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The PsbP protein, but not the PsbQ protein, is required for normal thylakoid architecture in *Arabidopsis thaliana*

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ABSTRACT

Interfering RNA was used to suppress the expression of the genes At1g06680 and At2g30790 in *Arabidopsis thaliana,* which encode the PsbP-1 and PsbP-2 proteins, respectively, of Photosystem II. A phenotypic series of transgenic plants was recovered that expressed intermediate and low amounts of PsbP. Earlier we had documented significant alterations in a variety of Photosystem II parameters in these plant lines [Yi, X., Liu, H., Hargett, S. R., Frankel, L. K., Bricker, T. M. (2007). The PsbP protein is required for photosystem II complex assembly/stability and photoautotrophy in *Arabidopsis thaliana*. J. Biol. Chem. 34, 24833–24841]. In this communication, we document extensive defects in the thylakoid membrane architecture of these plants. Interestingly, strong interfering RNA suppression of the genes encoding the PsbQ protein (At4g21280 and At4g05180) was found to have no effect on the architecture of thylakoid membranes.

1. Introduction

In higher plants, algae and cyanobacteria, at least six intrinsic proteins appear to be required for oxygen evolution by Photosystem II (PS II) [1–3]. These are CP47, CP43, the D1 and D2 proteins, and the α and β subunits of cytochrome b_{559}. Insertional inactivation or deletion of the genes for these components results in the disassembly of the PS II complex and the complete loss of oxygen evolution activity (for review, see [4]). Additionally, a number of low molecular mass intrinsic membrane protein components are associated with PS II [5–7], although the functions of many of these proteins remain obscure. Although PS II complexes containing only these intrinsic components can evolve oxygen in vitro, they do so at low rates (about 25–40% of control), are extremely susceptible to photoinactivation, and require high, non-physiological levels of calcium and chloride for maximal activity [1,3].

In higher plants and green algae, three extrinsic proteins, with apparent molecular masses of 33, 24, and 17 kDa, are required for high rates of oxygen evolution at physiological inorganic cofactor concentrations [8,9]. The 33 kDa component, the PsbO protein, has been termed the manganese-stabilizing protein (MSP) due to its stabilization of the manganese cluster during exposure to low chloride concentrations or to exogenous reductants. In vitro, the 24 and 17 kDa proteins (termed the PsbP and PsbQ proteins, respectively) appear to modulate the calcium and chloride requirements for efficient oxygen evolution. The precise roles of these proteins in oxygen evolution and PS II assembly/stability in vivo, however, remain unclear. These three extrinsic components interact with intrinsic membrane proteins and possibly with each other to yield fully functional oxygen-evolving complexes.

RNAi is a post-transcriptional gene-silencing process in which double-stranded RNA induces the degradation of homologous mRNA sequences [10]. RNAi has been successfully applied as a powerful gene-silencing tool in a variety of organisms, including *Caenorhabditis elegans* and *Drosophila melanogaster,* and in mouse oocytes. It has also become a popular research methodology for investigating the physiological functions of target genes in plants [11]. With respect to PS II membrane proteins, RNAi has been used to study the function of the PsbO, PsbP, and PsbQ proteins in arabidopsis [12–15] as well as the PsbP and PsbQ proteins in tobacco [16].

The mature PsbP protein is highly conserved in higher plants [8]. In arabidopsis, there are two genes, At1g06680 and At2g30790, which encode PsbP-1 and PsbP-2, respectively. In an

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MS medium, Murashigi and Skoog medium; PS I, Photosystem I; PS II, Photosystem II; LiDS-PAGE, lithium dodecyl sulfate polyacrylamide gel electrophoresis

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earlier study, we had established that strong RNAi suppression of PsbP expression led to significant alterations in Q_A^- reoxidation kinetics both in the absence and presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [14]. Significant alterations in fluorescence induction patterns were also observed. Similar results have been observed in tobacco [16]. Finally, low levels of the PsbP protein led to significant loss of assembled PS II reaction centers. Interestingly, in arabidopsis, strong suppression of Photosystem I (PS I) reaction center assembly/stability, as monitored by accumulation of the *psaB* gene product, was also observed to correlate with low levels of the PsbP protein [14]. The nature of this apparent pleiotrophic effect was unclear. The mature PsbQ protein also is highly conserved in higher plants [8]. In arabidopsis, there are two genes, At4g21280 and At4g05180, which encode PsbQ-1 and PsbQ-2, respectively. In both arabidopsis and tobacco, strong RNAi suppression of PsbQ expression yields no apparent phenotype in plants grown at normal light intensities [13,16].

