Vitis **54**, 21–28 (2015)

Modulation of gene expression in *ex vitro* grapevine (*Vitis vinifera* L.) by hormonal, oxidant and anti-oxidant stimuli

L. C. Carvalho B. J. Vilela*) and S. Amâncio

LEAF, Instituto Superior de Agronomia, Universidade de Lisboa, Lisboa, Portugal

Summary

When *in vitro* plantlets developed under low light conditions are exposed to high light upon transfer to *ex vitro*, it is common to observe symptoms of oxidative stress. In order to unravel more of what takes place in this phase of transition, paramount for plant survival, the present study focused on the expression of genes coding for proteins related with the metabolic pathways most affected upon transfer to *ex vitro* and monitored their expression in response to hormones and chemicals inducing alternative sinks for photosynthetic electron transport (PET) and reactive oxygen species (ROS) production. The expression of eight genes was significantly downregulated after all the treatments: catalase, beta-glucosidase, cytochrome P450, vegetative storage protein 2, wak1, a calcium binding protein, a meprin and TRAF homology domain-containing protein and a serine/threonine kinase. It was possible to verify that abscisic acid (ABA) was able to revert light induced gene expression and that the PET inhibitors DCMU and DPI had the same effect as ABA. ABA and jasmonic acid showed parallel effects, as both induced the expression of the same set of genes. Finally, exogenous cytokinin, instead of enhancing the plant´s response to high light led to the downregulation of light-responsive genes.

K e y w o r d s : abscisic acid, cytokinins, DCMU, DPI, gene expression, jasmonate, oxidative stress.

Introduction

Different environmental signals drive plant growth and development. When in excess, external stimuli can act as stressful agents, induce oxidative stress symptoms and promote the accumulation of reactive oxygen species (ROS) (Apel and Hirt 2004). However, ROS contained within certain limits, both temporally and spatially (Apel and Hirt 2004, Mullineaux *et al.* 2006) are integrated into signalling pathways, including those mediated by plant hormones (Cheong *et al.* 2002, Foyer and Noctor 2005, Fujita *et al.* 2006). Natural hormones can function as signals that communicate between organs and coordinate growth and development (Jaillais and Chory 2010). Cytokinins are a class of plant hormones that play a role in many aspects of growth and development, including apical dominance, the formation and activity of shoot meristems, delaying of senescence, nutrient mobilization, seed germination, root growth and stress responses (Brenner *et al.* 2005). They also mediate the response to variable external factors including light conditions in the shoot (Werner and Schmülling 2009) thus participating in a number of functional processes under light control (Brenner *et al.* 2005), such as de-etiolation and chloroplast differentiation (Roitsch and Ehness 2000). An increasing number of genes have been reported as cytokinin responsive. It is the case of some immediate-early response proteins which may subsequently act as transcriptional regulators and trigger a second wave of gene expression changes controlling more specific developmental or physiological processes. Therefore, it is important to analyse the transcriptional changes during an extended span of time (Brenner *et al.* 2005).

Jasmonic acid (JA) and methyl jasmonate (MJ) (collectively termed jasmonates) are ubiquitous plant signalling compounds (Wierstra and Kloppstech 2000). A defect in JA biosynthesis or in a signalling component of the JA pathway is accompanied by a defect or decrease in the expression of Jasmonate Responsive Genes, increasing the sensitivity to biotic and abiotic stresses (Sasaki-Sekimoto *et al.* 2005). JA can down-regulate genes encoding proteins required for photosynthesis (Mackerness *et al.* 1999) while MJ can induce genes involved in secondary metabolism, leading to the formation of terpenoid indole alkaloid precursors (van der Fits and Memelink 2000), suggesting that stress tolerance in plants requires jasmonate-mediated transcriptional activation of biosynthetic pathways of defence compounds (Sasaki-Sekimoto *et al.* 2005).

It is well known that ABA modulates a variety of important growth and developmental processes, adaptation to environmental cues and response to stress (Volodarsky *et al.* 2009; Nambara and Kuchitsu 2011 and references therein). ABA perception and signal transduction pathways have been unravelled through fine reverse genetic techniques (e.g. Israelsson *et al.* 2006, Li *et al.* 2006) and through *in vitro* reconstitution of an ABA signalling pathway (Fujii *et al.* 2009). ABA may intermediate the accumulation of hydrogen peroxide (H_2O_2) in the chloroplasts of Arabidopsis bundle sheath cells at the time of the acclimation of low light grown plants to high light (Fryer *et al.* 2003, Mullineaux *et al.* 2006). Recent studies also implicate the involvement of endogenous ABA in MJ signal transduction leading to stomatal closure in guard cells (Hossain *et al.* 2011).

Correspondence to: Dr. L. C. Carvalho, LEAF, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal. Fax: +351-21-3653-383. E-mail: lcarvalho@isa.ulisboa.pt

*) Present address: Centre for Research in Agricultural Genomics, Parc de Recerca UAB, Edifici CRAG, Campus UAB, Bellaterra (Cerdanyola del Vallés), 08193 Barcelona, Spain.

ROS accumulation is more prevalent in organelles where electron transfers operate, namely in chloroplast and mitochondria. Diphenylene iodonium (DPI) is an inhibitor of flavoprotein oxidases (OROZCO-CÁRDENAS *et al.* 2001) with a reversible effect, used especially in work related with plant development. Its range of inhibition includes NADPH oxidase and its application prevents ROS accumulation. Blocking NADPH oxidase activity with DPI inhibits ROS formation (FOREMAN *et al.* 2003). The herbicide DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, inhibits photosystem II (PSII) by displacing the secondary quinone acceptor, Q_B, from its binding site at the D1 protein of PSII. As DCMU is not redox active the re-oxidation of Q_A^- by forward electron transport is thus prevented (SASAKI-SEKIMOTO *et al.* 2005).

