Reactive oxygen species and transcript analysis upon excess light treatment in wild-type *Arabidopsis thaliana* vs a photosensitive mutant lacking zeaxanthin and lutein

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**Abstract**

**Background:** Reactive oxygen species (ROS) are unavoidable by-products of oxygenic photosynthesis, causing progressive oxidative damage and ultimately cell death. Despite their destructive activity they are also signalling molecules, priming the acclimatory response to stress stimuli.

**Results:** To investigate this role further, we exposed wild type *Arabidopsis thaliana* plants and the double mutant *npq1lut2* to excess light. The mutant does not produce the xanthophylls lutein and zeaxanthin, whose key roles include ROS scavenging and prevention of ROS synthesis. Biochemical analysis revealed that singlet oxygen (1O2) accumulated to higher levels in the mutant while other ROS were unaffected, allowing to define the transcriptomic signature of the acclimatory response mediated by 1O2 which is enhanced by the lack of these xanthophylls species. The group of genes differentially regulated in *npq1lut2* is enriched in sequences encoding chloroplast proteins involved in cell protection against the damaging effect of ROS. Among the early fine-tuned components, are proteins involved in tetrapyrrole biosynthesis, chlorophyll catabolism, protein import, folding and turnover, synthesis and membrane insertion of photosynthetic subunits. Up to now, the *flu* mutant was the only biological system adopted to define the regulation of gene expression by 1O2. In this work, we propose the use of mutants accumulating 1O2 by mechanisms different from those activated in *flu* to better identify ROS signalling.

**Conclusions:** We propose that the lack of zeaxanthin and lutein leads to 1O2 accumulation and this represents a signalling pathway in the early stages of stress acclimation, beside the response to ADP/ATP ratio and to the redox state of both plastoquinone pool. Chloroplasts respond to 1O2 accumulation by undergoing a significant change in composition and function towards a fast acclimatory response. The physiological implications of this signalling specificity are discussed.

**Background**

Plant growth is inhibited by many forms of abiotic stress, including intense light [1], nitrogen and phosphorus starvation [2,3], water stress/high salinity [4] and extreme temperatures [5,6]. Excess light induces the re-organization of the photosynthetic apparatus to facilitate light harvesting while avoiding potentially damaging effects. Concomitantly, metabolism is redirected towards the synthesis of protective compounds such as flavonoids [7,8], tocopherol and carotenoids [9,10], which participate directly in stress responses.

The chloroplast is a crucial intersection for environmental stimuli [11-13]. Short-term responses to excess light, elicited in a timeframe of seconds to minutes, include enhanced thermal dissipation of light energy [14-16] and detachment of the outer antenna system from the photosystem II (PSII) reaction centre [17,18]. Longer-term acclimation responses include an increase in the PSI/PSII ratio, and the production of Rubisco,
cytochrome b6/f complexes and ATPase at higher levels in order to increase the rate of O2 evolution under saturating light conditions and avoid plastoquinone (PQ) over-reduction. Moreover, the capacity for thermal energy dissipation (Non-Photochemical Quenching, NPQ) increases as PsbS accumulates [19,20].

Although cytochrome b6/f, ATPase and Rubisco are encoded by chloroplast genes, the vast majority of plastid polypeptides are encoded by nuclear genes and are imported as precursors through the plastid envelope [21,22]. Acclimatory responses therefore require the coordinated regulation of plastid and nuclear genes, which involves a retrograde signal [12,23-27]. In the last decade transcriptome analysis has confirmed the importance and sophistication of this regulatory network [13,28-30], but the signals and transduction pathways are not yet fully understood. Proposed signalling molecules include Mg-protoporphyrin IX [31], which couples the rate of chlorophyll synthesis to the expression of plastid proteins crucial for 1O2-mediated stress responses [30]. Further results showed that Executer1/2 are chloroplast proteins crucial for 1O2-mediated stress responses [51]. However, xanthophyll mutants have been recently used to study the effect and the signalling pathway of 1O2 [46,52]. We are clearly dealing with two different systems that accumulate 1O2. The most studied that depends on 1O2 steady-state accumulation from chlorophyll precursors and the second one that depends on the photoprotective activity of xanthophylls in thylakoid membranes. In the first case the toxic effect of 1O2 has a major role in defining the phenotype, while in npq1lut2 its effect as signal molecule is more important. We applied stress conditions within a physiological range, leading to acclimation rather than the apoptotic responses reported in previous studies [30,53]. By limiting cross-talk between the apoptotic and acclimatory signal transduction pathways, we found that 1O2 can function as a signal in both wild-type and npq1lut2 mutants under oxidative stress.

Results
Genes regulated by intense light at low temperatures in wild-type and mutant plants

An Affymetrix GeneChip® Arabidopsis ATH1 Genome Array was used to compare the transcriptional footprints of wild-type Arabidopsis thaliana plants and the npq1lut2 mutant when both were transferred at 10°C and exposed to either very low light levels (time 0, before the application of stress) or very high light levels (1000 μmol m⁻² s⁻¹) for 2 or 24 h (Figure 1). Three biological replicates were analyzed in each treatment group. These conditions (low temperature associated to high light) were carefully chosen in order to emphasize the double mutant npq1lut2, which lacks violaxanthin de-epoxidase (VDE) and lycopene-ε-cyclase (LUT2) activities, and therefore cannot synthesize two major photoprotective xanthophylls: lutein and zeaxanthin. These molecules help to quench chlorophyll triplet states (3Chl*) and scavenge 1O2 released within the thylakoid membrane [47,48]. Due to the defect in xanthophyll composition, the npq1lut2 mutant exhibits a remarkable sensitivity to high light [49] and accumulates higher levels of 1O2 than wild-type plants, while the accumulation of other ROS is unaffected as are other putative retrograde signals such as the PQ redox state and the ATP/ADP ratio. The system that gave a great breakthrough in the study of 1O2 accumulation in plants is the conditional flu mutant. This mutant in the dark accumulates protochlorophyllide that acts as a photosensitizer upon illumination and generates 1O2 in the stroma of chloroplasts [50]. In flu, 1O2 accumulation mediates the activation of a stress response [29] that is different from those induced by other ROS such as superoxide anion (O2⁻) or hydrogen peroxide (H₂O₂) [30]. Further results showed that Executor1/2 are chloroplast proteins crucial for 1O2-mediated stress responses [51]. However, xanthophyll mutants have been recently used to study the effect and the signalling pathway of 1O2 [46,52]. We are clearly dealing with two different systems that accumulate 1O2. The most studied that depends on 1O2 steady-state accumulation from chlorophyll precursors and the second one that depends on the photoprotective activity of xanthophylls in thylakoid membranes. In the first case the toxic effect of 1O2 has a major role in defining the phenotype, while in npq1lut2 its effect as signal molecule is more important. We applied stress conditions within a physiological range, leading to acclimation rather than the apoptotic responses reported in previous studies [30,53]. By limiting cross-talk between the apoptotic and acclimatory signal transduction pathways, we found that 1O2 can function as a signal in both wild-type and npq1lut2 mutants under oxidative stress.
effect caused by the lack of the two photoprotective xanthophylls [47].

We noted that many genes were similarly regulated by light at low temperatures regardless of the genetic background, i.e. they were not influenced by the mutations. We have first compared different time points for each genotype to identify genes responding in the same way in both genotypes. These genes represent the response to high-light and low-temperature conditions in our experiment. Among the rapidly-responding genes (reaction to stress within 2 h), 812 were modulated in both wild-type and mutant plants, all showing the same directional response in both backgrounds (476 up-regulated and 336 down-regulated; Additional file 1: Table S1). Among the delayed-response genes (reaction to stress within 24 h), 1128 genes were modulated in both backgrounds, again all showing the same directional response (611 up-regulated and 517 down-regulated; Additional file 1: Table S2).

Functional classification of the above genes was carried out using FunCat version 2.1 [54] and the most significant results (p < 0.005) are summarized in Table 1. A complete list with subcategories is provided in Additional file 1: Table S3. Many of the genes (up-regulated and down-regulated) fell into the Cell Rescue, Interaction with Cell Environment and Interaction with the Environment categories, which are generally associated with stress responses or hormone signalling. Among the down-regulated genes, there was a significant over-representation of those in the Control of Transcription and Cell Wall Biogenesis functional categories, whereas many genes involved in Primary and Secondary Metabolism were up-regulated (176 after 2 h, 210 after 24 h). For example, a change in L-phenylalanine metabolism, reflecting the overexpression of chloroplast chorismate mutase (AT3G29200; CM1) and phenylalanine ammonia-lyase 1 (AT2G37040; PAL1), could serve as a secondary pathway for the synthesis of phenylpropanoids and flavonoids. Additional file 1: Table S3 shows that photosynthesis, energy conversion and regeneration, and light absorption are down-regulated after 24 h, possibly because energy pathways are overloaded and therefore feedback-inhibited when constantly exposed to intense light.

The ten most strongly modulated genes after 2 h included several with a regulatory function, which are likely to be involved in the activation of a stress response according to their GENEVESTIGATOR response profiles (Additional file 1: Table S1). These comprised three transcription factors (AT4G28140, AT1G56650 and AT2G20880), two heat shock proteins (AT3G12580 and AT2G20560) and one putative allene oxide cyclase (AT3G25780). After 24 h we observed the strong induction of genes known or suspected to be involved in flavonoid biosynthesis or modification, i.e. dihydroflavonol 4-reductase, DFR, AT5G42800; anthocyanin 5-aromatic acyltransferase, AAT1, AT1G03940-AT1G03495; anthocyanin pigment 2 protein, PAP2, AT1G66390; anthocyanin 5-O-glucosyltransferase, AT4G14090; flavonoid 3’-hydroxylase, F3’H, AT3G07990; MYB family transcription factor, MYB75/PAP1, AT1G56650; UDP-glucosyl transferase,
AT5G54060; and anthocyanidin synthase, AT4G22870 (Additional file 1: Table S2). These genes are known to be important checkpoints in flavonoid biosynthesis as shown by microarray experiments performed under various abiotic stress conditions [7].

**Genes regulated by intense light at low temperatures in mutant plants only**

Only 20 genes were found to be differentially expressed when unstressed wild type and mutant plants were compared (18, considering that two of them are responsible for npq1lut2 mutation). All 18 genes were down-regulated in the mutant, suggesting that the two backgrounds are metabolically very similar when there is no stress and that the 18 genes may be directly influenced by the lack of NPQ1 and LUT2 enzyme activities, or of the corresponding products (Figure 1).

Following exposure to intense light, the number of differentially expressed genes increased dramatically. After 2 h, 121 genes were up-regulated in the mutant and 69 down-regulated, and after 24 h, 270 genes were up-regulated and 144 down-regulated (Figure 1). The distribution of functional categories among these genes was similar to the genes modulated in the same manner in both backgrounds. However, a distinct group of 67 genes specifically repressed in the wild type plants after 2 h of stress but not repressed in the mutant (p = 1.12 × 10^-9) was shown to encode chloroplast proteins (Table 2), 38 with no known function and others identified as transcription factors and pentatricopeptide repeat-containing proteins (PPR), possibly participating in ROS signal transduction from the chloroplast to the nucleus and vice versa [55]. This indicates that nuclear gene expression might be influenced by carotenoid composition and anti-oxidant activity in thylakoid membranes, especially when plants are placed under oxidative stress.

