

# Three PsbQ-Like Proteins are Required for the Function of the Chloroplast NAD(P)H Dehydrogenase Complex in Arabidopsis

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Arabidopsis has three PsbQ-like (PQL) proteins in addition to the PsbQ subunit of the oxygen-evolving complex of PSII. Recent bioinformatic and proteomic studies suggested that the two PQL proteins, PQL1 (At1g14150) and PQL2 (At3g01440), might function in the chloroplast NAD(P)H dehydrogenase (NDH) complex; however, their molecular function has not been characterized. In this study, we examined the function of the chloroplast NDH in the Arabidopsis *pql1* and *pql2* mutants. Post-illumination increases in Chl fluorescence, which are caused by an NDH-dependent cyclic electron flow, were absent in both mutants, indicating that PQL1 and PQL2 are required for NDH activity. In the thylakoid membranes of wild-type plants, PQL1 and PQL2 were tightly associated with the NDH-PSI supercomplex and protected from protease treatments, while unassembled PQLs were not stably accumulated in mutants lacking known NDH subunits. Subunit stability of the NDH complex was affected differently in the thylakoid membranes of the *pql1* and *pql2* mutants. These data indicate that PQL1 and PQL2 are novel NDH subunits and differ in their functional roles and in their binding sites in the NDH complex. Furthermore, functional analysis on PQL3 (At2g01918) using the *pql3* mutant suggests that PQL3 is also required for NDH activity. Proteins homologous to each PQL protein are found in various plant species, but not in cyanobacteria, algae, mosses or ferns. These results suggest that seed plants that have NDH activity in chloroplasts specifically developed three PQL proteins for the function of the chloroplast NDH complex.

**Keywords:** *Arabidopsis thaliana* • Cyclic electron transport • Oxygen-evolving complex protein • Thylakoid lumen.

**Abbreviations:** AL, actinic light; BN, blue-native; cyt, cytochrome; NDH, NAD(P)H dehydrogenase; OEC, oxygen-evolving complex; PPL, PsbP-like; PQL, PsbQ-like; RT-PCR, reverse transcription-PCR.

## Introduction

Oxygen-evolving complex (OEC) proteins are membrane-extrinsic luminal subunits of PSII. Despite their location close to the catalytic center, OEC proteins have undergone drastic changes during evolution (Enami et al. 2008): higher plants and green algae have three extrinsic proteins (PsbO, PsbP and PsbQ), whereas oxygenic photosynthetic bacteria are thought to have a different set of proteins (PsbO, PsbU, PsbV, and PsbP-like and PsbQ-like homologs) (Roose et al. 2007). PsbP and PsbQ in higher plants are believed to have evolved from their cyanobacterial homologs, cyanoP and cyanoQ, respectively (De Las Rivas et al. 2004). In addition to authentic OEC proteins in PSII, genomic and proteomic studies have identified multiple homologs of PsbP and PsbQ in higher plants (Roose et al. 2007). These OEC homologs are all nuclear-encoded proteins, and they most probably emerged as a result of gene duplication.

In a previous study, we investigated the functions of the two PsbP homologs, PsbP-like protein 1 (PPL1) and PPL2, in Arabidopsis (Ishihara et al. 2007). Analysis of gene expression suggests that PPL1 is expressed under stress conditions and that PPL2 is co-expressed with the subunits of the chloroplast NAD(P)H dehydrogenase (NDH) complex, which functions in PSI cyclic electron flow. Further genetic and biochemical studies demonstrated that PPL1 is required for the efficient repair of PSII photodamage, and that PPL2 is a novel thylakoid luminal subunit required for the assembly of the NDH complex. These results indicate that, during evolution, a PsbP homolog in ancient cyanobacteria evolved into PPL1, PPL2 and PsbP, and that each of these proteins has a distinct role in supporting photosynthetic electron transfer in higher plants (Ifuku et al. 2008).

Genes for the three PsbQ homologs have been found in the Arabidopsis genome [At1g14150, At3g01440 and At2g01918; hereafter referred to as PsbQ-like protein 1 (PQL1), PQL2 and PQL3, respectively] (Roose et al. 2007), and PQL1 and PQL2

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were predicted to be located in the thylakoid lumen (Peltier et al. 2002, Schubert et al. 2002). Analysis of the chloroplast membrane proteome of maize bundle sheath chloroplasts, which are enriched in the NDH complex, showed that the PQL1 and PQL2 homologs co-migrated with subunits of the NDH complex, as determined by blue-native-PAGE (BN-PAGE). These findings indicated that PQL1 and PQL2 might function in the NDH complex together with other thylakoid luminal components, such as PPL2, Tlp20 (CYP20-2) and FKBP16-2 (Majeran et al. 2008). There has been no report on the accumulation of PQL3 proteins in chloroplasts. A recent proteomic study of Arabidopsis chloroplasts also suggested that two PQLs bound to the NDH complex; different terminology for these proteins was used in this study (PsbQ-F1 and PsbQ-F2 for PQL1 and PQL2, respectively) (Peng et al. 2009). However, PQL1 and PQL2 have not yet been analyzed genetically and biochemically, and the molecular function of PQL3 is completely unknown.

In this study, we firstly characterized T-DNA insertional mutants of PQL1 and PQL2. NDH activity, as indicated by a post-illumination increase in Chl fluorescence caused by NDH-dependent cyclic electron flow, was absent in both *pql* mutants. Further, the assembly and organization of the NDH complex were affected differently in the absence of PQL1 and PQL2. These results suggest that PQL1 and PQL2 are distinct subunits of the chloroplast NDH complex. Furthermore, activity and accumulation of the chloroplast NDH complex were severely impaired in the absence of PQL3 in the *pql3* mutant. These results suggest that all of the three PQL proteins are required for the function of the NDH complex in chloroplast.

## Results

### Amino acid sequences of PQL proteins in Arabidopsis

All Arabidopsis PQL proteins showed limited sequence identity to the PSII protein PsbQ (Table 1), and they are predicted to have a four-helix bundled structure based on the crystal structure of spinach PsbQ (PDB\_ID, 1nze; Calderon et al. 2003) (Fig. 1A). Searches of genomic and expressed sequence tag (EST) databases identified putative orthologs of PQL1, PQL2 and PQL3 in many plant species, with highly conserved

amino acid sequences (Supplementary Fig. S1), but not in cyanobacteria, red algae, green algae, mosses and ferns. A phylogenetic tree for PsbQ homologs based on the sequence alignment of the four-helix bundled region showed that PQL proteins did not cluster with PsbQ in PSII, indicating that PQL proteins independently evolved from cyanoQ (Fig. 1B). Since PQL3 was identified in the latest version of data in The Arabidopsis Information Resource (TAIR), there is limited information for mRNA expression and protein accumulation on PQL3. We therefore first focused on the functional characterization of PQL1 and PQL2.

