Posttranslational Modification of Oat Phytochrome A: Phosphorylation of a Specific Serine in a Multiple Serine Cluster†

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ABSTRACT: Phytochrome A (phyA) is a photoreceptor of higher plants which mediates a variety of biochemical and physiological processes in response to red/far-red light. By detailed structural analysis of the peptides of the total tryptic digest of oat phyA, we found that the photoreceptor isolated from red light irradiated seedlings contains only one site of phosphate attachment, in the N-terminal Ser-rich region. The N-terminal tryptic phosphopeptide (residues 1−12) contains eight serine residues, any of which may be phosphorylated. Direct fast atom bombardment mass spectrometry (FAB MS/MS) analysis of the phosphorylated peptide as well as of its phosphate-containing fragment (residues 1−9) was not successful due to their hydrophilic nature and instability of the phosphate bond. β-Elimination of the phosphorylated tryptic peptide in the presence of ethanethiol converted the phosphoserine residue to *S*-ethylcysteine that is stable under FAB MS/MS. FAB MS/MS analysis of the modified peptide clearly showed that the phosphate group was attached to Ser_7. The *in vivo* phosphorylation site at Ser_7 in oat phyA is discussed for its possible regulatory role in phyA function.

Phytochrome is a photochromic switch that mediates many biochemical and physiological processes in higher plants in response to red/far-red light (Furuya, 1993; Quail, 1991, 1994; Quail *et al.*, 1995). Among several phytochromes, phytochrome A (phyA)[1] predominant in etiolated tissue is best characterized (Somers *et al.*, 1991). PhyA possesses a tetrapyrrole chromophore, phytochromobilin, which is covalently linked to a cysteine residue (Lagarias & Rapoport, 1980). Phytochrome exists in two photochromic isomers, red-light absorbing (Pr) and far-red-light absorbing forms (Pfr). The latter is considered to be a signal-mediating, "switch on" form. The mechanism of phyA action is unknown, but available evidence suggests that phosphorylation may be involved in the signal transduction and/or regulation of phyA activity [Bierman *et al.*, 1994; Doshi *et al.*, 1992; Otto & Schafer, 1988; Park & Chae, 1989; Park & Song, 1990; Romero *et al.*, 1991a,b; Wong & Lagarias, 1989; for a review, see Singh and Song (1990)]. PhyA is a good substrate for many protein kinases from mammalian tissues (Wong *et al.*, 1986). Ser_{17} and Ser_{598} were found to be the sites of protein kinase A-catalyzed phosphorylation of oat phyA *in vitro* (McMichael & Lagarias, 1990; Lapko *et al.*, 1996). PhyA has been shown to be a phosphoprotein according to chemical analysis (Hunt & Pratt, 1980) and by *in vivo* labeling experiments (Quail *et al.*, 1978). However, the site(s) of phosphate attachment is (are) unknown.

We have shown recently that oat phyA (isolated as Pfr) contains 0.4 mol of phosphate per monomer (Lapko *et al.*, 1996). Mapping of the total tryptic digest of oat phyA showed that native phyA has only one site of phosphate attachment, in the N-terminal fragment 1−12. Since this peptide fragment contains three- and five-serine clusters, determination of the specific serine residue for phosphorylation is a challenging task. We describe a method for the isolation of the tryptic peptide containing the site of *in vivo* phosphorylation. We report here the results of the sequence analysis of the phosphopeptide using fast atom bombardment mass spectrometry (FAB MS/MS). Implications of the *in vivo* site of phosphorylation are also discussed.

MATERIALS AND METHODS

Materials. Proteolytic enzymes, subtilisin (protease type XXVII, Nagarse), thermolysin (protease type X), and papain were purchased from Sigma Chemical Co. (St. Louis, MO). Alkaline phosphatase (from calf intestine) was supplied by Calbiochem (La Jolla, CA). Bio-Gel P-6 (fine) was purchased from Bio-Rad Laboratories (Oakland, CA). All other reagents were obtained from Sigma Chemical Co.

