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Kinetics of retrograde signalling initiation in the high light response of *Arabidopsis thaliana*

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High light acclimation depends on retrograde control of nuclear gene expression. Retrograde regulation uses multiple signalling pathways and thus exploits signal patterns. To maximally challenge the acclimation system, *Arabidopsis thaliana* plants were either adapted to 8 (low light (L-light)) or 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (normal light (N-light)) and subsequently exposed to a 100- and 10-fold light intensity increase, respectively, to high light (H-light, 800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$), for up to 6 h. Both L \rightarrow H- and N \rightarrow H-light plants efficiently regulated CO_2 assimilation to a constant level without apparent damage and inhibition. This experimental set-up was scrutinized for time-dependent regulation and efficiency of adjustment. Transcriptome profiles revealed that N-light and L-light plants differentially accumulated 2119 transcripts. After 6 h in H-light, only 205 remained differently regulated between the L \rightarrow H- and N \rightarrow H-light plants, indicating efficient regulation allowing the plants to reach a similar transcriptome state. Time-dependent analysis of transcripts as markers for signalling pathways, and of metabolites and hormones as possibly involved transmitters, suggests that oxylipins such as oxophytodienoic acid and jasmonic acid, metabolites and redox cues predominantly control the acclimation response, whereas abscisic acid, salicylic acid and auxins play an insignificant or minor role.

1. Introduction

Acclimation to changing environmental conditions optimizes plant growth and fitness. Plants encounter biotic and abiotic stress if such changes occur suddenly and with large amplitude. Stress is defined as pronounced deviation from optimum growth condition and is caused by direct effects of the stressor or as indirect consequence of interference of the stressor with metabolism. The acclimation response must be specific in order to react in an appropriate manner and to preserve resources. This specificity is realized by signal integration in signalling cascades that deploy distinct input signals.

High light (H-light) conditions trigger over-reduction of the photosynthetic electron transport (PET) chain with subsequent formation of reactive oxygen species (ROS) [1]. Non-photochemical quenching (NPQ) reduces light-driven electron pressure and ROS generation at photosystems PSII and PSI [2,3]. Singlet oxygen is generated at PSII and superoxide anions at PSI [4,5]. Detoxification of ROS counteracts redox imbalances and maintains plant fitness. In the chloroplast, superoxide dismutase catalyses the synthesis of H_2O_2 and O_2 from two molecules of O_2^- . H_2O_2 is detoxified by the ascorbate-dependent pathway using ascorbate peroxidase (Apx) [6,7] or the ascorbate-independent pathway using thiol oxidases such as peroxiredoxins [8,9].

Acclimation to H-light or excess light has been studied in quite some detail owing to its ecological significance, but also because it is experimentally straightforward with a precisely defined starting point. Transcripts of *Apx 1* and

