Light-harvesting mutants show differential gene expression upon shift to high light as a consequence of photosynthetic redox and reactive oxygen species metabolism

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The amount of light energy that is harvested and directed to the photosynthetic machinery is regulated in order to control the production of reactive oxygen species (ROS) in leaf tissues. ROS have important roles as signalling factors that instigate and mediate a range of cellular responses, suggesting that the mechanisms regulating light-harvesting and photosynthetic energy transduction also affect cell signalling. In this study, we exposed wild-type (WT) Arabidopsis and mutants impaired in the regulation of photosynthetic light-harvesting (stn7, tap38 and npq4) to transient high light (HL) stress in order to study the role of these mechanisms for up- and down-regulation of gene expression under HL stress. The mutants, all of which have disturbed regulation of excitation energy transfer and distribution, responded to transient HL treatment with surprising similarity to the WT in terms of general ‘abiotic stress-regulated’ genes associated with hydrogen peroxide and 12-oxo-phytodienoic acid signalling. However, we identified distinct expression profiles in each genotype with respect to induction of singlet oxygen and jasmonic acid-dependent responses. The results of this study suggest that the control of excitation energy transfer interacts with hormonal regulation. Furthermore, the photosynthetic pigment–protein complexes appear to operate as receptors that sense the energetic balance between the photosynthetic light reactions and downstream metabolism.

1. Introduction

The fluency of photosynthetic electron transfer reactions (i.e. the redox state of the electron transfer chain; ETC) can affect the level of reactive oxygen species (ROS) produced both by excited chlorophylls and by reduction of molecular oxygen. Blockage in electron transfer slows the photochemical quenching of excited chlorophylls, potentially leading to production of singlet oxygen ($^1$O$_2$). Additionally, inhibition of the free flow of electrons to acceptors (i.e. to stromal redox enzymes, NADP$^+$ or carbon dioxide) or saturation of electron acceptors can lead to reduction of molecular oxygen, resulting in the production of superoxide and hydrogen peroxide (H$_2$O$_2$; for reviews, see [1,2]).

Under excess light, energy conversion efficiency is downregulated in order to keep photosynthesis within the capacity of energy-consuming metabolism and to minimize the production of ROS. This regulation is based on two factors; the redox state of the ETC and the proton gradient across the thylakoid membrane ($\Delta$pH). The distribution of energy from light-harvesting complex II (LHCII) between photosystem I (PSI) and photosystem II (PSII) is regulated by reversible phosphorylation of several LHCII proteins by STN7 kinase [3,4] and TAP38/PPH1 phosphatase [5,6], according to the redox state of the ETC (for reviews, see [7,8]). This distribution mechanism is coupled...
with the ΔpH-dependent regulation of light-harvesting efficiency (for review, see [9]), which relies on the PSBS protein for rapid thermal dissipation of excess excitation energy [10].

Interaction between LHCII phosphorylation and thermal dissipation can be disturbed by knocking out the genes encoding the responsible proteins [11]. The psbs mutant over-exites photosystems under high light (HL), leading to a high reduction state of the plastoquinone (PQ) pool [12,13]. The lack of the PSBS protein also increases the lifetime of excited chlorophylls in the light-harvesting system [14], increasing the production of triplet chlorophylls that are able to react with molecular oxygen and produce $^{1}\text{O}_2$. Lack of PSBS also amplifies and disturbs the light intensity-dependent changes in thylakoid protein phosphorylation [15], which may be associated with a breach of the LHCII system [11] and generation of unconnected and unquenched LHCII fractions in the psbs mutant under HL. In the tap38/pph1 mutant, PSI excitation is favoured and thus the PQ pool remains more oxidized than in the wild-type (WT) [5,6], as opposed to the highly reduced ETC in npq4. Excitation imbalance in tap38 is emphasized under HL where LHCII is not dephosphorlated as it is in the WT. Similar to the situation in npq4, hyperphosphorylation of thylakoid proteins in tap38 under HL leads to the breakdown of PSI–LHCII complexes and severe problems in the transfer of sufficient excitation energy to PSI (NR Mekala, M Tikkanen, E-M Aro 2013, unpublished data). It is likely that this abrogates pigment–protein complex connectivity and enhances the production of $^{1}\text{O}_2$. The stn7 mutant is missing LHCII phosphorylation that is required for efficient energy transfer from LHCII to PSI under low light [3,4], but the lack of PSI excitation is compensated in stn7 by an increase in the amount of PSI [13,16]. In HL, stn7 plants have similar light-harvesting properties to those in the WT due to induction of thermal dissipation of excess excitation energy (qE) in increased light intensity [11], which occurs concomitantly with dephosphorylation of LHCII proteins in the WT [17]. In stn7, $^{1}\text{O}_2$ production is likely to be greater than in the WT only under low and moderate lights when the energy transfer from LHCII to PSI is limited. Additionally, the PQ pool in stn7 is reduced under low and moderate lights [3,16], but is more highly oxidized than in the WT upon shift to HL owing to the higher relative amount of PSI centres [13,16].