Recently, it has been hypothesized that the extrinsic proteins of PS II play significant roles in maintaining normal thylakoid membrane architecture, particularly with respect to the integrity of the grana stacks [17,18]. In this communication, we document dramatic alterations in thylakoid structure from plants which express low and intermediate amounts of the PsbP protein under normal growth light conditions. Plants which express low amounts of the PsbQ component, however, exhibit normal thylakoid membrane architecture.

2. Materials and methods

2.1. Plant material and growth conditions

The use of the pHANNIBAL vector [19] to construct intron-spliced hairpin RNA constructs designed to suppress either PsbP or PsbQ expression has been previously described [13,14]. Since the loss of significant amounts of the PsbP protein leads to the loss of normal photoautotrophy [14,16], the RNAi-PsbP plant lines were grown on sterile solid Murashigi and Skoog medium (MS medium) (0.7% agar) with 2% sucrose, 50 mg/l kanamycin, and 400 mg/l carbenicillin at 23 °C under ~50 μmol photons $m^2\ s^{-1}$ light. At normal growth light intensities the RNAi-PsbQ plants exhibit no apparent phenotype [13,16]. Consequently, after germination these plants were grown on a soilless potting mix at 23 °C under ~50 μmol photons $m^{-2}\ s^{-1}$ light. The wild-type plants were grown either on solid MS medium with 2% sucrose or on a soilless potting mix at 23 °C under ~50 μmol photons $m^{-2}\ s^{-1}$ light, serving as controls for the RNAi-PsbP or the RNAi-PsbQ plants, respectively.

2.2. Semi-quantitative analysis of the PsbP and PsbQ proteins

Immunological quantification of the PsbP and PsbQ proteins present in mutant plant lines was performed on thylakoid membranes. Leaves were ground in a glass homogenizer with a thylakoid isolation buffer (300 mM sorbitol, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 20 mM HEPES/KOH, pH 8.0, 10 mM $NaHCO_3$). The homogenate was then passed through two layers of Miracloth® (Calbiochemical Co.), and the thylakoids were pelleted by centrifugation at 6000×*g* for 5 min. The thylakoids were then resuspended in a small amount of isolation buffer and the chlorophyll concentration was determined by the method of Arnon [20]. Lithium dodecyl sulfate polyacrylamide gel electrophoresis (LiDS-PAGE) was performed in 12.5–20% polyacrylamide gradient gels. "Western" blotting, blocking, and probing with primary and secondary antibodies were as previously described [14]. A polyclonal antibody directed against the mature spinach PsbP component and a monoclonal antibody directed against the PsbQ protein [21] were found to cross-react with the PsbP and PsbQ proteins from arabidopsis and were used in these studies. For detection of the immobilized antibodies, a chemiluminescent substrate (SuperSignal® West Pico Chemiluminescent Substrate, Pierce) was used, and the X-ray film was exposed to the blots. After development, the X-ray films were scanned with an Epson Perfection V700 Photo Scanner at 600 dpi resolution and an 8-bit color depth. Quantification of these images was performed using ImageJ version 1.38 with local background correction.