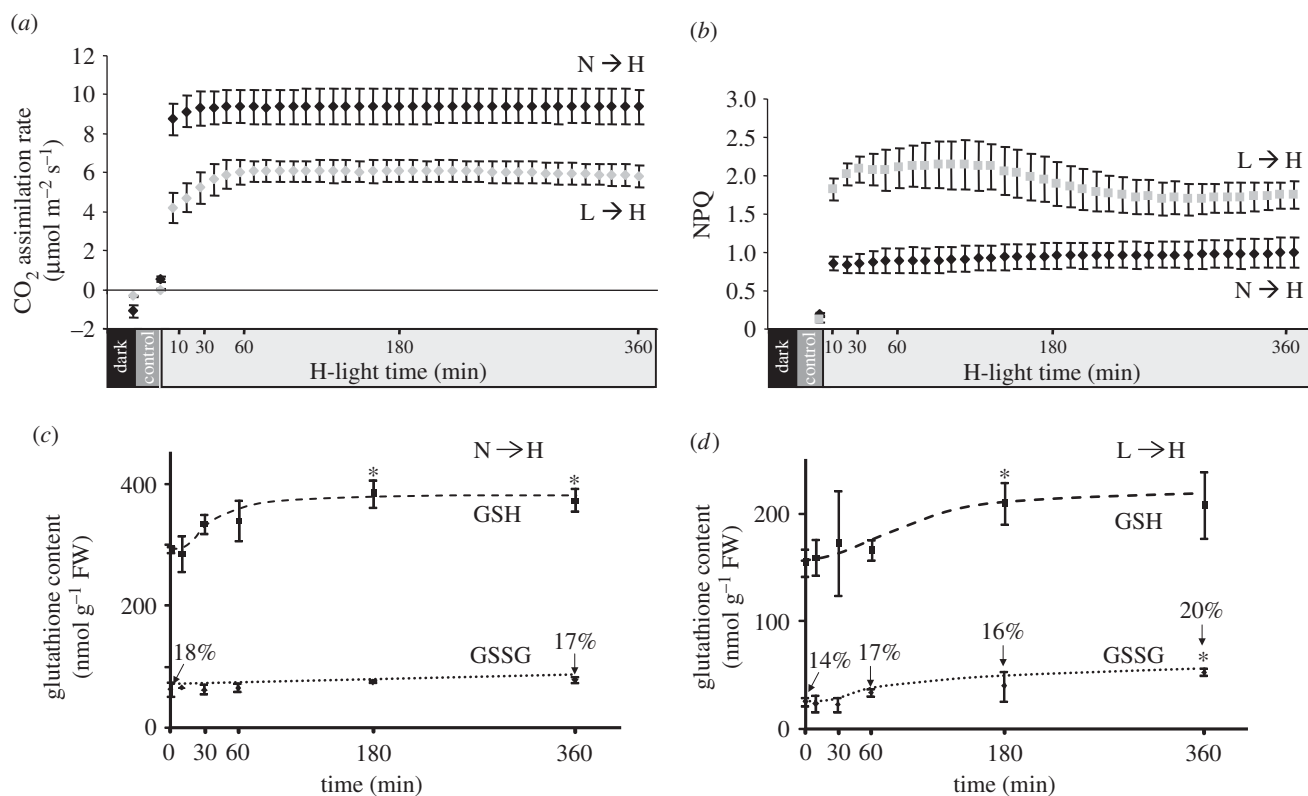


Figure 1. Time kinetics of photosynthetic parameters and the glutathione pool following the shift to H-light. (a) CO₂ assimilation rate and (b) non-photochemical quenching (NPQ) were determined in L-light and N-light plants after transfer to H-light. Prior to the measurement, plants had been dark-acclimatized for 30 min. (c,d) Reduced glutathione (GSH) and oxidized glutathione (GSSG) were quantified in (c) (N → H)- and (d) (L → H) plants, respectively. Data are means ± s.d. of *n* = 3 independent experiments. In the case of glutathione, asterisks indicate significant differences to the controls, Student's *t*-test, *p* < 0.05.

Apx 2 are upregulated within short time periods [10]. Systemic signalling is indicated by regulation of *Apx* in shaded distant leaves [11]. ROS amounts determine whether ROS act as signalling molecules or react with sensitive cell components, e.g. proteins, DNA or cell walls [12]. In addition, all chloroplast antioxidative enzymes are encoded in the nucleus [13] and have to be transported to the organelles [14]. The separation of the sites of ROS production and nuclear gene expression reinforces the need for retrograde signalling for information exchange to optimally balance gene activity with chloroplast metabolic state [15–19]. In contrast to earlier concepts that assumed the participation of only few signalling pathways, nowadays a signalling pattern is discussed to indicate retrograde signalling. Among the interesting cues are abscisic acid (ABA) [20], jasmonic acid (JA) [21], H₂O₂ [22,23] and ¹O₂ [24], the PET redox state, including the plastoquinone pool [25,26], and associated components such as STN7 [27]. Precursors that are conditionally synthesized in the chloroplast and afterwards transferred to the cytosol or other compartments such as xanthoxin for ABA synthesis [28] or oxophytodieneic acid (OPDA) for JA synthesis in the peroxisome [29] might be good candidates for signal transmission. Up to now, a 'plastid factor' as a distinct signalling molecule with a master function in retrograde signalling has not been identified. Thus, retrograde signalling more likely is recognized as a complex network of fine-tuned mechanisms with crosstalk and different pathways of signal transmission [30].

In a previous study, *Arabidopsis* (*A.*) *thaliana* was grown under normal light (N-light) or acclimatized to low light (L-light), and both transferred to high light (H-light) that corresponded to a 10-fold or 100-fold light shift. Analyses were performed after 6 and 24 h. The transcript abundances of the water–water cycle enzymes were highly similar 6 h after

transfer, irrespective of whether plants were subjected to a 10-fold or 100-fold shift, despite extremely different starting points from L- or N-light acclimatized conditions [31]. To understand the dynamic nature of acclimation, we focused our research on the involvement of possible signalling pathways, the time-resolved kinetics of responses and the degree of acclimation at the global transcript scale.

2. Results

H-light acclimation proceeds in various steps and on different time scales. In the first step, light-dependent photochemical and metabolic events are converted into signalling cues. As basic characterization, we measured CO₂ assimilation, quantum yield of PSII, photochemical quenching (qP) and NPQ in a time period between 0 and 6 h after transfer to H-light [31]. The CO₂ assimilation rate increased rapidly within 10 min and reached a constant level after 30 min in the N → H-light transfer, and after 60 min in the L → H-light transfer. The rates per leaf area were about 30% lower in the previously L-light acclimatized plants, but were constant until the end of the measuring time at 6 h (figure 1a). Apparently, neither plant suffered severe inhibition. Likewise, the photosynthetic yield declined as expected directly after the H-light shift, but remained mostly constant during the subsequent 6 h (see electronic supplementary material, figure S1). The yield and qP were remarkably lower after the L → H shift, accordingly NPQ was higher for the L-light plants (figure 1b). Constant values were measured for the N → H-light plants, whereas there was a peak between 60 and 120 min in the L → H plants, indicating some relaxation of photoinhibition to maintain constant CO₂-fixation rate.

