The essential role of sugar metabolism in the acclimation response of Arabidopsis thaliana to high light intensities

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Abstract

Retrograde signals from chloroplasts are thought to control the expression of nuclear genes associated with plastidial processes such as acclimation to varying light conditions. Arabidopsis mutants altered in the day and night path of photoassimilate export from the chloroplasts served as tools to study the involvement of carbohydrates in high light (HL) acclimation. A double mutant impaired in the triose phosphate/phosphate translocator (TPT) and ADP-glucose pyrophosphorylase (AGPase) (adg1-1/tpt-2) exhibits a HL-dependent depletion in endogenous carbohydrates combined with a severe growth and photosynthesis phenotype. The acclimation response of mutant and wild-type plants has been assessed in time series after transfer from low light (LL) to HL by analysing photosynthetic performance, carbohydrates, MgProtoIX (a chlorophyll precursor), and the ascorbate/glutathione redox system, combined with microarray-based transcriptomic and GC-MS-based metabolomic approaches. The data indicate that the accumulation of soluble carbohydrates (predominantly glucose) acts as a short-term response to HL exposure in both mutant and wild-type plants. Only if carbohydrates are depleted in the long term (e.g. after 2 d) is the acclimation response impaired, as observed in the adg1-1/tpt-2 double mutant. Furthermore, meta-analyses conducted with in-house and publicly available microarray data suggest that, in the long term, reactive oxygen species such as H2O2 can replace carbohydrates as signals. Moreover, a cross-talk exists between genes associated with the regulation of starch and lipid metabolism. The involvement of genes responding to phytohormones in HL acclimation appears to be less likely. Various candidate genes involved in retrograde control of nuclear gene expression emerged from the analyses of global gene expression.

Key words: Arabidopsis mutants, carbon partitioning, light acclimation, metabolomics, photosynthesis, retrograde signalling, sugar metabolism, transcriptomics.

Introduction

Light is one of the most prominent environmental factors for plants. It not only determines the rate of photosynthesis and biomass production (Sharkey, 1985), but it also controls various aspects of development (FrankhaUSER and Chory, 1997). Apart from these beneficial effects, excess light can trigger metabolic changes as well as redox imbalances, and thus has the potential to damage thylakoid structures in the chloroplasts. Therefore, the activity and composition of thylakoid proteins, which
determine photosynthetic electron transport rates (ETRs), have to be adapted to changing light intensities over both short- and long-term periods. Long-term adaptation can be ascribed as acclimation (Eberhard et al., 2008; Kleine et al., 2009).

The vast majority of chloroplast-localized proteins are nuclear encoded (Leister, 2003). In order to adjust their expression to plastome-encoded photosynthesis genes, a constant flow of information from the chloroplast to the nucleus seems to be required (Beck, 2005; Koussevitzky et al., 2007; Eberhard et al., 2008). It has been proposed that this ‘retrograde signalling’ is based on a variety of components such as tetrapyrroles (Surpin et al., 2002; Beck, 2005; Czarnecki et al., 2012), reactive oxygen species (ROS: Kim et al., 2008; Foyer and Noctor, 2009; Miller et al., 2009; Triantaphylidès and Havaux, 2009; Balazadeh et al., 2012), the redox state of plastoquinone (Pfannschmidt et al., 1999; Pfalz et al., 2012), plastidial gene expression (Ahlert et al., 2003), the metabolic state of the mesophyll including sugars (Smeekens, 1998, 2000; Rolland et al., 2002, 2006; Brüttigam et al., 2009), and plant hormones, such as abscisic acid (ABA), as a response to an enhanced xanthophyll cycle activity (Baier et al., 2004; Baier and Dietz, 2005). More recently phosphoadenosine phosphophosphate (PAP; Estavillo et al., 2012) and 2-C-methyl-d-erythritol-2,4-cyclopentane-1,3-diol-DOPE (MecPP; Xiao et al., 2012), an intermediate of the plastidial mevalonate, which then use known components, for example those involved in sugar or hormonal signalling (for a review, see Rolland et al., 2006). Mutants defective in the day and night path of photoassimilate export from the chloroplasts can serve as a model system to test this assumption.

The export of triose phosphates (TPs) during the day (day path) is blocked by a knockout of the TP/phosphate translocator (TPT; Schneider et al., 2002). The absence of a functional ADP-glucose pyrophosphorylase (AGPase) in the adg1-1 mutant in turn results in the absence of starch (Lin et al., 1986) and therefore a block in maltose and glucose delivery and export during the night (night path). In contrast, the adg1-1 mutant accumulates high amounts of soluble sugars during the day (Kunz et al., 2010; Schmitz et al., 2012). While a defect in the TPT can be compensated for by an increased starch turnover in the light (Häusler et al., 1998; Schneider et al., 2002; Walters et al., 2004), a simultaneous block in TPT and AGPase in the adg1-1tp-2 double mutant results in severe growth retardation, high chlorophyll fluorescence, inhibition of the ETR, and depletion of carbohydrates (Häusler et al., 2009; Schmitz et al., 2012). This harsh phenotype conditionally occurs only under high light (HL) conditions, but is absent in low light (LL)-grown plants (Schmitz et al., 2012). Marked differences in the abundance of plastome- and nuclear-encoded thylakoid proteins in adg1-1tp-2 were also HL dependent and could be rescued by feeding sugars to the plants (Heinrichs et al., 2012; Schmitz et al., 2012), suggesting that (i) HL acclimation is impaired in the double mutant and (ii) soluble sugars can restore the acclimation response.

The response of wild-type and mutant plants to continuous growth in HL or LL has been extensively characterized by Schmitz et al. (2012). Here, time series rather than different light intensities (Walters et al., 2004) have been used to study the impact of impaired carbon partitioning and leaf carbohydrate metabolism on the acclimation response of Arabidopsis thaliana towards HL. Photosynthesis parameters, metabolite levels, and global gene expression were measured after LL to HL (LL/HL) transfer in mutant and wild-type plants. The data suggest that endogenous sugars and ROS play a pivotal role in HL acclimation in A. thaliana.

Materials and methods

Plant material, growth conditions, and photosynthesis measurements

Plant material and growth conditions in HL [photon flux density (PFD)=300 μmol m−2 s−1] or LL (PFD=30 μmol m−2 s−1) have been described in detail by Schmitz et al. (2012). For LL/HL transfer experiments, plants were grown under long-day conditions (i.e. 16 h light/8 h dark) for 3 weeks. Photosynthesis parameters at photosystem II (PSII) were determined according to Schreiber et al. (1986) and Genty et al. (1989) with an IMAGING-PAM fluorometer (Walz, Effeltrich, Germany).

Determination of metabolites

Carbohydrates were extracted and measured enzymatically as described by Schmitz et al. (2012). Anthocyanins were determined according to Giraud et al. (2008). For gas chromatography–time of flight-mass spectrometry (GC-ToF-MS) profiling, primary metabolites were extracted and derivatized according to established procedures (Lisec et al., 2006). Raw data were then normalized following the procedure outlined in Roessner et al. (2001); peaks were identified using the software TagFinder (Luedemann et al., 2012), with the support of the mass tags deposited in the Golm Metabolome Database (Kopka et al., 2005). Further details regarding the metabolic profiling are reported in Supplementary Table S1 available at JXB online, which is compliant with the recommendations reported in Fernie et al. (2011).

