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Photosynthetic Antenna Size in Higher Plants Is Controlled by the Plastoquinone Redox State at the Post-transcriptional Rather than Transcriptional Level*[S]

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We analyze the effect of the plastoquinone redox state on the regulation of the light-harvesting antenna size at transcriptional and post-transcriptional levels. This was approached by studying transcription and accumulation of light-harvesting complexes in wild type *versus* the barley mutant *viridis zb63*, which is depleted in photosystem I and where plastoquinone is constitutively reduced. We show that the mRNA level of genes encoding antenna proteins is almost unaffected in the mutant; this stability of messenger level is not a peculiarity of antenna-encoding genes, but it extends to all photosynthesis-related genes. In contrast, analysis of protein accumulation by two-dimensional PAGE shows that the mutant undergoes strong reduction of its antenna size, with individual gene products having different levels of accumulation. We conclude that the plastoquinone redox state plays an important role in the long term regulation of chloroplast protein expression. However, its modulation is active at the post-transcriptional rather than transcriptional level.

Sunlight is the only energy source for plants: light is absorbed by chlorophylls and carotenoids bound to the pigment-protein complexes composing photosystems I and II (PSI and -II),[2] and it is converted into chemical energy. Both photosystems are composed by two distinct moieties: (i) a core complex, responsible for charge separation and for the first steps of the electron transport, and (ii) an antenna system that increases light-harvesting capacity. In photosynthetic eukaryotes, the antenna system is composed by the members of a multigenic family called *Lhc* (light-harvesting complexes) (1). To avoid the over-accumulation of excitation energy in photosystems, light-absorption capacity needs to be related to the electron transport rate. To this purpose, the number of antenna proteins associated to photosystems is regulated according to the environmental conditions (2). More recently it was shown that the regulation of antenna size is restricted to PSII, whereas PSI-LHCI stoichiometry remains constant (3). Besides antenna size regulation, other mechanisms, like the state transition, the adjustment of PSI/PSII ratio, and regulation of carotenoid biosynthesis, are also involved in the plant's long term acclimation to different conditions (4). The limiting step of electron transport chain from PSII to PSI is the oxidation of plastoquinol (PQ) by cytochrome b_6f (5); the PQ pool redox state depends on PSII excitation pressure, on the donor side, on the PSI capacity as acceptor, and on the rate of PQ reduction by cyclic electron transport (6). Thus, the ratio between reduced and oxidized plastoquinone is considered a good indicator of the balance between light absorption and electron transport rate: PQ over-reduction, in fact, suggests that electron transport is unable to use all the energy absorbed by PSII, where accumulation of excited states easily leads to the formation of harmful reactive oxygen species (ROS) (7). Thus, it is not surprising that the PQ pool redox state has been identified as a key signal modulating the expression of photosynthesis-related genes in response to light conditions (8). Studies on algae like *Dunaliella tertiolecta* and *Chlamydomonas reinhardtii* (9) showed that PQ redox state influences dramatically the expression level of photosynthetic genes. In particular, the largest effects were observed in the genes encoding members of the *lhc* family, whose expression is strongly repressed upon exposure to strong light. Besides plastoquinone, also thioredoxin, glutathione, and ROS were proposed as signals for regulation of gene expression (for a review see Ref. 10). The picture is even more complicated in the case of multicellular organisms like vascular plants, where the redox signal represents, in each cell, the state of 80–100 chloroplasts rather than a single organelle (10, 11).

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[2] The abbreviations used are: PSI, -II, photosystems I and II; α(β)-DM, *n*-dodecyl-α(β)-D-maltoside; Lhca (b), light-harvesting complex of PSI (II); LHCI, antenna complex of PSI; PQ, plastoquinone; ROS, reactive oxygen species; WT, wild type; Rubisco, ribulose-bisphosphate carboxylase/oxygenase; RT, reverse transcription; MS, mass spectrometry; HPLC, high-performance liquid chromatography; SAM, significance analysis on microarray; E, einstein.

Despite these studies, the role of gene expression regulation in long term acclimation of the photosynthetic apparatus to different light conditions is still unclear. In fact, while the regulation of the polypeptide level was reported by several laboratories (2, 12), data for mRNA level are still incomplete. This lack of information is probably due to the fact that treatments with electron transport inhibitors, like DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and DBMIB (2,5-3-methyl-6-isopropyl-*p*-benzoquinone), widely used to simulate different PQ redox conditions, cannot be prolonged indefinitely. In addition, it was recently shown that the repression of *lhc* gene transcription induced by strong illumination is reversible after ~24 h, even if the light treatment is maintained, thus suggesting that regulation mechanisms could be different on the longer term with respect to short term stress (13).

In this work we analyzed the regulation of antenna system in barley mutant *viridis zb63*, which has a chronically over-reduced PQ pool even if grown at very low light intensities (14), to obtain new insights on the role of plastoquinone redox state both on gene and protein long term regulation. The use of this mutant allows the study of a long term PQ over-reduction without the need of inhibitor treatments. *Viridis zb63* was recently shown to have a PSII antenna system reduced to its minimum level: a dimeric PSII reaction center core surrounded by one copy of each monomeric Lhcb4, Lhcb5, and the trimeric light-harvesting complex II (14). We found that, despite these strong effects on Lhc protein accumulation, transcription of corresponding genes was substantially unaltered in the mutant, as compared with WT, whose PQ is oxidized in low light (14). Stability of transcription level was not restricted to *lhc* genes, but it was observed for most photosynthesis-related genes. The PQ pool redox state, thus, plays a role in long term regulation of the expression of antenna proteins mainly at the post-transcriptional level, whereas its effect on gene transcription is relevant only a few hours after alteration in light conditions.

EXPERIMENTAL PROCEDURES

Genetic Material and Growth Conditions—A spring barley (*Hordeum vulgare* cv Bonus) and a mutant lacking PSI, *viridis zb63*, obtained by chemical mutagenesis on the cv Bonus genetic background, were used. Plants were grown for 9 days at 23 °C with 8-h photoperiod and 100 μmol m^{-2} s^{-1} of light. For RNA extraction leaves, in the middle of the light period, were harvested and immediately frozen into liquid nitrogen. This experiment was conducted three times to obtain three independent biological replicates.

RNA Isolation and Array Hybridization—Total RNA was prepared using TRIzol reagent according to the method published at the *Arabidopsis* functional genomics consortium website and further cleaned using an RNeasy Minikit (Qiagen, Valencia, CA) following the manufacturer's instructions. Disposable RNA chips (Agilent RNA 6000 Nano LabChip kit) were used to determine the concentration and purity/integrity of RNA samples using Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

Double-stranded cDNA was synthesized starting from 5 μg of total RNA using the GeneChip® Expression 3′-amplification reagents-One Cycle cDNA Synthesis Kit (Affymetrix) according to the standard protocol. This double-stranded cDNA was used as template to generate biotin-labeled cRNA from an *in vitro* transcription reaction (IVT), using the GeneChip® Expression 3′-amplification reagents-For IVT Labeling Kit (Affymetrix) following manufacturer instructions. The resulting biotin-labeled cRNA was fragmented into strands of 35–200 bases in length following Affymetrix protocols, and 15 μg of fragmented target cRNA was hybridized on an Affymetrix Barley Genome Array according to the standard protocol (16 h at 45 °C with rotation in the Affymetrix GeneChip Hybridization Oven 640). The arrays were washed and stained on an Affymetrix Fluidics Station 450 according to the standard protocol, then scanned on a Affymetrix GeneChip Scanner 3000.

Data Analysis—Scanned images were analyzed using the Gene Chip Operating Software 1.4 (Affymetrix). Expression analysis was done using default values. Quality control values, present calls, background, noise, scaling factor, spike controls, and the 3′/5′ ratios of glyceraldehyde-3-phosphate dehydrogenase and tubulin showed low variation between samples (15). Raw data files (CEL files) were background-adjusted and normalized, and gene expression values were calculated using Robust Multichip Analysis (RMA) algorithm implemented in the statistical package R 2.3.1 (R foundation) with the dedicated affy library (R foundation). Normalized data were imported into the Genespring GX 7.3.1 (Silicon Genetics) software for analysis. Each gene was normalized to the median of the measurements. We used detection calls (present) as an initial filtering step: a present-absent filter was applied to remove probe sets with less than two present cells. A second filter was applied to eliminate probe sets with normalized signal value between 0.66 and 1.5, to evaluate significant the -fold change.

In the comparison (wild type *versus viridis zb63*) the baseline was set as wild type; genes with at least a 2-fold change value in the comparison were further considered. Lists of differentially expressed genes were analyzed for statistical significant changes by a Welch *t* test (analysis of variance) with the Benjamini and Hochberg false discovery rate correction; the false discovery rate-adjusted *p* value cutoff was set to 0.05. For the functional probe sets annotation BLAST search results were exported from HarvEST 1.50.