### Genes co-expressed with PQL1 and PQL2 mRNA

Genes that were co-expressed with each PQL gene were identified using the *Arabidopsis thaliana* trans-factor and cis-element prediction database (ATTED-II) program based on Pearson's correlation coefficient (*r*) for neighboring pairs of genes (Obayashi et al. 2007). While the majority of genes that were co-expressed with authentic *PsbQ* genes encoded subunits and peripheral antenna proteins of PSII or PSI, the genes that were co-expressed with PQL1 and PQL2 mRNA were involved in the NDH pathway in cyclic electron transfer. As listed in Supplementary Tables S2 and S3, PQL1 was co-expressed with the genes encoding subunits of the NDH complex, NDH-N (Rumeau et al. 2005), CHLORORESPIRATORY REDUCTION 23 (CRR23 or NDH-L) (Shimizu et al. 2008), NDH-DEPENDENT CYCLIC ELECTRON FLOW 1 (NDF1 or NDH48) (Sirpiö et al. 2009a, Takabayashi et al. 2009), NDH-O (Rumeau et al. 2005), PPL2 (Ishihara et al. 2007), CRR3 (Muraoka et al. 2006) and NDF4 (Takabayashi et al. 2009), while PQL2 was co-expressed with the genes for NDH-N, PQL1 (this study), NDF4, CRR7 (Munshi et al. 2005) and PPL2. Both PQL genes were also co-expressed with the gene encoding Lhca6, a light-harvesting complex I protein required for functional NDH-PSI supercomplex formation (Peng et al. 2009). The gene expression patterns of the two PQLs suggest that their functions are likely to be related to the chloroplast NDH complex and different from that of PsbQ in PSII.

### Generation of mutant and transgenic lines

The functions of the PQL1 and PQL2 proteins were investigated in the respective Arabidopsis mutants. Two independent

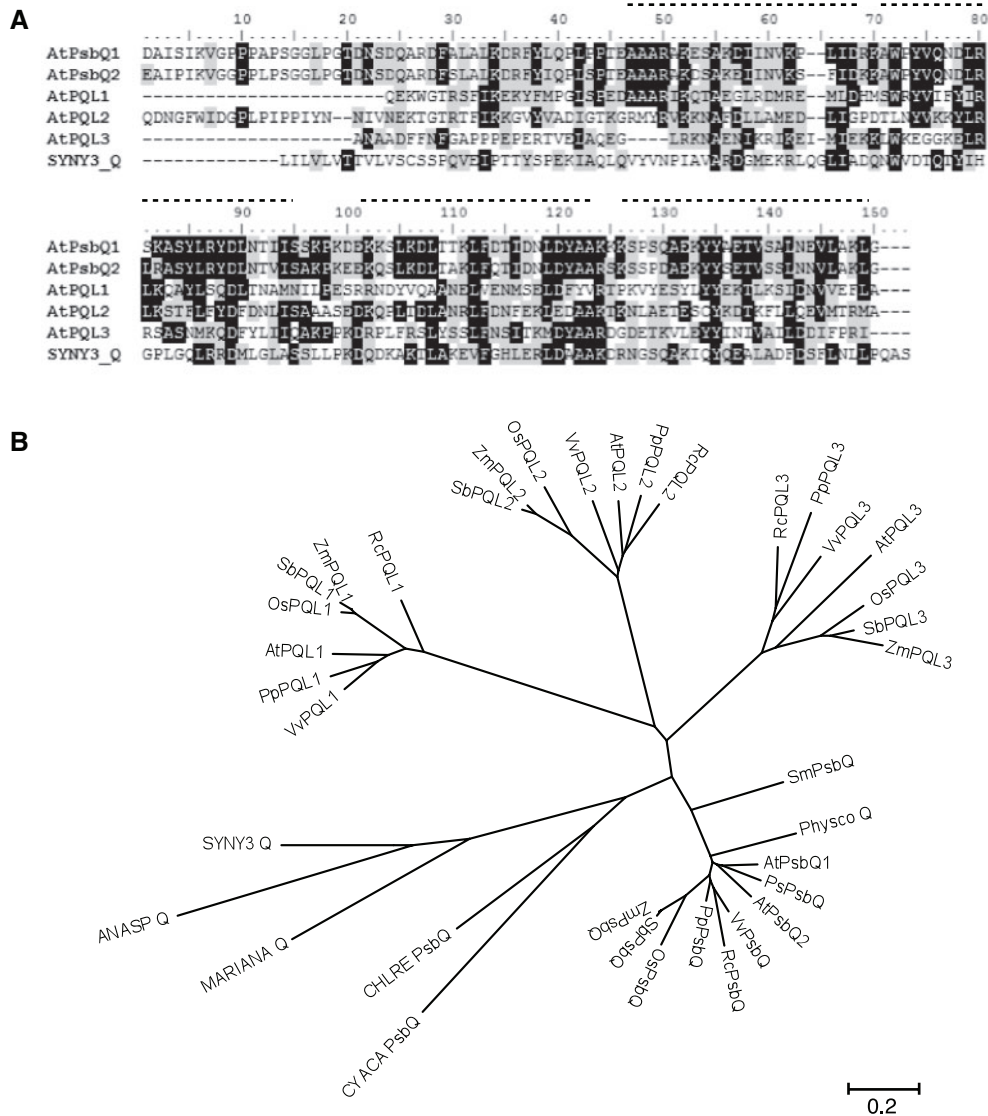
**Table 1** Percent identity and similarity of amino acid sequences among PsbQ, PQL, and cyanobacterial PsbQ proteins

Name	Locus	Accession No. <sup>a</sup>	PsbQ (I <sup>b</sup> )	PsbQ (+ <sup>c</sup> )	cyanoQ (I <sup>b</sup> )	cyanoQ (+ <sup>c</sup> )
PsbQ1	At4g21280	Q9XFT3	100	100	22.6	46.6
PsbQ2	At4g05180	Q41932	81.1	91.2	20.4	43.9
PQL1	At1g14150	Q9XI73	21.6	45.9	16.9	40.7
PQL2	At3g01440	Q9SGH4	28.2	50	20	39.7
PQL3	At2g01918	Q2V4B2	23.8	45.3	16.3	42.9

<sup>a</sup> UniProtKB/Swiss-Prot and TrEMBL accession number.