Ascorbic acid (Asc) and dehydroascorbate (DHA) were quantified with ascorbate oxidase according to Horling et al. (2003), and glutathione (GSH) and glutathione disulphide (GSSG) by enzymatic cycling with glutathione reductase and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Griffith, 1980) with some modifications, as described by Delze et al. (2012).

Mg-protoporphyrin IX (MgProtoIX) was extracted from 100 μg of frozen leaf powder in 2×200 μl and 1×100 μl acetone:methanol:0.1 N NH₄OH (10:9:1). After each centrifugation for 10 min at 4 °C, the supernatants were collected and aliquots analysed by HPLC-based fluorescence detection to quantify Mg-porphyrins by means of authentic standards (Frontier Scientific) according to Czarnecki et al. (2011).

RNA extraction for transcriptome analyses and quantitative reverse transcription–PCR (qRT–PCR)

Samples for transcriptome analyses were collected from two sets of independently grown plants, with 3–6 plants per sample. Total RNA was isolated according to Logemann et al. (1987), and for transcriptome analyses subsequently purified on Plant RNeasy extraction columns (Qiagen). Processing and hybridization to
GeneChip ATH1 Genome Arrays (Affymetrix) was conducted at the ‘Kompetenzzentrum Fluoreszente Bioanalytik’ (KFB, Regensburg). The complete set of microarray data can be obtained from http://www.ebi.ac.uk/miame/project/ (ArrayExpress accession: E-MEXP-3791). For qRT–PCR with Lhcb1 the following primer combination was used (RL_Lhcb1_fwd: TTCCCTGGAGACTACGGATG; RL_Lhcb1_rev: CCCACCTGCTGTGGATAACT).

Statistical evaluation of experimental data

Raw fluorescence probe signals of Affymetrix microarrays were evaluated and statistically analysed with the Robin software (Lohse et al., 2010). The data were normalized by the RMA-method (robust multiaarray averaging; Wu et al., 2004). Average log2-fold expression changes and adjusted P-values were calculated by Linear Models for Microarray Data (Limma; Smyth, 2004) and false discovery rate (FDR) corrected according to Benjamin and Hochberg (1995). The Tair10 genome release as well as a modified Mapman Binning (Thimm et al., 2004) were used to annotate the probe-IDs. Venn diagrams were constructed with the help of the web-based tool Venny (Oliveros, 2007). The R-tool 2.12.0 (package ‘pcaMethods’; Stacklies et al., 2007) has been implemented for multivariate analyses (principle component analysis (PCA)) as well as for Fisher’s exact tests.

Significant differences between more than two physiological data sets were analysed using single-site analysis of variance (ANOVA) combined with the post-hoc Tukey–Kramer test, which allows the comparison of unequal sample sizes and identifies values which are significantly different (Ludbrook, 1998). For data plotting and fitting, SigmaPlot10.0 for Windows (SPSS Inc.) was used.

Published microarray data were obtained either from NASC (http://affymetrix.arabidopsis.info) or from the supplementary data of the respective publications online. Differentially regulated genes were filtered by log2-fold changes at a threshold of ±1 (for time series at least one time point had to contain the candidate gene at a threshold of ±1) and commonly regulated genes were extracted. The relative abundance of coinciding genes was calculated from \( \frac{a_0}{X_T} \cdot \frac{a_0}{Y_T} \), where \( A_c \) is the coincidence coefficient, and \( a_0 \) equals the number of coinciding genes as a fraction of the total number of differentially regulated genes in in-house \( (X_T) \) or published \( (Y_T) \) experiments. The sum of the \( A_c \) values of all foreign arrays was set to 100% and, from the relative contribution, pie charts were constructed. The correlation of log2 ratios between in-house and published arrays was calculated according to Pearson (Galton, 1888).

Results and Discussion

LL/HL transfer leads to rapid photoinhibition followed by a slow recovery

Exposure of LL-grown plants to HL conditions rapidly leads to severe photoinhibition and a re-organization of the photosynthetic apparatus during acclimation (Bailey et al., 2004; Horton et al., 2008). The dynamics of these processes are reflected in changes of the photosynthetic light reaction determined by PAM fluorometry. Modulated chlorophyll \( a \) (Chl \( a \)) fluorescence in dark-adapted mutant and wild-type plants was monitored over a time period of 1 week (172 h) after LL/HL transfer (Fig. 1). HL exposure of LL-grown plants resulted in a drop in the \( F_v/F_m \) ratio within the first 3 h based on photoinhibition, followed by a slow recovery (Fig. 1A). In contrast to wild-type and single mutant plants, which exhibit a complete recovery of \( F_v/F_m \) within 1 week, the \( F_v/F_m \) ratio in \( adg1-1/tpt-2 \) decreased to a minimum value below 0.2 within the first 2 d upon HL exposure, followed by a small, but incomplete recovery thereafter. The decrease in \( F_v/F_m \) can be attributed to the dissociation of light-harvesting complexes (LHCs) from photosynthetic core complexes (Schmitz et al., 2012). The ETR in all plant lines increased only slowly with time after LL/HL transfer. In Col-0, \( tpt-2 \), and \( adg1-1 \), the maximum increase in ETR commenced after 48 h in HL (Fig. 1B), whereas in the \( adg1-1/tpt-2 \) double mutant the ETR remained significantly lower over the whole time course of the experiment. The statistical analysis of the above data is contained in Table 1 of Supplementary Document S1 available at JXB online.

LL/HL transfer results in sugar and anthocyanin accumulation

Dynamic changes in carbohydrate contents were assessed in wild-type and mutant plants within the first 8 h, and 38 h after LL/HL transfer (Fig. 2). Starch contents linearly increased with time only in wild-type and \( tpt-2 \) mutant plants and approached levels of ~100 \( \mu \)mol g \(^{-1}\) fresh weight (FW) at the end of day 2
Table 1. Distribution of functional categories following the ‘static’ assessment of expression profiles of adg1-1, tpt-2, and adg1-1/tpt-2 versus wild-type plants at t0 (LL) and at t4h, and t48h after LL/HL transfer

Only functional categories with five or more altered genes are displayed.

<table>
<thead>
<tr>
<th>Functional categories</th>
<th>adg1-1 versus Col-0</th>
<th>tpt-2 versus Col-0</th>
<th>adg1-1/tpt-2 versus Col-0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t0</td>
<td>t4h</td>
<td>t48h</td>
</tr>
<tr>
<td></td>
<td>Up</td>
<td>Down</td>
<td>Up</td>
</tr>
<tr>
<td>Amino acid metabolism</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cell wall</td>
<td>–</td>
<td>–</td>
<td>6 (2.7)*</td>
</tr>
<tr>
<td>CHO metabolism</td>
<td>–</td>
<td>–</td>
<td>5 (8.7)*</td>
</tr>
<tr>
<td>Development</td>
<td>5 (2.4)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hormone metabolism</td>
<td>–</td>
<td>5 (10.2)*</td>
<td>–</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Protein – degradation</td>
<td>–</td>
<td>11 (2.3)*</td>
<td>–</td>
</tr>
<tr>
<td>Protein – synthesis</td>
<td>–</td>
<td>–</td>
<td>7 (5.2)*</td>
</tr>
<tr>
<td>Protein – synthesis (plastome)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PS – light reaction (plastome)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>RNA – regulation of transcription</td>
<td>–</td>
<td>–</td>
<td>6 (1.2)</td>
</tr>
<tr>
<td>Signalling</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Stress</td>
<td>–</td>
<td>–</td>
<td>5 (8.1)*</td>
</tr>
<tr>
<td>Transport</td>
<td>–</td>
<td>–</td>
<td>5 (0.8)</td>
</tr>
</tbody>
</table>

The numbers of significantly (P<0.01) altered genes were taken from Supplementary Tables S2–S4 available at JXB online.

