Is Each Light-Harvesting Complex Protein Important for Plant Fitness?1

Ulrika Ganeteg*2, Carsten Külheim2, Jenny Andersson2, and Stefan Jansson
Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, S–901 87 Umeå, Sweden

Many of the photosynthetic genes are conserved among all higher plants, indicating that there is strong selective pressure to maintain the genes of each protein. However, mutants of these genes often lack visible growth phenotypes, suggesting that they are important only under certain conditions or have overlapping functions. To assess the importance of specific genes encoding the light-harvesting complex (LHC) proteins for the survival of the plant in the natural environment, we have combined two different scientific traditions by using an ecological fitness assay on a set of genetically modified Arabidopsis plants with differing LHC protein contents. The fitness of all of the LHC-deficient plants was reduced in some of the growth environments, supporting the hypothesis that each of the genes has been conserved because they provide ecological flexibility, which is of great adaptive value given the highly variable conditions encountered in nature.

Photosynthesis is one of Nature’s most complex biochemical processes, and many hundred proteins are probably involved in it. Many of these proteins have been studied in great detail, and their functions are well known, whereas others have functions that remain obscure. The photosynthetic proteins generally show very high degrees of conservation. Although several differ between higher plants and cyanobacteria, the differences between green algae and higher plants are relatively small. Among seed plants (angiosperms and gymnosperms), the major photosynthetic proteins, like subunits of PSI and PSII, the cytochrome b6f complex, the ATP synthase, and the enzymes of the Calvin cycle seem to be present in all plant species and highly conserved between them.

Although these observations indicate that strong selection pressure must be present to maintain the genes encoding each protein, surprisingly large numbers of the genes have been knocked out using reverse genetic approaches without giving rise to obvious growth phenotypes (for review, see Scheller et al., 2001). In such cases, it sometimes has been possible to record differences in specific photosynthetic parameters, but researchers have often concluded that the protein may have a function that is only apparent under certain conditions because evolutionary conservation is theoretically incompatible with functional redundancy. The situation for the light-harvesting complex (LHC) chlorophyll a/b-binding proteins is particularly intriguing. Ten homologous types (Lhca1–4 and Lhcb1–6) appear to have been conserved since before the separation of the evolutionary lines leading to angiosperms and gymnosperms, about 350 million years ago (Jansson, 1994), yet plants lacking one or many individual LHC proteins are fully viable and generally do not show a growth phenotype (Zhang et al., 1997; Andersson et al., 2001, 2003; Ganeteg et al., 2001), which is consistent with the hypothesis that they may have overlapping functions.

To test the hypothesis that individual genes are important for the plants, we recently have developed an assay to measure the fitness of Arabidopsis plants under natural conditions by measuring seed production (Külheim et al., 2002). Using this method, we have studied the npq1 and npq4 mutants, which lack proteins essential for feedback de-excitation (or the energy-dependent type of non-photochemical quenching; Niyogi et al., 1998; Li et al., 2000) and shown that even though these mutants grew indistinguishably from the wild type in a controlled environment, the removal of feedback de-excitation caused severe loss of fitness in the field (Külheim et al., 2002). We reasoned that the same might be true for other proteins of the LHC superfamily. To elucidate specific functions of the LHC proteins, we have constructed a collection of Arabidopsis plants in which individual LHC genes are repressed by antisense transformations (Zhang et al., 1997; Andersson et al., 2001, 2003; Ganeteg et al., 2001). Studies of these plants have given valuable information about the LHC proteins, but no major phenotypic differences from wild type have been seen in these plants except for Lhca4 (U. Ganeteg and S. Jansson, unpublished data) and Lhcb2 (Andersson et al., 2003). Therefore, in this study, we have measured the fitness in the field of these plants and other transgenic

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2 These authors contributed equally to the paper.
* Corresponding author; e-mail ulrika.ganeteg@plantphys.umu.se; fax 46–90–786–66–76.

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lines, where LHC proteins have been removed either by antisense inhibition or T-DNA tagging.

