β-Elimination Coupled with Tandem Mass Spectrometry for the Identification of in Vivo and in Vitro Phosphorylation Sites in Maize Dehydrin DHN1 Protein

Xinzhao Jiang and Yinsheng Wang*

Department of Chemistry, Mail Drop 027, University of California, Riverside, California 92521-0403

Received July 28, 2004; Revised Manuscript Received September 22, 2004

ABSTRACT: Dehydrins are a group of proteins that are accumulated during environmental stress such as drought and low temperature or during late embryogenesis. In the present study, we isolated dehydrin DHN1, also known as Rab17 protein, from maize kernel by an acid extraction method, removed the phosphoric acid groups from phosphorylated residues by β-elimination via treating the protein with barium hydroxide, and identified the sites of phosphorylation by tandem mass spectrometry. Our results showed that each of the seven contiguous serine residues (Ser78−Ser84) in the serine tract could be phosphorylated. The β-elimination procedure was shown to be essential for the detection and subsequent site mapping of the heavily phosphorylated peptide by mass spectrometry. We also found that protein kinase CK2 could catalyze the phosphorylation of the DHN1 protein in vitro and the level of phosphorylation was comparable to that of the DHN1 isolated from maize seeds. Moreover, the in vitro phosphorylation also occurred on the serine residues in the serine tract region, suggesting that CK2 might be involved in the phosphorylation of the serine track region in maize kernel in vivo.

Dehydrin proteins, also known as the late embryonic abundant (Lea) D-11 family, are a group of plant proteins that are overexpressed during dehydration-related environmental stress or late embryogenesis or upon treatment with abscisic acid (*1*−*6*). Dehydrin proteins assume a modular structure (*7*), and the K-segment (KIKEKLPG) is the highest consensus sequence domain in dehydrin proteins. This segment was suggested to be important for the lipid binding property of the maize dehydrin DHN1 protein (*8*). Some dehydrin proteins also contain a S-segment, and it has eight serine residues, among which seven are adjacent to each other in the protein chain (*7*) (Scheme 1 shows the amino acid sequence of the DHN1 protein).

Scheme 1: Sequence of the Dehydrin DHN1 Protein[a]

```
          10                  20                  30                  40
MEYGQQGQRG    HGRTGHVDQY    GNPVGGVEHG    TGGMRHGTGT

          50                  60                  70                  80
TGGMGQLGEH    GGAGMGGGQF    QPAREEHKTG    GILHRSGSSS

          90                 100                 110                 120
SSSSEDDGMG    GRRKKGIKEK    IKEKLPGGHK    DDQHATATTG

         130                 140                 150                 160
GAYGQQGHTG    SAYGQQGHTG    GAYATGTEGT    GEKKGIMDKI

         168
KEKLPGQH
```

[a] Serines in the serine-tract are highlighted in bold italics.

Dehydrin proteins can be phosphorylated. In this regard, some serine residues in the S-segment were shown to be phosphorylated in both dehydrin ERD-14 protein from *Arabidopsis* (*9*) and DHN1 protein, also known as Rab17, from mature maize kernels (*10*). In addition, a dehydrin-like, vacuole-associated protein, VCaB45, isolated from celery was also found to be phosphorylated (*11*). The S-segment in DHN1 protein is followed by a consensus protein kinase CK2[1] (CK2, previously known as casein kinase II) recognition sequence, which is composed of amino acid residues Glu85, Asp86, and Asp87 (Scheme 1). In this regard, Plana et al. (*10*) showed that CK2 can phosphorylate DHN1 in vitro, and CK2 was not able to phosphorylate this protein when these three acidic amino acids are replaced with alanines (*12*). In addition, phosphorylation of ERD-14 and VCaB45 was found to enhance their Ca^{2+} binding capabilities, whereas dephosphorylation of these two proteins abolishes their ion-binding properties (*9*, *11*). Moreover, Goday et al. (*13*) demonstrated that the phosphorylation of DHN1 protein is necessary for its preferential binding to nuclear localization signal (NLS) peptide.

