Identification of Three Previously Unknown in Vivo Protein Phosphorylation Sites in Thylakoid Membranes of Arabidopsis thaliana*

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The proteins in plant photosynthetic thylakoid membranes undergo light-induced phosphorylation, but only a few phosphoproteins have been characterized. To access the unknown sites of in vivo protein phosphorylation the thylakoid membranes were isolated from Arabidopsis thaliana grown in normal light, and the surface-exposed peptides were cleaved from the membranes by trypsin. The peptides were methylated and subjected to immobilized metal affinity chromatography, and the enriched phosphopeptides were sequenced using tandem nanospray quadrupole time-of-flight mass spectrometry. Three new phosphopeptides were revealed in addition to the five known phosphorylation sites in photosystem II proteins. All phosphopeptides are found phosphorylated at threonine residues implementing a strict threonine specificity of the thylakoid kinases. For the first time protein phosphorylation is found in photosystem I. The phosphorylation site is localized to the first threonine in the N terminus of PsAD protein that assists in the electron transfer from photosystem I to ferredoxin. A new phosphorylation site is also revealed in the acetylated N terminus of the minor chlorophyll a-binding protein CP29. The third novel phosphopeptide, composed of 25 amino acids, belongs to a nuclear encoded protein annotated as "expressed protein" in the Arabidopsis database. The protein precursor has a chloroplast-targeting peptide followed by the mature protein with two transmembrane helices and a molecular mass of 14 kDa. This previously uncharacterized protein is named thylakoid membrane phosphoprotein of 14 kDa (TMP14). The finding of the novel phosphopeptides extends involvement of the redox-regulated protein phosphorylation in photosynthetic membranes beyond the photosystem II and its light-harvesting antennae. Molecular & Cellular Proteomics 2:550–559, 2003.

Phosphorylation is one of the most common post-translational modifications of proteins, playing a major role in regulation of all cellular functions from gene expression to signaling and metabolic control. Phosphorylation of an array of proteins in plant photosynthetic thylakoid membranes occurs in a light-activated and redox-dependent manner and is implied in the adjustment of the photosynthetic reactions to the ambient light conditions (1, 2). The induction and alteration of thylakoid protein phosphorylation by light proceed on at least three different levels revealed for phosphorylation of the most abundant polypeptides of light-harvesting complex II (LHCCI).1 First, the membrane protein kinases are activated upon reduction of the membrane electron carrier plastoquinol and its binding to the quinol-oxidation site of a cytochrome bf complex, which serves as a redox sensor (3, 4). Second, phosphorylation is modulated by thiol disulfide redox state via the chloroplast ferredoxin-thioredoxin system (5, 6). Third, the light-induced conformational changes in the chlorophyll-binding membrane proteins expose their respective sites for phosphorylation (7, 8). The inverse dephosphorylation process of the thylakoid proteins by chloroplast protein phosphatases (9–11) is not directly regulated by light (12, 13). Reversible phosphorylation of LHCCI participates in the balancing of the absorbed light energy distribution between the two photosystems (14, 15). Phosphorylation of the minor chlorophyll a/b protein CP29 in chilling conditions has been associated with the resistance of plants to cold stress (16). Reversible phosphorylation of the photosystem II (PSII) reaction center proteins D1 and D2 controls their turnover in such a way that dephosphorylation by specific membrane protein phosphatase (11, 17) leads to proteolytic degradation of the worn-out D1 and D2 and replacement with the newly synthesized polypeptides (18, 19). The functions for phosphorylation of two other subunits of PSII, the chlorophyll a/b-binding protein CP43 and the 9-kDa PsbH gene product, are not yet understood.