When *in vitro* plantlets developed under low light conditions are exposed to high light at transfer to *ex vitro*, symptoms of oxidative stress are observed (CARVALHO *et al.* 2006, BAŤKOVÁ *et al.* 2008). This is most likely caused by absorption of light energy in excess of that required for very low levels of photosynthesis, leading to over-reduction of photosynthetic electron transport (PET) chain components, causing photoinhibition and thus ROS production (ORT and BAKER 2002, MULLINEAUX *et al.* 2006). *In vitro* grapevine (*Vitis vinifera* L.) transferred to *ex vitro* under a four-fold increase in photosynthetically active photon flux density (PPFD), shows an initial inhibition of PET (4-8 h) that is fully recovered after 96 h (CARVALHO *et al.* 2006). This acclimation seems to be associated with the induction of alternative electron sinks for PET that prevents over-reduction of quinone A (Q_A) (PFANNSCHMIDT *et al.* 1999). These alternative electron sinks may comprise the photoreduction of O_2 because PET inhibition is accompanied by an accumulation of H_2O_2 (CARVALHO *et al.* 2006), also suggested by the up-regulation of genes related to stress response, signalling, protein and hormone metabolism, at 24 h and 48 h after transfer (CARVALHO *et al.* 2011).

Several questions were raised by previous results (CARVALHO *et al.* 2006; CARVALHO *et al.* 2011), such as: Does ABA revert light induced gene expression? Do PET inhibitors have the same effect as ABA? As ABA and jasmonic acid induce the same sets of proteins in several plant species (PENA-CORTES *et al.* 1991), is it also the case in our system? Does an increased cytokinin level enhance the plant´s response to high light? In the current study we were able to address those questions and successfully answer them, by focusing on the genes coding for proteins related with the metabolic pathways most affected upon transfer to *ex vitro.* The expression of those genes was monitored in response to hormones and chemicals inducing alternative sinks for PET and ROS production, using quantitative real time RT-PCR.

Material and Methods

Plant material and growth conditions: Shoots of *Vitis vinifera* L. 'Touriga Nacional' were used as explants for *in vitro* multiplication. Explants were subcultured every four weeks into MS (Duchefa Biochemie, Haarlem, NL) (MURASHIGE and SKOOG 1962) 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 two weeks in the medium supplemented with 1.67 µM BA. For root induction, explants from the elongation phase had a supplement of 2 µM α-naphthaleneacetic acid (NAA) for four d (CARVALHO and AMÂNCIO 2002). Cultures were kept in a growth chamber under light from cool-white fluorescent lamps with 16/8 h photoperiod and 45 ± 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetic photon flux density (PPFD). Temperature was 25 ± 1 °C (light) and 22 ± 1 °C (dark).

Ex vitro treatments and material used*:* After *in vitro* induction root growth took place *ex vitro*. Microcuttings were transplanted to 6 cm diameter pots containing a sterilised 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 $\mu mol \cdot m^{-2} \cdot s^{-1}$ 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).

Exogenous applications of chemicals*:* JA (jasmonic acid) was applied to plants at a concentration of 1 mM. JA was mixed with 2 volumes ethanol and the resulting emulsion was diluted in water to the appropriate concentration. This suspension was sprayed onto the leaf surface of *in vitro* grown plants immediately before transplant. ABA (abscisic acid) was also sprayed onto the leaves before transplanting at a concentration of 0.1 mM. BA (6-benzylaminopurine; 1 mM) was applied by immersion of the shoot in a solution of the compound and immediately removed. Control plants were treated in the same way except that the exogenous product was replaced by distilled water. DPI (diphenyleneiodonium chloride) and DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea)) were both vacuum-infiltrated into plantlets for five minutes, in solutions with final concentrations of 50 µM and 10 µM, respectively. DPI solution was prepared in potassium phosphate buffer 10 mM, pH 7.8 and a 10 mM DCMU stock solution was prepared in 50 % ethanol and then diluted to the desired concentration with sterile water. In this case, the experimental control was performed with plants vacuum-infiltrated with sterile water.

Leaves were harvested for extraction at the moment of transfer (0 h) and every 24 h for 96 h after transfer to *ex vitro.* Two independent experiments were performed for each analysis and measurements were obtained from three biological replicates in each experiment.

RNA isolation and cDNA synthesis*:* Total RNA from leaves was extracted as in CARVALHO *et al.* (2011) using a modification of the method described by GEUNA *et al.* (1998). Nucleic acid concentration was quantified by spectrophotometry using the software Gen5 1.09 (Synergy HT, Bio-Tek Instruments, Winooski, USA). Total RNA quality was assessed and only samples with A_{260}/A_{280} between 1.8 and 2.1 and A_{260}/A_{230} between 2.0 and 2.2 were used. Total RNA integrity was checked through 1 % agarose gel electrophoresis under denaturing conditions. Samples

were then 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) in a 20 µL-reaction volume, according to the manufacturer's recommendations.

Real time PCR conditions and analysis*:* RT-qPCR was performed in 96 well clear reaction plates (Bio-Rad, Hercules, CA), using an IQ5 Real Time PCR (Bio-Rad, Hercules, CA) with three biological replicates and two technical replicates, as described in Carvalho *et al.* (2011).

