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Phylogeny-directed structural analysis of the *Arabidopsis* PsbS protein

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Abstract

Plant psbS proteins are essential for regulated thermal dissipation of excess light referred to as non-photochemical quenching of chlorophyll fluorescence yield (NPQ). Amino acid sequences derived from 65 *psbS* genes from 44 species were aligned to reveal extensive conservation consistent with of motifs that underlie intrinsic aspects of the NPQ mechanism. Site-directed mutagenesis was employed to block presumptive zeaxanthin or chlorophyll-binding sites in *Arabidopsis* psbS by disrupting ion-bonding between two pairs of non-adjacent glutamate and arginine residues. Transgenic *Arabidopsis* lines synthesizing only the altered psbS forms exhibited severely impaired NPQ capacity. In addition, the phylogenetic depth of the psbS database permitted identification of cryptic sites of adaptive evolution. Instances of localized positive selection were rare and largely limited to the family Poaceae (grasses). Specifically, adaptive evolution was detected in a hydrophilic stroma-exposed region and was correlated with the presence of the C4 pathway of carbon fixation.
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Keywords: *Arabidopsis thaliana*; Carotenoid; Chlorophyll; Expressed sequence tag; Fluorescence; Non-photochemical quenching

Non-photochemical quenching of fluorescence yield (NPQ) is a protective response to absorption of excess quanta that could damage photosystem II (PSII) reaction centers [1]. In higher plants the dominant component of NPQ (referred to as q_E) is profoundly enhanced by the xanthophyll pigment zeaxanthin, and is triggered by formation of a transthylakoid ΔpH [2,3]. A third essential component is the 22-kDa product of the nuclear *psbS* gene [4]. The central role of psbS in NPQ is evident as *Arabidopsis psbS* mutant lines show impaired quenching capacity yet exhibit normal photosynthesis and growth under moderate conditions [4–7]. Although spectroscopic evidence links psbS to a proposed *in vivo* dissipative charge transfer mechanism involving a carotenoid cation radical [8], the basis for the psbS-dependence of NPQ remains obscure.

A systematic comparison of protein sequences from a wide variety of sources is an effective first step in probing the relationship between structure and function. Highly conserved regions are likely to be essential for proper conformation or contain structural features directly related to mechanism. The psbS protein shares significant amino acid sequence similarity with major higher plant light-harvesting chlorophyll (Chl) proteins [9,10]. Four α-helical, hydrophobic regions indicate an association with the chloroplast thylakoid membrane [9]. Indeed, the majority of random point mutations described for *psbS* map to the transmembrane helices [5,6,11]. Likewise, the role of psbS in sensing the pH of the chloroplast thylakoid lumen involves specific glutamate carboxyls that when mutated result in a loss of NPQ capacity [5]. Finally, *in vitro* reconstitution studies with recombinant PSII antenna apoproteins LHCII and CP29 confirmed liganding of Chl by glutamate residues [12,13] at binding sites A1 and A4 in principal crystallographic models of the LHCII monomer [14]. Dual motifs occur in psbS that resemble motifs associated with the A1 and A4 sites [5,15].

We compiled over 1140 expressed sequence tags (ESTs) and cDNAs from publicly accessible internet sources to derive high quality contiguous *psbS* sequences for 65 genes

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from 44 species. Alignment of the encoded proteins confirmed the ubiquitous occurrence of the putative pigment-liganding motifs described above and site-directed mutagenesis established a functional role for these sites in the NPQ mechanism. Finally, application of the relative rate ratio test devised by Creevey and McInerney [16,17] revealed the existence of adaptive evolution in *psbS*, which sets the stage for rational analysis of structure in relation to function.