The ultimate evidence for phosphorylation consists of identification of the phosphorylated amino acid residue(s) in a protein sequence. In thylakoids the phosphorylation sites have been revealed in the PSII polypeptides D1, D2, CP43, and PsbH (20–22), in four subunits of the major LHCCI complex (23), in the minor chlorophyll a/b protein CP29 (24), and in thylakoid-soluble phosphoprotein of 9 kDa (TSP9) with yet

1 The abbreviations used are: LHCCI, light-harvesting chlorophyll a/b complex II; TMP, thylakoid membrane phosphoprotein; TSP, thylakoid-soluble phosphoprotein; CID, collision-induced dissociation; IMAC, immobilized metal affinity chromatography; PSII and -I, photosystem I and II; Ac-, N-terminally acetylated.
unknown function (25). All of these proteins were found to be phosphorylated at threonine residues with the only exception being one LHCII polypeptide from spinach phosphorylated at a serine residue (23). Two distinct phosphorylation sites were found in PsbH (22, 26), and three were found in TSP9 (25). TSP9 is uniquely released from the membranes after the light-induced phosphorylation that could probably serve in the cell signaling (25). All other identified thylakoid phosphoproteins are integral membrane polypeptides with their phosphorylation sites exposed to the outer surface of thylakoids. Notably, the phosphorylated peptides could be “shaved” from the surface of thylakoids by trypsin (11, 22). Analysis of the released peptide mixtures by liquid chromatography-mass spectrometry allows for simultaneous determination of phosphorylation states for multiple membrane proteins by measuring the ratios of phosphopeptide to dephosphopeptide from each of them (22). This approach has revealed the levels of in vivo phosphorylation for four PSII proteins in thylakoid membranes from the leaves of Arabidopsis thaliana exposed to a subset of different physiological conditions (22). The liquid chromatography-mass spectrometry provides an unprecedented tool for unraveling the dynamic protein phosphorylation network in vivo. However, these analyses could be accomplished only for the proteins with the localized phosphorylation positions. Thus, comprehensive studies on phosphorylation in the photosynthetic membranes require identification of all natural phosphorylation sites in thylakoid proteins.

In this paper we report identification of the three previously unknown in vivo phosphorylation sites in thylakoid proteins from A. thaliana. We enriched the phosphopeptides from the tryptic thylakoid peptides by immobilized metal affinity chromatography (IMAC) and successfully sequenced them using tandem nanospray quadrupole time-of-flight mass spectrometry. These novel phosphorylation sites are found in CP29 and in PsA,D, making it the first phosphoprotein found in photosystem I, and in a previously uncharacterized thylakoid membrane protein annotated in the Arabidopsis genome project as “expressed protein.”

**EXPERIMENTAL PROCEDURES**

Plant Growth and Preparation of Thylakoid Membranes—A. thaliana (ecotype Wassilewskija) was cultivated hydroponically (27) for 12 weeks in a 10/14-h light/dark regime. The thylakoid membranes were isolated from the leaves harvested in the daytime (1.5 h after the light was turned on) essentially according to the protocol (28). The isolated thylakoids were washed twice with 10 mM sodium pyrophosphate (pH 7.8), then were washed twice with 30 mM sodium phosphate (pH 7.8), 5 mM MgCl₂, 50 mM NaCl, 100 mM sucrose, and finally were washed twice with 25 mM NH₄HCO₃.

Protein Digestion with Trypsin—The thylakoids were resuspended in 25 mM NH₄HCO₃ to a concentration of 6.5 mg of chlorophyll/ml and incubated with sequencing grade-modified trypsin (Promega) (3 μg of enzyme/mg of chlorophyll) at room temperature for 2 h. The digestion products were frozen, thawed, and centrifuged at 15,000 × g. The supernatant was collected while the membranes were resuspended in water and centrifuged again. Both supernatants containing released thylakoid peptides were pooled.

Isolation of Phosphorylated Peptides—The peptides released from the thylakoid surface were methylated as described by Ficarro et al. (29). Phosphopeptides were affinity-enriched from the methylated peptides by IMAC with the use of immobilized Fe(III) (30). The IMAC procedure was modified in such a way that Fe(III) ions were bound to 5 μl of chelating Sepharose (Amersham Biosciences) in microcolumns prepared in GELoader tips (Eppendorf). The beads were washed twice with 20 μl of 0.1% (v/v) acetic acid, charged with 100 μl of 0.1 M FeCl₃, and washed twice with 20 μl of 0.1% (v/v) acetic acid to remove unbound iron ions. The mixture of methylated thylakoid peptides (20 μl) was loaded onto the column, and nonspecific bound peptides were removed by two washes with 20 μl of 0.1% acetic acid in water and 20 μl of deionized water. The bound phosphopeptides were eluted by five washes with 10 μl of 20 mM Na₂HPO₄ (pH around 9.0). The five eluted fractions were collected separately, and each of them was desalted using C₁₈ ZipTip (Millipore).