To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔRn) versus cycle number, baseline data were collected between the cycles 7 and 16. Amplification plots were analysed with an R_n threshold of 0.2 to obtain Cq and the data obtained were exported into a MS Excel workbook (Microsoft Inc.) for analysis. In order to compare data from different PCR runs or cDNA samples, Cq values were normalized to the C_q value of *Act2*, a housekeeping gene keeping a relatively high and constant level (Coito *et al.* 2012).

Data analysis: The prediction of phosphorylation sites was performed with the tool available at PREDIKIN (http://predikin.biosci.uq.edu.au). The Meta Analyser tool of GENEVESTIGATOR was used to study the expression profiles of the selected genes simultaneously in various conditions (Hruz *et al*. 2008). We selected abiotic stress and chemicals. For more information, see https://www.genevestigator.ethz.ch. The experiments underlying the results presented here are described in "step 1", "select arrays by annotations"; under the item "conditions", the titles of the experiments correspond to the list on the left in Fig. 4. The genes chosen are the *A*. *thaliana* orthologues of 8 *Vitis vinifera* genes, obtained using BLAST software in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). Further, the expression data obtained were submitted to the Hormonometer Database in order to build the hormone signature (http://genome.weizmann.ac.il/hormonometer/) (Volodarsky *et al.* 2009).

Results

The selection of the genes for this experiment was made based on a microarray analysis performed for time 0 and 48 h of *ex vitro* growth using the Affimetrix 14k Grape GeneChip that revealed changes in a panoply of genes related to signalling, response to stress and protein metabolism (Carvalho *et al.* 2011). To understand the transcriptional regulation of the primary response and recovery we chose a set of representative genes in the above-mentioned categories and performed an expression analysis in response to the application of DCMU, DPI, and the plant hormones BA (a synthetic CK), ABA and JA. The expression patterns were measured by RT-qPCR every 24 h up to 96 h after leaf treatment at time 0 of *ex vitro* growth.

For a number of genes the treatments induced significantly different expression patterns as compared with the controls. A random example is given in Fig. 1, comparing four genes under DPI and DCMU treatments with the controls, all of which were significantly down-regulated when compared with the respective controls.

The downregulation pattern was, for most genes, significantly more expressive than upregulation (Fig. 2). The

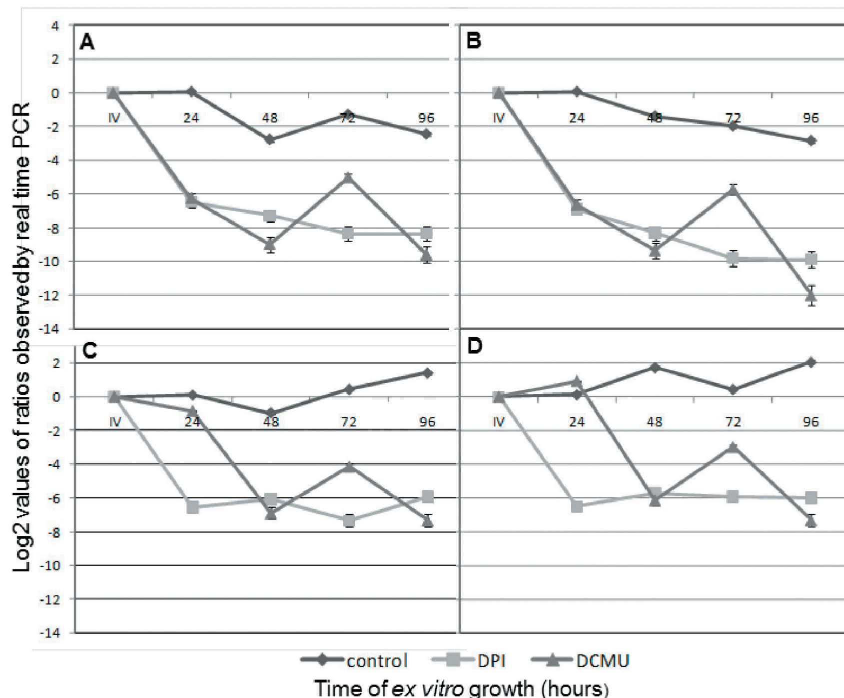

Fig. 1: Expression profiles obtained for four transcripts comparing kinetics during the light treatment alone (control) with DCMU and DPI treatments: **A** - myo-inositol 1-phosphate synthase isozyme-2 (XM_002272868.1), **B** - src2-like protein (XM_002272784.1), **C** - Acetyl Co-A synthetase (XM_002278482.1) and **D** - phospholipase D (DQ333882.1). Each line connects the different time points at which the samples were collected (from 0 to 96h). Expression data are shown in log_2(expression ratio) values obtained by real-time RT-PCR.

Fig. 2: Differentially regulated genes after the exogenous application of BA, ABA, JA, DCMU and DPI: temporal expression patterns in $\log_2$ (expression ratio) values obtained by real-time RT-PCR at 24, 48, 72 and 96 h after application and their level of response to the various chemical stimuli, according to the shade legend.

best example was the downregulation of almost all genes at 24 h after treatment with DPI (Fig. 2). This trend was kept up to 96 h except for four genes. The response to DCMU was bimodal for 45 genes, with several stress, hormone and nucleic acid metabolism related genes upregulated at 24 h and 72 h, and downregulated at 48 h and 96 h (Fig. 2).