Reduced and oxidized ascorbate (ASC/DHA) and glutathione (GSH/GSSG) are metabolites with functions as antioxidants, cosubstrates and regulators [6,7]. Their levels provide a basic characterization of cell redox state. Fresh-weight related GSH levels of N-light plants exceeded that of L-light plants by a factor of 3.5. Following the N → H shift and after a short delay, GSH increased by about 20%, which at $t = 180$ and 360 min was significantly different from $t = 0$ min. GSSG levels also increased slightly with time, thus the GSH/GSSG ratio remained unaffected (figure 1c). In a converse manner, GSSG increased more than GSH after L → H shift, and thus the GSH/GSSG ratio increased from 14 to 20%. Total ascorbate levels were more than twofold higher in N-light plants compared with L-light plants. After N → H shift, only a slight increase until $t = 30$ min could be detected. The proportion of DHA remained constant at 31% (see electronic supplementary material, figure S1c), similar to L → H shifted plants. Both ASC and DHA levels increased in L → H plants towards the end of the experiment (see electronic supplementary material, figure S1d).

Transcriptome profiling was intended to define the acclimation state after 6 h at a global scale. Thus, leaves from four conditions were harvested at 15.00, 6 h after the onset of H-light, namely L-, L → H, N- and N → H-light plants. Overall, approximately 12 times more genes were upregulated in their expression after the 100-fold light shift compared with the 10-fold light shift (see electronic supplementary material, figure S2a), and the number of downregulated genes was even 20-fold higher in the 100-fold shift (see electronic supplementary material, figure S2b). Categorization in functional groups with MAPMAN software [32] (see electronic supplementary material, figure S2e,f) revealed that most upregulated transcripts belong to the GO group of nucleic acid and protein synthesis. Surprisingly, stress-dependent genes were upregulated in N → H plants more than in L → H plants. The first analysis focused on 67 genes involved in ROS detoxification [33] and compared the transcript levels before the light-shift (N/L), during the light-shift (N → H/N, L → H/L; both compared with the untreated controls) and after the light shift (N → H/L → H; electronic supplementary material, figure S3). The transcript levels of water–water cycle enzymes were higher in N-light compared with L-light plants and both showed an upregulation after 6 h of H-light treatment. In comparison, *NADPH oxidases* showed mostly no changes in the transcript levels. A downregulation of the transcript amount was observed for *cat3* and most *glutathione peroxidases* during both shifts. Overall, only small differences in transcript levels of the ROS network were detected 6 h after the light shift of L-light and N-light plants.

Reference genes were selected from literature dealing with retrograde signalling and possible messenger molecules. The transcript abundances of these genes were assumed to give hints to involved retrograde signalling pathways after 6 h at H-light (figure 2). Only minor differential transcript accumulation was detected for selected marker genes of plastoquinone- and singlet oxygen-dependent signalling such as *PetE* and *Bap1*, respectively. Strongest regulation occurred for markers of sugar- and metabolite-signalling such as *β-amylase* and *chalcone synthase*, and also for oxylipin-dependent signalling as indicated by *lipoxygenase LOX3*. Some upregulation was seen for salicylic acid (SA) and ascorbate markers. Unexpectedly, considerable downregulation was observed for marker genes of ABA-, systemic and phytochrome-dependent signalling. At the end of the 6 h period of H-light acclimation, only

19 of the 64 transcripts were still differentially accumulated between former L-light and N-light plants; 12 were higher in N → H compared with L → H plants.

Five selected markers were analysed in detail to gain insights into the kinetics of regulation. *Monodehydroascorbate reductase (MDHAR, At1g63940)* as marker for the water–water cycle (figure 3a) doubled within 3 h after the L → H transfer, whereas little transcriptional change occurred after the N → H shift. The transcript level of the ABA-dependent reference gene *COR47 (At1g20440)* (figure 3b) was downregulated after the L → H transfer and reached the level of the N → H-shifted plants. The transcript of the H₂O₂-dependent marker *PKRP (At3g49160)* was twofold upregulated upon both light shifts (figure 3c). Interestingly, the response was delayed in both plants by about 30 min, and the rate of increase was slower in the L → H plants. The *palmitoyl protein thioesterase family protein (PPTE, At4g17470)* used as reference transcript for oxylipin signalling accumulated with ongoing H-light exposure and reached a greater than 20-fold (N → H) or greater than 30-fold (L → H) higher level in comparison with N- or L-light plants, respectively (figure 3d). The transcript level of the sugar marker *β-amylase (At4g15210)* increased more than 40-fold after the 10-fold shift but only approximately fivefold upon the 100-fold shift (figure 3e).

