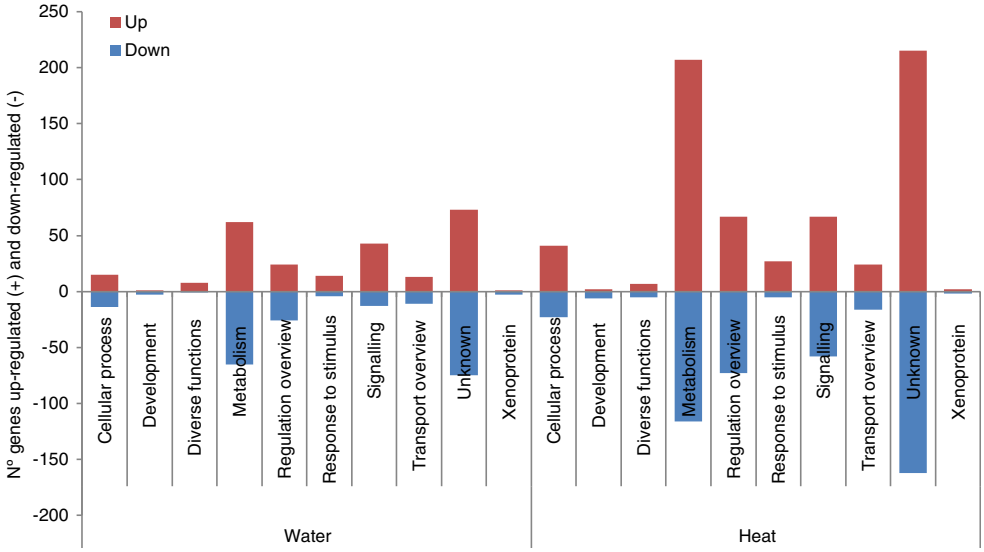

Fig. 3 Graphical representation of differentially expressed genes in water and heat stress. Classes are represented according to MapMan functional categories as in Table 1

gene is considered greatly, moderately, or slightly induced or inhibited as log2 fold change is, respectively, higher/lower than ±5, ±3, and ±1.5.

Most relevant functional categories

In the present study, the genes included in protein metabolism and assigned to protein folding (63 genes) exhibited the highest differential expression in response to HS (Fig. 4b and Supplementary Fig. 6); 22 greatly and 18 moderately up-regulated, while only one transcript was slightly down-regulated. Genes representative of all Hsp classes were heat responsive: Hsp70/DnaK genes XP_002282143 and CBI36549.3 were greatly and CBI16157.3, XP_002282802, and XP_002276268.2 were moderately up-regulated; most Hsp40/DnaJ transcripts, co-chaperones of Hsp70/DnaK, were slightly or moderately up-regulated, while XP_002283060.1 was slightly down-regulated. Among chaperonins differentially expressed upon HS, XP_002284449.1 and XP_002284449.1 were, respectively, strongly and moderately up-regulated; small heat-shock proteins, sHsp, were the most abundantly expressed Hsp class, with 18 strongly up-regulated transcripts in response to HS. Conversely to HS, the few WDS-responsive genes were down-regulated (Fig. 4a and Supplementary Fig. 6), and only the chaperonin XP_002284449.1 was present in common to HS (Supplementary Fig. 10). Among the genes assigned to the proteolysis subfunctional category, HS induced the up-regulation of the metalloprotease FtsH (XP_002283393.2) by more than 30-fold and of two ubiquitin-mediated F-Box protein genes by more than 20-fold, while a moderate response to WDS was the up-regulation of two cysteine proteases and the down-regulation of one ubiquitin-mediated protein gene.

The results obtained with the MapMan software assigned transcription factor (TF) genes to different families: AP2, bZIP, zinc finger (C2H2, C3H, C3HC4), GRAS, MYB, NAC, WRKY, and bHLH (Fig. 5 and Supplementary Fig. 7). As a whole, TF genes raised more responsive to HS than to WDS. However, the AP2/DREB gene CBI27772.3 and the AP2/ERF gene CBI32415.3 were greatly responsive to HS, but also moderately up-regulated upon WDS. The array probes hybridized with WRKY transcript sequences, namely WRKY 6, 18, 40, and 48, were moderately up-regulated, WRKY18 (encoding XP_002285255.1) responding to both stress treatments (Supplementary Figs. 7 and 10). Specific WDS response can be assigned to the slight repression of one RING C3HC4 transcript and the moderately up-regulation of WRKY11, 23, and 53 transcript sequences. Finally, a unique heat-shock factor annotated as heat-shock-activated transcription factor (Hsf) A-6b was moderately up-regulated after HS.