The threshold of log2 ratios was kept at ±1.0.

The data in parentheses refer to over- or under-represented functional categories (i.e. numbers above or below 1).

Significantly (P<0.05) over- or under-represented categories according to Fisher’s exact test are marked by an asterisk.

Over-represented categories are shown in bold.

(i.e. after 38 h; Fig. 2A). The soluble sugars sucrose (Suc), glucose (Glc), and fructose (Fru) dramatically increased in adg1-1 within the first 8 h and 38 h after LL/HL transfer, but showed only a transient increase in wild-type, tpt-2, and adg1-1/tpt-2 plants (Fig. 2B–D). Significant differences were observed for most time points only between adg1-1 and the other lines (see Table 2 of Supplementary Document S1 available at JXB online). The transient accumulation of carbohydrates in all lines reflects an increased capacity to assimilate CO2 under HL conditions. However, in adg1-1/tpt-2, a prolonged time in HL led to a depletion of soluble sugars (compare Schmitz et al., 2012).

LL/HL transfer also resulted in anthocyanin accumulation in wild-type and adg1-1 plants (Fig. 2E), which was, however, less pronounced in tpt-2 and adg1-1/tpt-2. Anthocyanin levels in HL-grown plants (most pronounced in adg1-1) appeared to be directly correlated with soluble carbohydrates (Fig. 2B–D, right panel). Suc is the most prominent inducer of anthocyanin production involving the MYB75/PAP1 (Teng et al., 2005) and HY5 transcription factors (Shin et al., 2013) as well as the COP1/SPA ubiquitin ligase (Maier et al., 2013). For further statistical data analyses, see Table 2 of Supplementary Document S1 available at JXB online.

Lhcb1 expression and MgProtoIX levels were not correlated in response to LL/HL transfer

The Chl biosynthesis intermediate, MgProtoIX, was proposed to act as a retrograde signal (Susek et al., 1993; Strand et al., 2003), although this idea has recently been challenged by more refined determinations of tetrapyrrroles and the lack of correlation of their content with the abundance of Lhcb1 transcripts (LHCII; Mochizuki et al., 2008; Moulin et al., 2008). Changes in MgProtoIX contents and Lhcb1 expression levels in mutant and wild-type plants in response to short- and long-term HL exposure (Fig. 3) lacked any direct correlation (Supplementary Fig. S1A available at JXB online). In all plants, MgProtoIX initially dropped to lower levels upon HL exposure compared with LL-grown plants (Fig. 3A–D). Long-term HL exposure resulted in higher levels of MgProtoIX in wild-type and tpt-2 plants (Fig. 3A, B), but not in adg1-1/tpt-2 (Fig. 3D), and was less pronounced in adg1-1 (Fig. 3C). Likewise, apart from small differences within the first 60 min after LL/HL transfer, the short-term response of Lhcb1 expression was similar between all plant lines (Fig. 3E–H). However, while Lhcb1 expression was suppressed to a different degree in HL-grown wild-type and both single mutant plants (Fig. 3E–G), it was slightly induced in adg1-1/tpt-2 (Fig. 3H). These data further indicate that acclimation to HL is perturbed in the double mutant. A statistical analysis of the data shown in Fig. 3 is given in Table 3 of Supplementary Document S1 available at JXB online. Although MgProtoIX did not correlate with Lhcb1 expression, there was a pronounced inverse correlation of Lhcb1 transcript levels with soluble carbohydrate contents in wild-type and mutant plants (Supplementary Fig. S1E available at JXB online). Hence, carbohydrates might play a profound role in the transcriptional acclimation response of A. thaliana. In contrast, MgProtoIX levels were directly correlated with the light reaction of photosynthesis (i.e. ETR; Supplementary Fig. S1F available at JXB online) rather than with products of the dark reaction (Supplementary Fig. S1B, C available at JXB online).
have the potential to leave the chloroplast and hence to act as a retrograde signal (Noctor et al., 2000; Karpinski et al., 2013). As H$_2$O$_2$ can also be produced in the peroxisomes during photorespiration and in the apoplast, the mesophyll has to distinguish between different sources of H$_2$O$_2$ signals (Shapiguzov et al., 2013).

The major H$_2$O$_2$ source in photosynthesis is the Mehler–peroxidase reaction or ‘pseudo cyclic’ electron transport (Badger et al., 2000). H$_2$O$_2$ can be detoxified by either the Asc–GSH cycle (Halliwell–Asada pathway; Noctor and Foyer, 1998) or the Asc-independent thiol peroxidase pathway (Dietz et al., 2006). Asc and DHA as well as GSH and GSSG levels after LL/HL transfer lack large differences in *adg1-1/tpt-2* (Fig. 4D, H) compared with wild-type plants (Fig. 4A, E). All plant lines show an increase in the total contents of both redox components depending on the time in HL. Significant changes in Asc and DHA levels were observed in the *tpt-2* mutant, which resulted in a drop of the Asc/DHA ratios below one (Fig. 4B). This was, however, not reflected in large changes in GSH/GSSG ratios (Fig. 4F).

Although the exposure of LL-grown *adg1-1/tpt-2* plants to HL resulted in severe photoinhibition, the redox components involved in the detoxification of ROS were hardly altered, most probably because photoinhibition also restricts the production of ROS at the thylakoid membrane. Hence, differential redox signalling as a cause of the phenotype of *adg1-1/tpt-2* appears to be unlikely. For statistical analyses, see Table 4 of Supplementary Document S1 available at *JXB* online.

### Assessment of transcriptomic changes in response to LL/HL transfer of mutants impaired in carbohydrate metabolism

The role of impaired carbon partitioning in HL acclimation has been assessed in wild-type and mutant plants by microarray-based transcriptomics in time series upon LL/HL transfer. Suitable time points for sampling were obtained from experiments shown in Figs 1 and 2, and were LL (*t*$_0$), as well as 4 h (*t*$_{4h}$; as short-term response) and 48 h (*t*$_{48h}$; as long-term response) after transfer to HL. At the latter time point, the ETR in wild-type and single mutant plants started to increase in response to HL exposure (Fig. 1B).

A multivariate analysis of the data sets (PCA; significance level, *P*>0.01; no threshold) revealed three individual clusters that were governed by the light regime and/or the time in HL rather than by metabolic defects of the respective mutants (Fig. 5). The microarray data were further analysed by ‘static’ and ‘dynamic’ comparisons of gene expression. For the ‘static’ approach (for details, see Supplementary Document 2 available at *JXB* online), expression levels in the mutants were referred to wild-type plants at each time point. Numbers of significantly altered genes are shown in the Venn diagrams (Fig. 6) and in more detail in Supplementary Tables S2–S4 available at *JXB* online. For a dynamic assessment of HL-responsive genes in wild-type and mutant plants, their expression level at *t*$_{4h}$ and *t*$_{48h}$ after LL/HL transfer were referred to *t*$_0$ (Fig. 7; Supplementary Tables S5, S6 available at *JXB* online).