<sup>b</sup> I, amino-acid sequence identity with PsbQ1 or cyanoQ (P73048).

<sup>c</sup> +, amino-acid sequence similarity to PsbQ1 or cyanoQ (P73048).



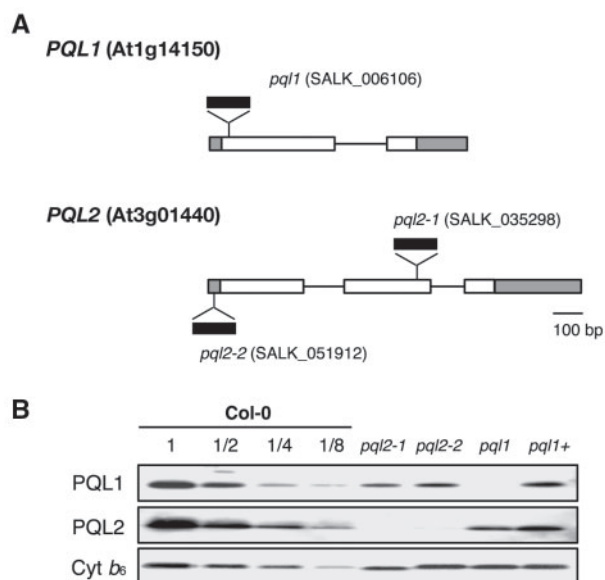
**Fig. 1** Amino acid sequence alignment and phylogenetic tree of PQL family proteins. (A) Multiple alignment of PsbQ family proteins created by Clustal W. Identical residues are shaded in black; similar residues are shaded in gray. Dotted lines indicate the regions predicted to adopt  $\alpha$ -helical structures. Structural predictions for PsbQ family proteins are available in the SWISS-MODEL Repository (<http://swissmodel.expasy.org/>). (B) Protein phylogenetic tree of PsbQ family proteins. The unrooted tree was produced by the Neighbor-Joining method in MEGA4. (A and B) The organisms from which cyanoQ (Q), PsbQ and PQL protein sequences were derived are as follows: At, *Arabidopsis thaliana*; Vv, *Vitis vinifera*; Pp, *Populus thoricocarpa*; Os, *Oryza sativa*; Sb, *Sorghum bicolor*; Zm, *Zea mays*; Rc, *Ricinus communis*; Sm, *Selaginella moellendorffii*; Physco, *Physcomitrella patens*; SYNY3, *Synechocystis* sp. PCC 6803; ANASP, *Anabaena* (*Nostoc*) sp. PCC 7120; MARIANA, *Acaryochloris marina* MBIC11017; CYACA, *Cyanidium caldarium*; CHLRE, *Chlamydomonas reinhardtii*. Accession numbers for PsbQ family proteins in the UniProtKB database are listed in **Supplementary Table S3**.

T-DNA insertion alleles were found for the *PQL2* gene (*pql2-1* and *pql2-2*), while only one mutant allele was found for *PQL1* (*pql1*) at the Salk Institute Genome Analysis Laboratory (Alonso et al. 2003) (**Fig. 2**). To confirm the effect of *PQL1* knockdown, the *pql1* plant was transformed with a genomic *PQL1* construct (*pql1+*). As shown in **Fig. 2B**, the specific absence of the *PQL1* and *PQL2* proteins in *pql1* and *pql2-1/pql2-2* plants, respectively, and the recovery of *PQL1* in complemented *pql1* plants were confirmed by immunoblot analysis.

The specific loss of *PQL1* or *PQL2* mRNA in the respective mutants was also confirmed by reverse transcription-PCR (RT-PCR) (**Supplementary Fig. S2**).

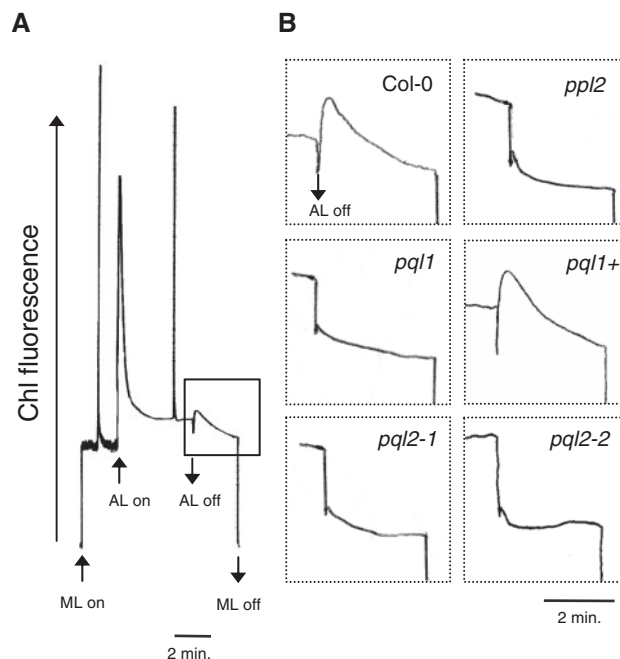
### Estimation of NDH activity by Chl fluorescence analysis

A transient increase in Chl fluorescence after exposure to actinic light (AL) is confirmed to be genetically correlated with the activity of the NDH complex in vivo, so that it has been



**Fig. 2** Positions of T-DNA insertions and accumulation of PQL proteins in the *pql1* and *pql2* mutants. (A) The positions of T-DNA insertions (black boxes) in the *pql1* and *pql2-1*, *pql2-2* mutants used in this study. White boxes indicate protein-coding regions, gray boxes indicate untranslated regions, and lines indicate introns. (B) Immunodetection of PQL proteins in the wild type (Col-0, including indicated serial dilution), in the *pql2-1*, *pql2-2* and *pql1* mutants, and in the *pql1* plant transformed with the PQL1 gene (*pql1+*). Thylakoid proteins were loaded on an equal chlorophyll basis (5  $\mu$ g of Chl per lane). Cyt *b*<sub>6</sub> is a loading control.