In the abiotic stress response, a MapMan onthology subfunctional category of response to stimulus, HS affected 41 out of the 45 expressed transcripts (Table 1, Fig. 6, and Supplementary Fig. 8), almost all up-regulated. In the MapMan annotation, one of the subcategories of abiotic stress response, temperature stress response, replicates the genes present in the subcategory protein folding in protein metabolism and modification, already addressed. HS also gave rise to a mainly moderate up-regulation of genes associated with several secondary stresses such as oxidative and osmotic stress, especially free radical scavengers such as peroxidases, glutathione transferases, ascorbate peroxidases, and glutaredoxins, and only two peroxidases suffered down-regulation. After WDS, a few genes coding for reactive oxygen species (ROS) scavengers, as laccases and glutaredoxins, were moderately induced while one dehydration-induced protein (ERD15) was down-regulated (Supplementary Fig. 8).

In what concerns the signaling functional category transcripts, HS affected more than twice as many (125) than WDS (56). From the subcategories, this functional category is divided in four which were significantly represented in this

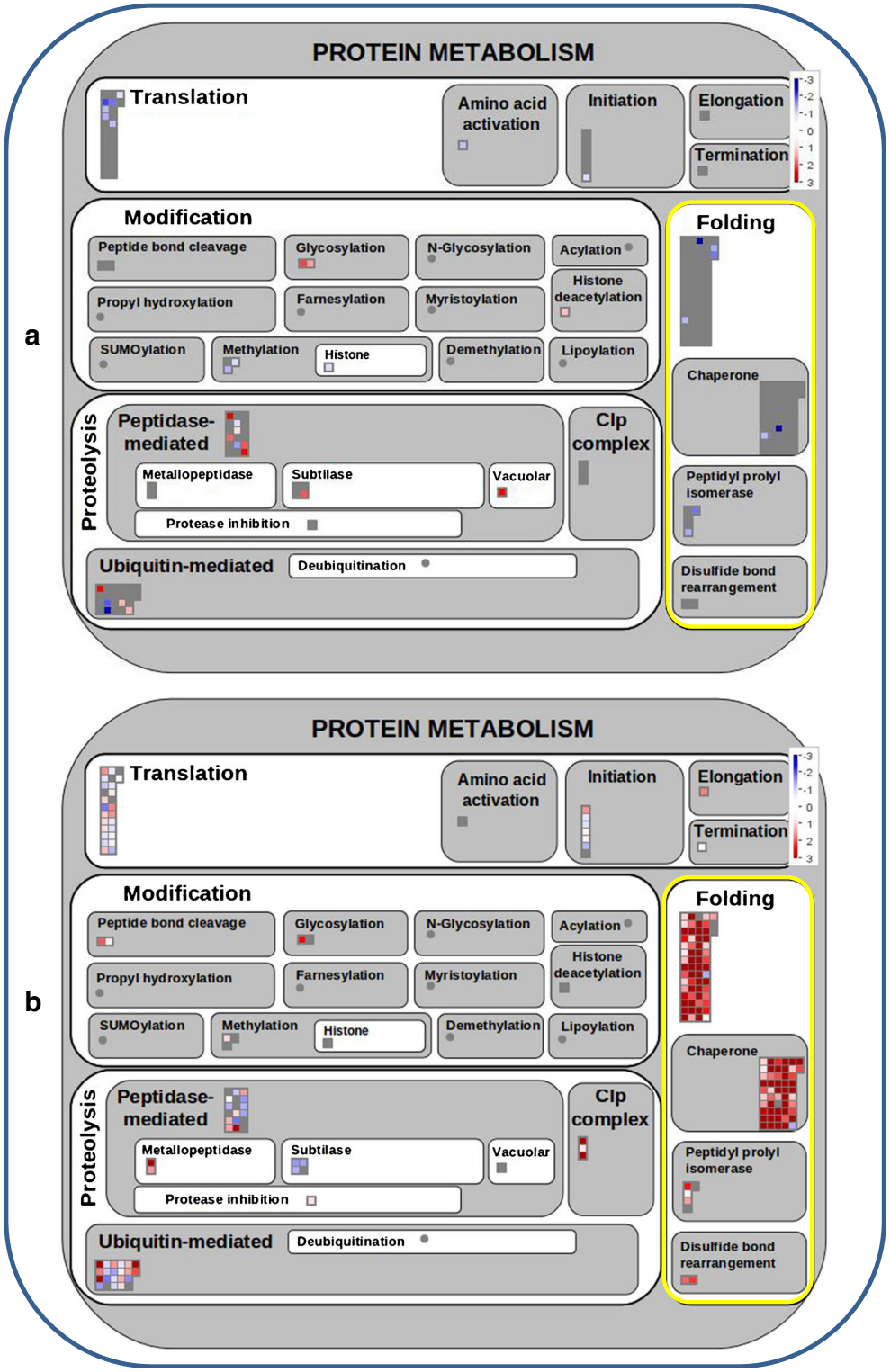

Fig. 4 Protein metabolism response. MapMan overview of "pathway" modulation under water (**a**) and heat stress (**b**). The genes shown are listed in Supplementary Fig. 6

