A pigment-binding protein essential for regulation of photosynthetic light harvesting

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Photosynthetic light harvesting in plants is regulated in response to changes in incident light intensity. Absorption of light that exceeds a plant's capacity for fixation of CO_2 results in thermal dissipation of excitation energy in the pigment antenna of photosystem II by a poorly understood mechanism. This regulatory process, termed nonphotochemical quenching, maintains the balance between dissipation and utilization of light energy to minimize generation of oxidizing molecules, thereby protecting the plant against photo-oxidative damage. To identify specific proteins that are involved in nonphotochemical quenching, we have isolated mutants of *Arabidopsis thaliana* that cannot dissipate excess absorbed light energy. Here we show that the gene encoding PsbS, an intrinsic chlorophyll-binding protein of photosystem II, is necessary for nonphotochemical quenching but not for efficient light harvesting and photosynthesis. These results indicate that PsbS may be the site for nonphotochemical quenching, a finding that has implications for the functional evolution of pigment-binding proteins.

Photosynthetic reactions in plants convert light energy into chemical energy that supports much of the life on Earth. However, the involvement of highly reactive intermediates necessitates regulation of photosynthesis to cope with the constantly changing quantity of light in natural environments and to protect against photo-oxidative damage[1–4]. Photosynthetic light harvesting in plants is subject to feedback regulation by the changes in pH (ΔpH) generated by electron transport in the chloroplast[3]. Whenever light absorption exceeds a plant's capacity for CO_2 fixation, accumulation of protons in the thylakoid lumen results in thermal dissipation of absorbed light energy in the pigment antenna of photosystem II. Absorption of excess sunlight occurs daily for many photosynthetic organisms, especially when growth is limited by environmental stresses such as extremes of temperature, drought, salinity and nutrient deprivation.

Photoprotective thermal dissipation of absorbed light energy is measured as nonphotochemical quenching of chlorophyll fluorescence (NPQ) during exposure of plants to high light[3]. Although several processes can contribute to NPQ, the major component in wild-type *Arabidopsis* and other plants is ΔpH-dependent NPQ, referred to as qE, which is characterized by rapid induction and relaxation kinetics (Fig. 1a, b). During induction of qE by light, the build-up of ΔpH activates synthesis of a specific xanthophyll pigment, zeaxanthin, through a xanthophyll cycle[5]. Allosteric binding of both H^+ and zeaxanthin to light-harvesting antenna proteins is suggested to cause a conformational change that leads to quenching of excitation energy[3,6,7]. The rapid relaxation of qE during subsequent darkness (Fig. 1a, b) reflects the decay of the light-induced ΔpH. Analysis of a mutant that is defective in the xanthophyll cycle, *npq1*, showed that zeaxanthin synthesis is necessary for about 70% of the total NPQ and 80% of the qE in *Arabidopsis*[8] (Fig. 1b). However, zeaxanthin is not sufficient for qE in the absence of the ΔpH, as shown using *npq2* (*aba1*) mutants that accumulate zeaxanthin constitutively[8–10].

Although qE has been the subject of intense research for more than a decade, its exact site and molecular mechanism are unknown. The possible involvement of specific proteins in the light-harvesting antenna of photosystem II has been addressed by identification of proteins that bind zeaxanthin[11] and dicyclohexylcarbodiimide (DCCD, an inhibitor of qE *in vitro*)[12] and by characterization of plants that are deficient in various antenna proteins[13,14]. These experiments have led to the suggestion that qE occurs in the Lhcb4 (CP29) and Lhcb5 (CP26) antenna proteins[3,7,11,15,16], but there has been no direct evidence for the function of any specific protein in qE.

npq4 is defective in nonphotochemical quenching

To identify components in addition to the ΔpH and xanthophylls that are necessary for qE, we used a chlorophyll fluorescence video-imaging system[8] to isolate *Arabidopsis* mutants with altered NPQ but normal pigment composition. Measurement of NPQ induction and relaxation kinetics showed that the *npq4-1* mutant was defective specifically in the qE component of NPQ (Fig. 1a, b). However, the *npq4-1* mutant exhibited zeaxanthin synthesis in high light that was indistinguishable in extent (Table 1) and kinetics (data not shown) from that of the wild type, in contrast to the previously characterized *npq1* mutants[8].