Phytochrome Preparations. PhyA with a specific absorbance ratio $A_{666}/A_{280} = 1.03$ (for the Pr form) was obtained as described previously (Lapko & Song, 1995).

Isolation of the Phosphorylated Tryptic Peptide (T1p) of phyA. A fast procedure for the isolation of peptide T1p included tryptic digestion of temperature-denatured phyA (70 °C, 3 min) in 20 mM potassium phosphate buffer, pH 8.1, with trypsin [trypsin:phytochrome ratio = 1:40 (w/w)] in the presence of 10 mM iodoacetamide for 5 h. The digest (1.4 mL) was applied directly to a Bio-Gel P-6 column (1.5 × 95 cm) equilibrated with 100 mM ammonium bicarbonate, pH 8.3. The elution volume, 91−108 mL (fraction VI), was

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[1] Abbreviations: ESIMS, electrospray ionization mass spectrometry; FAB MS/MS, fast atom bombardment MS/MS; phyA, phytochrome A; PKA, cAMP-dependent protein kinase A; Pr and Pfr, red and far-red absorbing forms of phytochrome, respectively; peptide T1, the N-terminal tryptic peptide of oat phyA; T1p, the N-terminal phosphate-containing tryptic peptide of oat phyA; TFA, trifluoroacetic acid.

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freeze-dried, redissolved in 0.1% trifluoroacetic acid (TFA), and subjected to reversed phase HPLC (Vydac 218TP54 column; 0.46 × 25 cm; A buffer: water, 0.1% TFA; B buffer: 90% acetonitrile, 0.1% TFA). Peptides were eluted from the reversed phase column at a flow rate of 1 mL/min using 100% solvent A for 6 min, followed by a gradient to 20% solvent B in 45 min, to 25% solvent B in 10 min, to 50% solvent B in 25 min, to 100% solvent B in 10 min, and finally holding at 100% solvent B for 10 min. Peptide T1p was found in fraction 11 (Figure 1b). The N-terminal tryptic peptide without phosphate (peptide T1) was eluted in fraction VII from the gel filtration column.

Dephosphorylation of Peptide T1p by Alkaline Phosphatase. Peptide T1p was treated with alkaline phosphatase in 20 mM ammonium bicarbonate for 2 h by using 5 ng of enzyme per approximately 200 pmol of peptide.

Subtilisin Digestion of Peptide T1p. Phosphorylated fragment (1−9) (MH^+ mass = 987.4) was obtained after 1 h incubation of peptide T1p (100−300 pmol) with 30 mL of subtilisin solution (A_{280} = 0.025) in 20 mM ammonium bicarbonate.

Chemical Modification of Peptide T1p. The unstable phosphoserine residue of peptide T1p was converted to *S*-ethylcysteine by a β-elimination reaction (50 °C, 1 h) in the presence of ethanethiol according to Meyer *et al.* (1991). The modified peptide was purified from the reaction mixture by reversed phase HPLC, as indicated earlier. The modified peptide eluted 3 min after peptide T1p.

Electrospray Ionization Mass Spectrometry (ESIMS) and FAB MS/MS. Fractions collected from the reversed phase column were dried and redissolved in 0.1% TFA. The molecular weights of the peptides in these fractions were determined via direct injection ESIMS by using a Micromass Platform II quadrupole mass spectrometer. Aliquots of the peptide solution were injected directly into a 5 mL/min flow of 50% acetonitrile as described previously (Jiang *et al.*, 1996). Molecular masses of peptides were routinely determined with an uncertainty of less than 0.3 Da. Collision-induced fragmentation of derivatized T1p was performed using a Micromass Autospec magnetic sector time-of-flight mass spectrometer operated in the fast atom bombardment ionization mode (Jiang *et al.*, 1996).