RT-PCR—RT-PCR was performed both on nuclear and chloroplast genes. For each reaction, first strand synthesis was done on 0.5 μg of total RNA (treated with DNase I) using 200 units of Moloney murine leukemia virus reverse transcriptase (Sigma) and a poly(T) primer, for nuclear genes, or a gene-specific primer, for chloroplast sequences. This synthesis was followed by RNase H treatment.

Transcripts were then amplified using the following primers: nuclear genes: *lhca3* (Contig1815_at), 5′-GCAGTACTTCCTGGGTCTCGAGAAG-3′ and 5′-GCAATGCACTAATTTTCATAGCACGATA-3′; *lhcb1* (Contig422_at), 5′-AGTTGCTTCGAGCAGCCCGTGGTA-3′ and 5′-AGTGGGCCACCGGCAATGCGGTA-3′; *lhcb6* (Contig1523_at), 5′-AAGCGATGCCGAGTTCATCAACCCGT-3′ and 5′-TACCCTTGCTCGCCGGTGAAGTTG-3′; *psaH* (Contig2250_at), 5′-CAGCTCGACCGCAATGGCGTCGCT-3′ and 5′-CGCGGCGAAGGTGTTGAAGAACTTGC-3′; ζ-carotene desaturase (Contig HW04B18_u_s_at), 5′-CTCAAGGTCGCCATCATAGGTGCC-3′ and 5′-GATCAACAAGAGCTTGAACAACTGGGC-3′; glycerophosphoryl diesterase (Contig 8049_at),

5′-TCGACTACGTCGAGTTCGACGTGC-3′ and 5′-AGTTTTCGCATGAGTTGCGCAGCATCG-3′; and copper-binding protein (Contig 17974_at), 5′-CCTTTTCACTTCAGAGCTTCACTGTTC-3′ and 5′-AGCACCTCATAGGGCGTCACGTC-3′. The chloroplastic genes were: *psaA* (HV_CEa0013J19f_at), 5′-CTTTATCCTAGTTTTGCCGAAGGAGCA-3′ and 5′-GGTATGGATAGGGAGGCATAGAATACA-3′; *psaC* (HV_CEb0010M11f_at), 5′-GTCTCTTGGCTCTTTTCACGCTTTCT-3′ and 5′-CATGCTACGGGTTGTTTCAGGCCCT-3′; *psbA*, 5′-TCCCTATTCAGTGCTATGCATGGTTCC-3′ and 5′-TCAACAGCAGCTAAGTCTAGAGGGAAG-3′; and *psbD*, 5′-GATGGGAGTTGCCGGAGTATTAGGC-3′ and 5′-CATGAGGCTGATCCTGAGCTGCCAT-3′.

The transcripts were amplified from 50 ng of cDNA as template and 2.5 units of TaqDNA polymerase (Sigma) using the followings cycles: 94 °C for 30 s, annealing for 30 s, 72 °C for 40 s, followed by a final extension step at 72 °C for 1 min. To highlight the exponential phase, the amplification was stopped after 24, 26, and 28 cycles, and 5 μl for each gene was collected. RT-PCR reactions were performed with primers designed on gene coding with β-actin used as the standard. Amplification products were separated on 2% agarose gel.

Thylakoid Isolation and Quantification—From 9-day-old plants, thylakoids were isolated as in a previous study (16). After the isolation, the membranes were further purified through a centrifugation on a sucrose step gradient: thylakoids were resuspended in 1.9 M sucrose, 25 mM Hepes, pH 7.5, 10 mM EDTA, and protease inhibitors then stratified at the bottom of a step gradient made in SW28 tubes with 1.3 M and 1.14 M sucrose solutions. After 1-h centrifugation at 27,000 rpm, the big green band containing purified thylakoids was recovered from the interface of the step gradient. Thylakoids were quantified both through chlorophyll absorption spectra and using a BCA assay (Sigma-Aldrich) for total protein content.

Proteomic Analysis—Proteins from thylakoids were prepared for isoelectric focusing as follows: first, proteins were de-phosphorylated by treating with calf intestine phosphatase) for 1 h at 37 °C. Then, after the precipitation with 14 volumes of tributylphosphate:acetone:methanol (1:12:1, v/v), proteins were resuspended using 7 M urea, 2 M thiourea, 40 mM Tris, 1% amidosulfobetaine-14, 1% carrier ampholyte, pH 3–8, reduced with 5 mM tributyl phosphine, and alkylated with 10 mM acrylamide.

Protein solution obtained was then used for passive re-hydration of the immobilized pH gradient strip (pH range 3–8, from Bio-Rad), then focused (3 h with linearly increasing voltage till 5 KV, then 5 KV till VH reach 65,000). The second dimension was performed with a gel system described before (17) with acrylamide gradient 12–18%. Gels were stained with Sypro Ruby (Sigma-Aldrich), and images for quantitative analysis with PDQuest software where acquired with a Versa-Doc detection system (Bio-Rad). Spots where cut from gels for MS analysis: the proteins from punched out spots were destained, alkylated with 55 mM iodoacetamide after reduction with 10 mM dithiothreitol, and digested in gel with trypsin at 37 °C overnight, and the peptides were extracted with 1% formic acid. For desalting, the solution was transferred to a nano-HPLC column and finally eluted from a 75-μm, 70-mm column with reversed-phase C18 material with a gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid. The mass spectrometer was a LTQ-FT (Thermo-Finnigan) with a linear ion trap and an ion cyclotron with a 7-Tesla magnet.

Lhcb1 Cloning and Expression—Sequences for different Lhcb1 where obtained from a cDNA library, amplified, and cloned in a pQE50His vector, using BamHI and HindIII as restriction sites (18). Transformed *Escherichia coli* cells (SG13009) where grown for 3 h at 37 °C then induced for 6 h with 1 mM isopropyl 1-thio-β-D-galactopyranoside; the recombinant proteins where purified as inclusion bodies following the protocol from Nagai and colleagues (19).

Reconstitution Procedure—The pigment-protein complexes refolding *in vitro* were performed as described before (20) with the following modifications: the 1.1-ml reconstitution mixture contained 420 μg of apoprotein, 240 μg of chlorophylls, and 60 μg of carotenoids. The chlorophyll *a*/*b* ratio of the pigment mixture was 2.3. All pigments used were purified from spinach thylakoids.

Analysis of Reconstituted Complexes—High performance liquid chromatography analysis was performed as described before (21). Chlorophyll to carotenoid ratio and chlorophyll *a*/*b* ratio were measured independently by fitting the spectrum of acetone extracts with the spectra of individual purified pigments (22). The absorption spectra at room temperature were recorded using a SLM-Aminco DK2000 spectrophotometer, in 10 mM HEPES, pH 7.5, 0.5 M sucrose, and 0.06% *n*-dodecyl-β-D-maltoside. The wavelength sampling step was 0.4 nm, scan rate 100 nm/min, and optical path length 1 cm.

Fluorescence emission spectra were measured using a Jasco FP-777 spectrofluorometer and were corrected for instrumental response. For emission spectra, samples were excited at 440, 475, and 500 nm with spectral bandwidths of 5 nm (excitation) and 3 nm (emission). For excitation spectra, emission was recorded at 685 nm, with bandwidths of 3 and 5 nm for excitation and emission, respectively. Chlorophyll concentration was ~0.02 μg/ml in 10 mM HEPES, pH 7.5, and 0.03% *n*-dodecyl-β-D-maltoside.

Western Blot Titration—For protein quantity titration using Western blot assay, thylakoids were loaded on minigels using the system described in a previous study (17), in four different dilutions to achieve a good reproducibility and avoid saturation effects. Different samples were quantified for their protein content using a BCA assay. For *viridis zb63*, on gels for Lhca3, Lhca4, Lhcb1, Lhcb4, Lhcb5, Lhcb6, PsbB, and PsbC, assays were loaded 11, 22, 33, and 44 μg of total proteins, whereas for Lhcb3, PsaE, and PsaF the protein amount was reduced to 1.2, 2.2, 4.4, and 8.8 μg of proteins. Similarly, for *viridis 115* on gels for Lhcb4 and Lhcb6, assays were loaded 4, 8, 12, and 16 μg of proteins, whereas for LHCII the amounts were 0.4, 0.8, 1.6, and 3.2 μg of proteins. One microgram of protein corresponded to 0.10 μg of Chls, 0.04, 0.06 per WT, *Viridis Zb63* and *115*, respectively. The results of the antibody reaction were acquired with a scanner and analyzed by densitometry using Gel-Pro Analyser 3.1.