The effects of the three hormones applied were significantly different. BA induced the downregulation of all but XM_002268804 at 24 h (Fig. 2). Two CK-responsive genes coding for serine/threonine-protein kinases involved in calcium-signalling showed opposite responses, XM_002265323.1 was up-regulated at 72 h and 96 h, an unusually delayed response to the hormone and XM_002280908.1 was downregulated for the whole period. Conversely, a group of genes was up-regulated at 48 h and 72 h (Fig. 2), namely acetyl-CoA synthetase, mitochondrial proline oxidase, tyrosine aminotransferase, cyclopropane fatty acyl phospholipid synthase, cytoplasmic aconitate hydratase and calcium-dependent protein kinase/ kinase (CDPK6) while quinone oxidoreductase was strongly upregulated at 96 h. After ABA treatment, all genes were significantly downregulated at 96 h, with a group of 16 genes, six of which stress related, downregulated all along the experiment. Conversely, 47 genes, comprising ten stress related, seven hormone and nucleic acid metabolism and five signalling genes had a gradual increase of expression until 72 h. The treatment with JA resulted in a steady increase in the expression of seven genes, two related with cell cycle, while another group suffered an immediate decrease (at 24 h) followed by a steady recovery of expression. *CAT*, *APX1* and *APX3* were significantly downregulated by JA.The expression of the following eight genes was significantly downregulated after all the treatments (Fig. 3): catalase (Gcat, AF236127), β-glucosidase

(XM_002285548.1), cytochrome P450 (XM_002263824.1) vegetative storage protein 2 (vsp2, XM_002283349.1), a calcium binding protein (CaBP-22, XM_002270889.1), a meprin and TRAF homology domain-containing protein (DQ914880), wak1 (XM_002282851.1) and a serine/threonine kinase (XM_002280908.1). All had repressions of up to 14 fold in log_2 (expression ratio) values. Most of these genes are potentially regulated by the calcium-dependent protein kinase (CDPK6), as indicated by the prediction of phosphorylation sites, PREDIKIN, a system that predicts the most likely phosphorylation site for a specific protein kinase and the most likely protein kinase for a phosphorylation site. This analysis predicted with high probability (score above 75) two sites of CDPK6 phosphorylation for catalase (position S9, site YRPSSAY and position S349 site FAYSDTQ), three for beta-glucosidase (T28, site ASDTPNY; S225, site ARCSAWQ and T368, site SYTTDPY) and for vsp 2 (S21, site RAFSDSL; S33, site DRRSSTK and S82, site RYRSDSE), and only one site for wak1 (T142, site KYKTGCL) and for CaBP-22 (T6, site DQLTDDQ), the latter being the highest probability site for CDPK6 activity. There were no phosphorylation sites predicted for Serine/threonine kinase. GENEVESTIGATOR allows an assessment of the expression of selected genes through numerous environmental and developmental conditions in *Arabidopsis* (Hruz *et al.* 2008). We compared the expression of the eight *Vitis* transcripts mentioned above with *Arabidopsis* homologs under abiotic stress and chemical treatments. The GENEVESTIGATOR light study (3 h white light) resulted in the upregulation of almost all the genes monitored in Fig. 3 while the application of heat, drought, cold, light/drought and the herbicide norflurazon resulted in downregulation of the eight genes (Fig. 4). Application of the inducer of systemic acquired resistance benzothiadiazole reverted the down-regulation of half the genes.

We ran our results through HORMONOMETER, a database specialized in the transcriptome response to hormones and growth regulators, in order to derive a coherent hormone signature to a particular hormone or PET inhibitor treatment in the transition of plants to high light. We compared our data by correlation analysis with a curated set of ATH1 arrays for different hormone treatments, namely, ABA, zeatin and Methyl Jasmonate (MJ). DPI and DCMU provided similar signatures (Fig. 5). With the exception of BA, all our treatments revealed a positive zeatin signature correlation and all had strong negative correlations with MJ signature. The ABA signature correlation was the most variable, negative with BA, positive with JA and changing along the period of the experiment with DPI and DCMU.

Discussion

In micropropagated grapevine plants transferred to *ex vitro*, high light triggers two main effects: an alternative path for electrons directly transferred to O_2, responsible for maintaining the oxidized state of Q_A; and the expression of nuclear-encoded genes for enzymes of the anti-oxidant system (Carvalho *et al.* 2006). In the present study, by feeding hormones and PET inhibitors enhancing ROS production we altered some of the most basic metabolic pathways, monitored through previously identified differentially expressed genes (Carvalho *et al.* 2011).

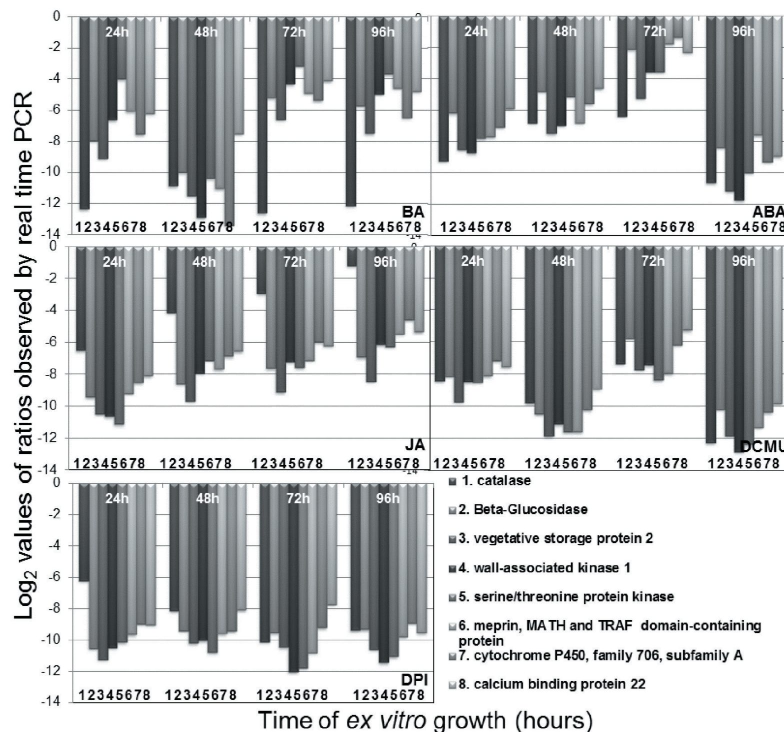

Fig. 3: Expression patterns of 8 genes after the exogenous application of BA, ABA, JA, DCMU and DPI. Expression in log_2(expression ratio) values obtained by real-time RT-PCR was monitored for 96 h after application of the various chemicals. Numbers 1 to 8 over the bars correspond to the gene names in the legend.