RESULTS

A Collection of LHC Protein-Deficient Arabidopsis Plants

To assess the specific function of each LHC protein, we have constructed and analyzed a collection of Arabidopsis plants that have suppressed levels of Lhca2, Lhca3, Lhca4, Lhcb1/Lhcb2, Lhcb4, or Lhcb5 and, thus, differ in LHC protein composition (Zhang et al., 1997; Andersson et al., 2001, 2003; Ganeteg et al., 2001). In this study, we have added two more plants to the collection: one in which Lhcb6 was repressed by antisense transformation and one in which Lhca1 was knocked out by T-DNA insertion. An earlier analysis of Lhca4-deficient plants has been described (Zhang et al., 1997), but the total LHC protein composition was not determined in the previous study. In the study presented here, we first characterized these three lines (Lhca1, Lhca4 and Lhcb6) completely in terms of LHC protein content under controlled conditions before subjecting them and the other lines to the fitness tests and other measurements described below.

Southern-blot analysis showed that the knockout Lhca1 line contains one T-DNA insert only (data not shown). The Lhca1 knockout plants have the T-DNA insert in the promoter region (205 bp upstream of the translation start) of the Lhca1 gene. As a consequence, the plants contain a small amount (less than 10% of wild-type levels) of Lhca1 protein (Fig. 1). Levels of Lhca4 are also much lower than wild type in these plants, apparently because the stability of this protein is reduced in the absence of Lhca1, but the Lhca2 and Lhca3 levels are not affected. All Lhcb proteins are present in wild-type amounts in the Lhca1 knockouts (data not shown). In the Lhcb4 transgenic plants (Zhang et al., 1997), the same interrelationship between Lhca1 and Lhca4 is also observed, i.e. when Lhca4 is absent the levels of Lhca1 decrease too. Again, we believe that this is because of decreased protein stability rather than lack of protein synthesis because levels of Lhca1 mRNA are not affected in the Lhca4 antisense lines (data not shown). This seems to be a common phenomenon in the light-harvesting antenna: Lhca2 and Lhca3 protein levels also seem to be interdependent and Lhcb6 is partially depleted in Lhcb4 antisense plants. All these effects appear to be caused by posttranslational events (Andersson et al., 2001; Ganeteg et al., 2001). The finding that Lhca1 is depleted in Lhca4 antisense plants appears to conflict with the results of a previous study (Zhang et al., 1997). This will be the focus of another report but, in brief, growth light conditions determine Lhca1 levels in the Lhca4 antisense plants, presumably via a posttranslational mechanism. In the Lhca4 plants, Lhca2 and Lhca3 are also severely depleted, although not to the same extent as the Lhca1 protein. The levels of the Lhcb proteins showed no significant change in the Lhca4 antisense plants (data not shown). In the Lhcb6 antisense plants, the only LHC protein affected is the corresponding protein, which is undetectable. All other Lhc proteins are present at wild-type levels.

We have summarized these data, together with previously published information, in Table I. It shows the LHC protein contents of the plants used in this experiment, grown under standard conditions in the growth chamber, on a reaction center basis. In the asLhcb2 plants, Lhcb1 is also lacking because of “cross-antisensing” (Andersson et al., 2003; Lhcb1 and Lhcb2 have very similar sequences). In all other cases, where a protein decreases as a consequence of the antisense inhibition of another protein, the effects seem to be because of posttranslational mechanisms, presumably related to the stability of the proteins being reduced by the absence of a neighboring protein with which they interact. In the Lhcb2 antisense plants, there is a compensatory increase in two other proteins, Lhcb5 and Lhca4 (Ruban et al., 2003), but this is not found in any other case. In Lhca4 antisense plants, there is a small increase in the PSI to PSII ratio (U. Ganeteg, S. Jansson, P. Horton, A. Ruban, unpublished data), whereas in the other lines, this ratio seem to be unchanged.