The energetic state of the photosynthetic light reactions is connected to the regulation of gene expression (for review, [18,19]) through a system known as ‘retrograde signalling’, which regulates the expression of genes involved in photosynthesis (for reviews, see [7,20–24]), plant development and stress responses (for reviews, see [2,25–27]). The redox states of the PQ pool [2,28,29] and PSI electron acceptors play a crucial role in retrograde signalling [30], but the impact of the mechanisms that regulate photosynthetic light reactions, as well as the identities of the signalling molecules, have remained largely unresolved.

In this study, we investigated the effect of light intensity and the importance of photosynthetic regulation mechanisms on chloroplast ROS signalling through analysis of gene expression in Arabidopsis thaliana WT and the stn7, tap38/pph1 and npq4 mutants under HL. Our analysis showed strong similarity between genotypes in the primary abiotic stress response, which appeared to be due to similar $\text{H}_2\text{O}_2$-initiated and 12-oxo-phytodienoic acid (OPDA)-mediated signalling networks [31], while $^{1}\text{O}_2$-initiated and jasmonic acid (JA)-mediated signalling differed between the genotypes [32], particularly in stn7. Our results demonstrate that strict regulation of photosynthetic light-harvesting and energy distribution to the photosystems according to light intensity maintains the hormonal landscape favourable for proper abiotic stress responses. This is an important factor to be taken into account when designing crop plants with enhanced photosynthetic performance.

2. Results

(a) Transcription response to high light in wild-type and the stn7, tap38/pph1 and npq4 mutants

We prepared microarrays from WT Arabidopsis thaliana and from the stn7, tap38/pph1 and npq4 mutants that were shifted from growth light (GL) to HL for 1 h, and analysed the genes with greater than twofold expression change following the change in light intensity. The major transcription response of mutant plants was generally similar to that of the WT, with around half of the differentially expressed genes appearing in all genotypes. Functional clustering of the 500 most responsive genes (figure 1) revealed that the largest clusters in all genotypes comprised genes responding to abiotic stress, predominantly heat shock proteins (HSPs), which
have a major role in protecting protein stability under stress conditions (reviewed in [33]). Notably, the mitochondrial and chloroplastic HSP20 families were enriched in all genotypes, suggesting a particular need for protein protection within these organelles under HL. The heat shock transcription factors (HSFs) that instigate cellular stress response at the transcription level were likewise strongly upregulated in all genotypes. The cytosolic H2O2 scavenger ‘ascorbate peroxidase 2’ (APX2) was one of the most strongly upregulated genes in all genotypes, and it was expressed at considerably higher levels in one of the most strongly upregulated genes in all genotypes, the cytosolic isoform, APX1, was upregulated only in npq4

To explore this further, we analysed the expression profiles of genes that responded to HL in our arrays from light-harvesting regulation mutants and WT plants under other experimental conditions available in GENEVESTIGATOR. The similarity in overall transcription response among all genotypes was reiterated using this approach (see electronic supplementary material, figure S1). However, the total number of genes upregulated by JA treatment in published arrays was clearly lower in our stn7 arrays, demonstrating that the transcription response in stn7 under HL may be more independent of JA than in WT and the other photosynthetic mutants. Furthermore, many genes that are upregulated in the conditional fluorescence (flu) mutant [39] did not respond to HL in stn7 in our arrays, suggesting a decrease in the capacity of 1O2 signalling, which triggers JA-mediated stress response [36].