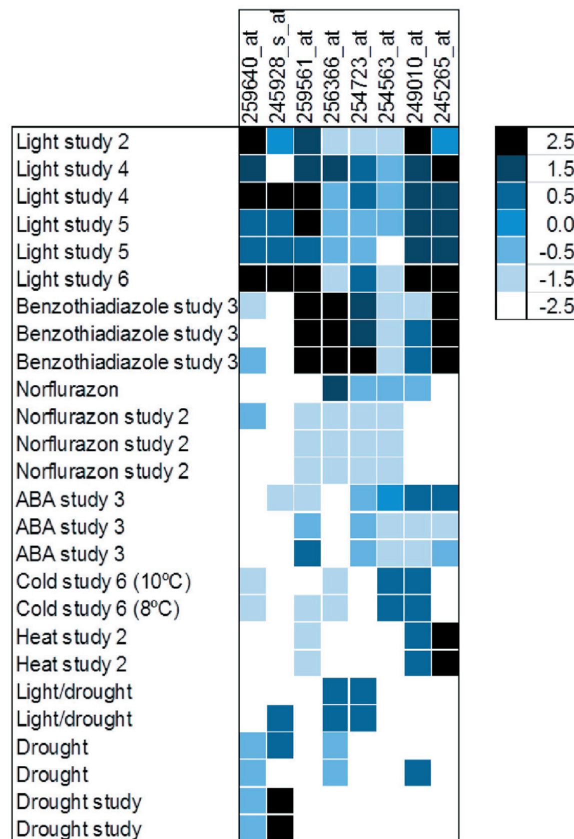

Fig. 4: Differentially regulated *Arabidopsis thaliana* orthologues of the 8 genes shown in Fig. 3 after exposure to two chemicals, benzothiadiazole and norflurazon and five abiotic stress conditions (light, cold, heat, drought and a combination of light and drought). Data were obtained with GENEVESTIGATOR (Hruz *et al.* 2008, https://www.genevestigator.ethz.ch) as described in Materials and Methods.

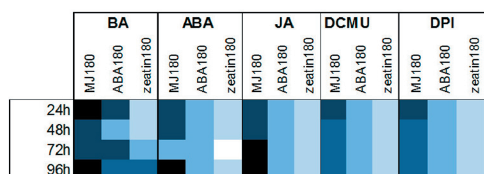

Fig. 5: Comparison of the transcriptional response in plants monitored for 96 h after application of BA, ABA, JA, DCMU and DPI with hormone responses. The response after the application of chemicals was analyzed using Hormonometer software (Volodarsky *et al.* 2009), which compares the behaviour of indexed genes to the response to different hormones (chosen here were MJ180, ABA180 and Zeatin180). The indexes of hormone action appear on the abscissa and the temporal monitoring of the response to chemical stimuli (every 24 h) appears on the ordinate. A positive correlation between the chemical response shift and a hormone treatment is denoted in dark, while lighter shades correspond to a negative correlation. Absolute values above 0.12 are the results of nonrandom correlations.

Jasmonic acid (JA) can down-regulate genes encoding proteins required for photosynthesis (Mackerness *et al.* 1999), as was the case of the gene coding for an oxygen binding protein (DQ914881) and of an electron carrier (DQ914883). Unlike other reports, e.g. (Sasaki-Sekimoto *et al.* 2005) *LOX2,* coding for a step in JA biosynthesis, was down-regulated by JA application, with a pattern similar to leaf senescence (He *et al.* 2002). ABA modulates a variety of important growth and developmental processes and responses to various stresses (Finkelstein *et al.* 2002). In micropropagated grapevine plantlets, ABA content is low until the protrusion of roots on day 7 (Vilela *et al.* 2007), and treatment with ABA induced the transcription of most antioxidant stress response genes, as expected, and its effect lasted until 72h post treatment, after that, a significant downregulation pattern was observed in almost all genes.

In a previous study we have shown that a group of genes was upregulated after 24 h and 48 h of irradiance four fold higher than *in vitro* (Carvalho *et al.* 2011). Interestingly, those genes were significantly downregulated after the treatments applied in the present study. Among them, it is worth to refer *CAT,* usually upregulated under drought, ABA and salt treatments (Xing *et al.* 2008), and with increased enzyme activity after CK application (Du *et al.* 2007). Stress-induced expression of *CAT1* depends on the production of H_2O_2, which is triggered by a mitogen-activated protein kinase (MAPK) (Xing *et al.* 2008) and both *CAT1* and *CAT3* are involved in ABA signalling in guard cells (Jannat *et al.* 2011). This suggests that various environmental stresses enhance the transcription of *CATs*. The significant downregulation of *CAT3* with all hormone treatments can be explained by the longer time-span than usually applied in this type of work and the sensitive biological system, clearly indicating a strong regulation of *CAT* transcription over extended periods. An extended regulation of up to 48 h had already been noticed in *in vitro* systems transferred to *ex vitro* (Carvalho *et al.* 2011). However, the regulation observed for these genes under the hormone and chemical treatments was sustained for twice that period (96 h), with effects that far from being transient, are kept for an important period of the plants development and adaptation to the environment.

