Molecular Plant • Volume 4 • Number 6 • Pages 1038–1051 • November 2011 RESEARCH ARTICLE

Comparative Transcriptomic Profiling of *Vitis vinifera* Under High Light Using a Custom-Made Array and the Affymetrix GeneChip

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ABSTRACT Understanding abiotic stress responses is one of the most important issues in plant research nowadays. Abiotic stress, including excess light, can promote the onset of oxidative stress through the accumulation of reactive oxygen species. Oxidative stress also arises when *in vitro* propagated plants are exposed to high light upon transfer to *ex vitro*. To determine whether the underlying pathways activated at the transfer of *in vitro* grapevine to *ex vitro* conditions reflect the processes occurring upon light stress, we used *Vitis vinifera* Affymetrix GeneChip (VvGA) and a custom array of genes responsive to light stress (LSCA) detected by real-time reverse transcriptase PCR (qRT–PCR). When gene-expression profiles were compared, 'protein metabolism and modification', 'signaling', and 'anti-oxidative' genes were more represented in LSCA, while, in VvGA, 'cell wall metabolism' and 'secondary metabolism' were the categories in which gene expression varied more significantly. The above functional categories confirm previous studies involving other types of abiotic stresses, enhancing the common attributes of abiotic stress defense pathways. The LSCA analysis of our experimental system detected strong response of heat shock genes, particularly the protein rescuing mechanism involving the cooperation of two ATP-dependent chaperone systems, Hsp100 and Hsp70, which showed an unusually late response during the recovery period, of extreme relevance to remove non-functional, potentially harmful polypeptides arising from misfolding, denaturation, or aggregation brought about by stress. The success of LSCA also proves the feasibility of a custom-made qRT–PCR approach, particularly for species for which no GeneChip is available and for researchers dealing with a specific and focused problem.

Key words: Quantitative real-time PCR; *Vitis vinifera* GeneChip; light stress; Heat Shock Proteins; oxidative and photo-oxidative stress.

INTRODUCTION

A sessile lifestyle renders plants subject to multiple abiotic stresses because this type of organism cannot survive unless it is able to cope with environmental changes. Common environmental factors or 'abiotic stresses' include strong light, high and low temperatures, freezing, drought, salinity, and acid soils. In the near future, extreme environmental conditions are thought to increase in intensity and frequency, because of global climate change. Therefore, understanding abiotic stress responses is now believed to be one of the most important topics in plant research (Hirayama and Shinozaki, 2010).

Abiotic stresses promote the onset of oxidative stress brought about by an accumulation of reactive oxygen species (ROS; Apel and Hirt, 2004). An extensive network of low-molecular-weight antioxidants and antioxidant enzymes is essential to protect the plant from ensuing oxidative stress (Mittler et al., 2004). However, ROS accumulation when contained within certain limits, both temporally and spatially, act as signaling molecules (Apel and Hirt, 2004; Mullineaux et al., 2006) and are integrated into signaling pathways, including those driven by hormones (Cheong et al., 2002; Fujita et al., 2006). For example, the accumulation of the ROS hydrogen peroxide (H_2O_2) in the chloroplasts of bundle sheath tissue of *Arabidopsis* leaves is important in initiating acclimation of low-light-grown plants to

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doi: 10.1093/mp/ssr027, Advance Access publication 15 April 2011
Received 14 December 2010; accepted 8 March 2011

high light and the hormone abscisic acid (ABA) may play a role in this process (Mullineaux et al., 2006). Further, evidence from microarray studies also shows that a much wider range of genes, as well as those coding for the antioxidant network, are important in the total cellular response to oxidative stress and to specific ROS (Gadjev et al., 2006).

In addition to environmental stress, oxidative stress can also arise when *in vitro* propagated plants developed under low-light conditions are exposed to high light during transfer to *ex vitro* conditions (Carvalho and Amâncio, 2002). In such a situation, among the many potential stresses to which the transferred plant can be exposed, oxidative stress is commonly experienced (Carvalho et al., 2006). This is most likely brought about by absorption of light energy in excess of that required for very low levels of photosynthetic metabolism, which leads to over-reduction of photosynthetic electron transport (PET) chain components, causing photoinhibition and production of ROS (Mullineaux et al., 2006). *In vitro* propagated grapevine (*Vitis vinifera* L.) when transferred to *ex vitro* conditions, typically with a fourfold increase in photosynthetically active photon flux density (PPFD) over *in vitro* conditions, shows an initial inhibition of PET (4–8 h) that recovers after 96h (Carvalho et al., 2006). This acclimation is associated with induction of alternative electron sinks for PET that prevents over-reduction of quinone A (Q_A; Pfannschmidt et al., 1999). Alternative electron sinks may include the photo-reduction of O_2, since PET inhibition is accompanied by an accumulation of H_2O_2, suggesting a signal for the up-regulation in gene expression and antioxidant enzyme activity, which peaked at 48h after transfer of *in vitro* grapevine to *ex vitro* growing conditions (Carvalho et al., 2006).

Structures emerging from tissues growing under dark or low-light conditions and shade leaves in the canopy can experience photo-oxidative stress caused by exposure to full sunlight (Kwak et al., 2006). The sensitivity of *Vitis vinifera* plants when transferred to *ex vitro* conditions can be contrasted with the field-grown grapevine's adaptability to a range of abiotic stresses such as high temperature and low water availability. Pot and field studies have shown that this species is quite resilient to photo-inhibition and, under drought stress, photorespiration may progressively replace photosynthesis as an electron-consuming process (Flexas et al., 2002). Other protective mechanisms observed in grapevine under excess light include non-photochemical energy dissipation and a fast D1 protein repair (Hendrickson et al., 2004). However, when stress conditions are prolonged, photo-protective mechanisms in grapevine leaves are insufficient to protect the photosynthetic machinery and photo-inhibition does take place. Therefore, this species is intrinsically resilient to high-light conditions that can promote photo-oxidative stress and a corollary of this is that *in vitro* plantlets transferred to *ex vitro* conditions must promote the induction of acclimatory processes.

Two sequencings of Pinot Noir grapevine were released in 2007 (Jaillon et al., 2007; Velasco et al., 2007). Cross-linking with data from the *Arabidopsis* genome (released in 2000) is helping to gather information from all-gene data providing the assignment of polypeptides detected by fine-tuned techniques, such as mass spectrometry-based protein analyses, the survey of metabolic pathways at the cellular and whole-plant levels (Hirayama and Shinozaki, 2010). However, there is still a huge effort to be made in order to curate and annotate the current version of the *Vitis* gene annotation, particularly regarding their function; thus, the cloning, sequencing, and identification of transcripts supplements the ongoing international effort of disclosing *Vitis* functional genomics.

This study set out to determine whether the underlying signaling pathways that are activated at the transfer of *in vitro* grown grapevine to *ex vitro* conditions reflect the processes equivalent to those that occur upon exposure to excess light, a photo-oxidative stress-inducing condition. We used the Affymetrix GeneChip '*Vitis vinifera* Genome Array' (VvGA), comprising 14 000 *V. vinifera* transcripts and 1 700 transcripts from other *Vitis* species, which accounts for circa half the predicted gene number (30 434; Jaillon et al., 2007) and an array of chosen genes known or expected to respond to high light (Light Stress Custom Array, LSCA). To construct the LSCA, we assembled primer pairs based upon a set of genes from *Arabidopsis thaliana* known to be responsive to high-light stress (Wang et al., 2000b; Ball et al., 2004; Gadjev et al., 2006) and chose sequences from genes also identified as high-light-responsive from analysis of light-responsive pathways from the PubMed and TAIR databases. The expression of the LSCA genes was surveyed using quantitative (q) real-time reverse transcriptase (RT) PCR. The results of both arrays were compared, exploring the feasibility of a custom-made real-time PCR approach that we believe should be of great interest, both for researchers working with species for which no GeneChip is available and for researchers dealing with a specific and focused problem.