Fitness Experiment

To assess the importance of the LHC proteins in nature, we planted the collection of LHC protein-deficient plants in the field in a randomized block design together with their corresponding wild types. Because light is a critical parameter for photosynthesis, we decided to carry out the experiments in two different light regimes: in full sunlight and in the shade of trees. The site in full sunlight was homogenous in all respects, including irradiation. The irradiance pattern on a typical day can be seen in the supplemental material. There were, not surprisingly, large variations in irradiance both between days and during the day (see also Külheim et al., 2002). It was not possible to find a shaded site that was as homogenous as the full sunlight site, so we split the shaded

![Figure 1](image-url). Figure 1. Relative LHC protein levels under controlled conditions in wild type (wt), koLhca1 plants (−a1), asLhca4, and asLhcb6 plants (−b6). Total leaf membrane preparations corresponding to 3 μg of chlorophyll were subjected to SDS-PAGE. The proteins were detected using antibodies specific for the different proteins.
site into two parts: Shade A and Shade B. The light regimes on a typical day at both Shades A and B are also shown in the supplemental material. The received light on a typical day at Shade A and Shade B was 14% and 16%, respectively, as compared with the Sun site. The maximum peak irradiance at the shade sites were 290 and 560 μmol m⁻² s⁻¹, respectively, and 1,141 μmol m⁻² s⁻¹ at the Sun site. Not only was the overall irradiance much lower at the Shade sites, but the variation over the day was also lower as compared with the Sun site because midday light was more shaded than morning and evening light.

The unavoidable differences in planting dates between the subexperiments resulted in some unexpected consequences. Most notably, the asLhca4 plants and the corresponding wild type (C24) performed very poorly at the Sun site. Because these were the last to be planted out, they were the least capable of coping with the drought, which intensified during the course of the experiment. Also, when larvae of diamondbacked moths (*Plutella xylostella*) appeared, these plants were smaller than those of the other genotypes, so they were much more heavily affected, and mortality was pronounced. The number of surviving plants was too small to allow a meaningful analysis, so these plants were not further considered in the fitness experiment. Therefore, data for the asLhca4 plants are only available for the shade experiment for the year 2002. In Figure 2 data from a pre-experiment, performed in 2001, are shown instead. The 2001 site was intermediate between the 2002 sun and shade sites in terms of light quantity and variation.

### Table 1. LHC protein content of LHC-deficient Arabidopsis plants

Results from SDS-PAGE and immunoblot analysis of total leaf membrane preparations. wt, Wild type level; 0, no protein detected; 0*, less than 10% of wild-type protein level; –, decreased protein level; +, increased protein level.

<table>
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<tr>
<th>Protein</th>
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**Figure 2.** Seed production in the field of wild type (wt) plants and plants with suppressed levels of Lhca1 (–a1), Lhca2 (–a2), Lhca3 (–a3), Lhca4 (–a4), Lhcb2 (–b2), Lhcb4 (–b4), Lhcb5 (–b5), and Lhcb6 (–b6). White/shaded panels show sun/shade experiments, respectively. Data shown are means ± se (n ≥ 30). A and B, Total seed count per plant. C and D, Number of siliques per plant. E and F, Number of seeds per siliquie. The statistical significance of deviations from wild type is showed by asterisks: (*), $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; §, experiment from 2001.
Growth (in terms of germination rate and timing, rosette diameter, time of bolting, and inflorescence height) was followed for all plants throughout the experiment. Most genotypes showed no differences in growth compared with the corresponding wild type, but the asLhca4 plants grew more slowly and flowered significantly later than the wild-type plants (data not shown).

The transgenic plants were grown in the field until they had finished producing flowers. At this stage, the leaves were in a late stage of senescence, and the plants appeared to be almost dead. Seed production for each plant was calculated by multiplying the average number of seeds from three mature siliques by the total number of siliques (Külheim et al., 2002). As in this study, the variation in seed production under natural conditions was very large. One plant produced 24,650 seeds, whereas some died or produced almost no siliques. Because it could not be taken for granted that mortality was genotype-independent, we calculated seed production both including and excluding plants that produced no seeds. However, the relative fitness was the same in both cases, so all figures presented hereafter are based solely on plants that did produce seeds.

At the Sun site, all Lhca lines produced fewer seeds than wild-type plants (Fig. 2A), although the difference was statistically significant only in the cases of Lhca2 and Lhca4. At the shade site, the koLhca1 plants produced the same number of seeds as the wild type, whereas Lhca2 produced fewer seeds and Lhca3 and Lhca4 significantly fewer seeds. The reduced seed production per plant was in most cases because of a decrease in the number of siliques per plant, but in a few cases, it was because of a decrease in seeds per silique or both of these factors (Fig. 2, C and E).