2.3. Electron microscopy

The leaves were dissected and fixed in 2% (V/V) glutaraldehyde and 1% (w/v) formaldehyde in 0.1 M cacodylate buffer, pH 7.2, for 3 h at room temperature. After being washed with the same buffer + glycerine, materials were post-fixed in buffered 2% osmium tertroxide (OsO_4) at room temperature for 1 h. Samples were rinsed with distilled water and stained using 0.5% uranyl acid in the dark for 1 h. Samples were then rinsed again in distilled water and dehydrated in an ethanol series, infiltrated in an ethanol: LR White resin series and embedded in LR White resin overnight at 60 °C. Embedded materials were sectioned with a Reichert–Jung Ultracut E microtome. Thin sections were mounted on collodion-coated copper grids, stained with Reynolds lead citrate, observed and photographed with a JEOL 100X transmission electron microscope (JEOL LTD Tokyo, Japan). The films were developed and scanned on an Epson Perfection V700 Photo Scanner. 15–30 images of chloroplasts for each plant line were examined. The chloroplasts shown in Figs. 2 and 3 are representative images from each plant line.

3. Results and discussion

3.1. Quantification of the PsbP and PsbQ proteins

Fig. 1 illustrates the experiments used to determine the amounts of the PsbP and PsbQ proteins present in the thylakoids of the RNAi-PsbP and RNAi-PsbQ mutants. In Fig. 1A, two RNAi-PsbP mutants (RNAi-PsbPA and RNAi-PsbPB) were examined. The RNAi-PsbPB mutant accumulated significant quantities of the PsbP protein and contained approximately 13% of the protein that was observed in wild-type. The RNAi-PsbPA mutant was found to accumulate <1% of the PsbP protein found in wild-type. In an earlier study, we found that arabidopsis RNAi-PsbP mutants which accumulated >5–10% of the PsbP protein exhibited a relatively mild phenotype, although they assembled somewhat fewer functional PS II reaction centers, and could grow photoautotrophically. RNAi-PsbP mutants which accumulated <5% of the PsbP protein were severely affected and assembled low amounts of functional PS II reaction centers and could not grow photoautotrophically [14]. Similar results were observed in tobacco [16]. It should be stressed that all of the RNAi-PsbP mutants which we examined in both this and our earlier study (see [14] for images of these plants) are green when grown on sucrose-containing media and show no apparent signs of necrosis or senescence. The RNAi-PsbPA line, which accumulates the smallest amount of PsbP protein, is very slightly less pigmented than the control wild-type plants. In Fig. 1B, the RNAi-PsbQ7 mutant was examined. This plant was found to accumulate <5% of the PsbQ protein observed in wild-type. Earlier, we had demonstrated that arabidopsis RNAi mutants containing low amounts of the PsbQ component exhibited no apparent defects when grown under normal light intensities (50–100 μmol photons $m^{-2}s^{-1}$) [13]. Identical results were obtained in tobacco [16].

