

Expression and functional analysis of the *PmPsbS* gene reveals its role in the regulatory mechanism of non-photochemical quenching (NPQ) in heteroblastic foliage of *Pinus massoniana* seedlings

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Abstract

To prevent severe oxidation damage to photosystems, excess absorbed light energy is dissipated as heat through a process known as non-photochemical quenching (NPQ). Heteroblastic foliage is observed during the first growth season of *Pinus massoniana*, giving rise to primary needle seedling (PNS) and secondary needle seedling (SNS). Notably, there are distinct differences in NPQ between PNS and SNS. However, the molecular regulatory mechanism of NPQ in the heteroblastic foliage of *P. massoniana* remains unclear. Here, *PmPsbS* was investigated and functionally analysed. The *PmPsbS* protein contains two conserved domains and four transmembrane regions and is primarily located in the thylakoid membrane of chloroplasts. The core region of the *PmPsbS* promoter is located between -852 and -330 bp, including predicted binding sites for light-responsive elements, G-box elements, and MYB transcription factors. Moreover, we predicted that *P. massoniana* would contain light-responsive MYB gene family members, encoding proteins that may bind to specific sites within the *PmPsbS* gene promoter to regulate its expression. Different photoprotective and light energy partitioning mechanisms in response to seasonal variation in the primary and secondary needles. However, under shade stress conditions alone, SNS was more susceptible to light inhibition, and the expression mechanism of *PmPsbS* differed between *P. massoniana* seedlings with heteroblastic foliage. These results indicated that *PmPsbS* can circumvent photoinhibition and enhance photoprotection, and plays an important role in the adaptation of the heteroblastic foliage of *P. massoniana* to light environments.

Keywords: *Pinus massoniana*; *PmPsbS* gene; GUS activity; Expression pattern; NPQ

Introduction

Photosynthesis is the process that uses light energy to assimilate carbon dioxide into carbohydrates, provid

ing almost all living organisms on Earth with the necessary substances and energy to survive (Monteith 1972; Blankenship 2021). Plants can maximize the absorption of light energy by combining high-density chlorophylls (Chls) and the special light-harvesting complexes (LHC) of carotenoids (Nicol and Croce 2021). However, excessive absorption of light energy under high-intensity light can lead to the formation of reactive oxygen species (ROS) and photodamage to cells (Correa-Galvis et al. 2016; Muller et al. 2001; Goss and Lepetit 2015; Ruban 2016). To prevent serious oxidative damage to photosystems, plants activate a photoprotective mechanism that dissipates excess harmful light energy as heat, known as non-photochemical quenching (NPQ) (Muller et al. 2001; Da et al. 2018; Ruban 2016; Goss and Lepetit 2015). NPQ is the sum of at least three different mechanisms, i.e., qE (energy quenching), qT (state transition) and qI (photoinhibitory quenching), which are defined on the basis of their relaxation kinetics (Muller et al. 2001). Additionally, qE-type energy dissipation occurs quickly, is rapidly reversible in the dark and is induced by a proton gradient across the thylakoid membrane (ΔpH) caused by excess light (Horton and Ruban 1992; Muller et al. 2001). High ΔpH protonates the protein PsbS and activates violaxanthin de-epoxidase (VDE), which converts violaxanthin (V) to zeaxanthin (Z) in the xanthophyll cycle (VAZ), thereby activating NPQ, inducing a conformational change in the main antenna complex (LHCII) of photosystem II (PSII) and switching its functional mode from light harvesting to heat dissipation (Goss and Lepetit 2015; Ruban 2016).

Research has shown that the PsbS protein enhances the dynamics of thylakoid membranes by reducing the ordering and increasing the fluidity of protein organization, which accelerates the reorganization and aggregation of LHCII (Kiss et al. 2008; Goral et al. 2012). Recent research results showed that PsbS interferes with the formation of densely packed aggregates of thylakoid membrane proteins, thus allowing easy exchange and incorporation of xanthophyll cycle pigments. The structures formed in the presence of zeaxanthin show enhanced excitation quenching, thus protecting the system against photodamage (Welch et al. 2021). Over the last 20 years research has confirmed that the PSII protein PsbS plays an important role in non-photochemical quenching (NPQ) (Fan et al. 2015). In *Nicotiana tabacum* and *Chlamydomonas*, NPQ is controlled by the complex dynamics of PsbS and zeaxanthin, and PsbS can respond rapidly to changes in light intensity in NPQ induction (Ruban 2017; Tibiletti et al. 2016). The mutant with knockdown of the *PsbS* gene in *Oryza sativa* (*npq4* mutant) is more easily photoinhibited under high light than the wild type, and PsbS can prevent PSII from producing superoxide radicals (Zulfugarov et al. 2014). In *Arabidopsis thaliana*, the *npq4* mutant has been confirmed to have defects in the fast response of NPQ (qE) and to produce more singlet oxygen ($^1O_2$) than *oePsbS* (overexpressed *PsbS* mutant) (Dall'Osto et al. 2014). Most of the current research focuses on the role of PsbS

in NPQ under high light intensity, but NPQ is activated not only under high light but also during weak light-induced photosynthesis in plants (Wolf et al. 2020; Zhang et al. 2011). Powles (1984) reported that low light intensities increase the photosynthetic carbon fixation of plants and lead to saturation above a certain threshold, so plants under low light are incapable of using all of the absorbed light energy and are also subject to excess light absorption (Powles 1984). Acidification of the thylakoid lumen under low irradiance contributes to the elevated stable qE (Ilik et al. 2010; Da et al. 2018; Wolf et al. 2020). Under weak light conditions, the m-type thioredoxin TRX-ms, which interacts with the xanthophyll cycle enzyme zeaxanthin epoxidase (ZE) in *Arabidopsis*, participates in regulating NPQ (Da et al. 2018). PsbS-inhibited rice produces a higher quantum yield of electron transport during the transition from high light to low light than wild-type rice, indicating that PsbS can regulate NPQ under low light conditions (Ikeuchi et al. 2014). In recent years, researchers have examined the downregulation of photosynthesis by evergreen conifers in autumn and winter, in which the dynamic xanthophyll cycle-mediated non-photochemical quenching (NPQ) is replaced by sustained NPQ (Fréchet et al. 2016; Porcar-Castell 2011). In evergreen conifers, such as Norway spruce, in addition to being capable of dynamic regulatory NPQ, sustained NPQ develops during winter provided that the temperature drops well below 0°C (Grebe et al. 2020). Conifers still use the NPQ mechanism under low light.

Pine seedlings have two kinds of leaves, primary and secondary needles, and exhibit heteroblasty during seedling development (Climent et al. 2011). Particularly large trait alterations occur upon the juvenile to adult transition, a change called heteroblasty (Zotz et al. 2011). *Juniperus flaccida* and *Pinus cembroides* are conifers of independent lineages known to exhibit leaf heteroblasty between the juvenile and adult life stages of development (Webster et al. 2022). Needle photosynthetic potentials differ strongly between primary and secondary needles (heteroblasty) in *Pinus* species (Kuusk et al. 2018a). Primary needles have a smaller cross-sectional area and lower leaf dry mass per unit area (Pardos et al. 2009; Kuusk et al. 2018b) but greater photosynthetic capacity (Wang et al. 2022), ultimately leading to a shorter needle payback time (Day and Greenwood 2011). The factors responsible for the greater assimilation potential of juvenile needles are poorly known. The main advantage of juvenile needles is more effective photosynthesis, which provides young seedlings with the necessary carbon building blocks for establishing extensive root systems and later for the development of adult needles (Kuusk et al. 2018a). Heteroblastic leaves play an adaptive role in *Pinus nigra*, *Pinus pinaster* and *Pinus brutia* by responding to complex environments (Climent et al. 2009). The juvenile needles of *Pinus halepensis* have greater efficiency of light interception due to greater exposed surface area for a given total needle surface

ce area, which likely also contributes to plant recruitment under the canopy of adult trees (Kuusk et al. 2018 b). The heteroblasty of coniferous leaves alters the photosynthetic capacity of plants.

Pinus massoniana Lam. is a major reforestation tree species in southern China and is widely distributed in tropical and subtropical monsoon climate areas (Yang et al. 2015). In our preliminary research findings, we observed both primary needle seedling (PNS) and secondary needle seedling (SNS) in the first growing season of *P. massoniana* (Wang et al. 2022; Wang et al. 2019). The morphological and photosynthesis-related physiological functions of heteroblastic foliage in *P. massoniana* differ significantly (Wang et al. 2022). Moreover, in a natural environment, insufficient sunlight caused by shade from broad-leaved tree species leads to the death of *P. massoniana* (Song et al. 2022). According to Siewiesiuk (2002), low light induces photodamage to photosystem (PS) II, possibly because the intermediate states of the water-oxidizing complex are reduced to lower degrees of oxidation at low light intensities (Siewiesiuk 2002). Low light stress restricts photosynthesis by inhibiting photosynthetic efficiency, promotes the production of reactive oxygen species (ROS), and causes oxidative damage (Suzuki et al. 2012; Zhang et al. 2011). High NPQ values alleviate photoinhibition by increasing the capacity of heat dissipation channels and reducing the excitation pressure of PSII reaction centres (Lu et al. 2019). However, the role of *PsbS* in NPQ in *P. massoniana* is unclear. Additionally, studies of the photosynthetic traits of these two leaf types in *P. massoniana* seedlings are lacking. Therefore, this study used heteroblastic foliage seedlings of *P. massoniana*, PNS and SNS to analyse the function of *PsbS* in *P. massoniana*. The gene structure, phylogeny, and expression pattern of *PmPsbS* and the *PmPsbS* promoter were further studied. The cis-acting elements and transcription binding sites of the *PmPsbS* promoter in *P. massoniana* were predicted, and promoter activity and subcellular localization were analysed through fusion expression. The expression patterns of *PmPsbS* in different tissues and under different shading conditions were determined.