Focusing on differences in expression levels (Additional file 1: Table S4), we noticed that genes encoding heat-shock proteins (AT3G12580, AT5G51440 and AT1G59860-AT1G07400) were more strongly up-regulated in the mutant after 24 h, as were those encoding antioxidant proteins such as 2-alkenal reductase (AER; AT5G16970), which catalyzes the reduction of the a,b-unsatured bond of reactive carbonyls [56], methionine sulfoxide reductase 3 (MSR3; AT5G61640), which promotes thioredoxin-dependent reduction of oxidized methionine residues in ROS-damaged proteins [57], and the oxidative stress protein rubredoxin (AT5G51010) [58]. A squalene monooxygenase 1,1 gene (SQP1,1; AT5G24150) is 12x more strongly repressed in wild type plants than in mutants and might be the base for changes in plant morphology or oxidative stress response in HL conditions [59,60].

**Gene clustering**

We next carried out a k-means cluster analysis, which organized all modulated genes into 11 clusters that differed little between wild-type and npq1lut2 mutant plants. Functional categories and their consistency were defined using MIPS functional catalogue (p ≤ 0.005). Up-regulated and down-regulated genes were analyzed after 2 and 24 h stress. For each subset, the number of genes is shown in brackets.

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### Table 1 Functional classification of genes regulated by intense light at 10°C

| Functional Categories                        | UP 2 h (476) | UP 24 h (606) | DOWN 2 h (336) | DOWN 24 h (370) |
|---------------------------------------------|--------------|---------------|----------------|-----------------|
| 01 Metabolism                               | 19.6 (176)   | 17.3 (210)    | -              | -               |
| 02 Energy                                   | -            | 2.2 (23)      | -              | -               |
| 11 Transcription                            | -            | -             | 7.5 (57)       | 4.5 (72)        |
| 14 Protein Fate                             | 5.4 (78)     | -             | -              | -               |
| 16 Binding Function                         | 7.0 (151)    | 5.3 (182)     | -              | -               |
| 20 Transport                                | 3.8 (59)     | -             | -              | -               |
| 30 Signal Transduction                      | -            | 7.99 (79)     | -              | 4.1 (47)        |
| 32 Cell Rescue                              | 14.1 (91)    | 6.89 (77)     | 7.2 (44)       | 9.5 (79)        |
| 34 Interaction with Cell Environment        | 12.4 (87)    | -             | 3.9 (22)       | 6.3 (43)        |
| 36 Interaction with the Environment         | 7.2 (47)     | -             | -              | 3.7 (27)        |
| 40 Cell Fate                                | -            | -             | 16.7 (326)     | 10.6 (160)      |
| 70 Subcellular Localization                | -            | -             | 16.7 (326)     | 10.6 (160)      |

Function and cellular localization of genes regulated by intense light (1000 μmol photon m^-2 s^-1, 10°C) in both wild-type and npq1lut2 mutant plants. Functional categories and their consistency were defined using MIPS functional catalogue (p ≤ 0.005). Up-regulated and down-regulated genes were analyzed after 2 and 24 h stress. For each subset, the number of genes is shown in brackets.
Table 2 Expression of genes down-regulated in response to intense light at low temperature exclusively in wild-type plants (2 h time point)

| Probeset | Locus identifier | Description | WT 0vs2 |
|----------|------------------|-------------|---------|
| 265067_at | AT1G03850 | glutaredoxin family protein | -2.83 |
| 264379_at | AT2G25200 | expressed protein | -2.67 |
| 248606_at | AT5G49450 | bZIP family transcription factor | -2.04 |
| 249932_at | AT5G22390 | expressed protein | -1.96 |
| 253305_at | AT4G33666 | expressed protein | -1.74 |
| 263674_at | AT2G04790 | expressed protein | -1.62 |
| 261196_at | AT1G12860 | basic helix-loop-helix (bHLH) family | -1.51 |
| 256698_at | AT3G20680 | expressed protein | -1.48 |
| 263209_at | AT1G10522 | expressed protein | -1.48 |
| 248285_at | AT5G2960 | expressed protein | -1.37 |
| 249750_at | AT5G24570 | expressed protein | -1.35 |
| 247574_at | AT5G61230 | ankyrin repeat family protein | -1.34 |
| 266899_at | AT2G34620 | mitochondrial transcription factor-related | -1.34 |
| 261118_at | AT1G75460 | ATP-dependent protease La (LON) | -1.31 |
| 263712_at | AT5G05985 | expressed protein | -1.27 |
| 248795_at | AT5G47390 | myb family transcription factor | -1.27 |
| 263593_at | AT2G1860 | pentatricopeptide (PPR) repeat-containing | -1.26 |
| 254688_at | AT4G13830 | DNAJ heat shock N-terminal (J20) | -1.24 |
| 261296_at | AT1G8460 | expressed protein | -1.24 |
| 257615_at | AT3G26510 | octicosapeptide/Phox/Bem1p (PB1) | -1.24 |
| 265457_at | AT2G46550 | expressed protein | -1.23 |
| 249472_at | AT5G39210 | expressed protein | -1.23 |
| 252136_at | AT5G50770 | calmodulin-related protein | -1.21 |
| 252922_at | AT4G39040 | expressed protein | -1.20 |
| 267591_at | AT2G39705 | expressed protein | -1.20 |
| 257856_at | AT3G12930 | expressed protein | -1.20 |
| 263264_at | AT2G38810 | histone H2A | -1.19 |
| 249929_at | AT5G2340 | expressed protein | -1.18 |
| 266329_at | AT2G01590 | expressed protein | -1.18 |
| 248762_at | AT5G47455 | expressed protein | -1.17 |
| 246506_at | AT5G16110 | expressed protein | -1.17 |
| 258250_at | AT3G15850 | similar to delta 9 acyl-lipid desaturase (ADS1) | -1.15 |
| 258683_at | AT3G08760 | protein kinase family | -1.15 |
| 259013_at | AT3G07430 | YGGT family protein | -1.14 |
| 253635_at | AT4G0620 | expressed protein | -1.14 |
| 246033_at | AT5G08280 | hydroxymethylbilane synthase | -1.14 |
| 248404_at | AT5G1460 | trehalose-6-phosphate phosphatase (TPPA) | -1.13 |
| 248402_at | AT5G21100 | dihydridopicolinate reductase family protein | -1.13 |
| 256728_at | AT3G25660 | glutamyl-tRNA(Gln) amidotransferase | -1.12 |
| 248663_at | AT5G48590 | expressed protein | -1.12 |
| 245984_at | AT5G13090 | expressed protein | -1.12 |
| 250663_at | AT5G07110 | prenylated rab acceptor (PRA1) | -1.11 |
| 254011_at | AT4G26370 | antitermination NusB domain | -1.10 |
| 261439_at | AT1G28395 | expressed protein | -1.10 |
| 259889_at | AT1G76405 | expressed protein | -1.10 |
| 253233_at | AT4G34290 | SWIB complex BAF60b domain | -1.10 |
| 259976_at | AT1G76560 | CP12 domain-containing | -1.10 |
| 260465_at | AT1G09101 | pentatricopeptide (PPR) repeat | -1.10 |
| 246205_at | AT4G39970 | remorin family protein | -1.09 |
| 257706_at | AT3G12685 | expressed protein | -1.09 |
wild-type and npq1lut2 plants, and is strongly enriched in chloroplast genes (Table 3). Indeed, among the 80 probes in the Arabidopsis ATH1 Genome Array representing genes in the chloroplast genome (ATC codes), five belong to cluster 18. One of these genes encodes a protein hypothetically involved in PSI assembly (AtYCF4, ATCG00520), two encode photosystem core complex proteins, PsbB from PSII (D2; ATCG00270) and PsaA from PSI (ATCG00350), and two encode ATPase subunits (ATCG00130 and ATCG00140). Other genes in cluster 18 encode a transcription factor regulating the cryptochrome response (AtCIB5, AT1G26260), an L-ascorbate oxidase (AT4G39830), a kinase (AT1G21270) and two unknown proteins (AT1G23850 and AT2G46640). All these genes are modulated by intense light at low temperature in the wild-type, while there is no response in the mutant.

ROS analysis in wild-type and npq1lut2 leaves

The npq1lut2 mutant was chosen because of its high sensitivity to photooxidative stress [47,49]. We determined the composition of ROS species released after the onset of illumination by infiltrating wild-type and mutant leaves with highly specific ROS-sensor probes: singlet-oxygen sensor green (SOSG) for $1O_2$, dichlorofluorescein (DCF) for $H_2O_2$ and OH., and proxyl-fluorescammine (ProxF) for $O_2^-$ and $OH$. Among all available probes specific for $1O_2$, we chose SOSG because, unlike other available fluorescent and chemiluminescent $1O_2$ detection reagents, it does not show any appreciable response to hydroxyl radical, $H_2O_2$ or superoxide anion; moreover, it was successfully applied to $1O_2$ detection in several systems, e.g. bacteria [62], diatoms [63], higher plants [48,63,64] and pigment-protein complexes isolated from higher plants [17,65]. Furthermore, C. Flors and co-workers applied SOSG to a range of biological systems that are known to generate $1O_2$ and in all cases, SOSG was confirmed as a useful in vivo probe for the detection of $1O_2$. Moreover, since highly sensitive probes for detection of $H_2O_2$, $O_2^-$ and OH. were also used in all measurements, any cross-detection Table 2 Expression of genes down-regulated in response to intense light at low temperature exclusively in wild-type plants (2 h time point) (Continued)

| Locus identifier | ATG expression | FC | Description |
|------------------|----------------|----|-------------|
| 245002_at        | ATCG00270      | -1.53 | Encode PSII D2 |
| 245007_at        | ATCG00350      | -2.22 | Encode PSI psaApsaB |
| 245018_at        | ATCG00520      | -1.20 | Hypothetical protein |
| 245025_at        | ATCG00130      | -1.41 | ATPase F subunit |
| 245026_at        | ATCG00140      | -1.30 | ATPase III subunit |
| 245873_at        | AT1G26260      | -1.05 | CBS, bhLL |
| 252862_at        | AT4G39830      | -2.50 | L-ascorbate oxidase putative |
| 259560_at        | AT1G21270      | -1.04 | serine/threonine protein kinase 2 (WAK2) |
| 263032_at        | AT1G23850      | -3.03 | expressed protein |
| 266320_at        | AT2G46640      | -1.01 | expressed protein |

This table shows the subset of genes in cluster 18. The ratio between npq1lut2 and wild-type plants after 2 h stress is expressed using a log2 scale. For each sample, the average of three repetitions is presented.

