DOI: 10.1007/s11099-011-0070-7 PHOTOSYNTHETICA 49 (4): 573-580, 2011

PsbO, PsbP, and PsbQ of photosystem II are encoded by gene families in *Nicotiana benthamiana*. Structure and functionality of their isoforms

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Abstract

The extrinsic proteins of photosystem II in plants (PsbO, PsbP and PsbQ) are known to be targets of stress. In previous work, differential regulation of hypothetical isoforms of these proteins was observed in *Nicotiana benthamiana* upon viral infection. Each of these proteins is encoded by a multigene family in this species: there are at least four genes encoding PsbO and PsbP and two encoding PsbQ. The results of structural and functional analyses suggest that PsbO and PsbP isoforms could show differences in activity, based on significant substitutions in their primary structure. Two psbQ sequences were isolated which encode identical mature proteins.

Additional key words: oxygen-evolving complex; photosystem II; PsbO; PsbP; PsbQ; stress.

Introduction

Photosystem II (PSII) is a multisubunit protein complex embedded in the thylakoid membrane of higher plants, eukaryotic algae and cyanobacteria. Its function is to harvest solar energy used for water oxidation. This reaction takes place in the oxygen-evolving complex (OEC), which is stabilized and protected by extrinsic membrane proteins attached to the lumenal side of PSII. These proteins are PsbO, PsbP, and PsbQ and are encoded by multigene families in higher plants such as *Arabidopsis*, pea, tomato, and tobacco (Seidler 1996, Peltier *et al.* 2000).

Despite considerable efforts put into the study of PSII structure, only the high-resolution crystal structure of a cyanobacterial but not plant PSII has been elucidated (Loll *et al.* 2005). Therefore, the exact binding sites for PsbO, PsbP, and PsbQ on plant PSII have not been resolved, and little is known about their protein interactions (Nield and Barber 2006, Suorsa and Aro 2007, Enami *et al.* 2008).

PsbO is involved in the regulation of PSII affinity for Mn and the stabilization of the OEC (Chu *et al.* 1994, Popelkova *et al.* 2008). It plays a role in the stabilization and turnover of the D1 during the PSII damage-repair cycle (Eisenberg-Domovich *et al.* 1995, Yamamoto *et al.* 1998, Komenda *et al*. 2010). In *Arabidopsis*, two PsbO proteins are found, which are encoded by distinct genes and show differences in functionality (Murakami *et al.* 2005, Lundin *et al.* 2007a,b).

The extrinsic protein PsbP plays an important role in Ca^{2+} and Cl^{-} retention (Ifuku and Sato 2002, Ifuku *et al.* 2004), providing Mn ions during the D1 turnover, and protecting the structural integrity of the PSII super-complexes (Bondarava *et al.* 2005, Suorsa and Aro 2007, Ido *et al.* 2009). Moreover, a function related to GTP metabolism has been proposed for PsbP in interaction with PsbO (de las Rivas and Roman 2005). In contrast with the differences in functionality described for the PsbO isoforms in *Arabidopsis*, the four PsbP isoforms found in *Nicotiana tabacum* appear to carry out the same functions *in vivo* (Ishihara *et al.* 2005). However, they may not be equivalent under stress, as indicated by previous studies on *N. benthamiana* plants with viral infection (Pérez-Bueno *et al.* 2004).

PsbQ is the most divergent of the extrinsic proteins

Received 15 February 2011, *accepted* 10 September 2011.
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Abbreviations: OEC – oxygen-evolving complex; pI – isoelectric point; PSII – photosystem II.
Acknowledgements: The authors thank Dr. Maite Serra and Mario García for critical reading of the manuscript. MPB was recipient of a JAE-Doc contract funded by CSIC. This work was supported by grants BIO2004-04968-C02-02, and MCINN-FEDER/AGL2008-00450 to IGL and BIO2004-04968-C02-02, MEC-FEDER/BIO2007-67874-C02-02, MCINN-FEDER/AGL2008-00214 and CVI 03475 (Junta de Andalucía) to MBA.

of PSII in higher plants. This feature could be related to its structural flexibility and its dispensable nature regarding oxygen evolution.