Materials and methods

Gas exchange, fluorescence, pigment, and Western analyses. Wild-type *Arabidopsis thaliana* (Columbia), line *Npq4-1* (*psbS* Δ) [4] and transgenic lines were grown as previously described [6,7]. Gas exchange and fluorescence measurements were performed as described previously [6,7,18], using a Walz PAM 101 Fluorometer equipped with an ED101BL emitter–detector unit. Total chlorophyll (Chl) and psbS content of leaf samples were assessed as described previously [7]. Four to six replicate leaves were processed for each line. A known mass of recombinant purified spinach psbS was included as an internal standard in anti-psbS Western blots.

DNA manipulations. The coding region of *Arabidopsis psbS* cDNA [10] was mutated using GeneEditor™ site-directed mutagenesis system (Promega, Madison, WI, USA) with oligos PSBS1A (5′-AAAGGCGAATGT GCTATTCGTTG-3′) and PSBS2A (5′-TTCGTTGGTCTTGTTGCT ATG-3′) to generate allele *npq4-E38V/R147L*, and with PSBS2B (5′-AA GGCGAACGTGTTATTCGTAG-3′) and PSBS1B (5′-GAGTTATTC GTAGGATTATTGGCACAGTTGGG-3′) to make allele *npq4-R43L/E142V* creating plasmids, pCC21 and pCC31, respectively. The *CaMV35S-psbS* mutant cassettes were cloned into pCAMBIA1200 (GenBank Accession No. AF234292), moved into *Agrobacterium tumifaciens* strain GV2260 and then transformed into *Arabidopsis npq4-1* line [4] by the floral dip procedure [19]. *Nicotiana benthamiana psbS* genes were isolated by amplifying genomic DNA with primers NTABS1 (5′-ATGGC TCAAACAATGTTGCTG-3′) and NTABS2 (5′-TCACTCCTCTTCCTC ATCAGTG-3′) and the sequences determined (GenBank Accession Nos. DQ340566 and DQ34057).

PsbS sequence analysis. The CAP3 Sequence Assembler [20] generated species-specific *psbS* unigenes using cDNA and EST sequences obtained from the National Center for Biotechnology Information, Chlamydomonas Resource Center (http://www.chlamy.org/) or derived from genomic sequences. CLUSTALW [21] was used to align mature psbS sequences, based on that from Arabidopsis [22]. Transmembrane spanning helices were predicted by HMMTOP [23]. DNA sequences were prealigned using CodonAlign [24]. Phylogenetic trees were constructed by the neighbor joining (NJ) algorithm [25] using PAUP 4.0 [26] and the Bayesian method [27] using *Physcomitrella patens* psbS as the outgroup. CRANN was used to search for sites of adaptive evolution [16,17] using NJ and Bayesian phylogenetic tree structures.

Results and discussion

PsbS sequences are highly conserved

A databank totaling 65 *psbS* genes from 44 species was constructed (see Supplementary materials). Fig. 1 shows an alignment of psbS proteins chosen for maximum phylogenetic divergence. Sequence conservation is high in membrane-spanning domains (horizontal bars Fig. 1) due to the importance of these regions in maintaining protein tertiary structure and orientation. Four domains (*) exhibited absolute conservation for all 65 sequences. Specifically, two conserved glutamates (E70 and E174 for *Arabidopsis* in Fig. 1) have previously been shown to exert an additive effect on NPQ capacity [5] presumably acting as sensors of lumen acidity [15]. Second, the dual NELFVGR domains in helices I and III resemble domains found in helices A and B of the LHCII monomer and CP29 [12,13]. These glutamates each serve as a central ligand to Chl *a* [14]. The apparent homology between the transmembrane regions I and III of psbS and the respective membrane-spanning helices B and A of spinach LHCII [14] suggests that corresponding putatively Chl-binding glutamates of psbS (E38 and E142 for *Arabidopsis*) are in the hydrophobic protein–lipid matrix *in vivo*.

