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Abscisic acid-induced modulation of metabolic and redox control pathways in *Arabidopsis thaliana*

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ABSTRACT

Abscisic acid (ABA) has been implicated as a mediator in plant responses to various environmental stresses. To evaluate the transcriptional and metabolic events downstream of ABA perception, *Arabidopsis thaliana* seedlings were analyzed by transcript and metabolite profiling, and results were integrated, using the recently developed BioPathAt tool, in the context of the biochemical pathways affected by this treatment. Besides the up-regulation of pathways related to the biosynthesis of compatible solutes (raffinose family oligosaccharides and certain amino acids) as a response to ABA treatment, we also observed a down-regulation of numerous genes putatively localized to and possibly involved in the reorganization of cell walls, an association that had not been recognized previously. Metabolite profiling indicated that specific antioxidants, particularly α-tocopherol and L-ascorbic acid, were accumulated at higher levels in ABA-treated seedlings compared to appropriate controls. The transcription of genes involved in α-tocopherol biosynthesis were coordinately up-regulated and appeared to be integrated into a network of reactions controlling the levels of reactive oxygen species. Based upon the observed gene expression patterns, these redox control mechanisms might involve an ABA-mediated transition of mitochondrial respiration to the alternative, non-phosphorylating respiratory chain mode. The presented data herein provide indirect evidence for crosstalk between metabolic pathways and pathways regulating redox homeostasis as a response to ABA treatment, and allowed us to identify candidate genes for follow-up studies to dissect this interaction at the biochemical and molecular level. Our results also indicate an intricate relationship, at the transcriptional and possibly post-transcriptional levels, between ABA biosynthesis, the xanthophyll cycle, and ascorbic acid recycling.

1. Introduction

Besides its role in plant development (reviewed in Barth et al., 2006), the phytohormone abscisic acid (ABA) serves as an endogenous signal to initiate adaptive responses of plants to changes in water availability that can be caused by drought, salt, osmotic, and/or cold stress conditions (reviewed in Xiong et al., 2002). In water-stressed leaves, ABA triggers a signaling cascade in guard cells that leads to a very rapid closure of stomata (within minutes of perception), thus reducing water loss by transpiration (Schroeder et al., 2001). In addition to these short-term effects, a modulation of the transcript levels of hundreds of genes has been reported for *Arabidopsis thaliana* subjected to salt (Sottosanto et al., 2004), drought/heat (Rizhsky et al., 2004) or cold stress (Fowler and Thomashow, 2002; Provart et al., 2003; Vogel et al., 2005). Massive changes in transcript levels have also been observed following treatment with exogenous ABA (Seki et al., 2002; Hoth et al., 2002; Wang et al., 2007). Most of these papers listed general functional categories for genes that were either up- or down-regulated by the various stresses (Kreps et al., 2002; Seki et al., 2002; Fowler and Thomashow, 2002; Sottosanto et al., 2004; Rizhsky et al., 2004) and only three studies provided an analysis at the biochemical pathway level (ubiquitin-proteasome system for proteolysis, Hoth et al. (2002); lipid and carbohydrate metabolism in chilling-sensitive mutants, Provart et al. (2003); transcription factors related to cold acclimation, Vogel et al. (2005)).

More specific information about the effects of abiotic stress on metabolic pathways had been obtained previously by classical analyses of metabolite levels. An accumulation of organic solutes,

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such as certain amino acids, polyamines and raffinose family oligosaccharides, has been suggested to prevent stress-induced water loss and reestablish cell turgor. In *Arabidopsis*, the dehydration-induced accumulation of proline is ABA-dependent, whereas the increases in leucine and isoleucine levels occur independent of ABA (Nambara et al., 1998). Under cold and osmotic (sorbitol) stress, proline appears to accumulate in an ABA-independent but under salt (NaCl) stress in an ABA-dependent fashion (Savouré et al., 1997). Other amino acid-derived metabolites also play important roles as compatible solutes (e.g., glycine betaine; Sakamoto and Murata, 2002) or signaling regulators (e.g., polyamines; Kasukabe et al., 2004) under various environmental stresses. Taji et al. (2002) have reported that drought-, high salinity-, and cold-treated *Arabidopsis* plants accumulate large amounts of raffinose family oligosaccharides and provided evidence the up-regulation of a galactinol synthase isogene with potential relevance in their biosynthesis. However, transgenic *Arabidopsis* plants overexpressing a galactinol synthase gene accumulated up to 20 times as much raffinose as wild-type plants, but did not show either enhanced freezing tolerance or increased ability for cold acclimation (Zuther et al., 2004), which casts doubts on the essentiality of raffinose in these processes.

Recent transcriptomic and metabolomic analyses have revealed that extensive changes in transcript and metabolite patterns occur under cold, heat and drought stress conditions, including increases in the biosynthesis of compatible solutes (Rizhsky et al., 2004; Kaplan et al., 2004; Cook et al., 2004). However, because of the widespread and dramatic changes, the interpretation of these studies in terms of the stress-mediated regulation of biochemical pathways becomes quite challenging. We have previously integrated global transcript and metabolite data from published reports on low-temperature responses in *Arabidopsis* using the recently developed BioPathAt tool (Lange and Ghassemian, 2005). The current paper complements and extends these studies with our own experiments assessing a detailed time course of ABA-mediated changes in transcript and metabolite patterns. Our data-dependent analysis focuses on the interface between stress perception and physiological responses, with an emphasis on the role of antioxidant metabolism and redox homeostasis in the ABA response.

2. Results and discussion

2.1. Experimental design and statistics

Our experiments were aimed at providing data, at the levels of steady-state transcript abundance and metabolite pools, to describe the early responses of *Arabidopsis* seedlings to ABA (first 24 h after transfer to ABA-containing media). We also assessed, by comparison with various publicly available datasets, similarities of transcript patterns and metabolite pools as obtained in our ABA experiments with those previously reported for various stress treatments. Seedlings were grown on plates (solid MS medium) under tightly controlled conditions with moderate light intensities (150 μmol photons m^{-2} s^{-1}; 16 h day/8 h night cycle) and, at day 14 after imbibition, were transferred to plates containing either MS medium (controls) or MS medium supplemented with 10 μM ABA. This transfer was performed at 3 h after onset of illumination, and samples were harvested from 0.5 to 24 h. The relatively low ABA concentration was chosen to avoid raising *in vivo* ABA levels (after uptake from the medium) far beyond physiological levels. The experiment was repeated three times (over a period of two months) with large seedling pools, which were kept separate for transcript and metabolite analyses. We did not obtain three replicate microarray data sets for all time points; instead we performed real-time PCR assays (using seedling pools from three independent ABA treatments and appropriate controls) to quantify the transcript levels of selected genes of interest.

The reproducibility of metabolite determinations had been tested previously with 10 independent extractions and analyses (data not shown). Only those metabolites for which absolute amounts varied less than 10% across all tests were taken into account. After determining transcript expression levels from microarray raw data, pair-wise comparisons were performed between the replicate samples (all genes detected as "present" in 0 h control sample 1 vs. 0 h control sample 2). The correlation coefficient of 0.93 indicated that biological variation across these replicates was quite modest. In addition, pair-wise comparisons were also conducted for all possible pairs of samples included in our time course study (Supplementary Material 1). In addition, two-way ANOVA analyses were employed to identify genes that are regulated by ABA treatment and those that show differences in the ABA vs. time interaction. For two-way ANOVA analyses, the two replicates at 0 h time points were used to estimate the within-group-error, and the general linear model was formulated as: expression = treatment + time + treatment × time + error. The variance for each gene in all ABA-treated samples (ABA-treated group) vs. control samples (control group) was estimated using the local pooled error method (Jain et al., 2003). Interestingly, a significant variance between the control group and the ABA-treated group was only detectable for a small percentage (<1%) of genes (*F*-test). It can be assumed that the errors for individual replicates would have been at similar levels, if more replicate data sets would have been obtained for each time point. Expression levels of genes from ABA-treated samples were then compared to the appropriate time course controls and genes were considered significantly up- or down-regulated over the control (with high confidence) when they were assigned a p-value <0.01 (after Bonferroni correction). On the basis of this analysis, 1403 genes were found to have p-values less than 0.01 (false discovery rate = $(25{,}447 \times 0.01)/1403 = 18.1\%$) (Supplementary Material 2). Over the last two decades, numerous ABA response-related genes have been identified and characterized using various molecular biological and biochemical methods. We used the expression patterns of known ABA-upregulated transcripts to assess the effectiveness of our experimental treatment. The p-values for 11 of these genes were equal to or lower than 0.01 (protein phosphatase HAB1 (Saez et al., 2004); protein phosphatases ABI1 and ABI2 (Leung et al., 1997); abscisic acid responsive element binding factor ABF1 (Choi et al., 2000); stress-responsive protein KIN1 (Gilmour et al., 1992); cold-responsive protein COR15A (Wilhelm and Thomashow, 1993); CBL-interacting protein kinase CIPK3 (Kim et al., 2003); diacylglycerol kinase DGK1 (Katagiri et al., 1996); low-temperature-responsive protein RD29A (Nakashima et al., 2006); and dehydrin RAB18 (Lång and Palva, 1992)), with an additional 10 genes that had p-values $\leqslant 0.05$ (dehydration-responsive proteins RD22, ERD6 and ERD15 (Yamaguchi-Shinozaki and Shinozaki, 1993; Sánchez et al., 2004; Kariola et al., 2006); phospholipase PLDP2 (Hallouin et al., 2002); calcium-binding protein RD20 (Takahashi et al., 2000); abscisic acid responsive protein ARA5 (Welin et al., 1994); abscisic acid responsive element binding factor ABF3 (Kang et al., 2002); ethylene-insensitive transporter EIN2 (Wang et al., 2007); cold-responsive protein COR15B (Wilhelm and Thomashow, 1993); dormancy-associated protein DRM1 (Sánchez et al., 2004) (Supplementary Material 2).