work: signaling (21 genes), G protein signaling (14 genes), hormone signaling (51 genes), and protein kinase signaling (77 genes) (Supplementary Fig. 9). Both stresses applied induced the transcription of calcium sensing and calcium signaling molecules (Fig. 7). In this subcategory, specific calmodulin-related genes were exclusively expressed after each stress and a sodium-inducible calcium-binding protein (ACP1) was expressed at a similar level after both stresses (Supplementary Fig. 10). From G protein signaling elements (Fig. 7), HS led to a strong up-regulation of Rab/YptGTPase Ara4-interacting protein, while WDS slightly down-regulated the transcription of two G proteins, one in common with HS

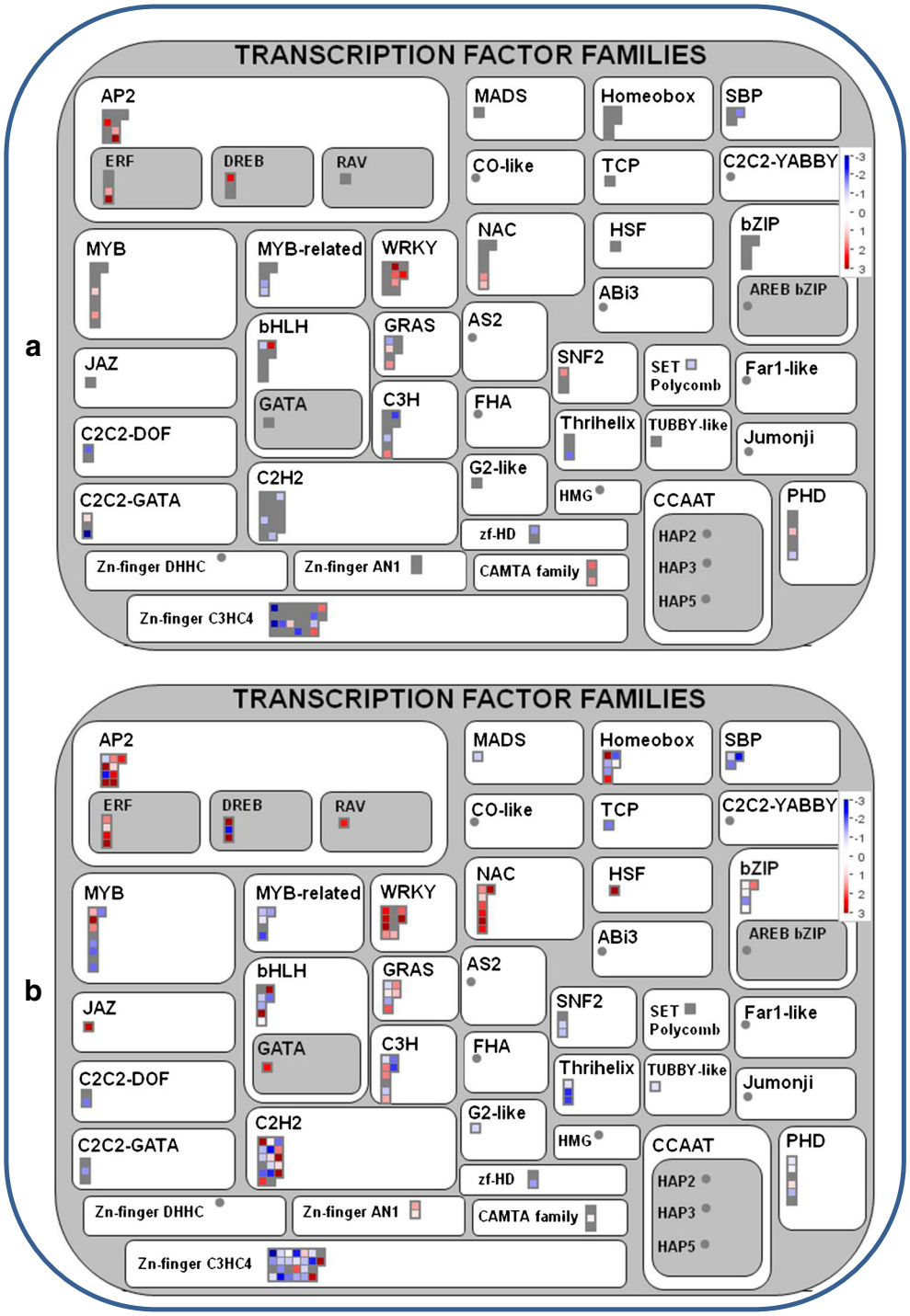

Fig. 5 Transcription factors response. MapMan overview of "pathway" modulation under water (**a**) and heat stress (**b**). The genes shown are listed in Supplementary Fig. 7