Genetic analysis showed that the phenotype of *npq4-1* plants was due to a single, semidominant, nuclear mutation (Table 2). The Npq^- phenotype of an *npq4-1 npq1-2* double mutant was indistinguishable from that of *npq4-1* (Fig. 1b), showing that the *npq4-1*

Table 1 Xanthophyll cycle pigments in wild-type and *npq4-1* leaves

		mmol per mol chlorophyll *a*				
		V	A	Z	V + A + Z	(A + Z)/(V + A + Z)
Wild type	before HL	53 ± 8	16 ± 2	13 ± 1	82 ± 9	0.36 ± 0.03
	after HL	18 ± 1	8 ± 1	43 ± 9	70 ± 10	0.73 ± 0.03
npq4-1	before HL	54 ± 2	12 ± 1	6 ± 1	73 ± 2	0.26 ± 0.02
	after HL	17 ± 1	8 ± 1	43 ± 5	68 ± 6	0.75 ± 0.02

Pigment composition of leaves was measured by high-performance liquid chromatography either before or after exposure to high light (HL, 640 μmol photons m^{-2} sec^{-1}) for 30 min. Values are means ± s.e. ($n = 4$).

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mutation blocks even the small amount of qE that is detected in *npq1-2* plants.

npq4 lacks the conformational change

The *npq4-1* mutant lacks the ΔpH- and zeaxanthin-dependent conformational change in the thylakoid membrane that is necessary for qE. This conformational change is monitored by a light-induced change in absorbance at 535 nm (ΔA_{535})[17,18], which appears in wild-type leaves as a shoulder on a major absorbance change at 505 nm (Fig. 2a). The ΔA_{505}, which is attributable to conversion of violaxanthin to zeaxanthin through the xanthophyll cycle[19], occurred in the leaves of both wild-type and *npq4-1* plants, consistent with the normal synthesis of zeaxanthin that was observed in *npq4-1* (Table 1). However, the ΔA_{535} was absent in the *npq4-1* mutant, as demonstrated by the lack of the shoulder on the ΔA_{505} peak (Fig. 2a).

To determine whether *npq4-1* retained any ΔA_{535} that had been obscured by the major ΔA_{505} signal, we examined an *npq4-1 npq2-1* double mutant. Plants carrying the *npq2-1* mutation accumulate zeaxanthin constitutively and lack the ΔA_{505} owing to the absence of xanthophyll cycling[8], allowing unambiguous visualization of the ΔA_{535} (Fig. 2b). In contrast to the *npq2-1* single mutant, the *npq4-1 npq2-1* double mutant completely lacked a detectable ΔA_{535} signal (Fig. 2b). Together, these results indicate that the *npq4-1* mutation may define a gene that is necessary for both the conformational change in the antenna and qE.

The *NPQ4* gene encodes PsbS

To isolate the *NPQ4* gene, we used a combination of molecular and visible genetic markers to map the *npq4-1* mutation to chromosome 1, ~0.7 cM south of the *ch1* locus (Fig. 3a). Hybridization with a *CH1* complementary DNA[20] identified several bacterial artificial chromosome (BAC) clones, some of which also contained the *Arabidopsis* gene encoding PsbS[21], an intrinsic pigment-binding photosystem II subunit (also known as CP22) of previously unknown function[22]. Polymerase chain reaction (PCR) amplification of the *psbS* gene from the *npq4-1* mutant was unsuccessful (data not shown), indicating that the fast neutron-induced *npq4-1* allele may contain a DNA rearrangement or deletion affecting *psbS*. DNA gel blot analysis confirmed that the *psbS* gene is completely absent from the genome of *npq4-1* (Fig. 3b). Four other alleles of *npq4*, which were isolated following mutagenesis with ethylmethane sulphonate, contained missense mutations in *psbS* (Fig. 3c). Complementation of the *npq4-1* (Fig. 1c) and *npq4-4* mutations (data not shown) by transformation with a wild-type copy of the *psbS* gene verified that the *NPQ4* gene is *psbS*.