Putative pigment-binding domains are essential for full NPQ

The question of whether psbS binds pigments is critical to an understanding of how psbS promotes NPQ,

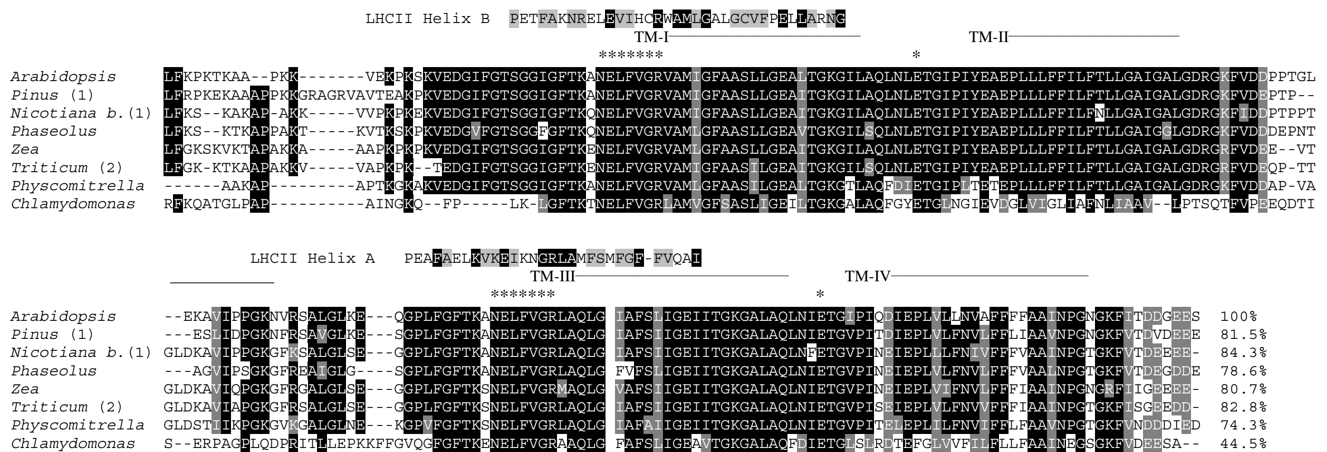

Fig. 1. Alignment of mature psbS sequences from taxonomically diverse species. Parentheses indicate psbS species paralog. Black and gray shading indicate amino acid identity or similarity. Asterisks indicate four conserved domains (see text). Percentages indicate residue similarity relative to *Arabidopsis*. GenBank Accession Nos.: *Arabidopsis*, NP_175092; *Zea*, AAQ55066; *Nicotiana b*, ABC59516; and *Chlamydomonas*, CRC EST 925067A11.y1.

acting directly as a site of quenching or as an allosteric activator in a more complex process [28]. We replaced the glutamate and arginine residues in the NELFVGR domains of *Arabidopsis* psbS with valine (V) and leucine (L), respectively (Fig. 2A). In LHCII and CP29 these changes blocked Chl-binding and maintained charge compensation for protein folding [12,13]. Anti-psbS immunoblots of leaf membrane proteins from transgenic *Arabidopsis* lines exhibited a single band with mobility identical to the wild-type control (Fig. 2B), indicating no apparent differences in post-transcriptional processing or targeting of the mutant proteins. PsbS is consistently absent in parental line *Npq4-1* due to a *psbS*Δ allele (Fig. 2B) [4]. Nevertheless, two-way analysis of variance (ANOVA) indicated that leaf-to-leaf differences were a major source of variation in psbS expression level ($p < 0.001$ for leaf and (leaf × genotype)). Typical responses of Chl fluorescence yield to a brief light stress treatment are shown in Fig. 2C. The diminished decline in signal for the *psbS* mutant lines compared to wild-type is diagnostic for a loss in NPQ capacity. A 90% loss in NPQ was observed for *Npq4-R43L/E142V*, a level indistinguishable from that observed for the deletion line. The loss of NPQ capacity for *Npq4-E38V/R147L* was a somewhat less, though substantial, 70% (Fig. 2D). We attribute these changes to psbS structure since, within the limits of analytical error, mean psbS expression levels for the transgenic lines did not differ from that of wild-type.