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**Redox components were similarly affected in mutant and wild-type plants after LL/HL transfer**

Light stress leads to the production of ROS, which can cause severe damage to membranes and the photosynthetic apparatus (Apel and Hirt, 2004; Vass, 2012). Besides ROS with a short lifetime such as singlet oxygen (Krieger-Liszkay, 2005), those with a longer lifetime such as, for instance, H$_2$O$_2$, would...
Table 2. Enrichment of functional categories among the commonly regulated genes in wild-type and mutant plants as a response to LL/HL transfer at \( t_{48h} \) versus \( t_0 \) and \( t_{48h} \) versus \( t_0 \)

The threshold of log2 ratios was kept at ±1.5. Only functional categories with five or more altered genes are displayed.

<table>
<thead>
<tr>
<th>Functional categories</th>
<th>No. of commonly regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( t_{48h} ) versus ( t_0 )</td>
</tr>
<tr>
<td></td>
<td>Up-regulated</td>
</tr>
<tr>
<td>Cell wall</td>
<td>–</td>
</tr>
<tr>
<td>Development</td>
<td>9 (1.12)</td>
</tr>
<tr>
<td>DNA synthesis/chromatin structure</td>
<td>9 (0.96)</td>
</tr>
<tr>
<td>Hormone metabolism</td>
<td>5 (0.87)</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>7 (1.54)</td>
</tr>
<tr>
<td>Major CHO metabolism</td>
<td>11 (8.87)*</td>
</tr>
<tr>
<td>Misc. – Cytochrome P450</td>
<td>5 (2.01)</td>
</tr>
<tr>
<td>Misc. – (UDP glucosyl- and glucoronyl transferases)</td>
<td>5 (2.48)</td>
</tr>
</tbody>
</table>

The numbers of significantly (\( P<0.01 \)) altered genes were taken from Supplementary Table S7 available at JXB online. The threshold of log2 ratios was kept at ±1.5.

Static assessment of global gene expression after LL/HL transfer

Under LL conditions, 523 genes were significantly altered in \( \text{adg1-1/tpt-2} \) compared with 88 in \( \text{adg1-1/tpt-2} \) and only two in \( \text{tpt-2} \) (Fig. 6A). After 4h in HL, the number of differentially regulated genes dropped to lower values in \( \text{adg1-1} \) and \( \text{adg1-1/tpt-2} \) (Fig. 6B), but increased again after 48h of HL exposure (Fig. 6C). In \( \text{tpt-2} \), the number of altered genes increased from 67 to 181 after 4h and 48h, respectively, in HL. However, the highest number was again found in \( \text{adg1-1/tpt-2} \) (388). The number of commonly regulated genes in the central overlapping regions of the Venn diagrams was small, suggesting that the alterations in gene expression compared with wild-type plants observed in the ‘static approach’ were related to the individual lesions in the mutants rather than to changing conditions. Static changes in expression of HL-responsive genes have been analysed and described in more detail in Supplementary Document S2 and in Supplementary Tables S2–S4 available at JXB online. Functional categories containing more than five genes are shown in Table 1. In \( \text{adg1-1/tpt-2} \), significantly over-represented genes of the categories ‘amino acid metabolism’, ‘cell wall’, and ‘CHO metabolism’ were down-regulated at \( t_{48h} \) after LL/HL transfer, whereas over-represented plastome-encoded genes belonging to the categories ‘protein – synthesis’ and ‘PS – light reaction’ were up-regulated. Remarkably, in \( \text{adg1-1/tpt-2} \), MYC4 (At4g17880), a basic helix–loop–helix (bHLH)-type transcription factor involved in jasmonate responses (Fernández-Calvo et al., 2011) and glucosinolate biosynthesis (Schweizer et al., 2013), was severely down-regulated in both LL and HL. According to the eFP browser (Winter et al., 2007), MYC4 is also inducible by sugars.

Dynamic changes in gene expression during LL/HL transfer experiments

Compared with the static assessment of global gene expression, which aimed at identifying differentially regulated genes between mutant and wild-type plants at each time point, the dynamic analysis displayed genes particularly responding to the LL/HL transfer and is therefore described in more detail.

Interestingly, 4h after HL exposure, the overlapping areas of all plants in this study comprised the highest number (1087) of significantly altered genes (Fig. 7A), which also showed a relatively high amplitude of differential expression (i.e. log2 ratios of ±3; Supplementary Table S5 available at JXB online). This observation indicates that the short-term response of HL-dependent regulation of a large set of genes was operational in wild-type and mutant plants. After 48h in HL, the number of genes in the central overlapping region of the Venn diagrams dropped by ~50% compared with \( t_{48h} \).
Acclimation to high light

but increased specifically in tpt-2 from 31 to 395 genes at t4h and t48h, respectively. Compared with tpt-2, the number of altered genes was appreciably smaller in adg1-1 (175) and adg1-1/tpt-2 (111).

Strategies for functional analyses of HL-responsive genes

Different strategies were applied to obtain additional information on HL-responsive genes in wild-type and mutant plants. The following observations and assumptions have been addressed. (i) It can be assumed that commonly regulated HL-responsive genes in all lines of this study should be independent from metabolic constraints by individual mutations. (ii) In contrast, specifically regulated or non-regulated genes after LL/HL transfer should be linked to the metabolic constraints in the individual mutant plants. Finally, (iii) comparisons of in-house with publicly available microarray data on hormone and stress treatments might help to identify components associated with signalling pathways involved in HL acclimation.

Wild-type and mutant plants comprise a large set of commonly regulated genes in response to LL/HL transfer

The central regions of the Venn diagrams shown in Fig. 7 comprise genes responding similarly to HL exposure in wild-type and mutant plants and can hence be considered as independent or less dependent than lesions in primary carbohydrate metabolism. These genes are listed in Supplementary Table S7.

Table 3. Pearson correlation coefficients calculated for comparisons of in-house with publicly available microarray data

In (A) a list of commonly regulated genes (Supplementary Table S7 available at JXB online) and up-regulated genes in tpt-2, 48 h after LL/HL transfer (Supplementary Table S8K* available at JXB online) have been used as basis for comparison. In (B) the gene lists for comparisons were obtained from Supplementary Tables SSA–C and SSA–C (available at JXB online).