Light-harvesting function in *npq4*

The PsbS protein is a member of the chlorophyll *a*/*b*-binding, light-harvesting complex (LHC) family of proteins[21,23,24]. However, PsbS is present in many oxygen-evolving photosystem II preparations[25] that are depleted of typical LHC proteins, indicating that PsbS may be closely associated with the photosystem II reaction centre,

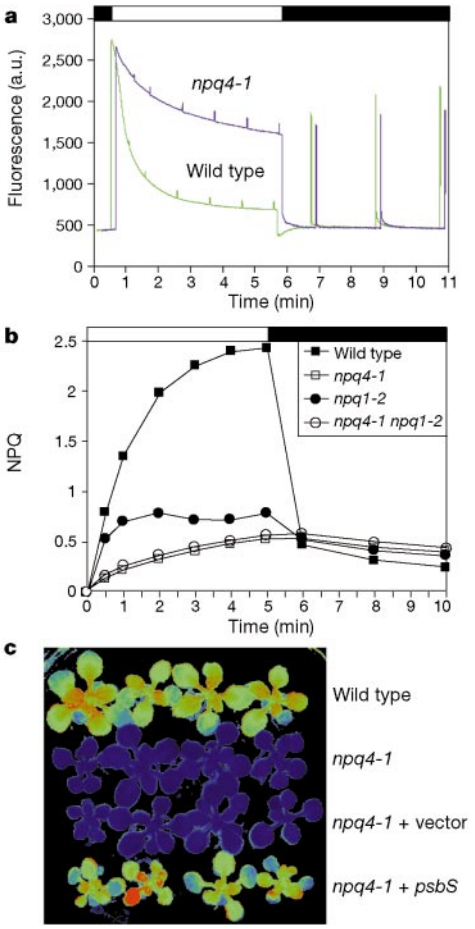

Figure 1 Nonphotochemical quenching phenotypes. White bars above graphs indicate periods of illumination with high light (1250 μmol photons m^{-2} sec^{-1}); black bars indicate darkness (with only very weak measuring light). **a**, Chlorophyll fluorescence during induction and relaxation of NPQ in wild type and *npq4-1*. The *npq4-1* trace is offset by 10 s relative to that of the wild type. **b**, NPQ calculated from fluorescence data for wild type, *npq4-1*, *npq1-2* and an *npq4-1 npq1-2* double mutant. **c**, Image of NPQ occurring after 1 min of illumination with 800 μmol photons m^{-1} sec^{-1}. *npq4-1* + vector, *npq4-1* plants transformed with pPZP121; *npq4-1* + *psbS*, *npq4-1* plants transformed with pXPL1. a.u., arbitrary units.