Material and methods

Study site and plant materials

The seeds originated from seed orchards of *P. massoniana* on the Duyun Ma'anshan State-owned Forest Farm (26°90'N, 107°180'E, 920 m elevation) in China. Its were disinfected and germinated before being planted in nursery pots with perlite as the medium. The artificial climate incubation box was set to parameters of 25°C/20°C (day/night), 65% relative humidity, and 12 hours of illumination (560 $\mu\text{molm}^{-2}\cdot\text{s}^{-1}$). After four weeks, approximately 1000 young seedlings were transplanted into nonwoven planting bags and placed in an outdoor seedbed to ensure uniform environmental conditions. The experiment was designed as follows: (1) In mid-August, PNS and SNS (approximately 60 each) were randomly selected for testing,

with the experimental method referring to Wang et al. (2022). (2) Four different light intensity gradients were set using shade net: natural light intensity (100% relative irradiance, 100% RI), 30% shading (70% RI), 50% shading (50% RI), and 80% shading (20% RI). Two weeks later, SNS and PNS were randomly selected from each treatment. The foliage samples from PNS and SNS, three biological replicates for each treatment, were frozen immediately with liquid nitrogen, and stored in a -80°C freezer.

Cloning of *PmPsbS* gene

The differential expression of the *PmPsbS* gene during the PNS and SNS processes was explored in our previous work (on publishing). Based on PacBio sequencing platform data from our previous research, cloning primers (Table 1) were designed for the complete CDS region of the gene using Primer Premier 5.0 software. First-strand cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo), followed by PCR amplification. The reaction mixture (50 µL) consisted of Prime STAR Max (25 µL), forwards and reverse primers (1 µL each, 10 µmol·L⁻¹), template cDNA (2 µL), and ddH₂O (21 µL). The amplification conditions were as follows: 98 °C for 10 sec, 55 °C for 15 sec, and 72°C for 10 sec, with a total of 35 cycles. The amplified products were purified using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa), and the purified target DNA fragments were subjected to 3' end A-tailing and then ligated to the pMDTM 19-T vector. The ligated vector was then transformed into *Escherichia coli* DH5α competent cells. Single clones obtained were subjected to bacterial liquid PCR detection, and positive clones were selected for sequencing validation by Shanghai Bioengineering Co. For full-length cloning of the *PmPsbS* gene, DNA from seedlings of *P. massoniana* was used as the template. And the method was the same as above.

Table 1 Primers for cloning of *PmPsbS* gene

Primer name	Primer sequence (5'→3')	Purpose
Pmpsbs-F1	ATGGCGCAGTCGGTCTCCCAT	CDS/full-length cloning
Pmpsbs-R1	TTATTCTTCTTCATCAACGTCGGTGA	
Pmpsbs-F2	GATAAGCGTGCTAAAGGGCAAGCGGG	Promoter cloning
PmPsbS-R2	CGTTTGGCAGCCGAATCTTGAGGC	
qPmpsbs-F	TCTGCTGCTCTTCTTTATT	Real-time fluorescence quantitative PCR (qPCR)
qPmpsbs-R	ATTCTTTCCAGGGTCTATC	
UBC-F	GATTATTTCATTGGCAGGC	Reference gene for qPCR
UBC-R	AGGATCATCAGGATTTGGGT	

G-F	CGGGATCCGATAAGCGTGCTAAAGGGCAAG	Promoter cloning
q1-F	CGGGATCCTGATGGTATCTATTTGGGAAGG	Missing fragment 1 cloning
q2-F	CGGGATCCTGAACTATGGGCCAACAGTA	Missing fragment 2 cloning
q3-F	CGGGATCCGCTCAGTTAAGGACTAATGCTC	Missing fragment 3 cloning
q4-4	CGGGATCCTCGCACAGATTACCATTGAT	Missing fragment 4 cloning
G-R	CCCATGGCGTCGTGTTAGCTGAATACACAG	Express vector reverse primers

Cloning of *PmPsbS* promoter sequence

Using the published *Pinus tabulaeformis* genome (<https://db.cngb.org/>) as a reference, the CDS of the *PmPsbS* gene was compared with the *P. tabulaeformis* genome to find the sequence that matches *PmPsbS*, and the upstream and downstream primers were designed based on the promoter sequence of *P. tabulaeformis* (Table 1). The promoter sequence of *PmPsbS* was amplified with a high-fidelity enzyme (Prime STAR Max), using DNA from seedlings as a template. The recovered target DNA fragments were ligated with the pMD™ 19-T vector and then transformed into *Escherichia coli* DH5α receptor cells. The positive clones were sequenced by Shanghai Bioengineering Co. The websites PlantCARE (<http://bioinformatics.psb.ugent.be/>) and PLACE (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>) were used to predict the cis-acting elements in the promoter sequence of the *PmPsbS* gene. Plant transcription factor-binding site sequence MEME files were downloaded using the JSPAR website (<https://jaspar.genereg.net/>), and transcription factor-binding site predictions on promoter sequences were analysed at the FIMO website(<https://meme-suite.org/>).

Physical and chemical properties of *PmPsbS* protein

The online software ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) was used to analyse the inferred amino acid sequence of the protein encoded by the gene. The online software ExPASy (<http://www.expasy.org/>) was used to analyse the amino acid number, theoretical isoelectric point, Molecular weight, number of aliphatic amino acids and hydrophilicity/hydrophobicity. Protein phosphorylation site prediction using NetPhos 3.1 Server (<http://www.cbs.dtu.dk/services/NetPhos/>). Secondary and tertiary structures using SOPMA and SWISS-MODEL (<https://swissmodel.expasy.org/>) for prediction analysis. Protein transmembrane structures were predicted by TMHMM (<https://dtu.biolib.com/app/DeepTMHMM/run>). Protein interaction analysis was performed using STRING software(<https://cn.string-db.org/>).

Evolutionary relationship and conserved domain of *PmPsbS* protein

The PsbS amino acid sequences of *Pinus lambertiana*, *Pinus taeda*, *Pseudotsuga menziesii*, *Populus trichocarpa*, *Zostera marina*, and *Physcomitrella patens* were obtained from the PlantGenIE database (<https://plantgenie.org/>). While the PsbS amino acid sequence of *Picea sitchensis* was obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/>). Functional validated PsbS protein sequences of *Arabidopsis thaliana*, *Solanum lycopersicum*, *Nicotiana tabacum*, *Spinacia oleracea*, *Physcomitrella patens*, *Oryza sativa subsp. japonica*, and *Chlamydomonas reinhardtii* were downloaded from the UniProt database (<https://www.uniprot.org/>). Then all the PsbS protein sequences were aligned using DNAMAN software to perform multiple comparisons, and a systematic phylogenetic tree was constructed using the ML method in MEGA6 software. The conserved structural domains of the proteins were predicted using NCBI's CD-Search (<https://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi>), and the conserved motifs were analysed by MEME-suite (<http://meme-suite.org/tools/meme>). Finally, evolutionary trees, conserved structural domains and conserved motifs were mapped using TBtools.

Construction of promoter expression vector

Based on the function and position of some cis-acting elements predicted by PlantCARE, 5'-end deletion cloning was performed on the basis of a 2 123 bp promoter fragment. Four pairs of primers were designed for amplification (Table 1). The plant expression vector pCAMBIA1304 was used as the base vector, with *GUS* reporter gene, to construct the plant expression vector. *Bam*HI, *Nco*I and *Bgl* II restriction endonucleases were used according to the polyclonal sites on the vector plasmid map. After the expression vectors were cleaved by the corresponding restriction enzymes, they were ligated overnight at 16°C using T4 DNA ligase. The recombinant plasmids were transformed into *E. coli* DH5 α receptor cells and sequenced after double digestion verification.

Transient expression of the promoter fragment of *PmPsbS*

The correctly sequenced recombinant plasmids were transformed into *Agrobacterium* GV3101 using liquid nitrogen freeze-thaw method and then injected into *N. tabacum* for transient expression. The injected *N. tabacum* plants were subjected to 24 hours of dark incubation without watering. After 24 hours of dark cultivation, different light intensity cultivations were performed: 100% RI, 70% RI, 50% RI, 20% RI. After being cultured for 60-72 hours, processed leaves were collected and subjected to GUS staining, and the method used was in reference to Lee and Yang (2006). At the same time, GUS activity in *N. tabacum* leaves was detected and the protein concentration was determined according to Kielkopf et al. (2020), while GUS activity detection was performed according to Norkunas et al. (2018). The results were expressed as the ratio of 4-methylumbelliferone (4-MU) produced to total protein content and time, represented as $\mu\text{mol (4-MU)} \cdot \text{g}^{-1} (\text{protein}) \cdot \text{min}^{-1}$, with 3 biological replicates used for each treatment.

Subcellular localization of PmPsbS

The online software ProtComp 9.0 (<http://linux1.softberry.com>) was used to predict the subcellular localization of PmPsbS. Experimental validation was performed by inserting the coding sequence of the cloned *PmPsbS* gene into the green fluorescent protein vector pBWA(V)HS-Glosgfp, thus constructing the fusion expression vector pBWA (V)HS-*PmPsbS*-Glosgfp containing the target gene (Liu et al. 2021). The vector pBWA-(V)HS-Glosgfp without any target gene was used as a control. The pBWA(V)HS-*PmPsbS*-Glosgfp fusion expression vector was transformed into *Arabidopsis thaliana* protoplasts using PEG-mediated transformation. After incubation in the dark at 28°C for 18-24 hours, the samples were mounted on slides and observed under a laser confocal microscope (Nikon C2-ER).