Electrospray Ionization Tandem Mass Spectrometry—The spectra were acquired on a hybrid mass spectrometer API QSTAR Pulsar i (Applied Biosystems, Foster City, CA) equipped with a nanoelectrospray ion source (MDS Protea, Odense, Denmark). The nanoelectrospray capillaries were loaded with 2 μl of peptide solutions in 50% acetonitrile in water with 1% formic acid. The collision-induced dissociation (CID) of selected precursor ions was performed using the instrument settings recommended by Applied Biosystems.

**RESULTS**

Isolation of Phosphorylated Thylakoid Peptides—Mass spectrometry allows for identification of natural protein phosphorylation without use of exogenous tracers such as radioactive labels. Thus, we isolated the photosynthetic thylakoid membranes from the leaves of A. thaliana exposed to normal growth light. Protein phosphorylation in thylakoids is restricted to the outer surface-exposed regions of membrane proteins, which could be removed from the membranes by trypsin (11, 22, 31). Accordingly, we shaved the isolated thylakoids with trypsin to release the surface-exposed peptides and remove the hydrophobic segments of the proteins imbedded in the membranes by centrifugation (Fig. 1A). The mixture of the released hydrophilic peptides contains more than a thousand peptides with the phosphopeptides compromising only about 1% of them (22). The characterization of the major thylakoid phosphopeptides (Fig. 1B) has been done after prior enrichment by IMAC with immobilized Fe(III) and Ga(III) cations (22). However, these IMAC procedures did not allow identification of some minor phosphopeptides (22). To increase the efficiency of the IMAC in the present work we performed methylation of carboxylic groups (29) to lower the number of negatively charged groups and decrease unspecific peptide binding during the IMAC with the immobilized Fe(III). This IMAC technique allowed for better enrichment of phosphorylated peptides, although some non-phosphorylated peptides were still present in the fractions finally eluted with the phosphate buffer. These fractions were subjected to thorough analyses by nanospray quadrupole time-of-flight mass spectrometry. The survey spectra of the peptide molec-
ular ions in two fractions are shown in Fig. 1, C and D. Each peptide ion present in the fractions was subjected to CID. For identification of the phosphorylated peptides we exploited the low stability of a phosphoester bond between a phosphoryl group and a peptide upon collision-induced fragmentation. This property of the phosphoester bond causes a prominent neutral loss of phosphoric acid species HPO₃⁻ (80 Da) and H₃PO₄⁻ (98 Da) from the positively charged peptide ions containing phosphorylated residues (22, 32). According to the observed neutral loss of the phosphoric acid and the calculated molecular masses, the major ion signals in the spectra shown in Fig. 1, C and D, were ascribed to the known phosphopeptides. The sequences of these phosphorylated peptides shown in Fig. 1B were confirmed by CID analyses (data not shown). Besides the known phosphopeptides we were able to identify three previously uncharacterized phosphorylated peptides marked with the letters X and Y in Fig. 1C and with the letter Z in Fig. 1D. The masses of the methylated phosphopeptides X, Y, and Z were determined to be 947.4, 1682.8, and 2765.4 atomic mass units, respectively. For further characterization each of these phosphopeptides was de novo sequenced after its collision-induced fragmentation, and the amino acid sequences were used in the BLAST database search (33) for identification of the corresponding proteins.