<table>
<thead>
<tr>
<th>Microarray data sets</th>
<th>A</th>
<th>t4h</th>
<th>t48h</th>
<th>tpt-2*</th>
<th>B</th>
<th>t4h</th>
<th>t48h</th>
<th>tpt-2</th>
<th>tpt-2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seedings+sucrose</td>
<td>0.759</td>
<td>0.901</td>
<td>0.479</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pho3 mutant</td>
<td>0.866</td>
<td>0.956</td>
<td>0.191</td>
<td>0.976</td>
<td>None</td>
<td>0.357</td>
<td>0.601</td>
<td>0.627</td>
<td>0.322</td>
</tr>
<tr>
<td>ABA feeding</td>
<td>−0.093</td>
<td>0.242</td>
<td>0.238</td>
<td>−0.026</td>
<td>0.692</td>
<td>0.299</td>
<td>−0.049</td>
<td>0.556</td>
<td>0.247</td>
</tr>
<tr>
<td>IAA feeding</td>
<td>−0.027</td>
<td>0.061</td>
<td>0.176</td>
<td>0.282</td>
<td>0.735</td>
<td>−0.063</td>
<td>−0.514</td>
<td>None</td>
<td>0.129</td>
</tr>
<tr>
<td>Zeatin feeding</td>
<td>0.110</td>
<td>−0.089</td>
<td>0.251</td>
<td>−0.369</td>
<td>−0.991*</td>
<td>−0.722</td>
<td>−0.230</td>
<td>−0.589</td>
<td>0.140</td>
</tr>
<tr>
<td>GA&lt;sub&gt;3&lt;/sub&gt; feeding</td>
<td>0.249</td>
<td>−0.527</td>
<td>0.808</td>
<td>None</td>
<td>None</td>
<td>0.136</td>
<td>0.641</td>
<td>None</td>
<td>0.190</td>
</tr>
<tr>
<td>ACC feeding</td>
<td>−0.232</td>
<td>0.178</td>
<td>0.533</td>
<td>0.375</td>
<td>0.520*</td>
<td>0.605</td>
<td>0.061</td>
<td>None</td>
<td>0.026</td>
</tr>
<tr>
<td>MJ feeding</td>
<td>0.600</td>
<td>0.609</td>
<td>0.279</td>
<td>0.874</td>
<td>0.795</td>
<td>0.113</td>
<td>0.345</td>
<td>−0.589</td>
<td>0.170</td>
</tr>
<tr>
<td>flu mutant+light</td>
<td>0.374</td>
<td>0.063</td>
<td>−0.077</td>
<td>0.317</td>
<td>−0.497</td>
<td>0.140</td>
<td>0.041</td>
<td>−0.863</td>
<td>0.353</td>
</tr>
<tr>
<td>Wild type+MV (24 h)</td>
<td>0.476</td>
<td>0.725</td>
<td>0.516</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Pearson correlation coefficient (0 to ±1)

The columns in (A) refer to threshold log<sub>2</sub>-fold change of ±1.5, whereas the columns in (B) are based on a threshold log<sub>2</sub> ratio of ±1.0. Pearson correlation coefficients above/below 0.7/–0.7 are displayed in bold.

ND, not determined; none, less than three commonly regulated genes.

*Less than four genes.
A number of metabolic genes associated with ‘lipid metabolism’, ‘major CHO metabolism’, and ‘transport’ were differentially regulated as a response to LL/HL transfer, specifically in the short term, namely at t₄h (Table 2). The detailed analysis of these genes, which is contained in Supplementary Document S3 available at JXB online, can be summarized as follows: most of the 11 genes associated with ‘major CHO metabolism’ are involved in starch metabolism (see also Walters et al., 2004). The transcriptional regulation of these genes is obviously independent from the presence or absence of starch, as it also works in the adg1-1 background. An analysis with ATTEDII (Obayashi et al., 2009) revealed that these genes are imbedded in a single regulatory network (Supplementary Fig. S2A, Supplementary Table S7A available at JXB online), which also comprises a chloroplast-localized AMP-activated protein kinase (At5g39790; Supplementary Table S7A available at JXB online). There appears to be a cross-talk between ‘CHO metabolism’ and ‘lipid metabolism’ as three members of this network are also contained in a network of up-regulated genes associated with ‘lipid metabolism’ (Supplementary Fig. S2B available at JXB online). Likewise, down-regulated genes involved in ‘lipid metabolism’ at t₄h versus t₀ (but not at t₄₈h versus t₀) form a large network (Supplementary Fig. S2C available at JXB online).

Inter- and intracellular transport processes are of particular importance in the adjustment of metabolism to the
Table 4. Contents of selected metabolites determined either enzymatically (starch, α-Suc, α-Glc, and α-Fru) or by GC-MS in leaf extracts of (A) Col-0 and tpt-2 or (B) adg1-1 and adg1-1/tpt2 grown either continuously in LL or HL or after LL/HL transfer at t_{4h} or t_{48h}

### Part A

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Col-0 t_{4h} (LL)</th>
<th>Col-0 t_{4h} (HL)</th>
<th>Col-0 (LL)</th>
<th>Col-0 (HL)</th>
<th>tpt-2 t_{4h} (LL)</th>
<th>tpt-2 t_{4h} (HL)</th>
<th>tpt-2 t_{4h} (LL)</th>
<th>tpt-2 t_{4h} (HL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>2.86 ± 0.29</td>
<td>33.04 ± 1.30</td>
<td>70.81 ± 3.66</td>
<td>122.50 ± 7.19</td>
<td>5.33 ± 0.23</td>
<td>36.93 ± 5.60</td>
<td>110.17 ± 3.74</td>
<td>148.49 ± 5.97</td>
</tr>
<tr>
<td>α-Suc</td>
<td>0.77 ± 0.06</td>
<td>1.97 ± 0.04</td>
<td>2.66 ± 0.25</td>
<td>3.90 ± 0.45</td>
<td>0.60 ± 0.03</td>
<td>0.16 ± 0.02</td>
<td>0.37 ± 0.02</td>
<td>0.70 ± 0.23</td>
</tr>
<tr>
<td>α-Glc</td>
<td>1.02 ± 0.12</td>
<td>2.05 ± 0.12</td>
<td>2.69 ± 0.25</td>
<td>8.32 ± 0.16</td>
<td>0.95 ± 0.05</td>
<td>1.14 ± 0.06</td>
<td>3.50 ± 0.22</td>
<td>5.95 ± 0.86</td>
</tr>
<tr>
<td>α-Fru</td>
<td>0.10 ± 0.01</td>
<td>0.56 ± 0.02</td>
<td>0.57 ± 0.06</td>
<td>1.69 ± 0.21</td>
<td>0.01 ± 0.01</td>
<td>0.12 ± 0.02</td>
<td>0.53 ± 0.07</td>
<td>1.20 ± 0.13</td>
</tr>
</tbody>
</table>

### Part B

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>adg1-1 t_{4h} (LL)</th>
<th>adg1-1 t_{4h} (HL)</th>
<th>adg1-1 t_{4h} (LL)</th>
<th>adg1-1 t_{4h} (HL)</th>
<th>adg1-1/tpt2 t_{4h} (LL)</th>
<th>adg1-1/tpt2 t_{4h} (HL)</th>
<th>adg1-1/tpt2 t_{4h} (LL)</th>
<th>adg1-1/tpt2 t_{4h} (HL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>0.27 ± 0.01</td>
<td>0.52 ± 0.03</td>
<td>0.67 ± 0.06</td>
<td>0.11 ± 0.06</td>
<td>0.42 ± 0.03</td>
<td>0.85 ± 0.03</td>
<td>0.68 ± 0.04</td>
<td>1.82 ± 0.79</td>
</tr>
<tr>
<td>α-Suc</td>
<td>1.74 ± 0.14</td>
<td>14.92 ± 0.45</td>
<td>8.67 ± 0.87</td>
<td>3.43 ± 0.74</td>
<td>1.34 ± 0.16</td>
<td>1.52 ± 0.10</td>
<td>1.99 ± 0.25</td>
<td>1.02 ± 0.09</td>
</tr>
<tr>
<td>α-Glc</td>
<td>2.40 ± 0.14</td>
<td>11.11 ± 0.08</td>
<td>7.89 ± 0.56</td>
<td>9.65 ± 0.58</td>
<td>2.05 ± 0.08</td>
<td>3.60 ± 0.04</td>
<td>4.00 ± 0.20</td>
<td>3.61 ± 0.15</td>
</tr>
<tr>
<td>α-Fru</td>
<td>0.89 ± 0.10</td>
<td>6.40 ± 0.16</td>
<td>2.68 ± 0.20</td>
<td>3.11 ± 0.16</td>
<td>0.66 ± 0.01</td>
<td>0.98 ± 0.06</td>
<td>0.78 ± 0.10</td>
<td>0.85 ± 0.06</td>
</tr>
</tbody>
</table>