RESULTS

We have previously established that grapevine transferred from heterotrophic growth conditions to *ex vitro* under PPFDs fourfold higher than *in vitro* experience reversible photo-inhibition symptoms within 4 h, followed by a transient rise in H_2O_2 (at 24 h) and increased activity of the ascorbate–glutathione cycle (at 24 and 48 h) determined by changes to ascorbate and glutathione levels and redox states, enzyme activities (Supplemental Table 1), and expression of the correspondent transcripts (Carvalho et al., 2006). Here, we set out to perform a comprehensive analysis of gene expression in the same experimental model at the same time points. For this purpose, we chose two distinct approaches; in the first, we used the Grapevine Affymetrix Genechip (further referred as VvGA); in the second approach, we designed a set of 380 primer pairs based upon *Vitis vinifera* ESTs that respond or would be expected to respond to a high-light stress situation (further referred to as Light Stress Custom Array, LSCA). This set was retrieved from the work of Wang et al.

(2000b) and Ball et al. (2004) and from the following databases: GenBank (www.ncbi.nlm.nih.gov/sites/entrez), Genoscope (www.genoscope.cns.fr/spip/Vitis-vinifera-whole-genome.html) and The Grape Gene Index (DFCI; http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=grape).

To confirm that the LSCA study detected the expected genes, 40 PCR products from the experiment were selected at random, cloned, and the sequenced fragments compared to the expected sequences. All sequences tested were the expected ones. As some of them were new in grapevine (15), they were submitted to GenBank and their accessions are summarized in Table 1, together with the respective fold change.

Vitis vinifera Affymetrix GeneChip

The array of Affymetrix GeneChip '*Vitis vinifera* Genome Array' (VvGA) was used to compare gene expression in control plants (time 0, immediately prior to transfer) to that in plants after 48-h growth under *ex vitro* conditions at 200 μmol m^{-2} s^{-1} PPFD and results were submitted to the GEO database (www.ncbi.nlm.nih.gov/geo/) under the Series Entry GSE27180. We used several criteria to validate the array data. First, two biological replicates were performed, and only genes that showed reliable signals and signal ratios in both replicates were considered. Second, we performed real-time quantitative PCR on a group of the 10 most up-regulated and the 10 most down-regulated genes and compared the results with those obtained from the array, using several reference genes (Table 2); El2 and L2 were the best, according to GENorm, and are the ones chosen for the correlation. As expected, the induction ratios from the PCR data in general exceeded those from the array, and the correlation between the PCR and array data is very good (Figure 1). The expression of those 20 genes was monitored by real-time quantitative PCR for 96 h after exposure to high light (Figure 2).

The scanning of grapevine's whole transcriptome with the Affymetrix GeneChip *Vitis vinifera* Genome Array (VvGA) revealed 125 up-regulated genes and 95 down-regulated. Of these, only seven up-regulated (two protease inhibitors, thaumatin-like protein VVTL1, class IV chitinase Chi4C, stilbene synthase, polygalacturonase inhibiter, and peptidegluta-thione transporter OPT1) and one down-regulated (class I extracellular chitinase Chi1b) sequences had been annotated. By comparison with data available in NCBI, TIGR, and Genoscope databases, it was possible to identify the majority of the remaining 212 sequences (Supplemental Table 2).

The expression of the 10 most up-regulated and of the 10 most down-regulated genes was monitored every 24 h for 96 h (Figure 2). None of these genes was common with the LSCA experiment (see below). It is clear that at 24 and 48 h, the expression pattern matched the one reported in VvGA (measured at 48 h) and that, from then on, four previously up-regulated genes changed expression pattern, two unknown genes, indole-3-acetic acid-amido synthetase (GH3.1), and the sugar kinase.

Table 1. Accessions of the *Vitis vinifera* Sequences Submitted to GenBank.

Accession	Gene putative function
DQ914882	Oxidoreductase/zinc ion binding
DQ914890	MPK13; MAP kinase
EF088505	Nitric oxide associated (NOA)
DQ914880	Meprin and TRAF domain-containing protein/MATH domain-containing protein
DQ914886	Inositol-3-phosphate synthase
DQ914887	Ubiquitin conjugating enzyme
DQ914879	WAK1 (CELL WALL-ASSOCIATED KINASE)
DQ914881	Heme binding/iron ion binding/oxygen binding
DQ914883	Electron carrier/electron transporter
DQ914884	CPHSC70-1 ATP binding
DQ914885	Unknown, similar to AT4G26920
EF088506	Calreticulin 2 (CRT2)
DQ914888	MYB transcription factor
DQ914889	Usp (universal stress protein)
EF088507	Putative acetyl-CoA synthetase

Light Stress Custom Array

The array of grapevine light-responsive ESTs (LSCA) was used to compare gene expression in control plants (time 0, immediately prior to transfer) to that in plants after 24 and 48-h growth under *ex vitro* conditions at 200 μmol m^{-2} s^{-1} PPFD. At 24 h, 158 genes were significantly up-regulated and 44 genes were down-regulated, while, at 48 h, only 27 genes were down-regulated and 192 were up-regulated (Table 4); 30 of these are still unknown. The number of sequences that were significantly regulated at both 24 and 48 h was 152. There was only one gene in common between the 10 most up-regulated at 24 and 48 h (HAT1), and SRG3 was up-regulated at 24 h and changed regulation pattern at 48 h. Among the 10 most down-regulated genes at 24 h, there are four that were still strongly down-regulated at 48 h (phospholipase C, zinc finger (CCCH-type) family protein/RNA recognition motif (RRM)-containing protein, MAPK13, and ELIP1) (Table 3).

VvGA versus LSCA

From the 380 genes analyzed in LSCA, 128 were common to the 16 436 genes in VvGA, corresponding to 33.6% of LSCA genes. When we chose the significantly regulated genes in LSCA at 24 and 48 h, the percentage of genes that were also present (but not necessarily significantly regulated) in VvGA was 34.6 and 33.3, respectively (Table 4), which is similar to the percentage of total genes in common. However, in the group of significantly regulated genes in VvGA, only eight up-regulated and one down-regulated genes were common to LSCA (Table 4).

Classification of the Gene Groups According to Biological Function

The set of differentially expressed genes was integrated with MapMan onthology (www.mapman.gabipd.org/). Briefly, MapMan ontology consists of a set of 36 hierarchical BINs, or functional categories, constructed around central metabolism, as well as other categories (e.g. stress, cell, etc.). Transcripts from the LSCA were then grouped into 29 functional categories, 16 of which were differentially represented at significant levels, while the differentially expressed genes obtained with VvGA were grouped into 20 functional categories, 16 of which were differentially represented at significant levels (Figure 3). In LSCA at 24 h, the most overrepresented up-regulated categories were 'nucleic acid metabolism', 'response to stress', 'protein metabolism and modification', 'hormones', and 'signaling'; at 48 h, 'signaling' genes had significantly increased. The most overrepresented down-regulated categories were 'response to stress' and 'signaling', on both time points. VvGA resulted in 'cell wall metabolism', 'response to stress', 'nucleic acid metabolism', 'hormones', and 'secondary metabolism' as the most overrepresented up-regulated categories, while the most overrepresented down-regulated categories were 'nucleic acid metabolism', 'cell wall metabolism', and 'lipid, fatty acid, steroid metabolism'. In VvGA, unknown genes accounted for 20% of up-regulated and 24% of down-regulated transcripts, while, in the LSCA study, this percentage never exceeded 13.