The OEC extrinsic proteins are some of the main targets of viral infection in *N. benthamiana* plants (Barón *et al.* 1995, Rahoutei *et al.* 2000, Pérez-Bueno *et al.* 2004). Furthermore, it was shown that the OEC polypeptide pattern in the thylakoid membranes was modified by viral infection, and that hypothetical PsbP isoforms were differentially affected. Takahashi *et al*. (1991) also described a differential down-regulation of PsbP in tobacco plants infected with cucumber mosaic virus.

The data presented here reveal that in *N. benthamiana* multigene families encode the PsbO, PsbP, and PsbQ proteins. The polypeptide sequences corresponding to the isolated cDNA sequences have been analysed and the impact of the differences in the proteins sequence are discussed.

Materials and methods

Plants of *Nicotiana benthamiana* Domin were grown in growth chambers under a regime of 16-h photoperiod at 25ºC, 80% relative humidity, and 200 μmol m^{-2} s^{-1} light intensity.

cDNA cloning: Two cDNA sets were used as templates for cloning cDNA sequences encoding the PsbO, PsbP, and PsbQ proteins: cDNA synthesised from total RNA isolated from *N. benthamiana* plants (Logemann *et al.* 1987) and a cDNA library of *N. benthamiana* cloned in pACT2, kind gift of Dr. J. Bol (Wageningen, The Netherlands). Sequences were amplified by: *SMART™ RACE* Amplification kit (*Clontech*, Mountain View, CA, USA), and RT-PCR by *Superscript II* RNase H reverse transcriptase (*Gibco BRL*, Barcelona, Spain). The primers for the downstream amplification were designed in a conserved region encoding the N-terminal region of the mature proteins (Pérez-Bueno *et al.* 2004). Further primers were designed based on alignments of the amplified sequences. Table 1 shows the primers and the amplification method used in each case.

Sequence analysis and bioinformatics tools: Nucleic acid and protein sequences were aligned by *CLUSTALW2* (Larkin *et al.* 2007) and edited by *ESPript 2.1* (Gouet *et al.* 1999). The corresponding amino acid sequences were deduced and analysed by the programs *TargetP* (Emanuelsson *et al.* 2000) for signal peptide prediction and *Compute pI/Mw* (Gasteiger *et al.* 2005) for theoretical calculations of pI and molecular mass.

Homology modelling of protein structures was carried out using *SWISS-MODEL* (Arnold *et al.* 2006) and the obtained models were displayed by *Swiss-PdbViewer v3.7*. (http://www.expasy.org/spdbv/). Loops of interest were modelled by *SWISS-MODEL*, using the database *LOOPDB* and energy computation by the *GROMOS96* implementation in *Swiss-PdbViewer*. The reliability of the loop modelling was assessed by the value of the B-factor, which provides information about the quality of the prediction.

Southern blot analysis: For the analysis of *N. benthamiana* genome, 10 μg of nuclear genomic DNA from young leaves (Sambrook *et al.* 1989) was digested with *EcoR*V, *Hind* III and *Xba* I and treated with DNase-free RNase A. Each digestion product was loaded onto a 1.2% agarose gel in TAE buffer and run overnight at 2.5 V cm^{-1}. Prior to blotting, the DNA was denaturalised and then transferred by capillarity to a *Hybond-N* membrane (*Pharmacia*, Barcelona, Spain). For maximum specificity, the blottings were hybridised overnight at 65ºC with [α-^{32}P]dCTP-labelled cDNA probes in 0.9 M NaCl, 90 mM sodium citrate, 5X Denhardt's solution, 0.1% SDS and 0.12 mg ml^{-1} salmon sperm DNA; and the washings were carried out at low stringency conditions (twice at 65ºC for 30 min in 0.9 M NaCl, 90 mM sodium

Table 1. Details of the amplification of *psbO*, *psbP*, and *psbQ* cDNAs from *N. benthamiana.*