Non-denaturing Deriphat-PAGE—Non-denaturing Deriphat-PAGE was performed as in a previous study (23) with the following modifications: the stacking gel had 3.5% (w/v) acrylam-

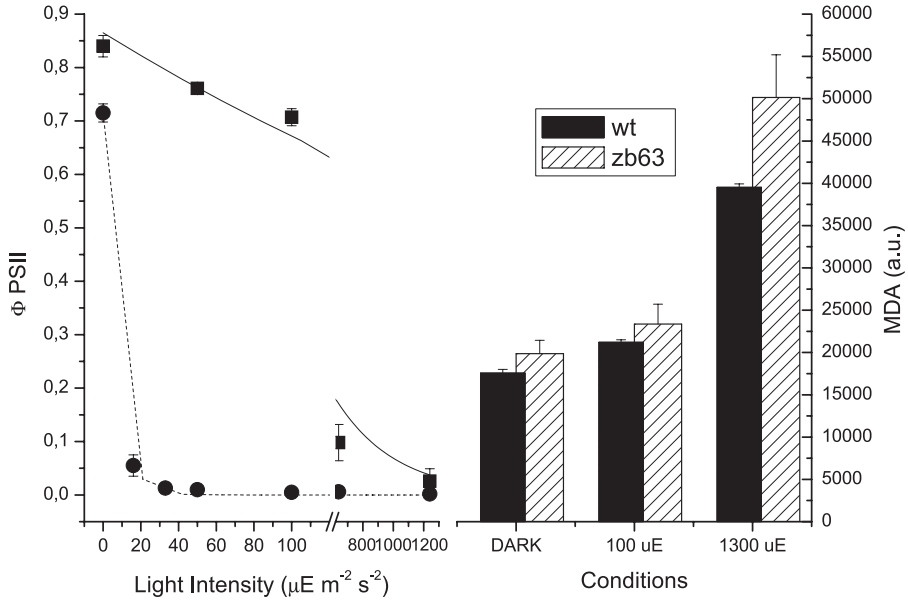


FIGURE 1. **Determination of PQ redox state and lipid peroxidation in WT *versus viridis Zb63* plants.** *Left*, determination of PSII (PSII) efficiency (μPSII) in wild-type (WT, *squares*) and mutant (*circles*) leaves illuminated with using increasing light intensities. *Right*, the reaction between ROS and lipids drives the accumulation of malondialdehyde (*MDA*): the graph shows the malondialdehyde-(thiobarbituric acid)$_2$ adduct formation in WT and *viridis zb63* (zb63) leaf discs, after exposure to different light intensities.

ide (48:1.5% acrylamide/bisacrylamide). The resolving gel had an acrylamide concentration gradient from 4.5 to 11.5% (w/v) stabilized by a glycerol gradient from 8% to 16% (w/v). 12 mM Tris and 48 mM glycine, pH 8.5, were also included in both gels. Thylakoids, at chlorophyll concentration of 1 mg/ml, were solubilized with an equal volume of 1.6% of α-DM (Anatrace®, Maumee, OH). Solubilized thylakoids were vortexed for 1 min, left for 10 min in ice, and then centrifuged at 13,000 rpm for 15 min for pelletting un-solubilized material. 30 μg of chlorophylls was loaded per each gel lane.

Biochemical Determination of Lipid Peroxidation—Lipid peroxidation was assessed indirectly by measuring malondialdehyde using HPLC, as before (24), with the following modifications. The samples (each one consists of 3 leaf discs of 5 mm in diameter) were exposed to 100 μE (control) and to 1300 μE light (high light) or incubated in the dark, for 4 h. After incubation, samples were frozen in liquid nitrogen, then ground in 150 μl of 175 mM NaCl and 50 mM Tris-HCl, pH 8.0. A volume of 0.5% w/v thiobarbituric acid in 20% w/v trichloroacetic acid was added, and samples were incubated at 95 °C for 25 min. After 20-min centrifugation, the malondialdehyde(thiobarbituric acid)$_2$ adduct was separated and quantified by HPLC, using 65% 50 mM KH_2PO_4-KOH, pH 7.0, and 35% methanol as elution buffer.

RESULTS

Differences in Transcription Rate between WT and Viridis zb63 Plants Grown in Control Conditions—Viridis zb63 is a barley mutant depleted in PSI but retaining a normal PSII activity (25, 26). The mutation is lethal, and it is maintained in a heterozygous state. Homozygous mutant plants grow up to 2 weeks on seed reserves, and they are easily distinguishable from WT because of their paler green color. The mutation causes a block of linear electron transport, which was shown to cause the chronic over-reduction of the PQ pool even in low light (20 μE) conditions (14). In this work we grew WT and mutant plants in dim light conditions (100 μE) ensuring the complete reduction of the PQ pool in the mutant but not in the WT. Because *viridis zb63* is depleted in PSI but still presents a residual 2% PSI activity (26), we verified the dependence of PQ redox state on light intensity in WT *versus* mutant leaves. As shown in Fig. 1, *left panel*, even very low illumination causes a saturation of PSII reaction centers in the mutant. On the contrary, in the WT saturation is achieved only with far stronger light intensities. This measure, thus, demonstrates that in growing conditions used here PQ pool is completely reduced in the mutant but not in the WT plants.

The same light intensity was also chosen, because it did not cause significant oxidative stress in the mutant: as shown in Fig. 1, *right panel*, the amount of lipid peroxides generated at 100 μeq with respect to dark conditions is similar in WT and *viridis zb63*, whereas larger accumulation of lipid peroxides is instead observed both in WT and mutant when leaves are exposed to high light (1300 μE), where the plastoquinone pool is reduced in WT plants as well.

The redox state of PQ and other electron transporters was proposed to play a key role in regulating the expression of photosynthetic genes (8, 27). However, these studies were all restricted to the effects of few hours of light treatment. In this work we used *viridis zb63* as a model to identify genes responding to the PQ redox state on a longer timescale, by comparing the mutant transcriptome to the wild-type one. After 9 days of growth at 100 μE, total RNA was extracted from plants and retro-transcribed using the poly(A) tail. The corresponding biotin-labeled cRNA was hybridized to the 22 k Barley 1 GeneChip (15) on three biological replicates for each genotype. A 2-fold change cut-off was applied, followed by analysis for statistically significant changes using Welch-*t* test and SAM (Significance Analysis on Microarray). Leaves used for sampling were maintained under continuous illumination during harvesting; this was an essential precaution in the case of the mutant, because we observed that the PQ redox pool is completely reduced even by very low illumination, but it is re-oxidized, possibly by alternative chloroplastic oxidases, after few minutes of darkness (data not shown).

In the described experimental conditions a total of 542 genes of 22,840 (from here forward we will refer to probe sets as genes) were differentially expressed between WT and mutant plants; in particular, 503 genes were up-regulated and 39 down-regulated in *viridis zb63* with respect to WT. Their classifica-

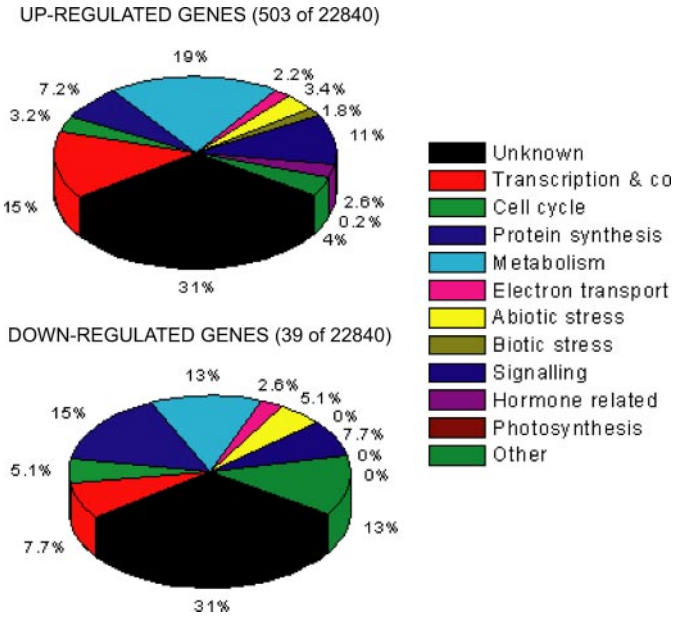


FIGURE 2. **Classification of up- and down-regulated genes in the *viridis zb63* mutant with respect to WT.** Pie graphs show a total of 503 up-regulated and 39 down-regulated genes, classified on the basis of their putative functional role.

tion is reported in Fig. 2; the complete list of up- and down-regulated genes is reported in the supplemental material.

Among them, 168 probe sets were annotated as an "expressed protein" of unknown function or as new genes without correspondence in the databases. 76 of the remaining genes (73 of which up-regulated) were associated to RNA transcription and maturation. 42 genes (36 up-regulated) were instead associated to protein synthesis or protein degradation, like ubiquitin and proteases. In addition, 16 genes related to cell cycle were up-regulated, including senescence-associated factors, apoptosis, and programmed cell death involved proteins. In the mutant, 53 genes involved in signaling were also induced, like kinases and phosphatases as well as 13 factors implicated in hormone biosynthesis or response. As expected, the mutation altered metabolism as well: in particular, 31 enzymes involved in sugar metabolism and translocation as well as 22 enzymes of lipid biosynthesis were up-regulated. Eighteen known stress-related genes were regulated and four are known to be involved in oxidative stress response: two peroxidases, one ferritin (28) and a protein induced by radicals (29). This result is consistent with previous data suggesting that mutant plants experience a chronic light excess condition, as confirmed by the large accumulation of zeaxanthin (14, 30).