2.2. ABA-mediated changes in metabolite pools

Previous metabolomics efforts using tissues of drought or cold-stressed *Arabidopsis* plants were geared toward providing a broad coverage of polar metabolites using a standardized sample prepa-

ration protocol followed by comprehensive GC–MS analysis (Rizhsky et al., 2004; Cook et al., 2004; Kaplan et al., 2004). Massive changes in the metabolome (hundreds of peaks) were observed in two of these studies (Cook et al., 2004; Kaplan et al., 2004), with the majority of peak identities remaining unknown. Among the identifiable metabolites were various sugars, organic acids and amino acids. In cold-acclimated (Cook et al., 2004) and cold-shocked (Kaplan et al., 2004) plants, the amounts of most of these metabolites increased dramatically (experimental period 14 and 4 d, respectively), whereas in drought-treated plants (experimental period 6–7 d) the reported metabolite changes were generally more subtle (Rizhsky et al., 2004). Exceptions were the increased accumulation (>4-fold vs. control) of glucose, sucrose, trehalose, mannose, and proline in water-stressed plants (Rizhsky et al., 2004). The present study was aimed at providing evidence regarding metabolite levels that are affected by treatment with ABA, and to provide comparisons with metabolite pools that accumulated under cold and/or drought stress (which are possibly mediated by ABA). Because of the difficulties in distinguishing primary from secondary effects in prolonged chemical treatments, we decided to obtain a time course of measurements over the course of one day (harvests at 0.5, 1, 3, 6, 12 and 24 h) using a relatively low ABA concentration (10 μM). In addition to the GC–MS analysis of sugars, organics acids, and amino acids as used in the above-mentioned metabolomics studies, we employed a yield-optimized protocol developed for small amounts of *Arabidopsis* seedlings (Ghassemian et al., 2006) to profile chlorophylls, carotenoids, isoprenoid quinones, tocopherols, fatty acids, and phytosterols, the levels of which, to our best knowledge, had not been determined before in this context. ABA-mediated increases were measured for α-tocopherol (membrane antioxidant), ascorbic acid (soluble antioxidant), several sugar solutes (galactinol, glucose, raffinose, sucrose and trehalose), unsaturated fatty acids (16:3, 18:1, 18:2, and 18:3; only transient changes observed!), and certain amino acids (L-Gly, L-Ile, L-Leu, L-Pro, and L-Val) (Table 1). Increased levels of sugar solutes and amino acids, as observed in our experiments with ABA-treated *Arabidopsis* seedlings, have also been reported as a response to cold, drought, and high salinity stress (Savouré et al., 1997; Nambara et al., 1998; Taji et al., 2002; Rohde et al., 2004; Rizhsky et al., 2004; Cook et al., 2004; Kaplan et al., 2004). The observed increase in the levels of the antioxidants α-tocopherol (α-Toc; 1.7-fold increase at 24 h; ABA vs. control) and L-ascorbic acid (3.1-fold increase at 24 h; ABA vs. control) is consistent with an earlier study reporting a dose-dependent α-Toc formation in ABA-treated maize seedlings (Jiang and Zhang, 2001). An integration of our transcript and metabolite data in the context of an ABA-mediated up-regulation of α-Toc biosynthesis is presented in Section 2.4.

2.3. ABA-mediated changes in transcript patterns

Previous microarray experiments with *Arabidopsis* indicated that ABA treatments lead to massive changes at the transcriptional level. Sánchez et al. (2004) exposed three-week-old plants to 50 μM ABA and observed an up-regulation of 1102 and a down-regulation of 767 transcripts. The general trend (up- or down-regulation) was the same for the majority of these genes in our experiment using 10 μM ABA, but the changes were not as pronounced and in many cases not statistically significant (Supplementary Material 3). Of the most highly up-regulated genes in cold-acclimated plants (>10-fold induction at 168 h vs. controls; Cook et al., 2004), roughly 25% were also found to be up-regulated by ABA in our experiments. A similar analysis for repressed genes in cold-acclimated plants (>10-fold repression at 168 h vs. control; Cook et al., 2004) indicated that roughly 30% were also repressed in our experiments as a response to ABA, demonstrating a substantial overlap in the changes of the most responsive transcripts in both experiments (Supplementary Material 3). Sánchez et al. (2004) reported an ABA-mediated up-regulation of genes encoding certain transcription factors, phosphatases, genes related to calcium signaling, CBL-interacting kinases and a down-regulation of genes involved in photosynthesis and in auxin and cytokinin responses. The same general trends were confirmed by our ABA experiments but are not discussed here, since the current study focuses on the regulation of biochemical pathways and processes that were not recognized as being ABA-regulated in previous transcriptomics experiments.

We used pathway-specific gene lists from the BioPathAt database (Lange and Ghassemian, 2005) to query for ABA up- and down-regulated biochemical pathways. A notable bias was found to exist for genes encoding enzymes putatively involved in cell wall remodeling, the expression of which was found to be largely repressed (Table 2). The most prominently down-regulated gene families were those encoding expansins (7 genes), extensins (7 genes), hemicellulose-modifying enzymes (pectate lyases (2 genes), polygalacturonases (3 genes), and esterases (7 genes)), peroxidases (14 genes), xyloglucan endotransglycosylases/hydrolases (XETs; 6 genes), and glycosyl hydrolases (7 genes). A similar repression of genes involved in cell wall metabolism was reported in an *in silico* analysis of previously published microarray experiments with plants grown under water deficit conditions (Bray, 2004). Yokoyama and Nishitani (2001) reported that, out of 33 Arabidopsis genes encoding putative XETs, only 2 genes were up-regulated, whereas 14 genes were down-regulated as a response to a short-term treatment with a low concentration (1 μM) ABA (expression of 12 genes did not change significantly). We hypothesize that in both sets of experiments, ABA and water deficit treatments, gene expression patterns point toward negative effects on cell expansion. Cell wall extensibility is controlled by cell wall modifying enzymes such as expansins (Takahashi et al., 2006), XETs (Wu et al., 2005; van Sandt et al., 2007), pectin methylesterases (Derbyshire et al., 2007), and various glucanases (Chanliaud et al., 2004). Although no specific enzymatic activity has been assigned to expansins, they have been implicated in the pH-dependent enlargement of cells (Li et al., 2003). Extensins are hydroxyproline-rich, rod-shaped glycoproteins cross-linked to cell wall polysaccharides, which may function as a scaffold for primary cell wall deposition (Cannon et al., 2008). Pectin methylesterases catalyze the demethylesterification of homogalacturonan, the main pectic component of the primary cell wall, which allows the formation of Ca^{2+} bonds and makes it accessible to pectin-degrading enzymes, thus affecting the texture and rigidity of the cell wall (Pelloux et al., 2007). Examples of such pectinolytic enzymes are pectate lyases and polygalacturonases, which cleave α-1,4-glycosidic linkages in homogalacturonan of demethylated pectins (Solbak et al., 2005; González-Carranza et al., 2007), thus leading to cell wall loosening in various biological processes. XETs are responsible for the cleavage (*endo*-hydrolysis) and reformation (*endo*-transglycosylaton) of bonds between xyloglucan chains (Saura-Valls et al., 2006), which results in the remodeling of the three-dimensional structure, and thus extensibility, of the primary plant cell wall. The down-regulation of these genes when plants are subjected to water deficit likely decreases the ability of cells to expand, which is in agreement with the known growth inhibition under these conditions (Bray, 1997). Water deficit has been demonstrated to increase endogenous ABA concentrations (Creelman et al., 1990), indicating that the response to dehydration might be mediated, at least in part, by ABA. One mechanism to decrease water loss is the opening and closing of stomata, a process that is regulated by ABA. The cell walls of guard cells are exceptionally strong and cell wall remodeling is a key process during stomatal opening and closing. It was shown that, when epidermal strips

