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Redox state of plastoquionone pool regulates expression of *Arabidopsis thaliana* genes in response to elevated irradiance★

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DNA microarray technology was applied to gain insight into the role of the redox state of PQ pool as a retrograde factor mediating differential expression of *Arabidopsis* nuclear genes during the acclimation to changing irradiance. DNA microarray chips containing probes corresponding to 24 000 *Arabidopsis* nuclear genes were screened with cRNA samples prepared from leaves of plants exposed for 5 h to low irradiance (control) *vs.* medium, high and excessive irradiances (MI, HI and EI, respectively). Six hundred and sixty three genes were differentially expressed as a result of an exposure to at least one elevated irradiance. Among 663 differentially expressed genes a total of 50 were reverted by DCMU — 24 ones modulated at medium irradiance, 32 ones modulated at high irradiance and a single one modulated at excessive irradiance. We postulate that their expression is regulated by redox state of plastoquinone (PQ) pool. Thus the PQ-mediated redox regulation of expression of *Arabidopsis* nuclear genes is probably limited to the irradiance window representing non-stressing conditions. We found that the promoter regions of the PQ-regulated genes contained conserved elements, suggesting transcriptional control by a shared set of *trans*-acting factors which participate in signal transduction from the redox state of the PQ pool.

Keywords: DNA microarray, elevated irradiance, gene expression, hierarchical clustering, plastoquinone, transcription factors

INTRODUCTION

The light environment fluctuates constantly on a time scale of seconds and minutes up to many days and plants must respond to the exposure to changing irradiances by mechanisms allowing them to harvest and use solar energy with a maximum efficiency under low irradiance and to avoid photodamage under elevated irradiance. When exposed to elevated irradiance, plants react with short-term, acute responses (up to 3 h) and long-term acclimative ones (longer than 3 h) are elicited at various levels, including changes in gene expression patterns (Leong & Andersson, 1987; Allen 1995; Bailey *et al.*, 2001; Jackowski *et al.* 2003; Kimura *et al.*, 2003). There are indications that expression of some nuclear genes is regulated in response to changing irradiance by signals originating from chloroplasts (retrograde signaling). For example, intermediates in chlorophyll biosynthesis pathway, namely Mg-protoporphyrin IX and Mg-protoporphyrin IX monomethylester have been suggested to regulate light-dependent accumulation of *Lhcb, RbcS*, *HSP70A* and *HSP70B* transcripts in *Chlamydomonas* (Johanningmeier & Howell, 1984; Kropat *et al.*, 1997) and of *Lhc* and *Lhcb1* transcripts in *Lepidium* and *Arabidopsis,* respectively (Oster *et al.*, 1996; Mochizuki *et al.*, 2001). The ChlH subunit of Mg-chelatase has been implicated to function in the chloroplast-nucleus signaling involving chlorophyll precursors in *Arabidopsis.*

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Abbreviations: bHlH, basic helix-loop-helix; bZIP, basic region/leucine zipper; cRNA, complementary RNA; DCMU, 3-(3′,4′-dichlorphenyl)-1,1′-dimethylurea; GBF, G-box binding factor; GSH, glutathione; MYB, myeloblastosis; PQ, plastoquinone; q_{NP}, non-photochemical quenching coefficient; q_P, photochemical quenching coefficient.

This enzyme, which is found in the chloroplast inner envelope, is hypothesized to bind — by ChlH — the excess pool of chlorophyll precursors accumulating in a light-dependent fashion in the envelope and to send a signal which arrests *Lhcb1* transcription in the nucleus (Mochizuki *et al.*, 2001).

Redox signals reflecting the intensity of photosynthetic electron flow are another group of factors found to be engaged in irradiance-dependent retrograde signaling, in particular the redox state of the chloroplast pool of GSH, which is linked to the rate of photosynthetic electron flow mainly by the Mehler-peroxidase reaction combined with the ascorbate-glutathione cycle (Polle, 1996). In an early work (Karpiński *et al.*, 1997) the GSH redox state was shown to participate — by an unknown mechanism — in the strong upregulation of the accumulation of *APX1* and *APX2* mRNAs which takes place in *Arabidopsis* plants exposed to excessive irradiance. More recently, GSH level and/or its redox state (as well as its metabolism) has been suggested to influence the expression of a number of stress-associated genes in *Arabidopsis* in response to excessive irradiance (Ball *et al.*, 2004). GSH may influence the expression of stress-associated genes by changing the redox state of thiol groups or by direct S-glutathionylation of potential regulatory proteins engaged in the chloroplast-to-nucleus signaling (Ball *et al.*, 2004). There are numerous reports pointing to a role of the redox state of the plastoquinone (PQ) pool which, unlike GSH, directly participates in photosynthetic electron transport, as another redox signal influencing the expression of nuclear genes in response to changing irradiance. The green alga *Dunaliella tertiolecta* acclimates to high light conditions by repressing the rate of transcription of *Lhcb* genes (3–4 fold within 9 h of acclimation) and the repression was found to be stimulated by reduced PQ pool. This conclusion was drawn from experiments in which the accumulation of *Lhcb* transcripts was modulated by changing redox state of PQ pool of *Dunaliella tertiolecta* cells solely by treatment with site-specific electron flow inhibitors (DCMU and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone), without a switch in irradiance (Escoubas *et al.*, 1995). Regulatory pathways originating from the redox state of the PQ pool have been shown to occur also in higher plants e.g. in high irradiance-acclimated *Lemna perpusilla* plants reduced PQ pool mediates the downregulation of *Lhcb* expression (Yang *et al.*, 2001). In *Arabidopsis*, by using DNA microarray technology, it was established that 185 genes, out of > 6000 analyzed, were differentially expressed during short term exposure to high irradiance (1 h) and that in the case of at least several of them the upregulation was repressed by DCMU treatment of the plants. This clearly pointed to an involvment of reduced PQ pool in the regulation of expression of *Arabidopsis* nuclear genes (Rossel *et al.*, 2002). It is unknown how the redox signal originating from the PQ pool may be transferred from the chloroplast to the nucleus, but a phosphorelay may be considered (Fey *et al.*, 2005). In a recent DNA microarray study involving 6 500 unique genes, the importance of the redox state of the PQ pool for differential expression of *Arabidopsis* nuclear genes that occurs during the acclimative phase of the response to changing irradiance could not be fully confirmed (Piippo *et al.*, 2006). Namely the expression of *Lhcb1* and *Lhcb2* decreased during the acclimation (3 h) of moderate light grown plants to both PSI light and high white light conditions in spite of the fact that the acclimative conditions induced contrasting redox states of PQ. Other recent data suggest that at least 8 h of acclimation to changing irradiance or PSI/PSII light shift is necessary for the redox state of the PQ pool to become a determinant of gene expression in higher plants and algae (Chen *et al.*, 2004; Fey *et al.*, 2005).