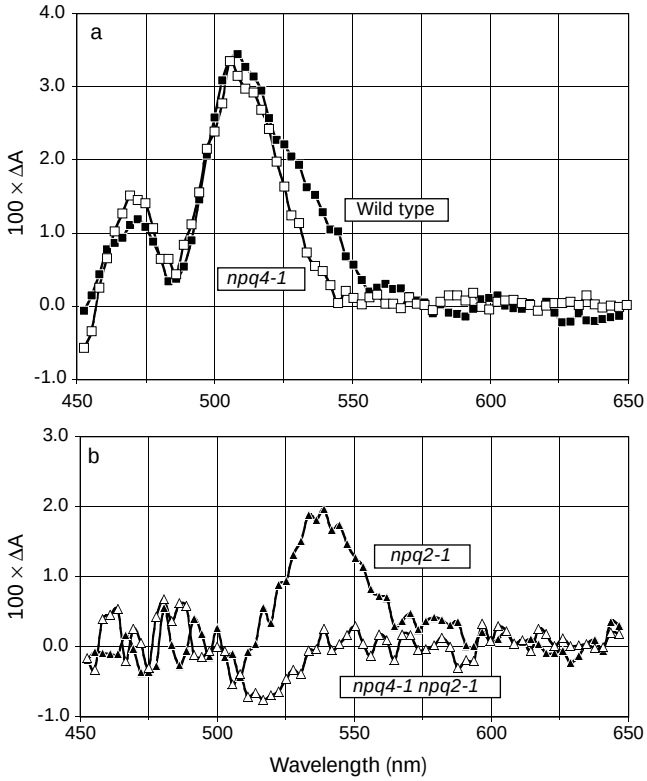

Figure 2 Light-induced spectral absorbance changes in leaves. **a**, Wild type (filled squares) and *npq4-1* (open squares). **b**, *npq2-1* (filled triangles) and *npq4-1 npq2-1* (open triangles).

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perhaps at the interface between the reaction centre core and the peripheral light-harvesting antenna[26]. Despite the absence of the PsbS protein in the *npq4-1* deletion mutant (Fig. 4), light harvesting and photosynthesis were not obviously affected. Both the quantum yield and the maximum rate of photosynthetic oxygen evolution in the *npq4-1* mutant were the same as those of the wild type (Table 3). The maximum quantum yield of photosystem II electron transport, determined from chlorophyll fluorescence measurements at room temperature, was similarly unaffected in *npq4-1* plants (Table 3). In addition, the fluorescence emission spectra of *npq4-1* and wild-type thylakoids at 77K were identical (data not shown). Growth of *npq4-1* plants at limiting light intensities was indistinguishable from that of the wild type (data not shown). The other LHC proteins that comprise the peripheral light-harvesting antenna of photosystem II (Lhcb1-Lhcb6) were all present at wild-type levels in *npq4-1* (Fig. 4).

Discussion

These results show clearly that the PsbS protein contributes to photoprotective energy dissipation rather than photosynthetic light harvesting. Although the exact role of PsbS in qE remains to be determined, the lack of both qE (Fig. 1) and the protonation-induced conformational change (ΔA_{535}) (Fig. 2) in the *npq4-1* mutant indicates that binding of one or more protons by PsbS may be a necessary feature of the qE mechanism. Several acidic amino-acid residues in PsbS on the lumen side of the thylakoid membrane are candidate proton-binding sites (Fig. 3c).

With the observation that the isolated PsbS protein binds chlorophylls and xanthophylls[22], our results suggest a model in which PsbS, rather than other pigment-binding LHC proteins in the antenna of photosystem II[3,7,11,15,16], is the site of ΔpH- and xanthophyll-dependent excitation quenching. Consistent with this model, plants that are deficient in LHC proteins but retain qE have wild-type levels of PsbS[27,28]. Upon protonation of PsbS, a conformational change could allow for quenching of single excited chlorophyll either by direct energy transfer from chlorophyll to zeaxanthin[5,29] bound to PsbS or by a zeaxanthin-dependent chlorophyll–chlorophyll[3,16] or chlorophyll–protein interaction occurring within PsbS. Alternatively, protonation of PsbS may be necessary for conformational changes and quenching that actually occur in adjacent LHC proteins of an antenna subcomplex. Indeed, isolated Lhcb complexes have been observed to exhibit pH-dependent quenching of chlorophyll fluorescence *in vitro* that is modulated by factors, such as zeaxanthin, that affect qE *in vivo*[30–32]. It is also possible that PsbS is simply necessary to maintain LHC proteins in a proper