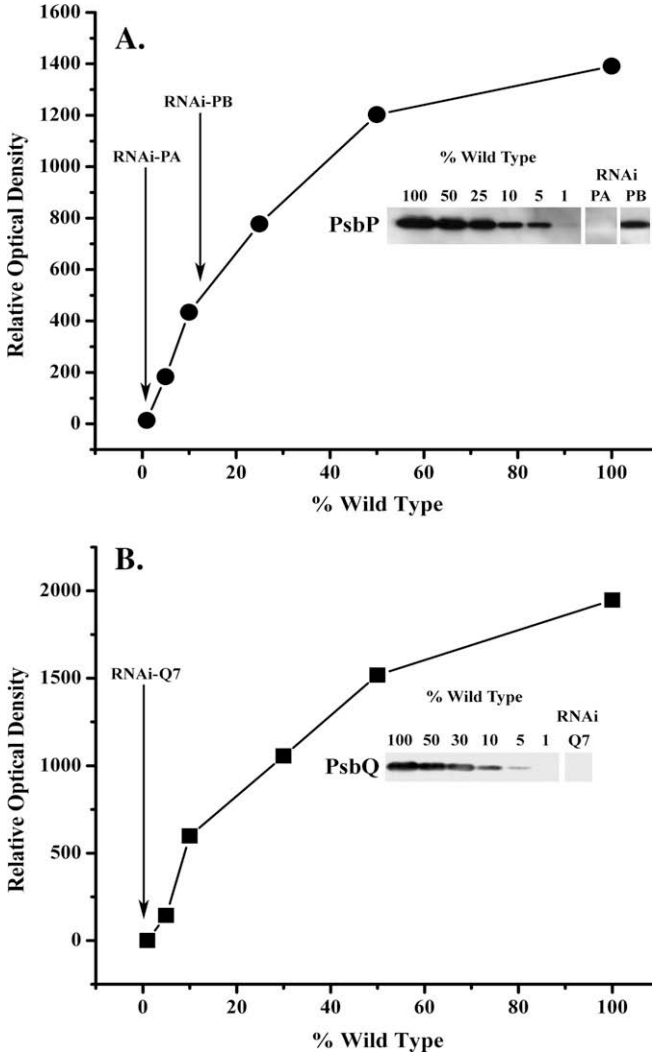

Fig. 1. Quantification of the PsbP and PsbQ proteins in RNAi mutants of *Arabidopsis*. (A) Quantification of the PsbP protein. Known amounts of wild-type thylakoids ranging from 5 μg Chl (100%) to 0.05 μg Chl (1%) were separated by lithium dodecyl sulfate polyacrylamide gel electrophoresis (LiDS-PAGE), "Western" blotted and detected by chemilumenescence. After immunodetection, the integrated optical densities of the wild-type PsbP bands were used to construct the standard curve shown. The amount of PsbP protein present in a sample containing 5 μg Chl of the RNAi-PsbP thylakoids was then determined in relation to the wild-type standard curve. The arrows indicate the amount of PsbP protein present in the thylakoids of RNAi-PsbPA (RNAi-PA) and RNAi-PsbB (RNAi-PB) thylakoids, respectively. The insert shows the "Western" blots used for this quantification. (B) Quantification of the PsbQ protein. Known amounts of wild-type thylakoids ranging from 10 μg Chl (100%) to 0.1 μg Chl (1%) were separated by LiDS-PAGE, "Western" blotted and detected by chemilumenescence. After immunodetection, the integrated optical densities of the wild-type PsbQ bands were used to construct the standard curve shown. The amount of PsbQ protein present in a sample containing 10 μg Chl of the RNAi-PsbQ7 thylakoids was then determined in relation to the wild-type standard curve. The arrows indicate the amount of PsbQ protein present in the thylakoids of RNAi-PsbQ7 (RNAi-Q7). The insert shows the "Western" blots used for this quantification.

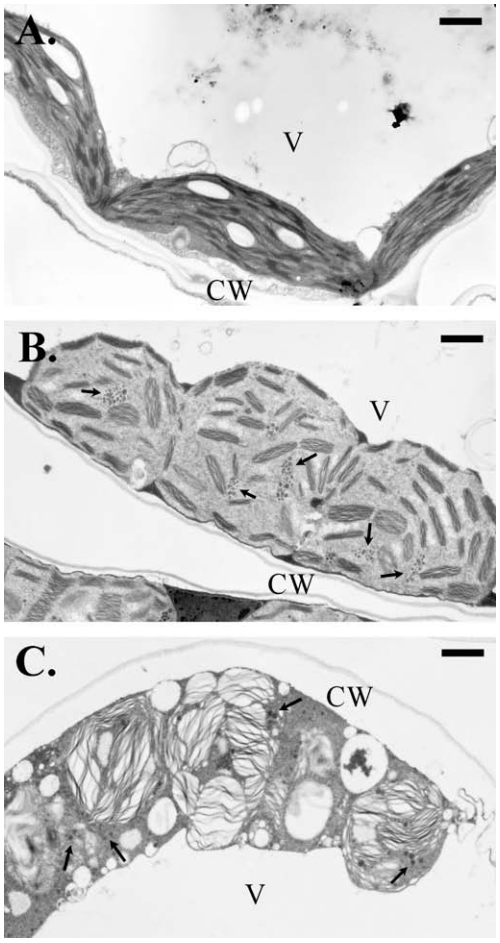

Fig. 2. Transmission electron microphotographs of wild-type, RNAi-PsbPA and RNAi-PsbPB chloroplasts. Green leaf tissue from the different plant lines which had been grown on agar in the presence of MS salts and sucrose were fixed, stained, embedded and sectioned as described in Section 2. (A) Wild-type chloroplasts, (B) RNAi-PsbPB chloroplasts, and (C) RNAi-PsbPA chloroplasts. Labels: V, central vacuole, CW, cell wall; arrows indicate osmiophilic granules, scale bar = 1 μm.