As mentioned, it was shown that PQ is over-reduced in *viridis zb63* even in low light conditions (14). Because the PQ redox state was shown to have a regulatory role in the acclimation of photosynthetic apparatus to different light conditions (8), we expected the mutant to have an altered expression of genes involved in the light phase of photosynthesis. On the contrary, among the differentially expressed genes only one could be identified as photosynthesis-related, and it does not encode a protein of the light-harvesting complex, but a ζ-carotene desaturase, an enzyme involved in the first steps of carotenoid biosynthesis. This observation is surprising if we consider that the composition of the photosynthetic apparatus of *viridis zb63* is largely modified with respect to WT. To get new insights, we analyzed in deeper detail the original data set for the expression level of all genes encoding PSI, PSII, and cytochrome b_6f subunits (31, 32); the complete list of these genes considered as involved in the light phase of photosynthesis identified in the array, together with their expression level, is reported in the supplemental material.

With the mentioned exception of ζ-carotene desaturase, which is expressed only 2.2 times more in the mutant, the other 231 genes considered show only small variation, below 2-fold, in expression between the two genotypes. In the few cases where average values are over the 2-fold limit, standard deviation is also higher and the statistical analysis classified these differences as non-significant with a p value of 0.05. This conclusion is also valid for chloroplast ATPase subunits: in fact, only one gene showed an altered expression in the mutant, and its sequence clearly misses any plastid localization peptide, supporting the conclusion that ATPase-encoding sequences are also not regulated in the experimental conditions.

Because this result was really unexpected, considering present literature in the field, we looked more closely to these 231 photosynthesis-related genes. We found that they can be distinguished in two classes: the majority, a total of 181, show a logarithmic -fold change of $<\pm 0.5$, thus they can be considered as unchanged. The remaining 46 genes, instead, are slightly regulated with a -fold change between 0.5 and 1. Interestingly, in all 46 cases the -fold change was negative, thus indicating a moderate repression. The fact that this regulation does not overcome the cut-off threshold could depend on experimental design, which does not allow discriminating confidently such small changes. However, the fact that all 46 genes are consistently repressed excludes the possibility that all differences are due to stochastic effects. These data thus support the view that, although the largest part of photosynthetic genes is not regulated, a sub-class is instead moderately repressed in the mutant. Among the latter we found 14 sequences encoding for Lhc proteins and in particular 12 encoding Lhcb1. These represent one-half of the total probe sets for this subunit and indicate the presence of a significant effect of the mutation, even if smaller than expected, on the regulation of Lhcb1 genes transcription. The remaining two regulated probe sets correspond to Lhcb4 and Lhca1: because for these polypeptides we found, respectively, another three and four unchanged probe sets, we cannot affirm confidently that they are regulated, and, even if such a regulation is present, it is surely smaller than in the case of Lhcb1.

To verify the quality of the array experiments and confirm the surprising absence of a significant variation in photosynthesis-related genes expression, we analyzed the expression level of some sequences by semi-quantitative RT-PCR. For this assay we chose four nuclear-encoded genes (*lhca3*, *lhcb1*, *lhcb6*, and *psaH*) among the different polypeptides composing both the core and the antenna system of PSI and PSII, the only photosynthesis-related sequence showing variation in microarray analysis (encoding ζ-carotene desaturase), one random gene strongly up-regulated and one down-regulated in the mutant (encoding, respectively, a glycerophosphoryl diesterase and a

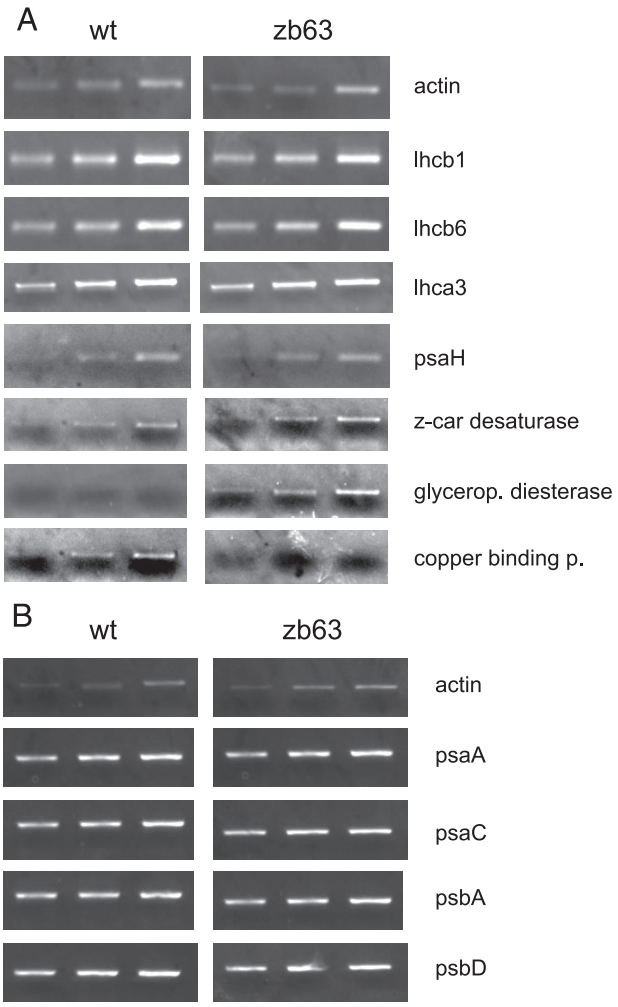


FIGURE 3. **Expression analysis by semi-quantitative RT-PCR of selected genes in WT and *viridis zb63* (zb63).** for each gene the amplification reaction was stopped after 24, 26, and 28 cycles. *A*, selected nuclear genes; *B*, chloroplast-encoded sequences. *β*-Actin was used as control for the total RNA amount.

copper-binding protein). The RT-PCR analysis (Fig. 3*A*) confirmed the absence of big differences in the photosynthesis-related genes, but in the case of *ζ*-carotene desaturase, which is under-regulated in the WT, glycerophosphoryl diesterase and the copper-binding protein also confirmed their large alteration of expression. In the case of Lhcb1, we observed a very small decrease of expression in the mutant, thus in the expected direction. Considering all the probe sets representing Lhcb1, the medium -fold change between WT and *viridis zb63* was only −0.43. Furthermore, in this case PCR analysis was complicated by the fact that several very similar Lhcb1 isoforms were present in the barley genome and they were probably not equally amplified during the reaction, possibly smoothing small variations in transcript levels. Even considering the limitations in the case of Lhcb1, however, PCR results are in good agreement with array experiments.

Several polypeptides composing the photosynthetic apparatus, especially the photosystems reaction centers, are encoded by the chloroplast genome, but they are excluded from array analysis because the total mRNA preparation exploits the poly(A) tail for retro-transcription. Therefore, we found it useful to verify whether our results could be explained by a different regulation of gene expression in the chloroplast and the nucleus. To verify this point, we evaluated the expression of four chloroplast-encoded sequences (two for each photosystem, *psaA*, *psaC*, *psbA*, and *psbD*) by RT-PCR as explained above, with the only difference of the individual retro-transcription for each gene. Also *β*-actin was individually retro-transcribed as the internal standard. No significant variation of gene expression regulation was observed between WT and the mutant (Fig. 3*B*). Thus, even considering the possible stochastic effects in PCR reactions, these results strongly support the hypothesis of a similar effect in the regulation of both chloroplast and nuclear photosynthetic genes.

It was previously shown that the *viridis zb63* mutant is depleted in PSI complex, but this mutation is not due to the absence of some of PSI polypeptides but to a still unknown factor for PSI assembly (31). In addition to what was observed so far, it is also interesting to point out that, 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.

Proteomic Analysis of Thylakoid Membranes—The results from transcriptome analysis appeared to be in striking contrast with a large number of previous reports showing the regulation of photosynthetic polypeptides during acclimation to different light conditions (2, 12). In the very same mutant, *viridis zb63*, the antenna content was recently shown to be strongly reduced even in the relatively low light conditions used in this work. Furthermore, it was shown that antenna size in the mutant is reduced to the minimum possible level, with Lhcb6 being completely absent and Lhcb1–3 strongly reduced. To further detail the difference between gene expression and protein accumulation level, we performed a proteomic analysis specifically focused on Lhc polypeptides. Polypeptides in thylakoid membranes from wild-type and mutant plants were separated by two-dimensional isoelectric focusing-SDS-PAGE. The comparison of different maps showed the presence of many spots less abundant (at least 2-fold) in mutant plants with respect to wild type, in the gel region corresponding to antenna proteins (Fig. 4). Western blot analysis with a mixture of antibodies against Lhc proteins confirmed that no Lhc proteins migrated out of the gel area analyzed in detail (data not shown).