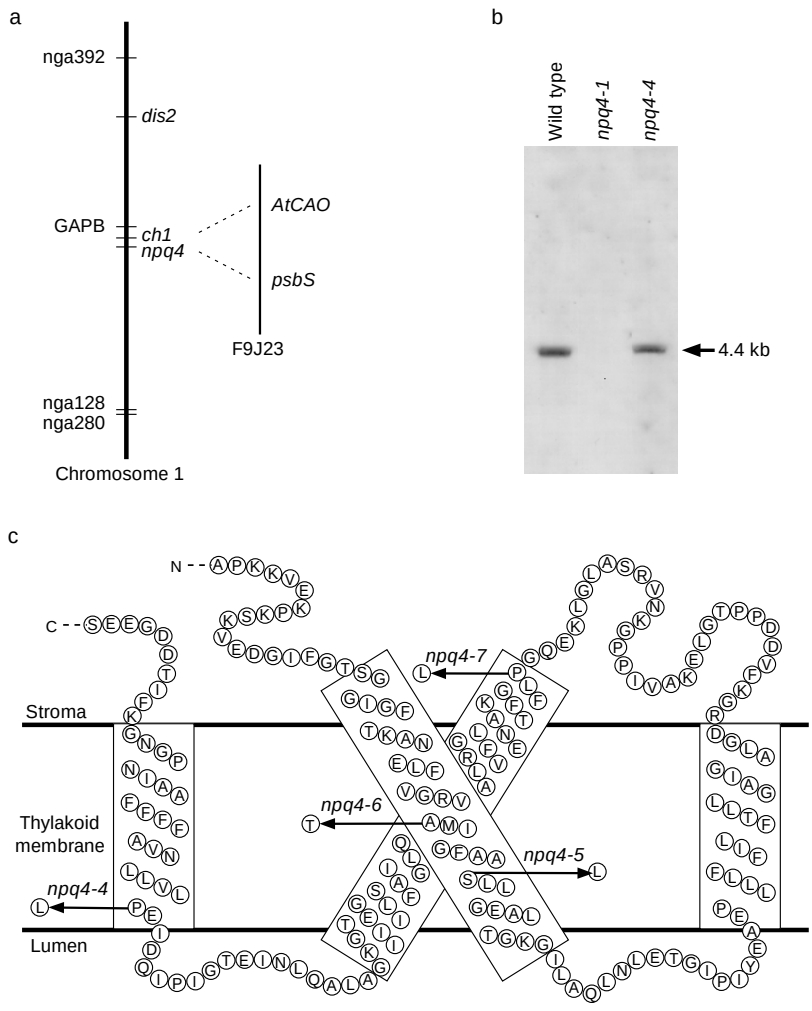

Figure 3 Cloning of *NPQ4*. **a**, Map position of *npq4-1*. Molecular markers are shown to the left of the vertical line that represents part of chromosome 1; visible markers are shown on the right. F9J23 is a BAC clone containing both *CH1* (*AtCaO*, ref. 20) and *psbS*. The relative positions of *AtCAO* and *psbS* are shown, but their exact locations within F9J23 were not determined. **b**, DNA gel blot analysis. Genomic DNA from wild type, *npq4-1* and *npq4-4* was digested with *Xba*I and hybridized with a *psbS* probe. **c**, Schematic representation of the PsbS protein. The positions of missense mutations in *npq4* point mutant alleles are indicated. The predicted topology of the PsbS protein is based on the crystal structure of the related LHC-II protein[47].

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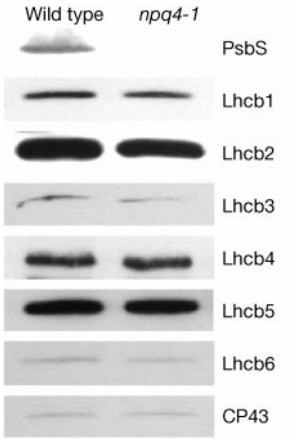

Figure 4 LHC protein levels in wild type and *npq4-1*. Thylakoid protein samples corresponding to equal amounts of chlorophyll were separated by SDS-PAGE, and immunoblot analysis was performed with polyclonal antibodies directed against each of the indicated photosystem II proteins. Lhcb1, Lhcb2 and Lhcb3 are components of trimers in the most peripheral antenna of photosystem II; Lhcb4, Lhcb5 and Lhcb6 are monomeric, peripheral antenna proteins (also known as CP29, CP26 and CP24, respectively)[21]. CP43 is a core antenna subunit of photosystem II.

supramolecular organization that allows qE to occur. However, efficient photosynthetic light harvesting by *npq4-1* in limiting light (Table 3) and accumulation of wild-type levels of other LHC proteins in *npq4-1* (Fig. 4) indicate that the qE defect of the mutant is not due to an obvious indirect effect on the photosystem II antenna. Further structural analysis of the PsbS protein may help to define possible proton- and/or zeaxanthin-binding sites and to elucidate the molecular mechanism of ΔpH- and xanthophyll-dependent regulation of light harvesting.