Table 1
Comparison of metabolite profiles in ABA-treated *Arabidopsis* seedlings with those obtained under various stress conditions

Metabolite	0.5 h: ABA vs. control	1 h: ABA vs. control	3 h: ABA vs. control	6 h: ABA vs. control	12 h: ABA vs. control	24 h: ABA vs. control
Chlorophylls and carotenoids						
beta-Carotene	1.46	0.98	1.48	0.92	1.01	n.a.
Chlorophyll a	1.21	1.30	1.23	1.19	0.72	n.a.
Chlorophyll b	1.39	1.60	1.43	1.46	0.97	n.a.
Violaxanthin	n.q.	n.q.	n.q.	1.20	1.90	n.a.
Lutein	1.29	1.55	1.67	0.75	1.07	n.a.
Isoprenoid quinones and tocopherols						
alpha-Tocopherol	1.24	1.20	1.22	1.47	1.20	1.71
Phylloquinone	1.33	1.06	1.06	0.87	1.07	1.15
Plastoquinone	0.77	1.00	0.80	0.83	0.91	0.72
Ubiquinone-9	1.26	1.11	1.10	1.06	1.02	0.71
Sugars and organic acids						
Aconitic acid	0.98	0.69	1.30	0.98	0.93	1.65
Ascorbic acid	0.62	0.69	0.85	0.97	2.09	3.10
Fructose	1.54	1.34	1.99	0.89	1.02	1.30
Fumarate	1.06	0.67	0.77	0.90	0.79	1.15
Galactinol	1.22	1.84	1.67	1.50	2.07	4.75
Glucose	0.88	1.56	1.33	1.51	2.54	2.32
Inositol	1.10	1.42	1.36	1.12	1.40	1.11
Malic acid	0.72	1.11	0.98	0.91	1.04	1.28
Raffinose	1.25	1.31	1.94	1.21	1.32	4.15
Ribose	1.00	1.76	1.19	1.03	0.95	1.36
Shikimic acid	0.98	1.55	1.12	1.05	0.88	1.23
Sucrose	0.97	1.28	1.30	1.85	2.21	2.63
Trehalose	1.43	1.93	1.58	1.06	1.43	4.30
Fatty acids						
16:0	0.95	n.a.	1.40	1.65	n.a.	1.07
16:1	1.08	n.a.	1.39	1.62	n.a.	1.12
16:3	1.03	n.a.	2.15	1.92	n.a.	0.97
18:0	0.90	n.a.	1.39	1.50	n.a.	0.81
18:1	1.30	n.a.	1.42	1.12	n.a.	0.83
18:2	1.19	n.a.	2.07	1.27	n.a.	0.99
18:3	2.79	n.a.	2.45	1.16	n.a.	1.01
Amino acids and related metabolites						
L-Ala	n.a.	n.a.	n.a.	n.a.	n.a.	1.10
L-Arg	n.a.	n.a.	n.a.	n.a.	n.a.	1.73
L-Asp	n.a.	n.a.	n.a.	n.a.	n.a.	1.51
L-Glu	n.a.	n.a.	n.a.	n.a.	n.a.	1.30
L-Gly	n.a.	n.a.	n.a.	n.a.	n.a.	1.98
L-His	n.a.	n.a.	n.a.	n.a.	n.a.	1.12
L-Ile	n.a.	n.a.	n.a.	n.a.	n.a.	2.43
L-Leu	n.a.	n.a.	n.a.	n.a.	n.a.	3.23
L-Lys	n.a.	n.a.	n.a.	n.a.	n.a.	1.14
L-Met	n.a.	n.a.	n.a.	n.a.	n.a.	1.41
L-Orn	0.58	1.14	1.02	0.74	0.77	1.24
L-Phe	n.a.	n.a.	n.a.	n.a.	n.a.	0.69
L-Pro	n.a.	n.a.	n.a.	n.a.	n.a.	6.42
L-Ser	n.a.	n.a.	n.a.	n.a.	n.a.	1.50
L-Thr	n.a.	n.a.	n.a.	n.a.	n.a.	1.25
L-Tyr	n.a.	n.a.	n.a.	n.a.	n.a.	1.18
L-Val	n.a.	n.a.	n.a.	n.a.	n.a.	2.04
Phytosterols						
Campesterol	0.86	0.83	0.53	0.72	0.76	0.84
Stigmasterol	0.70	0.72	1.24	0.71	0.89	1.05
beta-Sitosterol	0.99	0.89	0.60	0.80	0.74	0.76
Total sterols	0.94	0.87	0.61	0.78	0.76	0.80

Abbreviations: n.a., not available; n.q., amounts too low to be quantified accurately.

were incubated with arabinase, a pectin-degrading enzyme, stomatal opening was severely impaired (Jones et al., 2003). It can thus be hypothesized that the ABA-mediated down-regulation of genes involved in cell wall remodeling, as reported in this paper, might decrease the flexibility of cell walls, thus affecting guard cell function.

2.4. Activation of the redox control network

In guard cells of water-stressed seedlings, ABA causes the closing of stomata, mediated by the second messenger Ca^{2+}, thus reducing transpirational water loss. ABA-induced increases in Ca^{2+} levels are facilitated by influx through plasma membrane Ca^{2+} channels and release from internal stores (Wu et al., 1997; Leckie et al., 1998; Staxen et al., 1999; MacRobbie, 2000). Ca^{2+} channels are activated by reactive oxygen species (ROS; Pei et al., 2000; Köhler et al., 2003), the levels of which increase as part of the ABA signaling cascade (Guan et al., 2000; Jiang and Zhang, 2001; Zhang et al., 2001; Kwak et al., 2003). ABA treatment is known to induce the transcription of the gene encoding cytosolic ascorbate peroxidase (apx2), possibly to adjust the cytosolic redox poise (Chang et al., 2004; Davletova et al., 2005), and we observed