(Supplementary Fig. 10). In hormone signaling, twice the number of transcripts responded to HS as compared to WDS, mostly only moderately (Supplementary Fig. 9). Both HS and WDS led to moderate up-regulation of abscisic acid (ABA)-responsive genes. Concerning indole-3-acetic acid (IAA)-related genes, IAA 16 responded positively to both stresses, auxin-responsive factors were down-regulated after HS, and IAA-amidosynthetase gene was slightly down-regulated after both stresses. WDS led to an equivalent up- or down-regulation of two pathogenesis-related protein 1 salicylic acid-responsive genes. Ethylene response factor (ERF1, XP_002281052.1) may play a central role in stress response since its expression was up-regulated after both stresses (Fig. 7). The largest abiotic stress signaling subcategory, protein kinase signaling, is widespread and diverse, comprising mitogen-activated protein kinases (MAPKs), serine/threonine

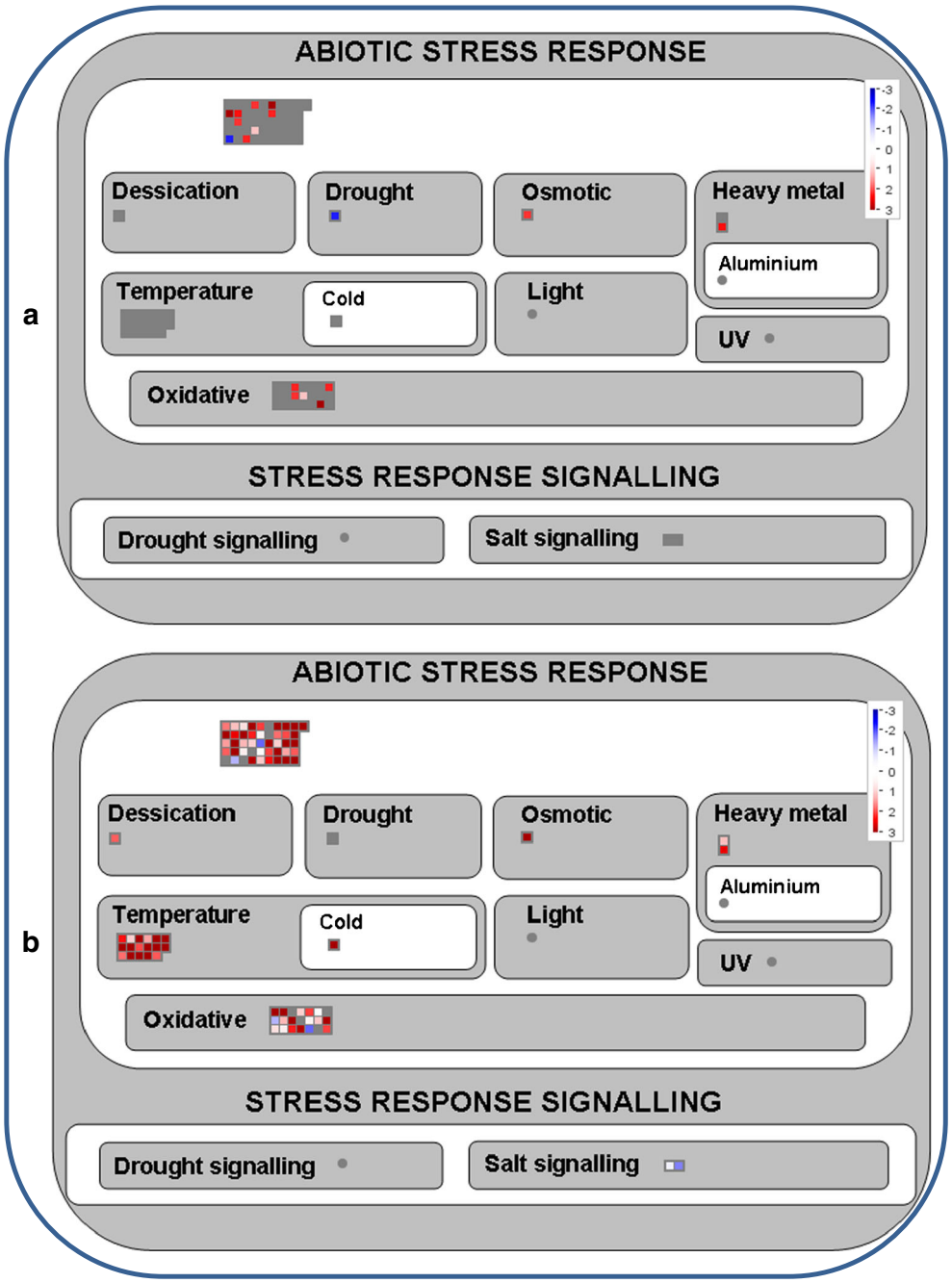

Fig. 6 Abiotic stress response. MapMan overview of pathway" modulation under water (**a**) and heat stress (**b**). The genes shown are listed in Supplementary Fig. 8

protein kinases, wall-associated kinases, among others (Fig. 7 and Supplementary Fig. 9). The highest level of up-regulation occurred for MAPKKK21 after HS and for serine/threonine kinase after WDS (Fig. 7).

