Intrinsically Unstructured Phosphoprotein TSP9 Regulates Light Harvesting in *Arabidopsis thaliana*[†]

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ABSTRACT: Thylakoid-soluble phosphoprotein of 9 kDa, TSP9, is an intrinsically unstructured plant-specific protein [Song, J., et al. (2006) *Biochemistry 45*, 15633−15643] with unknown function but established associations with light-harvesting proteins and peripheries of both photosystems [Hansson, M., et al. (2007) *J. Biol. Chem. 282*, 16214−16222]. To investigate the function of this protein, we used a combination of reverse genetics and biochemical and fluorescence measurement methods in *Arabidopsis thaliana*. Differential gene expression analysis of plants with a T-DNA insertion in the TSP9 gene using an array of 24000 *Arabidopsis* genes revealed disappearance of high light-dependent induction of a specific set of mostly signaling and unknown proteins. TSP9-deficient plants had reduced levels of *in vivo* phosphorylation of light-harvesting complex II polypeptides. Recombinant TSP9 was phosphorylated in light by thylakoid membranes isolated from the wild-type and mutant plants lacking STN8 protein kinase but not by the thylakoids deficient in STN7 kinase, essential for photosynthetic state transitions. TSP9-lacking mutant and RNAi plants with downregulation of TSP9 showed reduced ability to perform state transitions. The nonphotochemical quenching of chlorophyll fluorescence at high light intensities was also less efficient in the mutant compared to wild-type plants. Blue native electrophoresis of thylakoid membrane protein complexes revealed that TSP9 deficiency increased relative stability of photosystem II dimers and supercomplexes. It is concluded that TSP9 regulates plant light harvesting acting as a membrane-binding protein facilitating dissociation of light-harvesting proteins from photosystem II.

Chloroplast thylakoid membranes perform light-driven oxygenic photosynthesis via the coordinated action of several multiprotein membrane complexes: light-harvesting complex II (LHCII),[1] photosystem II (PSII), cytochrome *bf* complex, light-harvesting complex I, photosystem I (PSI), and ATP synthase. An ingenious environmentally modulated protein phosphorylation system has evolved in thylakoid membranes of plants and green algae (*1, 2*). The light-induced phosphorylation of thylakoid membrane proteins was discovered in 1977 (*3*), and for a long time it was considered to be restricted to the polypeptides of LHCII and PSII (*4*). However, the recent applications of novel experimental approaches have revealed phosphorylation of proteins in all thylakoid protein complexes, as reviewed in ref *1*, and in a number of previously uncharacterized thylakoid proteins (*5*−*7*). Many of these proteins become phosphorylated *in vivo* only under distinct environmental conditions (*6*−*9*). This implies the possible involvement of phosphorylation events in adaptive responses, while the exact mechanisms and functions of these reactions are still largely unknown (*1*).

The best characterized adaptive response is the phosphorylation-dependent regulation of light harvesting in the process of photosynthetic state transitions: balancing of absorbed light energy distribution between the two photosystems via reversible lateral migration of mobile LHCII between PSII and PSI (*2, 4, 10*). In photosynthetic state 1 mobile LHCII is bound to PSII, while in state 2 it migrates to PSI in a phosphorylation-dependent manner as a result of reduction of plastoquinone and activation of protein kinases (*2, 10*). The consequence of state 1 to state 2 transitions is thus a decrease in PSII antenna cross section and an increase in PSI antenna cross section. Thylakoid protein kinases Stt7 and STN7 have been shown to be essential for state transitions in the green alga *Chlamydomonas reinhardtii* and in plants (i.e., *Arabidopsis thaliana*), respectively (*11, 12*). It was assumed for a long time that phosphorylation and dephosphorylation of LHCII determine binding of mobile LHCII to either PSI or PSII, respectively. However, this model has been challenged by findings of

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[1] Abbreviations: LHCII, light-harvesting complex II; NPQ, nonphotochemical quenching; PSI, photosystem I; PSII, photosystem II; TSP9, thylakoid-soluble phosphoprotein of 9 kDa.

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nonphosphorylated LHCII bound to PSI (*13, 14*), as well as inability of phosphorylated LHCII to bind to PSI in several mutants of *Arabidopsis* (*10, 15*). Thus, it has been suggested that phosphorylation of proteins different from mobile LHCII could drive the state transitions (*10*); particularly, a plant-specific protein TSP9 has recently been proposed for such a role (*16*).

The thylakoid soluble phosphoprotein of 9 kDa, TSP9, was characterized in spinach as a strongly basic protein that becomes triply phosphorylated under illumination (*5*). The function of this protein is still unknown, but its mobile nature led to suggestions of its possible involvement in light- and phosphorylation-dependent cell signaling from photosynthetic membranes (*5, 17*). Genes for homologous proteins were found in *Arabidopsis* and 48 other plant species but not from any other organisms, making TSP9 a plant-specific protein (*5, 18*). NMR spectroscopy revealed that in aqueous solution TSP9 is an intrinsically unstructured protein that acquires certain structural features upon binding to membrane mimetic micelles (*18*). Fractionation of spinach thylakoid membranes and chemical cross-linking revealed that TSP9 is associated with LHCII as well as with the peripheries of PSII and PSI (*16*). These findings led to the suggestion that TSP9 can be involved in plant-specific regulation of light harvesting (*1, 16*) analogous to that operating by multiple differential phosphorylation of CP29 in the green alga (*6*).

In the present work, we demonstrated that recombinant TSP9 can be phosphorylated by thylakoid membranes isolated from the wild-type *Arabidopsis* plants and plants lacking STN8 kinase responsible for PSII phosphorylation (*19*) but not by the thylakoids deficient in STN7 kinase responsible for state transitions (*12*). We found that plants lacking TSP9 have distorted state transitions as well as decreased capacity for high light-induced nonphotochemical quenching (NPQ), another regulatory process of light harvesting. Using the microarray technique we also showed that plants deficient in TSP9 have reduction in the expression of specific genes, which supports the earlier hypothesis (*5*) that TSP9 may be involved in plant cell signaling as well.

MATERIALS AND METHODS

Plant Material. A. thaliana plants were grown hydroponically (*20*) at a photosynthetic flux of 120 μmol of photons m^{-2} s^{-1}, 23 °C, and 65−70% relative humidity. The photoperiod was 8 h light and 16 h dark. Ecotype Columbia was used for all experiments. The T-DNA insertion line in the Columbia background for TSP9 (GABI 377A12) from GABI-Kat was analyzed by PCR and immunoblotting to confirm that the mutant was homozygous and did not express any TSP9 protein. Primers were designed for the right (CAG TAG TAT ATA CGG TCG CAG ATT CCA TC) and left (AAT GCT AAT GCA ACT TTT TCT GGA GTA CA) flanking sequences of the TSP9 gene At3g47070 and for the T-DNA insert (CCC ATT TGG ACG TGA ATG TAG ACA C).