Table 2
ABA-mediated repression of genes involved in cell wall remodeling

Annotation	AGI number	Fold change vs. control					
		0.5 h	1 h	3 h	6 h	12 h	24 h
Expansions							
Beta-expansion, putative (EXPB1)	At2g20750	1.013	0.971	0.571	0.481	0.441	0.378
Beta-expansion, putative (EXPB3)	At4g28250	1.17	0.818	0.438	0.356	0.22	0.241
Expansion, putative (EXP1)	At1g69530	1.38	0.683	0.291	0.181	0.149	0.12
Expansion, putative (EXP14)	At5g56320	0.976	0.726	0.248	0.108	0.311	0.287
Expansion, putative (EXP15)	At2g03090	1.509	1.151	0.378	0.17	0.131	0.109
Expansion, putative (EXP6)	At2g28950	1.338	0.801	0.424	0.46	0.458	0.238
Expansion, putative (EXP8)	At2g40610	1.132	0.838	0.437	0.232	0.17	0.0894
Extensions							
Extension family protein	At1g28290	1.221	0.659	0.48	0.264	0.314	0.223
Extension family protein	At2g47540	1.164	0.971	0.439	0.345	0.468	0.549
Extension family protein	At3g28550	1.023	1.133	0.433	0.295	0.635	0.459
Extension family protein	At4g02270	1.218	0.705	0.248	0.138	0.305	0.249
Extension family protein	At5g05500	0.773	1.06	0.531	0.464	0.48	0.512
Extension family protein	At5g10130	1.184	0.701	0.506	0.577	0.332	0.48
Extension family protein	At5g35190	1.016	1.415	0.111	0.208	0.255	0.348
Pectate lyases, pectin methylesterases, pectin acetylesterases, pectinesterases, polygalacturonases							
Pectate lyase family protein	At1g67750	1.314	1.064	0.812	0.356	0.473	0.434
Pectate lyase family protein	At4g24780	1.646	0.721	0.42	0.365	0.422	0.225
Pectin methylesterase, putative	At1g11580	0.972	0.735	0.473	0.364	0.432	0.272
Pectinacetylesterase, putative	At4g19410	0.923	0.916	1.106	0.704	0.741	0.474
Pectinesterase family protein	At2g26440	0.769	0.516	0.515	0.558	0.429	0.385
Pectinesterase family protein	At2g26440	0.769	0.516	0.515	0.558	0.429	0.385
Pectinesterase family protein	At3g14310	0.941	0.708	0.553	0.313	0.4	0.275
Pectinesterase family protein	At5g04960	1.17	1.126	0.285	0.254	0.646	0.44
Pectinesterase family protein	At3g10720	0.964	0.957	0.891	0.712	0.451	0.285
Polygalacturonase (pectinase) family protein	At1g60590	0.632	1.284	0.977	0.666	0.531	0.364
Polygalacturonase (pectinase) family protein	At3g15720	1.364	0.997	0.486	0.924	0.362	1.657
Polygalacturonase (pectinase) family protein	At3g61490	1.44	0.853	0.535	0.578	0.673	0.434
Peroxidases							
Peroxidase 21 (PER21) (P21) (PRXR5)	At2g37130	0.918	1.576	0.327	0.367	0.204	0.208
Peroxidase 27 (PER27) (P27) (PRXR7)	At3g01190	1.269	0.738	1.344	0.653	1.002	0.411
Peroxidase 57 (PER57) (P57) (PRXR10)	At5g17820	1.062	1.299	0.704	0.439	0.422	0.246
Peroxidase 72 (PER72) (P72) (PRXR8)	At5g66390	1.077	0.579	1.05	0.663	0.252	0.176
Peroxidase 73 (PER73) (P73) (PRXR11)	At5g67400	1.199	0.844	0.111	0.171	0.299	0.312
Peroxidase, putative	At1g05240	1.497	0.719	0.284	0.166	0.14	0.144
Peroxidase, putative	At1g30870	1.079	1.256	0.386	0.297	0.562	0.257
Peroxidase, putative	At2g18980	1.407	0.652	1.188	1.15	0.938	0.439
Peroxidase, putative	At4g08770	1.601	0.403	0.185	0.193	0.0819	0.273
Peroxidase, putative	At4g11290	1.061	0.754	0.327	0.322	0.53	0.334
Peroxidase, putative	At4g26010	1.303	0.839	1.186	0.312	0.307	0.287
Peroxidase, putative	At4g30170	1.307	0.655	0.603	0.357	0.313	0.287
Peroxidase, putative	At5g64100	0.962	0.677	0.371	0.138	0.182	0.151
Peroxidase, putative	At5g64120	0.842	0.637	0.404	0.128	0.0951	0.143
Xyloglucan endotransglucosylases							
Xyloglucan endotransglycosylase, putative	At3g23730	1.013	0.658	0.769	0.752	0.713	0.391
Xyloglucan endotransglycosylase, putative	At3g44990	1.786	1.354	0.147	0.153	0.0695	0.191
Xyloglucan endotransglycosylase, putative	At4g14130	0.985	0.936	0.891	0.688	0.582	0.422
Xyloglucan endotransglycosylase, putative	At5g48070	0.808	3.717	0.762	0.265	0.129	1.39
Xyloglucan endotransglycosylase, putative	At5g57530	1.006	2.859	0.154	0.113	0.387	0.469
Xyloglucan endotransglycosylase, putative	At5g65730	1.081	1.22	0.437	0.616	0.581	0.221
Other							
Caffeoyl-CoA 3-*O*-methyltransferase, putative	At1g67980	0.915	0.291	0.715	0.288	0.324	0.381
Cinnamyl-alcohol dehydrogenase (CAD4)	At3g19450	1.156	0.946	0.568	0.556	0.422	0.382
Galactosyltransferase family protein	At1g11730	1.571	0.595	0.551	0.207	0.387	0.358
Glycoside hydrolase family 19 protein	At2g43610	0.886	0.776	0.841	0.325	0.387	0.182
Glycosyl hydrolase family 1 protein	At1g47600	1.366	1.033	0.788	0.806	0.305	0.228
Glycosyl hydrolase family 17 protein	At1g11820	1.014	0.845	0.773	0.486	0.54	0.355
Glycosyl hydrolase family 17 protein	At4g18340	0.715	0.605	0.431	0.595	0.481	0.262
Glycosyl hydrolase family 17 protein	At1g18650	1.242	1.009	0.713	0.811	0.462	0.462
Glycosyl hydrolase family 17 protein	At5g61130	1.406	0.869	0.46	0.467	0.533	0.489
Glycosyl hydrolase family 9 protein	At4g02290	1.433	1.018	0.658	0.34	0.408	0.364
Glycosyl transferase family 2 protein	At1g23480	0.877	0.609	0.621	0.474	0.528	0.306
Laccase, putative/diphenol oxidase, putative	At2g30210	1.208	0.67	0.514	0.625	0.483	0.306
Xyloglucan fucosyltransferase, putative (FUT5)	At2g15370	0.967	1	0.001	0.001	0.001	0.00976

The values shown represent fold change (treatment vs. control).

a strong up-regulation of apx2 transcript levels in our experiments (up to 6.6-fold at 6 h; Fig. 1). This finding was confirmed by quantitative real-time PCR analysis (apx2 4.5-fold up-regulated in ABA-treated seedlings at 24 h; Fig. 3).