The samples were harvested in the middle of the light period (i.e. after 8 h in the light). The data represent the mean of five independent samples ± SE; ND, not detected.
requirement of the plants. Among 32 differentially regulated genes associated with ‘transport processes’, the expression of two genes belonging to the phosphate translocator family was altered after 4 h in HL. The glucose 6-phosphate/phosphate translocator2 (GPT2; At1g61800) and the phosphoenolpyruvate/phosphate translocator2 (PPT2; At3g01550) were up- and down-regulated, respectively. Interestingly, GPT2 strongly responds to elevated soluble sugar levels (Kunz et al., 2010; Schmitz et al., 2012), for example in starch-free mutants or after feeding of exogenous carbohydrates to the plants (Heinrichs et al., 2012). Although GPT2 was highly up-regulated at t 4h versus t 0 in all plants (Supplementary Table S7A available at JXB online), its expression level at t 48h versus t 0 corresponded well to the sugar levels in the individual plant lines; that is, GPT2 expression was further increased in adg1-1, but was not significantly changed in the double mutant (compare Fig. 1). For more information, please refer to Supplementary Document S3 available at JXB online.

Fig. 5. Multivariate analysis (principle components) of the microarray data sets of LL/HL transfer experiments with Col-0 wild-type (circles), the tpt-2 (squares) and adg1-1 (triangles) single mutants, and the adg1-1/tpt-2 double mutant (diamonds). (This figure is available in colour at JXB online.)

Does the mutant-specific regulation or non-regulation of genes provide information on missing signals?

Here the question was asked of whether the analysis of genes specifically regulated or not regulated in the individual mutant plants in response to LL/HL transfer can provide information on the putative retrograde signals involved.

In a first approach, the intention was to identify significantly (Fisher’s exact test; P<0.05) over- or under-represented functional groups or clusters according to MapMan bins (Thimm et al., 2004). Significantly altered (P<0.01) HL-responsive or non-responsive genes in the adg1-1/tpt-2 double mutant (i.e. those genes responding to HL only in wild-type and single mutant plants) were categorized without threshold (compare Fig. 7). The major outcome of this approach is shown in Fig. 8 and can be summarized as follows: the MapMan binning of the expression data resulted in 36 major functional categories. In the short term (i.e. 4 h after LL/HL transfer; Fig. 8A), genes belonging to the categories ‘RNA binding/processing’ and ‘protein synthesis/targeting/folding’ were significantly over-represented in both groups, regulated and non-regulated genes, in adg1-1/tpt-2. All other significant alterations can be attributed to genes that were not differentially expressed in adg1-1/tpt-2, and these were the under-represented categories ‘biotic stress’ and ‘DNA’ as well as the over-represented categories ‘nucleotide metabolism’ and ‘metal handling’. In the long term (i.e. 48 h...
In a second approach, genes specifically responding to the constraints in the single and double mutants were separated and are listed in Supplementary Table S8 available at JXB online. For this purpose, the log2-fold change for genes assigned as non-regulated was limited to ±0.5. In particular, adg1-1/tpt-2 exhibited a strong phenotype upon HL exposure and hence further attention was focused on this line (compare MapMan binning, Fig. 8). At t₄₈h versus t₀ adg1-1/tpt-2 contained five up- and nine down-regulated genes (Supplementary Table S8A available at JXB online). Three of the five up-regulated genes were associated with histone proteins and hence ‘chromatin structure’ and ‘cell organisation’. Besides a gene of unknown function (At2g30600), a methylothioalkylmalate synthase gene (At5g23010) involved in methionine-derived glucosinolate biosynthesis (Textor et al., 2004) was up-regulated. Interestingly, of the nine down-regulated genes, four genes were associated with ‘biotic stress’ (three of these genes encode heat shock proteins; At1g53540, At3g46230, and At5g12020, which have also been recognized in the static approach; compare Supplementary Document S2 available at JXB online) and one gene each was linked to ‘calcium signalling’ (At2g41090), ‘RT’ (At5g61010), ‘mitochondrial electron transport’ (At4g05200), ‘minor CHO metabolism’ (At2g37760), and ‘lipid metabolism’ (At2g44300). In particular, heat shock proteins represent the interaction point of various stress response pathways (Swindell et al., 2007) and deserve further attention also with respect to retrograde signalling. In the category ‘RT’ an Exo70 type subunit of an exocytosis complex was down-regulated. Such organelle-like structures are involved in excretion processes by fusion with the plasma membrane (Li et al., 2010; Wang et al., 2010).

At t₄₈h versus t₀ there were five and six genes up- or down-regulated specifically in adg1-1/tpt-2 (Supplementary Table S8C available at JXB online). Amongst the up-regulated genes, plastome-encoded ribosomal proteins (two genes) and one gene encoding RNA polymerase were found. The further two up-regulated genes were associated with either ‘amino acid degradation’ (At3g16150) or ‘RT’, namely the heat shock transcription factor A9 (At2g26150), which under normal conditions is only weakly expressed in leaves (eFP browser, Winter et al., 2007), again bringing members of heat shock proteins or related transcription factors into focus.

Genes specifically not responding to HL exposure in the double mutant, but responding in the wild type and the single mutants, might provide information on missing signals for induction or repression of these genes. At t₄₈h versus t₀ only six HL-non-responsive genes were found in adg1-1/tpt-2 (Supplementary Table S8B available at JXB online). In the wild type and both single mutants, the three up-regulated genes are non-characterized transporters (At4g13800 and At5g26220) or are involved in developmental processes (At1g77450). The three down-regulated genes are associated with ‘cell wall degradation’ (At1g02640), ‘redox’ (At3g62950; i.e. a putative glutaredoxin), and a trehalose phosphatase/synthase (TPS) 9 (At1g23870; Leyman et al., 2001). A member of the same gene family (TPS1) is most probably involved in the initiation of flowering (Wahl et al., 2013). There is,
however, no direct link to the metabolic lesion in the mutant plant or to the putative signals involved. Likewise, the functional categories of genes not responding to HL in the double mutant are rather heterogeneous.

Further analyses of specifically regulated or non-regulated genes in the single mutant and the wild type are also presented in Supplementary Table S8 available at JXB online. Remarkably there were 87 genes specifically up-regulated in the tpt-2 mutant 48 h after LL/HL transfer. Of these genes, at least five genes were associated with Ca\(^{2+}\)-dependent signalling and four with glutathione-S-transferases. In wild-type plants, the expression of only a low number of genes was specifically altered (Supplementary Table S8M, N available at JXB online).