All the spots visible in this region were cut and analyzed by LTQ-FT (hybrid linear ion trap-Fourier transform-ion cyclotron resonance) mass spectrometry for identification. Digested peptides were first separated by HPLC and then their mass determined at high accuracy with by the Fourier transform-ion cyclotron resonance. All peptides were fragmented and analyzed by MS-MS scan at low resolution with the linear ion trap, allowing determination of peptide sequence. The high mass accuracy (<0.6 ppm) allows the discrimination of the individual superimposed traces of the peptides, which was critical to distinguish between very similar polypeptides. One example of such identification in the case of two peptides differing in only one amino acid (Asp in place of Glu) is shown in Fig. 5. Accurate mass determination together with the detection of an almost complete series of the N-terminal B-fragments and the C-ter-

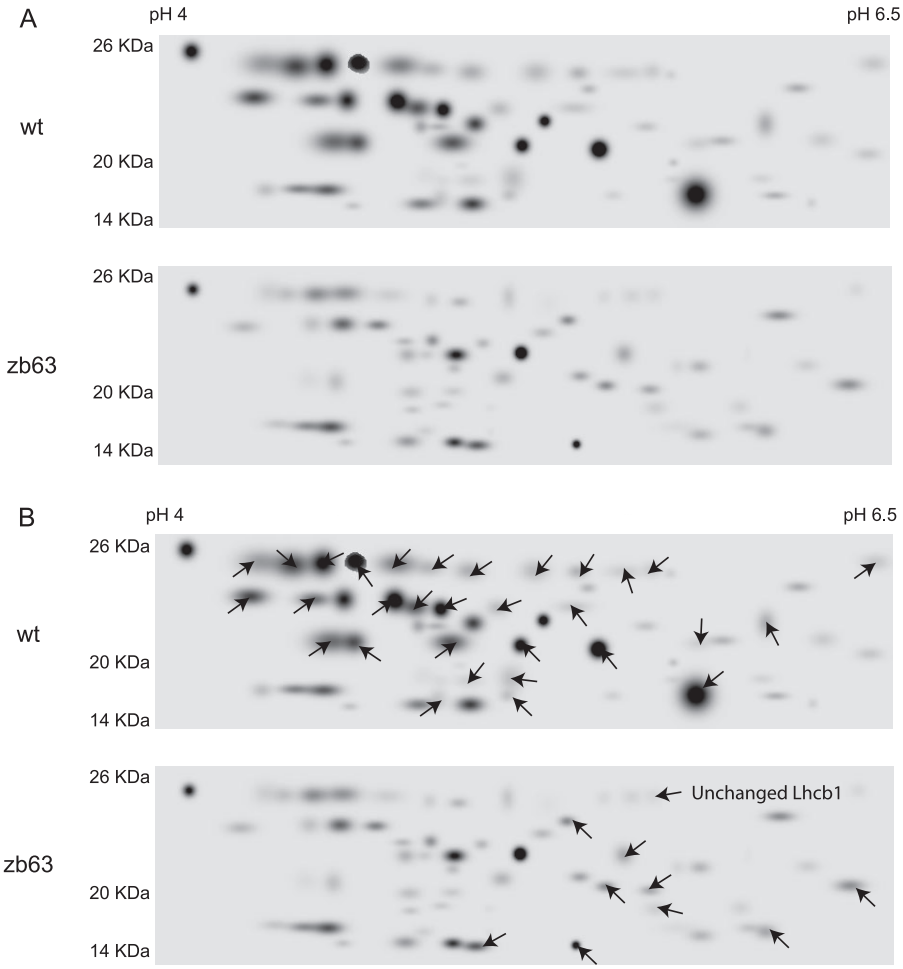


FIGURE 4. **Two-dimensional isoelectric focusing-SDS-PAGE analysis of WT and *viridis zb63* (zb63) thylakoids.** The gel area containing Lhc proteins is shown. *A*, Sypro Ruby-stained gel of WT and *viridis zb63*. *B*, the same gels with indication of spots overexpressed either in WT or in *viridis zb63* (marked with an *arrow*). The list of polypeptides identified in each *spot* by mass spectrometry is reported in the supplemental material. On the gel from mutant thylakoids, the *spot* corresponding to the only unchanged spot corresponding to a Lhcb1 isoform is also highlighted.

minal Y-fragments allowed unambiguous identification of the peptide sequence. Because identified peptides covered a large part of polypeptide sequences, it was thus possible to distinguish Lhc isoforms from one another, even if they differed in only a few amino acids.

The complete list of identified proteins overexpressed both in WT and in *viridis zb63* is reported in the supplemental material. Among the few proteins overexpressed in the mutant, we could identify different oxygen-evolving complex subunits, one ascorbate peroxidase, the ATPase δ-chain, a plastoquinone, and an NADH oxidoreductase. On the contrary, Lhca and Lhcb polypeptides were largely represented among the proteins underexpressed in the mutant, confirming that the antenna size is extensively regulated in this mutant.

To validate proteomic data, an immunotitration for different subunits was performed both for PSII and PSI antenna and core proteins. Thylakoids purified from WT and mutant plants were analyzed by SDS-PAGE and immunoblotting using specific antibodies. To avoid saturation effects, four different dilutions of each sample were loaded in the same gel (3). Because WT and mutant thylakoids have different chlorophyll to protein ratio, samples were normalized to their total protein content. In the case of PSII proteins, the assay for Lhcb1 confirmed a 2-fold down-regulation in *viridis zb63* with respect to WT. On the contrary, Western blots for Lhcb3 showed a stronger reduction: only traces of this subunit were detectable in the mutant membranes and only upon prolonged detection, when other signals were already saturated. Among minor antennas, Lhcb6 polypeptide was confirmed to be completely absent in the mutant, whereas Lhcb4 and Lhcb5 did not show significant changes between the two genotypes. Similarly, core proteins PsbB and PsbC were substantially unchanged in WT *versus* mutant. We were not able to detect PSI core subunits PsaE and PsaF, confirming their depletion in *viridis zb63*, in agreement with a previous report (14). On the contrary, the assay for Lhca3 and Lhca4 showed that these PSI antenna proteins were retained in mutant thylakoids despite the depletion of the PSI core, although it was at least three times lower than in wild type (Fig. 6).

To verify if the retaining of antenna polypeptides despite the core absence is a unique PSI feature, we also verified the presence of Lhcb polypeptides in a different barley mutant, *viridis 115*, which lacks PSII core complex. Western blot assays showed that both LHCII and the minor antennas Lhcb6 and Lhcb4 proteins were significantly accumulated, although to a reduced level with respect to WT. This result confirms that all antenna proteins are assembled even in the absence of the core complex both for PSII and PSI (Fig. 7).

After validation of MS results, we verified the case of Lhcb1 polypeptides in *viridis zb63* in deeper detail, because in barley there are at least fourteen different isoforms (18). These proteins are very similar to each other on the basis of sequence information, and it was not possible to completely distinguish each individual isoform based on peptides obtained by MS. However, we were able to discriminate five different classes, each containing one to three different polypeptides and indicated as Lhcb1a, Lhcb1b, Lhcb1c, Lhcb1d, and Lhcb1e. As in the case of array analysis, MS results showed that different isoforms have different expression profiles: many Lhcb1 are under-accumulated in the mutant, whereas a few others are retained at the same level than in wild type. In particular, we found that polypeptides belonging to classes from Lhcb1b to Lhcb1e were underexpressed in *viridis zb63*, while those of