Identification of the Phosphorylated N-terminal Peptide of CP29—For identification of the phosphopeptide X (Fig. 1C) with the mass 947.4 the doubly protonated molecular ion of this peptide with the m/z ratio of 474.7 ((947.4/2H₁⁺/H₁⁻)/2) was subjected to CID. The fragmentation spectrum shown in Fig. 2 contains a pronounced ion signal corresponding to the neutral loss of the phosphoric acid (H₃PO₄⁻, 98 Da) from the parent ion (m/z = 425.7, (947.4 + 2H⁺ – 98)/2)). This signal indicates the presence of the phosphorylated residue in the peptide. The clear series of b (N-terminal) and y (C-terminal) fragment ions (Fig. 2) revealed the complete peptide sequence, Ac-RFGFGtK, with t designating the phosphorylated threonine residue. Besides the phosphorylation the peptide was also N-terminally acetylated (Ac-). A search in the A. thaliana protein sequence database revealed that this sequence belonged to the N-terminal part of the LHCb4.2 precursor protein (gene with GenBank™/EBI accession number At3g08940), also called “PSII CP29 antenna protein.” A network-based tool TargetP (34) predicts the cleavage site of the chloroplast-targeting peptide after the amino acid residue 39 in the precursor protein. However, our experimental data...
show that only the first 31 amino acids of the initial translation product of lhcb4.2 are removed during the processing and that the resultant N-terminal arginine of the mature protein is acetylated. We also reveal the in vivo phosphorylation site in the mature CP29 protein from A. thaliana at threonine 6. This is a previously unknown phosphorylation site since the earlier found cold- and high light-induced phosphorylation of CP29 from maize was localized to the threonine 83 (24).

Identification of the Phosphorylated N-terminal Peptide of PsaD Protein—To reveal the sequence of the previously unknown phosphopeptide Y both doubly and triply charged molecular ions of this peptide (Fig. 1C) with \( m/z = 842.4 \) and 561.9, respectively, were subjected to CID. The fragmentation spectrum of the triply protonated peptide ion is shown in Fig. 3. The CID spectra of both triply (Fig. 3) and doubly charged (data not shown) ions allowed straightforward reading of 14 C-terminal amino acid residues of the peptide, TDSSAAAAAAPATK. The search in the A. thaliana database reveals that this sequence is present only in the N-terminal region of the PsaD1 subunit of PSI. Phosphorylation of this peptide was observed due to the neutral loss of phosphoric acid from both doubly and triply charged precursor ions and from the phosphorylated fragment ions. The triply charged precursor ion species before and after the loss of phosphoric acid are

**Fig. 2. Identification of the phosphorylation site in CP29.** Shown is the product ion spectrum obtained after electrospray ionization and CID of the phosphopeptide X. The selected parent molecular ion \( (M + 2H)^{2+} \) with \( m/z = 474.7 \) is indicated along with the ion at \( m/z = 425.7 \) that corresponds to the doubly charged parent ion with the neutral loss of phosphoric acid (mass 98). The b (N-terminal) and y (C-terminal) fragment ions are labeled in the spectrum. The indicated \( m/z \) differences of 17 and 28 between the fragment ions correspond to the loss of NH3 and CO from some of the b ions. The fragment ions that contained phosphorylated residue and underwent the neutral loss of \( H_3PO_4 \) (mass 98) are marked with asterisks. The peptide sequence is shown above the spectrum with the lowercase t designating acetylated N-terminal residue.