Wild-type plants were also transformed with a plasmid containing an RNAi construct based on the entire coding region of TSP9, essentially as described before (*21*). In short, a DNA fragment containing the coding region of the mature TSP9 was amplified from a full-length cDNA clone by PCR using primers that were based on a cDNA sequence for the TSP9 gene (At3g47070). The fragment was cloned in sense and antisense orientation separated by a fragment containing the intron coding region from a nitrite reductase gene from *Phaseolus vulgaris* (accession number U10419). The TSP9 construct was cloned downstream the enhanced CaMV 35S promoter in the pPS48 vector, and orientation of the insert was confirmed by sequencing. Subsequently, a fragment containing the enhanced 35S promoter followed by the TSP9 construct and the 35S terminator was excised with *Xba*I and ligated into the binary vector pPZP111. The vector construct was transformed by electroporation into the *Agrobacterium tumefaciens* strain C58. *Arabidopsis* plants were transformed by the floral dip method. The T1 transformants were screened for downregulation of TSP9 by immunoblotting. For immunoblotting a specific antibody, generated in rabbit against recombinant *Arabidopsis* TSP9 expressed in *Escherichia coli* and purified as described below, was used. The other used *Arabidopsis* plants were the earlier characterized mutants deficient in STN7 (*9*) and STN8 (*19*) protein kinases, as well as in PsbS protein (*npq4* mutant) (*22*). For state transitions and NPQ measurements, plants were grown in peat in a controlled environment *Arabidopsis* chamber (Percival AR-60 L, Boone, IA) at a photosynthetic flux of 100−120 μmol of photons m^{-2} s^{-1}, 20 °C, and 70% relative humidity with 8 h light and 16 h dark photoperiod.

Isolation of Thylakoid Membranes. The thylakoid membranes were isolated from ~4 g of *Arabidopsis* leaves harvested either after light adaptation (4 h after the light was on) or after dark adaptation (15 h after the light was off) essentially according to the protocol (*23*). Four-week-old plants were used for the preparation of chloroplasts and thylakoids. The isolated thylakoids were washed once with 50 mM Tricine, pH 7.8, 100 mM sorbitol, 5 mM $MgCl_2$, and 10 mM NaF and once with 25 mM NH_4HCO_3 and 10 mM NaF. NaF was used in the buffers when phosphorylation was quantified since NaF works as a phosphatase inhibitor.

Recombinant TSP9. A clone (MPIZp2000E012Q2) containing cDNA for mature *Arabidopsis* TSP9 was obtained from RZPD German Resource Center for Genome Research, Berlin, Germany. The gene for TSP9 was recloned and N-terminally fused to a construct containing maltose binding protein (MBP), a PreSci-protease cleavage site, and a His_6 affinity tag and expressed in *E. coli*. The fusion protein was isolated by the use of an amylose-resin (New England Biolabs), whereafter the MBP was cleaved off by PreSci protease (GE Healthcare) and finally the recombinant His_6-tagged TSP9 was collected by the use of Ni-NTA (Qiagen).

Phosphorylation of Thylakoid Membranes with and without Recombinant TSP9. ^{32}P-labeling of thylakoid proteins was performed by illumination (85 μmol of photons m^{-2} s^{-1}) of thylakoid membranes (0.2 mg of chlorophyll/mL) with or without recombinant TSP9 (0.1 mg/mL) in the presence of 0.25 mM ATP containing [γ-^{32}P]ATP (0.02 mCi/mg of chlorophyll) for 45 min at room temperature. The reactions were stopped by addition of 25 mM EDTA or SDS−PAGE sample buffer. The proteins were separated on 15% SDS−PAGE gels, which were stained with Coomassie blue and subjected to autoradiography for identification of ^{32}P-labeled proteins.

Immunoblotting. Thylakoid membrane proteins were separated by SDS−PAGE (6% acrylamide stacking gel + 14%

separation gel + 6 M urea), and the proteins were then transferred to a PVDF membrane (Immobilone; Millipore). For the anti-phosphothreonine antibodies, purchased from both Zymed and New England Biolabs, the membranes were blocked with 5% bovine serum albumin. For specific antibodies against TSP9, Lhcb1/Lhcb2 (Agrisera, Sweden) and PsbS blocking was done with 10% skimmed milk. For all antibodies used sample dilutions were made to verify that the signal was within linearity. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody, assayed using ECL detection kit (GE Healthcare), and developed by chemiluminescence imaging (LAS-1000). Quantification of the immunoblots was done using Fujifilm LAS-1000 software.

Blue Native PAGE (BN-PAGE)/SDS−PAGE. BN-PAGE was performed essentially as described (*16*). Thylakoid membranes were resuspended in 20% (w/v) glycerol and 25 mM BisTris-HCl, pH 7.0, at a chlorophyll concentration of 2 mg of chlorophyll/mL. An equal volume of 2% (w/v) *n*-dodecyl β-D-maltoside in resuspension buffer was added, and the mixture was incubated on ice for 5 min. After centrifugation at 14000*g* for 30 min the supernatant was supplemented with 0.1 volume of sample buffer (100 mM BisTris-HCl, pH 7.0, 0.5 M ε-amino-*n*-caproic acid, 30% (w/v) sucrose, 50 mg/mL Serva blue G) and subjected to BN-PAGE with a gradient of 5−13.5% acrylamide in the separation gel. The electrophoresis was performed at 4 °C at 100 V for 3−4 h. For separation of proteins in the second dimension, lanes were cut out and incubated with 5% β-mercaptoethanol in SDS sample buffer for 30 min at room temperature and then subjected to SDS−PAGE with 15% acrylamide in the separation gel.

Fluorescence Measurements, State Transitions, and Non-photochemical Quenching. The kinetics of state 1 to state 2 transitions was measured according to ref *10*, in short as follows. Instrument setup used was a pulse amplitude modulation 101−103 fluorometer (Walz, Effeltrich, Germany). A detached leaf from dark-adapted plants was fixed to the light fiber end, and maximum fluorescence yield was measured during exposure to a saturating flash (0.8 s, 6000 μmol of photons m^{-2} s^{-1}). The leaf was then illuminated for 15 min with 100 μmol of photons m^{-2} s^{-1} that favors PSII (blue light) from a Schott KL-1500 lamp equipped with a Corning 4-96 filter. From the blue PSII light (state 2), the maximal fluorescence yield (F_m^2) was measured, followed by induction of state 1 by switching on far-red light (Walz 102-FR) in addition to the blue light. After another 15 min the maximum fluorescence yield (F_m^1) was measured. Switching off the far-red light then allowed the kinetics of state 1 to state 2 transitions to be measured.

The fluorescence parameters for NPQ measurements were determined essentially according to ref *24*, in short as follows. A PAM 101-103 fluorometer was used with a standard setup. Plants were dark adapted before experiments. A detached leaf was placed around the end of the light fiber, and aluminum foil with moist tissue paper was placed around it. Switching on the measuring light allowed determination of the F_0 (minimal) fluorescence in darkness; the measuring light was also used for determination of the F_0', which is the minimal fluorescence in the light-adapted state. A saturation flash of white light (0.8 s, 6000 μmol m^{-2} s^{-1}) was then applied, allowing the measurements of F_m and F_m'. Actinic light was provided by a Schott KL-1500 lamp equipped with neutral gray filters. Three actinic light intensities were used: 150, 300, and 900 μmol of photons m^{-2} s^{-1}.

Xanthophyll Cycle Activity Assay. Wild-type and *tsp9* mutant plants were exposed to either growth or high light (120 or 900 μmol of photons m^{-2} s^{-1}, correspondingly); the leaves were cut and immediately frozen in liquid nitrogen. The samples were ground in ice-cold methanol, and the pigment extracts were clarified by centrifugation at 12000*g* for 15 min at 4 °C. The pigment separations were performed using the Agilent 1100 HPLC system equipped with a flow splitter on an Agilent reverse-phase C_{18} analytical column (150 × 0.5 mm i.d.; 3.5 μm particle size). The sample injection volume was 4 μL, and the flow rate was 7 μL/min. The mobile phase consisted of two solvents: A, composed of acetonitrile/water (80:20), and B, composed of acetonitrile/ethyl acetate (85:15). The pigments were eluted with a linear gradient from 100% A to 100% B over 29 min, followed by an isocratic elution with 100% B for 16 min. Online pigment analysis was carried out using an ion trap mass spectrometer HCT Ultra PTM discovery system from Bruker Daltonics (Bremen, Germany). The MS parameters were as follows: positive ion mode, dry gas N_2, MS/MS fragmentation energy 0.4V (when MS/MS analyses were used); mass spectra were acquired with a scan range of *m*/*z* from 300 to 1000. The pigments were identified on the basis of retention times, *m*/*z* values, and specific fragmentation ions from the corresponding standards. Violaxanthin, antheraxanthin, and lutein standards were purchased from DHI (Horsholm, Denmark); zeaxanthin was obtained from Sigma-Aldrich. Pigment quantification was performed by integration of the areas for extracted ion chromatograms at *m*/*z* 601 (violaxanthin), 585 (antheraxanthin), and 569 (zeaxanthin). The xanthophylls cycle deepoxidation ratio was calculated as (antheraxanthin + zeaxanthin)/(antheraxanthin + zeaxanthin + violaxanthin).