Hormone-related genes were up-regulated at 24 and 48 h in LSCA, especially those involved in ABA, ethylene, auxin, and cytokinin metabolisms. Sugar-binding proteins were also up-regulated at 48 h in LSCA and VvGA.

The category 'cell wall metabolism' is divided in two subcategories: 'cell wall biosynthesis' and 'cell wall modification'. The latter genes were up-regulated in LSCA whereas cell wall biosynthesis-related genes were down-regulated. VvGA retrieved 38 cell wall-related genes with differential expression, 15 related with cell wall biosynthesis, and four were down-regulated. From the 20 genes related with cell wall modification, half were up-regulated.

All the oxidative stress-related genes that were differentially expressed in the LSCA study were up-regulated, as were 18 biotic stress-related genes. Nevertheless, nine of these genes were HSPs, annotated as just 'biotic stress related'. Five anoxia-related genes were also up-regulated. The level of up-regulation of all these genes increased from 24 to 48 h (Table 5). One of the highly represented and significantly responsive gene family, both in VvGA and LSCA, was that of peroxiredoxins, some reaching levels of up-regulation higher than 100-fold in LSCA (Table 5). Electron transport-related genes were all up-regulated in the two analyses performed, with the sole

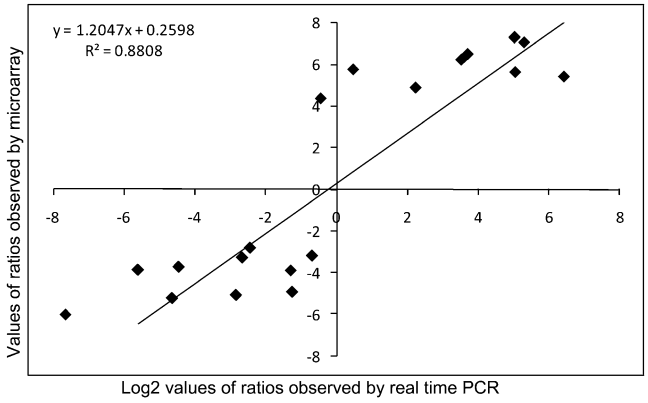

Figure 1. Comparison of Gene-Expression Ratios Obtained by Microarray and by Real Time RT–PCR.

Expression profiles are shown for the 10 transcripts whose expression was more significantly up-regulated in the microarray analysis and for the 10 transcripts whose expression was more significantly down-regulated. The microarray fold change are plotted on the YY-axis against the $\log_2$(expression ratio) values obtained by real-time RT–PCR on the XX-axis.

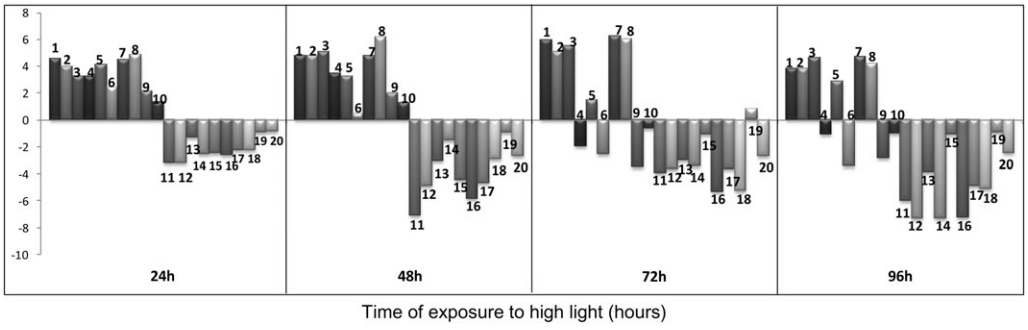

Figure 2. Gene-Expression Ratios Obtained by Real-Time RT–PCR, Monitored for 96 h After Transfer to HL.

Expression profiles are shown for the same 20 transcripts as shown in Figure 1. The name of the genes is shown in Table 2, in the same order as presented here. The $\log_2$(expression ratio) values obtained by real-time RT–PCR are plotted on the YY-axis against the time after exposure to HL on the XX-axis. Labels: 1. Protease inhibitor (PR6); 2. Endopeptidase inhibitor; 3. Glycosyl transferase (family 9); 4. Unknown; 5. Proline-rich cell wall protein; 6. Unknown; 7. Protease inhibitor (PR6); 8. Proline-rich cell wall protein (grip4); 9. Indole-3-acetic acid-amido synthetase; 10. Sugar kinase; 11. Adenine phosphoribosyl transferase; 12. LOB domain protein 4; 13. Putative auxin-regulated protein; 14. ABC transporter-like protein; 15. Gcap1 protein; 16. Major latex-like protein; 17. Unknown; 18. Unknown; 19. Unknown; 20. Unknown.

Table 2. Ten Most Up- and Down-Regulated Transcripts Obtained by Microarray (VvGA).

Probeset ID	GenBank annotation	Gene putative function	Fold change
1609875_at	AY156047.1	Protease inhibitor (PR6)	7.31
1616317_at	CD720960	Endopeptidase inhibitor	7.27
1615147_at	CB981835	Glycosyl transferase (family 9)	7.05
1609267_at	CK138210.1	Unknown	6.49
1608585_x_at	BQ797013	Proline-rich cell wall protein	6.22
1622396_at	CF202537.1	Unknown	5.75
1607288_at	CK136901.1	Protease inhibitor (PR6)	5.62
1617556_s_at	BQ797260	Proline-rich cell wall protein (grip4)	5.41
1620662_at	CB981820	Indole-3-acetic acid-amido synthetase	4.88
1617589_at	CF213123	Kelch repeat-containing F-box family protein	4.36
1614668_at	CF373733	Adenine phosphoribosyl transferase	–2.80
1621852_at	CF213721	LOB domain protein 4	–3.18
1613054_at	BQ794856	Putative auxin-regulated protein	–3.27
1622870_at	CB348576	ABC transporter-like protein	–3.72
1621879_at	CF215292	Gcap1 protein	–3.86
1617876_a_at	BQ797033	Major latex-like protein	–3.89
1621823_at	CF214240	Unknown	–4.91
1618245_at	CF212697	Unknown	–5.06
1617754_at	CB973158	Unknown	–5.21
1611371_at	CF215802	Unknown	–6.00

Genebank annotation is the accession number of the gene corresponding to the probe set.

exception of Nitric Oxide Associated (NOA) in the LSCA study and a copper–ion binding oxidoreductase in VvGA. Together with this increase in oxidative stress-related gene expression, a rise in H_2O_2 production at 24h was observed, followed by an increase in the activities of scavenging enzymes at 48h (SOD, CAT, and APX) (Supplemental Table 1).