	Primers	cDNA	Method
psbO	*33b* (*CGCTCACCACCAGGAAGCTGAACTG*), *NUP* (AAGCAGTGGTAACAACGCAGAGT)	*mRNA*	*cDNA* 5'RACE
	33a (GAAGGTGTTCCAAAACGT), *102* (AGATGGTGCACGATGCA)	cDNA library	PCR
	33a (GAAGGTGTTCCAAAACGT), *110* (CCAAGGCCT$_{18}$)	cDNA library	PCR
psbP	*24b* (*GATTCAAATCCACCCTCTGAATCAGT*), *NUP* (AAGCAGTGGTAACAACGCAGAGT)	*mRNA*	*cDNA* 5'RACE
	24a (CTGCAGATGCTGCTTATGGAGAAGC), *110* (5CCAAGGCCT$_{18}$)	cDNA library	PCR
	24c (CAAGCTGGTGACAAGAGATGG), *110* (CCAAGGCCT$_{18}$)	mRNA	cDNA AMV-RT
	24d (GTGCATAAAAAGCACAACTCATATGCTTAC), *101* (TACCACTACAATGGATG)	cDNA library	PCR
	24e (AGAGGGGGGAGAACTAAGTACATAC), *101* (TACCACTACAATGGATG)	cDNA library	PCR
	24f (TGCTTTAGGCCAAGCTCAATACTC),*101* (TACCACTACAATGGATG)	cDNA library	PCR
psbQ	*16b* (*GGGCTGTTCTTGGTCTTTGCTGCATGG*), *NUP* (AAGCAGTGGTAACAACGCAGAGT)	*mRNA*	cDNA *5'RACE*
	16a (AACTCGGATGAGGCAAGGGACTT), *102* (AGATGGTGCACGATGCA)	cDNA library	PCR
	16a (AACTCGGATGAGGCAAGGGACTT), *110* (CCAAGGCCT$_{18}$)	cDNA library	PCR

citrate and 0.1% SDS and twice for 30 min in 0.3 M NaCl, 30 mM sodium citrate and 0.1% SDS).

The cDNA sequences used as hybridization probes were synthesised by amplification of partial *psbO, psbP*, and *psbQ* cDNA clones isolated from *N. benthamiana.* These fragments cover most of the ORF and the full 3'UTR region of the corresponding mRNA.

Results and discussion

PsbO, PsbP, and PsbQ are encoded by small multigene families in *N. benthamiana*: The nuclear genome of *N. benthamiana* was analysed by Southern blotting (Fig. 1) in order to determine whether the extrinsic proteins PsbO, PsbP, and PsbQ were encoded by single genes or gene families. All sequences used as probes in the Southern blots correspond to partial cDNAs isolated from *N. benthamiana*, as described in Materials and methods. Taking into account the lengths of the probes used and the high sequence identities between them and the rest of the cDNA sequences from the same gene family here reported, especially in their fraction corresponding to the ORF, it is expected that the probes used would hybridize at least with the reported sequences.

Each probe hybridized to a small number of bands of different size (Fig. 1), thus suggesting that each extrinsic protein is encoded by a small multigene family in *N. benthamiana*.

PsbO proteins in *N. benthamiana*: Four cDNA were isolated which encode four PsbO proteins, NbPsbO1 to NbPsbO4 (accession numbers JF897603, JF897604, JF897605, and JF897606, respectively). The NbPsbO2 sequence is identical to the only *N. benthamiana* PsbO sequence in the databases (AY952375), except for a conservative substitution A3T in their signal peptide.

By comparing the theoretical pI and molecular mass of the mature PsbO proteins (Table 2) to the protein pattern described in Pérez-Bueno *et al*. (2004), as well as their N-terminal sequence, it is plausible to suggest that NbPsbO1, 2, 3 and 4 here reported correspond to PsbO-I, II, IV, and III, respectively.

The deduced mature proteins NbPsbO1 and NbPsbO2, as well as NbPsbO3 and NbPsbO4, share a 99% sequence identity, whereas the identity between them is 95–96%. Most of the amino acid substitutions (Fig. 2*A*) are located out of the highly conserved regions of PsbO from plants (de las Rivas and Barber 2004), but some are in positions which might be important for protein function: Glu residues in positions 33 and 140, as those in positions 1 and 188, are involved in electrostatic interactions with PsbP (Bricker and Frankel 2003). In these two positions, NbPsbO3 and NbPsbO4 present conservative replacements E/D with respect to the other two sequences. Moreover, E140 within the motif ^{137}GKP (E/D)^{140} would be directly involved in GTP binding (Lundin *et al.* 2007a,b). Similarly, the substitution K49N in NbPsbO2 is located within another GTP-binding motif ^{44}GKYNA(K/N)K^{50} (Larkin *et al.* 2007).