Microarray Analysis. For short-term light treatments, 4-week-old plants, grown under 120 μmol of photons m^{-2} s^{-1} at 20 °C, were shifted 3 h after the lights were turned on either to high light intensity of 500 μmol of photons $m^{-2}s^{-1}$, to darkness, or kept in growth light at 20 °C for 3 h. Global changes in gene expression were explored with spotted *Arabidopsis* 24k oligonucleotide arrays (MWG Biotech; http://www.mwg-biotech.com; ArrayExpress database accession number A-ATMX-2; http://www.ebi.ac.uk/arrayexpress). Twenty-five rosettes of wild-type and TSP9 mutant plants were collected, and total RNA was isolated with Trizol as described previously (*25*). Subsequently, DNA was removed with the Qiagen RNeasy mini kit, and cDNA synthesis was performed in the presence of 0.2 mM aminoallyl-dUTP using anchored poly(dT) primer (Oligomer, Finland) and the reverse transcriptase SuperScript III (Invitrogen). The aminoallyl-labeled cDNA was purified with the QIAquick PCR purification kit (Qiagen) and stained with CYTM postlabeling reactive dye pack (Amersham). cDNA corresponding to 15 pg of each dye was hybridized to the arrays in 50% formamide, 5 × SSC, 0.1% SDS, 0.1 mg/mL herring sperm, and 5 × Denhart's at 42 °C overnight. The arrays were scanned with an Agilent scanner (http://agilent.com), and the spot intensities were quantified with ScanArray Express Microarray Analysis system 2.0 (Perkin-Elmer Life Sciences, http://las.perkinelmer.com/). The data from

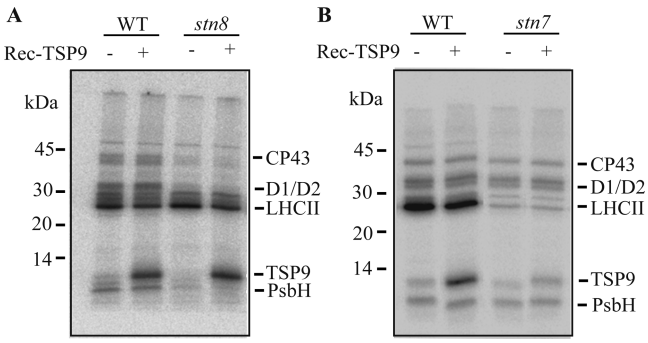

FIGURE 1: Phosphorylation of recombinant TSP9 protein in the presence of thylakoids from *stn8*, *stn7*, and wild-type *Arabidopsis* plants. Thylakoid membranes were illuminated for 45 min at room temperature in the presence of [γ-^{32}P]ATP with (+) or without (−) addition of recombinant TSP9 protein (Rec-TSP9). The proteins were separated by SDS−/PAGE and subjected to phosphorimaging. (A) Autoradiogram of ^{32}P-labeled thylakoids isolated from wild-type (WT) or *stn8* mutants (*stn8*). (B) Autoradiogram of ^{32}P-labeled thylakoids isolated from wild-type (WT) or *stn7* mutants (*stn7*).

three independent biological experiments (each experiment being a mean of three hybridizations on the arrays) from each three conditions (growth light, high light, and darkness) was analyzed with GeneSpring 7.2. Student's *t* test was used to analyze the data. Gene annotation was derived from *Arabidopsis* Information resource (http://www.arabidopsis.org) and was based on TAIR7.

RESULTS

STN7 Protein Kinase Is Involved in the Light-Induced Phosphorylation of TSP9. The two homologous protein kinases STN7 and STN8 have been found to be responsible for the light-dependent phosphorylation of several proteins in chloroplast thylakoid membranes of *Arabidopsis* (*9, 12, 19*). STN7 is required for state transitions (*12*) and phosphorylation of LHCII and CP29 (*9*), while STN8 is required for light-dependent phosphorylation of PSII core proteins (*19*). To find which protein kinase is involved in the light-induced phosphorylation of TSP9, we used the earlier characterized *Arabidopsis* mutant lines deficient either in STN7 (*9*) or in STN8 (*19*) kinase. We also obtained recombinant *Arabidopsis* TSP9 protein and assayed its light-dependent phosphorylation by radioactive ATP in the presence of thylakoid membranes isolated from wild-type and mutant plants. The autoradiograms in Figure 1 show the representative results of these *in vitro* phosphorylation experiments. The recombinant TSP9 appears as one of the major proteins phosphorylated under illumination by the thylakoid membranes from wild-type plants. The position of this phosphoprotein on the autoradiogram matched the exact position of the recombinant TSP9 in the corresponding gels stained with Coomassie blue. Figure 1A shows a significant reduction in phosphorylation of D1, D2, CP43, and PsbH proteins of PSII in the thylakoids lacking STN8 protein kinase. However, the loss of STN8 did not affect the level of LHCII phosphorylation, and importantly, it did not change the phosphorylation of recombinant TSP9 protein as compared to the wild type. In the thylakoids from plants lacking STN7 kinase the phosphorylation level of LHCII was greatly reduced, but that of D1, D2, CP43, and PsbH proteins was unaffected (Figure 1B). Notably, the absence of STN7 protein kinase resulted in significant reduction in phosphorylation of recombinant TSP9 (Figure 1B). The endogenously phosphorylated protein still visible in the thylakoids from STN7-deficient plants (Figure 1B) was also observed in similar experiments performed with the thylakoids from TSP9-knockout plants (data not shown) and consequently did not correspond to TSP9. Accordingly, the results of the *in vitro* phosphorylation assays with recombinant TSP9 and isolated thylakoid membranes show that the STN7 protein kinase is involved in light-dependent phosphorylation of TSP9.

Characterization of TSP9 Knockout Mutant. To study the function of the TSP9 protein in *Arabidopsis*, we obtained an *Arabidopsis* line with a T-DNA insertion disrupting the expression of the protein. The original T-DNA transformed GABI line with the insertion in the promoter region of the TSP9 gene locus At3g47070 was self-crossed to get homozygous seeds. Figure 2A shows a schematic drawing of the T-DNA insert. The homozygous plants were selected on the basis of the results of PCR on genomic DNA using the corresponding primers, positions of which are shown in Figure 2A. Figure 2B confirms the selection of homozygous plants: use of gene-specific primers flanking the TSP9 gene sequence results in a product of 1250 bp, while the use of gene-specific primer 1 and T-DNA left border primer 3 yields a PCR product of 833 bp. Total leaf protein extract was also prepared and analyzed by immunoblotting using an antibody against *Arabidopsis* TSP9. Figure 2C shows the absence of TSP9 protein in the mutant plants. As a control for equal loading on the gels the same protein extracts were assayed with an antibody against thylakoid immunophilin AtCYP38 (*26*) (Figure 2C). The mutant plants lacking TSP9 had no apparent phenotypical differences from the wild-type plants when grown under normal or high light conditions. The chlorophyll *a/b* ratios in the thylakoid extracts from the mutant and wild-type plants were also similar (3.3 ± 0.07).