The fitness reduction of the Lhcb lines at the Sun site was pronounced (Fig. 2B). All four lines produced significantly fewer seeds than wild-type plants. The effect in shaded conditions was less marked. asLhcb5 plants did not show any reduction in fitness, and the reduction for Lhcb2 was not statistically significant. The major effect found in the latter case was in the number of siliques per plant, but the number of seeds per siliques was significantly lower in the asLhcb6 line (Fig. 2, D and F). No significant difference in seed weight was observed for any of the lines (data not shown).

Lhca4 Antisense Plants Had a Severe Growth Phenotype

The fitness reduction was most pronounced for asLhca4 plants, and the growth of these plants was also severely impaired in terms of both inflorescence height and leaf rosette diameter, which in both cases reached only about 30% of wild-type levels in the shade (data not shown). Therefore, removal of Lhca4 by antisense inhibition seemed to affect Arabidopsis much more than removal of other Lhc proteins. In a pre-experiment to this study, performed in 2001, fitness was even more severely reduced, and the seed production of the Lhca4 antisense plants amounted to only 9% of the wild-type production (Fig. 2A). Thus, asLhca4 plants clearly performed much less well than wild-type plants.

Because growth was also heavily impaired in the asLhca4 plants, in contrast to the other lines where no apparent growth difference was seen, comparison of seed production between wild-type and asLhca4 plants is not as straightforward as with the other lines. In addition, flowering is somewhat delayed in this line, according to both a previous report (Zhang et al., 1997) and observations in this experiment. This finding prompted us to carefully measure growth of asLhca4 plants in a climate chamber under controlled conditions. Even though this is constant environment, its growth was clearly retarded (Fig. 3). The plants were obviously smaller, and the fresh weight of 6-week-old plants was equivalent to about 70% of the weight of corresponding wild-type plants. The dry weight to fresh weight ratio was the same for both genotypes (Fig. 3). Analysis of another antisense line lacking Lhca4 (line no. 23; Zhang et al., 1997) gave very similar results (data not shown), so lack of Lhca4 appears to severely affect plant growth. A likely explanation for the stronger phenotype of the asLhca4 plants is that the absence of Lhca4 resulted in depletion of several other proteins (Lhca1, Lhca2, and Lhca3), an effect observed to a lower extent in the other lines.

Figure 3. Growth (A) and morphology (B) under controlled conditions of wild type (wt) and asLhca4 plants (–a4). Plants were harvested at 7 weeks of age, and the rosette of each plant was weighed separately before and after drying. The results shown are means ± se (n = 6).
The Decreased Fitness Was Not a Consequence of Photo-Inhibition

In the field, plants are subjected to a wide range of stresses. Although stress can be manifested in many different ways, one useful parameter to monitor when stress to the photosynthetic apparatus occurs is the extent to which the plant is affected by photo-inhibition of photosynthesis. A convenient way to measure this, which can also be used in the field, is to record the chlorophyll fluorescence parameter ($F_v/F_m$), which decreases in all kinds of stress that result in increased excitation pressure of photosystem II. We have shown recently that plants that lack PsbS and, thus, feedback de-excitation, show reduced fitness in the field, and also increased levels of photo-inhibition (Kühlheim et al., 2002) If any of the LHC proteins studied here play an important role not only in light harvesting but also in photoprotection, we reasoned that we should be able to observe a decrease in $F_v/F_m$. Therefore, we assayed $F_v/F_m$ directly in the plants grown in the field during the experiment.

As expected, the level of photo-inhibition of wild-type plants varied with the weather (higher irradiance leads to increased photo-inhibition) and increased at the end of the experiment, when the leaves showed quite severe symptoms of senescence. The pattern resembled trends we have observed previously in Arabidopsis plants grown in the field and is probably typical of annual plants with accelerated life cycles, such as Arabidopsis.