Table 3 Relevant cluster isolated by QT clustering

| Locus identifier | ATG expression | FC | Description |
|------------------|----------------|----|-------------|
| 245002_at        | ATCG00270      | -1.53 | Encode PSII D2 |
| 245007_at        | ATCG00350      | -2.22 | Encode PSI psaApsaB |
| 245018_at        | ATCG00520      | -1.20 | Hypothetical protein |
| 245025_at        | ATCG00130      | -1.41 | ATPase F subunit |
| 245026_at        | ATCG00140      | -1.30 | ATPase III subunit |
| 245873_at        | AT1G26260      | -1.05 | CBS, bhLL |
| 252862_at        | AT4G39830      | -2.50 | L-ascorbate oxidase putative |
| 259560_at        | AT1G21270      | -1.04 | serine/threonine protein kinase 2 (WAK2) |
| 263032_at        | AT1G23850      | -3.03 | expressed protein |
| 266320_at        | AT2G46640      | -1.01 | expressed protein |

This table shows the subset of genes in cluster 18. The ratio between npq1lut2 and wild-type plants after 2 h stress is expressed using a log2 scale. For each sample, the average of three repetitions is presented.
of other ROS species than $^{1}\text{O}_2$ by SOSG can be excluded.

The results in Figure 2 show that only SOSG fluorescence differed according to the genotype, with significantly higher fluorescence in mutant leaves (Figure 2C); there was no significant difference in the DCF and ProxF signals (Figures 2A, B). These results show that the accumulation of $^{1}\text{O}_2$ is selectively enhanced in npq1lut2 mutant leaves whereas the other ROS are accumulated at the same level in both the mutant and wild-type. These data were confirmed by determining the extent of protein oxidation in thylakoids using the Millipore OxyBlot kit: npq1lut2 plants showed evidence of increased protein carbonylation after 1 day exposure to excess light, whereas wild-type plants took 5 days before an increase was detectable and the amplitude of the signal was far lower (Figure 2D).

It has been reported that the chloroplast can control the rate of transcription in the nucleus via the redox state of PQ [32], the ADP/ATP ratio and the redox state of stromal components [66,67]. In order to determine whether differences in gene expression between wild-type and mutant plants reflected differences in $^{1}\text{O}_2$ steady-state accumulation, we studied the kinetics of these parameters under the same stress conditions described above. There were no major differences in qP, ascorbate and glutathione redox state, and ADP/ATP ratio, but there was a significantly greater reduction in maximum PSIII photochemical efficiency (Fv/Fm) in mutant within the first 2 d, which reflects PSII damage induced by high $^{1}\text{O}_2$ levels (Table 4).

Nevertheless, acclimation to stress conditions led to the recovery of Fv/Fm in both wild-type and npq1lut2 plants within 3 days (Table 4). The levels of ascorbate and glutathione increased in both genotypes upon HL treatment. Ascorbate accumulates at even higher extent in wild-type leaves than npq1lut2 in response to HL. On the contrary, the total amount of ATP and ADP was only slightly affected by stress treatment in both genotypes (Additional file 2: Figure S3).

Regulation of photosynthetic pigment metabolism

We next investigated the transcriptional regulation of genes in the chlorophyll and carotenoid metabolic pathways, since these pigments play an important role in light harvesting and photoprotection, and pigment-protein complexes are the main sources of $^{1}\text{O}_2$ in thylakoids when the photosynthetic machinery is overexcited [46,68]. Specifically, we studied the carotenogenic genes (Additional file 1: Table S5) and the Lhc (Figure 3) and Psa/Psb gene families (Table 5) to see if their expression was sensitive to HL treatment.

We identified several genes in the chlorophyll biosynthetic pathway that were differentially regulated in wild-type and mutant plants exposed to excess light at low temperature. We found that heme oxygenase 3 (AT1G69720), which catalyzes the rate-limiting step in the degradation of heme, uroporphyrin III C-methyltransferase (AT5G40850), which is involved in siroheme biosynthesis, and glutamate-1-semialdehyde 2,1-aminomutase (AT3G48730) and uroporphyrinogen III synthase (AT2G26540), which catalyze steps in porphyrin and chlorophyll metabolism, were induced much more strongly in the mutant. In contrast, for a gene encoding protochlorophyllide reductase B (AT4G27440), which is involved in the light-dependent step of chlorophyllide a biosynthesis, was repressed specifically in the mutant (Additional file 1: Table S5). These results indicate that HL-treatment on npq1lut2 plants redirects the porphyrin biosynthetic pathway from chlorophyll synthesis to the production of heme and siroheme, thus reducing the total amount of chlorophyll in the overexcited system. Consistently, the chlorophyll content per leaf area decreased more rapidly in mutant plants than wild type plants exposed to excess light (Figure 4C).

Several genes in the xanthophyll biosynthesis pathway were up-regulated in both wild-type and mutant plants, with stronger induction after 24 h. These included phytoene synthase (AT5G17230), phytoene dehydrogenase (AT4G14210, AT1G57770), lycopene-β-cyclase (AT3G10230), β-carotene hydroxylase chy1 (AT4G25700) and zeaxanthin epoxidase (AT5G67030). The strong up-regulation of carotenogenic genes in response to elevated irradiation would sustain chloroplast acclimation. The carotenoid content of whole leaves supported this hypothesis, since mutant plants acclimated to a lower Chl/Car ratio than wild-type plants after 6 d exposed to excess light at low temperature (Figure 4B), suggesting that $^{1}\text{O}_2$ signalling can account for the modulation of xanthophyll content in the thylakoid membrane. The differential expression of VTE1 in wild-type and mutant plants (Additional file 1: Table S6) is consistent with the higher tocopherol production in the mutant plants exposed to stress conditions (Figure 4D).

Regulation of pigment-binding proteins

Lhc proteins are located within the thylakoid membranes, where they coordinate the chlorophylls and carotenoids. They are encoded by a superfamily of nuclear genes whose transcription [69], translation [70-72] and protein accumulation [20,35] are finely regulated in response to environmental cues. The expression profiles of most Lhc genes were very similar in wild-type and mutant plants exposed to excess light for 24 h (Figure 3). The genes significantly up-regulated in both genotypes were Lhcb4.3 (AT2G40100), Lhcb7 (AT1G76570), ELIP1 (AT3G22840) and ELIP2 (AT4G14690), indicating their involvement in
the general stress response. However, *Lhca4* (AT3G47470) was significantly down-regulated only in wild-type plants, whereas *Lhca6* (AT1G19150) was up-regulated only in the mutant.

Furthermore, many genes encoding PSII and PSI core complex subunits were significantly down-regulated in wild-type plants exposed to excess light, but up-regulated or marginally down-regulated in the mutant, i.e. *CP47* (ATCG00680), *D2* (ATCG00270), *PsbG* (ATCG00430), *PsbI* (ATCG00080), *PsbK* (ATCG00070), *PsaD* (AT1G03130), *PsaO* (AT1G08380) and *PsaN* (AT5G64040). Table 5 shows the gene expression ratios on the log2 scale. Marked fields represent probe sets showing a significant change. CP47 was more strongly...
repressed in wild-type compared to mutant plants, with a similar tendency observed for other probe sets such as D2 and PsaA, for which down-regulation or no modulation was observed in wild-type plants while up-regulation was observed in the mutant. These finding indicate that the main response to excess light at low temperatures is a general repression of photosynthesis-related genes, but HL treatment in mutant leaves results in specific transcriptional re-programming of the core subunits of both photosystems, relieving the transcriptional repression in wild-type leaves. Biochemical analysis of thylakoid pigment-protein composition during stress treatment showed that the photosynthetic machinery acclimates by reducing the PSI/PSII ratio (Figure 4E), but there is little change in the antenna size as detected by the LHClII/PSII ratio (Figure 4F). These results agree with previous reports showing that when PSI becomes rate-limiting for photosynthetic electron transport, changes in photosystem stoichiometry occur to counteract this inefficiency [32]. Although the redox state of PQ is the same in both genotypes (Table 4), genes encoding PS core complexes are differentially expressed and there are differences in the rate at which the PSI/PSII ratio declines. The faster reduction in the PSI/PSII ratio in mutant leaves, independent of PQ redox state or PSI photoinhibition (Table 4), suggests a ROS-dependent signal transduction pathway that facilitates the acclimatory modulation of thylakoid composition.

**Chloroplast reorganization in response to \( ^{1}O_2 \) accumulation**

Several signals are thought to pass from the plastid, either directly or indirectly, through the cytoplasm to the nucleus, where they modulate gene expression under stress [25]. After acetonic extraction, pigment analysis showed that the chlorophyll a/b ratio was higher in the mutant than the wild-type and this difference increased under stress (Figure 4A), reflecting the changing PSII/PSI ratio in the mutant upon HL treatment (Figure 4E) rather than a reduction in antenna size (Figure 4F). Under stress, Lhc transcription was inhibited to the same extent in both genotypes, whereas photosystem core genes were down-regulated more strongly in the wild-type plants. This is consistent with the significant increase in the Chl a/b ratio observed in the mutant, but there was no modulation of FtsH expression to explain the more rapid degradation of pigment-protein complexes (Additional file 1: Table S6). The Chl/Car ratio differs significantly between the two genotypes, with wild-type plants showing a 24% reduction under stress, and mutants showing a 38% reduction (Figure 4B). Evidence for oxidative stress was found in the pattern of antioxidant compounds, e.g. glutathione S-transferase, methionine sulfoxide reductase and tocopherol (Additional file 1: Table S6). Several genes showing induction in npq1lut2 only encoded chloroplast proteins, that might be involved in cell protection against the damaging effect of ROS (Figure 5). Since most were induced after 24 h in the mutant, it suggests that induction occurs only when \( ^{1}O_2 \) accumulation exceeds a threshold level (Additional file 1: Table S7).

**Discussion**

We have carried out a comparative analysis of wild-type Arabidopsis plants and the double mutant npq1lut2 in terms of mRNA levels, metabolite levels and physiological functions in response to conditions leading to oxidative stress. The npq1lut2 xanthophyll biosynthesis mutant was used to study the effect of \( ^{1}O_2 \) accumulation on physiological stress responses [47,49]. This mutant lacks violanthin de-epoxidase (NPQ1) and lycopene-\( \varepsilon \)-cyclase (LUT2) activities, and is susceptible

### Table 4 Time-course of main chloroplast parameters putatively involved in the regulation of gene expression, as previously reported [32,66,67]

| Time (hours) | WT     | nq1lut2 |
|-------------|--------|---------|
| 0           | 0.20 ± 0.06 | 0.13 ± 0.08 |
| 2           | 0.15 ± 0.07 | 0.12 ± 0.08 |
| 24          | 0.03 ± 0.02 | 0.01 ± 0.08 |
| 48          | 0.05 ± 0.03 | 0.02 ± 0.08 |
| 72          | 0.10 ± 0.03 | 0.07 ± 0.08 |
| 144         | 1.07 ± 0.03 | 0.06 ± 0.08 |

| Parameter          | WT     | nq1lut2 |
|--------------------|--------|---------|
| Fv/Fm              | 0.79 ± 0.01 | 0.46 ± 0.01 |
| ADP/ATP            | 2.2 ± 0.01 | 2.1 ± 0.01 |
| GSH/GSSH +GSSH     | 91.3 ± 0.01 | 75.6 ± 0.01 |
| Asc/(Asc +DHA)     | 74.5 ± 0.01 | 53.2 ± 0.01 |

Note: WT and npq1lut2 rosettes were pre-treated for 48 hrs at 10°C (see methods for details), then were exposed to photot oxidative conditions (1000 \( \mu \)mol photon m\(^{-2}\) s\(^{-1}\), 10°C, 16 h light/8 h dark). Leaves were harvested, then used for analysis of chlorophyll fluorescence parameters or immediately frozen in liquid nitrogen for measurements of metabolites, at the same time of the day over a 6-day-long stress period. Abbreviations: qP, photochemical quenching; Fv/Fm, maximal PSII photochemical efficiency; GSH/GSSG, glutathione reduced/oxidized; Asc, ascorbate; DHA, dehydroascorbate. Values that differ significantly between wild type and npq1lut2 mutant plants (Student’s t-test, p < 0.02) are marked by an asterisk.
the carotenoid content of the chloroplast affects gene expression under both normal and stress conditions, and affects chloroplast to nucleus communication [13,73,74]. Here, we show that $^{1}$O$_2$ accumulation in response to excess illumination within the physiological range is perceived as a signal to regulate significant number of nuclear genes encoding chloroplast proteins, facilitating acclimation to stress, but is not sufficient to induce apoptosis.