The semidominance of the loss-of-function *npq4-1* mutation (Table 2) indicates that the *psbS* gene dosage may be a critical determinant of qE capacity. The maximum extent of qE is considered to be an important factor for adaptation of plants to adverse environments, and plants growing in excessive light generally exhibit greater maximal qE than shade plants[33–35]. In *Arabidopsis*, the level of *psbS* messenger RNA is increased severalfold in response to high light (unpublished results), consistent with a possible relationship between *psbS* expression and qE capacity. Accumulation of PsbS protein in etiolated seedlings of spinach[27] may allow photoprotective qE upon exposure to light during the early stages of greening. In overwintering pine trees, which exhibit high levels of slowly reversible NPQ, increases in the amount of PsbS are associated with a reorganization of pigment-binding proteins[36], suggesting that PsbS is involved in more than one type of NPQ.

Unlike typical LHC proteins with three transmembrane α-helices, the PsbS protein has four helices that span the thylakoid membrane[26] (Fig. 3c). The first and third helices of PsbS are similar to each other and to the first and third helices of three-helix LHC proteins. In addition, the second and fourth helices of PsbS are similar to each other and to the second helix of three-helix LHC proteins, indicating that PsbS may have arisen by internal duplication of a gene encoding a two-helix protein and that the three-helix LHC proteins then evolved from a four-helix ancestor like PsbS[23,24,37]. Genes encoding one-helix proteins (similar to the first and third helices of PsbS), as well as two-helix proteins that resemble proposed intermediate forms in the evolution of LHC proteins, have been identified[21,38]. Expression of several genes encoding these 'ancestral' types of LHC protein is induced by high light stress[21,38], suggesting that they are photoprotective. Our finding that PsbS is involved in energy dissipation rather than light harvesting supports the hypothesis that, during the evolution of oxygenic photosynthesis, members of the LHC protein family with roles in photoprotection appeared before those involved in light harvesting[39,40]. □

Methods

Mutant isolation

Mutants of *Arabidopsis thaliana* (ecotype Col-0) were identified by chlorophyll fluorescence video imaging[8] of M_2 seedlings derived from mutagenesis with fast-neutron bombardment (Lehle Seeds) or 0.3% (v/v) ethylmethane sulphonate. Plants were grown on minimal agar medium in petri plates or in Sunshine growth mix (Sun Gro Horticulture) in pots in a greenhouse[8].

Chlorophyll fluorescence, spectroscopy and oxygen evolution

We measured chlorophyll fluorescence from attached rosette leaves at room temperature with an FMS2 fluorometer (Hansatech). NPQ was calculated as $(F_m - F'_m)/F'_m$. Measurements of light-induced spectral absorbance changes induced by illumination with 1,900 μmol photons m^{-2} sec^{-1} for 3 min were performed as described[8]. Photosynthetic oxygen evolution was measured as a function of incident photon flux density with a leaf disc electrode (LD2/2, Hansatech).

Pigment analysis

Chlorophylls and carotenoids were analysed by high-performance liquid chromatography (HP1100, Hewlett-Packard) on a Microsorb-MV column as described[41]. Carotenoids were quantified using standard curves of purified pigments (VKI).