The aim of the present study was to make a step towards ultimate establishing whether the redox state of the PQ pool mediates differential expression of higher plant nuclear genes during acclimation to changing irradiance. To do this the expression profiles of 24 000 *Arabidopsis* nuclear genes — represented by Affymetrix ATH1 genome platform — were monitored in plants grown in low irradiance and then acclimated (5 h) to medium, high and excessive (stressing) irradiances. Measurements of q_P and q_{NP} were used to determine the functional status of PSII at each irradiance studied. It was demonstrated that 663 individual genes yielded significant changes in expression in at least one of the three elevated irradiances applied. Genes for which the up- or downregulation occuring in response to elevated irradiance may be mediated by redox state of PQ pool were identified by comparing the expression levels of differentially expressed genes in DCMU-treated and DCMU-nontreated plants. A total of 24 genes modulated at moderate irradiance, 32 ones modulated at high irradiance and a single one modulated at excessive irradiance were reverted by DCMU treatment and it is implied that their expression is regulated by the redox state of the PQ pool which produces a chloroplast retrograde signal during 5-h acclimation. Thus the PQ-mediated redox regulation of expression of nuclear genes is probably limited to the irradiance window representing non-stressing conditions. Nine novel, conserved, short (6–8 bp) 5′ motifs were found which may participate in the transduction of the signal from the redox state of the PQ pool to downstream genes.

MATERIAL AND METHODS

Plant material and growth conditions. *Arabidopsis thaliana* (ecotype Columbia) plants were grown in plant growth chamber NBC 660 (Nema Industrietechnik, Germany) equipped with PLL type flouorescent tubes plus incandescent lamps for four weeks in 15 h-light/9 h-darkness conditions at a 22°C/18°C temperature cycle under 100 μmol quanta m^{-2} s^{-1} (low irradiance) and relative humidity of 70%. Then the plants were transferred for 5 h to 200 μmol quanta m^{-2} s^{-1} (medium irradiance), 400 μmol quanta m^{-2} s^{-1} (high irradiance) or 700 μmol quanta m^{-2} s^{-1} (excessive irradiance). As a control material one set of plants was maintained at 100 μmol quanta m^{-2} s^{-1}.

DCMU was applied to the plants directly before the irradiance switch by spraying them with solutions prepared by diluting the 100% ethanol stock solution (28 mM) in sterile water (+DCMU plants). The concentrations assayed were in the range of 4–40 μM. As a control the plants were sprayed with an appropriate ethanol solution (–DCMU plants). DCMU concentrations of 40 μM (medium irradiance) and 10 μM (high and excessive irradiance) were chosen as ensuring similar, low q_P values measured after 5 h of exposure to the elevated irradiances in +DCMU plants (not shown).

Chlorophyll *a* fluorescence measurements. Chlorophyll *a* fluorescence was measured at room temperature with a PAM FSM1 fluorometer (Hansatech, UK) run by Modfluor software essentially as described elsewhere (Jackowski *et al.*, 2003).

The photochemical fluorescence quenching coefficient q_P was calculated as $F_m' - F_s / F_m' - F_0'$ and the non-photochemical quenching coefficient q_{NP} as $1 - (F_m' - F_0')/ F_m - F_0$ (Bilger & Schreiber, 1986).

RNA isolation, labeling and hybridization with DNA microarrays. Total RNA was isolated from leaves with the Trizol reagent (Invitrogen, USA) and purified by using RNeasy Mini Kit (Qiagen, USA). Samples from two independent biological repeat experiments, each sample representing three separate preparations pooled together, were collected for each irradiance and each DCMU treatment. The quality of RNA samples was assessed electrophoretically by evaluating the 18S rRNA/28S rRNA ratio and spectrophotometrically by A_{260}/A_{280} measurements (Nanodrop ND-1000 spectrophotometer, Nanodrop Technologies, USA). Total RNA served as a template in first strand cDNA synthesis using T7-Oligo dT-primer and the second strand of cDNA was synthesized by consecutive administration of DNA polymerase I, DNA ligase and T4 DNA polymerase (One Cycle cDNA Synthesis Kit, Invitrogen, USA). Next, labeled cRNA was synthesized with direct incorporation of biotin (Gene Chip IVT Labeling Kit, Affymetrix, USA) and the resulting samples were heat-fragmented and hybridized with ATH1 DNA microarray chips (Affymetrix, USA) containing probes corresponding to 24 000 *Arabidopsis thaliana* nuclear genes. To obtain a fluorochrome signal hybridized chips were stained with phycoerythrin/streptavidin conjugates and biotinylated anti-streptavidin antibodies.

DNA microarray analysis. The DNA microarrays chips were scanned with Agilent Gene Array Scanner (Affymetrix, USA) and the hybridization data were analyzed using dChip software (http://biosun1.harvard.edu/complab/dchip/).

Raw signal intensities were normalized against an array with median overall intensity as the baseline array. Next, the PM/MM model fitting procedure was used to identify array and probe outliers (Cheng & Wong, 2001a; 2001b).

For each gene which was differentially expressed as a result of exposition for 5 h to at least one elevated irradiance the expression value at a given irradiance was standardized as follows: the mean expression value (involving expressions at all irradiances studied) was subtracted from the expression value measured at the given irradiance and the result was divided by standard deviation.

For each gene which was differentially expressed as a result of exposure for 5 h to medium irradiance or high irradiance and repressed by DCMU the expression value at a given irradiance was standardized as follows: the expression value found for control plants (LI) was subtracted from expression values measured at the given irradiance in the absence and in the presence of DCMU and the result was divided by standard deviation.

Normalized data were divided into groups containing replicate arrays for experiment conditions and the groups were compared using "Compare samples" function. The following criteria were specified for the comparisons of the normalized data received for the genes expressed at elevated irradiances *vs.* control conditions as well as those expressed at a given elevated irradiance with DCMU *vs.* without DCMU:

1. The fold change between the mean expression value (involving expressions at all irradiances studied) subtracted from the expression value measured at a given irradiance and the mean expression value was required to exceed the threshold of 2.

2. The absolute difference between B and E was required to exceed the threshold of 100.

3. The percentage of samples called "present" in the samples used in both groups was required to exceed the threshold of 20%.

For genes that satisfied the comparison filtering criteria (i.e. differentially expressed ones) annotation from The National Center for Biotechnology

Information was derived (http://www.ncbi.nlm.nih.gov) and hierarchical clustering was performed. The default hierarchical clustering algorithm used was based on 1 — r (r is the Pearson correlation coefficient between the standardized expression values of two genes) as the genes distance metrics and average linkage as the method of merging genes into supergenes.