3.2. Chloroplast ultrastructure of the RNAi-PsbP and RNAi-PsbQ mutants

In Figs. 2 and 3 the ultrastructure of the RNAi-PsbP and RNAi-PsbQ mutants, respectively, and their appropriate wild-type control plants, are shown. As Fig. 2 illustrates, reduction in the amount of the PsbP protein has a profound effect on overall chloroplast morphology. The wild-type plants contain chloroplasts with completely normal morphology exhibiting grana stacking, an extensive network of stroma thylakoids connecting the grana stacks, and numerous starch grains (Fig. 2A). In Fig. 2B, chloroplasts from the RNAi-PsbPB plant line, which contains 13% of the PsbP protein as wild-type, are shown. The chloroplasts appear swollen, no starch grains being evident. Additionally, the grana stacks are much wider, are disorientated and the membranes appear to be more loosely stacked (see below). There is almost a complete lack of recognizable stroma thylakoids, and numerous localized accumulations of osmiophilic granules are observed (arrows). In Fig. 2C, chloroplasts from the RNAi-PsbPA plant line, which contains <1% of the PsbP protein as wild-type, are shown. Again the chloroplasts are swollen, with no starch grains being evident. The grana stacks are highly distorted, with very swollen thylakoid lumens. Nevertheless, stacking is evident, with a parallel membrane organization observed within the distorted grana stacks. No stroma thylakoids are discernable and more disseminated populations of osmiophilic granules are observed (arrows).

Earlier, we had characterized a phenotypic series of plants which accumulated reduced amounts of the PsbP protein and concluded that the PsbP component was required for PS II assembly/stability [14]. Consequently, it is formally possible that the loss of assembled PS II could lead to the ultrastructural defects observed in the current study. In our earlier experiments, plants which accumulated 5–10% of wild-type levels of the PsbP protein

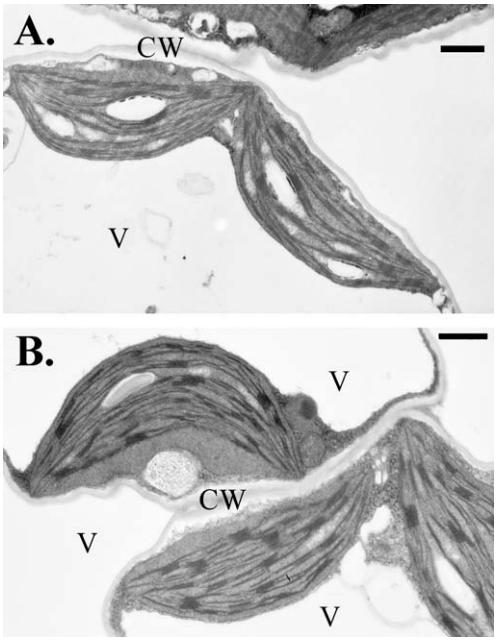

Fig. 3. Transmission electron microphotographs of wild-type and RNAi-PsbQ7 Chloroplasts. Green leaf tissue from the different plant lines which were grown on a soilless potting mix were fixed, stained, embedded and sectioned as described in Section 2. (A) Wild-type chloroplasts, and (B) RNAi-PsbQ7 chloroplasts. Labels: V, central vacuole, CW, cell wall, scale bar = 1 μm.

(comparable to the RNAi-PsbPB plant line examined in this study) exhibited a modest phenotype, accumulating near wild-type levels of several PS II core proteins, exhibiting a variable fluorescence yield which was 87% that of wild-type, and possessing near normal fluorescence decay kinetics both in the absence and presence of DCMU. Plants which accumulated undetectable amounts of the PsbP protein (comparable to the RNAi-PsbPA plant line examined in this study), however, exhibited severe alterations of in their PS II core protein complement, low variable fluorescence yield and defective fluorescence decay kinetics [14]. Consequently, the loss of functional PS II reaction centers may contribute to the ultrastructural defects observed in the RNAi-PsbPB and RNAi-PsbPA mutants.