Xanthophyll mutants are valuable for the analysis of $^{1}$O$_2$ signalling

The suitability of the lut2npq1 mutant for the analysis of $^{1}$O$_2$ signaling was confirmed by comparing physiological parameters and ROS accumulation in relation to wild-type plants. Previous results [47,75,76] showed that lut2 mutation in Arabidopsis only affected few physiological parameter (increase in PSI/PSI and Chl a/b ratios, reduced efficiency of state transitions and LHCII trimerization); however, photosynthetic efficiency and growth rate in lut2 plants were indistinguishable from wild-type. We cannot exclude that differences between the two genotypes at the onset of HL treatment could be responsible of some of the differential responses at transcriptome level. However, WT and npq1lut2 accumulate different amounts of $^{1}$O$_2$ from their chloroplasts before stress treatment (Figure 2, T = 0) as further confirmed by transcript levels at time 0 showing no major differences in gene regulation between WT vs mutant. Therefore, if a differential $^{1}$O$_2$ accumulation occurs even in low light, it is below the threshold level that makes $^{1}$O$_2$ a signal in the regulation of gene expression.

Present results demonstrate that $^{1}$O$_2$ is the only ROS differentially accumulated in the mutant with respect to WT upon HL treatment, while this mutations does not differentially affect the main parameters that, until now, have been related to gene expression regulation in HL. Indeed, following illumination at 1000 µmol m$^{-2}$ s$^{-1}$ and 10°C, the photosynthetic electron transport chain was reduced to the same extent in both genotypes (Table 4). This allowed us to monitor the impact of excess light on the redox state of the PQ pool, a physiological parameter that has been proposed to have a specific role in chloroplast to nucleus signalling during stress acclimation [32]; therefore, the differential gene expression in wild-type vs mutant plants cannot be attributed to changes in the PQ redox state, confirming data from previous studies [35]. Additional proposed signalling molecules, such as reduced forms of ROS, the redox state of the stoma redox component (GSH/GSSG, Asc/Asc+DHA), and the ATP/ADP ratio [67] were indistinguishable in the two genotypes (Table 4 and Additional file 2: Figure S3), suggesting they are not major transcriptional regulators in response to photo-oxidative stress when exposed to excess light at low temperatures [47]. Under normal growth conditions the gene expression profile of the mutant is almost identical to that of wild-type plants, but differences become evident following exposure to excess light (1000 µmol m$^{-2}$ s$^{-1}$) at low temperature (10°C). At time 0 (before stress), 18 genes were down-regulated in the mutant relative to wild-type plants, although the expression of those genes could be directly or indirectly regulated by the absence of lutein and zeaxanthin. Also, during high light treatments lutein and zeaxanthin could play a signalling role, directly or by compounds derived from them. The effect of individual carotenoids on transcription has not been analyzed in detail, but it is clear that
stresses conditions used in this report. Therefore, all data presented suggest that gene expression changes described could be reasonably ascribed to singlet oxygen, even if we cannot exclude that other factors could act as signal in npq1lut2 plants, together with singlet oxygen, in the modulation of gene expression.

The npq1lut2 mutant shows a selective loss of lutein, which is active in 3Chl* quenching [47], and of zeaxanthin, which is an 1O2 scavenger [47,48,77,78], therefore the mutant specifically accumulates 1O2 but not other ROS (Figure 2C) [47,79]. It should be noted that the change in xanthophyll composition marginally affects the composition of the photosynthetic apparatus in the mutant [47] while photosynthetic electron transport and growth rate are the same in both genotypes, therefore 1O2 steady-state accumulation in the npq1lut2 mutant occurs only in response to excess light conditions (Figure 1 and Additional file 1: Table S4). Thus, npq1lut2 mutant compares favourably with the flu mutant [29] in which 1O2 is produced through the accumulation of Chl biosynthesis precursors, eventually leading to complete chloroplast bleaching. The present study on npq1lut2 is the first case in which ROS generation has been elicited in its natural site (i.e. within thylakoid membranes) rather than provided from outside or produced by photosensitizing metabolic precursors soluble in the chloroplast stroma. The level of stress applied in our experiment is far lower from that described in (Op den Camp et al. Plant Cell 2003) and is followed by a successful acclimative response as in a physiological response. Therefore we strongly support the notion that in our experimental conditions, 1O2 acts primarily as a signal that modulates chloroplast acclimation to photooxidative stress.

The photosynthetic parameters and metabolic indicators discussed above (i.e. Fv/Fm, Chl a/b and Chl/Car ratios, PSI/PSII ratio) show that the chloroplast function and communication between the chloroplast and cytoplasm are impaired in the mutant, while the differential expression of nuclear genes encoding chloroplast proteins confirms that the chloroplast is a central switch of the plant's response to cold and light stress [13,74]. We can now decipher the contribution of 1O2 signalling to the stress acclimation response. A similar system was previously used with the mutant npq1lor1 of the green alga Chlamydomonas reinhardtii. Nevertheless, in Arabidopsis we identified a fast component of gene expression regulation by 1O2 at 2h that was not detected in Chlamydomonas [80].

The npq1lut2 transcriptome integrates the ROS signalling network

Oxidative stress is a complex process that can be triggered by a range of environmental, biotic and developmental factors. It is not surprising that different pathways can be induced, depending on the nature of the stress. Previous studies using a catalase-deficient mutant exposed to excess light identified genes that are differentially expressed in response to H2O2 accumulation, leading to the discovery that H2O2

| Locus identifier | Description | Fold Changes in WT | Fold Changes in npq1lut2 |
|-----------------|-------------|--------------------|-------------------------|
| ATCG00680       | CP47, subunit of PSII reaction centre | -0.9 | -0.1 |
| ATCG00020       | D1, subunit of PSII reaction centre | 0.3 | 0.5 |
| ATCG00270       | D2, subunit of PSII reaction centre | -0.1 | 0.5 |
| ATCG00430       | Photosystem II G protein | -0.8 | 0.1 |
| ATCG00080       | Photosystem II I protein | -1.0 | -0.7 |
| ATCG00070       | Photosystem II K protein | -1.4 * | -0.9 |
| AT4G05180       | PSBO2, oxygen-evolving enhancer protein 3 | -1.9 * | -1.4 * |
| AT5G64040       | PsaN | -1.2 * | -0.4 |
| AT1G03130       | Psd | -2.0 * | -1.0 * |
| AT2G20260       | Pse | -1.7 | -1.0 |
| ATCG00350       | PsaA | 0.0 | 0.8 |
| ATCG00340       | PsaB | 0.1 | 0.6 |
| AT1G08380       | Pso | -1.6 * | -0.9 |
| AT1G13330       | Psf | -1.0 | -0.7 |

This table presents the subset of genes belonging to each photosystem core complex. The ratio between npq1lut2 and wild-type plants after 24 h stress is expressed as a log2 scale. Marked fields represent probe sets with a significant changes after RMA analyses.
Figure 4 Biochemical characterization of thylakoid membrane composition under high light stress. Chlorophylls (A, C), carotenoids (B) and tocopherol (D) content of WT and lut2npq1 plants were measured on leaf acetone extracts as described in “Material and Methods”. (E, F) Stoichiometry between photosynthetic pigment-binding complexes under high light stress. PSII/PSI ratio (E) and biochemical antenna size (LHCl/PS ratio, F) were determined by both non-denaturing Deriphat-PAGE and immunoblot-titration using specific antibodies (see “Material and Methods” for details). Symbols and error bars show respectively means ± SD.
regulates anthocyanin biosynthesis [28]. Several reports have also proposed that \(^{1}\text{O}_2\) has a signalling role [81,82].

Here we have determined the photoprotective effect of two xanthophylls when plants are exposed to excess light at low temperatures. Only 18 genes were found to be differentially expressed between wild type plants and the npq1lut2 mutant under normal conditions, probably reflecting the absence of lutein and zeaxanthin in the mutant (Additional file 1: Table S4). However, when the plants were exposed to excess light at a low temperature, a group of 67 genes encoding chloroplast proteins was specifically repressed in wild type plants, whereas the same genes were not affected in the mutant. This is intriguing because a nuclear mutation affecting chloroplast xanthophyll composition is clearly able to regulate gene expression and ultimately chloroplast acclimation. We can thus conclude that the expression of some nuclear genes depends on the xanthophyll content directly or indirectly, via its impact on \(^{1}\text{O}_2\) accumulation (Figure 2C). We do not exclude that lutein, zeaxanthin and products of their metabolisms play a signalling role under stress. Indeed, carotenoids can play a clear signalling role [83]. Here we want to highlight the correlation between gene expression regulation and \(^{1}\text{O}_2\) steady-state accumulation in a mutant lacking two photoprotective xanthophylls. One possibility is that a subset of genes in Table 2 responds to the change in \(^{1}\text{O}_2\) accumulation within the thylakoid membranes, e.g. those encoding glutaredoxin (AT1G03850), ATP-dependent protease La (AT1G75460), DNAJ heat shock N-terminal (AT4G13830) and enzymes involved in phylloquinone and plastoquinone biosynthesis (AT1G60600). Functional annotation of the 38 uncharacterized genes in this list will help further to decipher how gene regulation by lutein and zeaxanthin occurs under oxidative stress, as shown in previous studies [84,85].

One group of genes specifically modulated in the npq1lut2 mutant overlaps with those regulated in flu (Additional file 1: Table S8), a reference mutant used in the study of \(^{1}\text{O}_2\) signals [50,86] in agreement with the high level of \(^{1}\text{O}_2\) accumulation measured in npq1lut2 (Figure 2C). Also in the attempt of comparing the
response in npq1lut1 vs flu, we performed a more sophisticated statistical analysis comparing npq1lut2 transcriptome and flu/executor transcriptome [53]. The conditions used in the two experiments are different as demonstrated by the high number of genes (2420 probe-sets) differentially expressed in the two wild-types (Additional file 2: Figure S4A). A low level of overlap between npq1lut2 and flu/executor transcript response was detectable (Additional file 2: Figure S4B) showing that transcriptomic analysis performed in different labs under different experimental conditions must be compared with precaution as shown by previous papers [7,36]. Comparative transcriptomic analysis of the 1O2 response signature showed that the cluster of genes regulated by 1O2 in both flu and npq1lut2 is not modulated in all oxidative stress cases analyzed to date. However, we identified a subset of genes affected by 1O2 and O2, whereas there is negative correlation between the genes modulated by 1O2 and those modulated by H2O2 (Table 6). This antagonistic transcriptional regulation mediated by 1O2 and H2O2 supports previous data showing cross-talk and antagonistic H2O2 and 1O2 signalling in flu mutants under stress overexpressing the thylakoid-bound ascorbate peroxidase [30]. The molecular basis of these opposing responses appears to reflect the presence of specific cis-regulatory elements responsive to either 1O2 or O2 within the corresponding promoters [87]. A new and close relationship among ROS was recently demonstrated, where each ROS species activates a specific response, but the pathways converge to produce a clear 1O2 signature in lipid peroxidation [52].