Expression of *PmPsbS* gene

According to the CDS of the *PmPsbS* gene, real-time fluorescent quantitative PCR primers were designed using Primer Premier 5.0 software (Table 1), and the expression levels of the *PmPsbS* gene in different tissues and under different treatments were examined. We performed detection using Bio-Rad's iTaq™ Universal SYBR® Green Supermix fluorescent dye and the CFX 96-well fluorescence quantitative PCR instrument. The reaction system consisted of 5 µL of Green Supermix, 0.25 µL of upstream and downstream primers (10 µmol·L⁻¹), 1 µL of template cDNA, and 3.5 µL of ddH₂O. The reaction program was as follows: predenaturation at 95°C for 30 s, denaturation at 95°C for 10 s, and annealing and extension at 61°C for 30 s for 40 cycles. The internal reference gene used was the *UBC* gene from *P. massoniana*. Each sample was run in triplicate, and the relative expression levels were calculated using the 2^{-ΔΔCt} method. The data were analysed and plotted using SSPS 24 and Origin 2019 software.

Results

Gene structure and Cis-acting elements in promoter of *PmPsbS*

The CDS of *PmPsbS* gene was cloned and found to be 882 bp (Fig. 1a). And the full-length of the *PmPsbS* DNA sequence was 1606 bp (Fig. 1b). Alignment of the CDS and full-length sequences (Fig. 1d) revealed that the *PmPsbS* gene consisted of two introns and three exons.

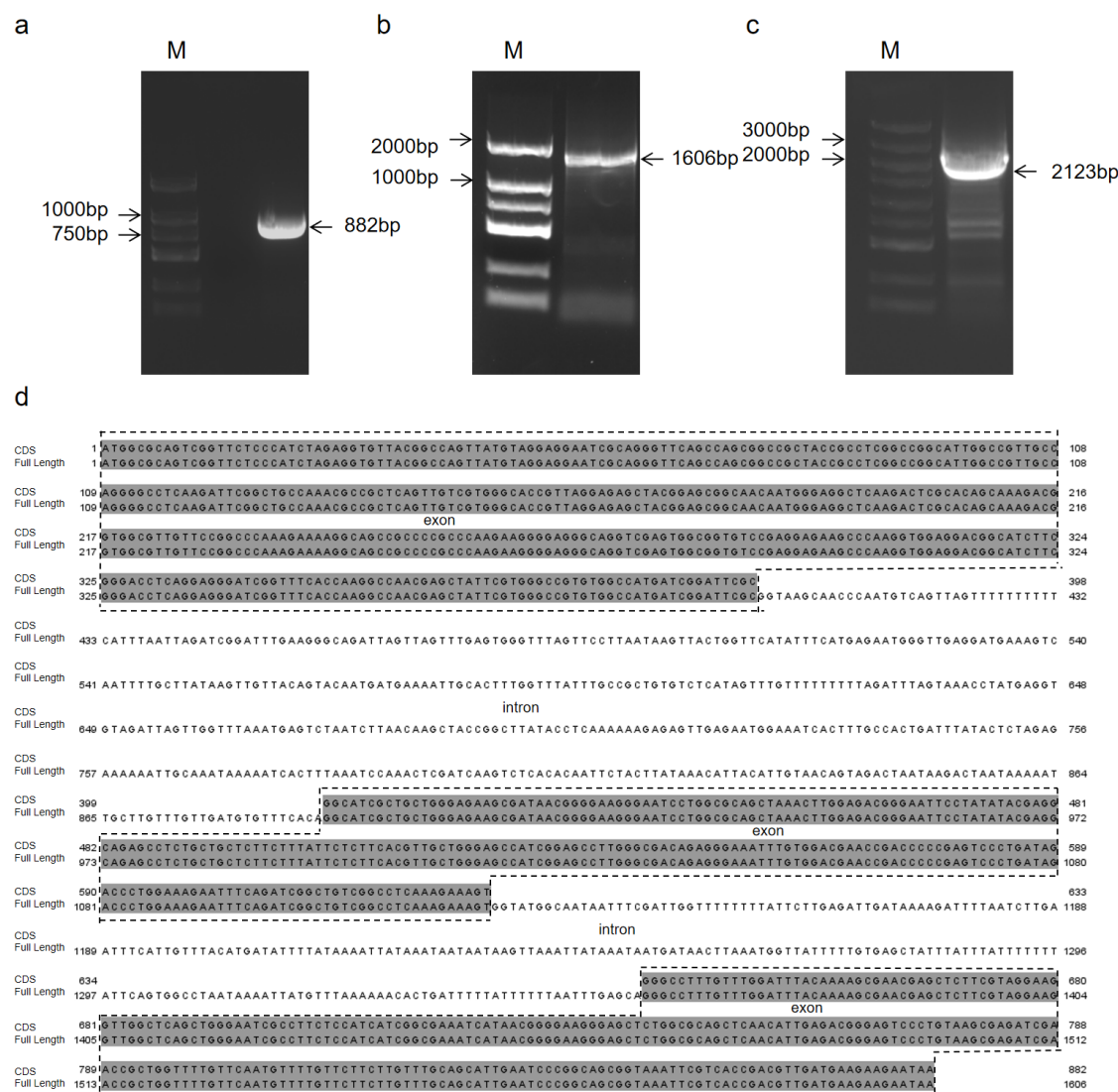


Fig. 1 Cloning and structure analysis of *PmPsbS*. **a.** Amplification of CDS region. M: DNA Marker. **b.** Amplification of full-length. **c.** Amplification of promoter. **d.** Comparison of CDS and DNA full-length sequences.

We obtained the *PmPsbS* promoter sequence with a length of 2123 bp (Fig. 1c). The cis-acting elements in the *PmPsbS* gene promoter were analysed with PlantCARE online software. We found that in addition to 33 TATA-box elements and 32 CAAT-box elements (Table 2 and Table S1), the promoter segment of the *PmPsbS* gene contains multiple light-responsive elements (A-box, G-box, GT1-motif, Box 4, and P-box), multiple hormone-responsive elements (AAGAA-motif, GARE-motif, P-box, and ERE), multiple transcription factor-binding sites (MYB and MYC), elements related to plant growth and development regulation (CCGTCC-box, CCGTCC-motif, Myb-binding site, and GCN4-motif). As well as elements related to abiotic stress (ARE and LTR), and elements involved in stress response (STRE). Using PLACE, we further predicted that the *PmPsbS* promoter not only contains the aforementioned cis-acting elements but also various tissue-specific cis-acting elements related to flowers, seeds, roots, embryos, and leaf flesh (Table 3).

Table 2 Partial cis-acting elements of *PmPsbS* gene promoter predicted by PlantCARE

element	position	sequence	function
A-box	-95; -174	CCGTCC	Light response element
G-box	-2	CACGAC	Light response element
GT1-motif	-1200; -1757	GGTTAA	Light response element
Box 4	-273	ATTAAT	Light response element
P-box	-599	CAACAAACCC CTT	Light response element
AAGAA-motif	-771; -947	GAAAGAA	Abscisic acid response element
GARE-motif	-818	TCTGTTG	Gibberellin response element
P-box	-2008	CCTTTTG	Gibberellin response element
TATC-box	-966; -651	TATCCCA	Gibberellin response element
ERE	-505	ATTTTAAA	Ethylene response element
ARE	-374; -1368, etc.	AAACCA	Anaerobic inducible element
MYB	-1301; -122, etc.	CAACAG/TAAC CA	MYB transcription factor
MBS	-663	CAACTG	Drought response element
MYC	-250; -1167, etc.	CATTTG/CAAT TG	MYC transcription factor
STRE	-606; -634, etc.	AGGGG	Stress response element
W-box	-1822	TTGACC	Stress response element
CCGTCC-box	-95; -174	CCGTCC	Meristem specific element
CCGTCC-motif	-95; -174	CCGTCC	Meristem expresses associated elements
Myb-binding site	-1300; -122, etc.	CAACAG	Growth and development regulatory element
GCN4-motif	-1152	TGAGTCA	Growth and development regulatory element
LTR	-898	CCGAAA	Involvement of low temperature homeopathic elements
TATA-box	-67; -270, etc.	TATA/ATTATA	Core element
CAAT-box	-239; -1169, etc.	CAAT/ CCAAT	Core element

Table 3 Tissue-specific expression of cis-acting elements of *PmPsbS* gene promoter predicted by PLACE

element	sequence	function	quantity
GTGANTG10	GTGA	Pollen specific expression	10
POLLEN1LELAT52	AGAAA	Pollen specific expression	12
RAV1AAT	CAACA	Flower specific expression	9
RYREPEATBNNAPA	CATGCA	Seed specific expression	1
CACTFTPPCA1	YACT	Mesophyll specific expression	16
OSE2ROOTNODULE	CTCTT	Root specific expression	6
ROOTMOTIFTAPOX1	ATATT	Root specific expression	7
NODCON2GM	CTCTT	Nodule specific gene	6
SITEIIATCYTC	TGGGCY	Anther specific gene	2
GCN4OSGLUB1	TGAGTCA	Endosperm specific expression	1

Transcription factor binding sites (TFbs) in the promoter region of the *PmPsbS* gene were examined using FIMO (Fig. 2). The database generated 293 TFbs (Table S2) from input searches, such as GARP, HSF, bZIP, C2H2, DOF, RAV, MYB, CPP, NAC, MIKC_MADS, and WRKY. These transcription factors are involved in regulating growth and development, light-responsive elements, as well as biological and abiotic regulation.