widely used to examine NDH activity in NDH-deficient mutants (for a review, see Shikanai 2007). This increase in Chl fluorescence is due to the reduction of plastoquinone by the stromal electron pool that accumulates during illumination with AL (Burrows et al. 1998, Kofer et al. 1998, Shikanai et al. 1998). Wild-type Arabidopsis exhibited an increase in Chl fluorescence after exposure to AL, indicative of NDH activity, whereas the *pql1* mutant and both *pql2* mutants did not have this response to AL, similar to the *ppl2* mutant (Fig. 3). The loss of NDH activity in the *pql1* mutant was completely recovered in the *pql1+* plant. These results suggest that PQL1 and PQL2 are independently required for NDH activity. As observed for other NDH-deficient mutants (Munekage et al. 2004), growth of *pql1* and *pql2* mutants was comparable with that of wild-type plants under normal growth conditions (Supplementary Fig. S2), while the PSII yield ( $\Phi_{\text{PSII}}$ ) of the *pql1* and *pql2* mutants under ambient light was slightly lower than that of wild-type plants (Table 2), which is consistent with the observation for *ppl2* and other NDH mutants (Ishihara et al. 2007, Ishikawa et al. 2008a, Peng et al. 2009). The maximal PSII efficiency ( $F_v/F_m$ ) and the Chl *a/b* ratio of *pql1* and *pql2* mutants were similar to those of wild-type plants (Table 2), indicating that each PQL has a function specific to NDH activity.



**Fig. 3** NDH activity monitored by Chl fluorescence analysis. The reduction of plastoquinone in the dark after exposure to actinic light (AL; 144  $\mu$ mol photons  $\text{m}^{-2} \text{s}^{-1}$  for 5 min) depends on NDH activity and can be monitored as a transient increase in Chl fluorescence. (A) A typical trace of Chl fluorescence in the wild type (Col-0). The curve in the boxes shows NDH activity. (B) NDH activity of wild-type, *ppl2*, *pql1*, *pql1+*, *pql2-1* and *pql2-2* plants as monitored by Chl fluorescence. ML, measuring light.

### The PQL1 and PQL2 proteins are tightly associated with the NDH complex

The localization of PQL1 and PQL2 in thylakoid membranes was addressed by BN-PAGE, followed by SDS-PAGE in the second dimension and subsequent immunoblot analysis using specific antibodies. As shown in Fig. 4A, signals for NDF1 (NDH48) and NDH-L subunits of NDH and PsaB subunits of PSI were detected in a large complex of  $\geq 1,000$  kDa, which corresponds to the NDH-PSI supercomplex (Peng et al. 2008). PQL1 and PQL2 were exclusively found in the same complex, indicating that they are specifically associated with the NDH-PSI supercomplex. Unlike OEC subunits in PSII (Ishihara et al. 2007, Ido et al. 2009), PQL proteins were not detected in smaller fractions on a BN-PAGE gel, indicating that neither PQL exists as a free protein in the thylakoid lumen.

Localization of PQL1 and PQL2 in the NDH complex was analyzed by digesting thylakoids isolated from wild-type plants with thermolysin. To digest the lumen-exposed parts of thylakoid membrane proteins, thylakoids were briefly sonicated to disrupt the membrane structure before protease treatment. When thylakoid membranes were not sonicated, PQL1 and PQL2 were not digested by protease as observed for PsbO in

**Table 2** Photosynthetic measurements of wild-type, *pql1*, *pql2*, and *ppl2* plants.

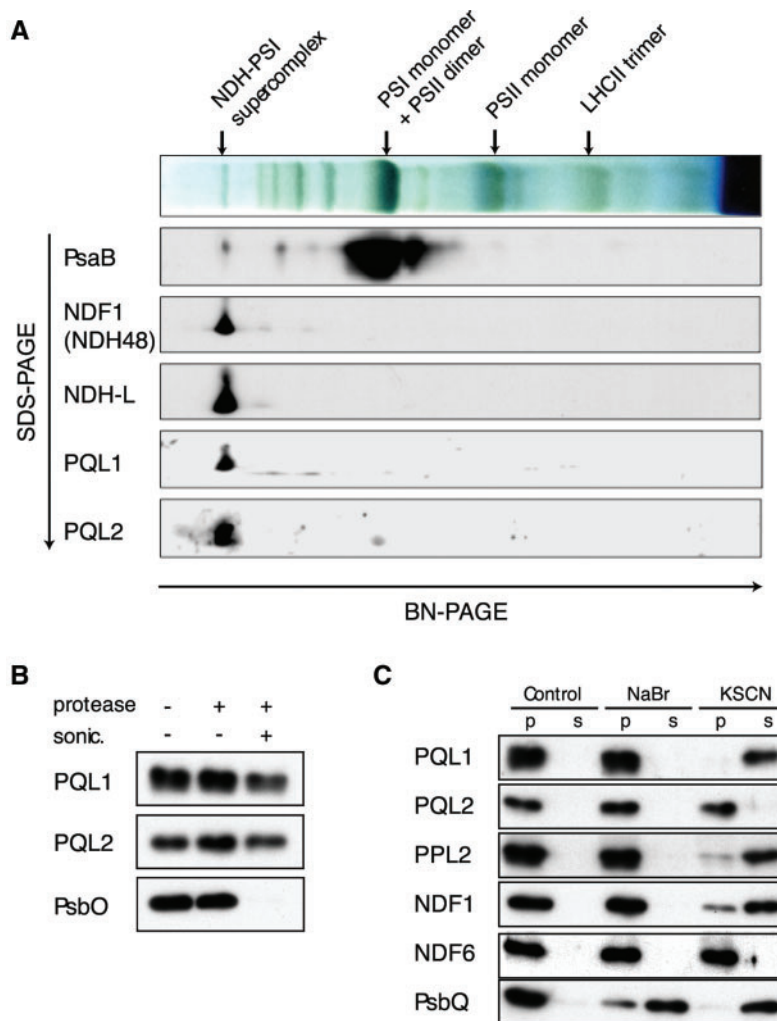
	Col-0	<i>pql1</i>	<i>pql2</i>	<i>ppl2</i>
$F_v/F_m^a$	0.805 ± 0.003	0.807 ± 0.003	0.807 ± 0.002	0.803 ± 0.002
$\Phi_{PSII}^b$	0.714 ± 0.008	0.665 ± 0.007*	0.671 ± 0.011*	0.667 ± 0.005*
Chl <i>a/b</i>	3.02 ± 0.12	2.99 ± 0.22	2.96 ± 0.23	3.00 ± 0.05

<sup>a</sup>  $F_v/F_m$  value was measured after incubation in the dark for 1 h.