Given the predicted high conservation of the structure of PsbO proteins along evolution (de las Rivas and Barber 2004), the crystal structure determined for PsbO from *Thermosynechococcus elongatus* [2AXT_O; (Loll *et al.* 2005)] was used as a template for homology modelling of *N. benthamiana* PsbO sequences. Fig. 2*B* shows two regions in NbPsbO1 and NbPsbO2 which could potentially adopt a different conformation compared to the other two proteins (as shown by the low values of the B-factor obtained): the region D/E33–K42, comprising position 33 (marked blue in Fig. 2*B*); and the loop A135–S141, containing the residue E140 (marked yellow in Fig. 2*B*). It is remarkable that the same conservative changes are present in position 33 of NtPsbO2, and in position 140 of the AtPsbO1 (Fig. 2).

NbPsbO3 and NbPsbO4 also show a conservative N66S substitution within a region suggested to interact with CP47 of PSII (de las Rivas and Barber 2004). Although there are no differences in the structure predicted for this region, the N66S substitution could have some impact on the binding affinity for CP47.

In *Arabidopsis*, the two PsbO proteins perform two functions with different efficiency, despite of having similar binding affinity for PSII: AtPsbO1 is more efficient on the structural role of stabilizing the lumenal

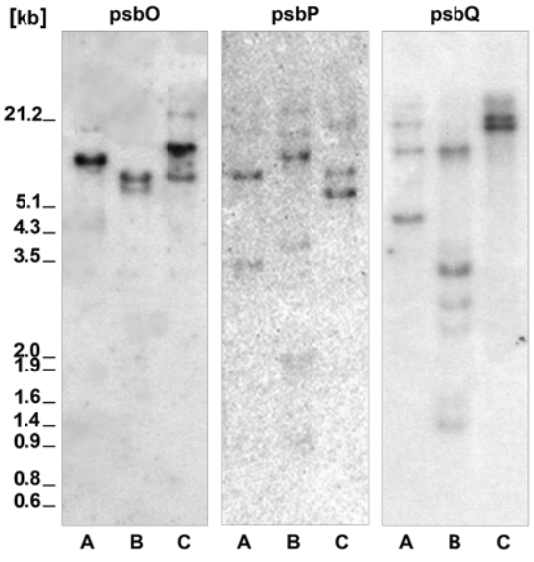

Fig. 1. Analysis by Southern blotting of the *N. benthamiana* genes *psbO*, *psbP*, and *psbQ* using as probes cDNA fragments isolated from the same species. Nuclear genomic DNA was digested with *EcoR*V (A), *Hind* III (B), and *Xba* I (C). The molecular mass markers are *EcoR* I and *Hind* III-digested λ phage.

Table 2. Theoretical molecular mass (MM) and pI of the extrinsic proteins PsbO, PsbP, and PsbQ from *N. benthamiana*.

	NbPsbO1	NbPsbO2	NbPsbO3	NbPsbO4	NbPsbP1	NbPsbP2	NbPsbP3	NbPsbP4	NbPsbQ1	NbPsbQ2
MM [kDa]	26.6	26.6	26.8	26.8	20.3	20.4	20.1	20.1	16.3	16.3
pI	5.05	5.04	4.94	4.94	5.27	5.10	5.28	5.28	9.48	9.48