Since the PsbP protein is required for the association of the PsbQ protein to PS II [8,9], the defects noted above could be due to an absence of PS II-associated PsbQ protein. Fig. 3 indicates that this is not the case. The chloroplasts of wild-type plants, which are shown in Fig. 3A, exhibit normal morphology. The chloroplasts of the RNAi-PsbQ7 plants, which accumulate <5% of the of the PsbQ protein present in wild-type, are indistinguishable from wild-type chloroplasts. This result indicates that the loss of the PsbQ protein is not responsible for the chloroplast defects observed in the RNAi-PsbP plant lines described above.

Fig. 4 provides a more detailed view of the thylakoids observed in the various plant lines. The thylakoids of wild-type plants (Fig. 4A) and the RNAi-PsbQ7 plants (Fig. 4B) appear very similar, with well-organized grana and stroma thylakoid membranes. Additionally, the diameter of the lumenal spacing is very consistent throughout the entire width of the grana stacks. In Fig. 4C, a typical grana stack from the RNAi-PsbPB plant line is shown. In addition to lacking apparent stroma thylakoids, the grana stacks are typically wider and taller than those observed in wild-type. The lumenal spacing is also highly irregular, with individual lumenal spaces exhibiting variable thicknesses throughout the width of the grana. In Fig. 4D, a grana stack from the RNAi-PsbPA plant line is shown. The grana appear extremely wide and very tall (note the

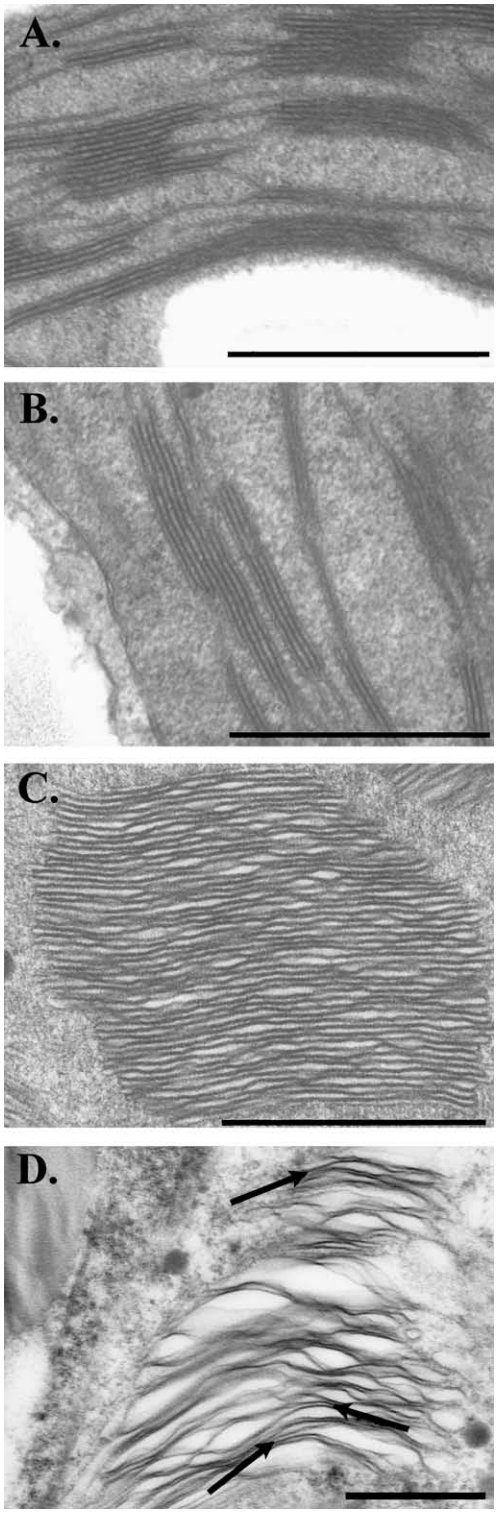

Fig. 4. Transmission electron microphotographs of wild-type, RNAi-PsbQ7, RNAi-PsbPA and RNAi-PsbPB grana stacks. Green leaf tissue was treated as described for Figs. 2 and 3. (A) Wild-type, (B) RNAi-PsbQ7, (C) RNAi-PsbPB, and (D) RNAi-PsbPA. Please note different magnification for (D) which was required to image a complete grana stack in this mutant. Arrows in (D) indicate regions of relatively normal lumen spacing, scale bar = 0.5 μm.