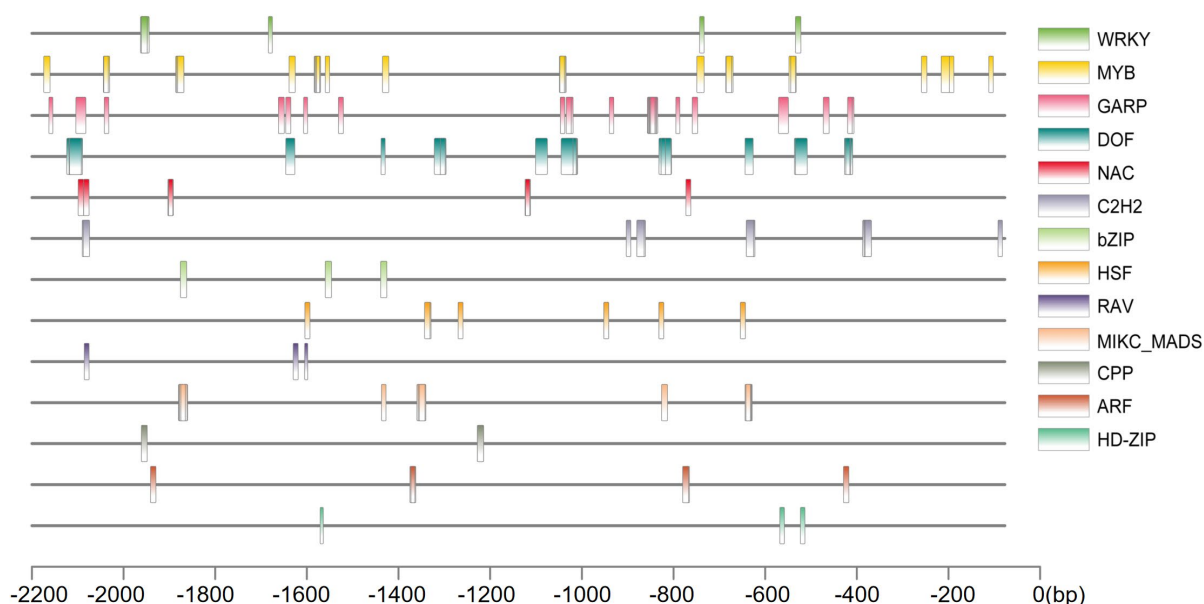


Fig. 2 Distribution of transcription factor binding sites (TFbs) in *PmPsbS* promoter regions.

Physicochemical properties and structure of PmPsbS protein

The PmPsbS protein was analysed using the online software ExPASy. It was made up of 293 amino acids with a relative molecular weight of 30797.83Da. The protein has a theoretical isoelectric point of 9.42 and a chemical formula of $C_{1409}H_{2262}N_{372}O_{395}S_2$, with a total of 4440 atoms. The PmPsbS was predicted to be stable with an instability index of 27.94 and a fatness index of 103.28. Using the online tool ProtScale, the average hydrophobicity index of the protein was predicted to be 0.24910, indicating that it was a hydrophilic protein (Fig. 3a). NetPhos 3.1 Server was used to predict phosphorylation sites, which revealed the presence of 8 serine phosphorylation sites, 9 threonine phosphorylation sites, and 1 tyrosine phosphorylation site within the PmPsbS (Fig. 3b).

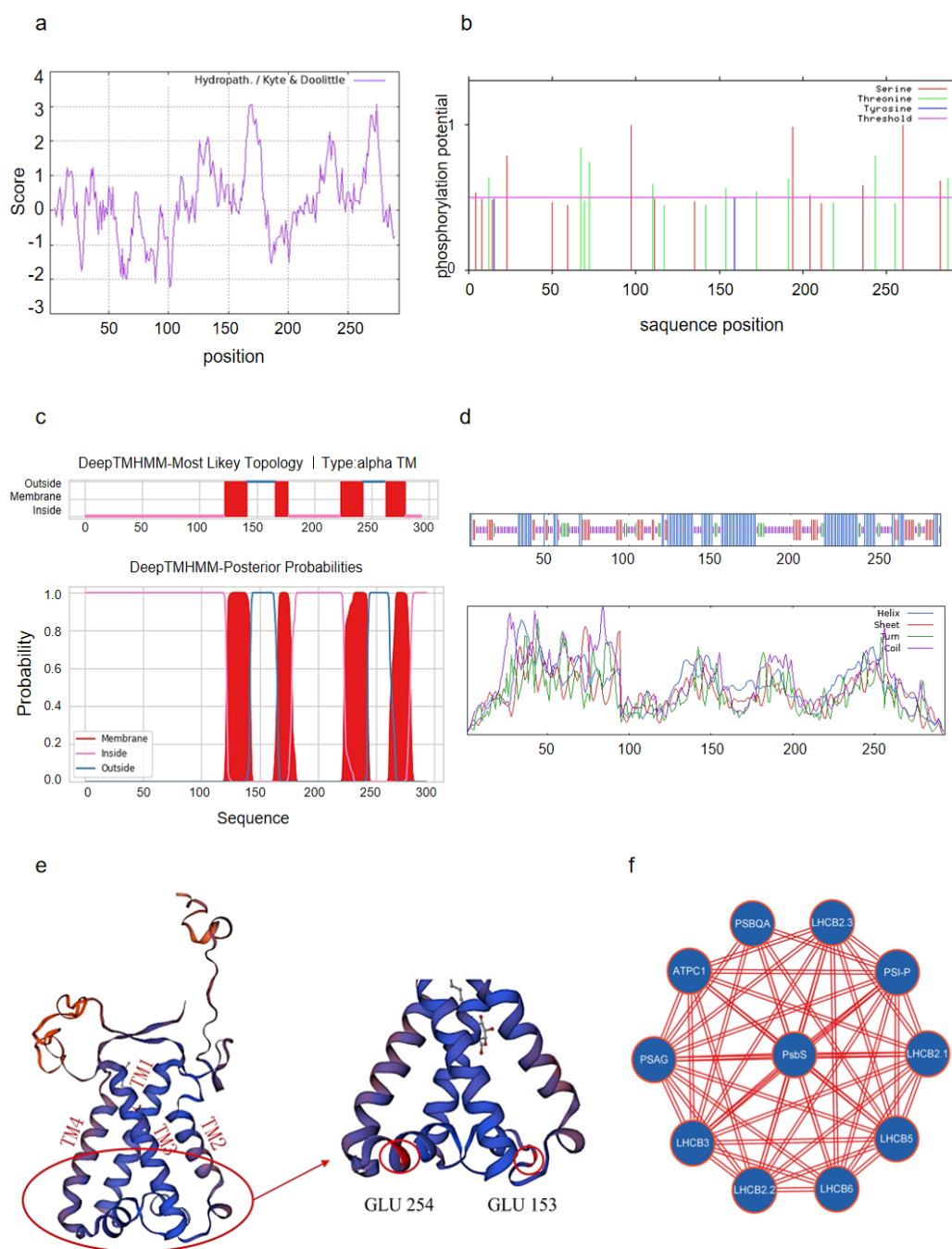


Fig. 3 Prediction of physical and chemical properties of PmPsbS protein. **a.** Hydrophilic and hydrophobic. **b.** Phosphorylation sites. **c.** Transmembrane structure. **d.** Direct view of secondary structure. **e.** Tertiary structure. **f.** Interaction protein.

Additionally, using TMHMM (Server-v2.0) online tool, the presence of 4 transmembrane regions in the PmPsbS protein was confirmed (Fig. 3c). The protein structure was analysed using the NCBI-CDD online tool, which revealed that the amino acid residues at positions 118-162 form a typical LHC protein structure (Fig. 5c). Furthermore, residues at positions 219-279 make up the LHC chlorophyll A-B binding protein complex. The secondary structure of the PmPsbS protein was predicted using the SOPMA online tool (Fig. 3d), which showed that α -helices (Hh) account for 34.81%, extended strands (Ee) account for 17.6%, beta turns (Tt) account for 8.53%, and random coils (Cc) account for 39.59%

of the protein. Finally, using the SWISS-MODEL online tool and X-ray crystallography, the tertiary structure of the protein was predicted with a high degree of similarity to the crystal structure of the *Spinacia oleracea* photoprotective protein PsbS (Fig. 3e). Using *Arabidopsis thaliana* PsbS as a reference, we found that PsbS interacts with ATPC1, LHCB2.1, LHCB2.2, LHCB2.3, LHCB3, LHCB5, LHCB6, PSAG, PSBQA, and PSI-P (Fig. 3f), with interaction coefficients greater than 0.9.

Homologous of PmPsbS protein

According to the multiple sequence alignment of the PsbS protein sequences (Fig. 4), the similarity coefficient between PmPsbS and PITA_32163 and PITA_32164 of *P. tabuliformis* was as high as 98.41%. The full chlorophyll a/b binding domain was present in PmPsbS, with several highly conserved regions. In *Arabidopsis*, the two glutamic acid residues Glu-122 and Glu-226 are essential for PsbS function (Mou et al. 2013). Multiple alignment analysis showed that the Glu-153 and Glu-254 residues in PmPsbS were conserved, indicating that the amino acid residues of PsbS proteins in coniferous trees play an important role in the light-harvesting complex. They are more conserved than those in angiosperms, and the receptor function in the NPQ mechanism may be intact.

An ML phylogenetic tree was constructed based on amino acid sequences (Fig. 5a). The results showed that the evolutionary divergence of PsbS proteins was consistent with the evolutionary status of these species, with mosses, algal plants, angiosperms, and gymnosperms clustering separately. Compared with that in angiosperm members, the evolutionary rate of PsbS in coniferous trees was significantly slower, and the differences accumulated between them were even smaller than those between two homologous genes in rice. Ten motifs were predicted by MEME to be conserved among PsbS proteins (Fig. 5b), with that motif 1, motif 2, motif 4, motif 5, and motif 9 being present in all PsbS proteins. The most primitive algal plants contained only the most basic five motifs, while mosses and ferns contained motifs 3 and 6. In addition, the low-complexity domain motifs 7 and 8 associated with the SCOP domain d1leha2 were found only in gymnosperms, indicating different degrees of evolution in gymnosperms and angiosperms. In addition, all PsbS proteins analysed were found to contain chlorophyll a/b binding domains (Fig. 5c).