We recorded photo-inhibition in all genotypes at both the sun and the shade sites. Typical results are shown in Figure 4, where $F_v/F_m$ levels found are compared for the Lhcb2 antisense plants and wild-type plants. As can be seen, there was no significant difference in the amount of photo-inhibition between the two genotypes, so the absence of Lhcb1 and Lhcb2 does not seem to increase the amount of excitation pressure on the photosynthetic apparatus. Corresponding graphs for the other genotypes can be found in the supplemental material. These graphs show that we did not detect any significant difference in photo-inhibition in any of the genotypes, including the Lhca4 antisense plants (despite their strong growth phenotype in the field), so none of the genotypes were adversely affected by higher levels of photo-inhibition.

**DISCUSSION**

Since the publication of Darwin’s theory on the survival of the fittest and Mendel’s discovery of genetics in the late 19th century, scientists have been aware that the interaction between genotype and environment is a key determinant of the fitness of an individual. Nevertheless, there have been very few studies to date in which the contribution of a single, known gene product to plants’ fitness in the natural environment has been quantified, partly for methodological reasons. Although huge numbers of plant mutants are available, there are far fewer cases where the mutation has been shown to result in the loss of a single defined gene product. More importantly, however, several different scientific traditions have to be combined. Molecular biologists grow mutants with known lesions in controlled environments without considering the fitness parameter, whereas ecologists generally grow poorly characterized plants in the natural environment and measure their fitness. To investigate the importance for plant fitness of single photosynthetic genes, we have developed an assay using Arabidopsis as a model plant (Kühlheim et al., 2002). For an annual plant like Arabidopsis, which flowers early in the season and produces seeds that germinate in the same year or the following spring, the ability to set seeds determines the survival of the species; therefore, relative seed production provides an accurate measure of relative fitness, provided that germination frequency is constant. Very recently, the same approach was used to study fitness effects of R-mediated resistance (Tian et al., 2003).

We already have reported a study of mutants lacking feedback de-excitation (Kühlheim et al., 2002). For most photosynthetic proteins, no mutants are available, but transgenic plants (typically antisense or T-DNA-tagged lines) lacking the protein of interest can be produced quite readily. To evaluate the structure, function, and regulation of the LHC proteins of higher plants, we have repressed individual LHC genes with antisense techniques and T-DNA knock-out approaches and, thus, constructed various lines of plants with different LHC compositions. In this study, we subjected these plants to the fitness assay to elucidate the importance of single LHC proteins.

The general issue addressed in this study is the importance of each single protein in the photosynthetic apparatus for the plants. Although many proteins are clearly essential for photosynthesis, plants lacking any one of surprisingly large numbers of the...
proteins in the photosynthetic apparatus have shown no apparent growth aberrations. The LHC proteins may be particularly interesting in this respect because they constitute a family of proteins that share the general function of maximizing the efficiency of photosynthetic light harvesting, but none of them are essential for photosynthesis. This raises intriguing questions because if they do not each have a specific function, their evolutionary conservation is contrary to theoretical expectations. In a number of investigations, plants lacking specific photosynthetic proteins have grown normally under stress-free conditions, and they then have been exposed to abiotic stress (often suboptimal light or temperature) under controlled conditions and differences have sometimes, but not always, appeared (e.g. Niyogi et al., 1998; Haldrup et al., 1999; Havaux and Niyogi, 1999). However, evolutionary selection takes place in natural, highly variable, environments, where plants have to cope not only with multiple stresses but also with rapid fluctuations in the environmental factors. Clearly, this places the plant under severe pressure to maintain a flexible and stress-resistant photosynthetic apparatus. Therefore, we performed this field study to challenge plants lacking individual photosynthetic proteins with conditions as close to natural as possible.