The genome-wide hypersensitive response is more strongly induced in flu mutants than in npq1lut2 mutants (Additional file 1: Table S9). Among 369 genes significantly up-regulated following infection with Pseudomonas DC3000 (avrRpm1) [88], 292 were also detected in the flu and npq1lut2 transcriptomes with 267 induced in flu and only 69 in npq1lut2 (resulting in a far less pronounced apoptotic response). In agreement with this, we did not observe cell death in Arabidopsis plants by vital staining and DNA fragmentation analysis (data not shown). Because npq1lut2 specifically showed higher 1O2 steady-state accumulation (Figure 2), this implies that cell death is not a specific or immediate response to 1O2 in the absence of the most effective photoprotection mechanisms present in wild-type plants, at least under our experimental conditions. However, we cannot exclude the possibility that higher levels of 1O2 accumulated under non-physiological conditions, might induce cell death.

Recent work by Apel and co-workers revealed that EXECUTER genes are involved in the early response to 1O2 in Arabidopsis by the transduction of 1O2 signals from the chloro plast to the nucleus in the flu mutant [51,53]. 1O2 accumulation in npq1lut2 induced the expression of ex2 but not ex1, but there was no effect in similarly-treated wild-type plants, confirming that 1O2 oxygen signals are measurable in the npq1lut2 transcriptome and that EX1 and EX2 might respond differently to environmental cues.

**Xanthophylls modulate the pigment composition of thylakoid membranes**

It is well documented that plants acclimate to different light conditions by regulating their carotenoid composition [89]. It is worth noting that the higher rate of 1O2 accumulation in npq1lut2 plants corresponds to the induction of genes representing the β-β branch of carotenoid biosynthesis (β-carotene hydroxylase, zeaxanthin epoxidase, lycopene β-cyclase; Table S5). In thylakoid membranes, the accumulation of β-β xanthophylls would increase the ability of plants to synthesize zeaxanthin and neoxanthin when needed, thus facilitating the response to excess light. Indeed, these β-β xanthophylls have both an important role in photoprotection [90-92] and mutants lacking such compounds undergo irreversible photo-oxidation when exposed to excess light [90]. Growth under intense light caused carotenoid levels to increase in npq1lut2 plants compared to the wild-type (Figure 4B), and because carotenoids scavenge 1O2 or directly quench 3Chl*, the increased Car/Chl ratio appears to be a protective mechanism [20,93].

Besides carotenoids, plants synthesize other antioxidants such as tocopherol (vitamin E). This lipophilic compound is localized exclusively in the lipid phase of the thylakoid membranes, and is an active 1O2 scavenger [10,94]. Higher levels of tocopherol were observed in the leaves of npq1 mutant plants after 3 d of excess light stress [95], and it was proposed to have a primary role in the prevention of lipid peroxidation promoted by 1O2. We found that npq1lut2 plants under chilling stress accumulated tocopherols to higher levels than wild-type plants when exposed to excess light for 6 d (Figure 4D) and contained ~70% more α-tocopherol. Tocopherol synthesis is therefore strongly induced by excess light in the mutant, particularly given the rapid consumption due to the increased rate of ROS accumulation. The biochemical analysis was consistent with the transcriptomic data, showing stronger and faster induction of tocopherol synthesis genes in the mutant, e.g. HPD (AT1G06570) and VTE1 (AT4G32770) (Additional file 1: Table S6).

Tetrapyrrole synthesis must also be regulated under excess light stress to prevent damage to the photosynthetic machinery, and when photo-oxidative stress accelerates the degradation of pigment-protein complexes, the synthesis of chlorophyll must slow down to
Table 6 Expression of genes up- and down-regulated in different ROS accumulating conditions

| Probeset | Locus identifier | OzoMV2h | OzoMV4h | fluMV2h | fluMV4h | vte2 | vte1 | cat | DCMU | Description |
|----------|-----------------|---------|---------|---------|---------|------|------|-----|------|-------------|
| 253259_at | At4g34410       | 0,24    | -1,13   | 0,20    | -0,93   |      |      |     |      | RRTF1, AP2 domain-containing transcription factor |
| 253832_at | At4g27654       | 1,53    | 0,88    | 1,29    | 0,33    |      |      |     |      | unknown protein |
| 248793_at | At5g47240       | 1,04    | 0,20    | -0,11   | -2,20   |      |      |     |      | ATNUDT8, Nudix hydrolase homolog 8 |
| 247360_at | At5g63450       | 1,08    | 0,19    | -0,80   | -0,81   |      |      |     |      | CYP94B1, oxygen binding cytochrome P450 |
| 266821_at | At2g44840       | 1,79    | 0,28    | 0,36    | -2,74   |      |      |     |      | Ethyne responsive element binding factor 13 |
| 262354_at | At1g64200       | 0,23    | -0,02   | -0,50   | 0,03    |      |      |     |      | Vacular H+-ATPase subunit 3 |
| 247030_at | At5g67210       | 0,11    | -0,10   | -2,48   | -1,03   |      |      |     |      | nucelic acid binding/putative ribonuclease |
| 256021_at | At1g58270       | 0,23    | 0,41    | -0,18   | -0,80   |      |      |     |      | ZW9 |
| 266977_at | At3g39420       | -0,05   | -0,10   | -0,27   | -0,75   |      |      |     |      | esterase/lipase/thioesterase family protein |
| 255941_at | At1g20350       | -0,18   | -2,10   | 1,03    | 1,08    |      |      |     |      | TIM17, mitochondrial inner membrane translocase |
| 263320_at | At2g47180       | 0,82    | 2,04    | 0,99    | 0,36    |      |      |     |      | AtGOLS1 Galactinol Synthase 1 |
| 266418_at | At2g38750       | -0,42   | -0,69   | -1,97   | 0,09    | -0,43 |      |     |      | ANNAT4, Annexin Arabidopsis 4, calcium ion binding |
| 264986_at | At1g27130       | -0,21   | -0,36   | 0,52    | 0,52    |      |      |     |      | ATGSTDU13, glutathione S-transferase 13 |

The transcription regulation of genes specifically responding to \( ^1\text{O}_2 \) in \( ^{12} \text{flu} \) and \( ^{14} \text{lpq}_{1l_2} \) mutants was compared to various experiments by using mutants and/or treatments. The ratio between treated and control plants is expressed as a log2 scale. For each sample, the average of three repetitions was considered.

We therefore measured changes in the total Chl content as well as in Chl a/b ratio. The tetrapyrrole pathway is regulated by metabolic intermediates at the transcriptional and post-translational levels [96]. In particular, heme is a well known repressor of early steps in the Chl synthesis pathway [97]. Crosstalk between tetrapyrrole biosynthesis and \( ^1\text{O}_2 \) was demonstrated in \( ^{16} \text{flu} \) mutants [98]. Our data clearly show that the higher levels of \( ^1\text{O}_2 \) accumulation in \( ^{14} \text{lpq}_{1l_2} \) mutants promote the expression of heme oxygenase 3 (AT1G69720) and uroporphyrin III C-methyltransferase (AT2G26540), resulting in higher levels of heme in mutant compared to wild-type plants (Additional file 1: Table S5). Furthermore, repression of protoclorophyllide reductase B (AT4G27440) in \( ^{14} \text{lpq}_{1l_2} \) could limit chlorophyll production, helping to reduce the number of pigment-protein complexes in the cell during chloroplast acclimation to excess light.

\( ^1\text{O}_2 \) therefore appears to participate in a fine-tuning system that modulates chlorophyll biosynthesis and the accumulation of carotenoids and lipophilic antioxidant compounds in excess light stress, thereby increasing plant fitness under normal illumination.

Xanthophylls affect the composition of the photosynthetic apparatus during acclimation

Light-harvesting complexes respond rapidly to changes in environmental conditions [99]. We showed that most \( ^{12} \text{Lhc} \) genes have similar expression profiles in wild type and \( ^{14} \text{lpq}_{1l_2} \) mutants, even though they encode...
proteins that bind lutein and zeaxanthin. Many Lhc genes were down-regulated, with Lhcb2.4 the most strongly repressed (Figure 3). The exceptions were Lhcb4.3, Lhcb7, PsbS and ELIPs, consistent with data showing that the four corresponding antenna proteins participate in photoprotection [69,100,101].

Interestingly, the different isoforms of Lhcb4 (CP29) were modulated in distinct ways despite their very similar polypeptide sequence. In particular, although Lhcb4.1 and Lhcb4.2 were down-regulated in both genotypes under stress conditions, Lhcb4.3 [102] was induced in both genotypes to the same extent. This is consistent with previous studies showing the evolutionary conservation of genetic redundancy in the Lhc superfamily [103], and it suggests that different CP29 polypeptides may play significant and specific roles in acclimation.

Our expression data also suggested that several signals intersect to regulate the Lhc superfamily and that transcriptional regulation is only one component of a more complex process. The most striking change in thylakoid composition under stress was the progressive reduction in the PSII/PSI ratio, which was more pronounced in npq1lut2 mutants (Table 5 and Figure 5). Such a reduction may be necessary to prevent the over-reduction of photosynthetic electron chains [20] and likely reflects changes to the rates at which the various substrates are synthesized and destroyed. PSI destruction is higher in the mutant because of the excessive photo-oxidation, and we have provided evidence that genes encoding several PSII core complex subunits (and to a lesser extent those in the PSI core complex) are induced in the mutant and repressed in wild-type plants (Table 5). It is well known that the transcription and the translation of PSII and PSI genes is extremely complex and often uncoupled. Analysis of the barley PSI-less viridis zb63 mutant showed an over-reduction of PQ pool and an increase in PSII core content into thylakoid with respect to WT (Frigerio 2007); all these changes in PSII content occurs without changes in PsAA mRNA levels. Furthermore, in the viridis zb63 mutant, despite the absence of fully assembled PSI complex and the missed accumulation of any core polypeptides, all genes encoding PSI subunits are substantially expressed at the same level with respect to wild-type plants. These evidences suggest that a) regulation of photosystems accumulation could not only involve chronic PQ reduction [32] and b) regulation of composition of photosynthetic components could be mainly at the level of protein turn-over.

In contrast to previous reports [20], the loss of PSII content was not accompanied by a dramatic loss of bulk LHCII, probably because more time might be needed to achieve a functional antenna size final state under our growth conditions. Finally, ^1O2 induces chloroplast ATP synthase protein 1 (AT2G31040) specifically in npq1lut2 mutants after 24 h exposure to excess light, and a higher level of ATP synthase was previously identified as one of the long-term responses that facilitate chloroplast acclimation to intense light [104].