In plants, the function of beta-glucosidase includes the hydrolysis of hormone precursors, pigment metabolism, seed development, and biomass conversion. Its dual activity leading both to the cleavage and to the synthesis of glycosidic bonds is pivotal in crucial biological pathways such as cellular signalling, biosynthesis and degradation of structural and storage polysaccharides and in host-pathogen interactions (Lee *et al.* 2008). Vegetative Storage Proteins (VSPs) are proteinaceous storage reserves that can account for up to half of the total soluble proteins in various vegetative storage organs. They can act as transient reserves that sequester unused amino acids during plant development. VSP transcripts are induced by mechanical wounding, JA, insect herbivory, osmotic and nutritional stresses (Lee *et al.* 2008). Interestingly, VSPs are known to function in tandem with beta-glucosidase in response to several biotic stresses. In this work, β-glucosidase (XM_002285548.1) coding for a member of the glycosyl hydrolase family 1 is apparently functioning in tandem with VSP in response to hormonal treatments, both showing significant levels of downregulation. Only after the application of an inducer of systemic disease resistance (benzothiadiazole) did VSP revert its downregulation pattern, however beta-glucosidase still remained downregulated (GENEVESTIGATOR data).

Interestingly, one of the genes upregulated upon CK treatment codes for a calcium-dependent protein kinase, homologous to CDPK6. In Arabidopsis, CDPK6 has been reported in the regulation of stomata movements mediated by ABA (Mori *et al.* 2006) and MJ (Munemasa *et al.* 2011) in a process that is parallel to ROS production. We have previously established that in *ex vitro* grapevine shoots without roots the regulation of stomata depends on ROS production induced by light stress (Vilela *et al.* 2008); CDPK6 could thus be a post-translational regulator of this response. As confirmed by *in silico* phosphorylation site predictions, CDPK6 is potentially capable of phosphorylating the majority of the proteins whose gene expression was downregulated by the different treatments. This seems to indicate a signalling pathway that goes beyond the transcriptional level and has effects on grapevine's response to light in excess and adaptation to *ex vitro* growth (Carvalho *et al.* 2006). Cytochrome P450 enzymes form a large superfamily of genes that can be induced in plants by many different stimuli, including chemicals, elicitors, wounding, pathogen attack, hormones (e.g. JA) and other stress factors (Keates *et al.* 2003). Cytochrome P450 enzymes can catalyze oxidative reactions that detoxify different compounds. However, some cytochrome P450 enzymes can be downregulated in response to stress. In a comprehensive microarray study involving several stress conditions and hormone feedings, ten out of 49 P450 transcripts were downregulated (Narusaka *et al.* 2004). In our study the responsive P450 transcript was one of the most strongly downregulated in all treatments.

The strong negative signature correlation between DPI and JA must be related to a reversal of the effects of this hormone, while cytokinins act in the same manner as DPI (Rakwal *et al.* 2004), as it is confirmed by the negative correlation of BA with MJ. Zeatin (a natural CK) is the only hormone showing a positive signature correlation with both PET inhibitors used. It has been reported that upon DCMU treatment, CK biosynthesis, namely zeatin, increases (Nyitrai *et al.* 2009). However, in the present work, DCMU inhibited the expression of high light induced genes and CK-induced genes usually show a pattern of expression similar to light induced genes (Werner and Schmülling 2009). DCMU was shown to lessen photodamage in isolated thylakoids by slowing the formation of stable forms of reduced Q_A, thereby allowing the occurrence of side-path reactions (Kirilovsky *et al.* 1994). As DCMU stops linear PET (Duysens 1972), it prevents H_2O_2 production in the Mehler-Asada pathway. Thus, it is not surprising that DCMU inhibited the expression of high-light-inducible genes. The similar effects that were obtained for DPI (acting as a NADPH-oxidase inhibitor) and DCMU suggest that the observed regulation events are not related to ROS, but to a growth acclimation to high light. If they were, removal of chloroplast ROS while keeping extraplastid ROS (through DCMU) would trigger different results than a situation when extraplastid ROS is not produced but electron transport ROS sources are present (in DPI treated leaves).

In conclusion, we were able to shed some light into the questions raised at the beginning of this work: ABA was proven to revert light induced gene expression and the inhibitors used in this experimental system had the same effect as ABA. ABA and JA hold a parallel effect, triggering the expression of the same sets of genes. Finally, exogenous cytokinin did not enhance the plant´s response to high light but downregulated the expression of genes upregulated by light.

Acknowledgements

This research was funded by Fundação para a Ciência e Tecnologia (FCT), through PestOE/AGR/UI0240/2011, and by grant SFRH/BPD/85767/2012 to LCC.

References

Apel, K.; Hirt, H.; 2004: Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. **55**, 373-399.

Batková, P.; Pospíšilová, J.; Synková, H.; 2008: Production of reactive oxygen species and development of antioxidative systems during i*n vitro* growth and *ex vitro* transfer. Biol. Plant. **52**, 413-422.

Brenner, W. G.; Romanov, G.; Köllmer, I.; Bürkle, L.; Schmülling, T.; 2005: Immediate-early and delayed cytokinin response genes of Arabidopsis thaliana identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. Plant J. **44**, 314-333.