The functional classification of differentially expressed genes was based on the MIPS (Munich Information Center for Protein Sequences) classification scheme and was performed using The MIPS Functional Catalogue Database (FunCatDB) (http://mips.gsf.de/projects/funcat)

Promoter motif searches. Sequences upstream of the differentially expressed genes (–500 to –1) were derived using TAIR Bulk Data Retrieval Tool (http://www.arabidopsis.org/tools/bulk/index.jsp). The derived sequences were analyzed for overrepresented short motifs (from 6 to 10 bp) by use of a stand-alone version of Motif Sampler algorithm, which is based on the Gibbs sampling method (Thijs *et al.*, 2001) (http://homes.esat.kuleuven.be/~thijs/Work/MotifSampler.html)

The following parameters were specified for promoter analysis:

1. A background model for *Arabidopsis thaliana* was indicated and its order was restricted to 1.

2. Both strands of DNA sequences were included in the analysis.

3. The number of different motifs to search for in a single run was set to 4.

The motif was considered as overrepresented *ergo* a potential regulatory element when the information content score (which reflects the Kullback-Leiber distance between the motif and single nucleotide frequency) was greater then 1.3 (Thijs *et al.*, 2002).

The MatInspector software (http://www.genomatix.de) was used to assess similarity of motifs identified as potential promoter regulatory elements to previously described transcription factor binding sites and to identify MYB and GBF recognition sequences in clusters of tightly coexpressed genes (Higo *et al.*, 1999). The analysis was performed using Plant IUPAC library and "matches to individual library strings" function. The maximum percentage of mismatches was set to 0.

RESULTS

Selection of elevated irradiance generating various PSII functional states

To select elevated irradiances which generate different functional states of PSII plants grown under low irradiance regime were transferred for 1 or 5 h to 200–800 μmol quanta m^{-2} s^{-1} and q_P and q_{NP} values were monitored. As illustrated in Fig. 1 no statistically meaningful changes in the q_P value were found to accompany 1 h or 5 h exposure of low irradiance-grown plants to 200 μmol quanta m^{-2}

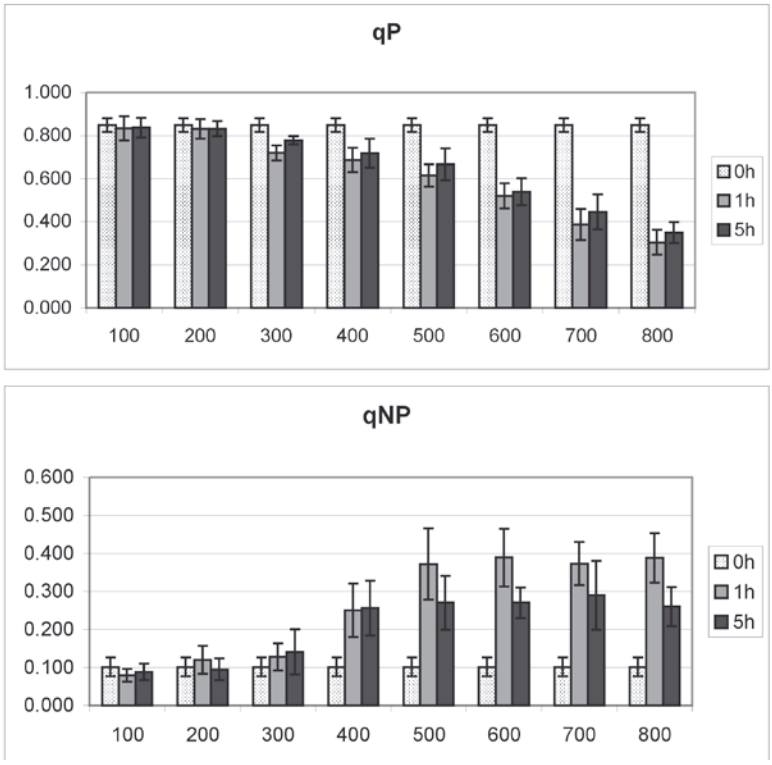


Figure 1. Time-course of changes of quenching coefficients q_P and q_{NP} as a function of acclimative irradiance. *Arabidopsis thaliana* plants were grown for four weeks at low irradiance (100 μmol quanta m^{-2} s^{-1}) and acclimated to 200–800 μmol quanta m^{-2} s^{-1} for 1 or 5 h. One set of plants (control material) was kept at 100 μmol quanta m^{-2} s^{-1}.

s^{-1} but as the q_{NP} value displayed a slight increase at 1 h (accompanied by a slight decrease in Fv/Fm, not shown) and recovered after 5 h of exposure, 200 μmol quanta m^{-2} s^{-1} was interpreted as a medium irradiance, near the inflexion point on the irradiance curve. The q_P value measured after 1 h of exposure to 300–500 μmol quanta m^{-2} s^{-1} was decreasing in a stepwise manner with an increase in irradiance (down to 0.62 at 500 μmol quanta m^{-2} s^{-1} *vs.* 0.83 in control plants), whereas q_{NP} exhibited the opposite tendency. This indicates that after 1 h of exposure to 300–500 μmol quanta m^{-2} s^{-1} a considerable part of the Q_A pool became reduced and this caused down-regulation of PSII in the form of an increase in q_{NP} – that is why it is implied that 300–500 μmol quanta m^{-2} s^{-1} reflects high irradiance conditions. Both q_P and q_{NP} were partially recoverable after 5 h of exposure, implying that this time point is, as expected, within the long-term acclimative phase of response to changing irradiance conditions, while 1 h time point is within the short-term acute phase of the response. q_P measured after 1 h of exposure to 600–800μmol quanta m^{-2} s^{-1} exhibited a further stepwise decrease (paralleled by high values of q_{NP}) to less than 0.6 (again partially recoverable after 5 h of exposure) and this reflects overreduction of Q_A. These data indicates that this irradiance window lies within the excessive irradiance range which poses a high risk of PSII photodamage.

The values of 200, 400 and 700 μmol quanta m^{-2} s^{-1} were selected for DNA microarray studies as representing medium, high and excessive irradiance conditions, respectively, and 5 h of exposure as representing the acclimative phase of the response to elevated irradiance.

DNA microarray-based identification of genes the expression of which is modulated in response to elevated irradiance in a redox state of PQ pool-mediated manner

Among the 24 000 genes analyzed, 663 ones (2.7%) were differentially expressed as a result of exposure to at least one elevated irradiance. The exposure to medium, high and excessive irradiance modulated the expression of 98, 321 and 564 genes, respectively (Fig. 2). At the medium irradiance alone 9 genes were upregulated *vs.* 10 downregulated whereas the relevant values for the high and excessive irradiances were 21 *vs.* 55 and 185 *vs.* 127, respectively. Sixty four genes were differentially expressed at all three irradiances studied.