In order to clarify this apparent deficiency in 1O2 and JA signalling in light-harvesting regulatory mutants, we focused on HL-responsive genes in the WT that are described as ‘hormone-responsive’ in TAIR database annotation. A clear difference in the expression of JA-responsive genes was identified in all mutants compared with the WT, and this difference was especially pronounced in stn7. We found 45 highly responsive JA-related genes in WT, of which only 11 had significant fold change in stn7 upon transfer to HL (figure 2). Most prominently COI1, which is a central component of many JA-dependent development, stress and pathogen signalling pathways [37], was not upregulated upon HL exposure in stn7, although 4.5–5.5-fold changes in COI1 expression occurred in WT and the other mutants. Additionally, the enzymes DAD1, LOX2 and AOC2, which are involved in early stages of JA biosynthesis in the chloroplast [38], showed around twofold upregulation in the WT in response to HL, but none of these were upregulated in stn7. Furthermore, several ‘jasmonate ZIM-domain’ (JAZ) proteins that are involved in downstream JA signalling were upregulated in the WT under HL, but not in stn7, and upregulation of JA-responsive transcription factors including MYB75 and MYB113, which

**Figure 2.** HL-induced expression of 45 genes involved in jasmonate signalling in WT and redox mutants. (a) Shows the expression changes in each genotype in our arrays after shift from GL to HL, and the comparative levels of expression in each mutant compared to the WT in both GL and HL, with genes expressed more highly in the mutant shown in red, and more highly in WT shown in green. (b) Shows the expression changes in the same 45 jasmonate-related genes in response to treatment with methyl jasmonate (MeJA; AT-00110), 12-oxo-phytodienoic acid (OPDA; AT-00293) or hydrogen peroxide (H2O2; AT-00185) accessed from the public array database.
upregulate anthocyanin production under HL stress [39], was also missing from stn7.

Considering the impairment of excitation energy transfer that exists in stn7 under conditions where thermal dissipation is not induced (i.e. under low and moderate light intensities, with the latter corresponding to our GL conditions), it is likely that $^1$O$_2$ production in stn7 is already high in GL conditions, and upon transfer to HL the capacity for a further increase in $^1$O$_2$ may be impaired. In light of the above results, we suspected that JA signalling pathways in stn7 may be upregulated under GL, and so we compared the expression levels of JA-responsive genes in the mutants with those in WT under both GL and HL conditions. As expected, we found that many genes were indeed higher than WT levels in stn7 under GL, and lower under HL, including COI1, LOX2, DADI and several JAZ proteins (figure 2). These results show that JA signalling of gene expression is overactive in stn7 under low light conditions, but is not upregulated to WT levels under light stress, further linking JA pathways with regulation of light energy-harvesting.

In contrast to the unique profiles of JA-responsive genes in each of the mutants, the expression of abiotic stress-responsive genes was similar in all genotypes. This was clear in the strong upregulation of 26 HSP genes in the WT under HL, which were expressed at approximately the same levels in all genotypes (see electronic supplementary material, figure S2). GENEVESTIGATOR analysis showed that these genes are generally unresponsive to JA treatment in other microarray experiments, while a subset is strongly upregulated by OPDA. OPDA sensitivity within the group of HL-responsive genes clearly overlaps with H$_2$O$_2$ response (figure 3), suggesting that H$_2$O$_2$-induced response to HL proceeds via OPDA signalling pathways, independently of JA signalling.

3. Discussion

(a) The role of light in reactive oxygen species signalling

ROS produced as by-products of energy metabolism, through chlorophyll excitation ($^1$O$_2$) and reduction of molecular oxygen (H$_2$O$_2$), can lead to oxidative damage of biomolecules, but on the other hand both types of ROS play crucial roles in regulation of cellular stress responses. ROS also play important roles in regulating growth and development [2], especially during ageing and senescence [40]. Light-driven production of ROS can take place only when enough light energy is available. Under limiting light, the ROS required for signalling responses against biotic stress are produced by catabolic metabolism outside the chloroplast [1,41]. Mutants with disturbed coordination between light-dependent and light-independent abiotic stress signalling have growth, defence and senescence phenotypes that are highly reactive to light intensity [42–44]. Under natural light conditions, light intensity fluctuates constantly in time scales from fractions of a second to diurnal and seasonal variations, and ROS signals emanating from chloroplasts must be tightly coordinated in order to prevent signalling imbalance that could encumber growth and lead to premature hypersensitive reactions, senescence, or indeed to uncontrolled growth at the expense of stress tolerance. As the major source of ROS in the light, the chloroplast must therefore not only regulate its own production of ROS, but also send a constant feed of information to the wider cell in order to maintain ROS homeostasis in the context of the entire cell.