Discussion

The expression of a meaningful number of genes assigned to the functional categories protein metabolism, transcription factors, signaling, and abiotic stress was significantly induced or inhibited and their roles deserve the discussion that follows. HS, applied as a short-term acute stress, gave rise to more than twice as many responsive genes than WDS. The most striking result obtained was the number and level of up-regulated genes related with protein folding in response to 1-h HS treatment. The genes coding for Hsps are highly conserved in both prokaryotes and eukaryotes and act as molecular chaperones assisting the folding of translated polypeptides and preventing the aggregation of nonfunctional proteins, without becoming part of the final structure. In *Arabidopsis*, the products of those genes (number in brackets) are distributed into families

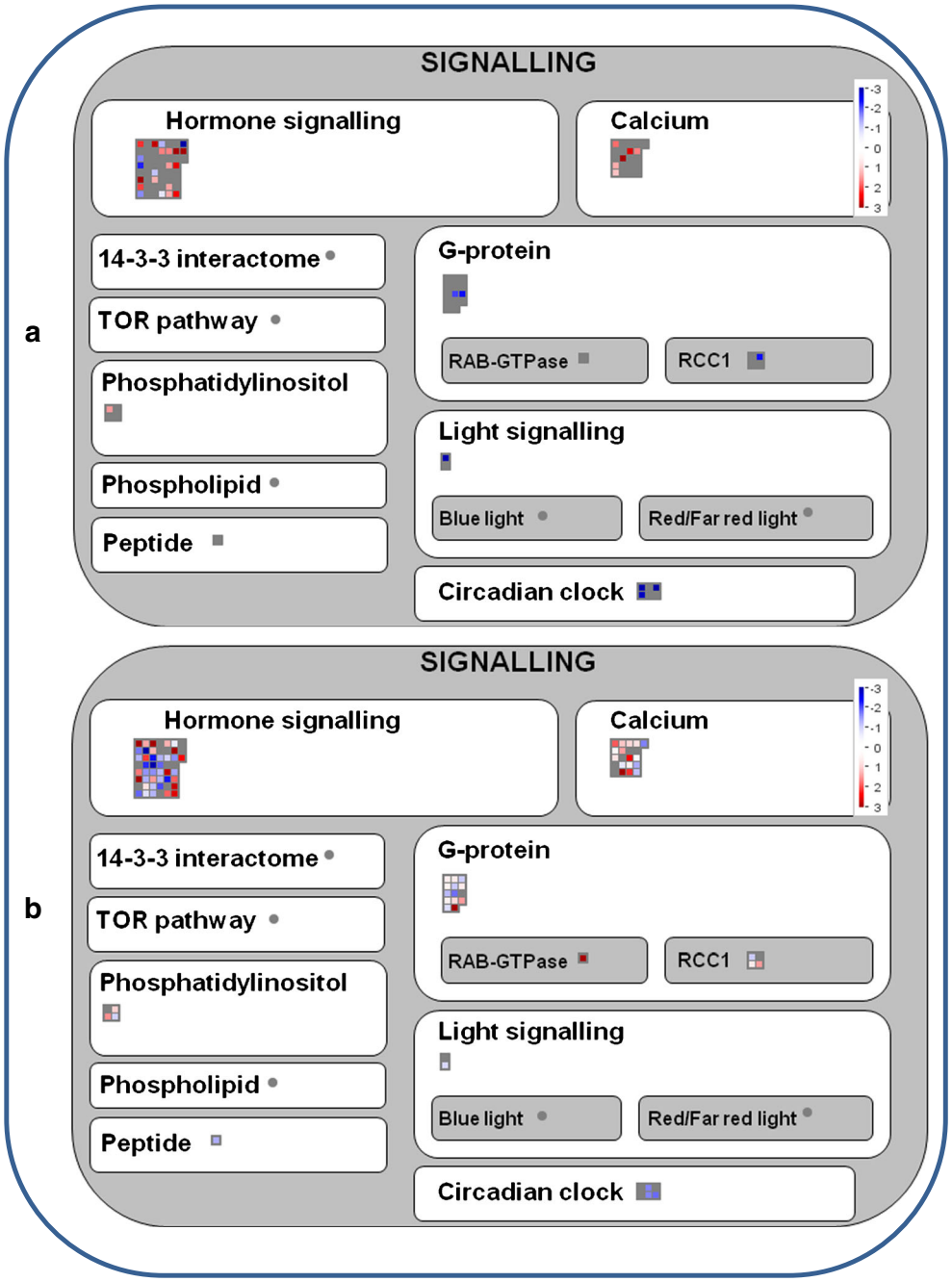

Fig. 7 Signaling response. MapMan overview of pathway modulation under water (**a**) and heat stress (**b**). The genes shown are listed in Supplementary Fig. 9