The differentially expressed genes were arranged according to similarity in the profile of their expression and hierarchical clustering was performed. Detailed data concerning the results of the clustering, functional classification and identity of the differentially expressed genes will be described elsewhere.

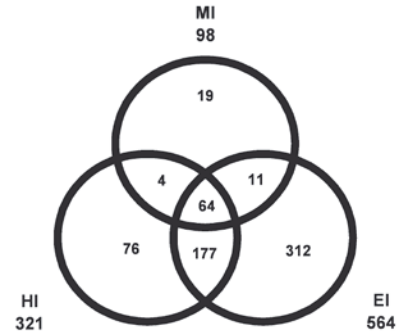


Figure 2. Total numbers of overlapping and non-overlapping genes differentially expressed in response to 5 h of exposure to medium, high and excessive irradiances (MI, HI and EI, respectively).
Samples from two independent biological repeat experiments, each sample representing three separate preparations pooled together, were collected for each elevated irradiance.

To search for the genes for which the up- or downregulation in response to elevated irradiance is mediated by the redox state of PQ pool, plants were sprayed with DCMU, a reagent specifically blocking the photosynthetic electron flow between Q_A and Q_B and in this way oxidizing the PQ pool. Of the 663 differentially expressed genes a total of 50 were reverted by DCMU, i.e. 24 ones modulated at medium irradiance, 32 ones modulated at high irradiance and a single one modulated at excessive irradiance (Fig. 3). No one differentially expressed gene was found to be reverted by DCMU at all three elevated irradiances although six genes modulated at both medium and high irradiance were among the DCMU-reverted ones — zinc bind-

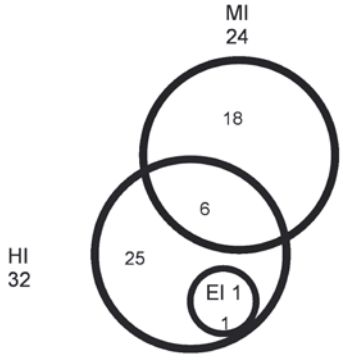


Figure 3. Total numbers of overlapping and non-overlapping genes differentially expressed in response to 5 h of exposure to medium, high and excessive irradiances and reverted by DCMU (MI, HI and EI, respectively).
Samples from two independent biological repeat experiments, each sample representing three separate preparations pooled together, were collected for each elevated irradiance (with or without DCMU treatment).

ing oxidoreductase (At5g51970), electron transporters (At1g11530 and At5g06690), starch synthase (At1g32900), legumain (At1g62710) and glucose 6-phosphate transporter (At1g61800). The single gene the expression of which was modulated at excessive irradiance and reverted by DCMU was found to be modulated at high irradiance and reverted by DCMU treatment as well (unclassified gene, At3g26510). Unclassified genes and metabolism-associated ones heavily dominated the pool of DCMU-reverted genes, both at medium and high irradiance. The remaining MIPS-based subcategories were represented by one or two genes.

The data regarding the expression profiles of the 50 differentially expressed genes which were found to be reverted by DCMU were analysed by hierarchical clustering. Figure 4 shows a clustered display of the data along with columns of coloured bars representing those genes as belonging to individual functional subcategories. When the data regarding the expression profiles of genes differentially expressed at medium irradiance in DCMU-treated and DCMU-untreated plants were analysed by hierarchical clustering, four clusters were identified designated M1–M4 which grouped 11 classified and 13 unclassified genes (Fig. 4a, Table 1). The M1 cluster, involving four classified genes activated 2.4–6 times at medium irradiance and reverted to a similar extent by DCMU-treatment, is remarkable since it includes PAP1 transcription factor. The genes which clustered with PAP1 at medium irradiance are: transferase transferring glycosyl groups (At1g32900), legumain (At1g62710) and glucose 6-phosphate transporter (At1g61800). Other upregulated genes (cluster M2) as well as all downregulated genes