Consistent with normal growth and appearance, ANOVA indicated no effect of psbS genotype on total Chl content ($p > 0.05$). Likewise, no effect was observed on intrinsic PSII quantum yield (average $\Phi_2 = 0.823 \pm 0.012$ (SE)). Mutational loss of psbS expression or function does not affect either the capacity or the ability to increase the transthylakoid ΔpH [18] for zeaxanthin accumulation in excess light [4,6]. We conclude that the glutamate/arginine motifs are fundamental to the mechanistic role of psbS in NPQ.

Since psbS reportedly binds zeaxanthin [29], evidence for binding of Chl *a* to E38 and E142 of psbS would buttress the concept that energy dissipation could occur solely within this complex. However, the question of whether native psbS binds Chl is controversial [28]. It is significant that a recently proposed three-dimensional model of psbS incorporates binding of two zeaxanthin molecules as part of an explanation of pH modulation of protein conformation [30]. Glutamates E38 and E142 were postulated to ligand to zeaxanthin in this structure. Hence, the loss of NPQ capacity in the site-directed mutants described here could result from inability to form a psbS–zeaxanthin complex. Certainly, losses in NPQ following replacement of glutamate/arginine pairs could result from altered protein conformation alone so that our results do not prove involvement of bound pigment in NPQ. However, normal and mutant forms of psbS all accumulated similarly in the leaf membrane fraction indicative of, at most, only subtle effects of residue replacements on conformation consistent with the previously established stability of this protein in the absence of pigments [31].