The effect of TSP9 deficiency on the phosphorylation of the major thylakoid proteins was analyzed by Western blotting with two different commercial anti-phosphothreonine antibodies (Figure 2D). Thylakoid membranes from both wild-type and *tsp9* mutant were isolated either from dark-adapted plants (15 h in darkness) or light-exposed plants (4 h in light). Quantification of the phosphorylation level for thylakoid membrane proteins showed that the phosphorylation of CP43 was not affected by the loss of TSP9 and that of D1 and D2 proteins was only slightly reduced in the mutant plants. Phosphorylation of LHCII, on the other hand, was significantly lower in TSP9 lacking thylakoids in comparison with those from wild-type plants. The phosphorylation level for LHCII was found to be $37 \pm 16\%$ lower in darkness and $22 \pm 14\%$ lower in light-exposed mutants compared to the wild-type thylakoids (results from seven immunoblotts for each of the two anti-phosphothreonine antibodies). The TSP9-deficient mutant and the wild-type plants did not differ in the expression of the major LHCII proteins Lhcb1 and Lhcb2 (Figure 2E), which confirmed the reduction in phosphorylation of LHCII, but not in the expression of these proteins in the *tsp9* mutant. Microarray analyses also showed that the TSP9 mutant was not affected in the expression of LHCII genes (see below).

Differential Gene Expression Microarray Analysis. TSP9 has earlier been suggested as a potential mediator protein in light-induced cell signaling from the plant photosynthetic membranes (*5, 17, 27*). To probe this hypothesis experi-

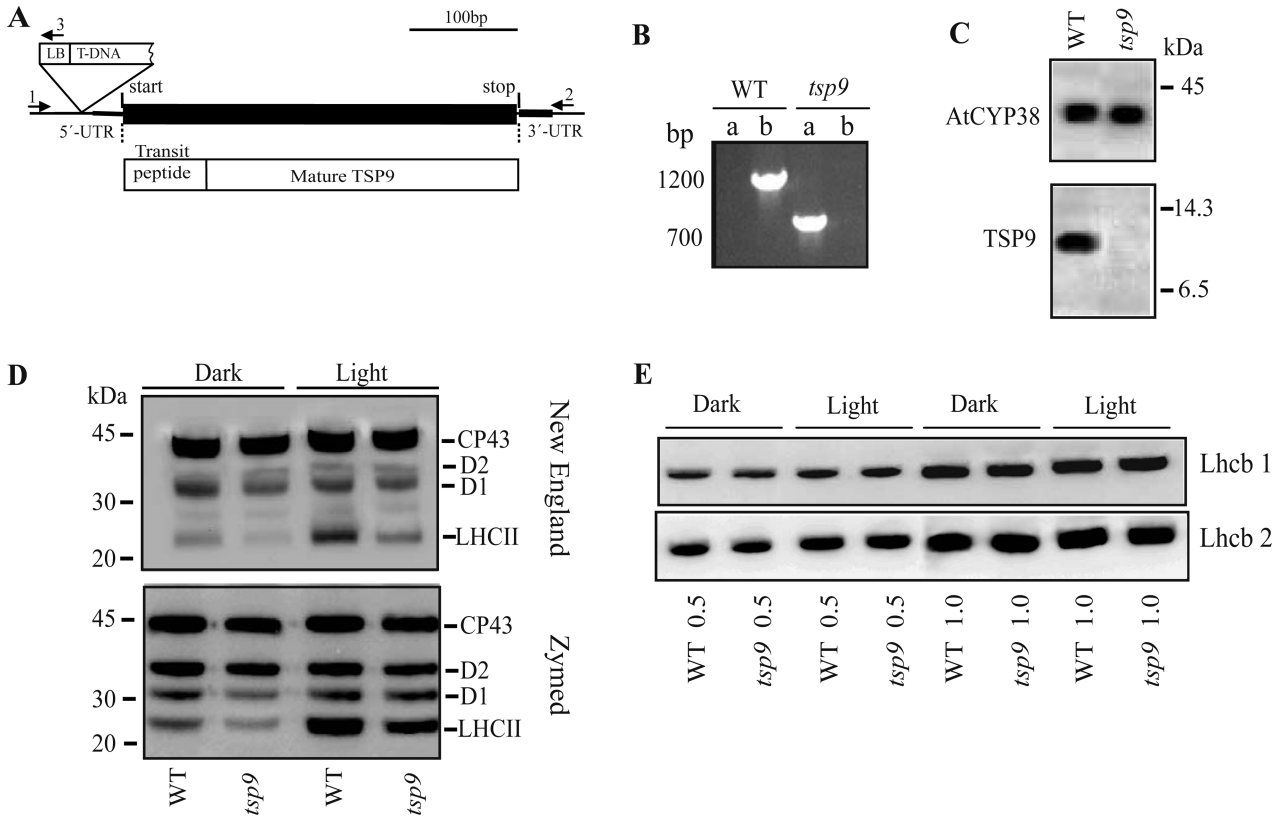

FIGURE 2: Characterization of the homozygous *tsp9* mutant. (A) Schematic figure showing the localization of the T-DNA insert in the At3g47070 gene promoter region. The primers used for PCR analysis are marked 1 (gene specific), 2 (gene specific), and 3 (T-DNA left border). (B) Ethidium bromide stained agarose gel with PCR products from genomic DNA isolated from homozygous mutant plants in comparison to wild-type plants. a, T-DNA left border and gene-specific primers 1 + 3; b, gene-specific primers 1 + 2. (C) Immunoblot of thylakoid membrane proteins separated by SDS−PAGE from wild-type (WT) and mutant (*tsp9*) plants. The upper blot was incubated with AtCYP38-specific antibody as a loading control, and the lower blot was incubated with TSP9-specific antibody, as indicated. (D) Immunoblot analysis of phosphorylation of thylakoid membrane proteins (0.5 μg of chlorophyll loaded in each well) from wild-type and mutant plants after 15 h of darkness (Dark) or 4 h of light (Light). The blots were incubated with phosphothreonine-specific antibodies from New England Biolabs or Zymed Laboratories Inc., as indicated. (E) Immunoblots of thylakoid membrane proteins as in (D) but with a use of specific antibodies against Lhcb1 or against Lhcb2, as indicated. The numbers 0.5 or 1.0 indicate sample amounts in μg of chlorophyll loaded in each well.

mentally, we analyzed the difference in gene expression between the *tsp9* mutant and wild-type plants using spotted *Arabidopsis* 24k oligonucleotide arrays (*25*). The total mRNA was extracted from plants exposed to growth light, high light, or darkness. We found that 54 genes in total had significantly different expression (p-value <0.05) in the TSP9 mutant versus wild-type plants when results from all three environmental conditions were combined (Supporting Information Table S1). These genes can be regarded as specific for the *tsp9* mutant. Importantly, all genes considered specific for TSP9 were expressed at lower levels as compared to the wild type. High light exposure of plants for 3 h revealed a reproducible and statistically significant additional difference between the *tsp9* mutant and wild type in the expression of a specific set of genes (Table 1). Only those genes for which the p-value calculated from three independent high light exposure experiments was 0.1 or less were considered. We found that these genes, which were not upregulated at high light in the *tsp9* mutant at a difference with the wild type, encode a number of signaling proteins (calcineurin, Ca-calmodulin, 14-3-3, C2 domain protein, different transcription factors, phosphatases, etc.) and also several unknown/hypothetical proteins.