Are sugar-, phytohormone-, or oxidative stress-related signals involved in HL acclimation of A. thaliana?

The contribution of putative retrograde signals to global gene expression was assessed by comparing in-house with publicly available microarray data. Genes found to be HL responsive in the experimental set-up were compared with those genes regulated by elevated ‘sugar levels’, ‘oxidative stress’, or ‘hormones’ (for details, see Supplementary Tables S9 and S10 available at JXB online). Elevated sugar levels either were achieved by feeding A. thaliana seedlings with 3% Suc in light series (Michael et al., 2008) or were obtained endogenously in the pho3 mutant defective in SUT2, namely the Suc transporter responsible for phloem loading (Lloyd and Zakhleniuk, 2004). Oxidative stress based on singlet oxygen (\(^{1}\text{O}_2\)) and \(^{3}\text{O}_2\)-dependent products from lipid or carotenoid oxidation (Shapiguzov et al., 2013) can be observed in the flu mutant (op den Camp et al., 2003). The FLU protein inhibits an early step in tetrapyrrole biosynthesis and thereby prevents the accumulation of the Chl precursor protochlorophyllide (Meskauskiene et al., 2001). The application of the herbicide methyl viologen (MV) to A. thaliana leads to the accumulation of \(\text{H}_2\text{O}_2\) (A. Ditzer, D. Bartels, and H.-H. Kirch; NASCARRYS-143; op den Camp et al., 2003). The effect of the phytohormones ABA, indole acetic acid (IAA), the cytokinin trans-zeatin (tZ), gibberellic acid (GA\(_3\)), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and methyl jasmonate (MJ) on global gene expression has been studied by Goda et al. (2008).

The major outcome of the microarray comparisons is summarized in Table 3, as well as in Fig. 9 and in Supplementary Fig. S3 available at JXB online. Genes responsive to external Suc feeding or enhanced endogenous carbohydrate levels in the pho3 mutant represent a large portion of the commonly de-regulated genes shown in Fig. 9. Remarkably, the overlap of genes responsive to Suc feeding and in the pho3 mutant was rather low (i.e. two out of 81 genes at \(t_{4\text{h}}\) and four out of 70 genes at \(t_{48\text{h}}\)). Still, the direction of regulation (i.e. up or down) of HL- and sugar-responsive genes coincided to a high degree. More than 50% of the commonly up-regulated genes in the present experiments responded to sugars in the same way (Fig. 9). Furthermore, the log2 ratios of the individual data sets correlated directly with each other and resulted in Pearson correlation coefficients well above 0.7 (Table 3). There was no such coincidence or correlation with genes responding to phytohormones such as ABA (Fig. 9; Table 3). However, genes responding to MV-induced oxidative stress exhibited a

![Fig. 8. MapMan binning-based distribution of up- and down-regulated genes in individual functional categories 4 h (A) and 48 h (B) after LL/HL transfer. The reference value (light grey bars) refers to the number of genes belonging to a certain category expressed as a percentage of all genes on the microarray, whereas intermediate grey and dark grey bars refer to the number of up- or down-regulated genes belonging to a certain category as a percentage of all differentially regulated genes. Intermediate grey bars refer to all genes not regulated in adg1-1/tpt-2 (i.e. genes regulated in Col-0 and the single mutants) and dark grey bars refer to genes specifically regulated in adg1-1/tpt-2. Significant differences (P<0.05) in the relative distribution amongst the individual categories were estimated by Fisher’s exact test and marked by either asterisks or circles for intermediate grey or dark grey bars, respectively. (This figure is available in colour at JXB online.)](image)
Acclimation to high light

positive long-term correlation (i.e. 48 h after LL/HL transfer, Table 3), although the portion of ROS-induced genes was lower compared with $t_{4h}$ versus $t_0$ (Fig. 9). Interestingly, an entirely different approach undertaken by Oelze et al. (2012) used plants acclimated to extremely LL close to the light compensation point and their transfer to a 10- and 100-fold higher light intensity, respectively. Regulation of selected marker transcripts measured 6 h after transfer to HL suggested that metabolite, redox, and lipid-derived signals are particularly important in realizing the acclimation.

Furthermore, the positive correlation with MJ is based on the fact that parts of the sugar-responsive genes also respond to MJ (e.g. 30% of the genes differentially regulated in pho3). It appears likely, therefore, that elevated sugar levels and ROS rather than phytohormones such as ABA are the predominant factors involved in the acclimation response in A. thaliana. However, the sugar-dependent response is obviously independent from the constraints in the mutant. Analyses of differentially regulated genes in the individual lines, including wild-type plants, revealed higher contributions of phytohormones or ROS to the HL response (Table 3: Supplementary Fig. S3 available at JXB online). To increase the number of genes for these comparisons, the gene lists from Supplementary Tables S5 and S6 available at JXB online [i.e. Venn areas (a), (b), (c), and (d) depicted in Fig. 7A and B; log2 ratio ±1.0] rather than from Supplementary Table S8 available at JXB online (log2 ratio ±1.5) were used.

Surprisingly, in the tpt-2 single mutant, >25% of the highly up-regulated genes at $t_{48h}$ and >30% of the moderately down-regulated genes also responded to $^{1}O_2$ (Fig. 9E; Supplementary
Fig. S3C available at JXB online). At $t_{4h}$, there was a highly negative correlation between ROS- and HL-dependent gene expression in $tpt-2$ (Table 3). This overall lower responsiveness towards ROS correlates well with aberrant levels of redox components (Asc and DHA) observed in this mutant (compare Fig. 4B). Genes individually up-regulated in $tpt-2$ at $t_{4h}$ after LL/HL transfer exhibited a good correlation with genes responsive to GA$_3$. However, the total number of overlapping genes was low (eight genes). In wild-type plants, positive correlations of HL-responsive genes were found for sugars and MJ as well as for the phytohormone IAA, whereas tZ exhibited a negative correlation (Table 3). In the above cases as well as in the case of the negative correlation of HL- and sugar-responsive genes in the $adg1-1/tpt-2$ double mutant, the portion of total genes available for these analyses was rather low (Supplementary Table S10 available at JXB online).

**Soluble sugars have a major impact on HL acclimation in A. thaliana**

Metabolomics can help to identify retrograde signals (Caldana et al., 2012). Profiles of 47 metabolites distributed amongst three major categories such as sugars (12), amino acids (18), organic acids (10), and others (seven) have been measured for all plants in this study. The plants were either grown continuously in LL or HL or were subjected to a LL/ HL transfer and samples taken at $t_{4h}$ and $t_{48h}$. Figure 10 shows an overview of the metabolic profiling. More detailed data are contained in Table 4 and in Supplementary Tables S1, S11, and S12 available at JXB online. PCA (Fig. 10A) indicates that the first component of the metabolic profiles was separated based on the light conditions applied, whereas the second component was separated due to metabolic constraints in the respective mutants. Strikingly, for $adg1-1/tpt-2$ there was neither a large separation in the first component nor was the second component clearly separated from the wild type, again showing that the double mutant is compromised in HL acclimation. The static and dynamic assessments of metabolite heat maps (Fig. 10B) indicate (i) that at $t_0$ (i.e. in LL) the most pronounced increase exists for sugars, but not for amino acids or organic acids in $adg1-1$ versus Col-0 and $adg1-1/tpt-2$ versus Col-0. (ii) Upon transfer to HL, sugars and organic acids appear to be mostly decreased (most pronounced in $adg1-1/tpt-1$ versus Col-0), whereas amino acids show a trend of only a transient decrease (at $t_{4h}$ and $t_{48h}$), but an increase at continuous growth in HL. (iii) As expected, most metabolites were elevated in Col-0 and both single mutants, at least transiently, in HL compared with LL. Strikingly, (iv) differences in metabolite contents are less marked in $adg1-1/tpt-2$ grown in HL compared with LL conditions.