according to their molecular weight and sequence homology, including homology to *Escherichia coli* proteins (eukaryotic/prokaryotic): Hsp100/Clp, (1–2); Hsp90, (7–8); Hsp70/DnaK, (10); Hsp40/DnaJ, (9); Hsp60/GroEL, (21); and sHsp (18) located in the cytoplasm, the nucleus, mitochondria, chloroplasts, and the endoplasmic reticulum (Feder and Hofmann 1999; Gupta et al. 2010; Kotak et al. 2007). Hsp70/DnaK are essential for normal cell function. Some are molecular chaperones expressed constitutively, while others are induced by heat or cold stress. Assuming that HS-induced transcripts are translated, their products must promote chaperone activity, although specific information on the contribution of Hsp70 to HS resistance in plants is still scarce (Wang et al. 2003). Hsp90 and their respective co-chaperones are abundant, evolutionarily conserved, and can account for 1 % of Hsp in unstressed conditions. As Hsp90 are not detected in association with polypeptide chains emerging from ribosomes, they are considered to have a general role in the refolding of misfolded proteins that can accumulate during plant development or in response to various biotic and abiotic stress conditions, sensing the environment and mediating appropriate phenotypic plasticity (Mayer and Bukau 1999; Sangster and Queitsch 2005).

Here, five transcripts coding for Hsp90 were induced by HS, two of them strongly up-regulated. Chaperonins, the protein complexes (Hsp10/GroES, Hsp60/GroEL) that assist the folding of newly synthesized proteins imported to the mitochondria and the chloroplast in an ATP-dependent manner (Mayer and Bukau 1999), were the only group within the functional category folding exhibiting the expression of a common transcript between the two types of stress. Moreover, this was the only transcript up-regulated after HS and down-regulated after WDS. This specific response to the two forms of abiotic stress points to an interesting gene for further analysis. Heat-shock response in plants underlies the expression of a high number of low molecular weight Hsps (sHsps) that can be large polymers of mostly 20 kDa monomers with a C-terminal-conserved domain of 80–100 amino acids; the "alpha-crystallin domain" first identified in vertebrate eye lens as a structural protein (Kotak et al. 2007). Plants have long been recognized as having the most complex sHsp gene family with 18 genes coding for putative sHsps in *Arabidopsis thaliana*, even larger numbers in rice and poplar (Ganea 2001; Siddique et al. 2008), and 13 in grapevine (Genoscope 12X, http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). sHsps can be present in the nuclear–cytoplasmic compartment and plastids, mitochondria, endoplasmic reticulum, and peroxisomes (Kotak et al. 2007). Functionally, sHsps participate in the response to different abiotic and biotic stresses and confer thermotolerance in an ATP-independent way by selectively binding and stabilizing proteins, preventing their aggregation at elevated temperatures, and protecting enzymes against heat-induced inactivation (Ganea 2001). Although not much evidence is available about the function of sHsps as chaperones in normal protein synthesis (Kotak et al. 2007), a cluster analysis in *Arabidopsis* revealed strong similarities among sHsp genes with respect to stress response patterns and development series (Swindell et al. 2007). The accumulation of sHsps transcripts during HS is well reported, although in vivo function of these Hsps is not fully clarified. However, the overexpression of a chloroplast sHsp protects photosystem II under some stress conditions and the overexpression of a mitochondrial sHsp enhances thermotolerance (Siddique et al. 2008). The number and expression levels reported here make sHsps interesting candidates for the identification of marker genes of HS response in grapevine.