Carvalho, L. C.; Amâncio, S.; 2002: Antioxidant defence system in plantlets transferred from in vitro to ex vitro : effects of increasing light intensity and CO_2 concentration. Plant Sci. **162**, 33-40

Carvalho, L. C.; Vilela, B. J.; Vidigal, P.; Mullineaux, P. M.; Amancio, S.; 2006: Activation of the ascorbate-glutathione cycle is an early response of micropropagated *Vitis vinifera* L. explants transferred to *ex vitro*. Int. J. Plant Sci. **167**, 759-770.

Carvalho, L. C., Vilela, B. J.; Mullineaux, P. M., Amâncio, S.; 2011: Comparative transcriptomic profiling of *Vitis vinifera* under high light using a custom-made array and the affymetrix GeneChip. Mol. Plant **4**, 1038-1051

Cheong, Y. H.; Chang, H. S.; Gupta, R.; Wang, X.; Zhu, T.; Luan, S.; 2002: Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. Plant Physiol. **129**, 1-17.

Coito, J. L.; Rocheta, M.; Carvalho, L. C.; Amâncio, S.; 2012: Microarray-based uncovering reference genes for quantitative real time PCR in grapevine under abiotic stress. BMC Res. Notes **5**, 220.

Du, L.; Jiao, F.; Chu, J.; Jin, G.; Chen, M.; Wu, P.; 2007: The two-component signal system in rice (*Oryza sativa* L.): a genome-wide study of cytokinin signal perception and transduction. Genomics **89**, 697-707.

Duysens, L. N.; 1972: 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) inhibition of system II and light-induced regulatory changes in energy transfer efficiency. Biophys. J. **12**, 858-863.

Finkelstein, R.; Gampala, S. L.; Rock, C. D.; 2002: Abscisic acid signaling in seeds and seedlings. Plant Cell **14**, 15-45.

Foreman, J.; Demidchik, V.; Bothwell, J. H. F.; Mylona, P.; Miedema, H.; Torres, M. A.; Linstead, P.; Costa, S.; Brownlee, C.; Jones, J. D. G.; Davies, J. M.; Dolan, L.; 2003: Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature **422**, 442-446.

Foyer, C. H.; Noctor, G.; 2005: Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell **17**, 1866-1875.

Fryer, M. J.; Ball, L.; Oxborough, K.; Karpinski, S.; Mullineaux, P. M.; Baker, N. R.; 2003: Control of Ascorbate Peroxidase 2 expression by hydrogen peroxide and leaf water status during excess light stress reveals a functional organisation of Arabidopsis leaves. Plant J. **33**, 691-705.

Fujii, H.; Chinnusamy, V.; Rodrigues, A.; Rubio, S.; Antoni, R.; Park, S. Y.; Cutler, S. R.; Sheen, J.; Rodriguez, P. L.; Zhu, J. K.; 2009: *In vitro* reconstitution of an abscisic acid signalling pathway. Nature **462**, 660-664.

Fujita, M.; Fujita, Y.; Noutoshi, Y.; Takahashi, F.; Narusaka, Y.; Yamaguchi-Shinozaki, K.; Shinozaki, K.; 2006: Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr. Opin. Plant Biol. **9**, 436-442.

Geuna, F.; Hartings, H.; Scienza, A.; 1998: A new method for rapid extraction of high quality RNA from recalcitrant tissues of grapevine. Plant Mol. Biol. Report. **16**, 61-67.

He, Y.; Fukushige, H.; Hildebrand, D. F.; Gan, S.; 2002: Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. Plant Physiol. **128**, 876-884.

Hossain, M. A.; Munemasa, S.; Uraji, M.; Nakamura, Y.; Mori, I. C.; Murata, Y.; 2011: Involvement of endogenous abscisic acid in methyl jasmonate-induced stomatal closure in Arabidopsis. Plant Physiol. **156**, 430-438.

Hruz, T.; Laule, O.; Szabo, G.; Wessendorp, F.; Bleuler, S.; Oertle, L.; Widmayer, P.; Gruissem, W.; Zimmermann, P.; 2008: Genevestigator V3 : A reference expression database for the meta-analysis of transcriptomes. Adv. Bioinform. **2008**, 1-5.

Israelsson, M.; Siegel, R. S.; Young, J.; Hashimoto, M.; Iba, K.; Schroeder, J. I.; 2006: Guard cell ABA and CO_2 signaling network updates and Ca^{2+} sensor priming hypothesis. Curr. Opin. Plant Biol. **9**, 654-663.

Jaillais, Y.; Chory, J.; 2010: Unraveling the paradoxes of plant hormone signaling integration. Nat. Struct. Mol. Biol. **17**, 642-645.

Jannat, R.; Uraji, M.; Morofuji, M.; Islam, M. M.; Bloom, R.; Nakamura, Y.; McClung, C. R.; Schroeder, J. I.; Mori, I. C.; Murata, Y.; 2011: Roles of intracellular hydrogen peroxide accumulation in abscisic acid signaling in Arabidopsis guard cells. J. Plant Physiol. **168**, 1919-1926.

Keates, S. E.; Kostman, T. A.; Anderson, J. D.; Bailey, B. A.; 2003: Altered Gene Expression in Three Plant Species in Response to Treatment with Nep1, a Fungal Protein That Causes Necrosis. Plant Physiol. **132**, 1610-1622.

Kirilovsky, D.; Rutherford, A.W.; Etienne, A. L.; 1994: Influence of DCMU and Ferricyanide on Photodamage in Photosystem II. Biochemistry **33**, 3087-3095.

Lee, K. H.; Kim, Y. S.; Park, C. M.; Kim, H. J.; 2008: Molecules and proteomic identification of differentially expressed proteins in Arabidopsis mutant ntm1-D with disturbed cell division. Mol. Cells **25**, 70-77.