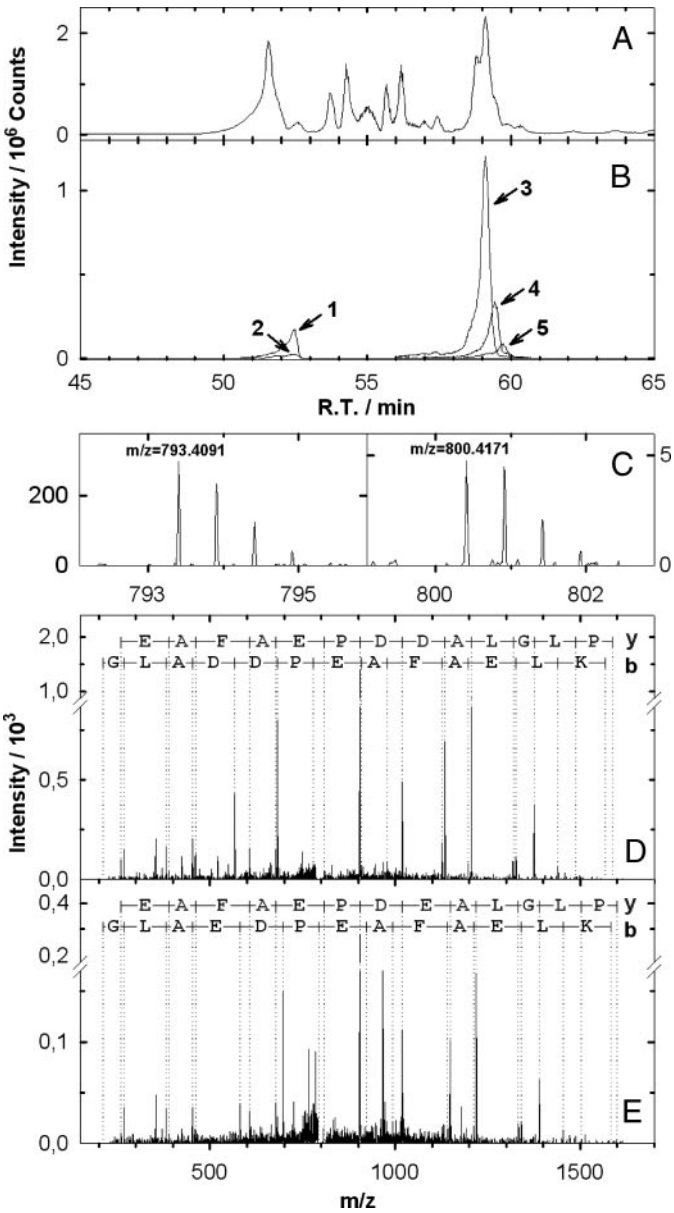
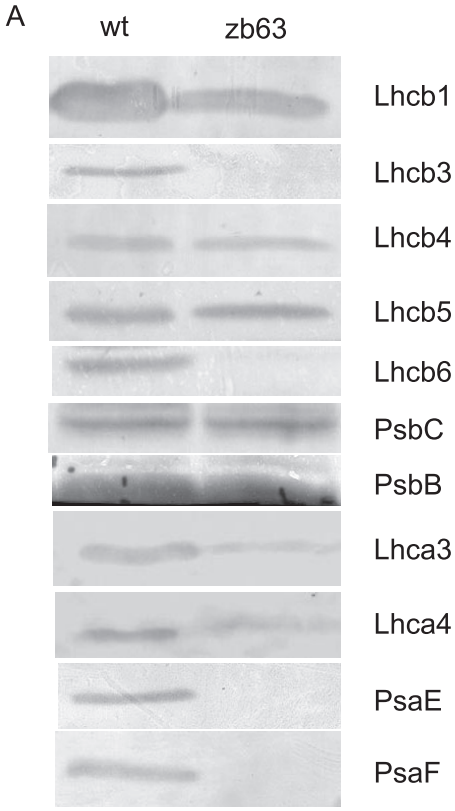


FIGURE 5. **Example of spot identification by mass spectrometry.** HPLC-MS data for tryptic digest of spot wt21. HPLC chromatogram for Base peak (*A*) and the following selected ions (*B*). *Lines 1* (*m/z* 793.4095) and *2* (*m/z* 800.4172) identify two isoforms with Asp-202 and Glu-202 of the doubly charged Lhcb1 peptide (197–211) PLGLA(D/E)DPEAFAELK with exact masses of M_r 1584.8044 and M_r 1598.8198, respectively, as detected by selected ion monitoring-MS (*C*, *left* and *right spectra*, respectively). Peptides labeled *1* and *2* are fragments of the larger peptides *3*, *4*, and *5* with a missed trypsin cleavage site. Exact masses are *m/z* 1176.2592 (*3*), *m/z* 1180.9319 (*4*), isobaric sequences, with Ile-177 to Asp-202 and Val-177 to Glu-202, both identified by MS/MS spectra (not shown), and *m/z* 1185.6039 (*5*). All peptides were fragmented and the MS-MS scan at low resolution in the linear ion trap. Sequence identification of C-terminal Y- and N-terminal B-ions for peptides 1 and 2 are shown in *D* and *E*, respectively.



B

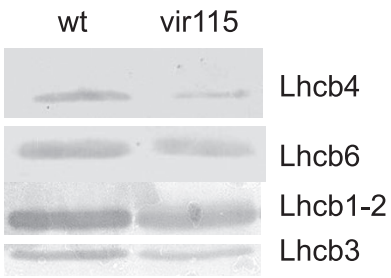
	WT	zb63
Lhcb1	1.00 ± 0.13	0.49 ± 0.11
Lhcb3	1.00 ± 0.30	n.d.
Lhcb4	1.00 ± 0.11	1.16 ± 0.41
Lhcb5	1.00 ± 0.29	1.03 ± 0.17
Lhcb6	1.00 ± 0.15	n.d.
PsbC	1.00 ± 0.28	1.37 ± 0.57
PsbB	1.00 ± 0.11	1.11 ± 0.28
Lhca3	1.00 ± 0.16	0.37 ± 0.14
Lhca4	1.00 ± 0.31	0.29 ± 0.14
PsaE	1.00 ± 0.34	n.d.
PsaF	1.00 ± 0.27	n.d.

FIGURE 6. **Western blot titration on *viridis zb63*.** Different polypeptides were quantified in WT and *viridis zb63* thylakoids by detection with specific antibodies. For each assay only one dilution is shown in *A* for clarity, but the quantification reported in *B* is based on four dilutions for each assay. All values reported are normalized to protein content in WT.

class Lhcb1a were retained. It would be interesting to verify if members of the Lhcb1a class present also mRNA expression levels different from the other isoforms. Unfortunately, isoforms are so similar to each other that it was not possible to establish a direct correspondence between Lhcb1 classes identified at the polypeptide level and array data sets, and thus we are obliged to leave the question open.

Nevertheless, the behavior of Lhcb1 is particularly interesting because it suggests that different isoforms are independently regulated, possibly because they fulfill specific physiological functions; for this reason they can be either retained or degraded in an over-reduced PQ genotype. Only two proteins belonged to the "retained" class: Lhcb1a.01 and Lhcb1a.02. These two sequences differ from all the others by three amino acid residues only, apart from the signal peptide (sequence alignment is reported in the supplemental material): Lhcb1a.01 carries Asn rather than Lys in position 91, Lhcb1a.02 carries Ser *versus* Ala in position 210, and both proteins carry Glu instead

A



B

	wt	vir115
Lhcb4	1.00 ± 0.35	0.11 ± 0.06
Lhcb6	1.00 ± 0.24	0.32 ± 0.18
Lhcb1-2	1.00 ± 0.08	0.46 ± 0.19
Lhcb3	1.00 ± 0.32	0.41 ± 0.04

FIGURE 7. **Western blot titration on viridis 115.** Different polypeptides were quantified in WT and viridis 115 thylakoids by detection with specific antibodies. For each assay only one dilution is shown in *A* for clarity, but the quantification reported in *B* is based on four dilutions for each assay. All values reported are normalized to polypeptide content in WT.

of Asp in position 168. According to sequence analysis (1), these substituted residues should not be involved either in chlorophyll, in carotenoid binding, nor in trimer formation.

Lhcb1 Reconstitution—To verify the hypothesis of a different functional role for Lhcb1 isoforms, their cDNAs have been amplified from a phage library and have been cloned in pQE50 (18). One representative polypeptide was chosen for each of the five classes, but in the case of the potentially most interesting class, Lhcb1a, both isoforms were analyzed. All polypeptides were expressed in *E. coli*, purified as inclusion bodies, and refolded *in vitro* using a well established procedure (20). Pigment-protein complexes thus obtained have been analyzed spectroscopically and biochemically. In Fig. 8, their absorption and fluorescence spectra as well as their pigment composition are shown. Despite their different protein-expression profiles, the only significant difference detectable between different Lhcb1 isoforms was a small variation in chlorophyll a/b ratio. Nevertheless, single isoforms do not show properties that could justify a specific functional role thus accounting for differences in expression level. However, it should be pointed out that in these experiments we could analyze only isolated monomeric complexes. Thus, the possibility that the different Lhcb1 could be distinct in the framework of supramolecular interactions within the photosynthetic membranes could not be verified.

DISCUSSION

Regulation of Antenna Size in Viridis zb63 Mimics the Acclimation to Extreme High Light—In the mutant *viridis zb63* we observed a strong regulation of the antenna size, as exemplified in Fig. 9, which shows the distribution of different pigment-binding complexes in mutant thylakoid membranes as compared with WT. It clearly appears that the ratio of antenna complexes to the PSII core is strongly reduced in the mutant. When the antenna proteins were analyzed in more detail by two-dimensional electrophoresis and Western blotting, we showed that Lhcb1–2-3 and Lhcb6 were the polypeptides showing the strongest decrease, whereas Lhcb4 and Lhcb5 content was essentially stable. Interestingly, this behavior is consistent with recent analyses in *Arabidopsis thaliana* plants acclimated to high light conditions, where it was observed that Lhcb6 and Lhcb1–2-3 were the major responsible of the changes in antenna size occurring during acclimation, whereas Lhcb4 –5 levels were maintained (3). The regulation of antenna size on *viridis zb63*, thus, is qualitatively similar but more extreme with respect to what observed was in *Arabidopsis* plants treated with intense light and low temperatures, suggesting regulatory mechanisms involved are very similar. Therefore, results obtained here with *viridis zb63* are not due to the specific mutation but rather reflect the normal regulation of antenna size in WT plants, making thus its analysis a powerful tool to yield information on antenna size regulation.