**Fig. 3. Identification of the phosphorylation site in PsaD.** Shown is the product ion spectrum obtained by electrospray ionization and CID of the phosphopeptide Y. The upper panel shows the full-size fragmentation spectrum for the triply charged molecular ion with \( m/z = 561.9 \). This ion is labeled in the spectrum with its \( m/z \) value along with the ion that underwent the neutral loss of phosphoric acid (mass 98) with \( m/z = 529.3 \). The lower panel shows a part of the same spectrum zoomed in the region \( m/z = 390–650 \) for indication of some doubly charged fragment ions. The detected b (N-terminal) and y (C-terminal) fragment ions are indicated in the spectra with the superscript 2 for doubly charged ions. The b ions marked with asterisks indicate the fragments produced after the neutral loss of \( H_3PO_4 \) (mass 98). The peptide sequence is shown above the spectra with the numbers corresponding to the observed b and y ions. The residue X designates the N-terminal modification corresponding to the addition of 72 atomic mass units. The lowercase t designates phosphorylated threonine residue in position 4 of the peptide.
indicated in Fig. 3 with their \( m/z \) ratios of 561.9 and 529.3, respectively (561.92 – 529.25 = 32.67 and 32.67 \( \times 3 = 98 \), which is the mass of \( \text{H}_3\text{PO}_4 \)). The peptide contains two serine and two threonine residues, and each of them could potentially be phosphorylated. We found phosphorylation of just the first N-terminal threonine residue in the peptide because only N-terminal fragment ions contained the phosphoryl modification and underwent the neutral loss of phosphoric acid. The C-terminal fragment ions from y1 to y11 (Fig. 3) and from y1 to y13 (after CID of the doubly charged precursor ion, data not shown) did not contain any modification. On the contrary, the spectrum in Fig. 3 demonstrates a subset of b4–b13 ions that correspond to either phosphorylated N-terminal peptide fragments or these fragments after the neutral loss of 98 (\( \text{H}_3\text{PO}_4 \)). The presence of b4 and -5 ions (b4* and b5* in Fig. 3) particularly, along with the absence of phosphorylation in the y1–y13 C-terminal fragments, localizes the phosphorylation site to the first N-terminal threonine residue in PsaD1 (Fig. 3).

There are two isoforms of nuclear encoded PsaD protein in \( \text{A. thaliana} \), which differ in the signaling peptides and the N termini of the mature proteins. The isoform PsaD1 (gene with GenBank\textsuperscript{TM}/EBI accession number At4g02770) has a signaling peptide of 45 amino acids as annotated in the \( \text{Arabidopsis} \) database. This corresponds to the N-terminal sequence of the mature protein EKTDSSAAAAAAPATK. The mass of this peptide, taking into account the methylation of Glu, Asp, and C-terminal Lys residues and the phosphorylation of Thr, is 72 atomic mass units (indicated as residue X in the sequence in Fig. 3) lower than that of the peptide we have sequenced. If one assumes that the signaling peptide was cleaved one amino acid residue before the annotated sequence predicted for the mature PsaD1 it would add an alanine and 71 atomic mass units to the peptide mass. However, such an assumption would still leave a difference of 1 atomic mass unit between the theoretical and experimentally found masses of the N-terminal peptide from PsaD1. The fragmentation mass spectra of neither doubly nor triply charged ions of this peptide allowed us to discover the exact final N-terminal part of its sequence. The reasons for this could involve some unidentified post-translational modification of the protein N terminus in vivo or some modification that occurred during the methylation procedure, which we performed before the IMAC. Nevertheless, our experimental data revealed the site of in vivo phosphorylation in PsaD protein and concomitantly the first protein in PSI found to be phosphorylated.

**Identification of the Phosphorylated Expressed Protein TMP14**—The calculated molecular mass of the phosphopeptide Z (Fig. 1D) is 2765.4 Da. The comprehensive fragmentation by CID and de novo sequencing of such a big peptide are challenging. Nonetheless, the fragmentation of the triply charged molecular ion of this peptide (\( m/z \) = 922.8) produced an easily interpretable series of the fragment ions and revealed the 25-amino acid-long sequence of the peptide shown in Fig. 4. The BLAST database search (33) identified this amino acid stretch as a tryptic peptide of the protein product of the nuclear encoded gene with GenBank\textsuperscript{TM}/EBI accession number At2g46820, annotated in the \( \text{Arabidopsis} \) database as “expressed protein.” The peptide contains seven
potential sites for phosphorylation on threonine residues (Fig. 4). The calculated mass for the peptide produced after digestion with trypsin was equal to the experimentally found mass, taking into account methylation and addition of one phosphate group. However, the neutral loss of the phosphoric acid from the precursor peptide ion was not observed because of its big size, allowing high freedom-of-energy disappearance during CID. Nevertheless, the neutral loss of phosphoric acid (H$_3$PO$_4$, 98 Da) was detected in the final N-terminal fragment ions (b ions with the asterisk in Fig. 4) showing that these N-terminal fragment ions contained the phosphorylated residue. The series of N-terminal fragments with the neutral loss pattern localized the phosphorylation site to threonine 2 or 3 in the peptide (Fig. 4). The analysis of the pattern for the b ions has not revealed convincingly which of these two threonine residues is phosphorylated.