Chloroplasts respond to the accumulation of ^1O2 by functional reorganization

We found that several genes showing dose-dependent induction by ^1O2 encoded chloroplast proteins whose function is to protect cells against the damaging effect of ROS. Most were induced after 24 h specifically in the mutant, suggesting induction occurs only when ^1O2 accumulation exceeds a threshold level (Additional file 1: Table S7).

Many of these proteins were thioredoxins, ^1O2-quenching proteins that respond to oxidative stress [105]. This is consistent with previous reports showing that thioredoxins are protective proteins that maintain the cellular redox environment [106]. Others are involved in chlorophyll catabolism (At4g22920 and At5g13800), and their induction correlates with both the down-regulation of genes involved in tetrapyrrole biosynthesis (Table S5) and the accelerated reduction of chlorophyll levels in mutant leaves under excess light stress compared to similarly-treated wild type plants. Others encode heat shock proteins (Hsps-p23like, sHsps, DNAJ, J8) and proteases (Clp serine-type endopeptidase, ATP-dependent Clp protease, OUT-like cysteine protease, MAPID Met-aminopeptidase), which function as molecular chaperones that suppress aggregation of proteins damaged by ROS, or to facilitate protein turnover (Table S7). Others are involved in either the synthesis or membrane-insertion of photosynthetic subunits, e.g. Hcf173 (At1g16720) is part of a thylakoid complex essential for the translation of psbA mRNA (encoding D1), and its induction in a mutant in which higher ^1O2 accumulation increases the rate of D1 turnover is consistent, and Alb3 (At2g28800) has a role in the insertion of a subset of light-harvesting complexes into thylakoids (Table S7). The induction of a lipase (At5g11650) and FAD7 (fatty acid desaturase 7, At3g11170) facilitates the production of jasmonic acid, an elicitor released by chloroplast membranes under photo-oxidative stress. EXECUTER2, whose role in coupling ^1O2 signalling from the chloroplast to nucleus has been described [53], was also up-regulated specifically in mutant plants.

The up-regulation of CIA2 (At5g57180) in the mutant after 2 and 24 h of excess light stress is particularly interesting because CIA2 is a transcription factor that specifically promotes the expression of genes encoding the translocon proteins Toc33 and Toc75, which are necessary for protein import into the chloroplast, and
chloroplast ribosomal proteins [107]. In addition, both Tic22 (At5g62650) and Tic55 (At2g24820) were up-regulated in the mutant, and these encode components of the translocon on the chloroplast inner envelope membrane. Taken together, these data suggest that 1O2 plays a key role in fulfilling the increased demand for protein import into the chloroplast during photo-oxidative stress, reflecting the higher rate of protein damage and turnover, by co-ordinately up-regulating both protein import and translation [107].

Conclusions
Xanthophylls accumulated within thylakoid membranes are compounds that participate actively to ROS scavenging and to the prevention of ROS synthesis. Our data provide evidences that xanthophylls modulate 1O2-dependent signals during the acclimation to high-light and low-temperature conditions. Indeed, in npq1lut2 double mutant 1O2 signalling facilitates the early fine-tuning of the expression of a group of genes encoding chloroplast proteins. This regulation does not correlate with the redox state of the PQ pool. Chloroplasts respond to these signals by a significant change in composition, resulting in rapid morphological and functional modifications. The response to 1O2 does not include cell death, even in the highly photosensitive npq1lut2 mutant.

Methods
Plant material and growth conditions
Arabidopsis thaliana plants, wild-type and T-DNA insertion mutants (Columbia ecotype) npq1 (At1G44446) and lut2 (At5G57030) were obtained from NASC collections [108]. Mutant npq1lut2 was obtained by crossing single mutant plants and selecting progeny by pigment analysis [47]. Plants were grown in pots filled with homogenous non-enriched compost and watered weekly with Coic-Lesaint nutrient solution [109]. They were grown in a growth chamber for 6 weeks under controlled conditions (~120 μmol photons m⁻² s⁻¹, 24°C, 8 h light/16 h dark, 70% relative humidity).

Micorarray experiments and statistical analysis of data
Before transcriptomic analysis, 6 weeks old plants were transferred from controlled conditions above described to a cold chamber (10°C) under low-light conditions (25 μmol photons m⁻² s⁻¹, continuous light) and maintained in this environment for 48 h in order to reduce the effect of the circadian clock [110]. Wild-type and npq1lut2 plants were then exposed to intense light (1000 μmol photon m⁻² s⁻¹) using 150 W halogen lamps (Focus 3, Prisma, Verona, Italy) at 10°C. Samples for transcriptome analysis were collected at 0, 2 and 24 h of excess light treatment, and rapidly frozen in liquid nitrogen prior to RNA extraction.

Three biological replicates per treatment were analyzed by using the Affymetrix GeneChip® Arabidopsis ATH1 Genome Array, which contains more than 22,500 probe sets representing 24,000 gene-specific tags (about 80 are chloroplast genes). For each biological repetition, RNA samples for a condition/genotype were obtained by extracting RNA from the entire rosette of eight pooled plants. Total RNA was quantified and then adjusted to a final concentration of 1 μg/μl. RNA integrity was assessed using the Agilent RNA 6000 nano kit and Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). RNA samples were processed following the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Inc., Santa Clara, CA). Scanned images were analyzed using the Gene Chip Operating Software v1.4. Expression analysis was carried out using default values. Quality control values, present calls, background, noise, scaling factor, spike controls, and the 3/5 ratios of glyceraldehyde-3-phosphate dehydrogenase (At3G04120) and actin (At5G09810) showed minimal variation between samples. Raw data files (CEL files) were background-adjusted and normalized, and gene expression values were calculated using the Robust Multichip Analysis (RMA) [111] algorithm implemented in the statistical package R2.3.1 (R foundation) with the dedicated “Affy” library [112].

The “Affy” library was used to run the MAS 5.0 algorithm on raw data to produce a detection call for each probe set. Because non-expressed genes (“absent”) represent experimental noise and can generate false positives, all the probe sets failing to show three “present calls” in at least one sample were removed from the analysis. Normalized data were imported into the GeneSpringGX7.3.1 (Agilent Technologies, Santa Clara CA) software for analysis. Each gene was normalized to the median of the measurements.

To identify differentially expressed probe sets, we applied a Welch t-test with Benjamini and Hochberg false discovery rate correction for multiple tests [113]. Differences in gene expression were considered to be significant when p < 0.05 and the ratio of expression levels was at least two-fold [114]. Clusters of genes with distinctive expression patterns were searched applying two algorithms: k-means [115] and QT (Quality Threshold) cluster analysis [116]. QT clustering algorithm groups genes into high quality clusters based on two parameters: “minimum cluster size” and “minimum correlation”. The minimum cluster size was set to 10 and minimum correlation to 0.75 (Pearson correlation). To determine if certain classes of genes were over-represented within selected clusters of genes compared to the functional categories on the entire array, the MIPS
Arabidopsis thaliana database (MatDB) (mips.gsf.de/projects/funcat) was employed [54].

Data from other experiments were obtained as additional data from published papers [36,85] or downloaded from the European Bioinformatics Institute [117]. For published microarray data comparing a test sample and a control sample, genes were considered to be differentially expressed when they showed a log2 ratio of either ≥1 or ≤-1 [7].

Quantitative real-time PCR (qRT-PCR)
Miroarray data were independently verified by qRT-PCR, using 3 μg total RNA from each sample. The RNA was reverse transcribed using an oligo(dT)18 primer with MoMLV Reverse Transcription Reagents (Promega) according to the manufacturer’s standard protocol. The reaction was incubated at 40°C for 10 min, then 45°C for 50 min, and then at 70°C for 15 min to inactivate the reverse transcriptase. The cDNA was quantified using a QbitTM fluorometer (Invitrogen), diluted and used for q-PCR amplifications with specific primers.

Each qRT-PCR was performed with SYBR Green fluorescence detection in a qPCR thermal cycler (ABI PRISM 7300, Applied Biosystems). Each reaction was prepared using 5 μl from a 0.2 ng/mL dilution of cDNA derived from the reverse transcription, 10 μl of SYBR Green PCR Master Mix (Applied Biosystems), and 0.5 μM forward and reverse primers in a total volume of 25 μl. The cycling conditions were: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curve analysis was performed to identify non-specific PCR products and primer dimers.

Primers were designed using Primer Express® Software for Real-Time PCR 3.0 (Applied Biosystems). Microarray data were validated by analyzing the expression profile at 0, 2 and 24 h excess light stress. The fold change between treated and untreated samples was compared to the transcriptomic data, and a linear correlation coefficient was calculated for each gene. The detailed qRT-PCR results for eight genes are shown in Additional file 2: Figure S2. Among 20 genes, 16 showed good correlation between qRT-PCR and microarray data (R² > 0.9).

ROS measurements
Steady-state accumulation of ROS in leaves was quantified using specific fluorogenic probes: singlet oxygen sensor green (SOSG), dichlorofluorescein (DCF) and proxylfluorescammine (proxF) (Molecular Probe, Eugene). SOSG is highly selective for 1O2, whose presence increases its 530 nm emission band [118]. DCF reacts with hydrogen peroxide (H2O2) and hydroxyl radicals (OH-), whereas proxF is selective for superoxide anions (O2-) and hydroxyl radicals, and their emission at 520 and 550 nm, respectively, increases upon exposure (Molecular Probe handbook). 6-weeks-old leaves were detached from plants grown at 120 μmol photons m⁻² s⁻¹, 24°C, 8 h light/16 h dark, kept at 10°C, 25 μmol photons m⁻² s⁻¹ for 48 hours. Leaves were infiltrated with the dye solution (SOSG 5 μM, DCF 1 mM and proxF 1 mM) and illuminated with strong red light (λ>600 nm, 1600 μmol m⁻² s⁻¹) at 10°C. We looked for increases in ROS-specific fluorescence to quantify ROS levels: SOSG (λex 480 nm, λemis 530 nm); DCF (λex 490 nm, λemis 525 nm); proxF (λex 420 nm, λemis 515 nm).

Extraction and measurements of metabolites
WT and npq1lut2 rosettes were pre-treated for 48 hrs at 10°C as above described, then were exposed to photooxidative conditions (1000 μmol photon m⁻² s⁻¹, 10°C, 16 h light/8 h dark). Leaves were harvested and immediately frozen in liquid nitrogen at the same time of the day over a 6-day stress period. Plant material was ground to a fine powder in liquid nitrogen and either used immediately for assays or stored at -80°C. Ascorbate and glutathione were extracted and assayed following the method developed by Queval and Noctor [119]. ATP and ADP were assayed as previously described [120]. Amino acids and sugars were extracted and quantified as described by [121].

In vivo fluorescence and NPQ measurements
Non-photochemical quenching of chlorophyll fluorescence (NPQ), maximum quantum efficiency of PSII (Fv/Fm) and photochemical quenching (qP) were measured with a PAM 101 fluorimeter (Walz, Effelterich, Germany) and were calculated according to [122]. Measurements were registered at the same hour every day over a 6-day-long stress treatment above described. For in vivo fluorescence measurements, leaves were illuminated for 25 min (1000 μmol photon m⁻² s⁻¹, 10°C) and photosynthetic parameters were determined during steady-state photosynthesis.