Although previous work demonstrated that the S-segment in dehydrin and dehydrin-like proteins can be phosphorylated (*9*−*11*), the exact sites of the phosphorylation remain to be elucidated. To understand better the biological and physiological function of the dehydrin proteins and their phosphorylation, we sought to identify the sites of phosphorylation in DHN1 protein by tandem mass spectrometry. Our results showed that there were 5−7 mol of phosphates incorporated per mole of DHN1 protein that was isolated from dry maize kernel. More interestingly, all seven serines, that is, Ser78−Ser84, in the serine-tract of the protein can be phosphorylated in vivo. This is a rare example of phosphorylations of up to seven contiguous amino acids in a protein. We also examined the phosphorylation of the

* To whom correspondence should be addressed. E-mail: yinsheng.wang@ucr.edu. Fax: (951) 827-4713. Tel: (951) 827-2700.

[1] Abbreviations: MS, mass spectrometry; MS/MS, tandem mass spectrometry; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; HMG, high-mobility group; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; CK2, protein kinase CK2; NLS, nuclear localization signal; CIP, calf intestinal alkaline phosphatase.

10.1021/bi0483965 CCC: $27.50
Published on Web 11/16/2004

DHN1 protein by protein kinase CK2 in vitro, and it turned out that both the extent and the sites of phosphorylation catalyzed by CK2 were very similar to those observed for the DHN1 protein isolated from maize kernel.

EXPERIMENTAL PROCEDURES

Plant Material. Maize kernel (*Zea mays* L., inbred B73, from Pioneer Hi-Bred International, Johnston, IA) was a gift from Prof. Julia Bailey-Serres at the Department of Botany and Plant Sciences, University of California at Riverside.

Protein Extraction and Purification. Approximately 10 g of dry maize kernel was ground into powder by using a mortar and pestle. Dehydrin DHN1 protein was extracted from the homogenized tissue by using 2% trichloroacetic acid (TCA, 100 mL). The supernatant was collected by centrifugation and filtered through a filter paper (Whatman Co., Middlesex, U.K.). The proteins were precipitated from the supernatant by increasing the TCA concentration from 2% to 25%. The pellet from centrifugation was collected, air-dried, reconstituted in water, and separated on a Surveyor HPLC system (ThermoFinnigan, San Jose, CA) by using a 4.6 mm × 250 mm C4 column (Varian, Walnut Creek, CA). The flow rate was 1.0 mL/min, and a 75-min linear gradient of 0−30% acetonitrile in 0.1% trifluoroacetic acid (TFA) was employed. The fractions containing the DHN1 protein were combined and dried by using a Savant Speedvac concentrator (Savant Instruments Inc., Holbrook, NY). The acid extraction mixture and DHN1-containing LC fractions were also analyzed by 15% SDS−PAGE.

Calf Intestinal Alkaline Phosphatase (CIP) Treatment. The dephosphorylation reaction was carried out by incubating 72.7 μg of DHN1 with 36 units of CIP at 30 °C for 30 min in a buffer containing 50 mM NH_4HCO_3. The dephosphorylated DHN1 protein was again purified by HPLC with a 0.50 mm × 150 mm C4 column (Vydac, Hesperia, CA) on an HP 1100 system (Agilent Technologies, Palo Alto, CA) equipped with a UV detector. The flow rate was 6.0 μL/min, and a 75-min gradient of 0−30% acetonitrile in 0.1% trifluoroacetic acid (TFA) was employed. The sample was then dried and subjected to MALDI-TOF MS analysis or enzymatic digestion.

Enzymatic Digestion. A 50-μg protein sample (purified from maize kernel) was digested by modified sequencing grade trypsin (Roche Applied Science, Indianapolis, IN) in 50 mM NH_4HCO_3 (pH 8.0) at an enzyme/substrate ratio of 1:50 overnight. In some cases, the HPLC-purified protein was digested by endoproteinase Glu-C (Roche Applied Science) in 100 mM phosphate buffer (pH 7.8) at an enzyme/substrate ratio of 1:25 overnight. The digestion was quenched by adding 0.1 M acetic acid.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry. The above dried digestion mixture was dissolved in 0.1% TFA and mixed with an equal volume of matrix solution, which was a saturated solution of α-cyano-4-hydroxycinnamic acid in a solvent mixture containing TFA, acetonitrile, and H_2O (0.1:50:50, v/v). After drying, the resulting sample was analyzed in reflectron mode on a DE-STR MALDI-TOF mass spectrometer equipped with a nitrogen laser (Applied Biosystems, Foster City, CA). Purified protein samples were analyzed in linear mode on the same instrument with the same matrix. The mass accuracies with external calibration were approximately 2000 and 100 ppm in linear and reflectron modes, respectively.