Phytohormones have been suggested to be involved in light acclimation [15,30,45]. Therefore, phytohormone levels were determined in order to identify candidate signals and to mechanistically link or validate our observations. OPDA, JA and its conjugate jasmonyl-isoleucine (JA-Ile) act as signalling molecules to stress [52,53]. Their concentration changed upon transfer to H-light with distinct kinetics and amplitude (figure 4a–c). Upon L → H transfer, OPDA, JA and JA-Ile increased until 60 min. Subsequently, JA and JA-Ile decreased again, whereas OPDA remained at a twofold higher level. In the N → H light experiment, OPDA increased fast, followed by JA and JA-Ile. In comparison, the JA antagonist SA [54] showed no significant regulation; if at all, there was a trend to decrease which will be important for the discussion (figure 4d). ABA plays a crucial role in control of development and stress acclimation [55,56]. The ABA level was unchanged during the first 30 min following the transfer. A significant increase by more than twofold was measured in N → H plants (figure 4e). Indole-3-acetic acid (IAA) levels were unaffected by H-light despite its connection to ROS [57,58] (figure 4f). The minor contribution of ROS signalling was confirmed by comparing the transcriptional regulation in our experiment with published ROS-related arrays using the ROSMETER [59] (see electronic supplementary material, figure S4). The heat map (please note that the colour scale covers only –0.4 to 0.4) indicates that the transcriptional differences between N/L, N → H/N and L → H/L were alike and that they were slightly coregulated with transcripts linked to H₂O₂ and singlet oxygen, but these differences were levelled in the N → H/L → H comparison as described above. It is also noteworthy that a negative correlation was observed with signatures derived from mitochondrial ROS.

3. Discussion

The experimental set-up of shifting plants to the same H-light condition from different preconditioning states was recently designed to maximally challenge the acclimation machinery