Table 2 Results of cross between *npq4-1* and wild-type plants

Cross	Type	Total	Npq$^+$	Npq$^{+/-}$	Npq$^-$
npq4-1/npq4-1 × *NPQ4/NPQ4*	F_1	8	0	8	0
	F_2	233	60	106	67

Pollen from the male parent (listed first) was crossed onto stigmas of the female parent to generate F_1 seeds. The F_1 plants were allowed to self-pollinate to generate F_2 seeds. The phenotypes of F_1 and F_2 plants were scored by fluorescence video imaging after 12 days of growth on agar medium. The heterozygous F_1 plants had an intermediate phenotype (Npq$^{+/-}$). The 1:2:1 segregation of the Npq phenotype in the F_2 generation is consistent with the hypothesis that *npq4-1* is a single semidominant, nuclear mutation ($\chi^2 = 2.3$, $P > 0.1$).

Table 3 Photosynthetic parameters of wild-type and *npq4-1* leaves

Parameter	Wild type	*npq4-1*
$\Phi(O_2)$	0.0818 ± 0.0024 ($n = 5$)	0.0812 ± 0.0033 ($n = 5$)
P_{max}	18.15 ± 1.06 ($n = 5$)	18.87 ± 0.92 ($n = 5$)
F_v/F_m	0.837 ± 0.003 ($n = 8$)	0.836 ± 0.004 ($n = 8$)

$\Phi(O_2)$, apparent quantum yield of O_2 evolution (O_2 evolved per incident photon); P_{max}, maximum rate of O_2 evolution (μmol O_2 m^{-2} sec^{-1}); F_v/F_m, maximum quantum yield of photosystem II electron transport. Values are means ± s.e.

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Genetic mapping and molecular biology

The *npq4-1* mutation was mapped initially by scoring PCR-based markers[42,43] on *npq4-1/npq4-1* F_2 progeny derived from a cross between *npq4-1/npq4-1* (Col-0 ecotype) and *NPQ4/NPQ4* (Ler-0 ecotype). Recombination fractions (number of crossovers/number of chromosomes scored) were 6/40, 8/44, 9/42 and 1/48 between *npq4-1* and nga392, nga128, nga280 and GAPB, respectively. Among 2,072 F_2 progeny of a cross between *npq4-1/npq4-1* and (*dis2-1 ch1-1*)/(*dis2-1 ch1-1*), we detected seven (*DIS2 CH1 NPQ4*)/(*dis2-1 ch1-1 NPQ4*) recombinant chromosomes. We used a *CH1* cDNA clone (103D24T7) as a hybridization probe (AlkPhos Direct, Amersham Pharmacia Biotech) to identify BAC clones containing the *CH1* gene. The *psbS* gene was amplified by PCR from BAC clones and genomic DNA of wild-type and *npq4* mutants using primers KN118 (5′-TCCTTCTCTCATCCTCAGAAA-3′) and KN119 (5′-CAACATGAAGAGAAGGTCACA-3′), and 1.5-kilobase (kb) PCR products were used as hybridization probes or for DNA sequencing. For complementation of *npq4-1* and *npq4-4*, a 4.4-kb *Xba*I fragment of BAC clone F9J23 containing the *psbS* gene was subcloned into the pPZP121 vector[44] to generate pXPL1. Plants were transformed[45] using *Agrobacterium tumefaciens* GV3101 containing either pPZP121 or pXPL1.

Immunoblot analysis

Thylakoid membranes were prepared and analysed by SDS-PAGE and immunoblotting using monospecific antibodies[46].

Received 12 October; accepted 6 December 1999.

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Acknowledgements

We thank A. K. Tran and V. Canale for technical assistance; J. Brusslan for unpublished data on *CH1*; T. Shikanai for the *npq4-4* allele; C. Funk, J. Knötzel and A. Staehelin for antibodies; R. Malkin for comments on the manuscript; and the Arabidopsis Biological Resource Center for strains and DNA clones. This work was supported by grants from the U.S. Department of Agriculture–National Research Initiative Competitive Grants Program and the Searle Scholars Program/The Chicago Community Trust to K.K.N., a grant from the National Science Foundation to A.G. and O.B., and grants from the Swedish Forestry and Agricultural Research Council and the Foundation for Strategic Research to S.J. When this work was initiated, K.K.N. was supported as a Department of Energy Biosciences Fellow of the Life Sciences Research Foundation.

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