Genes classified under the category 'protein metabolism and modification' and coding for proteins related with intercellular traffic, HSPs, chaperones, and ubiquitin-related were up-regulated at 24 and 48 h in the LSCA study. The typical pattern of HSP expression in LSCA was a gradual increase from 0 to 48h, with the exception of a HSP70, a HSP60, and BiP-2, which showed high levels of expression at 24h and then decreased (Figure 4). This major functional group was poorly represented in the VvGA study. Only 14 genes were responsive; four protease inhibitors were up-regulated.

Within the functional category 'signaling', the LSCA study revealed that calcium sensors were up-regulated at 24h whereas MAPkinases, phosphatases, and signaling molecules in general were down-regulated at 24h but up-regulated at 48h. Signaling proteins were also poorly represented in the VvGA study, no MAPK was found, and, from the four kinases present, two were up-regulated and two down-regulated, and only one calcium sensor was responsive (up-regulated).

DISCUSSION

We have previously characterized, at a physiological level, an oxidative stress response of micropropagated plants upon transfer to *ex vitro* conditions (Carvalho and Amâncio, 2002) and later described the reversible photo-inhibition symptoms and the oxidative stress at the level of ROS production, anti-oxidative metabolites, activities of scavenging enzymes, and the expression of key genes, determining that the most crucial moments are those between transplant and 48h of *ex vitro* growth (Carvalho et al., 2006). In this study, we proposed to verify at the molecular level that the response of micropropagated grapevine upon transfer to *ex vitro* growth mimics the photo-oxidative stress brought upon by excessive light (EEE, Karpinski et al., 1999). For this purpose, we used the Affymetrix GeneChip '*Vitis vinifera* Genome Array' (VvGA) and an array of chosen genes known or expected to respond to high light (LSCA). The experimental design applied in the study enabled the profiling of the light-responsive transcriptome of grapevine and to establish groups of genes that differ in their timing and intensity of expression. Although the sequencings of homozygous and heterozygous Pinot Noir grapevine were released in 2007 (Jaillon et al., 2007; Velasco et al., 2007), there is still a huge effort ongoing in order to identify and annotate genes; thus, the cloning, sequencing, and identification of *V. vinifera* transcripts supplements the ongoing international effort of disclosing *Vitis* functional genomics.

The major striking difference between VvGA and LSCA was the percentage of unknown/unclassified genes, reaching 25% of the VvGA down-regulated sequences, while, in the LSCA, this percentage was never higher than 13 (24-h down-regulated genes), which can be explained by the fact that LSCA is a custom array, built from many already identified sequences. Nevertheless, there is an interesting feature, common to both arrays: the percentage of down-regulated unknown genes is always higher than that of up-regulated genes. This must be a consequence of previous work/annotation efforts, repeatedly paying more attention and investing more work into up-regulated sequences. When comparing the number of common sequences between the two arrays, it was possible to divide LSCA into two distinct sets: the 200 genes that were retrieved from two previous *A. thaliana* arrays (Wang et al., 2000b; Ball et al., 2004) and the 180 genes obtained from known light-responsive pathways. The first yielded a percentage of common genes significantly higher than the latter, which enhances the suggestion that many genes comprised in the most important functional categories are still missing in the 14k Affymetrix array that in fact only comprises circa half of the predicted *V. vinifera* genes. Another conclusion to be taken from the comparison is that the custom-made array (LSCA) was much more sensitive than VvGA, which was already expected, for the sensitivity of real-time qPCR is much higher than that of microarrays (Gachon et al., 2004).

Table 3. Ten Most Up- and Down-Regulated Transcripts Obtained by Real-Time RT–PCR (LSCA).

GenBank annotation	Gene putative function	Fold Change 24h	Fold Change 48h
FN596494.1	Splicing factor Prp8, putative	8.60*	3.65*
XM_002272234	CDPK6 (CALCIUM-DEPENDENT PROTEIN KINASE 6)	8.41*	3.30
XM_002273432	SRG3 (SENESCENCE-RELATED GENE 3)	6.62*	–3.51*
XM_002266159	GT2 (trihelix DNA-binding protein / GT-2 factor)	6.71*	7.89*
XM_002264475	Unknown protein	6.33*	8.08*
XM_002282733	Mitochondrial proline oxidase	7.69*	10.61*
XM_002276984	Ceo protein	4.66*	5.16*
XM_002279078	GOLS2 (GALACTINOL SYNTHASE 2)	4.51*	1.53
XM_002268549	HAT1 (homeobox-leucine zipper protein 1)	4.43*	8.35*
XM_002278762	Integral membrane transporter family protein	5.67*	4.05*
XM_002276122	MPK14 (MITOGEN-ACTIVATED PROTEIN KINASE 14)	–8.47*	1.60
XM_002270194	Phospholipase C	–7.43*	–4.28*
XM_002280861	Zinc finger (CCCH-type) family protein/RNA recognition motif (RRM)-containing protein	–7.29*	–5.91*
XM_002277633	MPK12 (MITOGEN-ACTIVATED PROTEIN KINASE 12)	–7.32*	–0.34
XM_002284674	MPK13 (MITOGEN-ACTIVATED PROTEIN KINASE 13)	–5.10*	–0.42
XM_002282178	SKS6, pectinesterase	–7.15*	–3.12
XM_002278617	Rho-GTPase-activating protein 1	–3.12*	5.06*
XM_002270697	ELF4 (EARLY FLOWERING 4)	–2.76*	0.62
XM_002278012	MPK10 (MITOGEN-ACTIVATED PROTEIN KINASE 14)	–3.01*	0.40
XM_002283362	ELIP 1 (early light-induced protein 1)	–1.83*	–2.32*
XM_002267187	FAD-binding domain-containing protein, similar to reticuline oxidase precursor (Berberine-bridge-forming enzyme) (BBE)	3.73*	13.50*
XM_002274086	ATP binding/ATPase, coupled to transmembrane movement of substances	2.32	9.18*
XM_002273425	zinc finger (C3HC4-type RING finger) family protein	4.27*	11.25*
XM_002270449	Sex1 (regulator of starch metabolism)	1.03	11.46*
XM_002280582	HSFA7A (heat shock transcription factor A7A)	2.55	9.57*
XM_002281922	Cyclopropane fatty acyl phospholipid synthase	4.52*	10.25*
XM_002264475	Unknown protein	6.33*	8.08*
XM_002277386	HD2C (HISTONE DEACETYLASE 2C)	0.46	11.18*
XM_002262708	Allergen V5/Tpx-1-related family protein	2.94	6.21*
XM_002269844	Thaumatin-like protein (PR protein)	4.53*	–7.07*
EF088505	NOA1/NOS1 (NO ASSOCIATED 1)	0.64	–10.05*
XM_002280894	2-cysPRX A (2-cys peroxiredoxin)	-5.75*	–2.63*
XM_002285572	Hsp60 (heat shock protein 60 mitochondrial chaperonin)	1.89	–1.58*
XM_002282282	TRXh8 (thioredoxin h type 8)	0.57	–0.56

Genebank annotation is the accession number of the gene corresponding to the primer pair used. Fold change is expressed as $\log_2$. Values followed by * are significantly different at $p < 0.05$.

Comparing gene-expression profiles between the two experiments—protein metabolism and modification—signaling and anti-oxidative-related genes were more represented in the LSCA study than in the VvGA. This is obviously related to the fact that the custom array was built with the specific purpose of monitoring high-light-responsive transcripts and was based upon previous light-stress studies (Ball et al., 2004). Also, VvGA results significantly detected higher rates of variation in cell wall metabolism and secondary metabolism-related genes. These were overlooked in the LSCA study, probably due to its biased condition, although this array comprised 35 cell wall metabolism genes and 15 secondary metabolism genes. In general, the more represented functional categories in LSCA and VvGA have been mentioned in other studies involving other types of abiotic stresses in grapevine (for a review, see Cramer, 2010), and further enhancing the common attributes of abiotic stress defense pathways as a whole.