<sup>b</sup>  $\Phi_{PSII}$  was measured under illumination at 144  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ . Data presented are means ± SD of three independent measurements. Asterisks indicate the means that were statistically different from that of wild type ( $P < 0.01$ ; Student's *t* test).

thylakoid lumen (Fig. 4B). Furthermore, both PQL proteins were relatively resistant to protease, even when membrane integrity was disrupted by sonication. PQL1 and PQL2 were resistant to a harsher digestion with trypsin, in which both stroma- and lumen-exposed thylakoid proteins were digested (data not shown). These results suggest that both PQL1 and PQL2 are localized in thylakoid lumen and embedded inside the NDH complex.

We then examined the nature of the interaction between PQLs and the NDH complex by treating the thylakoids of wild-type plants with a moderate (2 M NaBr) or a strong (2 M KSCN) chaotropic salt (Fig. 4C). Treatment with either salt released the majority of thylakoid luminal PsbQ protein, indicating that these treatments were severe enough to extract



**Fig. 4** Localization and binding of PQL1 and PQL2 proteins in thylakoids isolated from wild-type Arabidopsis. (A) Thylakoid membrane complexes separated by BN-PAGE (top) were further subjected to second dimensional SDS-PAGE, and proteins were immunodetected with specific antibodies against PsaB, PQL1, PQL2, NDF1 (NDH48) and NDH-L. (B) Immunoblots from wild-type thylakoid membranes exposed to protease (thermolysin) digestion. When necessary, thylakoid membranes were briefly sonicated to disrupt membrane integrity. Antibodies against PQL1, PQL2 and PsbO were used. (C) Immunoblots of PQL1, PQL2, NDF1, NDF6 and PsbQ proteins in wild-type thylakoid membranes washed with 2 M NaBr and 2 M KSCN. Thylakoid-bound proteins in pellets (p) and proteins released in supernatants (s) were analyzed. Proteins corresponding to 5  $\mu\text{g}$  of Chl were loaded on each lane.

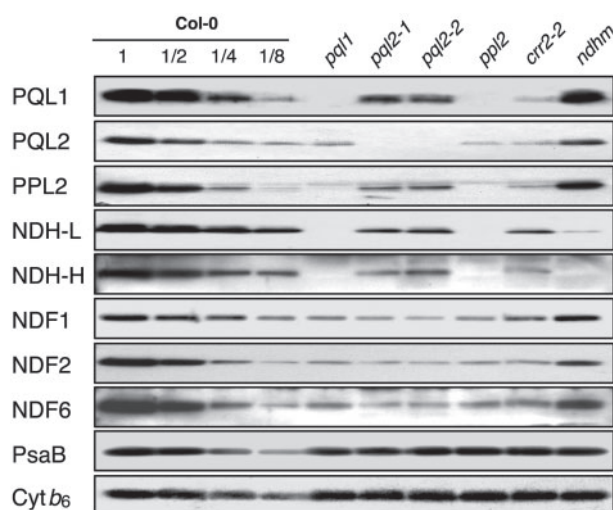
thylakoid lumenal components. PQL1 was not affected by 2 M NaBr, but was quantitatively released by 2 M KSCN. Two other peripheral subunits of the NDH complex, PPL2 on the lumenal side and NDF1 on the stromal side, showed a behavior comparable with that of PQL1. On the other hand, PQL2 was resistant to both salt treatments, as observed for the NDF6 protein, which has a membrane-spanning domain anchored to thylakoid membranes (Ishikawa et al. 2008b). Treatment with other salts, such as 0.1 M Na<sub>2</sub>CO<sub>3</sub> (pH 10.4) or 2 M CaCl<sub>2</sub>, did not affect the binding of PQL1 and PQL2 (data not shown). These results suggest that PQL1 and PQL2 tightly bind to the NDH complex: PQL1 mainly interacts with the NDH complex via a hydrophobic force, while multiple forces seem to be involved in PQL2 binding.

### NDH subunit stability in different NDH mutant backgrounds

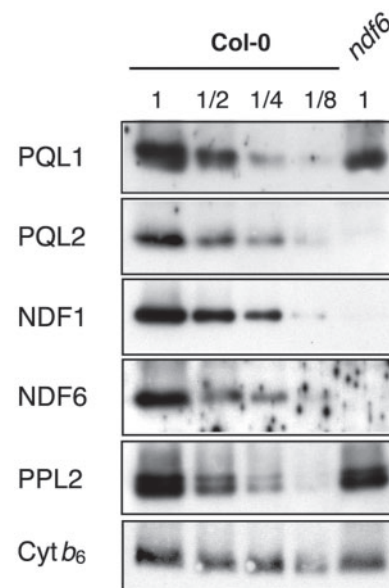
The stability of PQL1 and PQL2 in NDH-defective mutants and the effects of the absence of either PQL on the amounts of other NDH subunits were examined by immunoblot analysis (Fig. 5). The *crr2-2* mutant is defective in *ndhB*, which encodes a membrane subunit of the NDH complex. The *ppl2* mutant lacks the thylakoid lumenal PPL2 protein in the NDH complex (Ishihara et al. 2007), and the *ndhm* mutant lacks the NDH-M protein in the stroma-exposed peripheral hydrophilic domain. It was shown that the absence of NDH-B or PPL2 largely impairs the stability of the NDH complex, while a partial accumulation of many NDH subunits, except for NDH-H and NDH-L, was observed in the *ndhm* mutant (Peng et al. 2009). The levels of PQL1 and PQL2 were decreased to ~1/8 of the wild-type levels

in the *crr2-2* and *ppl2* mutants, and these levels were decreased to almost half of the wild-type levels in the *ndhm* mutant. This result indicates that unassembled PQL proteins are unstable and are degraded in thylakoid membranes, which is consistent with the absence of free PQL proteins as observed by BN-PAGE analysis (Fig. 4A).