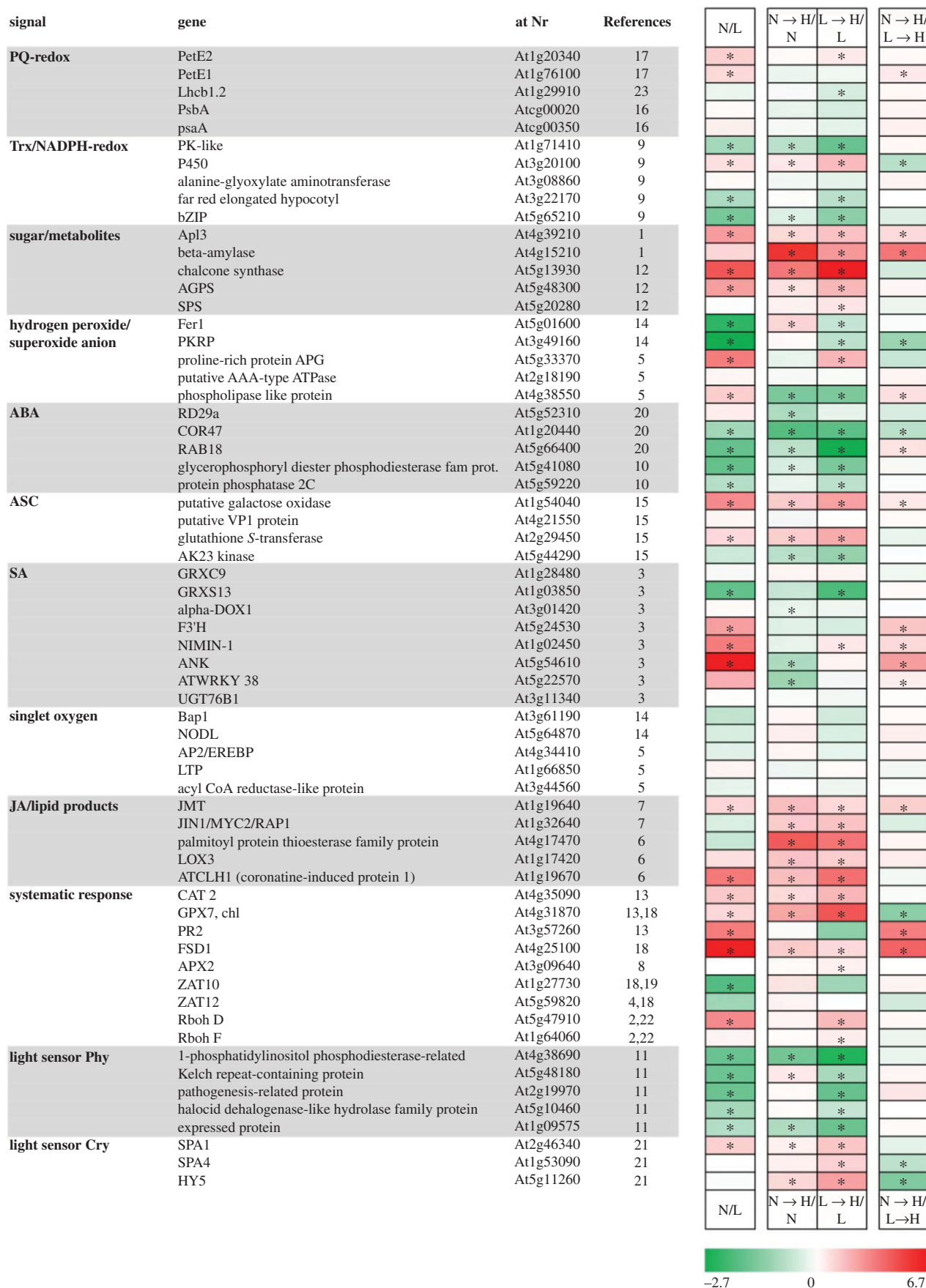


Figure 2. Transcript amounts of genes selected as markers for signalling pathways. Sixty-four reference genes known to respond to specific signalling cues were selected from the literature in order to determine their transcript amounts after 6 h of H-light treatment. Comparison before the light shift (N/L), after the light shift (N → H/N, L → H/L) and between the H-light treatments (N → H/L → H) was based on the data from the ATH1 whole genome microarray. \log_2 -fold; green, downregulation; red, upregulation; asterisks indicate significant differences, Student's *t*-test, $p < 0.005$, $n = 3$. PQ, plastoquinone; Trx, thioredoxin. References for selected marker genes: 1: Baier *et al.* [34]; 2: Bechthold *et al.* [35]; 3: Blanco *et al.* [36]; 4: Davletova *et al.* [37]; 5: Gadjev *et al.* [38]; 6: Goda *et al.* [39]; 7: cited in Goda *et al.* [39]; 8: Karpinski *et al.* [11]; 9: Lintala *et al.* [40]; 10: Matsui *et al.* [41]; 11: Mazella *et al.* [42]; 12: Muller *et al.* [43]; 13: Mullineaux *et al.* [44]; 14: op den Camp *et al.* [24]; 15: Pastori *et al.* [45]; 16: Pfanschmidt *et al.* [46]; 17: Pfanschmidt *et al.* [47]; 18: Pogson *et al.* [17]; 19: Rossel *et al.* [25]; 20: Sanchez *et al.* [48]; 21: Sellaro *et al.* [49]; 22: Torres *et al.* [50]; 23: Yang *et al.* [51]. (Online version in colour.)