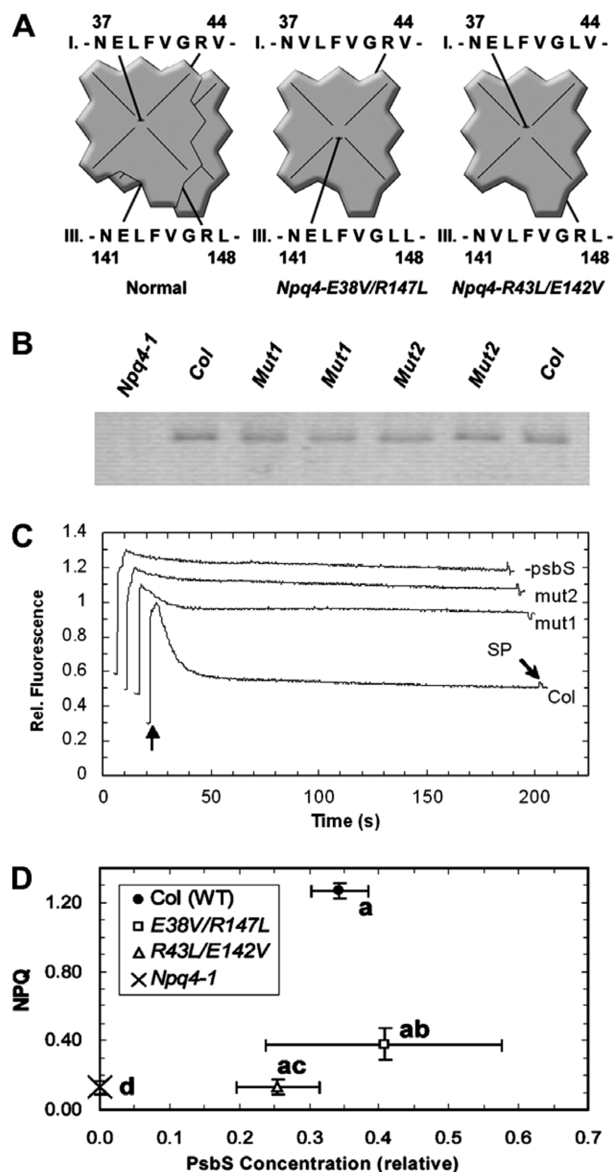

Fig. 2. Mutagenesis and characterization of pigment-binding motifs in *Arabidopsis* psbS. (A) Amino acid replacements to two putative Chl-binding domains in *Arabidopsis* psbS generate mutant *Npq4-E38V/R147L* (mut1) and *Npq4-R43L/E142V* (mut2). (B) Immunoblot of mutant proteins from duplicate leaf preparations with wildtype psbS. (C) Chl fluorescence transients (0.2-s intervals) for NPQ and Φ_2 in *Arabidopsis* leaves. Actinic illumination (↑) followed by a saturating pulse (SP) were introduced as indicated. Traces were displaced by five seconds along the abscissa scale and 0.1 units on the ordinate scale for clarity. "-psbS" refers to line *Npq4-1* (D) NPQ in relation to psbS level in normal and mutant leaves. The psbS and NPQ values were obtained from Western analyses and induction transients (B and C, respectively). Each mean is based on four to eight replicate leaves and bars indicate standard error. Letter designations refer to the results of a Tukey test that employed the error mean square from a two-way analysis of variance of psbS levels (genotype × leaf). Means sharing a common character are not significantly different ($p = 0.05$). Comparison of mean NPQ levels (not shown) indicated a highly significant ($p < 0.01$) difference between Col and each of the mutants.

Multiple psbS genes are present in some plant species

The range of available psbS sequences encompasses not only major taxonomic divisions (algae, mosses, gymnosperms, monocots, and eudicots), but also different photosynthetic biochemistry (C3 versus C4), ecology (wild versus cultivated, cold-tolerant), and morphology (herbaceous versus woody) (see Supplementary material). Significantly, phylogenetic relationships in Fig. 3 resemble the pattern of plant evolution (http://tolweb.org/tree/phylogeny.html) insofar as six major clades coincide with key taxa at the division and family level. The main deviation from this pattern is represented by an apparent early divergence of *psbS* leading to the present day grasses (Poaceae) that preceded the branching to gymnosperms (Pinophyta) and other flowering plants. The relatively short distance separating ancestral and contemporary *psbS* sequences of the Poaceae from that of the basal node suggest that negative selection was particularly stringent in this clade. A highly similar tree (see Supplementary material) was obtained using the NJ method [25].

A striking aspect of Fig. 3 is that among the 43 species shown 15 possessed multiple *psbS* genes (paralogs). Replicate psbS sequences within a species were very similar, except in *Oryza sativa* and *Pinus taeda*. The genomes of higher plants may have evolved through a series of genome