Conversely, all of the NDH subunits examined were significantly reduced in both *pql* mutants, with differences in NDH subunit accumulation in the *pql1* and *pql2* backgrounds. In the *pql1* mutant, NDH-H, NDH-L and PPL2 were undetectable. A similar accumulation pattern was observed in the *ppl2* mutant and was also reported for *fkbp16-2* mutants (Peng et al. 2009), suggesting that PQL1, PPL2 and FKBP16-2 function together in the NDH complex. In contrast, in the *pql2* mutant, PQL1, PPL2, NDH-L and NDH-H accumulated to ~1/4 of the wild-type level, whereas NDF proteins in the *pql2* mutant were more severely affected and accumulated to ~1/8 of the wild-type level. To confirm the functional interaction between PQL2 and NDF proteins, the accumulation of PQL proteins was examined in the *ndf6* mutant. Since the NDF6 protein has a transmembrane domain exposed in the thylakoid lumen, it is a plausible candidate for connecting stromal NDF proteins with a lumenal component. The results in Fig. 6 show that PQL1 and PPL2 stably accumulated in the *ndf6* mutant, suggesting that PQL1 and PPL2 do not directly interact with NDF6. On the other hand, PQL2 and NDF1 were absent in the *ndf6* mutant, indicating that PQL2 requires NDF6 to associate with the NDH complex. The levels of subunits of other



**Fig. 5** Accumulation of NDH subunits in thylakoids isolated from wild-type (Col-0, including indicated serial dilution), *pql1*, *pql2-1*, *ppl2*, *crr2-2* and *ndhm* plants. Isolated thylakoid proteins were analyzed by immunoblotting using the indicated antisera. Thylakoid proteins were loaded on an equal Chl basis (5 µg of Chl per lane). PsaB and Cyt *b*<sub>6</sub> were analyzed as internal controls.



**Fig. 6** Accumulation of PQL1 and PQL2 proteins in thylakoids isolated from wild-type (Col-0, including the indicated serial dilution) and *ndf6* plants. Isolated thylakoid proteins were analyzed by immunoblotting using the indicated antisera. Thylakoid proteins were loaded on an equal Chl basis (5 µg of Chl per lane). Cyt *b*<sub>6</sub> is a loading control.

thylakoid membrane complexes, such as PSI and PSII, were not affected in the *pql1* and *pql2* mutants (PsaB and Cyt *b<sub>6</sub>* in Fig. 5 and Supplementary Fig. S2B), which is consistent with specific functions for PQL proteins in the NDH complex.

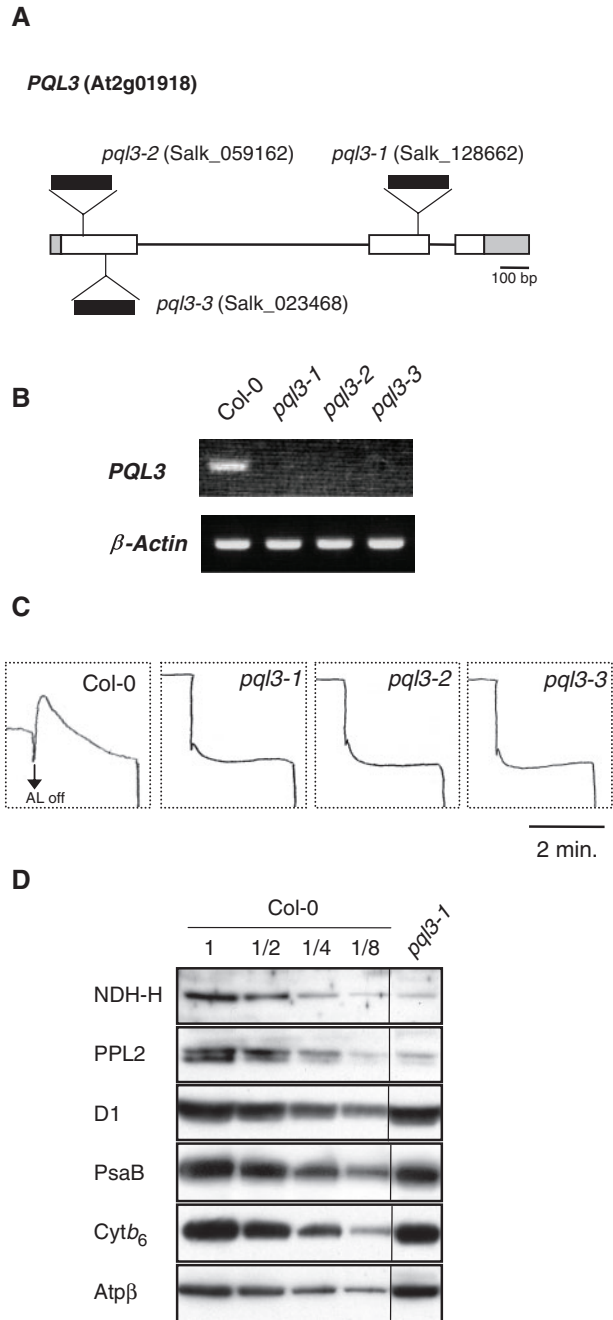
### Activity and accumulation of the NDH complex were impaired in the absence of PQL3

The involvement of PQL1 and PQL2 in the chloroplast NDH function indicated the possibility that PQL3 might also have a function in the NDH complex. We then examined the NDH activity in the *pql3* mutants lacking PQL3. Three independent T-DNA insertion alleles were found for the *PQL3* gene (*pql3-1*, *pql3-2* and *pql3-3*) (Fig. 7A). Although we could not produce a specific antibody for PQL3, the loss of *PQL3* mRNA in the homozygous mutant line was confirmed in all *pql3* mutants by RT-PCR (Fig. 7B). Interestingly, NDH activity was not observed in all of the *pql3* mutant alleles, suggesting that PQL3 is required for NDH activity (Fig. 7C). The level of the NDH-H subunit was decreased to ~1/8 of the wild-type levels in the *pql3-1* mutant (Fig. 7D). In addition, the amount of an NDH subunit in thylakoid lumen, PPL2, was greatly reduced in *pql3-1*. Similar observations were also confirmed in the *pql3-2* mutant (data not shown). The accumulation of D1 in PSII was not affected in *pql3-1*, indicating that PQL3 does not function in PSII. Similarly, the amounts of PSI, Cyt *b<sub>6</sub>/f* and the chloroplast ATPase subunits were not affected in the *pql3-1* mutant. These results suggest that PQL3 deficiency specifically impairs accumulation of the chloroplast NDH complex.

### Discussion

The recent discovery of thylakoid luminal components that are required for chloroplast NDH activity suggested the presence of a thylakoid luminal NDH subcomplex in higher plants. Proteome studies of the protein complexes in thylakoids showed that in addition to the two PQL proteins, CYP20-2 (Tlp20), PPL2 and FKBP16-2 bind to the thylakoid luminal side of the NDH complex (Majeran et al. 2008, Peng et al. 2009). In this study, we showed that the absence of PQL1 resulted in a severe reduction of NDH subunits, including NDH-H, NDH-L and PPL2, while the absence of PQL2 allowed partial accumulation of these NDH subunits. Knockdown or knockout of FKBP16-2 or PPL2 caused a severe reduction in the level of NDH subunits such as NDH-H, NDH-L and PPL2, while NDF1, NDF2 and NDH18 could accumulate in their absence (Peng et al. 2009), and the absence of CYP20-2 did not lead to a severe NDH malfunction (Sirpiö et al. 2009b). Since differences in subunit stability in different genotypes probably reflect the distinct locations of subunits in a protein complex, it is now possible to describe their functional interaction in the chloroplast NDH complex.