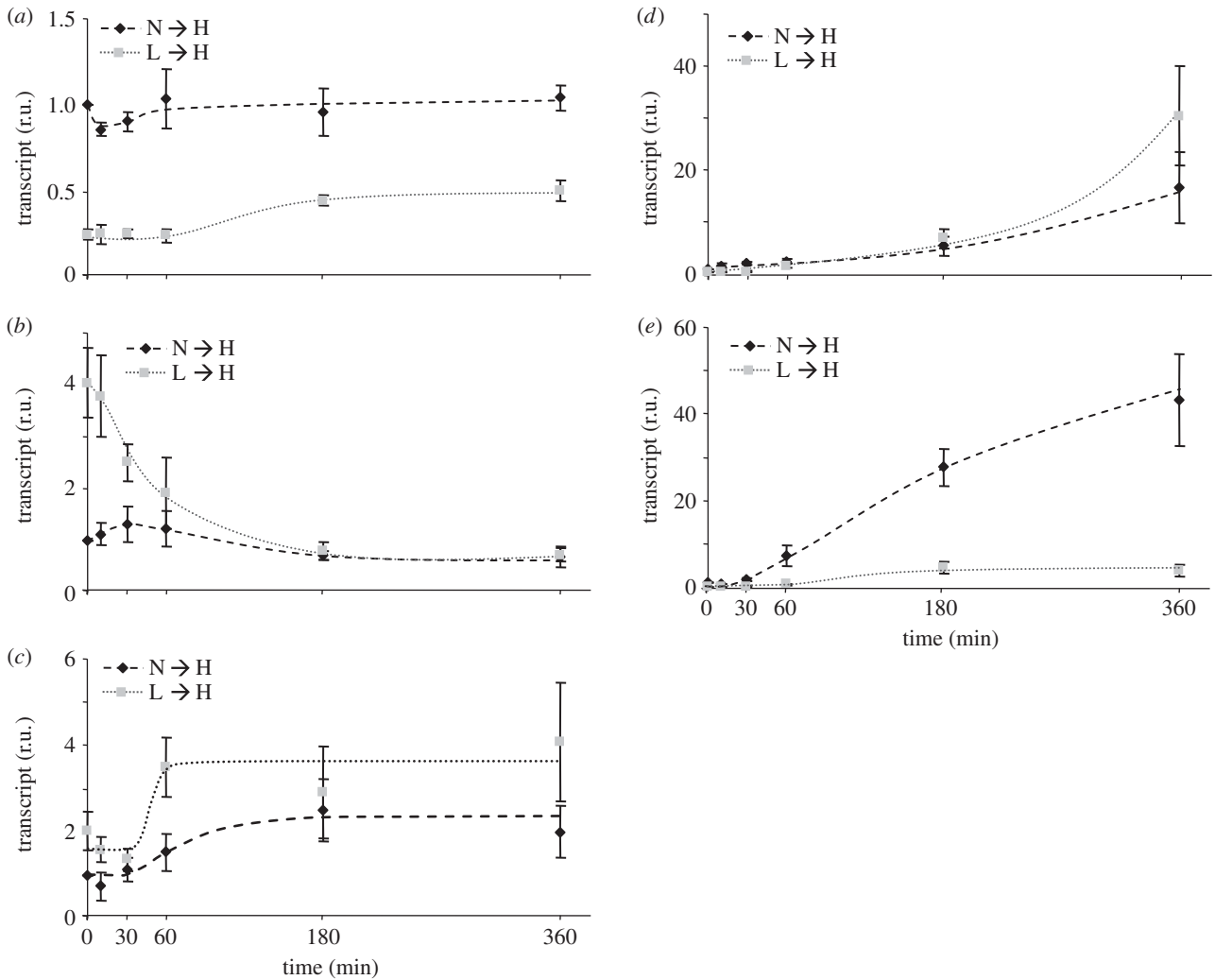


Figure 3. Time-resolved abundances of marker transcripts. Reference transcripts were quantified following the N \rightarrow H- or L \rightarrow H shift by qPCR (a) *monodehydroascorbate reductase (MDHAR)*, (b) ABA-dependent *cold regulated 47 (COR47)*, (c) ROS-dependent *pyruvate kinase related protein (PKRP)*, (d) oxylipin-indicating *palmitoyl protein thioesterase family protein (PPTE)* and (e) the sugar marker β -*amylase*. Data are means \pm s.d. of $n = 3$ independent experiments, each with replicates.

of leaves [31]. L-light plants growing in less than $10 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ for 10 days are completely shade-acclimatized with only 25% electron transport capacity of N-light plants. Focus of the previous study was directed to regulation of the enzymes of the water–water cycle at the levels of transcript, protein, enzyme activity and metabolite in N \rightarrow H and L \rightarrow H plants at 0, 6 and 24 h. The observed remarkably high and efficient plasticity prompted us to direct our attention to a time-resolved analysis. Here, it is shown that the plants reached and maintained constant CO_2 fixation rates in H-light within 60 min. This result in combination with the kinetics of NPQ and qP confirmed that activation of quenching mechanisms [60] was perfectly tuned to maintain efficient gross photosynthesis while avoiding damage development.

(a) Conditional acclimation after 6 h of high light treatment

Transcriptome analysis after 6 h H-light revealed differential transcript accumulation of approximately 7.5% in N \rightarrow H plants and 20% in L \rightarrow H plants, respectively. The high number of differentially accumulated transcripts by far exceeded that of paraquat-treated *flu* mutants [24] or H_2O_2 -treated *A. thaliana* wild-type [37] and indicates a profound reprogramming of L-light plants. The set of differentially

(twofold or more) regulated transcripts between N- and L-light plants comprised 2219 before the H-light transfer and had decreased to 205 genes between N \rightarrow H and L \rightarrow H plants at $t = 6$ h. Apparently, the global transcriptome adjustment of L-light plants upon H-light treatment occurred extremely efficiently to a similar state as in N-light plants. In contrast to previously analysed redox- and ROS-related transcriptomes [33], here also a large set of transcripts among the antioxidant network was upregulated. This indicates a role of ROS in response regulation or the preparatory activation of a programme for improved ROS scavenging in dependence on light. Also, in the ROS network, the majority of the significant differences were minimized after 6 h, leading to the hypothesis that ROS-dependent signalling does not play a major role 6 h or more after L \rightarrow H transfer [31]. However, ROS signalling might contribute to H-light response regulation at earlier time points as suggested previously [10,11]. This is in line with the upregulation of the ROS marker *PKRP* [24] between 30 and 60 min. The upregulation occurred faster in N \rightarrow H than in L \rightarrow H plants (figure 3). The difference in response speed tentatively correlated with lower expression of *RbohD* and *RbohF* in L-light plants (figure 2). Likewise, the levels of markers for cell signalling (figure 2) were highly similar between N \rightarrow H and L \rightarrow H plants, completing the picture of efficient transcriptional acclimation of *A. thaliana* to H-light.