(b) Induction of cell-wide H$_2$O$_2$ response in wild-type and stn7, tap38 and npg4 mutants

All genotypes tested here showed strong and uniform upregulation of abiotic stress-responsive genes. This suggests that the H$_2$O$_2$ signalling system is unaffected by impairment in the ability to regulate light-harvesting and the redox state of the ETC. A major portion of H$_2$O$_2$ in the plant cell is produced in the chloroplast as a result of reduction of the PSI acceptor side [45,46]. Strong induction of genes that are responsive to H$_2$O$_2$ signals upon increase in light intensity indicates that the source of H$_2$O$_2$ may be the photosynthetic machinery itself. The induction of cytosolic APX scavengers was previously found to be induced by ETC energy imbalance under sudden exposure to HL [47], although expression of APX1, APX2 and other heat shock factors is independent of chloroplast-derived H$_2$O$_2$ [34]. This suggests that HL-induced H$_2$O$_2$, which generated a comparable stress response in all of our genotypes, evolves from non-chloroplastic sources. Furthermore, strong upregulation of AOX and HSP20 genes suggests increases in H$_2$O$_2$ in the mitochondria under HL.
The reason for a cell-wide stress response to HL is unclear, and the messenger(s) used to communicate HL stress to the wider cell remain(s) unidentified; however, our work shows that H_2O_2 signalling is functional under HL in all genotypes. Notably, the redox state of the PSI electron acceptors and the capacity of PSI to produce H_2O_2 should be quite similar between the WT and mutants, because the main regulator of PSI reducing power under HL is photosynthetic control of Cyt b6f, which should function similarly in all genotypes. This would corroborate the instigation of HL-induced H_2O_2 response at the PSI acceptor side. In contrast to PSI, the redox state of PSII and the PQ pool differs between the genotypes. The excitation energy transfer to PSII is impaired in the npq4 mutant, leading to a highly reduced PQ pool under HL and a resistance against electron transfer from PSII, while in tap38 and stn7 the PQ pool is oxidized. This suggests that the H_2O_2 response under HL is independent of the redox state of the PQ pool.

(c) Regulation of light-harvesting complex II energy transfer is crucial in retrograde signalling

LHCCI antennae are the primary receivers and distributors of light energy, which is transferred from LHCCI to PSII and PSI according to the energetic state of the photosynthetic machinery. Energy transfer from LHCCI to PSII is fluent when the relative rate of incoming light energy is balanced with the energy-using reactions. In the fluent state, chlorophyll excitation is relaxed quickly and the probability of producing O_2 is low, but any imbalance interrupts the transfer fluency and increases the level of O_2. This system provides a perfect sensor for monitoring the ratio of light energy absorbed to light energy used, which is the ultimate indicator of the energetic state of the photosynthetic machinery. A constant flow of information about the chloroplast energetic state is vital for regulating growth and development and is especially important for the H_2O_2-driven defence responses that depend on the coordinated cooperation between H_2O_2 produced in different cellular compartments [44,48,49]. The connection between the energetic state of the chloroplast and other processes relies on retrograde signalling to coordinate plant growth, development and stress responses with the capacity for energy production [20,22,50].

Our results clearly demonstrate that the capacity to regulate light-harvesting is important for proper hormone signalling pathways. In stn7, the upregulated JA response under GL can be attributed to excess O_2 produced owing to under-excitation of PSI, and subsequent over-reduction of the ETC and slower quenching of excited LHCCI, while rapid oxidation of the ETC during HL-induced thermal dissipation by abnormally large amounts of PSI undermine the capacity for O_2-induced JA response in HL. In the npq4 mutant, the response of JA-induced genes was generally stronger than in the WT upon shift to HL, with several genes clearly upregulated to a greater degree than in the WT. This agrees with the concept that greater levels of O_2 are produced in npq4 owing to damaged, detached and unquenched LHCCI in HL.