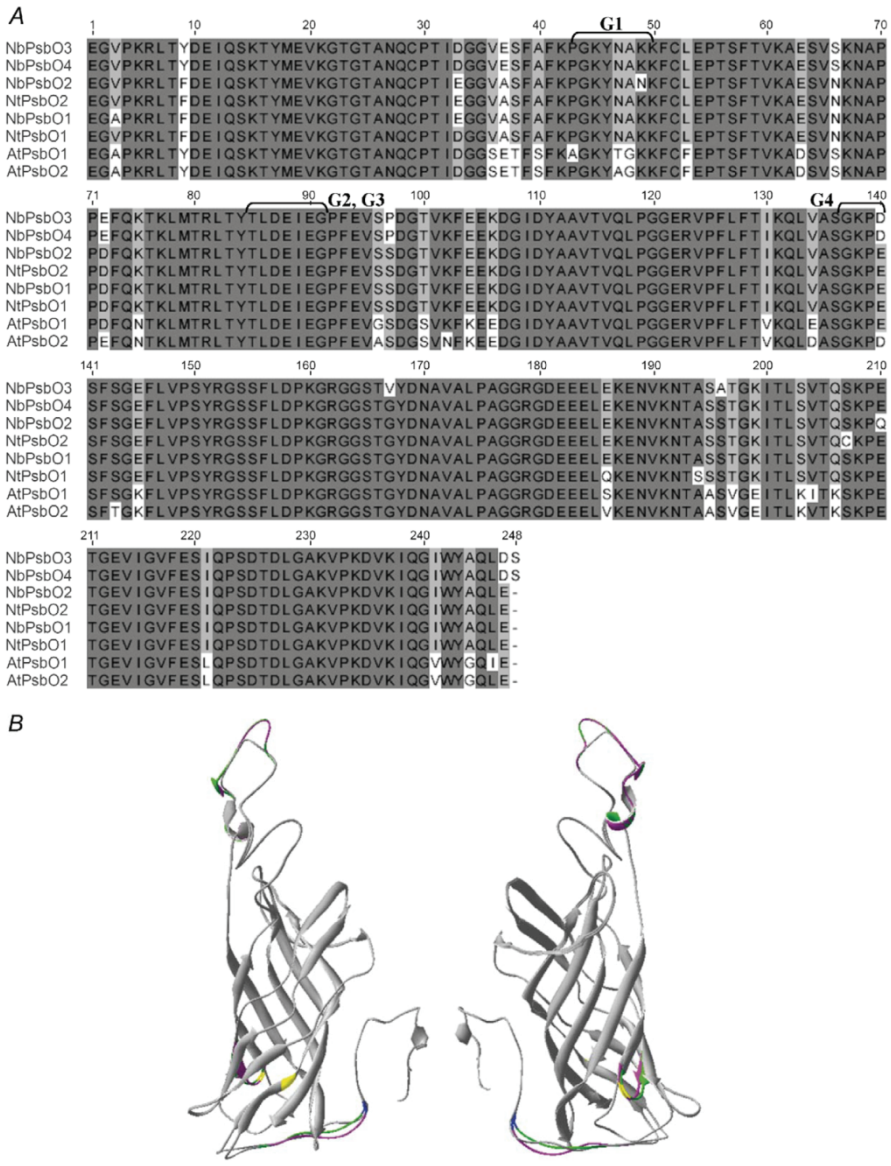

Fig. 2. *A*: Alignment of the mature PsbO protein sequences from: *N. benthamiana*, *Arabidopsis* (AtPsbO1 P23321; AtPsbO2 Q9S841), and tobacco (NtPsbO1 Q40459; NtPsbO2 AY0076). Putative GTP binding sites G1, G2, G3, and G4 (Lundin *et al*. 2007b) are marked. *B*: Homology modelling of the mature PsbO proteins from *N. benthamiana* (NbPsbO1 and NbPsbO2, lilac; NbPsbO3 and NbPsbO4, green) and the crystal structure of PsbO from *T. elongatus* (2AXT_O, grey). Yellow: residues K/N49 and E/D140. Blue: residue E/D33.

side of PSII whereas AtPsbO2, with GTPase activity, regulates the D1 dephosphorylation and turnover. The replacements V186S, V204I, and L246I at the C-terminus of the *Arabidopsis* PsbO protein sequences were suggested to be responsible for their functional differences (Murakami *et al.* 2005, Lundin *et al.* 2007a,b). Although these positions are conserved among the four PsbO proteins of *N. benthamiana*, replacements and changes in spatial orientation of other residues involved in the GTPase activity (K49N and E140D) (Lundin *et al.* 2007a) may be relevant.