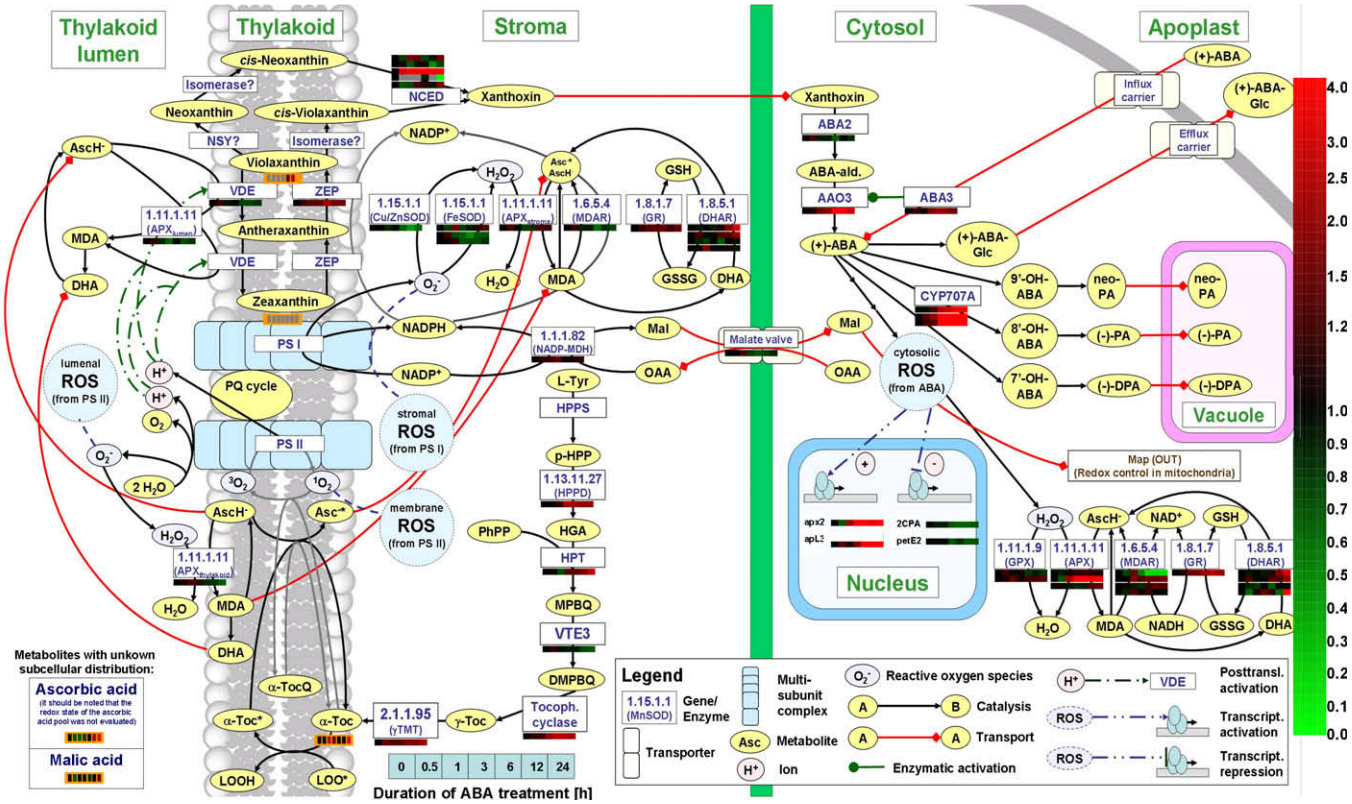

Fig. 1. BioPathAt map visualizing ABA-mediated redox control pathways using the graphical notation for biological networks suggested by Kitano et al. (2005). Enzyme names and EC numbers are shown in blue, specific subunits of enzymes are colored in red, and metabolite names are printed in black. Solid lines indicate anabolic reactions whereas broken lines are used to visualize catabolic reactions. Boxes representing changes in transcript abundance are positioned on top of the biochemical reaction arrow, whereas boxes indicating steady-state metabolite pools (orange frames) are shown below the compound name. On the horizontal axis of each box, the gene expression patterns or metabolite pool changes are shown for a time course of 14-day-old *Arabidopsis thaliana* seedlings subjected to ABA for 0, 1, 3, 6, 12 or 24 h (compared to dark-grown controls). Changes in transcript abundance and metabolite pools are displayed using colors to represent changes of expression from either a mean or control value (red for up-regulation and green for down-regulation) and color intensity to represent the level of the change. Black color is used when the transcript abundance or the metabolite pool size did not differ significantly from the dark-grown controls). Gray boxes indicate that the transcript level determinations for a specific gene did not meet either the selection criteria listed in Section 3 or that the level of a metabolite could not be detected with sufficient confidence. If no box is shown next to an enzyme name, the corresponding gene has not yet been cloned or is not represented on the GeneChip™ used to acquire the mRNA expression data. Abbreviations used for metabolites (very common abbreviations not included): $AscH^-$, protonated ascorbic acid; Asc^{-*}, ascorbic acid radical; DHA, dehydroascorbic acid; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinone; DPA, dihydrophaseolic acid; GSH, glutathione (reduced); GSSG, glutathione (oxidized); HGA, homogentisic acid; pHPP, *p*-hydroxypheynylpyruvic acid; LOOH, fatty acid (undefined chain length); LOO^*, fatty acid radical (undefined chain length); Mal, malic acid; MDA, monodehydroascorbic acid; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; OAA, oxaloacetic acid; PA, phaseolic acid; α-Toc, alpha-tocopherol; α-Toc^*, alpha-tocopherol radical; γ-Toc, gamma-tocopherol. The annotation of all genes represented in this figure is given in Supplementary Material 4.

At the transcriptional level, we observed an upregulation of genes involved in the biosynthesis of starch (two isogenes encoding ADP-glucose pyrophosphorylase [At4g39210; *p*-value = 0.01; At2g21590; *p*-value = 0.02]) and breakdown of starch (α-amylase [At4g25000; *p*-value = 0.01], β-amylase [At3g23920; *p*-value = 0.02], and isoamylase/starch debranching enzyme [At4g09020; *p*-value = 0.03]) (Supplementary Material 2). Rook et al. (2001) reported that ABA treatment led to a strong induction of the starch biosynthetic ADP-glucose pyrophosphorylase gene apL3 (encoding a large subunit isozyme) in detached leaves of adult *Arabidopsis* plants only in the presence of glucose, whereas our own results with seedlings indicated a >9-fold ABA-mediated induction of apL3 transcript in the absence of exogenous glucose (Fig. 1). It is conceivable that the ABA-mediated increase in endogenous glucose levels measured in our experiments lead to the induction of apL3 transcription, which might not occur when adult plants are transferred to the dark as reported by Rook et al. (2001). Our results indicate that the ABA treatment may have led to a transcriptional upregulation of starch turnover, which could be a cause of the measured increases in soluble sugar levels. Although our experimental design is not suited to provide direct support for such a hypothesis, there is precedent in the literature of a link between ABA and starch turnover. For example, an increase of starch turnover and enhanced accumulation of soluble sugars was also found to be involved in the ABA-induced freezing tolerance in the moss *Physcomitrella patens* (Nagao et al., 2005).

Promoters known to be negatively regulated by ABA, likely via signaling involving ROS, include those upstream of genes encoding a plastid-localized 2-*cys*-peroxiredoxin (2CPA; At3g11630; Baier et al., 2004) and a plastocyanin (petE2; At1g20340; Wöstemeyer and Oelmüller, 2003), the expression levels of which both decreased in ABA-treated samples in our experiments (*p*-values of 0.01 for 2CPA and 0.05 for petE2; *p*-values given for ABA vs. controls across time course) (Fig. 1). The expression of 2CPA (At3g11630), which is part of a larger gene family with divergent stress-dependent expression patterns, was also shown to be repressed by exogenous L-ascorbic acid (Horling et al., 2003), which is in agreement with the increase in endogenous L-ascorbic acid levels measured in our experiments. Kwak et al. (2003) demonstrated that ROS are second messengers of ABA signaling, and we found that both NADPH oxidases associated with this process (AtrbohD, At5g47910; AtrbohF, At1g64060) were up-regulated at 3–6 h after and onset of ABA treatment (Supplementary Material 2). In general, we observed an ABA-mediated up-regulation of genes