PmPsbS	MAQSVIFSRGVTS.YVGGIAGESCPPIFFFE..ALAVARGIKIRIFNAAQISWAFLEGFIRS.GNNGRIKTRTAKTVALE	76
PILAhq_071817	MAQSVIFSRGVTS..YVGGIAGESCPPIFFFE..ALAVARGIKIRIFNAAQISWAFLEGFIRS.ANAGIKTRTARTVLE	77
ABK25763.1	MAQSVIFSRVRS..YVGGIAAFSCRPPIPAPE..ALFVARGIRIRFFNAAETSWFLRFLIFS.ANAGINARTARTVLE	75
ABK20973.1	MAQSVIABSGGLSS..CVAGIAGENCRPPIFFSE..ALFVARGIRIRFFNAAQISWFLRFLIFS.ANAGINARTARTVLE	75
PITA_07006	MAQSVIABSGVSS..CVAGIARFRCRPPIFFSE..ALFVARGIRIRIFNAAQISWAFLEGFIRS.ANATGINAGIARTVLE	75
PITA_32163	MAKSVIFSRGVTS.YVGGIAGESCPPIFFFE..ALFVVRGLIKIRIFNAAQISWAFLEGFIRS.GNVGGLKTRTAKTVALE	76
PITA_32164	MAKSVIFSRGVTS.YVGGIAGESCPPIFFFE..ALFVVRGLIKIRIFNAAQISWAFLEGFIRS.GNVGGLKTRTAKTVALE	76
PSME_00032446	MAQSVIFSRGVTSYVGGIAGESCPPIFFFE..ALFVARGIRVRFNAAQISWFLRFLIFS.SGNAGISARTQCTVLE	78
PSME_00052084	MAQSVIVSRASS..CVAGSAGESCRPCEAST..ALFVARGIRIRFFTAACVSWFLRFLIFS.VNAGRNAGISKTVALF	75
Atlg44575	MAQTMILTS.....GVTAGHFLRNK.....SPLAQP..KVHHLFLSG.NSFVLESRR.....CSFVFLALE	54
Potri.002G083500	MAQTMVIMS.GVS...TRQVVDLKRPLILCFQ.....VQRIRP.AEFSRLIYNF..LFSKASS.....NAFTTALF	61
KMZ60119.1	MAQSMITSS.VLG...CLSSTVVRKTLILCFQ.....IQIRIP.TSSSTILFLSKSSFGRTCTST.....IFFQFLALE	64
NP_001296186.1	MAQTMILTA.....NAKVILRSKESL.....VERLKP.KELSSIFLESLEPIRFSSTNASSSKFTSTTVALE	62
NP_001312190.1	MAQTMILTA.....NAKVILRSKESL.....VERLKP.KELSSIFLESLEPIRFSSTNASSSKFTSTTVALE	61
XP_021856726.1	MAQTMILMEGVSS..TTNTIDLKRNAILKLQ.....IQIKIPKSSSTNLFSEF..LFSSSSSSS.....TVKTLALE	64
XP_024358294.1	MAQCAILIRIG...AAQALGGVACQSLTSTR.....RISLFAASSSTLFLVGLGNRRAERVSSG.....AVKTFALE	66
XP_015621169.1	MAQSMIVSG.....ANGTVAASTSR.....IQFVRP.TEFSRLVLSCESSIGRAVS.....VKTVALE	55
XP_015633953.1	MALCCSMA.....MEMVSV.SDLGTAFRSSEMVLCRMKK.....HLVVAAE	42
XP_001689476.1	MAMTISTKAFA.....RGVSARKNIVRVYAATTKVNEKLASK.....TEVERE	44
XP_001689923.1	MAMTISTKAFA.....RGVSARKNIVRVYAATTKVNEKLASK.....TEVERE	44
Consensus	maqs l s a q pl rgl r p pl l s tvalf	
PmPsbS	RFREKAAAFKKGRAGRVAVSEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	156
PILAhq_071817	RFREKAAAFKKGRAGRVAVSEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	153
ABK25763.1	RFREKAAAFKKGRAGRVAVSEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	155
ABK20973.1	RFREKAAAFKKGRAGRVAVSEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	155
PITA_07006	RFREKAAAFKKGRAGRVAVSEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	156
PITA_32163	RFREKAAAFKKGRAGRVAVSEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	155
PITA_32164	RFREKAAAFKKGRAGRVAVSEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	156
PSME_00032446	RFREKAAAFKKGRG...AVSEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	155
PSME_00052084	RFREKAAAFKKGRAGRVAVSEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	155
Atlg44575	KFKTKAA.....KQVEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	125
Potri.002G083500	KFKTKAA.....KQVEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	133
KMZ60119.1	KSKAKTIK.....KQVEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	134
NP_001296186.1	KSKAKTIK.....KQVEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	135
NP_001312190.1	KSKAKTIK.....KQVEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	133
XP_021856726.1	KSKAKTIK.....KQVEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	134
XP_024358294.1	GKTKPAFA.....KQVEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	138
XP_015621169.1	KSKAKTIK.....KQVEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	128
XP_015633953.1	KSKAKTIK.....KQVEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	115
XP_001689476.1	KQATGLFA.....KQVEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	106
XP_001689923.1	KQATGLFA.....KQVEKFKVELGIFGTSGGIGFTKANELFVGRVAMGFAASLIGBATGKGIACQNIETGI	106
Consensus	kpk kaaapp a kpkvdegiftsggigftkanelfvgrvami gfaasllgeaitgkqilaqinietgi	
PmPsbS	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	228
PILAhq_071817	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	225
ABK25763.1	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	228
ABK20973.1	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	232
PITA_07006	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	232
PITA_32163	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	228
PITA_32164	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	228
PSME_00032446	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	229
PSME_00052084	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	232
Atlg44575	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	200
Potri.002G083500	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	208
KMZ60119.1	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	208
NP_001296186.1	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	212
NP_001312190.1	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	210
XP_021856726.1	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	209
XP_024358294.1	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	214
XP_015621169.1	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	204
XP_015633953.1	PIYDAEFLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	189
XP_001689476.1	NGIEVEGIVIGLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	182
XP_001689923.1	NGIEVEGIVIGLIFLIFLITLGAIGALIGRKGVLE..FTF...ESLIDEGKNFRSAVGLKE...SGFLFEGFTKANELFVGR	182
Consensus	piyeaepilliflilitlgaigaligrkvledd pttgl avi pgkgfrsalglke gplfgftkanelfvgrl	
PmPsbS	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	293
PILAhq_071817	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	291
ABK25763.1	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	294
ABK20973.1	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	297
PITA_07006	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	298
PITA_32163	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	293
PITA_32164	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	293
PSME_00032446	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	295
PSME_00052084	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	298
Atlg44575	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	265
Potri.002G083500	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	272
KMZ60119.1	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	271
NP_001296186.1	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	276
NP_001312190.1	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	274
XP_021856726.1	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	274
XP_024358294.1	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	279
XP_015621169.1	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	268
XP_015633953.1	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	254
XP_001689476.1	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	245
XP_001689923.1	AQIGIAFSLIGIITGKGALACQNIETGVFVSEIEPIVLFNVIFLFAAVNEGCKRVTIVDEEE	245
Consensus	aqigiafsligaitgkqilaqinietgvpi etepilvlnv ifffaa npg gkfvtd dee	

Fig. 4 Multiple sequence alignment of PmPsbS protein with model plants and conifers. PILAhq_071817: *Pinus lambertiana*; ABK25763.1 and ABK20973.1: *Picea sitchensis*; PITA_07006, PITA_32163, and PITA_32164: *Pinus taeda*; PSME_00032446 and PSME_00052084: *Pseudotsuga menziesii*; Atlg44575: *Arabidopsis thaliana*; Potri.002G083500: *Populus trichocarpa*; KMZ60119.1: *Zostera marina*; NP_001296186.1: *Solanum lycopersicum*; NP_001312190.1: *Nicotiana tabacum*; XP_021856726.1: *Spinacia oleracea*; XP_024358294.1: *Physcomitrella patens*; XP_015621169.1 and XP_015633953.1: *Oryza sativa subsp. Japonica*; XP_001689476.1 and XP_001689923.1: *Chlamydomonas reinhardtii*. The dark shading represented absolutely conserved amino acid sites. The pink shading represented similar amino acids whose homology level >75%. Blue shading highlighted similar amino acids with homology levels >50%.

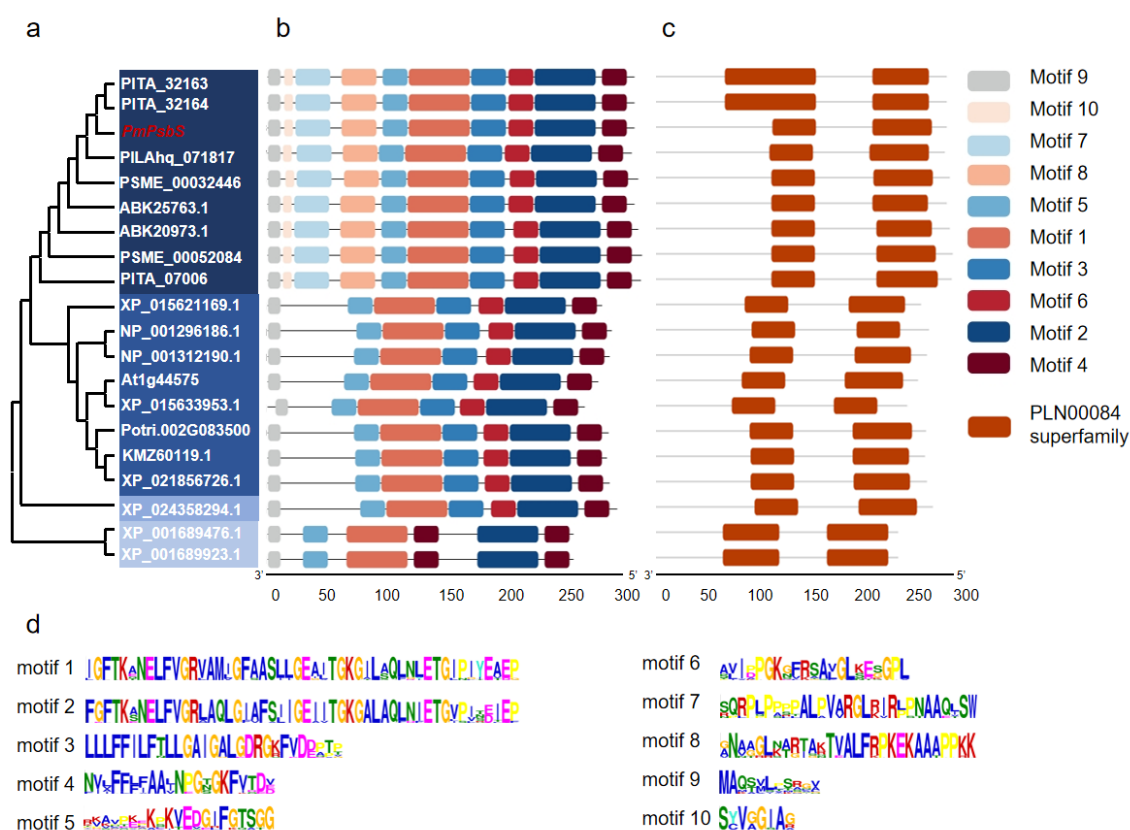


Fig. 5 Sequence analysis of PmPsbS protein. **a.** The colour of the evolutionary tree from dark to light represents: gymnosperms, angiosperms, bryophytes and algae, respectively. **b.** Conserved motif distribution map. **c.** Conserved domain. **d** Conserved motif sequence.