Pigment analysis
Pigments were extracted from whole leaves with 80% acetone (v/v), then separated and quantified by HPLC [10].

Membrane isolation and thylakoid protein separation
Unstacked thylakoids were isolated from dark-adapted leaves or leaves treated with intense light as previously described [123]. SDS-PAGE analysis was performed with the Tris-Tricine buffer system [124]. Non-denaturing Deriphat-PAGE was performed following the method developed by Peter and Thornber [125,126]. For the identification of oxidized proteins, polypeptides were transferred to nitrocellulose membrane and carbonylated
residues were identified by western blotting using the OxyBlot kit (Millipore). For immunoblot titration of CP47 (Psbc, PSIII inner antennae), LHCCI (Lhcb1, PSI outer antennae) and Psaa (PSI core complex), thylakoids corresponding to 0.5, 1, 2 and 4 μg of chlorophylls were separated by SDS-PAGE and the proteins detected by western blot with specific antibodies as described previously [20].

### Additional material

**Additional file 1: Tables describing WT and npq1lut2 transcriptome.**

**Additional file 2: Figures describing WT and npq1lut2 plant photosynthetic characterization, transcriptome analysis and transcriptome validation.**

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### Authors’ contributions

AA carried out the molecular genetic studies and drafted the manuscript; PC carried out metabolomic analysis; AA and ER participated in the biochemical and photosynthetic characterization of plants under photodynamic conditions, measurements of ROS and drafted the manuscript; PB carried out metabolomic analysis; AA, and ER participated in the RNA isolation, microarray experiments and statistical analysis of data, quantitative real-time qPCR, LC and RB conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

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### References

1. Barber J, Andersson B: Too Much of A Good Thing - Light Can be Bad for Photosynthesis. *Trends in Biochemical Sciences* 1992, 17:61-66.

2. Scheible WR, Gonzalez-Fontes A, Morcuende R, Lauender M, Geiger M, Glab J, Gojon A, Schulze ED, Stitt M: Tobacco mutants with a decreased number of functional nia genes compensate by modifying the diurnal regulation of transcription, post-transitional modification and turnover of nitrate reductase. *Plant Physiol* 1997, 106:304-319.

3. Desnos T: Root branching responses to phosphate and nitrate. *Current Opinion in Plant Biology* 2008, 11:82-87.

4. Munns R: Comparative physiology of salt and water stress. *Plant Cell and Environment* 2002, 25:239-250.

5. Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF: Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *Plant Journal* 1996, 16:433-442.

6. Lee JH, Hube A, Scholl F: Derepression of the Activity of Genetically-Engineered Heat-Shock Factor Causes Constitutive Synthesis of Heat-Shock Proteins and Increased Thermotolerance in Transgenic Arabidopsis. *Plant Journal* 1995, 8:605-612.

7. Lillo C, Lea US, Ruff P: Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. *Plant Cell and Environment* 2008, 31:587-601.

8. Lepiniec L, Debeaujon I, Routaboul JM, Baudry A, Pourcel L, Nesic N, Caboche M: Genetics and biochemistry of seed flavonoids. *Annual Review of Plant Biology* 2006, 57:405-430.

9. Dellapenna D, Posogni BV: Vitamin synthesis in plants: tocopherols and carotenoids. *Annual Review of Plant Biology* 2006, 57:711-738.

10. Haunaux M, Eymery F, Porfirio S, Rey F, Dornam P: Vitamin E protects against photoinduction and photooxidative stress in Arabidopsis thaliana. *Plant Cell* 2005, 17:9451-3469.

11. Dal Bosco C, Busconi M, Govoni C, Baldi P, Stanca MA, Croatti S, Basi R, Cattivelli L: cor gene expression in barley mutants affected in chloroplast development and photosynthetic electron transport. *Plant Physiology* 2003, 131:795-802.

12. Fernandez AP, Strand A: Retrograde signaling and plant stress: plastid signals initiate cellular stress responses. *Current Opinion in Plant Biology* 2008, 11:509-513.

13. Svensson JT, Croatti C, Campoli C, Bassi R, Stanca MA, Close TJ, Cattivelli L: Transcriptome analysis of cold acclimation in barley Albina and Xantha mutants. *Plant Physiology* 2006, 141:257-270.

14. Bassi R, Caffari S: Lhcb proteins and the regulation of photosynthetic light harvesting function by xanthophylls. *Photosynthesis Research* 2000, 64:243-256.

15. de Bianchi S, Ballottari M, Dall’Osto L, Bassi R: Regulation of plant light harvesting by thermal dissipation of excess energy. *Biochimica et Biophysica Acta* 2010, 1801:651-660.

16. Horton P, Johnson MP, Perez-Bueno ML, Kiss AZ, Ruban AV: Photosynthetic acclimation: Does the dynamic structure and macro-organisation of photosystem II in higher plant grana membranes regulate light harvesting states? *Fems Journal* 2008, 275:1069-1079.

17. Betterle N, Ballottari M, Zorzan S, de Bianchi S, Cazzaniga D, Dall’Osto L, Morosinotto T, Bassi R: Light-induced Dissociation of an Antenna Heteroligomer Is Needed for Non-photochemical Quenching Induction. *Journal of Biological Chemistry* 2008, 283:15323-15326.

18. Molinavina Y, Grouneva I, Lambrev PH, Leppett B, Goss R, Wilhelm C, Holzwarth AR: Ultrafast fluorescence study on the location and mechanism of non-photochemical quenching in diatoms. *Biochimica et Biophysica Acta-Photoenergetics* 2009, 1787:1189-1197.

19. Anderson JM, Choe WS, Park YK: The grand design of photosynthesis: Acclimation of the photosynthetic apparatus to environmental cues. *Photosynthesis Research* 1995, 46:129-139.

20. Ballottari M, Dall’Osto L, Morosinotto T, Bassi R: Contrasting behavior of higher plant photosystem I and II antenna systems during acclimation. *Journal of Biological Chemistry* 2007, 282:8947-8958.

21. Keegstra K, Cline K: Protein import and routing systems of chloroplasts. *Plant Cell and Environment* 1999, 11:557-570.

22. Leister D: Chloroplast research in the genomic age. *Trends in Genetics* 2003, 19:47-56.

23. Anderson LE, Levin DA: Chloroplast aldolase is controlled by a nuclear gene. *Plant Physiology* 1970, 46:819-820.

24. Batschauer A, Mosinger E, Kreuzk D, Dott I, Apel K: The implication of a plastid-derived factor in the transcriptional control of nuclear genes encoding the light-harvesting chlorophyll a/b protein. *European Journal of Biochemistry* 1986, 154:625-634.

25. Nott A, Hung HS, Koussevitzky S, Chory J: PLASTID-TO-NUCLEUS RETROGRADE SIGNALING. *Annual Review of Plant Biology* 2006, 57:739-759.

26. Woodson JD, Chory J: Coordination of gene expression between organelar and nuclear genomes. *Nature Review Genetics* 2008, 9:383-395.

27. Kiene T, Voigt C, Leister D: Plastid signalling to the nucleus: messengers still lost in the mist? *Trends in Genetics* 2009, 25:185-190.

28. VanDaele WA, Zimmermann P, Rombauts S, Vandenabeele S, Langebartels C, Gruissem W, Inze D, Van Breusegem F: Genome-wide analysis of hydrogen peroxide-regulated gene expression in Arabidopsis reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. *Plant Physiology* 2005, 139:806-821.

29. op den Camp RGL, Przybyla D, Ochsenbein C, Lalić C, Kim CH, Danon A, Wagner D, Heide E, Gobel C, Feussner I, et al: Rapid induction of distinct stress responses after the release of singlet oxygen in arabidopsis. *Plant Cell* 2003, 15:2320-2332.
30. Laloi C, Stachowiak M, Pers-Kamczyk E, Warzych E, Murga I, Apel K. Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in Arabidopsis thaliana. Proceedings of the National Academy of Sciences of the United States of America 2007, 104:672-677.

31. Strand A, Asami T, Alonso J, Ecker JR, Chory J. Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX. Nature 2003, 421:79-83.

32. Pfannschmidt T, Nilsson A, Allen JF. Photosynthetic control of chloroplast gene expression. Nature 1999, 397:625-628.

33. Moulin M, McCormac AG, Terry MJ, Smith AG. Tetrapyrrole profiling in Arabidopsis seedlings reveals that retrograde plastid nuclear signaling is not due to Mg-protoporphyrin IX accumulation. Proc Natl Acad Sci USA 2008, 105:15178-15183.

34. Fey V, Wagner R, Brautigam K, Wirtz M, Hell R, Dietzmann A, Leister D, Haehnel W, Cattivelli L, Morosinotto T, Bassi R. Photosynthetic antenna size in higher plants is controlled by the plastocyanine redox state at the post-transcriptional rather than transcriptional level. J Biol Chem 2007, 282:29451-29460.

35. Gadjev I, Vanderauwera S, Gecchey TS, Laloi C, Minkov N, Shulavev V, Apel K, Inze D, Mittler R, Van Breusegem F. Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. Plant Physiol 2006, 141:436-445.

36. Miller G, Shulavev V, Mittler R. Reactive oxygen signaling and abiotic stress. Physiol Plant 2008, 133:481-489.

37. Oelmüller R, Pfannschmidt T. Chloroplast-mediated regulation of nuclear genes in Arabidopsis. J Biol Chem 2005, 280:5318-5328.

38. Frigerio S, Campoli C, Zorzan S, Fantoni LI, Crosatti C, Drepper F, Oelmueller R, Cattivelli L, Morosinotto T, Bassi R. Photosynthetic control of chloroplast antenna size for efficient chlorophyll triplet quenching in the major LHCII antenna complexes of photosystem II - Role of individual xanthophylls in chlorophyll triplet quenching. Trends in Plant Science 2008, 13:793-804.

39. Potocky M, Jones MA, Bezvoda R, Smirnoff N, Zarsky V. Reactive oxygen species produced by NADPH oxidase are involved in pollen tube signaling but not due to Mg-protoporphyrin IX accumulation. Proc Natl Acad Sci USA 2008, 105:15178-15183.

40. Fey V, Wagner R, Brautigam K, Wirtz M, Hell R, Dietzmann A, Leister D, Cattivelli L, Morosinotto T, Bassi R. Chloroplast to nucleus of Arabidopsis thaliana. J Biol Chem 2005, 280:5318-5328.

41. Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Oelmüller R, Cattivelli L, Morosinotto T, Bassi R. Photosynthetic antenna size in higher plants is controlled by the plastocyanine redox state at the post-transcriptional rather than transcriptional level. J Biol Chem 2007, 282:29451-29460.

42. Pfannschmidt T, Nilsson A, Allen JF. Photosynthetic control of chloroplast gene expression. Nature 1999, 397:625-628.

43. Moulin M, McCormac AG, Terry MJ, Smith AG. Tetrapyrrole profiling in Arabidopsis seedlings reveals that retrograde plastid nuclear signaling is not due to Mg-protoporphyrin IX accumulation. Proc Natl Acad Sci USA 2008, 105:15178-15183.