TSP9 Deficiency Distorts Photosynthetic State Transitions. The balancing of the light-harvesting capacities of PSI and PSII is achieved by state transitions (*10, 28*) operating via redox activation of the protein kinase STN7 (*12*). To assay the capacity of state transitions in the *tsp9* mutant, we used the earlier developed setup (*10*). We analyzed state 1 to state 2 transitions in detached leaves of wild-type plants and plants lacking TSP9 or the STN7 protein kinase (*9*), as well as in leaves of the *npq4* mutant, which lacks the PsbS protein (*22*). PsbS is known to be involved in the nonphotochemical quenching (NPQ) of excess light absorbed by the thylakoid membranes, but not in the process of state transitions. Chlorophyll fluorescence was measured in dark-adapted leaves, and first the maximum fluorescence signal, F_m, was determined (Figure 3A). PSII light (blue light) was then applied to the leaf, and the steady-state fluorescence level was reached as photochemical and nonphotochemical quenching increased. The maximal fluorescence in state 2 (F_m^2) was determined. By applying far-red light, which favors PSI, in addition to the blue light the transition to state 1 was induced, and the maximal fluorescence in state 1 (F_m^1) was determined. The measurements of state transitions were expressed as the F_r parameter, which is the relative change in fluorescence when the PSI light is switched on in state 2 and turned off in state 1. It is calculated as $F_r = [(F_i' - F_i) - (F_{ii}' - F_{ii})]/(F_i' - F_i)$ (Figure 3A). State transitions can also be quantified by the difference between F_m^1 and F_m^2,

Table 1: Genes Found To Be Downregulated (<0.5×) in the *tsp9* Mutant after 3 h of High Light Treatment As Compared to Wild-Type Plants[a]

gene locus	change	*p*-value	description	Chloro P	Target P
At3g47070	0.039	0.0014	At3g47070, expressed protein (TSP9)		
At3g10880	0.485	0.087	hypothetical protein		
At2g16460	0.477	0.086	expressed protein	chloroplast	mitochondrion
At3g18640	0.483	0.094	zinc finger protein-related		
At3g52290	0.495	0.075	calmodulin-binding family protein		
At2g34570	0.498	0.106	expressed protein, contains Pfam profile	chloroplast	chloroplast
At1g48000	0.495	0.092	Myb family transcription factor		
At2g20875	0.493	0.084	expressed protein	chloroplast	chloroplast
At3g24860	0.458	0.089	hydroxyproline-rich glycoprotein family protein	chloroplast	chloroplast
At1g44180	0.469	0.087	aminoacylase, putative		secretion
At2g25260	0.401	0.066	expressed protein		secretion
At4g33890	0.489	0.072	expressed protein		
At4g23000	0.477	0.098	calcineurin-like phosphoesterase family protein		
At1g26480	0.488	0.105	14-3-3 protein GF14 iota (GRF12)		
At1g05630	0.467	0.105	inositol polyphosphate 5-phosphatase		
At5g05050	0.490	0.072	peptidase C1A papain family protein		
At4g19550	0.484	0.073	expressed protein		mitochondrion
At4g31250	0.465	0.077	leucine-rich repeat transmembrane protein kinase		secretion
At4g15340	0.475	0.090	pentacyclic triterpene synthase (04C11)		mitochondrion
At1g05000	0.454	0.072	tyrosine-specific protein phosphatase		
At1g03000	0.469	0.071	AAA-type ATPase family protein		mitochondrion
At4g23330	0.458	0.102	eukaryotic translation initiation factor-related		
At4g28365	0.483	0.107	plastocyanin-like domain-containing protein		secretion

[a] Results are means from three independently grown and high light (500 μmol of photons m^{-2} s^{-1}) treated sets of plants. The change values indicate the expression level in the *tsp9* mutant relative to that in wild type. The cellular localization of the corresponding proteins predicted by Chloro P (*44*) and Target P (*45*) programs is indicated in the corresponding columns.

but the F_r parameter is more robust and less influenced by other factors such as NPQ and photoinhibition. For the state transitions in wild type (Figure 3A) a value of 0.65 ± 0.05 was determined, while for the *stn7* mutant (Figure 3C) the value was -0.01 ± 0.02. The latter significantly lower F_r value is in agreement with the fact that STN7 is responsible for the induction of the state transitions (*12*). When the state transitions were measured in the *tsp9* mutant (Figure 3B), an intermediate value of 0.36 ± 0.04 was obtained, showing the reduced capacity for state transitions in the TSP9-deficient plants. Control measurement on the *npq4* mutant showed no difference from the wild type in the performance of state transitions (Figure 3F). State transitions were also measured in several independent RNAi plants with TSP9 protein undetectable by immunoblotting: in contrast to the wild type, no TSP9 was detected in the RNAi plant when proteins from thylakoid samples containing from 0.5 to 3 μg of chlorophyll were analyzed (Figure 3D). Figure 3E shows representative data obtained with RNAi plants. For the state transitions in the RNAi plants the F_r value of 0.30 ± 0.04 was determined (Figure 3F). These results demonstrated that RNAi plants with a significantly reduced amount of TSP9 exhibited a decrease in state transitions identical to that observed in the *tsp9* mutant (Figure 3F) and confirmed that TSP9 deficiency distorts photosynthetic state transitions.

TSP9 Deficiency Reduces NPQ. To compare the light-dependent NPQ process in mutant and wild-type plants, we used a standard setup (*24*) for the determination of the chlorophyll fluorescence. As illustrated in Figure 4A, the F_0 (minimum fluorescence in the dark-adapted state) and F_0' (minimum fluorescence in the light-adapted state) parameters were determined. Application of a saturating flash of light allowed measurement of the maximum fluorescence level F_m. Actinic light was applied at three different intensities: 150 μmol of photons m^{-2} s^{-1} (Figure 4A), 300 μmol of photons m^{-2} s^{-1} (data not shown), and 900 μmol of photons m^{-2} s^{-1} (Figure 4B). Another saturating pulse allowed measurement of the maximum fluorescence under illumination (F_m'). NPQ was calculated as $NPQ = (F_m - F_m')/F_m'$. This parameter was measured in leaves from wild type, *tps9* mutant, and *npq4* mutant lacking PsbS protein. In the latter mutant NPQ and particularly the fast quenching component qE that is observed in the first few seconds of illumination of dark-adapted leaves are largely absent (*22, 29*). The dependence of NPQ on the light intensities in these plant species is shown in Figure 4C. The NPQ value in the *npq4* mutant was extremely low at all light intensities (Figure 4C), as was expected (*22*). In the *tsp9* mutant we observed a reduction in the NPQ performance in comparison with the wild type at the high light intensity (Figure 4C). There was a clear difference between wild-type and TSP9-lacking plants in performing NPQ at 900 μmol of photons m^{-2} s^{-1}: wild type, 2.0 ± 0.3 ($\pm$SD, $n = 3$); TSP9, 1.3 ± 0.2 ($\pm$SD, $n = 3$). Using a portable PAM-2000 instrument for leaf fluorescence measurements (eight independent experiments for the mutant and wild-type plants), we also observed statistically significant reduction of NPQ in the *tsp9* mutant plants in comparison with wild-type plants at a light intensity of 800 μmol of photons m^{-2} s^{-1} (data not shown). To check that reduction of NPQ in the *tsp9* mutant was not due to reduced expression of PsbS, we assayed the content of this protein using a specific antibody. The immunoblots of the thylakoid samples containing from 0.6 to 2.2 μg of chlorophyll did not reveal any difference in the level of PsbS protein between the mutant and wild-type plants irrespective of the light conditions (Figure 4D and data not shown). We also examined if the reduction of NPQ in *tsp9* under high light was a consequence of the change in xanthophyll cycle, namely, in the deepoxidation state of violaxanthin. To this end we extracted pigments from wild-type and *tsp9* plants exposed to either growth or high light and analyzed the relative ratios of violaxanthin, antheraxanthin, and zeaxanthin in these samples. The results shown in Figure 4E demonstrate no significant difference between the xanthophylls cycle