RESULTS

The tryptic digest of oat phyA was completely mapped as a preliminary step for specific chemical modifications of Pr and Pfr forms of oat phyA. The sequence analysis of isolated peptides allowed us to confirm more than 97% of the sequence of the phyA3 isoform (unpublished results). These results showed that isolated oat phyA contains only one site of phosphate attachment within the N-terminal tryptic fragment 1−12 (T1p). The procedure fast isolation and purification of peptide T1p (MH^+ = 1317.4) included (1) tryptic digestion of phytochrome in the presence of iodoacetamide, (2) direct application of the digest to a Bio-Gel P-6 column, and (3) reversed phase HPLC (Figure 1). Unphosphorylated N-terminal tryptic peptide T1 (MH^+ mass = 1237.4) eluted from the gel filtration column in fraction VII, while the phosphorylated form eluted in fraction VI (Figure 1a). The molecular weights of both T1 and T1p indicate that the N-terminus was acetylated, as reported previously (Grimm *et al.*, 1988).

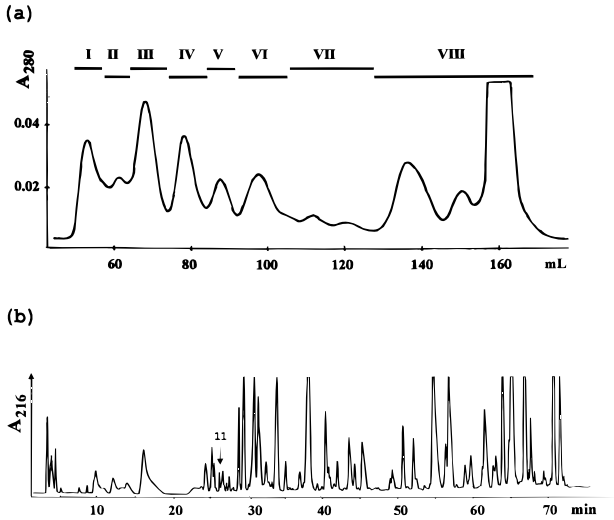

FIGURE 1: Isolation of phoshorylated tryptic peptide T1p of oat phy A. Oat phy A was digested with trypsin for 5 h at 37 °C as indicated under Materials and Methods. (a) The digest was applied to a Bio-Gel P-6 (1.5 × 95 cm) column equilibrated with 100 mM ammonium bicarbonate. Flow rate, 10.4 mL/h. (b) Reversed phase HPLC of fraction VI shown in Figure 1a (see Materials and Methods). The phosphopeptide was found in peak 11.

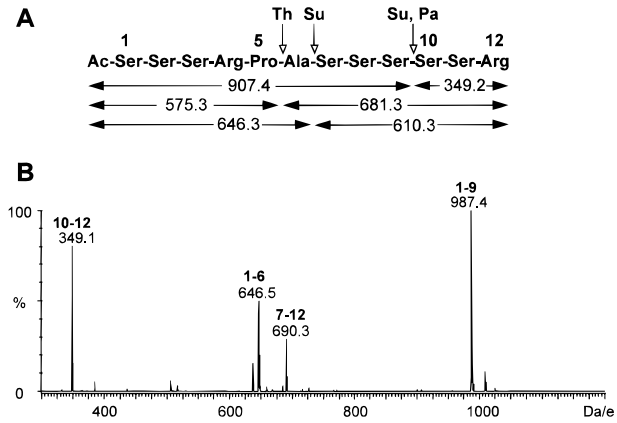

FIGURE 2: (A) Cleavage sites and MH^+ mass values of fragments produced by thermolysin (Th), subtilisin (Su), and papain (Pa) digests of peptide T1. (b) ESIMS of peptide T1p digested with subtilisin as indicated under Materials and Methods. Molecular ions at 690.3 Da/e (residues 7−12) and 987.4 Da/e (residues 1−9) indicate the presence of phosphate group in these fragments.

Identification of the peptides was confirmed by the molecular weights of peptides produced from the subdigestion of T1 and T1p. Subdigestion of T1 with thermolysin, papain, and subtilisin gave fragment peptides corresponding to the cleavage sites indicated in Figure 2A. Although the phosphorylated form of the peptide, T1p, was resistant to proteolysis by thermolysin and papain, it was easily fragmented by subtilisin (Figure 2B). Treatment of T1p with alkaline phosphatase gave the unphosphorylated peptide, T1 (MH^+ = 1237.4), confirming that the former was phosphorylated. Finding both unphosphorylated and phosphorylated N-terminal fragments is consistent with our previous study that only 40% of oat phyA is phosphorylated (Lapko *et al.*, 1996).