The core region of the *PmPsbS* promoter

To analyse the minimum active fragment of the promoter, four 5' end deletion fragments of different lengths were designed according to the promoter sequence (Fig. 6), and the cloned target fragment and the expression vector were separately enzymatically cleaved and ligated by T4 DNA ligase to replace the *CaMV35S* promoter upstream of the *GUS* gene of pCAMBIA1304 vector to construct the recombinant (Fig. 6d). The expression vector was transformed into *N. tabacum*. After 3 days of transient expression, GUS chemical staining was performed. The findings of the study revealed that no coloration was observed in the negative control (CK-) (Fig. 6e), signaling absence of background contamination. Conversely, the positive control (CK+) displayed a blue colour, indicating the correct expression of the *GUS* gene. Injection of the full-length promoter (-2 123 bp) and three truncated fragments, q1 (-1 744 bp), q2 (-1318 bp), and q3 (-852 bp), into *N. tabacum* leaves resulted in darker blue GUS staining, suggesting that these fragments could drive the expression of the *GUS* gene. However, the injection of the q4 fragment (-330 bp) did not elicit any blue staining, implying that this specific truncated fragment did not possess activity and could not induce GUS expression. As a result, the core region of the promoter was identified between q3 and q4, spanning from -852 bp to -330 bp.

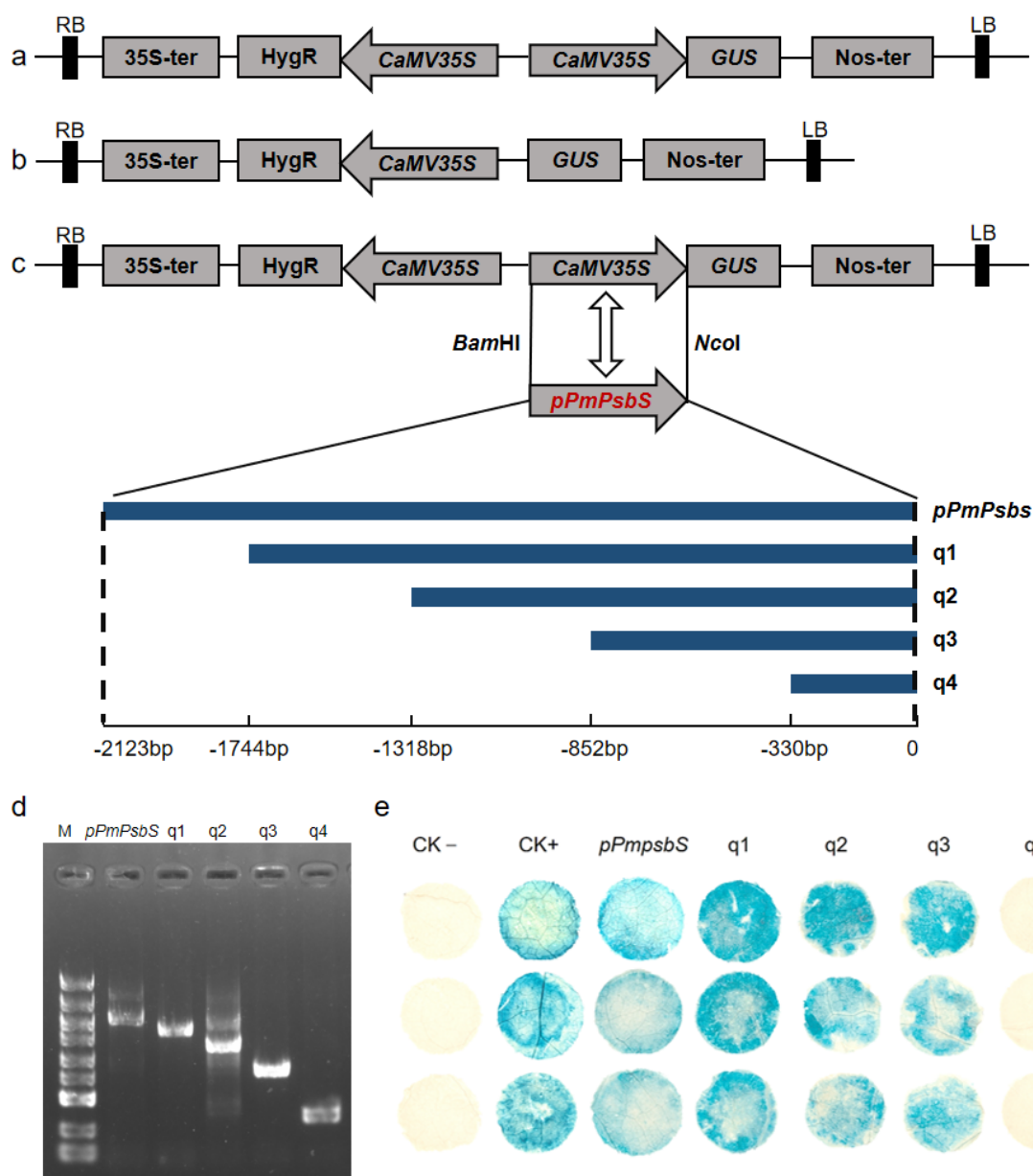


Fig. 6 Transient expression of the promoter fragment of the *PmPsbS* according to GUS staining method. **a.** pCambia1304 expression vector. **b.** pCambia1304 vector with removal of *CaMV35S* promoter. **c.** Constructed pCambia1304 expression vectors of different promoter fragments. *pPmPsbS*: -2123 bp *PmPsbS* promoter. M: DNA Maker 5000bp. q1: -1744 bp *PmPsbS* promoter. q2: -1318 bp *PmPsbS* promoter. q3: -852 bp *PmPsbS* promoter. q4: -330 bp *PmPsbS* promoter. **d.** Cloning of promoter fragments from the constructed expression vectors. **e.** GUS staining with full-length and deletion promoters. CK-: Negative control, injected with the pCambia1304 vector. CK+: Positive control, injected with pCambia1304 empty vector.

Subcellular localization of PmPsbS protein

The subcellular localization of PmPsbS was predicted using the online software ProtComp 9.0. Subsequently, the results were standardized and presented as heatmaps (Fig. 7a). The analysis indicated a higher probability of PmPsbS being localized in chloroplasts rather than extracellular regions. The *PmPsbS* gene was incorporated into a green

fluorescent protein (GFP) fusion transient expression vector, which was then introduced into *Arabidopsis thaliana* protoplasts in culture. The transformed protoplasts were subsequently examined using laser confocal microscopy. The results demonstrated successful expression of the pBWA(V)HS-Glosfp empty vector within *Arabidopsis thaliana* protoplasts. Furthermore, the fusion expression vector, pBWA(V)HS-PmPsbS-Glosfp, exhibited localization within the thylakoid membrane of the chloroplasts in the protoplasts. These results suggested that *PmPsbS* from *P. massoniana* likely functioned on the thylakoid membrane in the chloroplasts

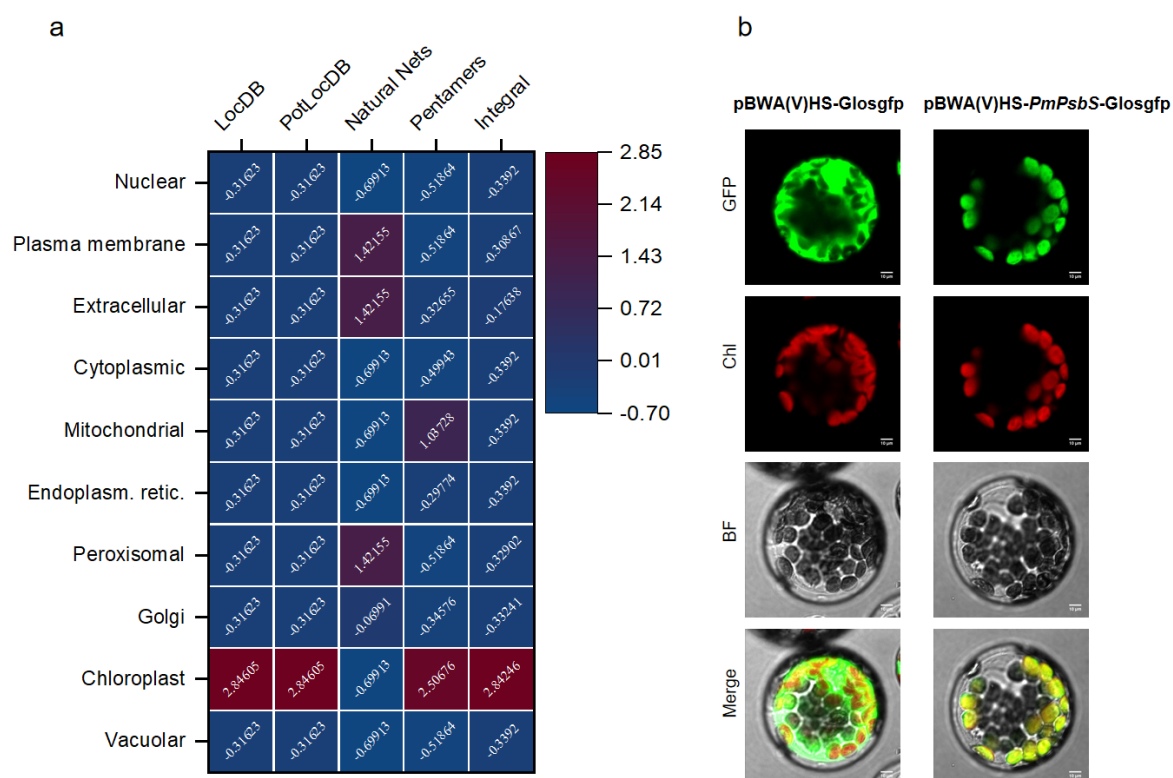


Fig. 7 Subcellular localization of PmPsbS protein. **a.** Heatmap predicting of PmPsbS subcellular localization. **b.** pBWA(V)HS-Glosfp: A vector overexpressing only the GFP tag (serving as a blank control). pBWA(V)HS-PmPsbS -Glosfp: *PmPsbS* gene overexpression vector fused with GFP (green fluorescent protein) tag. GFP: The signal emitted by the green fluorescent protein. Chl: Chloroplast fluorescence signal. BF: Clear field. Merge: Denoted the superimposed images or signals.