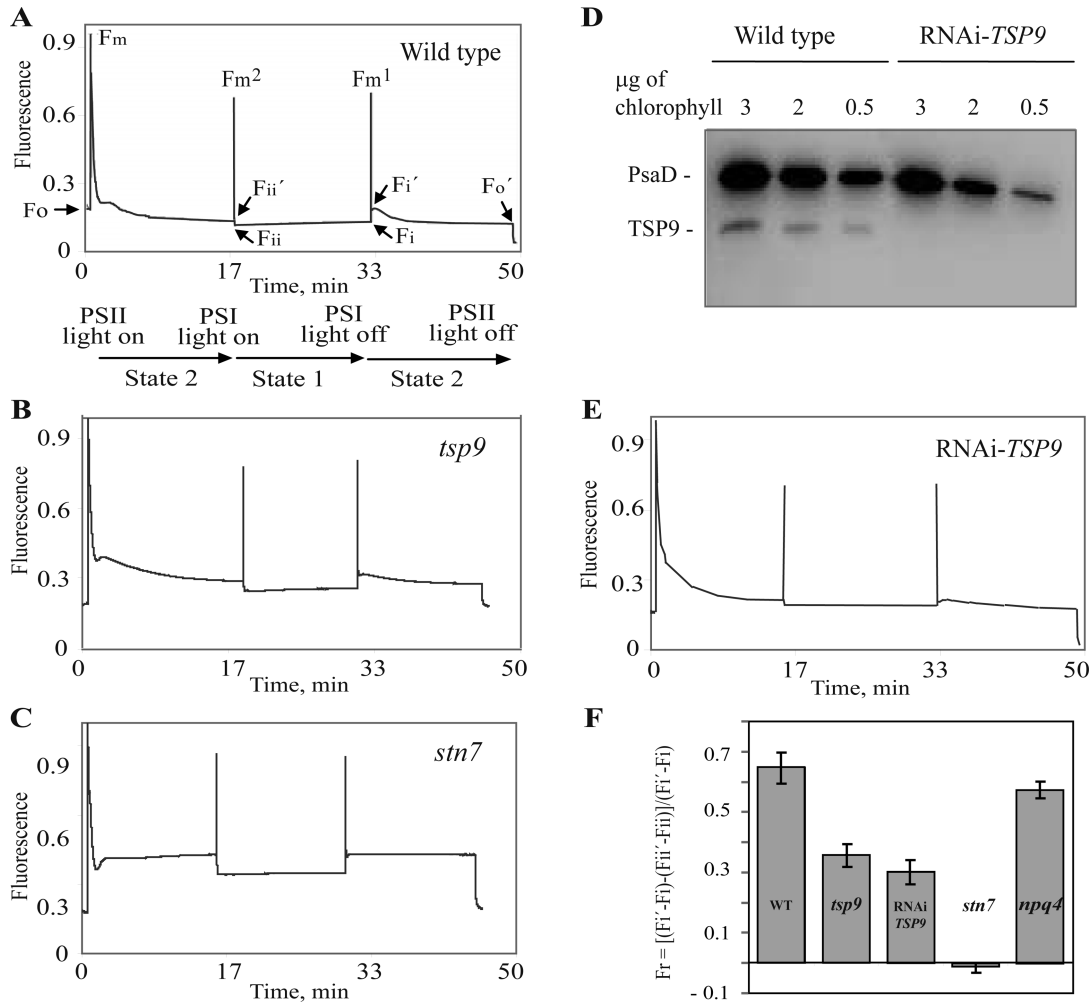

FIGURE 3: Changes in PSII chlorophyll fluorescence during state transition. (A) A trace of PSII fluorescence changes in wild type with indicated fluorescence parameters. The maximal PSII fluorescence (F_m) is determined in dark-adapted leaves and in state 1 (F_m^1) and state 2 (F_m^2). State transitions are represented by the relative changes in fluorescence when the PSI light (far-red light) is turned on or off. PSII light (blue light) is on continuously. The relative fluorescence change is calculated as $F_r = [(F_i' - F_i) - (F_{ii}' - F_{ii})]/(F_i' - F_i)$, with F_i, F_i' and F_{ii}, F_{ii}' parameters indicated on the fluorescence trace. (B) Fluorescence trace from the *tsp9* mutant. (C) Fluorescence trace from the *stn7* mutant. (D) Immunoblot of thylakoid membrane proteins separated by SDS−PAGE from wild-type and RNAi-*TSP9* plants with the indicated amounts of chlorophyll loaded in each well. The upper band was detected with PsaD specific antibody as a loading control, while the lower band was detected with TSP9-specific antibody, as indicated. (E) Fluorescence trace from the RNAi-*TSP9* plant. (F) Comparison of state transition values, F_r, for wild-type, *tsp9*, RNAi-*TSP9*, *stn7*, and *npq4* mutant plants. The values are averages ± SD of five independent fluorescence measurements for each of the genotypes. The F_r values for *tsp9* and RNAi-*TSP9* are not significantly different from each other but differ significantly from that for wild type ($p = 0.001$).

deepoxidation ratios determined for the mutant and wild-type plants. Thus, we conclude that TSP9 deficiency in *Arabidopsis* plants causes reduced performance of both state transitions and NPQ.

TSP9 Affects Stability of PSII−LHCII Supercomplexes. To investigate the possible effect of TSP9 depletion on the organization of the major protein complexes in the thylakoid membrane, we employed analyses by blue native (BN) electrophoresis, which has been successfully used in studies of thylakoid membranes (*30*) and particularly in the localization of TSP9 in the thylakoid membranes from spinach (*16*). 2D gel analysis of several independent membrane preparations revealed a reproducible difference between wild-type and mutant thylakoids in a subset of protein complexes. The membranes from the *tsp9* mutant showed markedly increased PSII dimer/monomer ratios compared to wild type (Figure 5A). Moreover, the relative amounts of PSII−LHCII supercomplexes and of "LHCII assembly", containing major LHCII polypeptides together with PSII linker proteins CP29, CP26, and CP24, were also increased in *tsp9*. The relative difference in the intensities of these protein complexes in thylakoid membranes from wild type and *tsp9* is clearly seen in Figure 5B, presenting blue native gels from three independent experimental preparations. The observed changes can reflect a reorganization within the thylakoid membrane resulting in tighter association of PSII with LHCII and in the increased stability of the PSII−LHCII supercomplexes and PSII dimers caused by the absence of TSP9.