Li, S.; Assmann, S. M.; Albert, R.; 2006: Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. PLoS Biol. **4**, 1732-1748.

Mackerness, S.; Surplus, S. L.; Blake, P.; John, C. F.; Buchanan-Wollaston, V.; Jordan, B. R.; Thomas, B.; 1999: Ultraviolet-B-induced stress and changes in gene expression in *Arabidopsis thaliana*: role of signalling pathways controlled by jasmonic acid, ethylene and reactive oxygen species. Plant Cell Environ. **22**, 1413-1423.

Mori, I. C.; Murata, Y.; Yang, Y.; Munemasa, S.; Wang, Y. F.; Andreoli, S.; Tiriac, H.; Alonso, J. M.; Harper, J.; Ecker, J. R.; Kwak, J. M.; Schroeder, J. I.; 2006: CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and $Ca^{(2+)}$-permeable channels and stomatal closure. PLoS Biol. **4**, 1749-1762.

Mullineaux, P. M.; Karpinski, S.; Baker, N. R.; 2006: Spatial dependence for hydrogen peroxide-directed signaling in light-stressed plants. Plant Physiol. **141**, 346-350.

Munemasa, S.; Hossain, M. A.; Nakamura, Y.; Mori, I. C.; Murata, Y.; 2011: The Arabidopsis calcium-dependent protein kinase, CPK6, functions as a positive regulator of methyl jasmonate signaling in guard cells. Plant Physiol. **155**, 553-561.

Murashige, T.; Skoog, F.; 1962: A revised medium for rapid growth and bio assays with Tohaoco tissue cultures. Physiol. Plant. **15**, 473-497.

Nambara, E.; Kuchitsu, K.; 2011: Opening a new era of ABA research. J. Plant Res. **124**, 431-435.

Narusaka, Y.; Narusaka, M.; Seki, M.; Umezawa, T.; Ishida, J.; Nakajima, M.; Enju, A.; Shinozaki, K.; 2004: Crosstalk in the responses to abiotic and biotic stresses in Arabidopsis: analysis of gene expression in cytochrome P450 gene superfamily by cDNA microarray. Plant Mol. Biol. **55**, 327-342.

Nyitrai, P.; Czövek, P.; Óvári, M.; Keresztes, Á.; 2009: Signaling mechanism mediating the anti-senescence effect of low-concentration chemical stressors sprayed onto bean seedlings. Environ. Exp. Bot. **66**, 501-506.

Orozco-Cárdenas, M. L.; Narváez-Vásquez, J.; Ryan, C. A.; 2001: Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell **13**, 179-191.

Ort, D. R.; Baker, N. R.; 2002: A photoprotective role for $O_{(2)}$ as an alternative electron sink in photosynthesis? Curr. Opin. Plant Biol. **5**, 193-198.

Pena-Cortes, H.; Willmitzer, L.; Sanchez-Serrano, J. J.; 1991: Abscisic acid mediates wound induction but not developmental-specific expression of the proteinase inhibitor II gene family. Plant Cell **3**, 963-972.

Pfannschmidt, T.; Nilsson, A.; Allen, J. F.; 1999: Photosynthetic control of chloroplast gene expression. Nature **397**, 625-628.

Rakwal, R.; Yang, G.; Komatsu, S.; 2004: Chitinase induced by jasmonic acid, methyl jasmonate, ethylene and protein phosphatase inhibitors in rice. Mol. Biol. Rep. **31**, 113-119.

Roitsch, T.; Ehness, R.; 2000: Regulation of source / sink relations by cytokinins. Plant Growth Regul. **32**, 359-367.

Sasaki-Sekimoto, Y.; Taki, N.; Obayashi, T.; Aono, M.; Matsumoto, F.; Sakurai, N.; Suzuki, H.; Hirai, M.Y.; Noji, M.; Saito, K.; Masuda, T.; Takamiya, K.; Shibata, D.; Ohta, H.; 2005: Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. Plant J. **44**, 653-668.

van der Fits, L.; Memelink, J.; 2000: ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science **289**, 295-297.

Vilela, B. J.; Carvalho, L. C.; Amâncio, S.; 2008: Integration of stress produced reactive oxygen species in the stomatal regulation of micropropagated *Vitis vinifera* L. plantlets impaired in ABA signalling. Plant Signal. Behav. **3**, 558-559.

Vilela, B.J.; Carvalho, L.C.; Ferreira, J.; Amâncio, S.; 2007: Gain of function of stomatal movements in rooting *Vitis vinifera* L. plants: regulation by H_2O_2 is independent of ABA before the protruding of roots. Plant Cell Rep. **26**, 2149-2157.

Volodarsky, D.; Leviatan, N.; Otcheretianski, A.; Fluhr, R.; 2009: HORMONOMETER: a tool for discerning transcript signatures of hormone action in the Arabidopsis transcriptome. Plant Physiol. **150**, 1796-1805.

Werner, T.; Schmülling, T.; 2009: Cytokinin action in plant development. Curr. Opin. Plant Biol. **12**, 527-538.

Wierstra, I.; Kloppstech, K.; 2000: Differential effects of methyl jasmonate on the expression of the early light-inducible proteins and other light-regulated genes in barley. Plant Physiol. **124**, 833-844.

Xing, Yu.; Jia, W.; Zhang, J.; 2008: AtMKK1 mediates ABA-induced CAT1 expression and H_2O_2 production *via* AtMPK6-coupled signaling in Arabidopsis. Plant J. **54**, 440-451.

Received May 16, 2014