The N-terminal peptide of phyA includes eight serine residues, making it difficult to sequence, and hence identify the phosphorylated serine. The molecular weights of peptides including residues 1−6 (*m*/*z* 646.5) and 1−9 (*m*/*z* 987.4) found in the subtilisin digest of T1p (Figure 2B) show that

a	102	189	276	432	529	600	**731**	818	905	992	1079	
b	130	217	304	460	557	628	759	846	933	1020	1107	
d_a	86	173	260			600	671	**802**	889	976	1063	
	Ser	Ser	Ser	Arg	Pro	Ala	**SER**	Ser	Ser	Ser	Ser	Arg
y″		1152	1065	978	822	725	654	523	436	349	262	175
w_a		1119	1032	876		708	**577**	490	403	316	229	73

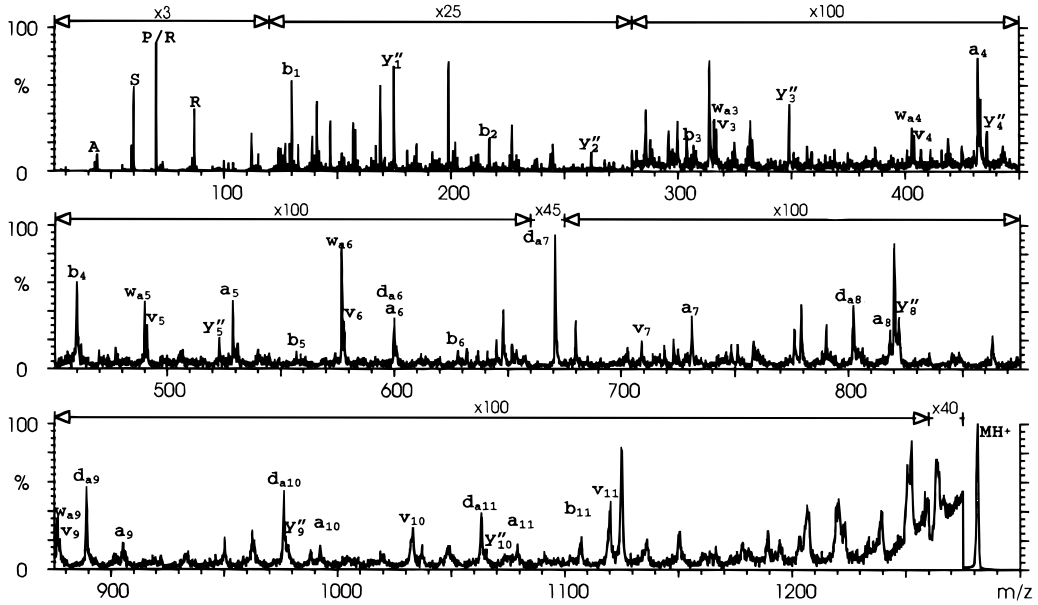

FIGURE 3: FAB MS/MS spectra of modified peptide T1p. Peptide T1p was subjected to a β-elimination reaction in the presence of ethanethiol and purified by reverse HPLC. Approximately 200 pmol of the HPLC-purified T1p with $MH^+ = 1281.6$ was analyzed by FAB MS/MS. Fragments of types b, y″, a, d_a, and w_a (Biemann, 1990) are shown above the amino acid sequence at the top of the figure. Fragment of types b and y″ have the general formulas $[H(NHCHRCO)_n]^+$ and $[H(NHCHRCO)_nOH]^+$, respectively. Fragment $a_n = b_n - CO$; fragment $d_n = a_n - R_n a$; fragment $w_n = y'' - NH_2 - R_n a$. R represents the side chains of the amino acids; R_na is the β-substituent of the *n*th amino acid. Ions observed in the mass spectra are underlined. The serine in boldface font was determined to be phosphorylated. Strong ions at *m*/*z* 731.4 (a-series), 802.4 (d_a-series), and 577.3 (w_a) indicate specific Ser_7 modification; ions at *m*/*z* 687.3, 758.4, and 621.3 for corresponding series (for hypothetical Ser_8 modification) and at *m*/*z* 774.4, 845.4, and 534.2 (for Ser_9 modification) were not observed.