The induction of cell wall genes including those coding for expansins, pectinesterases, and endoxyloglucan transferases

suggests an increase in cell wall biosynthesis and modification—cytological events required for the initial *ex vitro* growth, taking place, even under stress conditions. This particular trait, apparent in VvGA and LSCA, was the major difference between typical abiotic stress responses when cell division and growth are impaired (Baena-Gonzáléz, 2010) and our plant system at transfer from *in vitro* to *ex vitro*.

Oxidative Stress

The dual role of ROS in plants as by-products of aerobic metabolism and as endogenous signals may explain the tight control that maintains ROS levels below toxic or activating thresholds (Apel and Hirt, 2004; Mittler et al., 2004; Mullineaux et al., 2006). Here, between 17 and 24% of the genes significantly changed after transfer to *ex vitro* in the LSCA study could be assigned to pathways associated with a response to stress, many of which specifically related to ROS processing. However, some genes expected to be significantly changed upon transfer, such as those encoding isoforms of APX, were not responsive in the LSCA array. CAT was also missing from the LSCA-responsive genes and its up-regulation has been associated with severe stress over time (Cramer, 2010), so its absence is not surprising. The high levels of APX and CAT activity measured at 48 h must have been assured by the already existing pool of enzymes (Carvalho et al., 2006). Genes coding for various SOD isoforms, GSTs, several peroxiredoxin (Prx), and thioredoxin isoforms were significantly up-regulated, together with other genes indirectly associated with the oxidative stress responses such as *LOX2*, which codes for a lipoxygenase with a role in several stress and hormone-responsive pathways (Wang et al., 2000a). This partially mimics the response observed after excess light treatments in *Arabidopsis* (Karpinski et al., 1999).

Prxs are nuclear-encoded, abundant, low-efficiency peroxidases, with multiple sub-cellular locations and mostly dependent on thioredoxins for reductive regeneration, and which, unlike most peroxidises, are highly insensitive to oxidative inactivation (Muthuramalingam et al., 2009). The LSCA study comprised genes coding for peroxiredoxins targeted to the chloroplast (2-Cys Prx A and Prx Q), cytosol (type II Prx C), and mitochondrion

Table 4. Numbers of Up- and Down-Regulated Genes in Each Treatment as Well as Numbers and Percentages of Genes Common to the Two Arrays (VvGA and LSCA).

	VvGA48			LSCA24			LSCA48		
	T	C*	P	T	C**	P	T	C***	P
Up-regulated	125	8	6.4	158	51	32.3	192	62	32.3
Down-regulated	95	1	1.0	44	19	43.2	27	11	40.7
Total	220	9	4.0	202	70	34.6	219	73	33.3

T, total number of significantly regulated genes; C*, number of significantly regulated genes in VvGA48 that are present in LSCA; C**, number of significantly regulated genes in LSCA24 that are present in VvGA48; C***, number of significantly regulated genes in LSCA48 that are present in VvGA48; P, percentage of common genes in relation to the total number of genes in the respective array.

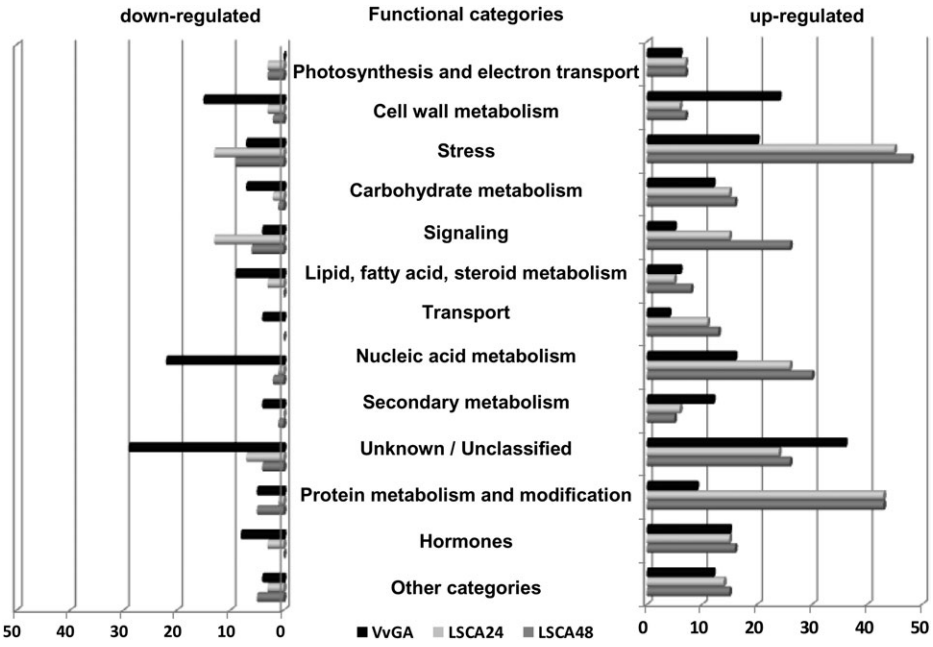

Figure 3. Functional Annotation of the 16 Functional Categories that Were Differentially Represented at Significant Levels (by Fold Change) in the Affymetrix GenChip (VvGA) at 48 h and in the LSCA study at 24 and 48 h.

For presentation purposes, the two functional categories ('unknown' and 'unclassified') were joined together. Genes from the functional categories 'nitrate metabolism', 'sulphur metabolism', 'phosphate metabolism', 'cell cycle', 'amino acid metabolism', and 'storage proteins' are presented together as 'other categories' because each of these categories individually comprised fewer than five genes.

Table 5. 'Response to Stress' Gene Expression Profiles in the LSCA Study.