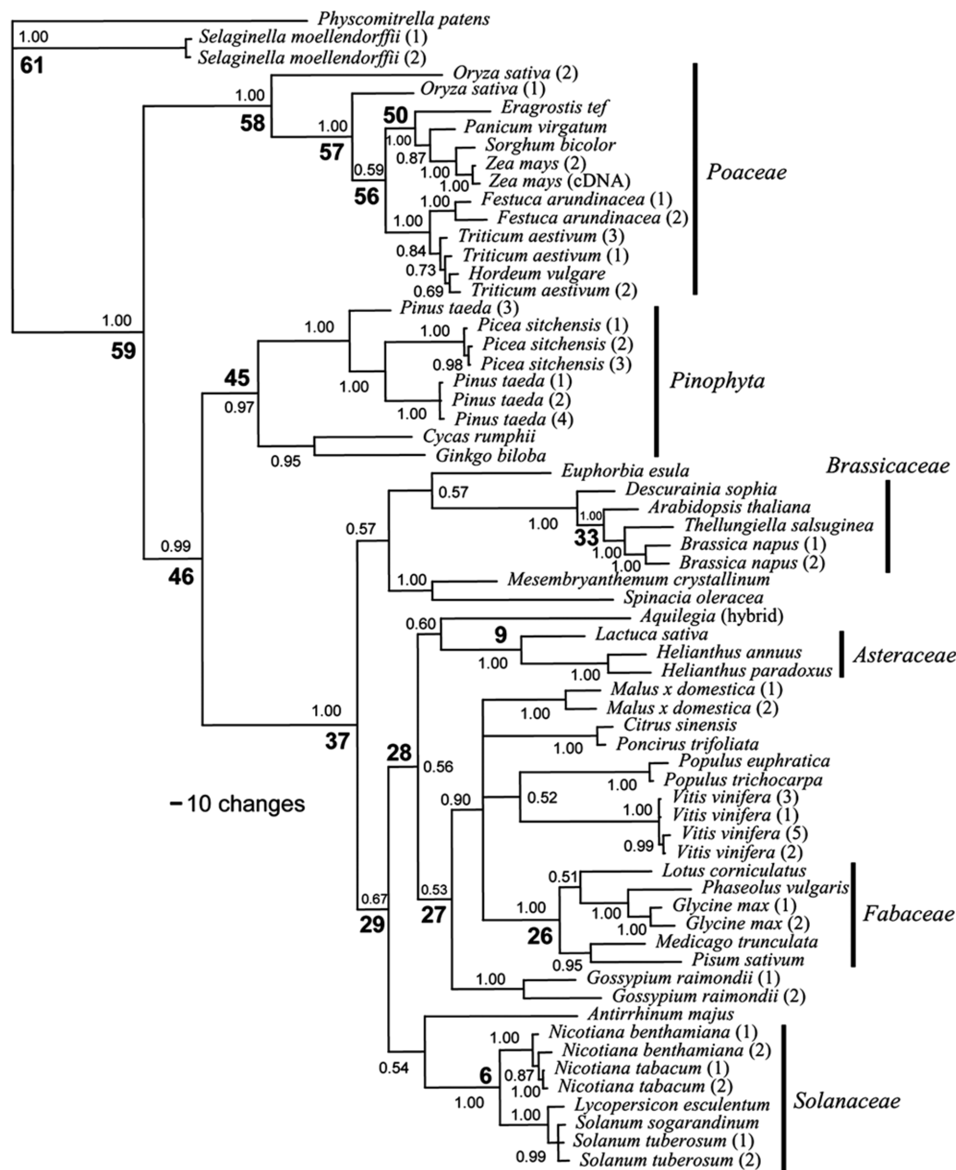

Fig. 3. Bayesian-derived phylogeny of 64 higher plant *psbS* sequences. The search for the best set of trees was based on a total of 125,000 generations and four Monte Carlo chains. A tree structure was saved each 100 generations and the consensus tree shown was based on the final 1000 structures. Designations in parentheses distinguish paralogous genes. The small (non-bold) nodal values refer to the probabilities of the respective clades. The large (bold) values identify nodes of particular relevance to this study as assigned by program CRANN. Note that major clades coincided with key taxonomic groups at the division (e.g. *Pinophyta*) and family level (heavy bars).

duplications, each followed by deletion and rearrangement of duplicated sequences during diploidization. The rate of loss for duplicated genes differs among species [32].

Sites of adaptive evolution are present in psbS from C4 grass species

The algorithm of Creevey and McInerney [16,17] provides a sensitive test for the presence of adaptive evolution. First, the neutrality test compares amino acid replacement (non-synonymous, R) versus silent (synonymous, S) nucleotide substitution rates summed over all lineages descending from each node of the tree (Fig. 3). Fig. 4 shows the distributions of replacement and silent substitutions versus codon position for the basal node (node 61). A conspicuous feature is an alternating pattern of replacement substitution frequency (Fig. 4A) that contrasts with the expected monotonous distribution of silent substitutions (Fig. 4B). The non-uniform distribution of replacement substitutions, which correlates with psbS topology (Fig. 4A), indicates that discrete domains in psbS are evolving differently. Second, the relative rate ratio test is used to detect adaptive (positive) evolution by classifying substitutions as invariant (I) or variable (V) depending on whether the associated codon change persists or changes in descendent lineages. Selective pressure to preserve an amino acid replacement substitution results in an invariant change (directional positive selection). Conversely, recurring pressure to change identity in response to environmental fluctuations leads to variable substitutions (non-directional positive selection). Since silent substitutions are not subject to selection, the SI/SV ratio serves as a reference. A significant difference (based on a *G*-test) in the RI/RV ratio relative to SI/SV indicates the presence of positive selection. The relative rate ratio test failed to detect significant positive evolution in any domain for the basal node. Statistical analysis provides support for the dominating influence of negative selection among *psbS* sequences from land plants (see Supplementary material C). However, positive directional selection was detected in seven major clades of the Bayesian phylogenetic tree (Fig. 3) across the six *psbS* domains examined (Table 1). All but one of the clades (Fabaceae) exhibiting a significant ratio test result occurred in the family Poaceae (grasses). We note that the same analyses based on the NJ tree confirmed that adaptive evolution occurred in the Poaceae (data not shown). Negative selection was strong in all clades of Table 1 except Domain 3 of node 50 (Fig. 3).