the site of phosphorylation is C-terminal to Ala_6, and N-terminal to Ser_{10}. These results clearly place the phosphorylation site within the segment containing residues 7−9. Initial attempts to locate the phosphorylation site by FAB MS/MS analysis of the segment including residues 1−9 (as well as peptide T1p) failed. The T1p fragment spectrum was weak and did not have enough peaks to unambiguously sequence the peptide.

We then derivatized the phosphorylated peptide, T1p, by β-elimination of the phosphate in the presence of ethanethiol. Analysis by HPLC indicated that derivatization was approximately 80% complete. The derivatized T1p eluted 3 min later than T1p (data not shown). Analysis of the derivatized T1p by FAB MS/MS gave a much stronger fragmentation spectrum (Figure 3), from which the site of phosphorylation was determined. Although a complete series of y″ and b fragments was not found, there are many strong ions belonging to the a, d_a, and w_a series [for the fragmentation pattern of peptides, see Papayannopoulos (1995)], indicating that Ser_7 is phosphorylated. Whereas the peak at *m*/*z* 731 corresponds to the modification of Ser_7 (a_7), similar a_7 fragments that would give peaks at *m*/*z* 687 and 774, if Ser_8 or Ser_9 were phosphorylated, are not present. Likewise, peaks at $m/z = 802$ (d_{a8}) and at 577.0 (w_{a6}) consistent with phosphorylation of Ser_7 are present, while the corresponding peaks for phosphorylation of Ser_8 or Ser_9 are not present (for details, see the Figure 3 caption). Although the phosphorylated peptide was obtained in a pure form, tandem FAB MS/MS allowed selection of only one precursor ion so all fragmentation could be attributed to the analyzed peptide.

DISCUSSION

Phosphorylation of serine and threonine residues in proteins is a common mechanism for modulating the protein activities in diverse cellular processes (Edelman *et al.*, 1987). In a visual system, phosphorylation of rhodopsin is a crucial step in the inactivation of the active form of the photoreceptor (Wilden *et al.*, 1986). Ser_{17} and Ser_{598} were identified previously as sites of oat phyA phosphorylation by protein kinase A *in vitro* (McMichael & Lagarias, 1990). Biological consequences of these *in vitro* phosphorylations are still unknown, though we have shown recently that phosphorylation of Ser_{17} and Ser_{598} is conformationally "non-silent" (Lapko *et al.*, 1996).

A structural analysis of the tryptic digest of oat phyA (isolated as Pfr) showed that phosphorylation at the N-terminal serine-rich region is the only or highly dominant site of phosphate attachment. Our tryptic mapping accounted for almost the entire amino acid sequence of the A3 isoform (1128 amino acid residues) except for short peptides of 1−2 residues for only 28 amino acid residues. Isolated tryptic peptides included all theoretically possible sites of phosphorylation. The search for hypothetical phosphopeptides was facilitated by the knowledge that a phosphopeptide usually eluted from the C_{18}-HPLC column 1−5 min earlier than the

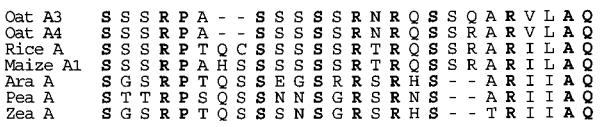

FIGURE 4: Comparison of the N-terminal sequences of phyA's from different plants. The amino acid sequence of the oat phyA3 isoform is used as the reference sequence. Numbering starts from the *N*-acetylserine residue as the posttranslationally modified N-terminus. Conserved amino acid residues are shown in boldface font.

corresponding unphosphorylated form. Most of the tryptic peptides were obtained in good yield; however, no corresponding phosphopeptide other than T1p was identified. For instance, the tryptic segment including residues 15−20 was found in two HPLC fractions as a result of cyclization of the N-terminal glutamine with loss of ammonia. Tryptic segment 595−607 was also identified in two HPLC fractions (not complete cleavage of Arg_{595}−Glu_{596} peptide bond). However, no phosphopeptides from these phyA regions, which included the sites of protein kinase A-catalyzed phosphorylation *in vitro*, were observed.