Table 4 shows that carbohydrate contents determined enzymatically or by GC-MC showed similar relative changes. Remarkably, Glc was the only soluble sugar that showed considerable variations in its content in $adg1-1/tpt-2$, in particular as a short-term response to LL/HL transfer (i.e. at $t_{4h}$). In contrast to $adg1-1/tpt-2$, Glc contents remained high or further increased in wild-type and single mutant plants 48h after LL/HL transfer and when grown continuously in HL.

It is hence tempting to speculate that a diminished Glc content in HL-grown $adg1-1/tpt-2$ might contribute to the lack of HL acclimation of the double mutant, a notion that is further supported by the rescue of the double mutant’s HL phenotype by feeding of Glc (Heinrichs et al., 2012; Schmitz et al., 2012). Like Glc, Fru and Suc accumulated in wild-type and single mutant plants (at least transiently; Table 4A, 4B), whereas there was no such increase in $adg1-1/tpt-2$. It recently could be demonstrated that diminished cytosolic Fru contents due to the deficiency of SWEET17, an A. thaliana
Fru exporter from the vacuole, resulted in plants with stunted growth (Chardon et al., 2013). Hence, besides Glc, Fru might probably also be considered as a signalling molecule. Moreover, glycine, proline, and myo-inositol showed a similar distribution pattern in adgl1-1/tpt-2 to Glc. Proline (Verslues and Sharma, 2010) and myo-inositol (Donahue et al., 2010) are involved in stress responses, and the latter is the structural basis for lipid signalling molecules (Valluru and van den Ende, 2011). Maltoolose contents were selectively increased in tpt-2 starting 48h after LL/HL transfer, but were close to the limit of detection in the starch-free background. Maltoolose results from starch degradation via β-amylase and thus supports the idea of daytime starch mobilization in the absence of the TPT. The complete list of metabolites (Supplementary Table S11 available at JXB online) was further evaluated by a static and dynamic assessment, similar to the microarrays, statistically analysed (Table 5 of Supplementary Document S1 available at JXB online) and listed in Supplementary Table S12 available at JXB online.

Conclusions

The genetic approach used here with mutants provides novel insight into the significance of carbohydrates and redox/ROS signalling in retrograde control of nuclear gene expression as a response of A. thaliana to HL exposure. Sugars are the first major products of photosynthesis and their contents are correlated with environmental conditions such as light and temperature. Even with an impaired day and night path of carbon export from the chloroplast in the adgl1-1/tpt-2 double mutant, some changes in Glc contents in response to HL were observed in the short term but not in the long term. In the group of commonly regulated genes, which respond to HL in the short term, a cross-talk of genes involved in carbohydrate and lipid metabolism exists. The impaired sugar-based long-term adaptation to HL observed in adgl1-1/tpt-2 most probably results in its severe growth and photosynthesis phenotype. To what extent severely down-regulated transcription factors such as MYC4 are involved in the lack of HL acclimation of adgl1-1/tpt-2 will be the subject of future experiments. Further elements, probably involved in the fine-tuning of the sugar response, have been identified amongst the genes which were either not or were specifically de-regulated in the single or double mutants. For instance, genes coding for heat shock proteins or genes that are involved in calcium signalling were specifically down-regulated in adgl1-1/tpt-2. Detailed analyses of candidate genes would hence be challenging in future research. Meta-analyses of transcriptome data such as, for instance, conducted by van Akelen and Whelan (2012) can also shed more light on the fine-tuning in retrograde signalling. The meta-analyses conducted here support the idea that carbohydrates play a pivotal role in HL acclimation of A. thaliana.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Correlation analyses of Lhcb1 expression levels, ETR, and contents of MgProtoIX, soluble sugars, and total carbohydrates.

Figure S2. Graphviz presentation of co-expression networks obtained with ATTEDII for data shown in Supplementary Table S7A and Supplementary Document S3.

Figure S3. Relative distribution of differentially regulated genes in publicly available microarray experiments compared with in-house expression data for Col-0 (A), tpt-2 (B), adgl1-1 (C), and adgl1-1/tpt-2 at t4h and t48h after LL/HL transfer.


Document S2. Detailed description of the static assessment of global gene expression data after LL/HL transfer between wild-type and mutant plants.

Document S3. Detailed analysis of commonly regulated genes following the dynamic assessment of global gene expression after LL/HL transfer between wild-type and mutant plants.

Table S1. Overview of the metabolite reporter list.

Table S2. Static comparison of global gene expression in adgl1-1, tpt-2, and adgl1-1/tpt-2 versus Col-0 at t0 before LL/HL transfer.

Table S3. Static comparison of global gene expression in adgl1-1, tpt-2, and adgl1-1/tpt-2 versus Col-0 at t4h after LL/HL transfer.

Table S4. Static comparison of global gene expression in adgl1-1, tpt-2, and adgl1-1/tpt-2 versus Col-0 at t48h after LL/HL transfer.

Table S5. Dynamic comparison of global gene expression in Col-0, adgl1-1, tpt-2, and adgl1-1/tpt-2 at t0 before LL/HL transfer versus t0.

Table S6. Dynamic comparison of global gene expression in Col-0, adgl1-1, tpt-2, and adgl1-1/tpt-2 at t4h after LL/HL transfer versus t0.

Table S7. Filtered list of significantly (P<0.01) altered genes in the central overlapping regions of the Venn diagrams shown in Fig. 7A and B.

Table S8. List of genes specifically regulated or not regulated in the individual lines. The gene lists are based on the Venn diagrams shown in Fig. 7.

Table S9. Comparison of HL-responsive genes within the group of commonly regulated genes (Supplementary Table S7) with publicly available microarray data on genes responding to elevated sugars, oxidative stress, or the phytohormones ABA, indole acetic acid (IAA), trans-zeatin (tZ), gibberellic acid (GA3), the ethylene precursor ACC, and methyl jasmonate (MJ).

Table S10. Comparison of HL-responsive genes within the group of individually altered genes (Supplementary Table S8) with publicly available microarray data on genes responding to elevated sugars, oxidative stress, or the phytohormones ABA, indole acetic acid (IAA), trans-zeatin (tZ), gibberellic acid (GA3), the ethylene precursor ACC, and methyl jasmonate (MJ).

Table S11. Contents of metabolites determined by GC-MS in leaves of (A) Col-0, (B) adgl1-1, (C) tpt-2, and (D) adgl1-1/tpt-2 grown either continuously in LL or HL or after transfer from LL to HL at t4h or t48h.
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References


Acclimation to high light