It is believed that most adaptive changes in sequences

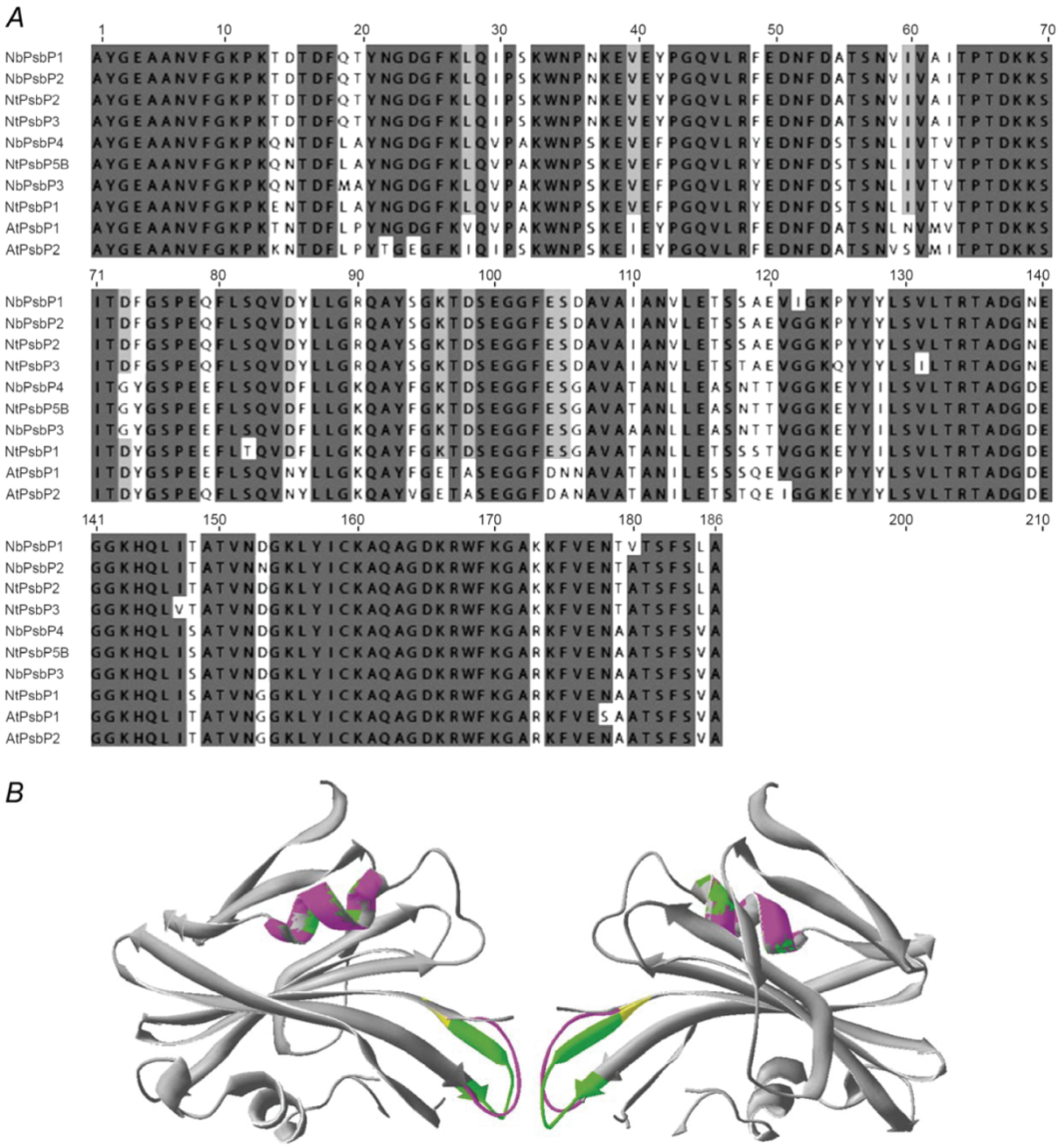

Fig. 3. *A*: Alignment of mature PsbP protein sequences from: *N. benthamiana*, *Arabidopsis* (AtPsbP1 Q42029; AtPsbP2 O49344), and tobacco (NtPsbP1 Q7DM39; NtPsbP2 P18212; NtPsbP3 Q04127; NtPsbP5B Q04126). *B*: Homology modelling of the mature PsbP proteins from *N. benthamiana* (NbPsbP1 and NbPsbP2, lilac; NbPsbP3 and NbPsbP4, green) and the crystal structure of NtPsbP3 (1V2B, grey). Yellow: residue K143.

occur after gene duplication. The resemblance between the PsbO sequences of both species, despite their evolutionary distance, seems remarkable and suggests a differential functionality for NbPsbO1 and NbPsbO2 with respect to the NbPsbO3 and NbPsbO4 isoforms of *N. benthamiana*, as reported for AtPsbO1 and AtPsbO2 in *Arabidopsis* (Murakami *et al.* 2005, Lundin *et al.* 2007a,b).