a

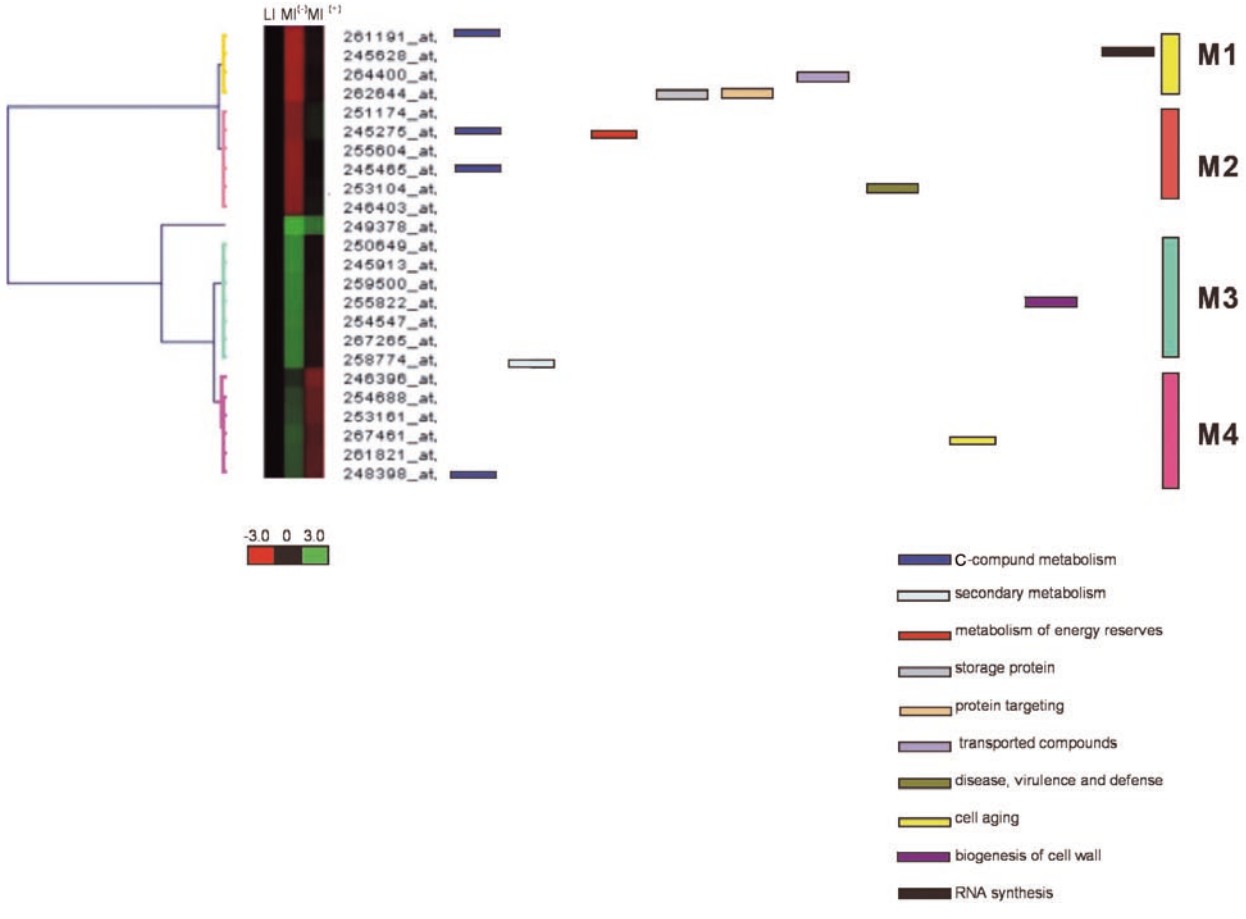
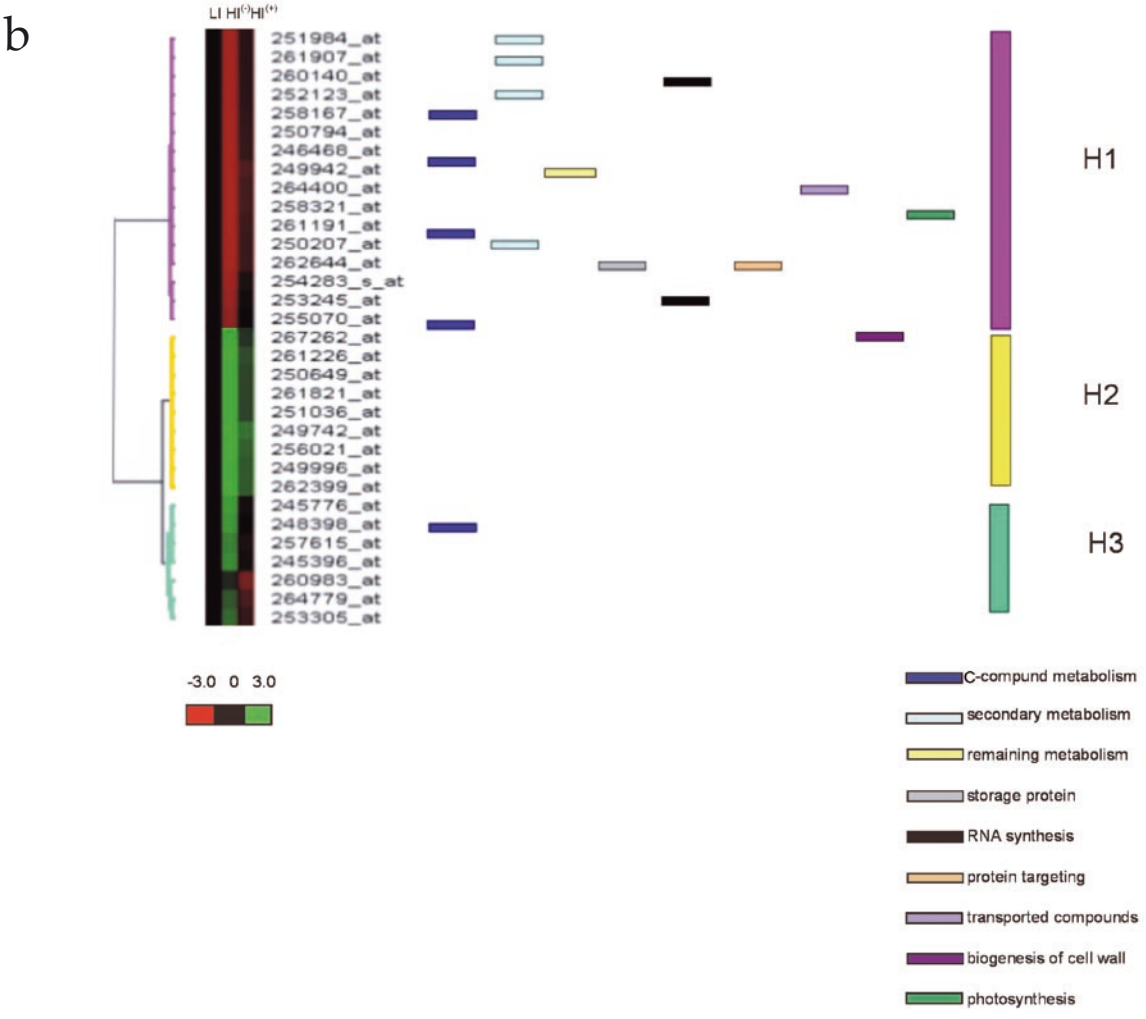


Figure 4. Hierarchical clustering of genes differentially expressed after exposure to medium irradiance (a) and high irradiance (b) and reverted by DCMU.
The genes belonging to individual MIPS-based functional subcategories are shown as columns of small coloured boxes, on the right in relation to the clustered display of the data. Separate clusters were marked by coloured bars and by the same colouring of the corresponding region of dendrogram both in (a) and (b). Standardized expression of each gene is displayed in colour as fold change between the mean expression value (involving expressions at all irradiances studied)

(M3 ands M4) did not cluster tightly with any transcription factor. The upregulatory nature of PAP1 suggests that this factor is engaged in signal transduction pathway(s) occurring in response to MI by activating the transcription of at least three above-mentioned genes, transducing the upstream signal dependent on the redox state of PQ pool. Other genes which were differentially expressed at medium irradiance and reverted by DCMU (those belonging to M2–M4 clusters) may be regulated, using the PQ signal, by pre-existing transcription factors, possibly along with PAP1.

The hierarchical cluster analysis of the data regarding the expression profiles of genes which were differentially expressed at high irradiance and repressed by DCMU disclosed the existence of three clusters (Fig. 4b, Table 2). One of them (designated H1) comprises all genes which are upregulated in response to high irradiance while the genes which are downregulated are grouped in two clusters, designate H2 and H3. Cluster H1 comprises as many as 14 classified and two unclassified genes activated 2.01–24.51 times in response to high irradiance and reverted under DCMU treatment. Four of them code for enzymes participating in the flavonoid/anthocyanin biosynthesis pathway (4CL — At1g65060, PAL2 — At3g53260, F3H — At3g51240, CHS — At5g13930) whereas the other classified ones are: oxidoreductase/zinc ion binding — At5g51970, nitrilase 4 — At5g22300, UDP-glucosyltransferases — At3g21560 and At5g17050, hydrolase hydrolyzing O-glycosyl compounds — At4g09020, PAP2 — At1g66390, GBF6 — At4g34590, transferase transferring glycosyl groups — At1g32900, legumain — At1g62710 and glucose 6-phosphate transporter — At1g61800.



subtracted from expression value measured at given irradiance and the mean expression value. Since the expression level for each gene is standardized to have a mean of 0 and standard deviation 1, the standardized expression values most likely fall within (–3, 3). The colour scale ranges from green (downregulation, –3), through black (no differential expression, 0) to red (upregulation, +3). LI — low irradiance (control), $MI^{(-)}$ — medium irradiance without DCMU treatment, $MI^{(+)}$ — medium irradiance with DCMU treatment, $HI^{(-)}$ — high irradiance without DCMU treatment, $HI^{(+)}$ — high irradiance with DCMU treatment.