The sequence analysis of the expressed protein product of the gene with GenBank™/EBI accession number At2g46820 (Fig. 5) predicts that this is a precursor protein with a high chloroplast-targeting probability of 0.966 (34). The signaling peptide is predicted to consist of 45 amino acids (34). Accordingly, the mature protein would comprise the residual 129 amino acids and would have a mass of 13,944 Da in the non-phosphorylated form. Most of the computer programs for prediction of protein secondary structure (www.expasy.org) predict two transmembrane helices in this protein with the N
terminus oriented to cytoplasm (stroma of chloroplast). The protein was also annotated as a potential membrane protein with two transmembrane spans in the database of Arabidopsis membrane proteins (aramemnon.botanik.uni-koeln.de). The function of this protein is unknown as it has not been characterized before and has no homology to any classified protein domains. On the basis of the determined properties of this protein we have named the protein thylakoid membrane phosphoprotein of 14 kDa (TMP14). The homology database search revealed proteins similar to TMP14 in a number of plant species and cyanobacteria. The alignment of the sequences for TMP14 and its homologs is shown in Fig. 5 and discussed below.

**DISCUSSION**

We have identified three previously unknown sites of in vivo protein phosphorylation in the photosynthetic membranes of Arabidopsis. Taking advantage of the capacity of mass spectrometry to reveal naturally phosphorylated phosphopeptides we isolated thylakoid membranes from the plants exposed to normal growing light and cleaved the surface-exposed parts of the membrane proteins by trypsin as described previously (22). The challenge of this approach consists of the identification of phosphopeptides from the very complex resultant mixture of the peptides cleaved from the membrane. It could be addressed by specific enrichment for the phosphorylated peptides. The previous enrichment by traditional IMAC with immobilized Fe(III) or Ga(III) ions allowed characterization of the major phosphopeptides, while a number of other phosphorylation sites resisted identification (22). The conventional IMAC procedures with either Fe(III) (30) or Ga(III) (35) suffer from nonspecific binding of peptides containing multiple carboxylic acid groups. However, the recent improvement of the method by conversion of carboxylic acids to methyl esters allows overcoming this problem (29). Using this technique we obtained a better enrichment of the phosphorylated peptides in the present work. We have obtained the mixtures of the major known phosphopeptides and three previously uncharacterized phosphopeptides from thylakoid proteins. The sequences of these newly identified phosphopeptides were revealed by successful mass spectrometric analyses. The phosphorylation sites in these peptides were mapped to threonine residues as in the other characterized phosphoproteins from thylakoids of Arabidopsis (22). The discovery of these new in vivo phosphorylated species has the potential to lead to the uncovering of molecular events for regulation of plant photosynthesis, and these findings are discussed below individually for each of these phosphorylated proteins.

We isolated and sequenced the phosphorylated peptide from CP29 protein, isoform LHCb4.2. The first residue (arginine) in this peptide was acetylated, which indicated that the peptide corresponded to the N terminus of the mature protein. CP29 belongs to the family of nuclear encoded light-harvesting chlorophyll a/b-binding thylakoid proteins that contain three membrane-spanning helices (36). The transit peptide cleavage sites for most of these proteins in Arabidopsis were predicted by homology but not experimentally verified. We found that mature CP29 (LHCb4.2) starts from amino acid 32 of the initial translation product of lhcb4.2 and is phosphorylated at threonine 6. CP29 (LHCb4) is the minor chlorophyll a/b antenna protein present probably in one copy/PSII unit and represented by three isoforms in Arabidopsis (36). Two genes, lhcb4.1 and lhcb4.2, have about the same level of expression, whereas the third, lhcb4.3, is expressed at the lower level and also differs from the first two by lacking a large part of the C-terminal region located in the thylakoid lumen (36). Mature LHCb4.1 and LHCb4.2 are 91% identical in amino acid sequences and differ mostly in N-terminal regions. In particular, in LHCb4.1 there is no arginine corresponding to the first position of the mature LHCb4.2, and the threonine 6 phosphorylated in LHCb4.2 is also absent in LHCb4.1.