There is a wealth of data demonstrating that retrograde signalling is influenced by the redox states of the PQ pool [28,29] and PSI acceptors [30], as well as the metabolic state of PSI electron acceptors in the stroma [2,51] and the intermediates of chlorophyll biosynthesis [35,52,53]. These factors therefore exert control over the function of the photosynthetic machinery as a part of the green cell. Here, we show that dynamic regulation of energy harnessed and transferred by the LHCCI system, through phosphorylation of LHCCI proteins and protonation of PSBS, is crucial for the proper signalling responses and synchronization of photosynthesis with cellular metabolism. We propose that the ROS produced by the photosynthetic machinery is balanced with that from other cellular compartments by oxylipin signalling (see also [25,27]). As depicted in figure 4, our results provide evidence that JA signalling is initiated in the chloroplast by O_2 according to the fluency of excitation energy transfer to PSII and PSI from the light-harvesting system. By contrast, OPDA signalling appears to be controlled by the redox state of the ETC, most probably the PSI acceptors, and may therefore depend on production of H_2O_2. It is conceivable that interaction between these two ROS-initiated oxylipin-mediated signalling pathways, and the interaction between cellular ROS and oxylipin networks, synchronize the photosynthetic machinery with systemic hormonal regulation of plant growth, development and defence responses (see figure 4). Further studies are required to integrate the signalling mechanism proposed here with previously established retrograde signalling networks, and to better understand the role of the chloroplast in the wider networks that regulate plant growth, development and defence. It is also important to recognize the robust interactions between light-harvesting, hormonal signalling and stress responses when designing plants with enhanced photosynthetic performance.

4. Material and methods

(a) Plant growth

WT Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0 and stn7, tap38/pph1 and npq4 mutant plants were grown for six weeks in a phytotron at 23°C, relative humidity 60%, 8-h photoperiod under constant white GL of 130 μmol photons m^{-2} s^{-1}. Plants used for HL samples were shifted to 1000 μmol photons m^{-2} s^{-1} for 1 h.

(b) RNA isolation and microarray preparation

RNA samples contained pooled RNA from three plants of each genotype under either GL or HL. using a Plant RNA Isolation Mini Kit (Agilent Technologies) according to the manufacturer’s instructions. RNA concentration and quality were verified spectrophotometrically. Three replicate RNA samples were prepared from separate plants for each genotype and each light condition. Microarrays were prepared from each RNA sample (i.e. triplicate arrays for each genotype under each light condition). A total of 100 ng of RNA was amplified and Cy3-labelled using the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies) and hybridized to Arabidopsis 4 × 44K chips (Agilent Technologies). Microarrays were prepared and analysed by the Finnish Microarray and Sequencing Centre (Turku, Finland).

(c) Bioinformatics methods

Pre-processing of microarrays was performed using the ‘normexp’ background correction method to avoid negative or zero corrected intensities, followed by between-array normalization using the quantile method to make all array distributions identical. Control probes were filtered and then within-array replicate spots were replaced with their average. Pair-wise comparisons between groups were conducted using the Linear Models for Microarray Data (Limma) package from Bioconductor (http://www.
Figure 4. Hypothetical scheme describing: (a) ROS-mediated interaction of the light-harvesting and electron transfer machinery with hormone-responsive gene expression; (b) Abnormal production of ROS in various light-harvesting mutants under normal GL (stn7) and under HL (tap38 and npq4) conditions as described in the text. Fluent excitation energy transfer in the LHClII system minimizes the production of $^{1}\text{O}_2$ from excited chlorophylls (WT). This is dependent on control over the interactions between LHClII with other LH molecules, PSII and PSI (through LHClII phosphorylation), and on relaxation of excited chlorophylls via thermal dissipation of excitation energy (through PSBS protonation). The redox state of PSI depends on the availability of light energy and on regulation of light-harvesting efficiency and linear electron transfer (reviewed in [54]). Based on results of this study and available literature, we propose that: (i) the redox state of the PSI acceptor side controls $\text{H}_2\text{O}_2$ production that triggers the abiotic stress pathway proceeding through OPDA signalling; (ii) increased production of $^{1}\text{O}_2$ from excited chlorophylls upregulates JA synthesis and promotes biotic stress responses. Under increases in light intensity, these two pathways are upregulated in parallel and they must be balanced by light-harvesting regulation to ensure proper acclimation to biotic and abiotic stresses that overlap in nature. Proper function of $\text{H}_2\text{O}_2$- and OPDA-based signalling is not dependent on the regulation of excitation energy transfer. By contrast, proper $^{1}\text{O}_2$- and JA-dependent signalling is dependent on fluent and properly regulated excitation energy transfer and distribution, provided by the STN7 kinase, TAP38/PPH1 phosphatase and the PSBS protein.