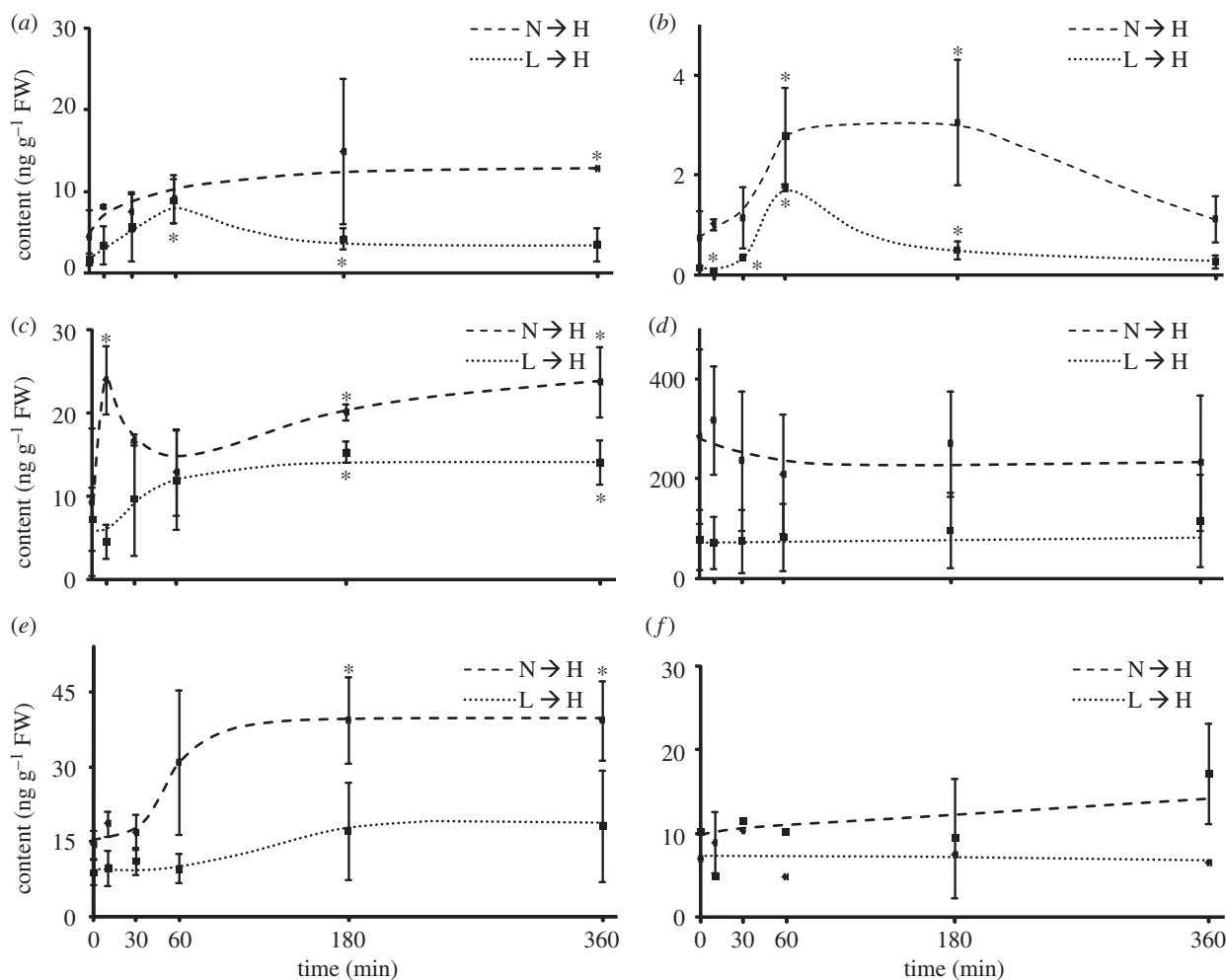


Figure 4. Changes in phytohormone contents in plants transferred to H-light as quantified by LC–MS/MS. (a) Jasmonic acid (JA) and (b) jasmonyl-Ile, (c) 12-oxophytodienoic acid (OPDA), (d) salicylic acid (SA), (e) abscisic acid (ABA) and (f) indole-3-acetic acid (IAA) were quantified in a time kinetics after transfer to H-light. Data are means \pm s.d. of $n = 3$ independent experiments (IAA $n = 2$), asterisks indicate significant differences to the controls, Student's *t*-test, $p < 0.1$.

(b) Involvement of oxylipins and redox regulators

Up-to-date concepts on retrograde control of nuclear gene expression assume the activation of different signals that combine to a specific pattern which in turn optimizes the acclimation response [15,17,30]. This study presents the kinetic dissection of (i) marker transcript regulation, and (ii) the accumulation of metabolite signals and phytohormones. Except for JA and OPDA, ASC and GSH, which increased readily in N → H plants, all other markers showed a delay of at least 30–60 min. OPDA is synthesized from α -linolenic acid in the chloroplast and exerts signalling function independent of JA and JA-Ile regulation [61,62]. Following binding to cyclophilin 20-3, the Cyp20-3/OPDA complex activates cysteine synthesis, increases thiol levels and reduction state and induces defence gene expression [53]. Accumulation of GSH may be explained by this regulatory mechanism and could be part of a general acclimation response. In line with this result, *A. thaliana* deficient of Cyp20-3 revealed a H-light sensitive phenotype [63]. Furthermore, OPDA-triggered signalling involves activation of TGA transcription factors [64]. The upregulation of *PSTE*, a reference gene for oxylipin signalling [39], might be the consequence of higher OPDA levels after the H-light transfer.

The JA-antagonist SA decreased during N → H and remained constant during L → H, which argues against SA as transmitter in H-light acclimation. SA suppresses JA signalling [65] by inhibition of the E3 ubiquitin-ligase SCF (COI1) which is

required for JA signalling [66]. Suppression of SA may enable oxylipin signalling in the H-light response [54,67]. RBOHD/F activity, ROS accumulation and SA control systemic H-light acclimation and the cell death programme [68]. The lack of up-regulation in the L → H transfer experiments indicates that retrograde signalling can use different signalling patterns.

In N → H plants, ABA increased to about twice the control level after 3 h. The trend to increase was also seen in L → H plants, but was insignificant and occurred with a slight delay. Surprisingly, an anti-parallel response could be detected between ABA-regulated *COR47* [48] and ABA amount. The discrepancy could be explained by alkaline trapping of deprotonated ABA in the stroma in H-light as modelled by Slovik & Hartung [69] and discussed before [31]. As a consequence of ABA anion accumulation in the alkaline stroma, the effective ABA concentration in the nucleoplasm and cytosol could be low despite the overall increase in ABA accumulation of the leaves (figure 4e).