44. Fey V, Wagner R, Brautigam K, Wirtz M, Hell R, Dietzmann A, Leister D, Oelmüller R, Pfannschmidt T. Retrograde plastid redox signals in the expression of nuclear genes for chloroplast proteins of Arabidopsis thaliana. J Biol Chem 2005, 280:5318-5328.

45. Frigerio S, Campoli C, Zorzan S, Fantoni LI, Crosatti C, Drepper F, Haehnel W, Cattivelli L, Morosinotto T, Bassi R. Photosynthetic antenna size in higher plants is controlled by the plastocyanine redox state at the post-transcriptional rather than transcriptional level. J Biol Chem 2007, 282:29451-29460.

46. Gadjev I, Vanderauwera S, Gecchey TS, Laloi C, Minkov N, Shulavev V, Apel K, Inze D, Mittler R, Van Breusegem F. Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. Plant Physiol 2006, 141:436-445.

47. Miller G, Shulavev V, Mittler R. Reactive oxygen signaling and abiotic stress. Physiol Plant 2008, 133:481-489.

48. Oelmüller R, Pfannschmidt T. Chloroplast-mediated regulation of nuclear genes in Arabidopsis. J Biol Chem 2005, 280:5318-5328.

49. Potocky M, Jones MA, Bezvoda R, Smirnoff N, Zarsky V. Reactive oxygen species produced by NADPH oxidase are involved in pollen tube signaling but not due to Mg-protoporphyrin IX accumulation. Proc Natl Acad Sci USA 2008, 105:15178-15183.

50. Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Oelmüller R, Cattivelli L, Morosinotto T, Bassi R. Photosynthetic antenna size in higher plants is controlled by the plastocyanine redox state at the post-transcriptional rather than transcriptional level. J Biol Chem 2007, 282:29451-29460.

51. Pfannschmidt T, Nilsson A, Allen JF. Photosynthetic control of chloroplast gene expression. Nature 1999, 397:625-628.
The protective functions of carotenoid and nonphotochemical quenching of chlorophyll fluorescence is due to changes in Photosystem II antenna size and stability. Biochimica et Biophysica Acta-Bioenergetics 2002, 1533:309-319.

82. Kim C, Meskauskiene R, Apel K, Laloi C: Dall Havaux M, Kloppstech K: The protective functions of carotenoid and nonphotochemical quenching in Arabidopsis mutants. Proceedings of the National Academy of Sciences of the United States of America 1998, 95:13324-13329.

83. Havaux M, Dall Ootto L, Bassi R: Zeaxanthin has enhanced antioxidant capacity with respect to all other xanthophylls in Arabidopsis leaves and functions independent of binding to PSI antenna(II)(CW). Plant Physiology 2007, 145:1506-1520.

84. Niyogi KK: Photoprotection revisited: Genetic and molecular approaches. Annual Review of Plant Physiology and Plant Molecular Biology 1999, 50:333-359.

85. Rinalducci S, Pedersen JZ, Zolla L: Genetic mutants of Arabidopsis thaliana: altered nonphotochemical quenching of singlet oxygen signaling in Chlamydomonas reinhardtii. FEBS Letters 2005, 575:555-560.

86. Ledford HK, Baroli I, Shin JW, Fischer BB, Eggen RI, Niyogi KK: Comparative profiling of lipid-soluble antioxidants and transcripts reveals two phases of photo-oxidative stress in a xanthophyll-deficient mutant of Chlamydomonas reinhardtii, Mutat Genet Genomics 2004, 272:470-479.

87. Fischer BB, Krieger-Liszkay A, Hideg E, Snyrychova I, Wiesendanger M, Eggen RI: Role of singlet oxygen in chloroplast to nucleus retrograde signaling in Chlamydomonas reinhardtii. FEBS Lett 2007, 581:5555-5560.

88. Kim C, Meskauskiene R, Apel K, Laloi C: No single way to understand singlet oxygen signalling in plants. EMBO Rep 2008, 9:435-439.

89. Cazzonelli C, Popson BJ: Source to sink regulation of carotenoid biosynthesis in plants. Trends Plant Sci 2010.

90. Ichihashi A, Richly E, Noutsos E, Salamin F, Leister D: Analysis of 101 nuclear transcriptomes reveals 23 distinct regulons and their relationship to metabolism, chromosomal gene distribution and co-ordination of nuclear and plastid gene expression. Gene 2005, 344:34-41.

91. Kharel A, Chaturvedi S, Cser M: Transcriptome analysis of the Rpf F-box-mediated response to pathogen elicitor. Plant Physiol 2009, 150:1006-1016.

92. Alberte A, Ainsworth EA: Altered xanthophyll metabolism, chromosomal gene distribution and co-ordination of transcriptomes reveals 23 distinct regulons and their relationship to metabolism, chromosomal gene distribution and co-ordination of nuclear and plastid gene expression. Gene 2005, 344:34-41.

93. Bailey S, Walters RG, Jansson S, Horton P: The protective functions of carotenoid and nonphotochemical quenching of singlet oxygen signaling in plants. FEBS Letters 2007, 553:119-124.

94. Scho N, Kropf-Fischler G, Schrodta M, Beck CF: A reporter system for the individual detection of hydrogen peroxide and singlet oxygen: its use for the assay of reactive oxygen species produced in vivo. Plant Journal 2007, 50:475-487.

95. de Torres M, Sanchez P, Fernandez-Delmond I, Grant M: Expression profiling of the host response to bacterial infection: the transition from basal to induced defence responses in RPM1-mediated resistance. Plant J 2003, 33:663-676.

96. Havaux M, Kloppstech K: The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in Arabidopsis npq and tt mutants. Plant Cell 2001, 213:933-941.

97. Alboresi et al: Analysis of 101 nuclear transcriptomes reveals 23 distinct regulons and their relationship to metabolism, chromosomal gene distribution and co-ordination of nuclear and plastid gene expression. Gene 2005, 344:34-41.

98. Caffarri S, Frigerio S, Olivieri E, Righetti PG, Bassi R: Differential accumulation of Lbch gene products in thylakoid membranes of Zea mays plants grown under contrasting light and temperature conditions. Photosynthesis 2005, 5:758-768.

99. Delatorre WR, Beuley KD: Acclimation of Barley to Changes in Light-Intensity - Photosynthetic Electron-Transport Activity and Components. Biosynthesis Research 1990, 2:127-136.

100. Li XP, Bjorkman O, Shih C, Grossman AR, Rosenquist M, Jansson S, Niyogi KK: An pigment-binding protein essential for regulation of photosynthetic light harvesting. Nature 2000, 403:391-395.

101. Tsvetkova-Choveloua T, Franck F, Alavady A, Dall Ootto L, Cariere F, Bassi R, Grimm B, Nussaume L, Havaux M: The light stress-induced protein ELIP2 is a regulator of chlorophyll synthesis in Arabidopsis thaliana. Plant J 2007, 50:795-809.

102. Jansson S: A guide to the Lhc genes and their relatives in Arabidopsis. Trends in Plant Science 1999, 4:236-240.

103. Cassar A, Fregoni S, Ollivier E, Righetti PG, Bassi R: Differential accumulation of Lbch gene products in thylakoid membranes of Zea mays plants grown under contrasting light and temperature conditions. Photosynthesis 2005, 5:758-768.

104. Lee KP, Kim C, Lee DW, Apel K: TIGORDA d, required for regulating the light-regulated changes in photosynthetic light harvesting. FEBS Lett 2007, 581:879-888.

105. Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen HM, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al: Genome-wide insertion mutagenesis of Arabidopsis thaliana. Science 2003, 301:653-657.

106. Coic Y, Lesaint C: Dissection of oligonucleotide array probe level data. Biostatistics 2007, 8:297-304.

107. Sun CW, Huang YC, Chang HY: Tetrapyrrole biosynthesis in higher plants. Photosynthesis Research 1999, 50:333-359.

108. Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen HM, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al: Genome-wide insertion mutagenesis of Arabidopsis thaliana. Science 2003, 301:653-657.

109. Kreps JA, Simon AE: Environmental and genetic effects on circadian clock-regulated gene expression in Arabidopsis. Plant Cell 1997, 9:297-304.

110. Itzinger RA, Hobbis B, Collin F, Beazer-Bradley YD, Antonellis KJ, Scherf U, Speed TP: Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003, 4:249-264.

111. Irizarry RA, Hobbs B, Collin F, Beazer-Bradley YD, Antonellis KJ, Scherf U, Speed TP: Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003, 4:249-264.

112. Gauthier L, Cope L, Bolstad BM, Irizarry RA: affy-analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 2004, 20:317-319.

113. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I: Controlling the false discovery rate in behavior genetics research. Behav Brain Res 2001, 125:279-284.
115. Soukas A, Cohen P, Socci ND, Friedman JM: Leptin-specific patterns of gene expression in white adipose tissue. Genes & Development 2000, 14:963-980.

116. Heyer LJ, Kruglyak S, Yooseph S: Exploring expression data. Identification and analysis of coexpressed genes. Genome Research 1999, 9:1106-1115.

117. Parkinson H, Kapushesky M, Shojatalab M, Abeygunawardena N, Coulson R, Farne A, Holloway E, Kolesnykov N, Lilja P, Lukk M, et al: ArrayExpress - a public database of microarray experiments and gene expression profiles. Nucleic Acids Research 2007, 35:D747-D750.

118. Flors C, Fryer MJ, Waring J, Reeder B, Bechtold U, Mullineaux PM, Nonell S, Wilson MT, Baker NR: Imaging the production of singlet oxygen in vivo using a new fluorescent sensor, Singlet Oxygen Sensor Green. J Exp Bot 2006, 57:1725-1734.

119. Queval G, Noctor G: A plate reader method for the measurement of NAD, NADP, glutathione, and ascorbate in tissue extracts: Application to redox profiling during Arabidopsis rosette development. Anal Biochem 2007, 363:58-69.

120. Gibon Y, Vieigolas H, Tiesen A, Geigenberger P, Stitt M: Sensitive and high throughput metabolite assays for inorganic pyrophosphate, ADP-Glc, nucleotide phosphates, and glycolytic intermediates based on a novel enzymic cycling system. Plant J 2002, 30:221-235.

121. Carllo P, Mastroiannardo G, Nacca F, Fuggi A: Nitrate reductase in durum wheat seedlings as affected by nitrate nutrition and salinity. Functional Plant Biology 2005, 32:209-219.

122. Van Kooten Q, Snell JHF: The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynth Res 1990, 25:147-150.

123. Bassi R, Rigoni F, Barbatto R, Giacometti GM: Light-harvesting chlorophyll a/b proteins (LHCCI) populations in phosphorylated membranes. Biochimica et Biophysica Acta (BBA)-Bioenergetics 1988, 936:29-38.

124. Schagger H, von Jagow G: Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem 1991, 199:223-231.

125. Peter GF, Thomber JP: Biochemical Composition and Organization of Higher-Plant Photosystem-II Light-Harvesting Pigment-Proteins. J Biol Chem 1991, 266:16745-16754.

126. Garcion C, Baillanger R, Fournier T, Pasquier J, Schnitzer MA, Gabriel JP, Metraux JP: FiRe and microarrays: a fast answer to burning questions. Trends Plant Sci 2006, 11:320-322.

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