The CP29 antenna protein from Zea mays has been found phosphorylated after exposure of the plants to high light in the cold (16). This maize protein resisted N-terminal sequencing by Edman degradation being N-terminally blocked (16), probably acetylated as we determined for CP29 (LHCb4.2) in the present work. However, the phosphorylation site in the maize CP29 has been mapped to threonine 83 (24). There is no threonine residue in the maize CP29 at the position corresponding to the phosphorylated threonine 6 in LHCb4.2 from Arabidopsis. The other important difference between the phosphorylation of CP29 in these species is in the physiological conditions inducing this modification. We found phosphorylation of Arabidopsis CP29 in these species is in the physiological conditions induced by high light in the cold (24). Moreover, no induction of CP29 phosphorylation by cold has been found in Arabidopsis with the use of either immunoblotting or 33P-labeling for the phosphorylation analyses (37). The phosphorylation of CP29 in winter rye was also induced by very high light intensities, although the exact phosphorylation site in that protein has not been determined (38). Thus, in the present work we found a previously unknown site for in vivo phosphorylation of CP29 in Arabidopsis and concomitantly revealed the N-terminal sequence of the mature CP29 and its acetylation.

The second previously unknown phosphorylated peptide that we isolated and sequenced belonged to the N terminus of the PsAD1 subunit of PSI. Remarkably, this is the first phosphorylated protein found in PSI. PsAD is a product of a nuclear encoded gene that contains a predicted transit peptide of about 30–45 amino acid residues, depending upon species. The biogenesis and assembly of PsAD with PSI was extensively studied and was found to proceed in a rather unusual way when the precursor PsAD protein is first assembled with PSI and then the precursor protein is processed, yielding mature PsAD associated with PSI (39–41). The N-terminal sequence of the mature PsAD has never been determined for either plant or cyanobacterial protein. We revealed the se-
quence of 14 amino acid residues in the N-terminal peptide from *A. thaliana* PsaD but have not resolved the structure of the final N-terminal region of the protein, which included two or three additional residues with a probable modification. Nevertheless, we have found the exact site of PsaD phosphorylation in vivo corresponding to the first threonine residue in the mature protein. This finding opens a new direction in studies of possible PSI regulation by protein phosphorylation. The three-dimensional structure of cyanobacterial PSI has been recently revealed at a 2.5-Å resolution (42). The core of this large membrane protein complex consists of the intrinsic membrane subunits PsaA and PsaB. The extrinsic protein subunits PsaC, PsaD, and PsaE do not contain any transmembrane α-helices but closely contact the stromal loops of PsaA and PsaB. These peripheral polypeptides PsaC, PsaD, and PsaE form the “stromal ridge” of PSI involved in the docking of ferredoxin, an electron carrier at the stromal side of the photosynthetic membrane (43). Ferredoxin receives electrons from the iron-sulfur clusters of PSI. The properties of these iron-sulfur clusters depend upon the presence of the PsaC and PsaD subunits (44). PsaD is a hydrophilic protein of about 18 kDa and has only a few elements of secondary structure and no stable three-dimensional structure in solution (44). When PsaD is bound in PSI, it forms a well defined three-dimensional structure with an antiparallel four-stranded β-sheet followed by a second two-stranded β-sheet and a short loop connecting the fourth β-strand to the only α-helix in PsaD (43, 44). Thus, significant structural changes and flexibility of PsaD together with its control position at the electron acceptor site of PSI may be employed by regulatory mechanisms operating via protein phosphorylation. The finding of PsaD phosphorylation also implies that regulatory functions for protein phosphorylation in photosynthetic membranes are probably not restricted to PSII and its light-harvesting antennae.