DISCUSSION

In this work we studied the plant-specific phosphoprotein TSP9 in *A. thaliana*. The gene for this protein in *Arabidopsis* (locus At3g47070) was identified in the genome sequencing project and annotated as a gene for "unknown" protein because of lack of homology to any classified protein domain. TSP9 was then characterized in spinach thylakoids as a strongly basic protein that becomes triply phosphorylated in

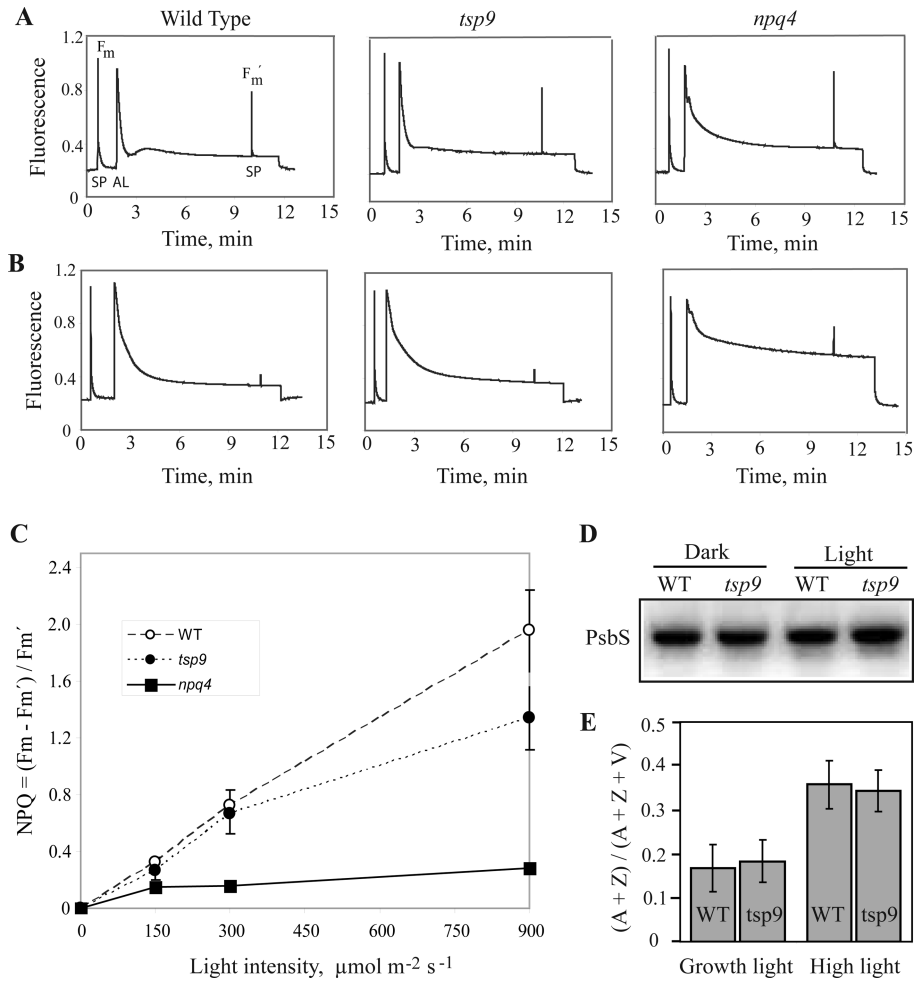

FIGURE 4: Nonphotochemical quenching analyses. (A, B) Representative traces of PSII fluorescence changes in the leaves of wild-type plants and mutants lacking TSP9 (*tsp9*) or PsbS protein (*npq4*), as indicated. (A) Fluorescence traces at light intensity of 150 μmol m^{-2} s^{-1}. A saturating pulse (SP) allows measurement of the maximum fluorescence level (F_m). Actinic light to drive photosynthesis (AL) is applied, and after a period of time when steady state is reached another saturating light flash (SP) allows maximum fluorescence in the light (F_m') to be measured. (B) Fluorescence traces at light intensity of 900 μmol m^{-2} s^{-1}. (C) Dependence of NPQ on light intensities in the leaves of wild type and *tsp9* and *npq4* mutants, as indicated. (D) Representative Western blot analysis of the PsbS protein content in thylakoid membranes from dark- or light-adapted leaves of wild-type plants and *tsp9* mutant, as indicated. Thylakoid proteins were separated by SDS−PAGE and immunoblotted using PsbS-specific antibody. (E) The xanthophylls cycle deepoxidation ratios (A + Z)/(A + Z + V), where A is antheraxanthin, Z is zeaxanthin, and V is violaxanthin, determined after pigment extraction from the leaves of wild-type and *tsp9* mutant plants. The plants were exposed to either growth light (120 μmol of photons m^{-2} s^{-1}) or high light (900 μmol of photons m^{-2} s^{-1}), as indicated. The values are averages ± SD of three independent pigment extract characterizations for each of the genotypes and each light condition.

the middle of its sequence when the thylakoids are exposed to light (*5*). The previous studies of TSP9 have not revealed its biochemical function or its physiological role. Analysis of publicly available microarray data using the Genevestigator database (https://www.genevestigator.ethz.ch/at/) shows that TSP9 in *Arabidopsis* is expressed only in the green photosynthetic tissues. Furthermore, TSP9 is differentially expressed under different light stresses, which points to a specific role of TSP9 in photosynthesis, in good agreement with the plant-specific occurrence of the protein.

Structural analyses of spinach TSP9 by NMR spectroscopy revealed that it is an unstructured protein disordered under aqueous conditions but adopting a partially ordered conformation in the presence of detergent micelles (*18*). The micelle-induced structural features of TSP9 consist of an N-terminal α-helix and a less structured helical turn near the C-terminus. These structured elements contain mainly hydrophobic residues, which may represent the primary modules for interaction with membrane protein partners, while the rest of the TSP9 molecule is highly flexible either in the presence or in the absence of the micelles (*18*). Analyses of all known sequences of TSP9 orthologues from different plant species and NMR spectroscopy study of spinach TSP9 unambiguously place this protein in a family of intrinsically disordered/unstructured proteins (*31, 32*). These proteins containing long regions lacking fixed structure are found to occur in 2% of archaebacterial, 4% of eubacterial, and 33% of eukaryotic proteins (*33*). In eukaryotic cells intrinsic disorder dominates in proteins associated with signal transduction, cellular regulation, and assembly of large macromolecular complexes (*31, 32*).

Using the *Arabidopsis* mutants deficient in the light-regulated protein kinases STN7 and STN8 in the present work, we show that TSP9 is not phosphorylated by thylakoids lacking STN7 kinase. The STN7 protein kinase is required for state transitions (*12*) and for the phosphorylation of LHCII and CP29 (*9*), while the STN8 kinase phosphorylates proteins of the PSII core (*19*). We conclude that STN7