The use of *viridis zb63* as a model system, however, has a significant advantage, because such a strong effect on the antenna size was achieved without subjecting plants to oxidative stress conditions like high light and low temperature. In fact, we verified that the light intensity used in this experiment was sufficient to reduce the PQ redox pool without generating oxidative stress, as determined by the quantification of lipid peroxides accumulation (Fig. 1). This analysis of *viridis zb63* thus allows analyzing the effects of PQ reduction without any overlap with those induced by ROS, a distinction that is not possible in a high light-treated plant where both responses are overlapping.

PQ Redox State Is a Signal for Post-transcriptional Regulation—Photosynthesis is a complex phenomenon, composed by many reactions of different nature (chemical and physical) that need to be synchronized and regulated in response to environmental conditions. For instance, the capacity of light absorption must be balanced with the photosystems capacity of using excitation energy harvested. The need for such a regulation is due to the choice of chlorophylls as chromophores for light harvesting: a porphyrin with long living excited states and high triplet yield. Because of these properties, energy absorbed in excess may lead to the formation of harmful ROS. The redox state of the electron transport chain between PSI and PSII is an indicator of the overall efficiency of the photosynthesis light phase, and it was suggested to play a key role in the regulation of antenna size. In particular, it was shown that PQ redox state is implicated in the down-regulation of the *lhc* genes expression in high light conditions (8).

In this work we used the barley mutant *viridis zb63*, a genotype with a chronically reduced PQ, as a model for clarifying the role of its redox state in long term regulation of gene expression. By analyzing both gene transcription and polypeptides steady-state levels, we observed that, although polypeptides levels were drastically affected (14), gene expression showed only small variations and restricted to Lhcb1. To understand this apparent discrepancy with literature data, it is worth considering that our work covered a more extended timescale with respect to previous experiments. In fact, we analyzed plants at the end of 9 days of chronic plastoquinone reduction, while previous experiments suggesting the key role of PQ redox state for *lhc* gene expression were performed upon treating with light stress for few hours (8). A recent study, however, pointed out that, when *lhc* expression was analyzed up to 24 h of high light treatment,

A

Sample	No chl	Chl a/Chl b	Chl/Car	No Car	Neo	Viola
Lhcb1a.01	12	1.22	4.2	2.9	0.7	0.3
Lhcb1a.02	12	1.19	4.3	2.8	0.7	0.2
Lhcb1b	12	1.31	4.0	3.0	0.7	0.3
Lhcb1c	12	1.31	3.8	3.2	0.8	0.3
Lhcb1d	12	1.21	4.2	2.8	0.7	0.2
Lhcb1e	12	1.28	4.2	2.9	0.7	0.2
Max dev st		0.05	0.2	0.2	0.1	0.1

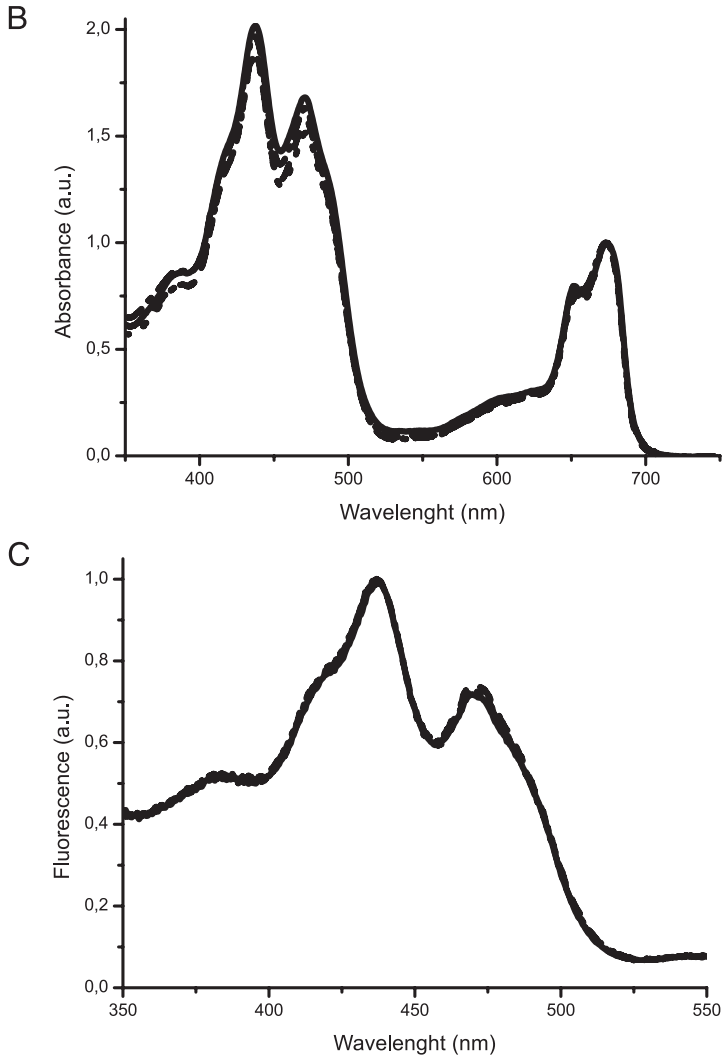


FIGURE 8. **Characterization of Lhcb1 isoforms reconstituted *in vitro*.** One member for each class of Lhcb1 isoforms identified from proteomic analyses (*lhcb1a–e*) was reconstituted *in vitro*. In the case of class *a* all putative members were included and indicated as *01* and *02*. *A*, pigment binding properties of refolded complexes. Data are normalized to 12 total chlorophylls per molecule as in a previous study (18). *B* and *C*, absorption and fluorescence excitation spectra of refolded complexes. Given that all spectra are very similar, only the ones for Lhcb1a.01, Lhcb1a.02, Lhcb1b, and Lhcb1d are reported, respectively, in *solid*, *dashed*, *dotted*, and *dash-dotted lines*.

the transcription inhibition was transitory and final mRNA levels recovered to roughly the starting levels (13), consistent with our results. This behavior, however, could be alternatively explained with a rapid induction of oxidative stress and consequently a transient accumulation of ROS, as suggested previously (33): the treatment with norfluorazon, which blocks chloroplast development, leads to accumulation of chlorophyll precursors that easily interact with oxygen, generating ROS, and results in the repression of *lhcb* genes.

In our work, instead, we can exclude ROS effects and confidently conclude that *lhc* gene expression does not play a key role for the regulation of polypeptide accumulation during long term acclimation. Furthermore, not only the *lhc* but all genes involved in the light phase of photosynthesis and encoding the four large complexes in the thylakoid membranes (PSI, PSII, cytochrome b_6f, and ATPase) shared the same transcriptional behavior. This suggests that, although differences between individual genes may exist in the short term, long term mechanisms induced by the over-reduction of PQ pool do not consist of strong transcription inhibition.

Despite lack of relevant transcriptional regulation, however, the level of Lhc polypeptides is indeed strongly decreased in the mutant with respect to the WT (Figs. 4 and 6). We conclude that the steady-state level of Lhc proteins depends on post-transcriptional rather than on transcriptional regulation. It is worth emphasizing that this dependence is really strong: in *viridis zb63*, in fact, the antenna size is reduced to minimum level (14) due to strong decrease of Lhcb1–3 and Lhcb6, whereas, according to array analysis, transcription is slightly affected in *lhcb1* only.

Several mechanisms could be responsible for the observed post-transcriptional regulation: first, transcript stability could be decreased, but such a variation would have been detected in array analysis where stationary mRNA levels are analyzed. Further possible regulation mechanisms can instead operate at translation level. In fact, a mutant in a RNA-binding protein, Nab1, was recently shown to affect Lhcb translation in *Chlamydomonas* (34). In addition, polysome association of photosynthetic mRNA, including *lhc* transcripts, was shown to be affected by exposure to stress conditions (35). However, according to these data the effect on translation appears to be common to whole mRNA rather than restricted to photosynthesis-related genes.

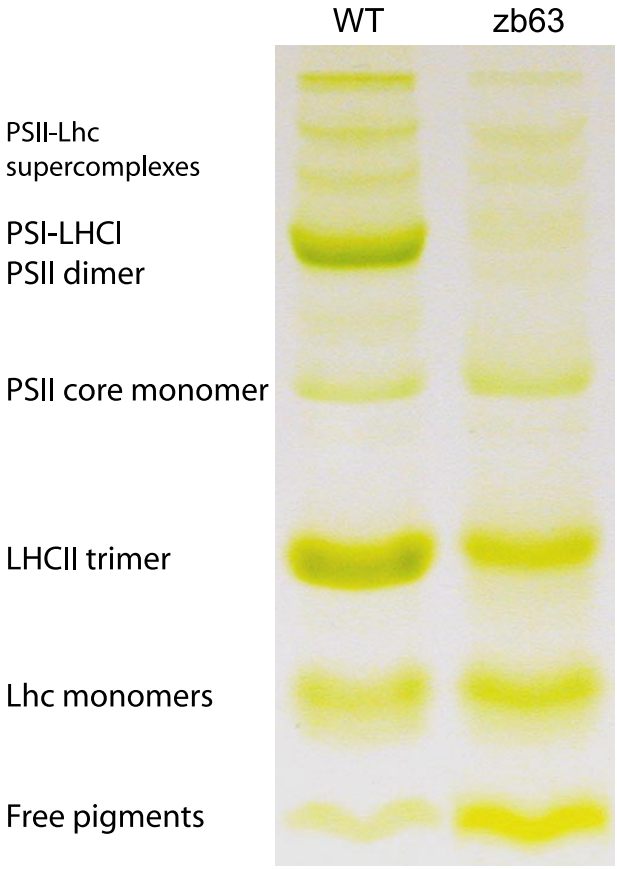


FIGURE 9. **Non-denaturing Deriphat-PAGE of WT and *viridis zb63* (zb63) thylakoid membranes.** 30 μg of chlorophylls was loaded for each sample.