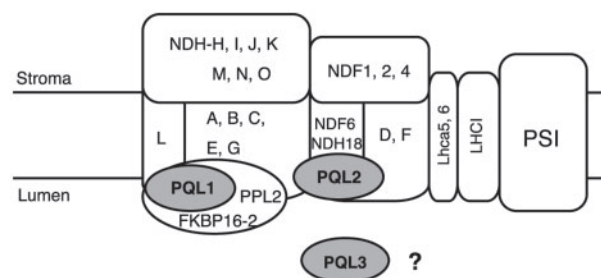
Recently, Peng et al. (2009) proposed a model of the NDH-PSI supercomplex based on a matrix analysis of protein blots using various NDH mutants and antibodies against



**Fig. 7** Functional analysis of PQL3 protein in the *pql3* mutants. (A) The positions of T-DNA insertions (black boxes) in *pql3* and *pql3-1*, *pql3-2* mutants. White boxes indicate protein-coding regions, gray boxes indicate untranslated regions, and lines indicate introns. (B) RT-PCR analysis of the expression of the *PQL3* gene in wild-type (Col-0) and *pql3* plants.  $\beta$ -Actin was analyzed as a control. (C) NDH activity of *pql3-1*, *pql3-2* and *pql3-3* as monitored by Chl fluorescence. (D) Accumulation of NDH-H and PPL2 in thylakoids isolated from wild-type (Col-0, including indicated serial dilution) and *pql3-1* plants. Thylakoid proteins were separated by SDS-PAGE and analyzed by immunoblotting using the indicated antisera. Thylakoid proteins were loaded on an equal Chl basis (5  $\mu$ g of Chl per lane).

known NDH subunits. In their model, the chloroplast NDH is divided into four subcomplexes. PPL2 and FKBP16-2 form a subcomplex on the lumen side and stabilize a hydrophilic subcomplex (subcomplex A) that consists of NDH-H–NDH-K and NDH-L–NDH-O. A transmembrane NDH-L protein was absent in the *ppl2* and *fkbp16-2* mutants, suggesting that NDH-L connects the lumen subcomplex and the hydrophilic subcomplex A. NDF1, NDF2 and NDH18, together with NDF4 and NDF6, were proposed to form another subcomplex (subcomplex B) and to associate with a membrane subcomplex consisting of NDH-A–NDH-G. They speculated that PQL1 and PQL2 might also be included in the same lumen subcomplex together with PPL2 and FKBP16-2. However, our present study clearly suggests that PQL1 and PQL2 have different binding sites in the NDH complex. Besides, we suggest that PQL3 is also required for NDH activity; however, we could not examine association of PQL3 with the NDH complex because of the lack of a specific antibody.

According to our results, a schematic model for the chloroplast NDH complex modified from that in Peng et al. (2009) is shown in Fig. 8. PQL1, PPL2 and FKBP16-2 should be close to each other, because PPL2 was absent in *pql1*, PQL1 was significantly affected in *ppl2* and the subcomplex A subunits NDH-H and NDH-L were similarly absent in the *pql1*, *ppl2* and *fkbp16-2* mutants (Fig. 5 in this study, and Peng et al. 2009). PQL1, PPL2 and FKBP16-2 can bind to the NDH complex in the absence of NDH-L (see immunoblots for *ndhm* in Fig. 5 and *ndhl* in Peng et al. 2009), suggesting that they also interact with other unidentified membrane proteins. Since the subcomplex A consists of the NDH subunits commonly found in cyanobacteria, it may have a conserved function for NDH, while the chloroplast-specific lumen subcomplex including PPL2, FKBP16-2 and PQL1 has become essential for stabilizing the subcomplex A in chloroplasts. The PQL1 protein was identified in a proteome analysis of thylakoid-associated polysome nascent chain complexes (Sirpiö et al. 2007), so it should be incorporated into the NDH complex at an early step of assembly, and hence localize inside the protein complex, which is consistent with PQL1 protease resistance in thylakoid membranes (Fig. 4B). On the other hand, PQL2 is likely to localize near subcomplex B containing NDF proteins and NDH18, because the amount of NDF proteins is relatively low in the *pql2* mutant (Fig. 5), and PQL2 was absent from the *ndf6* mutant (Fig. 6). Since the subcomplex B stabilized by PQL2 consists of chloroplast-specific subunits, which are not found in cyanobacteria, it may be important for the NDH function specifically developed in chloroplasts, such as association with PSI. PQL2 was barely extracted by chemical treatments and digested by protease, so it is likely that PQL2 is also surrounded and shielded by other unidentified NDH subunits. PQL3 seems to be localized in the thylakoid lumen because the precursor for PQL3 has an apparent bipartite pre-sequence containing a TAT-type lumen-targeting signal peptide (The Plant Proteome Database, <http://ppdb.tc.cornell.edu/>); however, its association with the NDH complex needs to be confirmed in the future.



**Fig. 8** A schematic model of the NDH–PSI supercomplex in chloroplasts. The model was modified from that proposed by Peng et al. (2009). PQL1 together with PPL2 and FKBP16-2 is required for the stable accumulation of the stromal subcomplex, including NDH-M–NDH-O and NDH-H–NDH-J, probably via NDH-L. Since these luminal subunits bind to the NDH complex in the absence of NDH-L, other unidentified membrane proteins also interact with them. The membrane subunit NDF6 is essential for PQL2 binding, and the absence of PQL2 affects the stability of the subcomplex including NDF proteins. PQL3 protein is required for NDH activity and may be localized in thylakoid lumen, while its association with the NDH complex was not examined. CYP20-2 was not included in this model, because it does not appear to be essential for NDH functions (Sirpiö et al. 2009b). The model is designed to aid the reader in visualizing the locations of PQLs in the NDH complex, but by no means does it show the exact location or stoichiometry of each NDH subunit or of PSI. See the Discussion for details.