The expression of Hsps is mainly regulated at the transcriptional level by Hsfs (Iba 2002). The induction of Hsps depends on the temperature at which each species ordinarily grows. In higher plants, *Hsps* are generally induced by a short exposure to a temperature at or above 40 °C (Larkindale et al. 2005). Among the 21 Hsfs identified in the *Arabidopsis* genome and divided in three evolutionary classes, A, B, and C, based on their oligomerization domains, evidence only confirms six to be heat induced (Koskull-Döring et al. 2007 ; Larkindale and Vierling 2008; Nover et al. 1996). In the present HS experimental conditions applied to grapevine, only one Hsf revealed ad HS responsive. The ubiquitination pathway is closely associated with abiotic stress tolerance and F-box proteins recognize substrates through the Skp1-Cullin-F-box complex for degradation by ubiquitin-26S proteasome (Mazzucotelli et al. 2008). A study comparing the F-box gene number in the genome of herbaceous and woody plants found a much more expanded F-box gene family in the former species (Yang et al. 2008). The authors proposed that in woody plants, a smaller F-box gene family complex may reflect a comparatively reduced need for ubiquitination-mediated protein turnover in long-lived perennial species (Yang et al. 2008). In our study, F-box protein genes responded sharply to HS. Proteases are active in the renovation and processing of cell-impaired proteins (Simova-Stoilova et al. 2010). FtsHs are ATP-dependent thylakoid membrane integral Zn metalloproteases which remove unassembled or oxidatively damaged proteins from photosystem II (Adam et al. 2001). The net up-regulation of FtsH transcript may indicate that these proteins contribute to HS chloroplast tolerance. The moderate response induced by WDS is in accordance with other experiments. Although Tattersall et al (2007) obtained WDS through the use of PEG, and therefore attained much more rapidly than in the present work, the authors report a lower number of significantly affected genes, when compared with chilling. The hypothesis they put forward is that a more natural, gradually increasing stress (chilling) allowed for a more complex response in the acclimation process than the rapidly applied stresses (WDS and salinity) and therefore gave rise to a higher number of affected genes. Nevertheless, this does not seem to be the case in the present study since WDS was gradually imposed and the tendency for a lower number of responsive genes was maintained, which can lead to the hypothesis that the amount of affected genes is in fact related to the type of stress itself. Cysteine proteases are associated to both senescence and abiotic stresses, although the signature of cysteine protease polypeptides in response to WDS is described as unique (Khanna-Chopra et al. 1999). The transcripts of cystatins, which are regulators of endogenous cysteine proteinases playing roles in defense against biotic and abiotic stresses, were significantly up-regulated in the leaves of grapevine plants under WDS ψ=−0.75 MPa, a value similar to our WDS plants (Cramer et al. 2007). However, the lower activity and expression of certain cysteine proteases in leaves of a drought-resistant wheat cultivar under WDS was reported as an indicator of WDS resistance (Simova-Stoilova et al. 2010). Therefore, cysteine proteases and their corresponding regulator genes deserve further attention for the identification of specific markers for WDS tolerance. From the perception of stress signals to the expression of stress-responsive genes, TFs act as switches of transcription regulatory cascades for plant adaptation to environmental changes (Riechmann et al. 2000; Yamaguchi-Shinozaki and Shinozaki 2006). That was the rationale to analyze the results obtained in the functional category transcription factor families.

Transcription factor genes were assigned to different families (Fig. 5 and Supplementary Fig. 7), some directly associated to plant abiotic stress—AP2, zinc finger, MYB, bZIP, and WRKY (Chen et al. 2002)—and a number of them only found in plants—WRKY, NAC, and GRAS (Riechmann et al. 2000). Previous studies describe AP2/EREBP as mediating a rapid response to drought and cold stress in a direct interplay with ABA (Chen et al. 2002; Liu et al. 1998). AP2 and ERF transcription factors are overrepresented after ROS signaling events (Gadjev et al. 2006). The activity of a number of antioxidant genes responding to the HS point to a deregulation of redox homeostasis, what could explain the up-regulation of *AP2* transcription factors in conditions different from cold stress (Chen et al. 2002). WRKY belongs to one of the largest TF families in plants playing roles either in the repression or the de-repression of important plant processes (Rushton et al. 2010). In our study, WRKY40 and WRKY18, described as transcriptional repressors in *Arabidopsis* and taking part in plant response to abiotic stress (Chen et al. 2010), were up-regulated, the former after HS and the latter after both stress treatments.

Once again, HS affected significantly more stress-related transcripts than WDS. The up-regulation of the desiccation stress response protein LEA after HS can be explained by its function in maintaining the integrity of membranes and the stabilization of macromolecules, paramount in a response to severe heat stress (Umezawa et al. 2006). Also, the effects of primary stresses are often interconnected and can cause cellular damage and secondary stresses, explaining the HS-induced responses of genes associated with several secondary stresses such as oxidative and osmotic stress. Typically, after WDS, the above-referred genes are induced (Wang et al. 2003). In our work, among the up-regulated genes were two genes for laccases, proteins implicated in plant responses to ABA-mediated abiotic stress (Liang et al. 2006). The multiplicity of information embedded in abiotic stress signals underlies the complexity of stress signaling that mainly results from the coordinated action of various genes in a single pathway or in diverse pathways. Both stresses applied induced the transcription of calcium sensing and calcium signaling molecules. The proteins which sense cytoplasmic Ca^{2+} alterations and relay this information to downstream molecules act as an important component of signaling (Knight 2000; Knight and Knight 2001). A large number of hormones, local mediators, and sensory stimuli exert their effects on cells and organisms by binding to G protein-coupled receptors which play important roles in determining the specificity and temporal characteristics of cellular responses to signals (Hamm 1998). HS led to a significant up-regulation of Rab/YptGTPase Ara4-interacting protein while WDS affected the transcription of two G protein signaling elements.