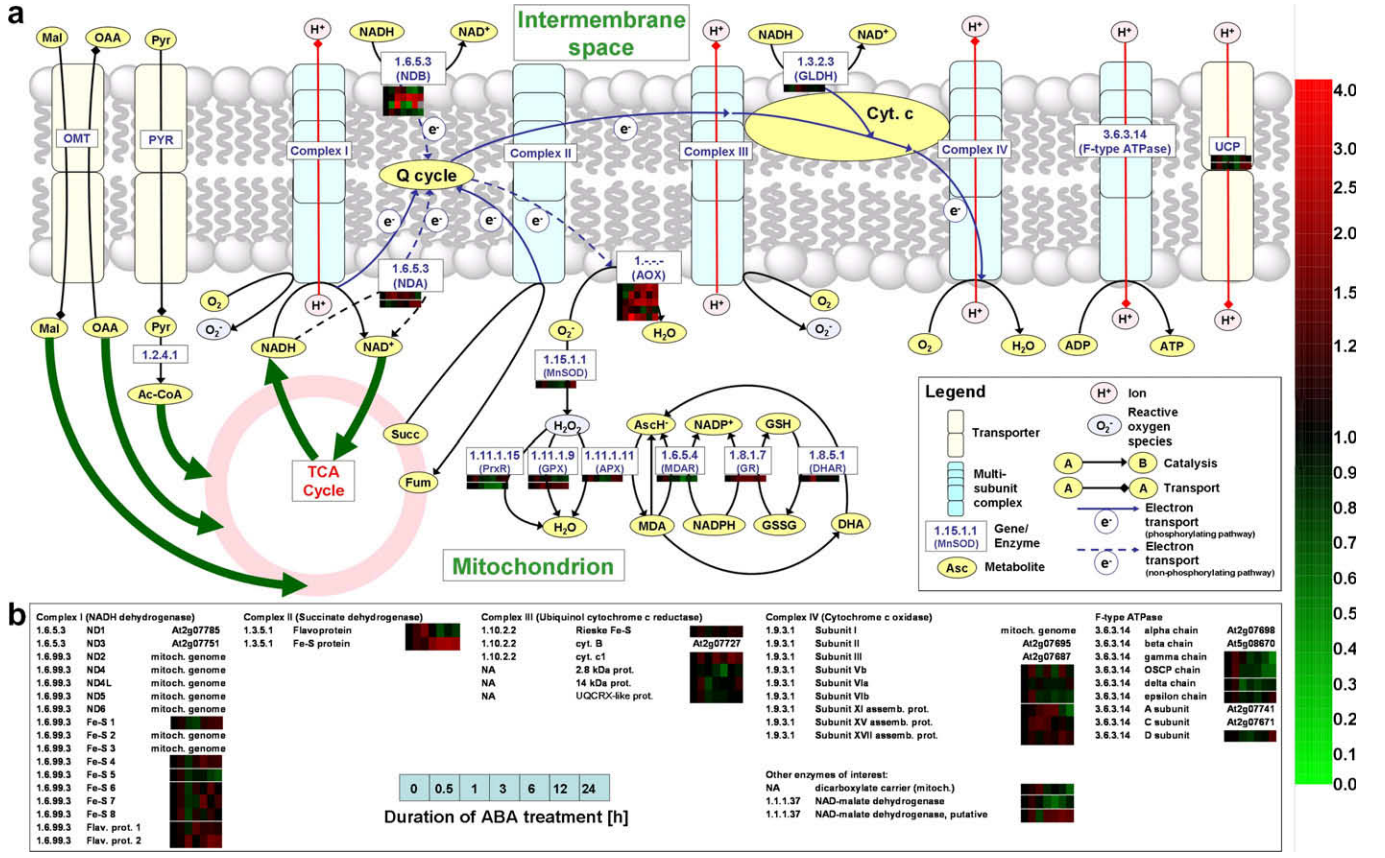

Fig. 2. BioPathAt map visualizing ABA-mediated changes in the expression patterns of genes related to mitochondrial respiration. The graphical notation follows the suggestions for representing biological networks published by Kitano et al. (2005). For a more detailed legend see Fig. 1. Abbreviations used for metabolites (very common abbreviations not included): Ac-CoA, acetyl-coenzyme A; $AscH^-$, protonated ascorbic acid; DHA, dehydroascorbic acid; Fum, fumaric acid; GSH, glutathione (reduced); GSSG, glutathione (oxidized); HGA, homogentisic acid; Mal, malic acid; MDA, monodehydroascorbic acid; OAA, oxaloacetic acid; Pyr, pyruvic acid; Succ, succinic acid. The annotation of all genes represented in this figure is given in Supplementary Material 4.

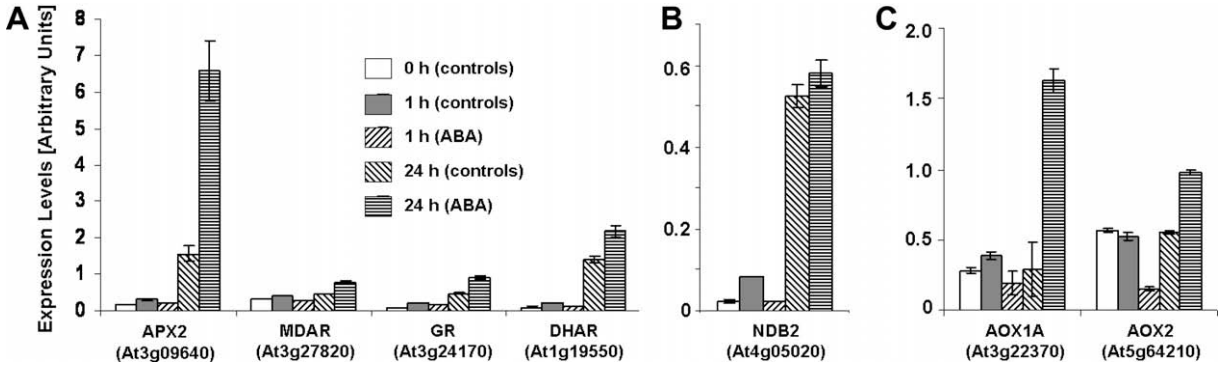

Fig. 3. Quantitative real-time PCR analysis of the abundance of transcripts encoding enzymes involved in the redox control network. Because the absolute expression levels of genes of interest were quite different, we used two endogenous controls: β-actin (At3g18780) for genes expressed at medium levels and ubiquitin-conjugating enzyme E2 (At5g25760) for genes expressed at low levels. The average signal intensity of the 0 h samples was used as the calibrator. For details see the Section 3.

encoding the cytosolic enzymes involved in the ROS-scavenging ascorbate-glutathione cycle (ascorbate peroxidase (APX2; At3g09640; p-value = 0.01), monodehydroascorbate reductase (MDAR; At3g27820; p-value = 0.03), glutathione reductase (GR; At3g24170; p-value = 0.06 and thus weakly significant), and dehydroascorbate reductase (DHAR; At1g19550; p-value = 0.02)), whereas expression levels of genes encoding the plastidial and mitochondrial isozymes did not change significantly (Fig. 1). Quantitative real-time PCR confirmed the ABA-mediated increases of these transcripts (APX2, up 4.5-fold; MDAR, up 1.8-fold; GR, up 2-fold; DHAR, up 1.6-fold; fold change reported for ABA vs. control at 24 h). We thus hypothesize that, based on the gene expression patterns observed in our experiments, the ABA treatment led to an activation of the cytosolic transcriptional redox control network in *Arabidopsis* seedlings.

2.5. ABA induces its own biosynthesis

Several studies have shown that, under certain conditions, ABA regulates its own biosynthesis and catabolism at the transcriptional level (Krochko et al., 1998; Iuchi et al., 2001; Xiong et al., 2001a, 2001b, 2002), indicating that feedback processes might play

a role in adjusting ABA levels. In agreement with these results, we also observed an ABA-mediated upregulation of transcripts for zeaxanthin epoxidase (ZEP; *p*-value of 0.03), aldehyde oxidase (ASCORBIC ACID3; *p*-value = 0.05), and ABA 8′-hydroxylase (CYP707A; *p*-value of 0.01) (Fig. 1). In contrast with previous findings suggesting that an ABA treatment had no effect on the expression of 9-*cis*-epoxycarotenoid dioxygenase mRNA (NCED; Xiong et al., 2001b), we detected a strong upregulation of the NCED3 transcript (At3g14440; *p*-value = 0.04) (Fig. 1), which is in accordance with its established role in the response to drought and salt stress, when increased levels of ABA (and the genes involved in its biosynthesis) have been measured (Audran et al., 1998; Seo et al., 2000; Iuchi et al., 2001; Xiong et al, 2001a, 2002; Tan et al., 2003). The expression levels of the ABA2 gene, which encodes an aldehyde dehydrogenase converting xanthoxin to abscisic aldehyde, were not altered by ABA (Fig. 1), which has also been reported to be the case under salt, osmotic and dehydration stress (Gonzáles-Guzmán et al., 2002; Cheng et al., 2002). Endogenous ABA pools are controlled by balancing biosynthesis and catabolism, both of which were activated at the level of steady-state transcript abundance in our experiments, but the overall regulation of these processes involves multiple levels of control that are poorly understood (Nambara and Marion-Poll, 2005). Mutants impaired in the biosynthesis of the antioxidant ascorbic acid (AscA) contain increased ABA levels (Pastori et al., 2003), indicating that redox control might play a role in ABA biosynthesis. The de-epoxidation of violaxanthin to zeaxanthin requires reduced, protonated ascorbic acid (AscH) in the thylakoid lumen (Bratt et al., 1995). In our experiments, the genes encoding luminal and thylakoid ascorbate peroxidases (APXs) as well as violaxanthin de-epoxidase (VDE) were slightly (but not significantly) down-regulated (Fig. 1). In contrast, transcripts for ZEP and NADP-dependent malate dehydrogenase (provides NADPH for ZEP) were up-regulated (*p*-values of 0.03 and 0.04, respectively), which may explain our detection of slightly increased violaxanthin levels (Table 1). It has been hypothesized that when the Halliwell–Foyer cycle for the regeneration of AscH is pushed off balance, the xanthophyll cycle becomes uncoupled, which may lead to an accumulation of violaxanthin and subsequently ABA (Baier and Dietz, 2005). Although an ABA treatment increased levels of ROS in maize seedlings, oxidative damage such as lipid peroxidation was not observed, possibly due to enhanced α-Toc levels (Jiang and Zhang, 2001). In our experiments, we also measured an increased accumulation of α-Toc (Table 1). In addition, we observed for the first time that this change is accompanied by a concomitant increase of the steady-state transcript levels of almost all known genes involved in the α-Toc biosynthetic pathway (Fig. 1 and Supplementary Material 2; 4-hydroxyphenylpyruvate dioxygenase (HPD), *p*-value = 0.0004; homogentisate phytylprenyltransferase, *p*-value = 0.005; tocopherol cyclase, *p*-value = 0.02; γ-tocopherol methyltransferase, *p*-value = 0.05).