LC-Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS). LC-ESI-MS/MS analysis of the enzymatic peptides was carried out by coupling directly the effluent from a 0.32 mm × 50 mm C18 capillary column (300 Å in pore size, 5 μm in particle size, Micro-Tech Scientific, Vista, CA) to an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). The flow rate to the column was approximately 6 μL/min, which was obtained from a 120 μL/min pump flow by using a homemade precolumn splitter. A 65-min gradient of 0−70% acetonitrile in 0.6% acetic acid was delivered by a Surveyor HPLC pump (ThermoFinnigan). MS/MS was done in data-dependent scan mode by selecting the first to third most abundant ions as precursors for fragmentation. In some cases, LC-MS/MS experiments were also performed by monitoring the fragmentation of a few preselected precursor ions.

β-Elimination and MALDI-MS/MS. A 0.15-M $Ba(OH)_2$ solution (200 μL) was added to a 10-μM tryptic digestion mixture of the protein (100 μL). The solution was incubated at room temperature for 2 h, and the reaction was stopped by adding glacial acetic acid. The resulting mixture was dried by a Savant Speedvac concentrator and cleaned up by using C18 ZipTip (Millipore, Bellirica, MA) or purified by HPLC with a 0.50 mm × 150 mm Zorbax C18 capillary column (Agilent Technologies). A linear gradient of 0−30% acetonitrile in 0.1% TFA was employed for the purification of the peptide mixture. The purified peptides were analyzed by MALDI-MS and the $[M + H]^+$ ions of the phosphopeptides, which had undergone β-elimination, were selected for fragmentation on a QSTAR XL hybrid quadrupole time-of-flight mass spectrometer equipped with an oMALDI ion source (Applied Biosystems). The mass accuracy in MS/MS mode was approximately 100 ppm with external calibration.

Phosphorylation of DHN1 Protein by Protein Kinase CK2 in vitro and Identification of the Sites of Phosphorylation. The rephosphorylation of the CIP-treated, HPLC-purified DHN1 protein was conducted by using 20 units of human recombinant CK2 (New England Biolabs, Beverly, MA) per microgram of protein. The mixture was incubated in the kinase buffer supplied by the vendor, and aliquots were removed after the mixture had been incubated for different periods of time. These sample aliquots were desalted by using C4 Zip-Tip (Millipore) and examined by MALDI-MS. For the identification of the sites of in vitro phosphorylation, CK2 was added to the CIP-treated DHN1 protein at a ratio of 100 units per microgram of protein and the resulting reaction mixture was incubated at 30 °C for overnight. The protein was then purified by employing a Vydac C4 column under the same conditions as described above. The purified DHN1 protein was digested by trypsin, subjected to β-elimination, and analyzed on the Q-STAR XL instrument as discussed above for the DHN1 isolated from maize seeds.

RESULTS

Purification of DHN1 Protein from Maize Kernel. Dehydrin proteins are intrinsically unstructured, and they exhibit high solubility in boiling water (*14*). A heat-and-cool