GenBank annotation	Gene putative function	Fold Change	
		24h	48h
XM_002277525.1	Low-temperature and salt-responsive protein(Lti6A like)	1.01	3.05*
XM_002269844.1	Pathogenesis-related thaumatin family protein	4.53*	–7.07*
XM_002271118.1	Universal stress protein (USP) family protein	1.85	5.88*
XM_002273432.1	SRG3 (Senescence-Related 3)	6.62*	–3.51
XM_002273495.1	Fibrillin, plastid-lipid associated protein	–2.25	2.56*
XM_002273752.1	PR-1	0.64	1.77
XM_002274584.1	Class IV chitinase	6.50*	5.64*
XM_002273495.1	FIB (FIBRILLIN)	–0.34	0.48
XM_002272579.1	Pyruvate decarboxylase	1.74	8.04*
XM_002283569.1	Dehydrin RAB18 (responsive to ABA 18)	–0.82	2.36*
XM_002282110.1	Cell division cycle protein 48 (CDC48)	0.29	1.29
XM_002282710.1	C2H2 zinc finger protein FZF	4.39*	1.90
XM_002273425.1	C3HC4-type zinc finger protein	–1.34	1.84
XM_002277494.1	Zinc finger (C3HC4-type RING finger)	4.27*	7.92*
XM_002278686.1	RCD1 (RADICAL-INDUCED CELL DEATH1)	4.65*	5.16*
XM_002270326.1	DnaJ (j3)	1.97	4.78*
XM_002270326.1	DNAJ heat shock protein	1.37	1.90
XM_002279496.1	DNAJ heat shock N-terminal domain-containing protein	–1.09	1.40
XM_002281908.1	Heat shock protein 70	5.39*	–1.52
XM_002273208.1	Heat shock protein 81.4	–0.17	3.39*
XM_002280899.1	HSP17.4	1.49	2.39
XM_002281224.1	Heat shock protein 17.6	1.32	2.06
XM_002281358.1	Heat shock protein 17.6	0.70	2.52
XM_002270287.1	Myrosinase binding protein	0.95	2.25
XM_002263087.1	Coronatine-induced protein 1 (CORI1)	0.94	2.53
XM_002281358.1	Class I small heat shock protein	0.99	3.69*
XM_002281153.1	Plant defensin (PDF1.3)	2.66	3.87*
XM_002278101.1	Disease resistance protein (TIR-NBS-LRR class)	–3.84*	2.68
XM_002271434.1	Lipase	–0.62	2.70*
HM004362.1	Early light-induced protein 1	–1.83	–2.32
XM_002274762.1	Peroxidase (prxr1)	–1.20	0.15
XM_002282290.1	Thioredoxin 2	6.78*	8.23*
XM_002263922.1	Catalase	0.36	1.94
AF501625.1	Glutathione transferase 8	1.53	2.60
XM_002275399.1	Glutathione transferase (103–1A)	–0.55	0.99
EF088687	Glutathione transferase 6	–0.60	0.77
XM_002274169.1	Thioredoxin h1(TRXh1)	–6.20*	1.58
XM_002282282.1	Thioredoxin h7 (TRXh7)	1.93	0.93
XM_002274627.1	Thioredoxin h type 8 (TRXh8)	0.57	0.84
XM_002279159.1	Thioredoxin h9 (TRXh9)	1.38	4.79*
XM_002265561	2-cys peroxiredoxin (2-cysPRX B)	3.48*	6.50*
XM_002275900.1	PRXQ	–3.47*	4.82*
XM_002284141.1	Type 2 peroxiredoxin	3.77*	6.99*
XM_002284141.1	Peroxiredoxin-2C (PRXIIC)	1.23	4.29*
XM_002283616.1	Peroxiredoxin-2E	–2.98*	1.33
XM_002280975.1	Type II PRX F	–3.18*	1.29
XM_002267363.1	Iron superoxide dismutase 3	1.93	3.47*

Table 5. Continued

GenBank annotation	Gene putative function	Fold Change 24h	Fold Change 48h
XM_002275399.1	Glutathione transferase	1.89	2.26*
XM_002281621.1	Glutathione transferase (103–1A)	1.28	3.72*
XM_002278884.1	Methionine sulfoxide reductase domain-containing protein	4.11*	7.20*
AF236127.1	Catalase 3	0.33	2.01
XM_002282363.1	Dehydroascorbate reductase (DHAR1)	–0.89	1.70
XM_002282964.1	Monodehydroascorbate reductase, MDHAR	–1.64	–0.11
XM_002262806.1	Glutathione-S-transferase (pm24.1)	–2.06	0.16
XM_002274385.1	Copper/zinc superoxide dismutase (CSD2)	0.90	2.03
XM_002280486.1	Fe superoxide dismutase (FSD1)	4.26*	7.42*

Genebank annotation is the accession number of the gene corresponding to the primer pair used. Fold change is expressed as $\log_2$. Values followed by * are significantly different at $p < 0.05$.

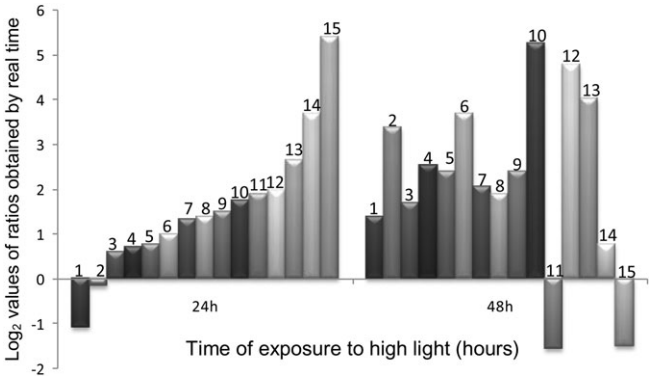

Figure 4. 'Heat Shock Proteins' Gene Expression Profiles in the LSCA Study.

The horizontal axis of the plot corresponds to the time points at which gene-expression measurements were obtained (24 and 48 h). The vertical axis indicates the $\log_2$ (expression ratio) associated with each gene under the stress treatment. Labels: 1. DNAJ heat shock N-terminal domain-containing protein; 2. Heat shock protein 81.4; 3. Heat shock protein 18.2; 4. Heat shock protein 17.6; 5. Hsp17.6; 6. 17.6-kDa class I small heat shock protein (HSP17.6B-CI); 7. Heat shock protein 17.6; 8. DNAJ heat shock protein; 9. HSP17.4; 10. Heat shock protein 100/ClpB; 11. Heat shock protein 60 mitochondrial chaperonin (hsp60); 12. DnaJ (j3); 13. HSP81-1 (Heat Shock Pprotein 81–1); 14. Luminal binding protein 2 precursor (BiP-2); 15. Heat shock protein 70.

(type II Prx F), which were all significantly up-regulated, with a tendency to increase with time. It is interesting to note that a 10-fold excess light had only a small stimulating effect on *Arabidopsis Prx* expression, while a 10-fold drop in PPFD suppressed the expression of all *Prx* within 4–8 h (Horling et al., 2003). This down-regulation was explained by a low balance between photosynthetic activity versus oxidative metabolism. 2-Cys Prx A, 2-Cys Prx B, and type II Prx E were also up-regulated in *Arabidopsis* under medium-intensity-light-stress induction (Heiber et al., 2007). The variation in *Prx* expression in response to changes in light intensity could be related to its dual function, either in antioxidant defense or signaling (Dietz, 2003). In a study using reporter gene lines under the control of 2-Cys Prx-A promoter, the promoter activity correlated with the availability of electron acceptors in PSI, ABA suppressing that activity (Baier et al., 2004). Five isoforms of cytoplasmic thioredoxins (h-type), which are linked to type II Prx (Yamazaki et al., 2004), were comprised in the LSCA study, and the isoforms h2 and h9 were up-regulated up to 30-fold at 48 h—a result significantly higher than the one obtained in a gene-wide grape berry transcriptomics study (Goes da Silva et al., 2005).

A few stress-related genes were down-regulated, the most significant of which was one coding for a thaumatin-like protein, up-regulated at 24 h and down-regulated at 48 h. Even though thaumatin-like genes are most often responsive to biotic stress, they can also be up-regulated under abiotic stress. The response of our experimental system differs from the usual behavior under stress; the likely cause for the down-regulation at 48 h may be the result of ABA accumulation (Swindell, 2006), typical of the initial phase of *ex vitro* growth, when roots begin to develop (Vilela et al., 2007). The same behavior was shown by SRG3 (SENESCENCE-RELATED GENE 3), also under ABA regulation (Swindell, 2006).

The VvGA resulted in only 20 up-regulated stress-related genes, such as those coding several ELIP, chitinase, and isoforms of dirigent proteins. From these genes, only four were related to oxidative stress: glyoxalase I, hydroperoxide lyase, one hydrolase, and one laccase.