The three previously uncharacterized 25-amino acid-long peptide that we isolated and sequenced in this work belonged to the unknown protein, which we named thylakoid membrane phosphoprotein of 14 kDa. This protein has never been studied before but was annotated as “expressed protein” in the *Arabidopsis* database and as a potential membrane protein with two transmembrane regions in the database for *Arabidopsis* membranes proteins. TMP14 is encoded by the nuclear gene with GenBank™/EBI accession number At2g46820. We identified the product of this gene just because it was phosphorylated in vivo. Interestingly, our approach for mass spectrometric characterization of phosphoproteins makes TMP14 already a second novel thylakoid protein identified due to its post-translational modification. We have recently characterized a novel plant-specific protein TSP9 undergoing multiple phosphorylation by light-activated protein kinase(s) and subsequent release from the thylakoid membrane (25). TMP14, like TSP9, has no homology to any classified protein domains. However, unlike hydrophilic TSP9, TMP14 contains two transmembrane regions. Besides this, if TSP9 is a plant-specific protein, TMP14 has homologous proteins encoded in the genomes of plants and cyanobacteria as well. The sequence alignment of *A. thaliana* precursor TMP14 with the deduced sequences for cognate proteins from other plants and cyanobacteria is shown in Fig. 5. As expected, the degree of TMP14 homology to the proteins from other plants is higher than the homology to the cyanobacterial analogs. All of the plant proteins contain two potential transmembrane helices, as predicted for each of them, and could be considered integral membrane proteins. However, the computer programs do not unambiguously predict two transmembrane regions for the cyanobacterial proteins. The TMP14s from plants have well defined signaling peptides with the predicted (34) high probability for chloroplast targeting. Our results experimentally confirmed the localization of TMP14 in thylakoid membrane of chloroplast. Topology prediction for the plant proteins places their N termini to the stromal side of thylakoid membrane, which is in agreement with phosphorylation of TMP14 at the membrane surface exposed to the stroma of chloroplast. We have confined the phosphorylation site in TMP14 to one of two N-terminal threonine residues in the sequenced peptide. These residues correspond to positions 65 and 66 in the sequence of the precursor protein shown in Fig. 5. The alignment shows that threonine 65 is conserved in five plant species but is not present at this position in the protein from rice. The threonine at position 66 is found only in *A. thaliana*. According to the alignment it looks more likely that threonine 65 could be the site of TMP14 phosphorylation in *Arabidopsis* and probably four other plants. On the other hand the alignment-based positioning of the phosphorylation site should be taken with caution because the regulatory modifications of similar proteins in different plants could be rather diverse (25, 45). Nevertheless, the discovery of phosphorylated TMP14 in the photosynthetic membrane raises a lot of new questions about the function of this protein, its binding partners, association with one of the photosynthetic protein complexes, and the role of its in vivo phosphorylation.

We determined that all of the three newly identified phosphopeptides were phosphorylated at threonine residues, as were the phosphopeptides from other previously characterized thylakoid proteins (20–22, 24, 25). These data reinforce the fact of high specificity of protein kinases in thylakoid membranes toward the threonine residues in the substrate proteins. Such a high specificity to threonine residues is rather unusual for the majority of eukaryotic Ser/Thr protein kinases. Moreover, the thylakoid protein kinases are special in their activation by light and redox regulation by the electron carriers in the photosynthetic membrane (1, 2). There are more than 1100 genes encoding protein kinases in the genome of *A. thaliana* (46), and the hunt for thylakoid protein kinases is still in the beginning. At present there are five candidate genes for membrane protein kinases that could phosphorylate LHClI: a family of three TAK kinases (47, 48) and two kinases...
homologous to Sf7 kinase from *Chlamydomonas reinhardtii* (49). The mechanism for the redox regulation of these potential LHCCI kinases is elusive. The identities and the number of protein kinases involved in the phosphorylation of the other thylakoid proteins are also unknown. The major challenge in unraveling the complex network for redox-dependent protein phosphorylation in thylakoids is caused by the requirement of integrity of the functional membrane system for its operation (1, 2). The feasible strategy to address this challenge could be to use the available protein kinase knockout lines in *A. thaliana* and mass spectrometry technique for analyses of in vivo phosphorylation states for thylakoid proteins (22). Identification of all natural protein phosphorylation sites in the photosynthetic membranes is one of the prerequisites for these analyses. Thus, our present work makes a clear step forward in this direction.

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