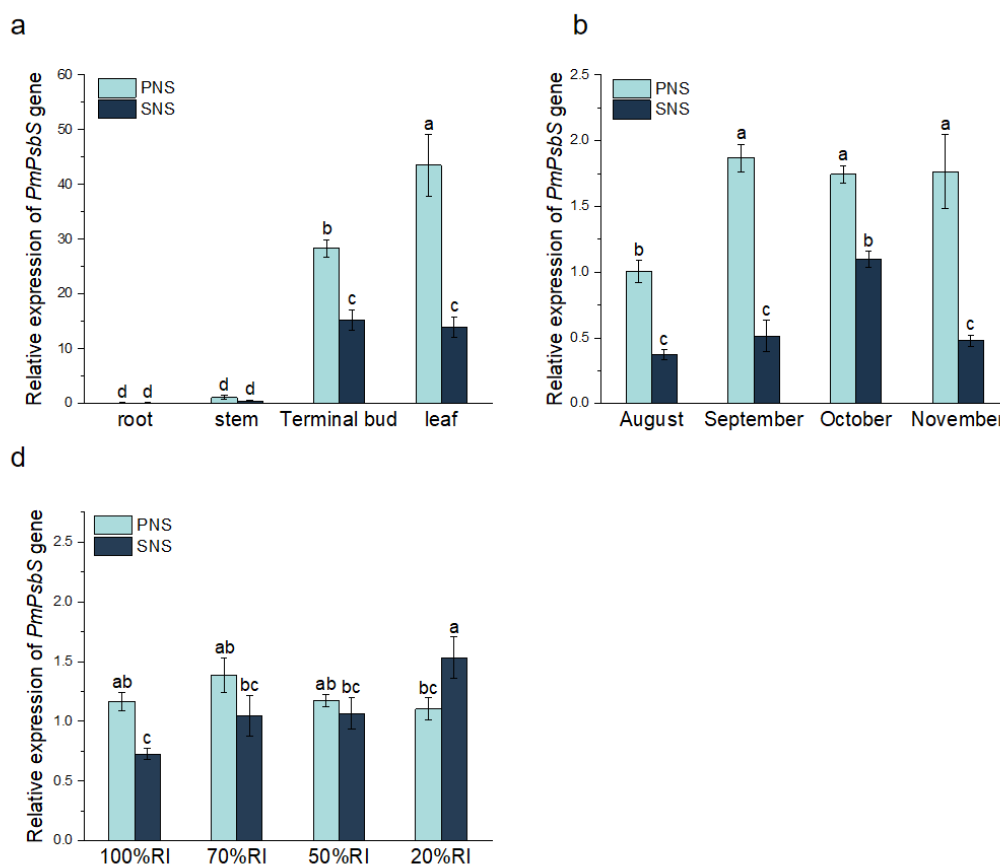
The expression pattern of *PmPsbS* in PNS and SNS

Relative expression levels of *PmPsbS* in different tissue of PNS and SNS were analysed using RT-qPCR (Fig. 8a). The expression of *PmPsbS* in the apical bud and leaves of both types of seedlings was significantly higher compared to the stem and roots, with only a minimal expression observed in the stem and negligible expression in the roots. The expression levels of the *PmPsbS* in the leaves, terminal buds, stems, and roots in PNS followed the sequence: leaves > terminal buds > stems > roots. However, in SNS, there was no significant difference in the expression levels between the

leaves and apical buds. Compared to SNS, the relative expression levels of the *PmPsbS* gene were significantly upregulated in leaves and apical buds in PNS.

Furthermore, we investigated the expression of the *PmPsbS* gene in the seedlings during the autumn and winter seasons. From August to November, the expression level of the *PmPsbS* in SNS was significantly lower compared to that in PNS, with the expression level in SNS ranging from 27.2% to 64.0% of that in PNS (Fig. 8b). During the transition from autumn to winter, we observed an increase in the expression levels in both PNS and SNS. However, after acclimatization to the external environment, the expression of the *PmPsbS* exhibited a decrease in SNS.

Fig. 8 The relative expression level of the *PmPsbS* gene, analysed by RT-qPCR. **a.** Expression in different tissues PNS and SNS. **b.** Expression from August to November in PNS and SNS. **c.** Expression under different shade conditions. The different lowercase



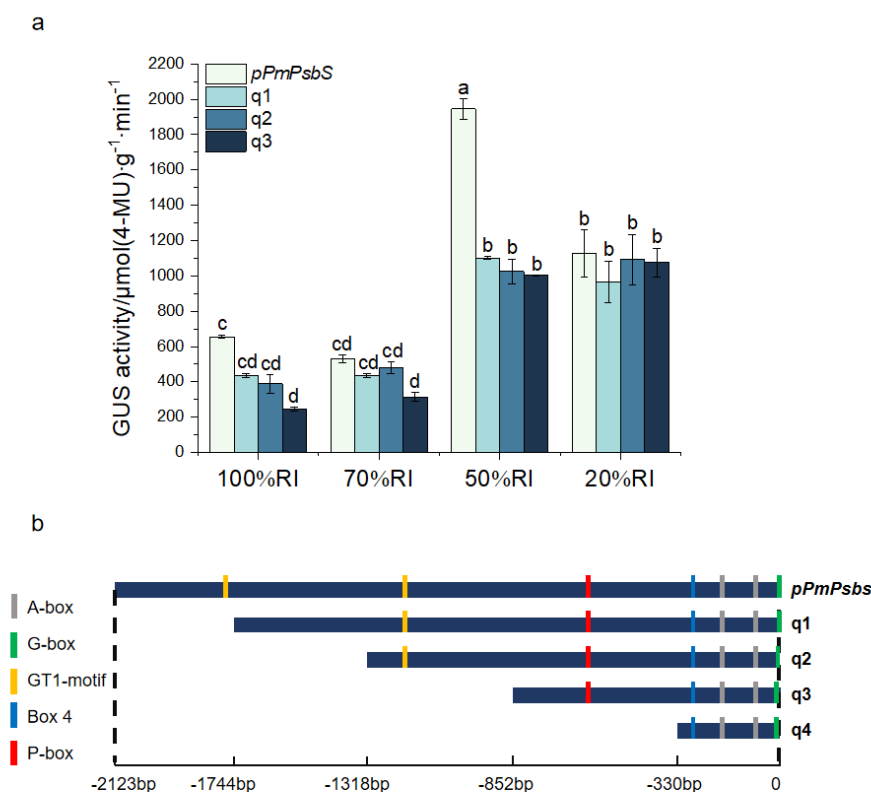
letters indicated a significant difference ($p < 0.05$).

We conducted a study by exposing the PNS and SNS to varying light intensities. The results showed that the expression level of the *PmPsbS* in PNS was higher compared to SNS under 100% RI (Fig. 8c). However, as the level of shading increased, the expression level of the *PmPsbS* in PNS gradually decreased below that in SNS. It was worth noting that there was no significant change in the expression of the *PmPsbS* in PNS under different shading conditions. In contrast, in SNS, the expression level of the *PmPsbS* significantly increased at 20% RI. Consequently, the response of the *PmPsbS* to shading conditions differed between PNS and SNS.

Expression activity of the *PmPsbS* promoter under shade conditions

To further investigate the function of the *PmPsbS* promoter, we constructed expression vectors containing the promoter fragments and transferred them to *N. tabacum* for transient expression. The results obtained from the GUS activity assays (Fig. 9a) demonstrated a significant upregulation of expression for all the designed promoter fragments as the level of shading gradually increased up to 50% RI and 20% RI. Notably, under 50% RI conditions, the full-length promoter exhibited significantly higher GUS activity compared to the other promoter fragments. This observation suggested the probable presence of an enhancer within the -1744 bp to -2123 bp region, which effectively activated promoter activity. Interestingly, as the light intensity decreased from 50% RI to 20% RI, the GUS activity of the full-length promoter reduced to the level observed for the other three promoter fragments.

Fig. 9 Expression activity of *PmPsbS* promoter fragments under shade conditions. **a.** GUS activity of different length



of promoter fragments. **b.** Schematic design of the missing promoter fragments. The different lowercase letters indicated a significant difference ($p < 0.05$).