Heat Shock Proteins

Heat shock proteins (Hsps) and transcription factors (Hsfs) are central components of the heat shock regulatory network. It has long been recognized that these elements are also involved in response to cold and non-thermal stress treatments (Feder and Hofmann, 1999), and Hsp/Hsf are elicited by a broad range of types of stress, including oxidative. In fact, heat shock transcription factors can function as reactive oxygen species sensors in plants (Miller and Mittler, 2006).

Genome-wide transcriptional profiling did not allow the monitoring of the expression of Hsf and Hsp genes under the present stress conditions. However, the custom array (the LSCA study) approach provided us with a broad picture of the response of this large gene family. Heat shock proteins were strongly up-regulated, especially at 48 h—up to 40-fold, as in the case of HSP100, HSP70. This is an atypically late response for HSPs, which usually display strong levels of up-regulation in the early stages of response, usually before 6 h, up to 24 h, but never as far as 48 h (Kilian et al., 2007; Swindell et al., 2007). Only two HSP70s and one HSP60 were significantly down-regulated at 48 h, after an up-regulation at 24 h. These families, together with HSP80 and HSP100, are usually correlated with small-magnitude response to stress. However, in the LSCA study, they were up-regulated at the same levels of magnitude as HSP20s. Interestingly, the HSP100 family, rather than the regular chaperone function of preventing protein aggregation and misfolding, exerts its action in protein degradation. The removal of non-functional but potentially harmful polypeptides arising from misfolding, denaturation, or aggregation (Wang et al., 2004) that is bought upon by stress is of extreme importance for the maintenance of cellular homeostasis and for the recovery period, thus the delayed response of Hsp100.

This protein-rescuing mechanism also involves the cooperation of another ATP-dependent chaperone system: the Hsp70. The Hsp100 family solubilizes the aggregated protein and releases it in a state that can be refolded with the assistance of the Hsp70 system. They perform dual chaperone and regulatory activities, thereby influencing the eventual fate of selected protein substrates, which are either fully degraded or unfolded and released (Wang et al., 2004).

The oxidative stress imposed in this study had a strong impact on heat shock genes. Hsf activation and, consequently, Hsp expression observed after 48 h of high-light exposure are probably a response to the production of reactive oxygen species, such as H_2O_2 (Carvalho et al., 2006; Vilela et al., 2007).

CONCLUSIONS

Integrating the results obtained so far, some interesting features come up: micropropagated grapevine transferred to *ex vitro* under a PPFD fourfold higher activates signaling pathways up to 48 h after transfer, which is a clearly delayed and time-prolonged response when compared with the universal responses to 'typical' abiotic stress (Mittler, 2006; Cramer, 2010). A vast number of genes related to functions such as stress defense pathways, hormones, protein metabolism, and modification are also activated in the same timeframe.

The use of a custom-made array (LSCA) was extremely helpful in this case study, helping to give an insight into the plant's behavior—more enlightening than the information provided by the Affymetrix GeneChip. This does not seem to be simply motivated by the lack of annotation of the GeneChip, for all the significantly up- and down-regulated genes were annotated by us. However, it was still not possible to annotate 33% of the significantly expressed genes. It is thus apparent that many genes comprised in the most important functional categories are still missing in the 14k Affymetrix array. The use of custom-made arrays is becoming straightforward, with the automation of sample manipulation and the use of new data analysis programs. The LSCA proved to be an important tool to be used in other stress studies, its plasticity allowing for constant updates and adjustments that can be made for each specific case.

Taken individually, the responses obtained in the present study show similarities to those reported in excess-light treatments (Karpinski et al., 1999; Ball et al., 2004) but also to mechanisms controlling the expression of genes for antioxidant enzymes induced by moderate-light stress.

METHODS

In Vitro Plant Growth

Shoots of *Vitis vinifera* L., var. Touriga Nacional, were used as explants for *in vitro* multiplication. Explants were sub-cultured every 4 weeks into Murashige and Skoog (1962), MS (Duchefa Biochemie, Haarlem, NL) basal medium supplemented with 0.5 μM α-naphthaleneacetic acid (NAA) and 5.0 μM 6-benzylaminopurine (BA). Before root induction, shoots were elongated for 2 weeks in the same medium but with 1.67 μM BA. For root induction, explants from the elongation phase received a supplement of 2 μM α-naphthaleneacetic acid (NAA) for 4 d (Carvalho and Amâncio, 2002). Cultures were maintained in a growth chamber under light from cool-white fluorescent lamps with 16/8-h photoperiod and 45 ± 5 μmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD). Temperature was 25 ± 1°C (light) and 22 ± 1°C (dark).

Ex Vitro Treatments and Material Used in the Experiments

After *in vitro* induction, root growth took place *ex vitro*. Microcuttings were transplanted to 6-cm-diameter pots containing a sterilized mixture of hydrated peat and perlite (1:1, v/v) and placed in 450-L glass chambers (500E, Aralab, PT). A PPFD of 200 ± 10 μmol m^{-2} s^{-1} was provided by cool-white fluorescent lamps under a photoperiod of 16/8 h. The programmed relative humidity (RH) inside the glass chamber (98%) was obtained by an ultrasonic fog system controlled by a hygrometer. Temperature was kept between 25 ± 2°C (light) and 22 ± 1°C (dark). Leaves were harvested into 0.5-g samples from *in vitro* material at the moment of transplant (time 0) and after 24, 48, 72, and 96 h.

RNA Isolation and cDNA Synthesis

Total RNA from leaves was extracted using a modification of the method described by Geuna et al. (1998). In the presence of liquid N_2, 0.5 g leaf tissue were ground to powder and added to 6 ml extraction buffer (0.1 M glycine, 0.001 M EDTA, 2% (w/v) SDS, 1% (w/v) sodium lauryl sarkosine, 200 mM NaCl,

pH 9.5 with NaOH; 1.6% (w/v) BSA, 16 mM DTT, and 10% (w/w) PVPP were added just before use). Following the addition of 200 μl proteinase K (10 mg ml^{-1}), the samples were placed in a rotary shaker at 37°C for 20 min. This step was followed by a centrifugation at 14 000 *g*, for 5 min, at room temperature. The aqueous phase was transferred to a new tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added. After centrifugation under the same conditions, this step was repeated. The supernatant was transferred to a new tube containing 450 mg cellulose fiber and a 0.55 equivalent volume of absolute ethanol was added. The samples were incubated at room temperature for 45 min in a rotary shaker, to allow binding of nucleic acids to the cellulose matrix. Cellulose-bound nucleic acids were collected by centrifugation (5 000 *g*, 3 min, room temperature) by re-suspending the matrix twice in 3 ml washing buffer consisting of STE buffer (0.1 M Tris-HCl, pH 8.0, 0.002 M EDTA) in 35% ethanol. Nucleic acids were then recovered by centrifugation (5 000 *g*, 3 min, room temperature) after re-suspending the matrix in 1.8 ml STE buffer. The recovered supernatant, added of 1/10 (v/v) sodium acetate (3 M, pH 5.2) and kept on ice for 10 min, was cleaned by centrifugation (14 000 *g*, 10 min, 4°C). For nucleic acid precipitation, ethanol (2.5 vol. of 100%) was added and the samples were incubated for 1 h at –80°C and then washed with ethanol 70%. After drying, the pellets were re-suspended in water to the desired volume. Samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI) and reverse-transcribed using random hexamers and Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

Assembly of the Custom Array of Light Stress Genes

Of the 380 EST chosen for the real-time PCR study, 200 were obtained in an earlier study of high-light stress that used an Incyte microarray containing 8 000 unigene EST probes (Wang et al., 2000b) and from a study of glutathione-responsive genes (Ball et al., 2004). The further 180 cDNA sequences were chosen from genes also identified as high-light-responsive from analysis of known light-responsive pathways in *Arabidopsis* from the PubMed and TAIR databases. Known sequences of *V. vinifera* antioxidative genes, AF236127, AF056622, and AF019907, coding for catalase (Gcat), CuZnSOD precursor, and glutathione reductase (GOR), respectively, were also added to this array. Primer pairs used for amplification of the sequences studied were obtained with the primer design tool Beacon Designer (Premier Biosoft Int.) and are shown in Supplemental Table 3. This custom-made array is referred to in the text as LSCA (Light Stress Custom Array).