Phytohormones are essential for the ability of plants to adapt to abiotic stresses by mediating a wide range of adaptive responses. They often alter gene expression by rapidly inducing

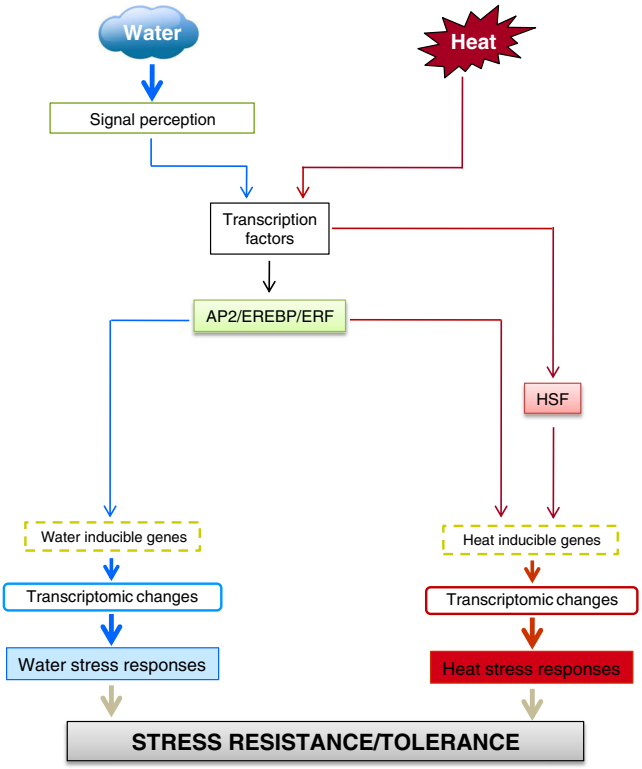

Fig. 8 A schematic diagram of transcriptional networks of transcription factors involved in heat and water stress responses. AP2/EREBP/ERF, Apetala2/ethylene-responsive element binding protein/ethylene response factor; *HSF* heat-shock factor

or preventing the degradation of transcriptional regulators (Santner and Estelle 2010). ABA signaling is described as a typical response to WDS (Wilkinson and Davies 2002), and ABA-responsive genes have been proposed as targets for genetic engineering for enhanced drought tolerance. In fact, the first response of plants when faced with severe water deficit is the closure of stomata, through an ABA-dependent mechanism. ABA-induced gene expression often relies on the presence of *cis*-acting elements ABRE, in target gene promoters (Liang et al. 2006). In this experiment, both HS and WDS led to moderate up-regulation of some ABA-responsive genes and ABA-related transcription factors. This renders ABA signaling genes as important targets for abiotic stress resistance in a broad concept, not just for drought resistance.

Ethylene seems to play a central role in both stresses pointing to a stress-mediated cross-talk in the ethylene signaling pathway (Sreenivasulu et al. 2007), evidenced by the expression of ethylene-responsive genes (Supplementary Fig. 5), such as ERF1 up-regulated in both conditions.

Conclusions

To understand and differentiate the multigenic traits that regulate grapevine drought and heat stress responses, we performed

a chip analysis in the leaves of Aragonez variety after a short-term (1 h) acute heat stress and a gradually increasing water stress (4 day). Surprisingly, HS affected 70 % of total Aragonez differentially expressed transcripts with a significant number and level of up-regulated genes related with protein folding, the molecular chaperones which prevent the aggregation of nonfunctional proteins. This is an expected behavior for HS since Hsps protect plants against heat damage, but a surprising result for WDS where a higher expression of genes assisting the processing of damaged proteins was anticipated and suggests that in fact the expression of Hsps is a unique signature of HS. The distinctive transcription regulation between HS and WDS was further put in evidence by the number of differentially expressed TF transcripts regulating inducible genes involved in stress tolerance. Out of the few genes with a common response (12 % of up-regulated and 9 % down-regulated), the only highly up-regulated common TF gene, AP2/ERF, can represent the cross-talk between the response pathways to both stresses (Fig. 8). In terms of survival, grapevine Aragonez variety responded to acute heat stress with an explosion of gene expression that must have high energetic costs, while it seems to be more tolerant to the lack of water, withstanding WDS by reducing gene expression to a minimum and increasing only those genes necessary to survive.

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Conflict of interest The authors declare that they have no competing interests.

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