There are several potential problems with such a study. The plant material should be genetically homogenous. The antisense effect has to be stable under field conditions. It must be shown that seed production is an adequate measure of fitness in the test species. The genotype differences need to be large enough to be detectable despite the huge variations in growth (and, thus, seed production) in the field. The possibility that the transformation event per se may reduce fitness needs also to be considered, i.e. could the presence of the rest of the T-DNA be harmful for the plant under natural conditions? Also, the significant amount of manpower necessary to conduct the field experiments made it impossible to analyze more than one transgenic line lacking each protein. We believe that all of these potential problems have been addressed in this study, enabling us to draw rigorous conclusions from the results. First, inhomogeneity of genetic material is in normal experiments minimized by germinating seeds on selective plates. Although this was not possible here, errors introduced in this way could only result in an underestimation of the reduction in fitness for a transgenic line since some of the “transgenic” plants would be wild type. Second, loss of the antisense effect in the field in some plant individuals will also result in an underestimation of fitness. Third, we believe that Arabidopsis seed production is a reasonably accurate measure of fitness. Several problems are associated with measuring the germination frequency of plants grown in the field. To maximize germination frequency, Arabidopsis seeds should be left on the plants until the siliques open and the seeds are dispersed. Here, we could not do this because all seeds were harvested at the same time; hence, germination tests would not have been reliable. However, plants with reduced seed production are also likely to produce a smaller proportion of viable seeds, which will probably result in an underestimation of the true fitness reduction. Fourth, a strict experimental approach was adopted, in which the genotypes were grown in a fully randomized block design because even plants of the same genotype growing close to each other can often display very different traits because of stochastic events, e.g. attacks by herbivores. We do not believe that the rest of the T-DNA (e.g. the resistance marker) has any negative effect on the fitness. We performed an identical analysis with similar transgenic lines lacking proteins with less obvious functions and lines overexpressing different proteins. None of these plants exhibited any reduction in fitness, suggesting that the rest of the T-DNA is unlikely to have an effect on fitness. In addition, the data are consistent with theoretical expectations: Plants lacking a large portion of the antenna (e.g. asLhca4) show a more pronounced reduction in fitness than those with deficiencies where the overall effects on protein content were more minor, e.g. Lhca1. Finally, in all cases but the koLhca1 line, where only one line is available, we have in the original publications analyzed several lines of each construct in the lab, and we always observed consistent phenotypes. This is also true for asLhcb6 (data not shown). The data for Lhca1, therefore, are weaker, but because the Lhca1 plants were the ones where we saw the smallest effect, we think that this will not be important for the conclusions of the paper. Therefore, we believe that the effects we observed are, genuinely, consequences of the loss of individual LHC proteins. We cannot exclude the possibility that the effect is indirect (i.e. because of decreased integrity if the whole antenna system in the absence of the target protein) but that does not change the main conclusion that each protein has a unique function.

The LHC contents in the field may differ because the total and relative levels of LHC protein vary with changes in conditions (Bailey et al., 2001), and in plants lacking individual proteins, such variations may cause secondary effects. However, this is not important for the general conclusions of this study.

Plants in the field are subjected to a wide range of stresses, such as differences in light intensity and quality, drought, and grazing. As expected, the experimental plants showed a variety of stress responses, such as early flowering, small, thick leaves, pronounced anthocyanin production, and leaf necrosis. However, none of the transgenic lines exhibited more photo-inhibition of photosynthesis than the corresponding wild type, unlike mutants lacking PsbS or violaxanthin de-epoxidase, which are pro-
teins with a photoprotective function (Kühlheim et al., 2002). This indicates that adequate photoprotective mechanisms are still present in all the plants tested in this experiment, and that none of these LHC proteins has a direct role in photoprotection. Therefore, in contrast to the situation in Chlamydomonas reinhardtii (Elrad et al., 2002), no LHC proteins appear to be directly involved in feedback de-excitation in higher plants (in which, presumably, only the PsbS protein has this function). Instead, we interpret the measured loss in fitness as being because of the plants having reduced flexibility in the systems evolved to cope with changes in the light environment in the field.