Within the C-terminus of the protein, which could be involved in the interaction with the PSII core (de las Rivas and Barber 2004), the region including the α-helix E182–N189 appears to be shifted in the *N. benthamiana* PsbO proteins with respect to the crystal structure for the cyanobacterial PsbO (Fig. 3). Within this region, E188 takes part in interactions with PsbP (Bricker and Frankel 2003) and would adopt a different orientation in *N. benthamiana* PsbO with respect to cyanobacterial PsbO, as supported by its low B-factor value. These differences are in accordance with previous suggestions of differences in the binding sites on PsbO for PSII from bacteria compared to those from higher plants.

PsbP proteins in *N. benthamiana*: Four cDNA sequences were isolated which encoded the proteins termed NbPsbP1 to NbPsbP4 (accession numbers JF897607, JF897608, JF897609, and JF897610, respectively). NbPsbP1 corresponds to the only PsbP sequence from *N. benthamiana* available in the database (AY952374),

which lacks the first residues of the leader peptide, although they differ in one conservative replacement H10Q in the leader peptide.

The mature proteins NbPsbP1 and NbPsbP3 share with NbPsbP2 and NbPsbP4 a 98% identity on their amino acid sequence, respectively, whereas sequence identity between the pairs is only 81%. Multiple amino acid substitutions are scattered along the entire sequence (Fig. 3*A*), even in the first 15–19 N-terminal residues, essential for the stability of the protein structure (Ifuku *et al.* 2005): the replacement D15N, which confers higher activity regarding oxygen evolution (Ifuku and Sato 2001), was found in NbPsbP1 and 2. On the other hand, the four PsbP sequences show several replacements within positively charged domains involved in electrostatic interactions with PsbO: ^{11}KPK(T/Q)^{14}, ^{27}KLQ(I/V)P(S/A) KWNP(S/N)K^{38}, ^{90}(R/K)QAY(S/F) GK^{96}, ^{143}KHQLI(T/S)ATVN^{152}, and ^{166}KRWFKGA (K/R)K^{174} (Tohri *et al.* 2004). Many of the replacements among the four PsbP sequences of *N. benthamiana* are also found among the four known sequences of tobacco and nine of these replacements are also found in the two sequences of *Arabidopsis* (Fig. 3*A*).

Within the α-helix in the region K174–T181, important for the overall structure of the protein (Ifuku *et al.* 2004), there are two replacements among the *N. benthamiana* PsbP proteins (positions 179 and 180, Fig. 3*A*).

Structural differences in the proteins PsbP of *N. benthamiana* were assessed by homology modelling (Fig. 3*B*) using as template the crystal structure of NtPsbP3 (1V2B) (Ifuku *et al.* 2004, 2005), from which some regions are missing due to high disorder (the first 15 residues in the N-terminus, regions R90–A107 and T135–G142).

The region T135–G142 could be modelled using templates from the LOOPDB database, showing differences in the possible conformation, based on the low values for the B-factor obtained (Fig. 3*B*). Since the residue K143 (in yellow, Fig. 3*B*), next to this loop, could be involved in electrostatic interactions with negative charged residues on the PsbO protein (Tohri *et al.* 2004), its spatial conformation might affect the accessibility of PsbO to K143 of PsbP and in consequence the affinity binding.

In tobacco, full expression of the four PsbP proteins was required for optimal PSII activity (Ishihara *et al.* 2005) and possible differences in functionality were reported for them (Ifuku and Sato 2001): isoforms with Asn in position 15 exhibited a higher ion binding activity compared to Asp in such position. Thus, NbPsbP3 and NbPsbP4 could have a higher activity than the other two isoforms. It is important to note that these two proteins, which would correspond to the spot named PsbP-D in Pérez-Bueno (2004), attending to their pI and MM in Table 2. The proteins in the spot PsbP-D were the first ones to disappear in plants infected with virus, whereas the spots PsbP-B and C (Pérez-Bueno *et al.* 2004), corresponding to NbPsbP1 and NbPsbP2, with lower activity, would be more stable along the infection.