Acclimation to H-light involves diverse mechanisms at different time scales such as rapid photoinhibition and antioxidant expression, and long-term acclimation, including adjustment of chlorophyll synthesis [3,10,70,71]. The complex acclimation process, here underscored by reorganization of 20% of the transcriptome in L → H plants, suggests that no single and exclusive pathway mediates the response. ROS [10], SA [68], ABA [72], redox signals [46] and others have been suggested to mediate the acclimation. Our study provides

a detailed inventory after 6 h following the N → H and L → H transfer and a framework of the timing of signalling events. The increase in oxylipin levels was among the fast responses followed by an increase in GSH levels in the N → H plants. The glutathione increase was delayed in L-light acclimatized plants, possibly owing to resource limitation and starvation. Likely, L-light plants build up cell constituents such as carbohydrates and proteins before investing in defence. This is also indicated by the delayed upregulation in L → H plants of *β-amylase*, a reliable sugar marker [34]. Interestingly, also the ROS marker *PKRP* revealed the faster and more intense response in the N → H plants. SA, ABA and IAA appear to play no major role in our experimental system. We conclude from this study that oxylipins, metabolites and redox cues, but possibly less ROS, should be investigated in the future to understand rapid processes in H-light acclimation.

4. Methods

(a) Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (wild-type) was grown as described in [31] (10 h light with 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, and 21°C/14 h dark and 18°C, 50% relative humidity). After three weeks, plants were either transferred to 8 (low light, L-light) or kept at 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (normal light, N-light) for 10 days. Plants (4.5 week old) were transferred to high light (800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, H-light) for time periods as indicated, or remained in L- or N-light. Rosettes were harvested at 15.00 (± 30 min), frozen in liquid nitrogen and stored at -80°C .

(b) Assimilation rate and photosynthetic parameters

Assimilation rate, PSII quantum yield, qP and NPQ were determined with the gas exchange fluorescence system GFS-3000 according to the manufacturer's instructions [31] (Walz, Germany) with plants acclimatized to darkness for 30 min. Following 10 min at 8 and 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ for L- and N-light controls, respectively, the light intensity was increased to 800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ for H-light treatment.

(c) Whole genome ATH1 microarray

Total RNA was isolated using RNeasy mini kit (Qiagen). KFB (Regensburg, Germany) was commissioned for hybridization of the Affymetrix ATH1 genome array. Each condition was hybridized three times from independent experiments. Microarray data were imported into ROBIN (MPI Golm, Potsdam, Germany) and normalized according to the RMA algorithm [73,74]. Differentially regulated subsets were defined as those with twofold or greater difference and an adjusted *p*-value cut-off of less than

or equal to 0.005 [75,76]. The gene list was imported into the MAPMAN IMAGEANNOTATOR 1.6.3 [32] to analyse pathways. The transcriptome data were analysed for ROS signatures by the ROSMETER according to Rosenwasser *et al.* [59] as implemented in the bioinformatics tool that is available at http://app.agri.gov.il/NOA/ROS_desc.php.

(d) Transcript profiling

RNA isolation and cDNA synthesis were performed as by Wormuth *et al.* [77], and quantitative real-time PCR (qPCR) as by Oelze *et al.* [31] with the single difference of using MESA blue MasterMix plus SYBR assay (Eurogentec, Belgium). *Actin2* (At3g18780) was used as reference gene.

(e) Ascorbate and glutathione content

Reduced (ASC) and oxidized ascorbic acid (DHA) were determined as by Horling *et al.* [78]. For the determination of reduced and oxidized glutathione (GSH/GSSG), an enzyme cycling assay based on sequential oxidation of GSH by 2,2'-dinitro-5'-dithiodibenzoic acid (DTNB) and reduction by NADPH in the presence of glutathione reductase was performed as by Oelze *et al.* [31].

(f) Analysis of oxylipins, salicylic acid, indole acetic acid and abscisic acid in *Arabidopsis thaliana*

The analysis of hormones was performed according to Stingl *et al.* [79]. Plant material was extracted with 950 μl ethyl acetate/formic acid (99:1, v/v). Dihydrojasmonic acid (50 ng), JA-nor-Val (50 ng), [$^{18}\text{O}_2$]OPDA (50 ng), [D_6]ABA (50 ng), [D_4]SA (50 ng) and [D_2]IAA (50 ng) were added as internal standards. Samples were homogenized with a ball mill for 3 min at 20 Hz. Pellets following centrifugation were re-extracted, and the pooled supernatants were dried in a vacuum concentrator. After evaporation of the solvent, samples were dissolved in 40 μl acetonitrile/water (50:50, v/v) and analysed by liquid chromatography (LC)–tandem mass spectrometry (MS/MS).

A. thaliana ID numbers: *Actin* (At3g18780), *β-amylase* (At4g15210), *COR47* (At1g20440), *MDHAR* (At1g63940), *PKRP* (At3g49160), *PPTE* (At4g17470).

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