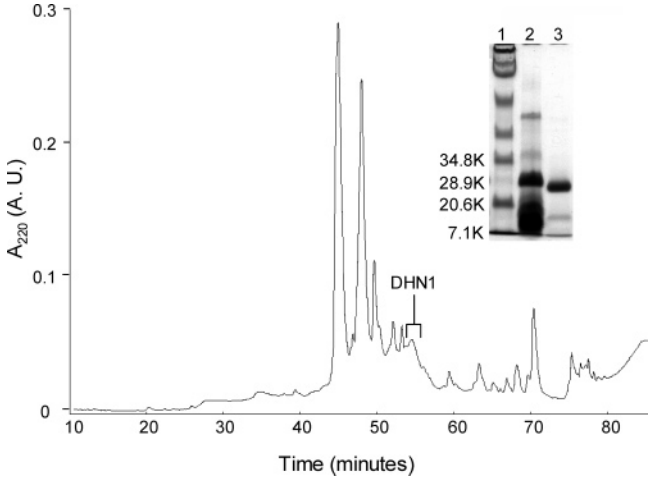


FIGURE 1: HPLC trace for the separation of the protein mixture extracted from maize kernel. The inset shows SDS−PAGE (15%) of the HPLC fraction containing dehydrin DHN1 protein: lane 1, molecular weight marker; lane 2, crude acid extraction mixture of maize seeds; lane 3, DHN1-containing HPLC fraction.

procedure or precipitation with 65% ammonium sulfate was previously employed as an initial enrichment step, which was followed by purification with several steps of chromatographic separations (*10*, *14*, *15*). In the process of isolating plant high-mobility group (HMG) proteins (*16*), which are acid-soluble, we found that the DHN1 protein can be extracted by using a 2% aqueous solution of TCA at room temperature (details given in Experimental Procedures). HPLC separation of the resulting extraction mixture with a reversed-phase C4 column gives DHN1 that is approximately 90% pure as estimated by SDS−PAGE (Figure 1) and MALDI-TOF MS analysis (Figure 2). DHN1 protein, similar to most other dehydrin proteins, showed increased apparent molecular weight on SDS−PAGE over its true molecular weight (Figure 1) (*3*, *9*, *11*, *14*).

We also tried the heat-and-cool procedure, and MALDI-MS analysis showed that the DHN1 protein purified by heat-and-cool procedure contained approximately 1 mol less phosphate per mole of DHN1 protein than that of the protein purified by the acid extraction procedure (data not shown). This result suggests that the high-temperature conditions employed in the heat-and-cool approach can result in the loss of a phosphate group from DHN1.

DHN1 Protein Isolated from Maize Kernel Is Phosphorylated. The identity of the protein was confirmed by LC-ESI-MS/MS and MALDI MS/MS combined with database search with SEQUEST (ThermoFinnigan) and Mascot (Matrix Sciences Inc., Boston, MA) (Table 1). MALDI-MS (Figure 2) of the HPLC-purified DHN1 protein showed elevated molecular masses ($[M + H]^+$ ions) at *m*/*z* 17531, 17617, and 17708 compared to that of the unmodified DHN1 protein (*m*/*z* 17161). The differences between the measured and calculated masses indicated that there might be 5−7 mol of phosphate groups incorporated to 1 mol of protein, and the hexaphosphorylated protein appears to be the most abundant as represented by peak intensities in MALDI-MS. To confirm that the molecular weight increase is indeed due to phosphorylation, we treated the DHN1 protein with CIP. MALDI-MS of the CIP-treated protein showed that the molecular mass of the protein dropped to 17144 Da, which is close to the calculated mass of the unmodified protein