Under the uncontrolled conditions used here, the huge variation in performance of individuals of the same genotype necessitates the use of large numbers of plants to detect significant differences between the genotypes. Scaling up creates logistic problems, but for seven of the eight genotypes, we found a statistically significant decrease in fitness under some conditions. Not surprisingly, the effect differed greatly between the subexperiments. In a pre-experiment, performed in 2001 (see supplemental material), the Lhca4, Lhcb2, Lhcb4, Lhcb5, and Lhcb6 lines all showed a greater decrease in fitness, especially in the shade, probably because the weather conditions were less favorable and/or grazing was different. By far, the largest effect in 2002 was found in the asLhca4 line, which produced only 32.5% of the wild-type amount of seeds (shade). In 2001, fitness reduction was even more pronounced: asLhca4 plants produced on average only 9% as many seeds as the wild type. These plants also showed growth retardation in the climate chamber, unlike the other genotypes. Presumably, the loss of most of the light-harvesting antenna of PSI caused an imbalance in electron transport, which impaired the performance of the plants, and this will be the subject of a coming study. It may appear surprising that we were able to detect a clear effect on fitness in plants that did not differ in growth rate. We believe that the higher sensitivity of the fitness assay is because of the fact that the production of reproductive structures puts high demands on the photosynthetic apparatus. Before flower formation, photosynthetic efficiency may be slightly affected without affecting growth rates, but when flowers and later seeds are formed, very strong sinks are created. Under such circumstances, even minute differences in light harvesting could reduce maximum photosynthetic capacity, leading to significantly reduced flower formation and, thus, seed set.

It may be surprising, given that the missing proteins are antenna proteins, that the reductions in fitness were higher under high-light conditions in some cases, but we believe that this finding should be treated with caution. In the pre-experiment in 2001, fitness reduction was much more pronounced under shaded conditions. However, the site used that year was different from those used in 2002, and the shading was much more irregular, resulting in larger variations in irradiance. If data from both years are taken into consideration, it may be concluded that the LHC proteins, like PsbS, have a major role in adjusting the light-harvesting antenna in response to variations in light conditions, but more detailed studies are required to determine the types of light conditions that are particularly harmful to the different genotypes. At present, we can only conclude that most, and probably all, of the studied proteins have a significant effect on plant performance under natural conditions, supporting the view that each LHC protein is important for plant fitness.

MATERIALS AND METHODS

Plant Material

Arabidopsis ecotypes Columbia or C24 were used as genetic background for the construction of LHC protein-deficient antisense plants as previously described. The antisense (as) Lhca4 (line 22) is described by Zhang et al. (1997), asLhca2 and asLhca3 are described by Ganeteg et al. (2001), asLhcb4 and asLhcb5 are described by Andersson et al. (2001), and asLhcb2 is described by Andersson et al. (2003). The Lhca1 T-DNA insertion mutant (koLhca1) was obtained from the Syngenta SAIL collection (Garlic 870 E9, Syngenta, TMRI, San Diego). Twenty-eight seeds were sown, of which eight produced plants that were found to have decreased levels of Lhca1 according to western-blot analysis and 77K fluorescence analysis. One homozygous plant with one T-DNA insert was selected for seed collection, and these seeds were used in this study. Lhcb6 antisense plants (asLhcb6) were constructed by cloning EcoRI-digested full-length CDNA of Lhcb6, amplified by PCR from Arabidopsis expressed sequence tag clone 23AI17 using oligonucleotides T7 (Life Technologies, Grand Island, NY) and PWE (Ganeteg et al., 2001), into the vector pSJ10 (Ganeteg et al., 2001). Antisense orientation of the inserts was confirmed by sequencing, and a successful clone was used for Agrobacterium tumefaciens-mediated transformation of Arabidopsis as described earlier (Ganeteg et al., 2001). Positive transformants were selected on Murashige and Skoog plates supplemented with 50 μg ml⁻¹ kanamycin and screened for decreased levels of Lhcb6 using immunoblotting. In our experience, segregation analysis of antisense plants does not always give clear-cut results, but asLhcb2, asLhca4, and koLhca1 are homozygous and give 100% such progeny, and asLhcb1, asLhcb5, and asLhcb6 are heterozygous and give 75% such progeny. The asLhca3 plants give 100% and asLhca2 plants approximately 75% such progeny, but the genetics have not been established. In the experiments under controlled conditions, kanamycin-resistant plants were used, but in the experimental design applied in the field experiment, we chose to sow out seeds directly to test the plants under conditions that were as natural as possible.

Plant Growth

For experiments under controlled conditions, Arabidopsis plants were grown under fluorescent lamps in a growth cabinet with a day/night temperature regime of 23°C/18°C, a photosperiod of 8 h with a light intensity of 150 μmol m⁻² s⁻¹ quanta, and 75% humidity.