Table 1. Genes differentially expressed at medium irradiance and reverted by DCMU

Category	Probe set	Accession number	Description	Fold change	
				$MI^{(-)}/LI$	$MI^{(+)}/MI^{(-)}$
C-compound and carbohydrate metabolism	261191_at	At1g32900	transferase, transferring glycosyl groups	5.04	–3.47
	245275_at	At4g15210	ATBETA–AMY (β-amylase)	2.38	–2.04
	245465_at	At4g16590	ATCSLA01 glucosyltransferase/ transferase, transferring glycosyl groups	2.49	–3.04
	248398_at	At5g51970	oxidoreductase/ zinc ion binding	–2.13	3.58
secondary metabolism	258774_at	At3g10740	ASD1 hydrolase, acting on glycosyl bonds	–2.62	2.91
metabolism of energy reserves	245275_at	At4g15210	ATBETA–AMY (β-amylase)	2.38	–2.04
storage protein	262644_at	At1g62710	BETA–VPE cysteine-type endopeptidase/ legumain	2.4	–2.33
protein targeting	262644_at	At1g62710	BETA–VPE cysteine-type endopeptidase/ legumain	2.4	–2.33
transported compounds	264400_at	At1g61800	antiporter/ glucose-6-phosphate transporter	6	–4.15
disease, virulence and defense	253104_at	At4g36010	pathogenesis-related thaumatin family protein	2.27	–2.36
cell aging	253161_at	At4g35770	SEN1 (dark inducible 1)	–11.2	6.27
biogenesis of cell wall	255822_at	At2g40610	ATEXPA8 (*Arabidopsis thaliana* expansin A8)	–2.66	2.71
RNA synthesis	245628_at	At1g56650	PAP1 (production of anthocyanin pigment 1)	2.63	–2.51
unclassified proteins or classification not yet clear-cut	261821_at	At1g11530	electron transporter/ thiol-disulfide exchange intermediate	–2.38	3.5
	259500_at	At1g15740	protein binding	–2.47	2.84
	246396_at	At1g58180	carbonate dehydratase/ zinc ion binding	–2.37	2.17
	267265_at	At2g22980	SCPL13 catalytic/ serine carboxypeptidase	–2.61	2.97
	251174_at	At3g63200	nutrient reservoir	2.23	2.12
	255604_at	At4g01080	unknown protein	2.2	–2.84
	254688_at	At4g13830	J20 (DNAJ-LIKE 20) heat shock protein binding / unfolded protein binding	–2.53	2.2
	254547_at	At4g19860	phosphatidylcholine-sterol *O*-acyltransferase	–2.32	2.01
	250649_at	At5g06690	electron transporter/ thiol-disulfide exchange intermediate	–2.01	2.69
	245913_at	At5g19860	unknown protein	–2.27	2.24
	249378_at	At5g40450	unknown protein	–5.64	2.31
	246403_at	At1g57590	carboxylic ester hydrolase	2.08	–3
	267461_at	At2g33830	unknown protein	–8.25	7.31

It is interesting to note that two of the genes belonging to the cluster code for transcription factors, namely PAP2 (MYB family, highly homologous to PAP1) (Borevitz *et al.*, 2000) and GBF6 (bZIP family). Interestingly, PAP2 coexpresses at high irradiance in DCMU-untreated and DCMU-treated plants with three genes found to cluster with PAP1 at medium irradiance with and without DCMU treatment, i.e. transferase transferring glycosyl groups (At1g32900), legumain (At1g62710) and glucose 6-phosphate transporter (At1g61800).

The genes which were downregulated at high irradiance and reverted by DCMU (16 genes, vast majority being unclassified) were grouped in clusters H2 and H3 (Fig. 4b, Table 2) and did not coexpress with any transcription factor.

The only gene which was differentially expressed at EI and reverted by DCMU (At3g26510, downregulated 4.78 times at EI and reverted 2.03 times by DCMU treatment at EI) coded for unknown, octicosapeptide/Phox/Bem1p (PB1) domain-containing protein.

Table 2. Genes differentially expressed at high irradiance and reverted by DCMU

Category	Probe set	Accession number	Description	Fold change	
				$HI^{(-)}/LI$	$HI^{(+)}/HI^{(-)}$
c-compund metabolism	248398_at	At5g51970	oxidoreductase/ zinc ion binding	–2.46	2.11
	261191_at	At1g32900	transferase, transferring glycosyl groups	8.58	–2.41
	246468_at	At5g17050	UDP-glycosyltransferase/ transferase, transferring glycosyl groups	3.93	–2.05
	258167_at	At3g21560	UDP-glycosyltransferase/ transferase, transferring hexosyl groups	3.21	–2.37
	255070_at	At4g09020	ATISA3/ISA3; alpha-amylase/ hydrolase, hydrolyzing O-glycosyl compounds	2.01	–2.22
secondary metabolism	250207_at	At5g13930	CHS (chalcone synthase); naringenin-chalcone synthase	7.71	–2.12
	261907_at	At1g65060	4CL3; 4-coumarate-CoA ligase	4.11	–2.25
	252123_at	At3g51240	F3H naringenin 3-dioxygenase	13.08	–2.88
	251984_at	At3g53260	PAL2; phenylalanine ammonia-lyase	2.05	–2.22
remaining metabolism	249942_at	At5g22300	NIT4 (nitrilase 4)	3.40	–2.08
storage protein	262644_at	At1g62710	BETA–VPE; cysteine-type endopeptidase/ legumain	3.09	–2.08
RNA synthesis	260140_at	At1g66390	PAP2 (production of anthocyanin pigment 2)	9.99	–3.45
	253245_at	At4g34590	GBF6	2.30	–2.49
protein targeting	262644_at	At1g62710	BETA–VPE; cysteine-type endopeptidase/ legumain	3.09	–2.08
transported compounds	264400_at	At1g61800	glucose-6-phosphate transporter	9.85	–2.30
biogenesis of cell wall	261226_at	At1g20190	ATEXPA11 (*Arabidopsis thaliana* expansin A11)	–3.43	2.21
photosynthesis	258321_at	At3g22840	ELIP1 (early light-inducable protein 1)	7.49	–2.48
unclassified proteins or classification not yet clear-cut	264779_at	At1g08570	electron transporter/ thiol-disulfide exchange intermediate	–2.65	2.21
	261821_at	At1g11530	electron transporter/ thiol-disulfide exchange intermediate	–3.71	2.02
	245776_at	At1g30260	AGL79	–2.59	2.35
	262399_at	At1g49500	unknown protein	–6.16	4.81
	260983_at	At1g53560	unknown protein	–2.02	2.15
	256021_at	At1g58270	ZW9	–3.23	2.26
	267262_at	At2g22990	SNG1 (sinapoylglucose 1); serine carboxypeptidase	–4.29	2.55
	257615_at	At3g26510	octicosapeptide/Phox/Bem1p (PB1) domain-containing protein	–3.41	2.71
	245396_at	At4g14870	unknown protein	–2.04	2.09
	254283_s_at	At4g22870	unknown protein	24.51	–4.07
	253305_at	At4g33666	unknown protein	–3.44	3.73
	251036_at	At5g02160	unknown protein	–4.54	2.85
	250794_at	At5g05270	unknown protein	8.89	–2.46
	250649_at	At5g06690	electron transporter/ thiol-disulfide exchange intermediate	–3.15	2.10
	249742_at	At5g24490	structural constituent of ribosome	–7.26	2.89
	249996_at	At5g18600	arsenate reductase (glutaredoxin)/ electron transporter/ thiol-disulfide exchange intermediate	–5.92	2.55