The N-terminal tryptic fragment of oat phyA contains eight serine residues that could be potential sites of phosphorylation. FAB MS/MS of the phosphorylated peptide showed that the peptide represents a homogeneous component and *in vivo* phosphorylation of phyA is highly specific for Ser_7. Phosphorylation at serine residues other than Ser_7 within the serine-rich region could affect to some extent the position of the resulting peptide on gel filtration or HPLC profile. The peptide T1p, peak 11 of Figure 1b, however, was the only phosphopeptide found upon tryptic mapping. No phosphopeptide that included two or more phosphate groups attached was detected.

Light treatment of plant materials before homogenization is a very important factor that could significantly affect the phosphate content of the isolated photoreceptor. We showed previously that the phytochrome preparations isolated as Pfr had a higher phosphate content than the phytochrome isolated as Pr (Lapko *et al.*, 1996). This raises the possibility of phytochrome phosphorylation *in vivo* after activation by red light. This is qualitatively similar to the activation of G-protein-coupled receptors resulting in their phosphorylation by specific kinases (Inglese *et al.*, 1993). Red light/phytochrome also appears to modulate phosphorylation of plant proteins (Grimm *et al.*, 1991; Harter *et al.*, 1994; Romero *et al.*, 1991a; Tong *et al.*, 1996), possibly via a G-protein (Bowler *et al.*, 1994; Romero *et al.*, 1991b; Romero & Lam, 1993).

So-called "phytochrome-associated kinase", stimulated by polycations, phosphorylates *in vitro* the N-terminal serine-rich region of oat phyA, but the exact position of the modification was not determined (McMichael & Lagarias, 1990). It is tempting to assume that the phosphorylation site coincides with the site determined in the present study. The N-terminal part of phyA from different plants (Figure 4) contains, however, several conserved serine residues (*N*-acetylserine$_1$, Ser_7, and Ser_{10}), and phosphorylation at a serine residue other than Ser_7 by "phytochrome-associated kinase" (Wong *et al.*, 1989) cannot be ruled out.

The N-terminal region of phyA is important for the spectral and structural integrity of the chromoprotein (Chai *et al.*, 1987; Sommer & Song, 1990; Vierstra & Quail, 1983) and its biological function (Boylan *et al.*, 1994; Cherry *et al.*, 1992; Jordan *et al.*, 1996; Wagner *et al.*, 1996). Clusters of serine residues in the N-terminal region of oat phyA are a characteristic feature of phyA sequences conserved among the chromoproteins from different plant species. It has been shown that Ser-to-Ala substitutions in the Ser-rich N-terminal region of phyA resulted in an increased biological activity of phyA expressed in transgenic tobacco (Emmler *et al.*, 1993; Stockhaus *et al.*, 1992). Furthermore, removal of segment 7−12 in the N-terminus of oat phyA expressed in transgenic tobacco resulted in a hyperactive photoreceptor (Jordan *et al.*, 1996). Identification of the apparent phosphorylation site at Ser_7 *in vivo* suggests that the N-terminus of the photoreceptor is involved in negative regulation of biological activity of phyA via phosphorylation. The hypothesis that phyA is down-regulated by phosphorylation was stated recently by Jordan *et al.* (1996). One possible mechanism is the interaction of phosphorylated phyA at Ser_7 with a repressor protein. Phosphorylation of the activated receptors with subsequent binding with arrestin-like protein is a general mechanism for the quenching of the transduction cascade in G-protein-coupled receptor systems (Hargrave & McDowell, 1992). Work is in progress to identify protein(s) which may interact with the phosphorylated phyA in a "phosphate"- and/or light-dependent manner.

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