From the multiple replacements and the differences in spatial conformation comprising regions involved in protein interactions, we conclude that the four PsbP proteins of *N. benthamiana* may play the same roles within the PSII but differ in their activity rates/binding affinities to other PSII subunits and/or ions. Differential regulation of the accumulation levels of the PsbP isoproteins in *N. benthamiana* upon viral infection could be a mechanism to modulate the activity of PSII and therefore the activity of the electron transport chain under stress conditions.

PsbQ proteins in *N. benthamiana*: The analysis of genomic DNA isolated from *N. benthamiana* showed that PsbQ is encoded by a gene family. Two isolated cDNA sequences (accession numbers JF897611 and JF897612) share a 97.3% identity in their ORF sequence and encode the proteins NbPsbQ1 and NbPsbQ2 which only differ in one semiconservative substitution in position 24 of the

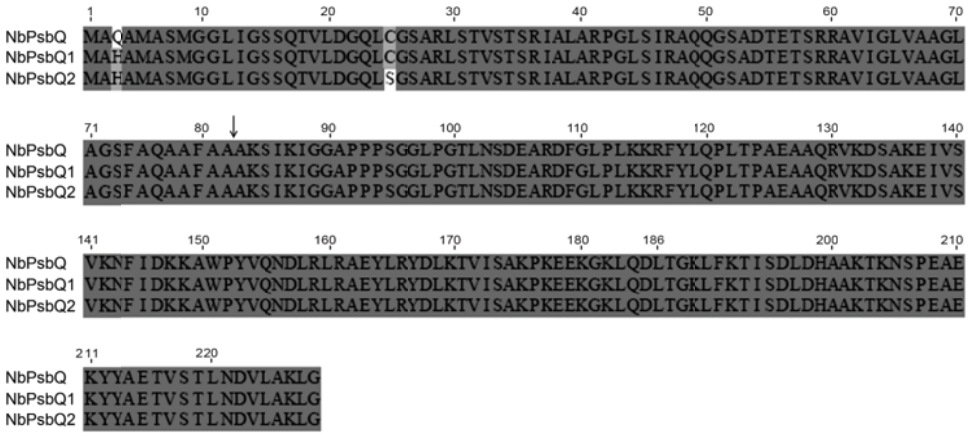

Fig. 4. Multiple alignment of the PsbQ precursors from *N. benthamiana* here reported and those available in the data base (NbPsbQ, Q5EFR5). *Arrow* – cleavage site of the signal peptide. Sequences are sorted by pairwise identity.

signal peptide (Fig. 4). Their theoretical molecular mass and pI are shown in Table 2.

The nucleic acid sequences NbPsbQ1 and NbPsbQ2 reported in this work have a 99% and 97% identity with the only sequence from *N. benthamiana* on the database (Q5EFR5), respectively. The encoded protein sequences differ on a conservative substitution (H3Q) and a semi-conservative (C24S) in their signal peptide.

Conclusions: The homology models presented in this work show that most of the possible differences in structure among isoforms of the PsbO, PsbP, and PsbQ proteins here reported involve loops. Loops are often a very important part of the protein surface and determine the binding affinity to other proteins, ligands or substrates. Moreover, functional differences among members of the same protein family are usually due to structural differences in their loop regions (Blouin *et al.* 2004), suggesting that functionality and/or binding affinity of PsbO and PsbP isoforms depends upon the particular conformation of these regions in each protein.

Multigene families are thought to be a possible adaptative response to variable ambient conditions. Our results are compatible with an scenario in which genes of the same family were differentially regulated in *N. benthamiana* upon viral infection at a transcriptional and/or translational level, and the isoforms of PsbO and PsbP could perform different functions, as it has been already described for the PsbO proteins in *Arabidopsis*, or could perform the same functions with different efficiencies (Murakami *et al.* 2005, Lundin *et al.* 2007a,b). Given that the extrinsic proteins of PSII are especially sensitive to stress and their accumulation pattern is differentially regulated upon viral infection, it is suggested that isoforms of PsbO and PsbP proteins might differ in functionality and/or binding affinity to other PSII subunits or ions. This would tune the activity of PSII according to environmental conditions.

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