It is noteworthy that OEC homologs bind together with immunophilins in both the PSII and the NDH complexes. The immunophilins comprise a peptidyl prolyl domain and are believed to function as auxiliary proteins facilitating the rate-limiting *cis*–*trans* isomerization of the peptidyl prolyl bond during protein folding. A recent study showed that a thylakoid luminal immunophilin, AtCYP38, guides folding of the D1 protein in PSII, thereby allowing the correct assembly of the water-splitting  $Mn_4$ –Ca cluster (Sirpiö et al. 2008). The PsbP homolog PPL1 was shown to be required for the efficient repair of the damaged D1 protein in PSII (Ishihara et al. 2007). PsbP functions in PSII to induce protein conformational changes around the catalytic manganese cluster to stabilize the water-splitting reaction (Tomita et al. 2009), and PsbQ supports this function (Ifuku and Sato 2002). Taking all the above information together, it is likely that OEC homologs and immunophilins cooperatively assist in the correct folding of the membrane proteins on the thylakoid luminal side to facilitate the functional assembly and organization of membrane protein complexes, such as the PSII and NDH complexes. During the evolution of higher plants, environmental stresses probably have increased the extent of fluctuations in the physical status of the chloroplast thylakoid lumen. The recruitment of PQLs and PPL2 to the thylakoid lumen may allow higher plants to cope with significant physiological changes in this space, such as alterations in pH and ion composition. Originally, the



molecular function of PQLs and PPL2 might have been 'auxiliary', to support the assembly of the NDH complex, as proposed in a recent review (Suorsa et al. 2009). However, the association of PQL1, PQL2, and PPL2 with the NDH-PSI complex is apparently not transient, and it is necessary for NDH activity (Figs. 3, 4). Furthermore, PQL1, PQL2, and PPL2 were unstable in the NDH-deficient mutant (Figs. 5, 6). These results suggest that these OEC homologs can indeed be regarded as subunits of the NDH complex.

Putative orthologs of three PQLs and PPL2 have been found in many higher plants, particularly in angiosperms, but not in mosses and ferns (Fig. 1B). Mosses and ferns have other NDH genes in their nuclear and chloroplast genomes, indicating that modification of the thylakoid lumenal side of the chloroplast NDH complex occurred recently in evolution, and that seed plants, at least angiosperms, developed a novel mechanism to support NDH activity. Analysis of the NDH complex in mosses and ferns, which have NDH genes but do not have PQLs and PPL2, may provide a clue as to why higher plants developed and utilize these OEC homologs in the chloroplast NDH complex.

In summary, we showed that all the of three PQL proteins are required for the chloroplast NDH function in higher plants: PQL1 and PQL2 function as NDH subunits, while further research is needed to elucidate the function of PQL3 fully.

## Materials and Methods

### Analysis of co-expression profiles

Genes that are co-expressed with each of the PsbQ family genes were identified using Pearson's correlation coefficient ( $r$ ), which was calculated using the 771 Affymetrix ATH1 array data from AtGenExpress (Schmid et al. 2005), publicly available in ATTED-II (<http://www.atted.bio.titech.ac.jp/>) (Obayashi et al. 2007).

### Plants and growth conditions

Seeds for the T-DNA insertion mutants, *pql1* (SALK\_006106), *pql2-1* (SALK\_035298), *pql2-2* (SALK\_051912C), *pql3-1* (SALK\_128662), *pql3-2* (SALK\_059162) and *pql3-3* (SALK\_023468), were obtained from a collection developed at the Salk Institute Genomic Analysis Laboratory (Alonso et al. 2003). The positions of the T-DNA insertion were confirmed by genome-based PCR, and expression in each mutant was assessed by RT-PCR and immunoblotting. For genetic complementation of *pql1* mutants, the *PQL1* gene with its native promoter was amplified from wild-type genomic DNA, subcloned into the vector pGWB501 (Nakagawa et al. 2007) and introduced into the *pql1* mutant by *Agrobacterium*-mediated transformation. Detailed information about the primer sets used in this study is described in **Supplementary Table S4**. Arabidopsis plants were grown in soil under growth chamber conditions (10/14h photoperiod at 50–100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 22°C) for 3–4 weeks.

### Chl fluorescence analysis

Chl fluorescence was monitored by conventional methods using a PAM2000 Chl fluorometer (Walz, Effeltrich, Germany). NDH activity in vivo was analyzed as previously described (Ishihara et al. 2007). Briefly, after illumination with AL (144  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for 4 min, AL was turned off, and the transient increase in Chl fluorescence was monitored under ambient lighting.

### Specific antibody production against the PQL proteins

PCR-amplified cDNAs for the mature PQL1, PQL2 and PQL3 proteins were inserted in-frame into the expression vector pET-22b (Novagen, Nottingham, UK) such that the His tag was fused to the C-terminal end. The PQL1-His, PQL2-His and PQL3-His fusion proteins were expressed in the *Escherichia coli* strain Rosetta-gami B (DE3) (Novagen) and purified on a TALON Metal Affinity Resin (Clontech, Basingstoke, UK). Polyclonal antibodies were raised in rabbits with the purified antigens.

### SDS-PAGE and immunoblot analysis

Thylakoid membranes were isolated from leaves as previously described (Ishihara et al. 2007). An amount of protein corresponding to 5  $\mu\text{g}$  of Chl was solubilized and then separated by SDS-PAGE using 15% acrylamide gels. Proteins were transferred to a polyvinylidene fluoride (PVDF), membrane using a semi-dry blotting system (Bio-Rad), and immunoreactive proteins were detected using ECL plus (GE Healthcare, Buckinghamshire, UK). Treatment of isolated thylakoid membranes with salt solutions was performed as previously described (Karnauchov et al. 1997). Digestion of isolated thylakoid membranes with thermolysin (100  $\mu\text{g ml}^{-1}$ ) was performed for 30 min on ice.

### BN-PAGE analysis

BN-PAGE was performed as described (Ishihara et al. 2007). Thylakoid samples containing 7.5  $\mu\text{g}$  of Chl were solubilized with 1% (w/v) *n*-dodecyl- $\beta$ -D-maltoside and separated on 4–14% acrylamide gradient gels. For two-dimensional BN/SDS-PAGE analysis, a lane was excised from the gel, denatured and run in the second dimension by SDS-PAGE with 15% acrylamide and 6 M urea, followed by immunoblot analysis.

## Notes Added in Proof

Note that in this paper, AT1G14150 and AT3G01440 are named as PQL1 and PQL2, respectively. On the contrary, in Suorsa et al. (Plant Cell Physiology, this issue, 877–883), the PQL1 and PQL2 proteins are named just the opposite (PQL1 referring to AT3G01440 and PQL2 referring to AT1G14150).

## Supplementary data

Supplementary data are available at PCP online.

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