different size scales) and no identifiable stroma thylakoids are observed. These grana stacks are highly deformed, although the generally parallel arrangement of thylakoids is still evident. The apparent lumenal spaces of individual thylakoids are highly vari-

able in thickness throughout the entire grana width and exhibit large areas of localized swelling. Nevertheless, some areas of relatively normal lumenal spacing are observed and are indicated by arrows.

4. Discussion

These results clearly demonstrate that the PsbP protein has a pronounced effect on thylakoid membrane architecture. Recently it has been hypothesized that the extrinsic proteins of PS II, in particular PsbP and PsbQ, play an important role in the light-induced contraction of the lumen under conditions where PS II is functionally active [17,18]. These authors suggest that in the light, protein–protein interactions (specifically PsbQ–PsbQ interactions) occur across the diameter of the contracted lumenal space. Our results constitute a formal test of this hypothesis and, at least partially, support it. First, since chloroplasts depleted of the PsbQ protein exhibit normal membrane thylakoid architecture under standard growth light conditions (50 μmol photons m^{-2} s^{-1}), we believe that it is unlikely that this protein plays an important role in either the contraction of the lumenal space or in enhancing grana stability. It should be noted, however, that under low light conditions (5 μmol photons m^{-2} s^{-1}), the functional phenotype of RNAi-suppressed PsbQ plants is quite dramatic, with the plants losing the ability to grow photoautotrophically [13]. Chloroplast ultrastructural changes in the RNAi-PsbQ plants under these conditions have not been examined at this time. Our results do indicate, however, that PsbP–PsbP (or PsbP-X) interactions may be involved in the hypothesized process. Certainly, as PsbP is depleted, the diameter of the lumenal space becomes increasingly large, leading ultimately, in the RNAi-PsbPA plant, to dramatically deformed grana stacks.

Other aspects of the effects of PsbP protein depletion on chloroplast morphology are also quite interesting. The grana increase dramatically in size and appear more disordered, stroma thylakoids are lost, and the chloroplasts appear enlarged and more globular (compare Fig. 2A and B). Some shade plants, *Alocasia macrorrhiza* for instance, when grown in very low light environments, contain enlarged and disordered grana stacks, very small amounts of stroma thylakoids, and have a globular appearance [22,23]. Indeed, when these plants are grown under very low light conditions, their chloroplasts have an overall appearance which is quite similar to those observed in the RNAi-PsbPB plants (compare our Fig. 2B with Fig. 1, upper, of [23]). Even more striking, high magnification images of the enlarged grana stacks from *A. macrorrhiza* appear nearly identical to those of the RNAi-PsbB plants examined in this study (compare our Fig. 4C with Fig. 1, lower, of [23]) and exhibit highly variable lumenal spacing across the width of their grana stacks. These results may indicate that the RNAi-PsbPB plant is "locked" in a state that approximates the very low light growth state of *Alocasia*. As noted above, some of these responses may be a result of decreased levels of PS II. *Alocasia*, when grown under very low light conditions, assembles significantly fewer PS II reaction centers than those grown at higher light intensities [24]. Similar results have been obtained for many other species, including *Arabidopsis thaliana* [25,26]. The RNAi-PsbP plants that we previously examined [14] also exhibited a decrease in PS II reaction centers in tandem with the down regulation of the PsbP component.

5. Conclusions

Our results support the hypothesis that an extrinsic protein of PS II, specifically the PsbP component, is involved in the modulation of normal grana architecture. The mechanism for this modulation is, however, unclear, and is a subject for further study.

Acknowledgement

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