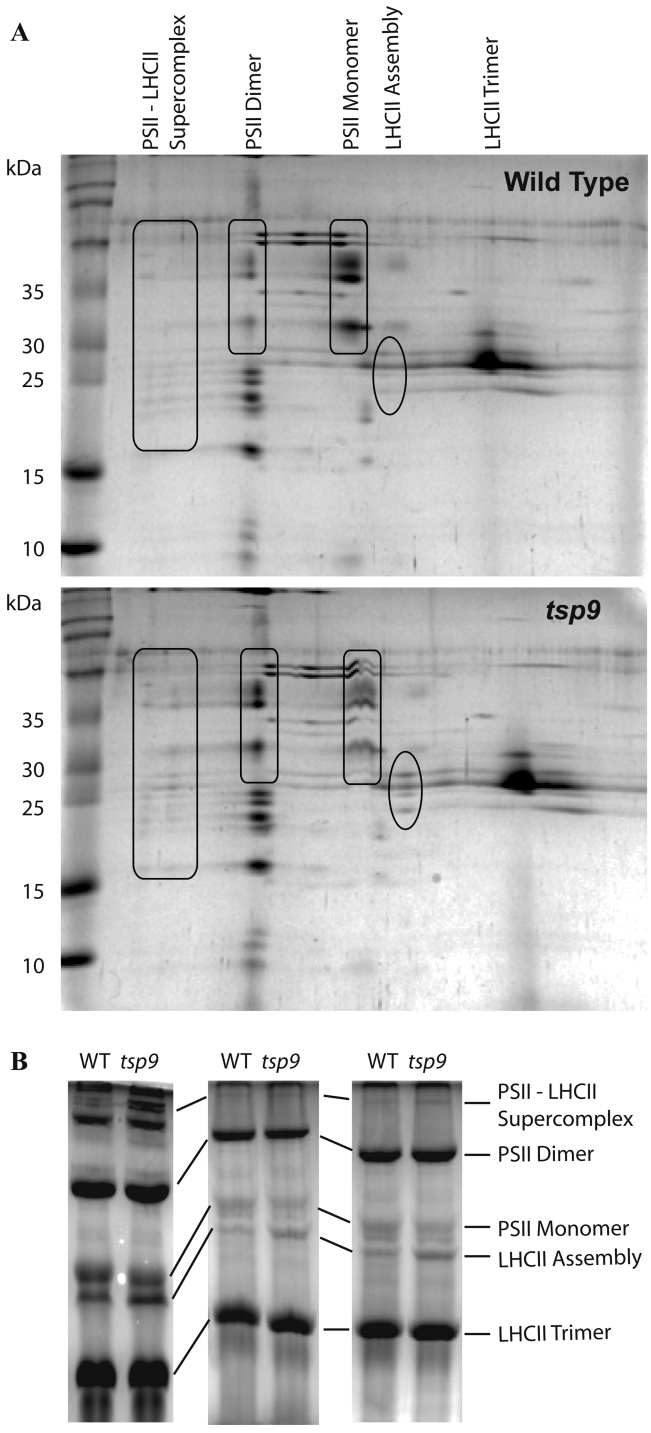

FIGURE 5: Comparative analyses of PSII and LHCII complexes in thylakoid membranes of wild-type and *tsp9* mutant plants. (A) Two-dimensional gel analysis of thylakoid protein complexes from wild-type and *tsp9* plants. Thylakoids were solubilized with 1% *n*-dodecyl β-D-maltoside and separated by native BN electrophoresis in the first dimension. Lanes were cut out and subjected to denaturing SDS−PAGE in the second dimension. The gels were stained with Coomassie blue. The positions of the major protein complexes verified by immunoblotting are indicated for PSII−LHCII supercomplexes, PSII dimers, PSII monomers, "LHCII assembly", containing major LHCII polypeptides together with PSII linker proteins CP29, CP26, and CP24, and LHCII trimers. (B) BN electrophoresis gels of three independent thylakoid membrane preparations from wild-type (WT) and *tsp9* (*tsp9*) plants. The positions of the major protein complexes are indicated as in (A).

is also required for phosphorylation of TSP9. It has been shown that lack of STN7 results in differential expression of some genes (*9, 34*), which, however, did not overlap with those found downregulated in our present study. It was also suggested that TSP9 could be a part of a signaling cascade induced by light-dependent thylakoid protein phosphorylation, which causes a partial release of TSP9 from the photosynthetic membranes (*5*). Our results from the differential gene expression study identify a specific set of mostly signaling or unknown/hypothetical proteins that are not upregulated in high light in the mutant lacking TSP9. These data could provide a direction for the elucidation of a light-dependent signal transduction pathway from plant photosynthetic membranes. The changes in gene expression could be due to TSP9 itself being involved in this signaling pathway or due to an interaction between TSP9 and the STN7 kinase. We also found reduced LHCII phosphorylation in plants lacking TSP9, which may indicate that either TSP9 is important for a proper interaction between LHCII and STN7 kinase or at some stage TSP9 is involved in the regulation of LHCII phosphorylation.

The fluorescence measurements on plants lacking TSP9 or downregulated in TSP9 expression clearly show a decrease in the capacity to perform photosynthetic state transitions, even if it is not as dramatic as in the case of the STN7 kinase mutant. A lack of TSP9 could affect the process of state transitions at several levels. The lowering of LHCII phosphorylation in the plants lacking TSP9 is one factor; another is the possible role of TSP9 as a linker protein between LHCII and PSII. In *C. reinhardtii* exposed to state 1 or state 2 the linker protein CP29, which connects LHCII to PSII (*35*), was found doubly or quadruply phosphorylated, respectively (*6, 36*). Moreover, in state 2 the phosphorylated CP29 migrated to PSI together with the mobile LHCII (*36*), and it was suggested that in green algae multiple protein phosphorylation at the interface of PSII and LHCII causes migration of the latter to PSI (*1, 6*). In plants this mechanism could differ because the state transitions are significantly less pronounced than in green algae (*2, 37*). Moreover, only a single site of phosphorylation has been found in CP29 from *Arabidopsis* (*38*), opening the possibility for a different mechanism. The multiply phosphorylated TSP9 protein in spinach thylakoids has been localized at the interface of LHCII and PSII (*16*). TSP9 migrated together with LHCII upon separation of thylakoid protein complexes by either native gel electrophoresis or sucrose gradient centrifugation. Studies with a cleavable cross-linking agent revealed the interaction of TSP9 with both major and minor LHCII proteins (*16*). Some of the cross-linked complexes contained in addition to TSP9 peripheral PSII subunits CP29, CP26, and PsbS, which comprise the interface between LHCII and the PSII core. Notably, the interaction of TSP9 with the peripheral PSI subunits PsaL, PsaF, and PsaE was also found, and this interaction was increased upon light-induced phosphorylation of spinach TSP9 (*16*). Thus, multiple phosphorylation of TSP9 could contribute to plant-specific state transitions via decreasing the docking of TSP9−LHCII to PSII and migration of the TSP9−LHCII complex to PSI.

The plants lacking TSP9 also showed a decreased capacity to perform NPQ at high light intensity, while this effect was not as dramatic as in the *npq4* mutant. The exact mechanism for the process of NPQ is not completely known, but the transthylakoid ΔpH is an important factor, as well as the PSII antenna protein PsbS (*39, 40*). The recently published

data (*41*) provided evidence that PsbS controlled the organization of PSII and its association with LHCII. The protonation of PsbS resulting from the ΔpH under NPQ was proposed to induce PsbS detachment from PSII along with LHCII. Notably, the cross-linking experiments revealed the interaction of TSP9 with PsbS, as well as with other peripheral subunits of PSII in spinach (*16*). In the present work we observed distinct changes in the organization and/or stability of PSII complexes in the TSP9-deficient plants, which could be the structural basis for the distortions in both NPQ and state transitions. The increase in LHCII−PSII supercomplexes in the *tsp9* mutant could be a direct consequence of a lower ability of LHCII to dissociate from PSII when TSP9 is not present. The dimer to monomer transitions of PSII also require the detachment of at least part of LHCII (*42*), and consequently, a greater stability of PSII supercomplexes affects these transitions as well. Our data show that TSP9 is involved in adjustments of plant light harvesting to changing quality and quantity of light, and the mechanism of TSP9 action is most probably consisting of facilitation of LHCII dissociation from PSII.

TSP9 has been identified in many different plants, but surprisingly, no similar genes were found in the sequenced genomes of cyanobacteria or green algae. TSP9 could be a lately evolved protein which is specifically involved in the regulation of the photosynthetic light-harvesting process in plants. It is an intrinsically unstructured protein (*18*). The functional benefits of unstructured proteins consist of a greater capture radius for specific binding sites, weak binding at relatively large distance followed by folding as the protein approaches the binding site (*43*), and an ensemble of alternative structures allowing binding to several partner molecules (*31*). In the specific case of TSP9 its hydrophobic N-terminal α-helix (*18*) formed upon binding to thylakoid membrane can intercalate between hydrophobic subunits of photosynthetic complexes facilitating their dissociation. Binding of TSP9 to LHCII and peripheries of PSII and PSI is light- and phosphorylation-dependent (*16*). Thus, we postulate that TSP9 regulates photosynthetic state transitions and NPQ in plants, facilitating dissociation of LHCII from PSII and affecting dynamic interactions of LHCII with PSII and PSI under changing ambient light.

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SUPPORTING INFORMATION AVAILABLE

One table with the list of differentially expressed genes in *tsp9* mutant versus wild-type plants exposed to three environmental conditions: growth light, high light, or darkness. This material is available free of charge via the Internet at http://pubs.acs.org.

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