An alternative possibility is that protein regulation is achieved by activation of polypeptides degradation. Some findings in the literature already suggested this mechanism is active in modulating Lhc accumulation: for instance it was shown that transition from low to high light activates the proteolysis of LHCII trimer subunits (36, 37). Consistent with this idea, we observed that the transcription of two different proteases was induced in the mutant with respect to WT. One of them plays some role in regulation of photosynthesis, being a chloroplastic Clp protease, which was already shown to be active against OE33 (38).

Other chloroplast proteases, belonging to the FtsH family, were shown to be associated to the thylakoid membrane and to be active in degrading antenna proteins (39). Their activity *versus* antenna polypeptides was shown to be influenced by activation of the senescence program (40) or by acclimation to high light conditions (41). This family of proteases was represented in array data set (see supplementary material), but none of the five FtsH sequences showed regulation in the mutant with respect to WT. However, this result was expected, because previous reports already showed that these proteases are not overexpressed in high light (42). On the contrary, activation of proteolysis was proposed to be triggered by the exposure of an unfolded region at the N terminus of target proteins (43), as in the case of photo-oxidative stress (44). We are presently verifying the possibility that these proteases are indeed differently activated in *viridis zb63* with respect to the WT plants.

A further suggestion that Lhc degradation might play a major role in antenna size regulation in this mutant derives from the observation that *viridis zb63* largely accumulates zeaxanthin in Lhc proteins (thylakoids de-epoxidation index is 0.58). In fact, it was previously shown in the *lut2npq2 Arabidopsis* mutant, which presents zeaxanthin as the only xanthophyll, that the accumulation of this carotenoid induces a decrease in antenna size because of selective destabilization of trimeric LHCII (45). Similar results have been reported also in the *lor1npq2* mutant of *C. reinhardtii* and thus appears to be a general regulatory mechanism of Lhc proteins (46). Binding of zeaxanthin to Lhc proteins was shown to induce conformational changes, as demonstrated by spectroscopic as well as biochemical methods (47, 48) and protease-sensitive sites might become more exposed as a consequence. Thus, zeaxanthin accumulation in excess light conditions, in addition to its other effects, could have a photoprotective role by inducing the reduction of antenna size and reducing light-harvesting capacities.

Epistatic Regulation of Translation in Higher Plants Is Effective for Core Complexes but Not for Antenna Proteins—We observed little or no effect on the transcription of genes encoding the PSI core complex subunits in the *viridis zb63* mutant. On the contrary, the corresponding polypeptides were all concomitantly undetectable. This phenotype could be due to translational regulation, to protein degradation, or both. Previous reports showed the role of assembly factors on protein accumulation for both PSI (49) and PSII (50) core complexes in *C. reinhardtii* and demonstrated the presence of control by epistasis of synthesis. It was shown, in fact, that the absence of PsaB caused a down-regulation of PsaA synthesis due to translation auto-regulation. Similarly, in the case of PSII, the absence of D1 or D2 proteins led to inhibition of translation initiation of other PSII subunits, without alteration of mRNA levels. In different species, however, this regulatory mechanism is not always conserved: in cyanobacteria, in fact, mutations affecting PSII assembly were shown to increase the proteolytic susceptibility of other subunits, without alteration of transcription or translation rate (51).

In the case of plants direct evidences are still lacking. However, we consider the hypothesis of an epistatic regulation as being more likely considering the more recent divergence from green algae and the conservation in photosystem organization in *Viridiplantae*. Consistently, a recent report in *A. thaliana* suggested the presence of a translational mechanism similar to the one evidenced in algae: the mutant *lpa1* accumulates lower level of PSII proteins due to a strong reduction in translation, despite normal RNA level and polysome association (52). In addition, very recently, control by epistasis of synthesis has been shown to play a major role in tobacco at least in the case of Rubisco large subunit (53). The composition of photosynthetic apparatus observed in *viridis zb63* mutant is consistent with the hypothesis that a similar mechanism of epistatic regulation is active in PSI and PSII assembly in higher plants, although protein degradation cannot be ruled out.

In addition to previous works, moreover, this study also allowed insights into the assembly of antenna proteins in photosystems. In fact, while we observed such a regulation (either due to epistasis or protein degradation) in the case of core pro-

teins, specific Lhc subunits were maintained in both mutants affected in PSI or PSII core complexes, although the overall pool of antenna proteins was reduced. These antenna proteins were not only present but also properly folded, as shown in the case of *viridis zb63*, where the peculiar red fluorescence typical of PSI antenna is detected despite the absence of PSI core (14).

The evidence for a distinct mode of regulation for core *versus* antenna assembly is even more striking if we consider the case of Lhcb6, a PSII subunit that is reduced in the PSII-less mutant *viridis 115* while completely absent in *viridis zb63*. This suggests the presence of a finely regulated process: Lhcb6 is the antenna protein undergoing the strongest changes in abundance in response to growth conditions (3); its reduction in high light might trigger disconnection and degradation of peripheral LHCII population leading to reduction in antenna size. This down-regulation is extreme in *viridis zb63*, because Lhcb6 polypeptide is undetectable; on the contrary, in *viridis 115*, despite the absence of a functional PSII, the light excess experienced by the cells is not high enough to drive its complete deletion.

In conclusion, our analysis suggests that antenna proteins are independently folded in the thylakoid membranes and only successively associated to the assembled core complex. If this association fails, antenna proteins undergo proteolytic degradation, but their synthesis and assembly are not prevented. This distinct regulation of core and antenna subunit assembly appears to be a common mechanism of all antenna proteins for both PSI and PSII, because *viridis zb63* and *viridis 115* showed a common phenotype in this respect.

Role of Different Specific Lhcb Polypeptides—In *viridis zb63* we observed a strong regulation of Lhcb1–2-3 and Lhcb6 content, whereas Lhcb4–5 are essentially maintained. This observation is consistent with a previous report (14) showing that the minimal PSII antenna is composed by a LHCII trimer (S trimers, from Strongly bound (54)), Lhcb5 and Lhcb4, while Lhcb6 is absent. Here we also present evidences that Lhcb3 as well is absent in the LHCII trimers (S trimers) closely connected to PSII core and maintained in *viridis zb63* (54), whereas it is included in M trimers, where it participates to an Lhcb4-Lhcb6-LHCII complex (55, 56).

It is also worth observing that in *viridis zb63* some Lhcb1 isoforms have a specific regulatory behavior, despite their very similar polypeptide sequences. This is consistent with similar regulation of different Lhcb1 polypeptides in maize acclimated to different light conditions (57). We suggest that these conserved Lhcb1 isoforms are preferential components of the LHCII trimers in S position that are retained in the mutant (14). Despite this conserved regulation, however, the monomeric pigment-protein complexes refolded *in vitro* did not show differences in their biochemical and spectroscopic properties. This is consistent with their high sequence homology, and it suggests that the intrinsic function in light harvesting and photoprotection is the same. It cannot be excluded, however, that the very small differences in primary sequence may cause differential accessibility for LHCII-degrading proteases, also because of variable interaction with other subunits, like Lhcb5 or Lhcb4. One further possibility to explain the reason for the different regulation of Lhcb1 isoforms can be inferred from the analysis of Lhcb sequences in different organisms: sequence analyses show that the presence of multiple isoforms of LHCII components is a conserved feature in the green lineage (58). However, these isoforms show conserved species-specific characteristics in sequence properties rather than conserved features between different species. This suggests that there might be a selective advantage in having multiple copies of very similar genes. One possible explanation may be proposed on the basis of the dual role of Lhc proteins in light harvesting and photoprotection. Lhcb1, as mentioned are down-regulated in high light condition, leading to a reduction of the antenna size. The presence of few, similarly regulated, Lhcb subunits would lead to complete degradation of the antenna system under strong high light, thus leaving PSII core complex with little protection as in the *ch1* mutant (59). On the contrary, multiple Lhcbs with distinct sensitivity to degradation still provide the possibility of modulating antenna size while ensuring the maintenance of photoprotection: in *viridis zb63*, in fact, a minimal antenna size is retained even in extreme stress conditions (14).

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