2.6. ABA-mediated ascorbic acid accumulation appears to be regulated by enhanced recycling

In our experiments, AscA levels increased to roughly 3-fold (ABA vs. control) at 24 h (Table 1) and this is the first report in which steady-state transcript data for all genes encoding known biosynthetic enzymes have been evaluated as well, thus allowing a correlation analysis. An interesting question is whether the ABA-mediated rise in AscA levels are brought about by either transcriptional up-regulation of the AscA biosynthetic pathway or by increased recycling? Interestingly, we observed no significant ABA-mediated changes (compared to appropriate controls) in the steady-state levels of genes encoding GDP-mannose 3,5-epimerase (At5g28840), L-galactose 1-phosphate phosphatase (At3g02870), L-galactose dehydrogenase (At4g33670), and L-galactono-1,4-lactone dehydrogenase (GLDH; At3g47930). Little is known about the regulation of most of these genes under conditions in which the synthesis of AscA is induced and only the role of GLDH has been studied in a little more detail. Recent studies have shown that L-galactono-1,4-lactone dehydrogenase (GLDH), the last enzyme of the *de novo* pathway for AscA biosynthesis, co-localizes with mitochondrial complex I and requires oxidized cytochrome c as a substrate to deliver electrons to the ETC between complexes III and IV (Bartoli et al., 2000). GLDH levels are coordinately regulated with stress-induced changes in the rate of respiration (Millar et al., 2003). The depiction of the ETC in Fig. 2 as an assortment of separate complexes has been chosen to allow details of components to be visualized; however, it should be noted that respiratory chain complexes in plants appear to form one (or more) supercomplex(es) (Eubel et al., 2003), possibly including GLDH (Millar et al., 2003), thus allowing a coupling of mitochondrial respiration and AscA synthesis. Since mitochondria are susceptible to oxidative damage (Sweetlove et al., 2002) and play a key role in modulating antioxidant crosstalk of the entire cell (Dutilleul et al., 2003), the adjustment of antioxidant pools is important to maintain redox homeostasis. Since the steady-state transcript levels of GLDH, and other genes involved in AscA biosynthesis, in our ABA-treated seedlings remained largely constant throughout the experimental period, AscA formation might not be regulated at the transcriptional level under these conditions. A lack of response at the transcriptional level has also been reported for the GLDH gene in hormone and stress-treated melon seedlings (Pateraki et al., 2004), whereas a concomitant increase in biosynthetic gene levels and AscA pools were observed in jasmonic acid-induced *Arabidopsis* cell suspension cultures (Wolucka et al., 2005) and in tobacco plants grown under various light conditions (Gatzek et al., 2002). Although our experimental design, which combines global transcript and metabolite profiling, is not suited to test for AscA turnover directly, the up-regulation of genes encoding enzymes involved in AscA recycling, as discussed in Section 2.5 (with reference to Fig. 1), provides indirect evidence for the hypothesis that the balance of recycling activities might be more important than the regulation of biosynthesis under the experimental conditions used in the present study.

2.7. Potential impact of ABA treatment on ascorbic acid-dependent enzyme activities

The ABA-induced increases in AscA levels (Table 1) may have effects beyond the redox control network (as discussed in Section 2.5). Of particular interest is the fact that AscA has been recognized as a co-substrate of several dioxygenases (Arrigoni and De Tullio, 2000, 2002). We thus assessed if the transcript levels of genes encoding such dioxygenases were up-regulated by ABA. 1-Aminocyclopropane-1-carboxylate oxidase, the rate-limiting enzyme of ethylene biosynthesis, and gibberellin 3β-hydroxylase, which is involved in gibberellin biosynthesis, are encoded by small gene families in *Arabidopsis*. However, the expression levels of the genes coding for these enzymes (At1g05010, At1g12010, At1g62380, At1g7730, At2g19590 and At1g15550, At1g80330, At1g80340, At4g25420, respectively) did not change significantly as a response to ABA. In contrast, a 2.5-fold increase in the expression levels of the gene encoding HPD (At1g06570), which catalyzes the formation of homogentisic acid (the aromatic precursor of plastoquinone and tocopherols), was measured as a response to ABA. This observation is in agreement with the overall induction, at the transcriptional level, of α-Toc biosynthesis, as supported by both gene expression and metabolite profiling data (see Sections 2.2 and 2.5 for details). Another enzyme that requires AscA for activity is NCED, and we observed that one isogene encoding NCED (At3g1440) was dramatically induced (17-fold) by ABA. NCED is

involved in ABA biosynthesis and the effects of ABA on its own biosynthesis have been discussed in Section 2.5. It can thus be hypothesized that, in addition to this transcriptional control mechanism, ABA biosynthesis could be further increased by enhanced availability of the NCED co-substrate AscA. Interestingly, slightly increased expression levels of NCED isogene (At4g191170) were measured in the Arabidopsis *vtc1* mutant, which is impaired in AscA biosynthesis (Pastori et al., 2003). The authors hypothesized that increased NCED transcript levels (compared to wild-type) might result in increased enzyme levels, thus compensating for a loss of NCED activity due to low AscA levels. The correlation between AscA levels and NCED transcript abundance were confirmed by growing *vtc1* in the presence of AscA, which resulted in decreases of NCED transcripts to wild-type levels (Pastori et al., 2003). In the experiments reported here, we observed an overall up-regulation of ABA biosynthesis at the transcriptional level and increased levels of AscA, possibly because of increased recycling as discussed in Section 2.6, which may have additive effects on the enzyme activities of the ABA pathway.

Is has also been shown that, based on a comparison of data sets generated with wild-type *Arabidopsis* and an AscA deficient mutant (vtc2-2), ascorbate availability can be a limiting factor for VDE activity (Müller-Moulé et al., 2002). VDE is a key enzyme in the xanthophyll cycle, which is a component of a mechanism called non-photochemical quenching (NPQ). This process leads to dissipation of excess light energy (for example when plants are grown under high light conditions) as heat (Dreuw et al., 2005) and is important to limit the formation of dangerous activated oxygen species, thus preventing photodamage. In the context of our experiments, it can be hypothesized that the increased accumulation of AscA as a response to ABA treatment might result in increased VDE activity (as discussed in Section 2.5), and possibly increased NPQ. Support for such a hypothesis is provided by the work of Omasa and Takayama (2003), who demonstrated that a topical ABA treatment of bean leaves resulted in increased NPQ at high light intensities. Our results thus indicate an intricate relationship, at the transcriptional and possibly post-transcriptional levels, between ABA biosynthesis, the xanthophyll cycle, and AscA recycling.

2.8. A role for the alternative respiratory chain in the ABA response?

The use of ROS as signaling molecules obviously requires tight control due to the toxicity of large amounts of ROS. In addition to the above-mentioned mechanisms to regulate ROS accumulation, plant mitochondria have evolved a non-phosphorylating bypass of the electron transport chain (ETC) that consists of alternative oxidase (AOX), alternative NAD(P)H dehydrogenases (NDA and NDB), and uncoupling proteins (UCP) (Fig. 2a). The AOX bypass has been hypothesized to act as an overflow mechanism that prevents the overreduction of the ETC in the presence of ROS (Purvis and Shewfeldt, 1993; Maxwell et al., 1999; Møller, 2001; Mittler, 2002; Affourtit et al., 2002; Juszczuk and Rychter, 2003; Fernie et al., 2004). In experiments with various abiotic stressors, including those that lead to ROS formation, the steady-state transcript levels of genes encoding ETC components remained largely unchanged (Clifton et al., 2005), which was also the case in our experiments with ABA (Fig. 2b; Supplementary Material 2). This behavior at the transcriptional level was also observed for genes encoding UCP (Fig. 2a), which is in accordance with reports that UCP is primarily regulated by post-transcriptional biochemical activation (Considine et al., 2003). In contrast, an upregulation of the AOX1a, AOX2 and NDB2 genes was reported for several abiotic treatments (Clifton et al., 2005). In the experiments presented herein, ABA treatments resulted in increases in steady-state transcript levels for NDB2 (At4g05020; p-value = 0.05 for ABA vs. control across time course), two AOX isogenes (AOX1a, At3g22370, p-value = 0.01; AOX2, At5g64210, p-value = 0.02), and a very slight (statistically not significant) up-regulation of NDA1 and NDA2 (Fig. 2a). Based on quantitative real-time PCR assays, NDB2 was slightly up-regulated by ABA (1.2-fold at 24 h), whereas the ABA-mediated upregulation of AOX1 and AOX2 transcript levels (5.4-fold and 1.8-fold, respectively, at 24 h). Taken together, these results indicate an ABA-mediated induction, at the transcriptional level, of the ETC bypass pathway.