Real-Time PCR Conditions and Analysis

Polymerase chain reactions were performed in an optical 96-well plate with an Opticon2 DNA Engine (MJ Research Inc.). The 20-μL reaction mixture was composed of cDNA, 0.5 μM gene-specific primers, and two master mix DyNAmo SYBR Green qPCR Kit (Finnzymes, MJ Bioworks). Amplification of PCR products was monitored via intercalation of SYBR-Green (included in the master mix). The following thermal profile was used for all PCRs: initial polymerase activation, 95°C, 3 min; 40 cycles at 94°C 10 s (denaturation), 63°C 20 s (annealing), 72°C 15 s (extension), with a single fluorescence reading taken at the end of each cycle. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the lack of primer dimers. Further, RT–PCR products were resolved on 2% (w/v) agarose gels, run at 4 V cm^{-1} in Tris-acetate-EDTA buffer (TAE), along with a 50-bp DNA-standard ladder (Invitrogen Gmb H) to confirm the existence of a single product of the desired length.

To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔRn) versus cycle number, baseline data were collected between cycles 5 and 17. All amplification plots were analyzed with an R_n threshold of 0.2 to obtain C_T (threshold cycle) and the data obtained were exported into a MS Excel workbook (Microsoft Inc.). In order to compare data from different PCR runs or cDNA samples, several housekeeping genes were tested using geNorm (Vandesompele et al., 2002) and, in the end, C_T values were normalized to the C_T values of *Act2*, *EL2*, and *L2*.

RNA Isolation, Target Synthesis, and Hybridization to Affymetrix GeneChips

Total RNA was extracted using the method described above. Concentration and purity were determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). Each GeneChip experiment was performed with biological duplicates.

RNA was processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip *Vitis vinifera* Genome Arrays, according to the manufacturer's One-Cycle Target Labeling Assay. Briefly, 5 μg of total RNA containing spiked in Poly-A RNA controls (GeneChip Expression GeneChip Eukaryotic Poly-A RNA Control Kit; Affymetrix) was used in a reverse transcription reaction (One-Cycle DNA synthesis kit; Affymetrix) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an *in vitro* transcription (IVT) reaction to generate biotinylated cRNA (GeneChip Expression 3'-Amplification Reagents for IVT-Labeling; Affymetrix). Size distribution of the cRNA and fragmented cRNA, respectively, was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay.

Fragmented cRNA (10 μg) was used in a 200-μl hybridization solution containing added hybridization controls. The mixture (130 μl) was hybridized on arrays for 16 h at 45°C. Standard post-hybridization wash and double-stain protocols (Midi_euk2v3) were used on an Affymetrix GeneChip Fluidics Station 400. Arrays were scanned on an Affymetrix GeneChip scanner 3000.

GeneChip Data Analysis

Scanned arrays were analyzed first with Affymetrix MAS 5.0 software to obtain Absent/Present calls and for subsequent

analysis with DNA-Chip Analyzer (dChip) Version Release (15 April 2005) (www.dchip.org, Wong Lab, Harvard). The arrays were normalized to a baseline array with median CEL intensity by applying an Invariant Set Normalization Method (Li and Wong, 2001a). Normalized CEL intensities of the four arrays were used to obtain model-based gene-expression indices based on a PM (Perfect Match)-only model (Li and Wong, 2001b). Replicate data for the same sample type were weighted gene-wise by using inverse squared standard error as weights. Only genes called 'Present' in at least one of the four arrays and within replicate arrays called 'Present' within a variation of $0 <$ Median (Standard Deviation/Mean) < 0.5 were kept for downstream analysis (12 222 genes). Thus, genes called 'Absent' in all arrays and genes with highly inconsistent expression levels within replicate arrays were excluded. 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 (Median false discovery rate of 6.4%). 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 (2001b) 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. Annotations for the 14 000 *Vitis vinifera* transcripts and 1 700 transcripts from other *Vitis* species that are represented on the GeneChip *Vitis vinifera* Genome Array were obtained from the NetAffx database (www.affymetrix.com) as of April 2005 and imported into dChip using ChipInfo software (Zhong et al., 2003).

The results obtained for the 10 most relevant up- and down-regulated genes were confirmed by real time RT–PCR, under the conditions already described. Primer pairs used for amplification of the sequences studied were obtained with the primer design tool Beacon Designer (Premier Biosoft Int.) and are shown in Supplemental Table 2.

Cloning and Sequencing

Forty products amplified by real-time PCR were cloned with the pMOS*Blue* Blunt Ended Cloning Kit (GE Healthcare, Lifesciences) and purified with the Wizard Plus SV Minipreps DNA Purification System (Promega) prior to sequencing. Sequences of amplification products were compared to GenBank sequences and to TIGR sequences in the Grape Gene Index using BLAST software (www.ncbi.nlm.nih.gov/BLAST/ and http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=grape). All the sequences that were not found on both databases were submitted to GenBank as new ESTs.

LSCA Data Analysis

Two independent experiments were performed for each analysis and the measurements were obtained from randomly chosen plants. For real-time PCR, three independent measures were made for each time point ($n = 3$).

Expression values were obtained by averaging the normalized results and comparing them with time 0. Data were arranged in increasing levels of transcription and their ranking was obtained using the method of Breitling et al. (2004) to obtain a global view of the transcriptional changes of the genes after 24 and 48 h.

Functional Annotation of Differentially Expressed Genes

The sets of differentially expressed genes in VvGA and LSCA were integrated with MapMan ontology (mapman.gabipd.org/). Briefly, MapMan ontology consists of a set of 36 hierarchical BINs, or functional categories, constructed around a central metabolism, as well as other categories (e.g. stress, cell, etc.).

Vitis vinifera Gene Index sequences were downloaded from TIGR and blasted (BLASTx, version 2.2.14) against *Arabidopsis* proteins that release TAIR (www.Arabidopsis.org/) under default settings. Blasts against SwissProt/Uniprot plant proteins PPAP (www.uniprot.org/program/plants/), InterProScan (www.ebi.ac.uk/Tools/InterProScan/), and the Conserved Domain Database CDD (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) were also performed. The results of all Blast hits were compiled and an initial classification into MapMan BINs was achieved and then checked manually based on the annotations provided by TIGR.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

FUNDING

This research was funded by Fundação para a Ciência e Tecnologia (FCT), co-financed by FEDER, project POCTI/AGG/37968/2001 that includes a research grant to B.J.V., by project PTDC/AGR-GPL/099624/2008, by Plurianual funds to CBAA, and by the FCT-awarded post-doc grant SFRH/BPD/5707/2001 to L.C.C. No conflict of interest declared.

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