Discussion

Plants need to protect themselves from excess light, which causes photo-oxidative damage and lowers the efficiency of photosynthesis. The primary role of PsbS is thought to be a protective one, as a key player in some aspects of nonphotochemical quenching (NPQ) (Nicol and Croce 2021; Kiss et al. 2008; Niyogi et al. 2005; Muller et al. 2001). For

full-length of the *PmPsbS* gene consisted of 3 exons and 2 introns. Moreover, the PmPsbS stood out from other members of the LHC superfamily due to its possession of four transmembrane domains and two conserved structural domains (Kim et al. 1994). It is hypothesized that PsbS protein originated as a single helix known as the highly light-induced protein (HLIP) and subsequently gained four additional helices through gene duplication within the *PsbS* gene (Montané and Kloppstech 2000; Wallbraun et al. 1994). In recent years, a new hypothesis model has been proposed that the LHC and PsbS may have originated from different homologous members of the stress-enhanced protein subfamily, and the independent evolution of the LHC and PsbS families has been strongly supported by phylogenetic evidence (Engelken et al. 2010). The evolutionary divergence of the *PmPsbS* gene was consistent with the evolutionary status of these species, with mosses, algal plants, angiosperms, and gymnosperms clustering separately. Furthermore, in *Arabidopsis*, the PsbS is localized on the chloroplast thylakoid membrane, and the protonation of the Glu122 and Glu226 residues binds with zeaxanthin, resulting in the qE state, which promotes the dissipation of excess excitation energy (Niyogi et al. 2005; Sacharz et al. 2017). Similarly, we found Glu-153 and Glu-254 of PmPsbS were conserved and localized on the chloroplast thylakoid membrane. The lumen-faced Glu have unusually high pKa values, providing an explanation for the pH responsiveness to thylakoid lumen acidification of this protein domain (Krishnan-Schmieden et al. 2021). PmPsbS had short β -folds that were not found in LHCII, making the structure of PsbS dense and difficult to interact with other internal pigment molecules (Fan et al., 2015). Under neutral and alkaline conditions, PsbS protein is mostly found in a dimeric form, but under acidic conditions, PsbS commonly binds to the LHCII complex in a monomeric form (Correa-Galvis et al., 2016). However, whether PsbS interacts with quenching pigments to actively participate in the main mechanism of NPQ remains a debated issue in the field of NPQ research.

In eukaryotic organisms, elaborate mechanisms have evolved to control the spatial and temporal patterns of transcription during growth, differentiation, and development (Hochheimer et al. 2003; Smale 2001). Transcriptional activation represents a fundamental mechanism for regulating gene expression. (Hochheimer et al. 2003). *PmPsbS* promoter sequences in this study showed that the core promoter region was from -852 bp and -330 bp, while the transcription start site (TSS) was located at -311 bp. Besides, there was a TATA-box present at -26 bp upstream of the TSS, 192 bp away from the nearest CAAT-box upstream. These were similar to the situation observed in *Suaeda liaotungensis* (Li et al. 2007). The promoter region of *PmPsbS* contained various types of responsive elements and transcription factor-binding sites. These suggested that the promoter of the *PmPsbS* gene may be induced for expression by multiple factors. Among them, we particularly focused on light-responsive elements such as A-box, G-box, GT1-motif, and Box 4, as well as transcription factors related to light response such as MYB, WRKY, NAC, bZIP, and C2H2. *BIT1* and *SmMYB35* possess typical R2R3 DNA binding motifs (Hong et al. 2008; Li et al. 2022). In *Arabidopsis*, *BIT1*

regulates promoter activity of *PsbS* in a blue light-dependent manner and exhibits specific binding to a MYB-binding consensus sequence in the promoter region of *PsbS* (Hong et al. 2008). The expression of the photoresponsive R2R3-MYB transcription factor SmMYB35 is controlled by SmHY5 through direct binding to the G-box in the promoter region (Li et al. 2022). Recent research on *Oryza japonica* demonstrates that the novel cis-element CTAATC facilitates specific binding of OsMYBS2 to the *OsPsbS1* promoter, leading to the suppression of its expression (Fu et al. 2021). Therefore, it is speculated that members of the MYB family related to light response may bind to specific binding sites in the promoter of the *PmPsbS* gene in *P. massoniana*, thereby regulating gene expression. In low light conditions, the activity of the *PmPsbS* promoter increases, with the shortest responsive fragment being -852bp, which contains several stress-responsive elements including an ARE, but only one P-box (part of a light-responsive element), contradicting the predictions of the promoter's cis-acting elements. It is possible that there are some unidentified elements that can respond to light regulation (Arguello-Astorga and Herrera-Estrella 1996). We found multiple MYB transcription factor binding sites in the -330bp to -852bp region of the *PmPsbS* promoter, and it does not specifically bind any chlorophyll or xanthophyll pigments (Fan et al. 2015), suggesting that the *PmPsbS* gene may not be directly regulated by light. The specific locations and regulatory mechanisms of the light-responsive elements in the *PmPsbS* promoter require further investigation and exploration as the regulation of gene expression through cis-acting elements is a highly complex process.

PsbS expression levels reflect the plant's photoprotective state to a certain extent. In this study, we found that *PmPsbS* is mainly expressed in the terminal bud and leaves, which is consistent with the results of the study in *Phyllostachys edulis* (Lou et al. 2018). Leaves are considered the primary site of photosynthesis, but green nonleaf tissues are capable of significant carbon assimilation through photosynthesis (Lawson and Phytologist 2022). The localization of *PmPsbS* on the thylakoid membrane within chloroplasts supports the notion of high expression of *PmPsbS* in leaf tissues and suggests that the roots, stems, and leaves of *P. massoniana* seedlings also have distinct patterns of NPQ accumulation and demand. The expression levels of *PmPsbS* in the apical buds and leaves of the PNS are significantly higher compared to those in SNS. This may be due to the luxury growth strategy of increasing the absorption and conversion of light energy to quickly obtain high yields, which increases light protection and allows *PmPsbS* to be easily induced in PNS (Wang et al. 2022).

It is often assumed that plants can only activate NPQ as a protective mechanism under high-intensity light (Nicol and Croce 2021; Goss and Lepetit 2015; Niyogi et al. 2005; Muller et al. 2001). However, in weak light conditions, when photosynthetic carbon fixation is saturated (Sielewiesiuk 2002; Powles 1984), plants are unable to utilize all the absorbed light energy, resulting in acidification of the thylakoid lumen and thus triggering NPQ (Powles 1984). In fact, the NPQ mentioned here still addresses plant's inability to absorb excessive light energy. The triggering factor of NPQ is the perception of the transmembrane proton gradient by the PsbS protein (Nicol and Croce 2021; Goss and Lepetit 2015).

Low temperature (LT) plays a synergistic role with excess illumination by limiting electron transport and carbon fixation rates. In these conditions, even a relatively weak light may exceed the energy utilization rate with a consequent photoinhibitory effect (Huner et al. 1998; Gerotto et al. 2011). It has been confirmed that an increase in the *PsbS* expression of *Arctostaphylos uva-ursi* in winter was particularly pronounced in the shaded population (Zarter et al. 2006). In this work, we found that PNS and SNS were continuously induced to express *PmPsbS* from autumn to winter. The capacity of *P. massoniana* to respond to changing autumn and winter climate is here clearly demonstrated by the increase in their photochemical capacity, similar to what was previously observed in other plants (Gerotto et al. 2011; Chang et al. 2015). Although the de-epoxidation of the xanthophyll cycle (DEPS) and sustained NPQ are increased during late autumn, the transition from energy dependent to sustained NPQ is not complete until winter (Porcar-Castell 2011; Demmig-Adams et al. 2012). Interestingly, in the given environmental conditions during the autumn and winter seasons, it has been observed that PNS and SNS continue to experience varying degrees of light inhibition. Studies have shown that the differences in shape, size, colour, leaf composition, chlorophyll content, leaf structure, and arrangement of heteroblastic foliage can affect the performance of NPQ (Zhang et al. 2021; Forster and Bonser 2009). Wang et al. (2022) have proved that there were differences in photosynthetic capacity between heteroblastic foliage in *P. massoniana*. The NPQ of primary needles was higher, and the ability to convert excess light energy into thermal energy increased protect the structure of the optical system structure under low temperature. Studies of *Juniperus sabina* and other plants have led to similar conclusions (Zhang et al. 2021; Forster and Bonser 2009). Therefore, different photoprotective and light energy partitioning mechanisms in response to seasonal variation in the primary and secondary needles. The effects of shade on leaf shape are subtle with respect to individual traits but are combinatorially strong (Chitwood et al. 2015). In our shading experiment, without the addition of low-temperature conditions, the SNS seedlings showed a significant induction of *PmPsbS* gene expression under the 20%RI condition. Interestingly, the *PmPsbS* gene in the PNS did not show significant changes throughout the shading process. But the primary needles responded more sensitively to high light intensity than the secondary needles (Wang et al. 2022). In explaining the different light intensities, it was evident that PNS and SNS have distinct NPQ mechanisms. Under low light conditions, SNS is more prone to reaching photosynthetic carbon saturation and activating photoprotective states.

In summary

The *PmPsbS* protein contains two conserved domains and four transmembrane regions and is primarily located in the thylakoid membrane of chloroplasts. The core region of the *PmPsbS* promoter is situated between -852 to -330 bp, including predicted binding sites for light-responsive elements, G-box elements, and MYB transcription factors.

Additionally, we predict the presence of light-responsive MYB gene family members in *P. massoniana*, which may bind to specific sites within the *PmPsbS* gene promoter to regulate its expression. Through the analysis of gene function and expression of *PmPsbS* in *P. massoniana*, we discovered its crucial role in non-photochemical quenching (NPQ). However, under low light conditions, SNS is more prone to reaching photosynthetic carbon saturation and activating photoprotective states. These results indicated that *PmPsbS* can circumvent photoinhibition and enhance photoprotection and plays an important role in the adaptation of heteroblastic foliage of *P. massoniana* to light environments.

Author contribution statement

F.W. conceived and designed the research, provided the funding. Y.Y. and Y.X. conducted the experiments. Y.Y. and F.W. wrote the manuscript. All authors read and approved the final manuscript.

Supplementary Information

Prediction of cis-acting elements in *PmPsbS* promoters (Table S1); Prediction of transcription factor binding sites in *PmPsbS* promoters (Table S2)

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Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

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