A search for conserved elements in promoters of genes the expression of which is mediated by the redox state of the PQ pool

A promoter motif analysis of 50 genes which were differentially expressed as a result of exposure to at least one elevated irradiance and reverted by DCMU was performed to search for conserved known and novel regulatory elements which may participate in signal transduction from the redox state of the PQ pool. As shown in Table 3 twelve motifs were identified. When the occurrence of the 5′ motifs were compared with regard to the genes which were differentially expressed at medium *vs.* high irradiance and up *vs.* downregulated ones no clear trends were found, i.e. the promoters possessing the motifs included all subsets of the 50 genes analysed (not shown).

One of the motifs, GAGArA, is very similar to the bwGAGAGA element found earlier as overrepresented in *Arabidopsis* core promoters (Molina & Grotewold, 2005) and thus can not be regarded as specifically involved in elevated-irradiance responsiveness mediated by the redox state of the PQ pool. Two out of the remaining eleven resemble known motifs. The AyACGT motif closely resembles ATACGTGT, described earlier as Z-box (one of light-responsive elements) suggested to be activated in *Arabidopsis* by a *trans*-acting factor designated ZBF (which remains to be characterized) in cooperation with HY5 of bZIP family (Yadav *et al.*, 2002). The second motif, ATATAT, resembles *Arabidopsis* TGTATATAT sequence (designated SORLREP 3), one of a few elements demonstrated to be overrepresented in light-responsive, phytochrome A-repressed promoters, binding yet-to-be-discovered bHLH repressors and other, still unknown, transciption regulators (Hudson & Quail, 2003). Finally, nine motifs found by us have not been identified previously.

To further define the *cis*-acting elements engaged in irradiance responsivness mediated by the redox state of the PQ pool we searched for the presence of binding sites for PAP1, PAP2 (both belonging to the MYB family) and GBF6 (bZIP family). These transcription factors were found among the 50 genes regulated in a PQ-dependent manner and we anticipated that the factors would be involved in the regulation of expression of at least a subset of coexpressed genes. As it is overviewed in Table 4, wAACCA (one of the MYB recognition sequences) (Abe *et al.*, 2003) and G-box (a ubiquitous CACGTG motif recognized by GBF proteins) (Menkens *et al.*, 1995) are frequently found in the 5′ motifs of the genes tightly coexpressed with *PAP1*/*PAP2* and *GBF6*, respectively. Namely, 100% of promoters of genes tightly coexpressed with *PAP1* and 69% of

Table 3. Common promoter motifs from 50 genes which were differentially expressed on exposure to at least one elevated irradiance and reverted by DCMU.

The motifs were selected so that their information contents did not fall below 1.30. The degenerate symbols are the following: b = GTC, m = AC, n = any nucleotide, r = AG, w = AT, y = TC.

Motif	Number of promoters containing motif	Information content	Similar to		
			Motif	Description	Recognized by
GTGGmT	19/50	1.87			
GAGArA	28/50	1.83	bw**GAGAGA**	overrepresented in *Arabidopsis* core promoters	no data available
AyACGT	13/50	1.83	**ATACGT**GT	Z-Box (Z-DNA–forming sequence)	ZBF (+HY5)
AnCCAC	25/50	1.77			
TTTTGT	29/50	1.72			
TTTGGT	31/50	1.68			
TTTTTT	34/50	1.53			
ATATAT	19/50	1.52	TGT**ATATAT**	SORLREP3 (Seqence Over-Represented in Light-Repressed Promoters)	no data available
TAATTA	23/50	1.52			
GTwGGTTT	11/50	1.48			
TTTyTTyT	30/50	1.36			
ArArAAAA	34/50	1.31			

Table 4. Distribution of G-boxes and MYB1AT MYB recognition sites among the promoters of the genes belonging to clusters M1 and H1.

Genes possessing and not possessing given site are shown as grey and open cells of the table, respectively. Degenerate symbol w means AT.

Cluster	Gene accession number	G-box (CACGTG)	MYB1AT (wAACCA)
M1	At1g32900		■
	At1g56650	■	■
	At1g61800		■
	At1g62710	■	■
H1	At1g32900		■
	At1g61800		■
	At1g62710	■	■
	At1g65060		■
	At1g66390	■	
	At3g21560		■
	At3g22840	■	■
	At3g51240	■	■
	At3g53260		■
	At4g09020	■	■
	At4g22870		■
	At4g34590		
	At5g05270	■	
	At5g13930	■	
	At5g17050		
	At5g22300		■

those coexpressed with *PAP2* (clusters M1 and H1, respectively) possess MYB recognition sites (including *PAP1* promoter itself) while G-box is present in 44% of promoters of the genes coexpressed with GBF6 (including *PAP2,* cluster H1). Thus all three factors may in fact upregulate the transcription of subsets of genes regulated in a PQ-dependent manner. In spite of this, the MYB and GBF recognition sequences were not retrieved among the conserved, PQ-specific promoter motifs (Table 4) since these recognition sequences yield very low information content scores when promoters of genes which do not coexpress tightly with *PAP1*/*PAP2* and *GBF6* are analysed (not shown).