However, it should be noted that there is no direct correlation of AOX expression and its activity in respiration as its activity is known to be largely determined by various posttranscriptional mechanisms, including the redox poise of the of the ubiquinone pool (Hoefnagel and Wiskich, 1998; Jarmuszkiewicz et al., 2005), the mitochondrial pool sized of certain keto-acids (Millar et al. 1993), and the enzyme's intermolecular disulfide bond status (Umbach et al., 1994). It is noteworthy that the total ubiquinone pool did not change significantly as a response to ABA treatment in our experiments (Table 1). Umbach et al. (2005) reported that in transgenic *Arabidopsis*, plants with lowered expression levels of AOX increased levels of ROS were measurable in the presence of the respiratory chain inhibitor KCN. In these AOX antisense plants, the expression of genes putatively encoding enzymes involved in cell wall remodeling (e.g., expansins) was slightly upregulated (Umbach et al., 2005). We observed ABA-mediated increases in AOX and decreases in expansin steady-state transcript levels, thus confirming an inverse correlation. Because measurements of *in vivo* AOX enzyme activity adjusting for all posttranslational modifications are currently not available (Juszczuk and Rychter, 2003) but AOX steady-state transcript changes appear to be at least loosely correlated with alternative respiratory chain activity, transcriptional profiling might be a crude yet useful indicator of AOX modulation changes in the context of the alternative respiratory chain.

2.9. Concluding remarks

Metabolite profiling of ABA-treated *Arabidopsis* seedlings and appropriate controls revealed an ABA-mediated increase in the pools of compatible organic solutes as well as the antioxidants α-Toc and ascorbic acid. Transcript profiling and subsequent analysis using the BioPathAt tool (Lange and Ghassemian, 2005) indicated that these antioxidants might be integrated into a biochemical network that controls the redox status in several subcellular compartments. In a recent review article, Foyer and Noctor (2005) suggested that redox homeostasis and antioxidant pools might act as a metabolic interface adjusting responses to various environmental cues. Our results, although mostly descriptive in nature, fully support such a notion and the data discussed herein allowed identification of specific genes putatively involved in the ABA-induced redox regulatory network. Our results also indicate that there might be a relationship between ABA biosynthesis, the xanthophyll cycle and AscA recycling. The testing of this hypothesis will provide valuable insights into the crosstalk between different pathways to protect plants from excess irradiation.

3. Experimental

3.1. Plant growth, ABA treatments and tissue harvests

Wild-type *Arabidopsis* (ecotype Col) were grown on agar plates. Seeds were stratified for 4 d at 4 °C, and placed in one line in the top third of rectangular Petri plates containing ½ MS media, 10 mM MES/KOH (pH 5.7) and 0.9% agar (supplemented with 50 ug/mL carbenicillin to suppress bacterial growth). The plates were stacked vertically and maintained under a 16 h day

(150 μmol photons × m^{-2} × s^{-1})/8 h night regime for 14 d. Seedlings were then transferred onto 15 mm round Petri dishes containing ½ MS media, 10 mM MES/KOH (pH 5.7) and 0.5% agar (controls). Half of the plates also contained 10 μM (+)-abscisic acid (ABA; Sigma) as a treatment. Seedlings were harvested at 0.5, 1, 3, 6, 12, and 24 h after onset of ABA treatment, immediately shock-frozen in N_2, transferred to a mortar, ground to a fine powder under N_2 in the dark, and stored as a homogenate at −80 °C in a 50 ml Ar-flushed Falcon reaction tube. Further MIAME- and MIAMET-compliant details (Brazma et al., 2001; Bino et al., 2004) are given in Supplementary Material 1.

3.2. RNA isolation, cRNA synthesis, microarray hybridization, and data analysis

For *Arabidopsis* GeneChip™ (Affymetrix) experiments, mRNA was extracted as described in Supplementary Material 1. Subsequent cRNA synthesis for microarray hybridization was performed as described previously (Zhu et al., 2001). An *Arabidopsis* whole-genome oligonucleotide microarray representing roughly 26,000 genes was used to assess transcriptome changes. Information regarding the design, sensitivity, dynamic range and reproducibility of this custom GeneChip™ are available at http://www.tmri.org/EN/downloads/arabidopsis_exon_array.pdf. The probe arrays were scanned with a GeneArray™ scanner (Agilent Technologies), and expression levels for all genes were determined using Microarray Analysis Suite 5.0 (Affymetrix) (Zhu et al., 2001). MIAME-compliant details (Brazma et al., 2001) describing protocols are provided in Supplementary Material 1 and all microarray and statistical data are given in Supplementary Material 2. Further information on the statistical methods used in the present study is presented in Chen et al. (2005).

3.3. Quantitative real-time PCR analysis of transcript expression

RNA was isolated from frozen leaf homogenate using the Trizol® reagent (Invitrogen). An aliquot of the RNA extract was treated with DNase-I prior to cDNA synthesis using Superscript III reverse transcriptase according to the manufacturer's instructions (Invitrogen). RNA isolation and cDNA synthesis were carried out with three independent biological replicate samples. For quantification of mRNA abundance, a diluted cDNA mixture was used as a template in a quantitative RT-PCR assay (40 cycles at 60 °C annealing temperature) performed using a 7500 PCR system (Applied Biosystems). Fluorescence intensities of three independent measurements were normalized against a reference dye. Because the absolute expression levels of genes of interest were quite different, we used two endogenous controls: beta-actin2 for genes expressed at medium levels (At3g18780; forward: TCGGTGGTTCCATTCTTGCT; reverse: GCTTTTAAGCCTTTGATCTTGAGAG) and ubiquitin-conjugating enzyme E2 for genes expressed at low levels (At5g25760; forward: GTCTGTGTGTAGAGCTATCATAGCATTG; reverse: GATTCCCTGAGTCGCAGTTAAGA). The following primers were used to amplify genes in interest: APX2 (At3g09640; forward: AAGGAGCGTTCAGGATTCGA; reverse: CGCTTAGTATCTCTTTGAAATAGGAGTTG); MDAR (At3g27820; forward: TTCCCAGAGGCTCACTGCAT; reverse: AATTTTACGCCTTTAGCCCTGTAG); GR (At3g24170; forward: TGTAGTGGGTCTCAGCGAAGAA; reverse: TGGATTAAAGCCTGAGGTGAAGA); DHAR (At1g19550; forward: GAGCTTTCCCCATGTCCATAAC; reverse: CCTCGGTCTTAGTTTTCTCAAAAGA); NDB2 (At4g05020; forward: CCGTCTACGCCAGTAAGCAAGT; reverse: AACGCCTCATCCAATCTGAAA); AOX1A (At3g22370; forward: TTCCTGCTGATGCGACACTT; reverse: TGGTAGTGAATATCAGATGCAAAATG); and AOX2 (At5g64210; forward: CTTAAAGATATCGACAATGGGAAGATC; reverse: GGCAATCTCCAATAATCAATAGCA). For each experimental sample, the amount of target and endogenous reference were determined from a standard curve for average fluorescence signal intensities. The calculated target amount was divided by the endogenous reference amount to obtain a normalized target value. The 0 h time point was selected as the calibrator sample. Each of the normalized target values was divided by the calibrator normalized target value to generate the relative levels of expression. Thus, the normalized amount of target is expressed as an unitless number and all quantities are expressed as an x-fold difference relative to the calibrator.

3.4. Metabolite extractions and analyses

All metabolite analyses were carried out in duplicate or triplicate following separate extractions/derivatizations/analyses. Mean values of relative abundance were calculated for each identified compound, and the fold change between ABA-treated samples and appropriate controls was calculated. Chlorophylls, carotenoids, tocopherols and isoprenoid quinones were extracted and analyzed using a protocol modified after Fraser et al., 2000. Fatty acids were extracted from seedling tissue and derivatized to methyl esters by heating to 80 °C for 90 min in 2.5% sulfuric acid in MeOH (tridecanoic acid was added as internal standard). Fatty acid methyl esters (FAMEs) were extracted with hexane and analyzed by GC–MS based on a protocol modified after Dyer et al. (2002). For the extraction of carbohydrates and organic acids, frozen tissue homogenate (120–150 mg) was processed and analyzed according to Roessner et al. (2000). Phytosterols were extracted and analyzed as previously described (Bligh and Dyer, 1959; Laule et al., 2003). Amino acids were extracted from frozen tissue homogenate (450–500 mg) and analyzed using an amino acid analyzer (L-8800; Hitachi, San Jose, CA) according to Inaba et al. (1994). MIAMET-compliant details (Bino et al., 2004) of all analytical protocols and metabolite profiling data are given in Supplementary Material 1 and Table 1, respectively.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.09.020.

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