For the fitness experiment, plastic trays with drainage holes were filled with soil and pots without bottoms (with three seeds from either wild-type or antisense plants) were randomly positioned in the tray to avoid position-dependent differences in growth affecting the results. The seeds were stratified for 4 d and then placed in the field. When seedlings appeared, one pot was chosen for the experiment, and the others were removed. The field experiments were performed in the experimental garden of Umeå University (Sweden; 63° 50’N, 20° 20’E). Because these plants are genetically modified organisms, permission to grow the transgenic lines in the field was applied for and given by the Swedish Board of Agriculture (Jordbruksverket). Details about the permit and measures taken to prevent contamination of the environment by transgenic seeds or pollen are available on request. Three sites were cleared of vegetation: one in full sunlight (hereafter named sun) and two in the shade of trees growing at the site (Shades A and B). The
distance between the shade sites was less than 5 m, and they were about 50 m from the sun site. Ten centimeters of soil were replaced with new soil, and the plastic trays were placed out in the field, giving soil-to-soil contact. For practical reasons, not all genotypes could be planted at the same time on all sites. The Lhcb2, Lhcb4, Lhcb5, and Lhcb6 antisense plants and Columbia wild type were planted out on sun and Shade B sites on June 24. A second set of genotypes (Lhca1, Lhca2, and Lhca3 antisense plants plus Columbia wild type) was transferred to the sun and Shade A sites on July 12. Finally, Lhca4 and C24 wild type were planted out at the sun and Shade B sites on July 17. The irradiation of the three sites was recorded for typical days using a quantum sensor (Skye SKP 215, Skye Instrument Ltd., Llandrindod Wells, UK), and data were recorded and stored with a data logger (CR 10, Campbell Scientific Ltd., Logan, UT), other weather parameters were logged about 200 m from the sites, and these data are available at http://www.tfe.umu.se/weather/arkiv.asp. In general, the summer in the area was unusually dry and warm.

The intention was to expose the plants in the field experiment to conditions that were as natural as possible, without watering, fertilization, or protection against pests. However, to avoid all the small seedlings dying through drought, some water had to be added early in the experiments, and after a heavy attack of diamond-backed moths (Plutella xylostella), insecticide (Pyrex N, Wikholm & Co., Stockholm) was applied once. When the plants started to flower, the site was covered with a net to prevent insects reaching the plants and, thus, reduce the probability of spreading transgenic pollen. The experiment was terminated between mid-August and early September, depending on the genotype and experimental site. When harvested, most plants appeared to be dead, and no more flowers were produced.

Thylakoid Protein Preparation and Immunoblotting

Leaves of Arabidopsis plants (6–8 weeks old) grown under controlled conditions were taken for thylakoid protein preparation 3 h into the growth period according to Zhang et al. (1997), and the proteins were separated and transferred to nitrocellulose membranes according to Ganeteg et al. (2001). The antibodies used to detect the LHC proteins were collected as described earlier (Ganeteg et al., 2001).

Experimental Design and Statistical Treatment

A block design was used in which all genotypes in a subexperiment were fully randomized in a tray containing 30 plants. For example, at “sun”, six seedlings each of the Lhcb2, Lhcb4, Lhcb5, Lhcb6, and Columbia genotypes were placed in each tray. Three to nine trays, all individually randomized, were used in each subexperiment in each area, the total number of plants of each genotype was at least 40 per area, and the total number of plants in the whole experiment was 1,050.

Silique and Seed Production

For each experimental plant, the total number of siliques was counted, and seeds were counted from three randomly chosen mature siliques. The total number of seeds was then calculated for each plant by multiplying the average number of seeds per siliqua by the number of siliques. The seeds from the three siliques were pooled and weighed (to the nearest 0.001 mg), giving an estimate of total seed weight.

Photo-Inhibition Measurement

A plant stress meter (Techtum Lab, Umeå, Sweden) was used to measure the fluorescence parameter Fv/Fm in intact plants in the field according to Öquist and Wass (1988). For each date, we measured Fv/Fm in three to five randomly chosen plants for each permutation of genotype and experimental site.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

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LITERATURE CITED


Havuux M, Niyogi KK (1999) The violaxanthin cycle protects plants form photooxidative damage by more than one mechanism. Proc Natl Acad Sci USA 96: 8762–8767