One characteristic of positive Darwinian selection is an association between a protein structure and a particular environment [33]. A comprehensive phylogeny of the Poaceae based on molecular markers and morphology [34] shows that a common ancestor is shared by the C3 subfamilies Ehrhartoideae (genus *Oryza*) and Pooideae (genera *Festuca*, *Hordeum*, and *Triticum*) and the C4 species-containing subfamilies Chloridoideae (genus *Eragrostis*) and Panicoideae (genera *Sorghum*, *Zea*, and *Panicum*)

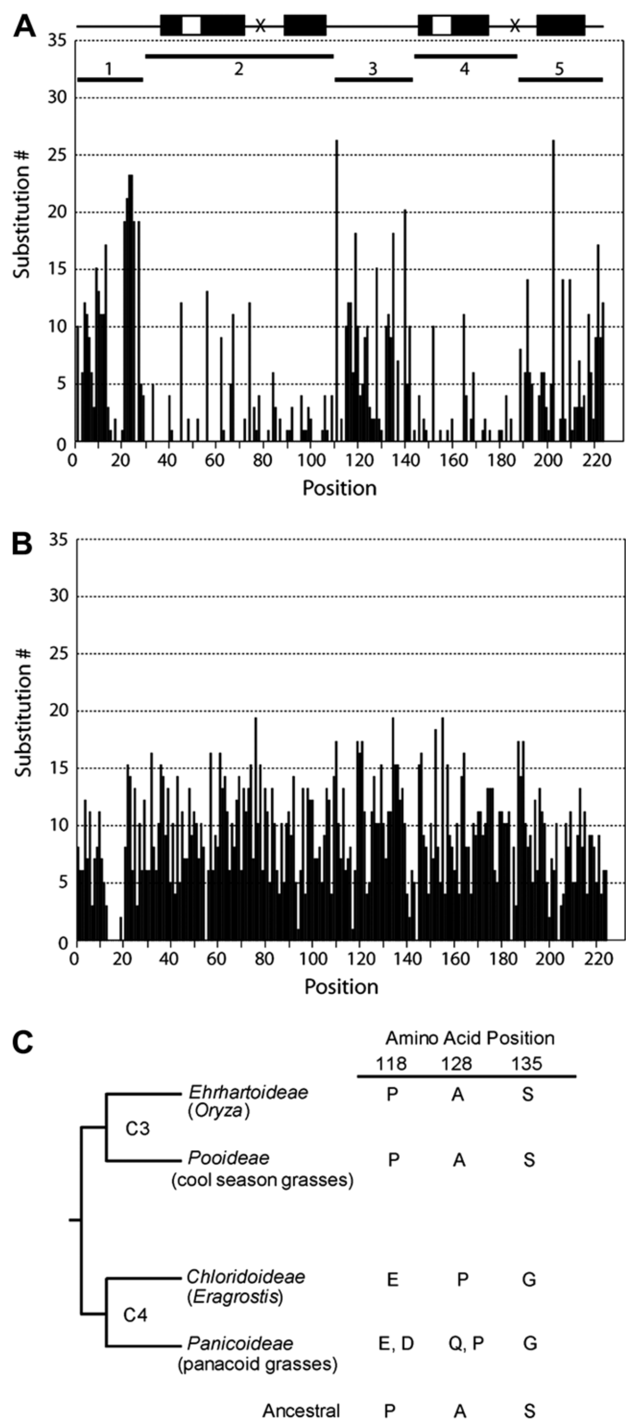

Fig. 4. Distribution and nature of base substitutions in *psbS* genes reported by CRANN. (A) shows the number of amino acid replacements and (B) the number of silent substitutions versus position in the psbS CLUSTAL alignment. PsbS topology domains include transmembrane helices (heavy black bars), putative Chl-binding domains (white bars), and H^+-sensing glutamates (×). (C) Adaptive evolution in psbS within the family Poaceae. The cladogram shows the relationships among four major subfamilies of C3 and C4 species used in this study. Also shown are the amino acid identities at three positions in Domain 3 where positive selection is likely to have occurred (Table 1). The RI/RV ratio was 3/1, 2/0, and 2/0 for positions 118, 128, and 135, respectively for node 57 (see Fig. 3). In instances where multiple amino acid identities occurred within a subfamily, the residues are listed in order of their prevalence. Also shown is the ancestral sequence as predicted by CRANN.

Table 1
Application of the Creevey–McInerney tests to taxon-specific clades

Node	50	50	56	57	57	58	26
Taxon	Poaceae	Poaceae	Poaceae	Poaceae	Poaceae	Poaceae	Fabaceae
Domain	Full	3	3	3	4	4	3
Neutrality test							
Observed							
R	50	12	19	20	6	8	29
S	78	9	21	28	30	43	31
Expected							
R	97.5	16.0	30.5	36.5	27.5	38.9	46.6
S	30.5	5.0	9.5	11.5	8.5	12.1	13.4
	**		**	**	**	**	**
Ratio test							
RI	22	8	9	9	3	3	10
RV	28	4	10	11	3	5	19
SI	21	1	3	4	3	3	4
SV	57	8	18	24	27	3	27
	*	*	*	*	*	*	*