DISCUSSION

Almost 3% of the 24 000 genes analysed were found to be differentially expressed (i.e. the fold change between the baseline group mean and the experimental group mean exceeded 2) in response to exposure to at least one elevated irradiance with excessive irradiance regulating the transcription of the highest number of genes and generating the highest values of fold changes.

Our data allow attributing a significant role to the redox state of PQ pool in retrograde signaling in *Arabdopsis* in response to 5 h of exposure to non-stressing elevated irradiances since as many as 50 genes differentially expressed at medium or high irradiance (and just a single gene differentially expressed at excessive irradiance which was also differentially expressed at high irradiance) have been reverted by oxidation of PQ pool induced by DCMU treatment. Most probably other retrograde plastid signals may influence the expression of nuclear genes at excessive irradiance window when the majority of the PQ pool becomes reduced, e.g. the redox state of GSH pool, as has convincingly been demonstrated recently by showing that the expression of more than 30 genes is affected in high irradiance-stressed *Arabidopsis* mutants with lowered level of foliar glutathione (Ball *et al.*, 2004). Our data demonstrate that at medium and high irradiance 5 h of acclimation is enough for the changing redox state of PQ pool to become operational as a retrograde signal regulating the expression of *Arabidopsis* nuclear genes, in contrast to the events taking place within 3 h of acclimation seemingly involving other mechanisms of signaling (Piippo *et al.*, 2006).

In spite of the existence of a few reports pointing to a role of the redox state of the PQ pool as a redox signal influencing the expression of *Lhcb* genes in response to changing irradiance (Escoubas *et al.*, 1995; Chen *et al.*, 2004), we were not able to support this idea since no *Lhcb*s, with an exception of *Lhcb2.4* and *Lhcb4.2*, were differentially expressed at the threshold level 2 at elevated irradiances (not shown) and this precluded the analysis of their expression pattern in experiments with DCMU. In the case of *Lhcb2.4* and *Lhcb4.2* the redox state of the PQ pool does not mediate their downregulation occurring at excessive irradiance since the downregulation was not reverted by DCMU.

At medium irradiance genes which were differentially expressed and repressed by DCMU formed four clusters (M1–M4). It is noteworthy that one of them (M1) comprises the gene coding for PAP1 (MYB75) which has been suggested to contribute to activation of not only numerous genes controling general flavonoid, anthocyanin and flavonol biosynthetic pathways but also of many genes coding for proteins loosely related or unrelated to general flavonoid and anthocyanin biosynthetic pathways (Borevitz *et al.*, 2000; Tohge *et al.*, 2005). Our data show that in response to DCMU treatment the gene coding for PAP1 coexpresses closely with three other ones probably unrelated to general flavonoid and anthocyanin biosynthetic pathways and listed neither by Borevitz *et al.* (2000) nor by Tohge

et al. (2005), namely those coding for transferase (At1g32900), legumain (At1g62710) and glucose 6-phosphate transporter (At1g61800). Thus it is reasonable to suggest that PAP1 is involved in a signal transduction pathway dependent on the redox state of PQ pool responsible for activation of those novel genes under medium irradiance as well.

The genes which were activated at high irradiance and reverted by DCMU formed a major cluster designated H1, highlighted by the presence of genes encoding two transcription factors — PAP2 (MYB90) and GBF6. The cluster comprises additionally four genes controling enzymes of general flavonoid biosynthesis pathway and nine others. *PAP2* has been shown recently to be upregulated in response to high light treatment (1–8 h) and suggested to be a member of a transcriptional cluster (regulon) involved in *Arabidopsis* in the anthocyanin and flavonol biosynthesis pathway (Vanderauwera *et al.*, 2005). As *PAL2, 4CL3, CHS* and *F3H* are found both in our H1 cluster and the putative regulon, our data support this suggestion at least with regard to genes coding for CHS, PAL2, F3H and 4CL3, and additionally provides evidence that a signal pathway leading to elevated irradiance- and PAP2-dependent activation of *CHS, PAL2, F3H* and *4CL* is mediated by the redox state of the PQ pool. In the case of nine other genes belonging to cluster H1, the signal of the redox state of PQ may be transduced at high irradiance by PAP2 or GBF6, depending on whether the individual gene contains a MYB-binding domain or a G-box (see below).

The single one gene was found, coding for the putative PB1 domain-containing protein, to be modulated (downregulated) at excessive irradiance and reverted by DCMU. The involvement of the redox state of the PQ pool in the signal transduction pathway leading to downregulation of this gene at excessive irradiance is interesting since PB1 domain is considered to be a novel protein binding module involved in a variety of cellular signaling proteins of origins ranging from yeast to human (Yoshinaga *et al.*, 2003).

A search was performed for conserved (overrepresented) promoter regulatory elements which may participate in the transduction of the signal from the redox state of the PQ pool to downstream genes. Besides GAGArA, very similar to the bwGA-GAGA sequence found recently among thirteen *cis*-acting elements overrepresented in *Arabidopsis* core promoters (Molina & Grotewold, 2005) and two motifs similar to known elements (AyACGT and ATATAT — Table 3) nine entirely novel motifs were identified. The occurrence of such PQ-specific motifs will assist us in further analysis of transcription regulatory phenomena engaged in the PQ-mediated response to changing irradiance. Anyhow it may already be strongly suggested based on the data presented in this work that members of the MYB (PAP1 and PAP2) as well as bZIP (GBF6) families of activators are among the *trans*-acting factors involved. This suggestion concerns those PQ-regulated genes which coexpress tightly with the three factors and possess MYB- or GBF-binding elements in their promoters. For example, all genes belonging to the M1 cluster do contain MYB1AT, one of the MYB-binding elements, and thus may be upregulated by PAP1 in a way dependent on the redox state of PQ pool. Furthermore, those of genes belonging to cluster H1 which coexpress most stringently with GBF6 (the ones coding for hydrolase hydrolyzing O-glycosyl compounds, legumain, CHS and ELIP1) do contain G-boxes and those coexpressing most closely with PAP2 (4CL3, F3H and PAL2) contain MYB1AT elements (Table 4). Promoters of other genes that are responsive to the redox state of the PQ pool may bind (by nine novel motifs and possibly other, not listed in Table 3 due to having the information contents below 1.30) also other, still unidentified factors which themselves do not require PQ-mediated upregulation for activation of the *cis*-acting elements.

It is worth noting that promoters of both *PAP1* and *PAP2* contain G-boxes and that of *PAP1* contains MYB1AT, a MYB-recognizing element as well. This may indicate that *PAP1* and *PAP2* may function as downstream targets of another set of transcription factors belonging to MYB and bZIP families in the signal transduction pathway dependent on the redox state of the PQ pool, leading to the modulation of genes expression at medium and high irradiance.

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