Note. The node designation refers to the phylogenetic tree of Fig. 3 and the domains are defined in Fig. 4. Statistically significant instances of selection based on a *G*-test or Fisher's exact test are indicated by asterisks [*($p < 0.05$), **($p < 0.005$)]. See text for further explanation of terms.

(Fig. 4C). Although the phylogenies of *psbS* in the Poaceae differs slightly between Figs. 3 and 4C, a clear segregation among *Oryza*, the cool season grasses, and the C4 types is evident. We provide evidence that adaptive evolution in Domain 3 has contributed to the evolution of *psbS* in the grasses (Table 1). Marked changes in the type of amino acid occur at positions 118, 128, and 135, each of which exhibits an excess of RI relative to RV substitutions (Fig. 4C). Replacements involving proline (positions 118 and 128) alter α-helical structure and orientation of the polypeptide backbone. Likewise, substitutions involving glycine (position 135) change the flexibility of the chain [35].

Since the sequences for the C3 species do not differ from the predicted ancestral sequence for node 57 (Fig. 3) we must consider the possibility that the changes in amino acid identity of Fig. 4C are associated with acquisition of the C4 syndrome. Specifically, these replacements in psbS may augment the well-known tolerance of C4 species to environmental stress (e.g. improved water use efficiency) by enhancing safe dissipation of excess illumination. The C4 photosynthetic mechanism had multiple origins during evolution of the family Poaceae and its repeated occurrence extends into tribes within the subfamily Panicoideae [34,36]. Thus, several potential opportunities exist to test the correlation between carbon fixation biochemistry and psbS structure among species more closely related than those examined here.

Finally, adaptive changes may result from direct selection or be correlated to evolutionary changes in other proteins involved in function. Candidate "partner" proteins for psbS include the major PSII light-harvesting Chl-proteins (LHCII, CP24, CP26, and CP29) that share homology with psbS [10,37] and serve as energy donors to a presumptive psbS–zeaxanthin quenching complex.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.01.173.

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