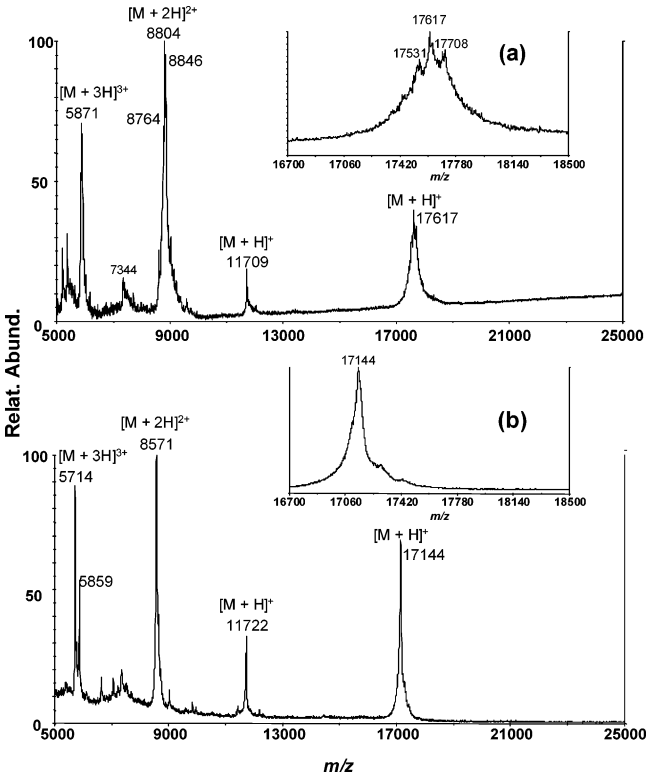


FIGURE 2: MALDI-MS of (a) the HPLC-purified dehydrin DHN1 protein from maize kernel and (b) the CIP-treated DHN1 protein. The portions of the spectra containing the singly charged ion of dehydrin are enlarged and shown in the insets. The ion of 11.7 kDa is an unknown protein coeluting with DHN1.

(Figure 2). This result demonstrates without ambiguity that the protein is phosphorylated.

DHN1 Is Heavily Phosphorylated in the Serine Tract Region in Vivo. To determine the sites of phosphorylation, we digested the DHN1 protein with either modified trypsin or endoproteinase Glu-C (V8) and subjected the resulting peptides to both MALDI-MS and LC-ESI-MS/MS analyses. We, however, were unable to find any phosphorylated peptide (Figure 3a shows a portion of the MALDI-MS of the tryptic digestion mixture of DHN1). Considering that high level of phosphorylation may accumulate in the serine tract region (*17*), we suspected that the ionization efficiency of the phosphopeptide(s) could be too low for the peptide to be detected in MS. In addition, it is also possible that the molecular ion(s) of the phosphopeptide(s) might not be stable. If either or both of these two factors are responsible for the failure in observing the phosphopeptide(s), removal of the phosphate groups in DHN1 should facilitate the detection of the S-segment tryptic peptide. To validate this, we dephosphorylated the DHN1 protein by CIP and digested the resulting protein with trypsin. Indeed, MALDI-MS analysis of the tryptic peptide mixture showed the appearance of the unmodified S-segment tryptic peptide (ion of *m*/*z* 1589.5, corresponding to amino acid residues 76−92, Figure 3b).

The above results demonstrated that the S-segment of the DHN1 protein is likely to be heavily phosphorylated. It is desirable, therefore, to establish the extent and locate the sites of the phosphorylation in this peptide. To this end, we removed the phosphoric acid groups from phosphorylated serines by using β-elimination reaction (*18*, *19*). Comparing

Table 1: A Summary of the Dehydrin Peptides Identified by MS/MS

sequences	theoretical mass, $[M + H]^+$	measured mass[a]	modifications[b]	enzyme	identification
1−9	1138.5	1138.6	*N*-acet	trypsin	MALDI-MS/MS; LC-ESI-MS/MS
29−49	1998.9	1000.3 (2+)	Met-34 ox	V8	LC-ESI-MS/MS
36−64	2711.2	1356.3 (2+)	no	trypsin	LC-ESI-MS/MS
36−68	3234.5	1618.3 (2+)	no	trypsin	ESI-MS/MS
50−65	1572.7	787.2 (2+)	no	V8	LC-ESI-MS/MS
69−75	753.4	753.5	no	trypsin	MALDI-MS/MS; LC-ESI-MS/MS
76−92	1499.5	1499.5	phosphorylation (5P, 6P, and 7P)	trypsin	MALDI-MS/MS after β-elimination
	1481.5	1481.5			
	1463.5	1463.5			
76−92	1497.5	1497.5	phosphorylation (6P and 7P)	trypsin	MALDI-MS/MS after β-elimination
	1515.5	1515.5	Met-89 ox		
105−110	608.4	609.4	no	trypsin	LC-ESI-MS/MS
105−153	4771.1	1591.5 (3+)	no	trypsin	LC-ESI-MS/MS
155−159	563.3	563.3	no	trypsin	LC-ESI-MS/MS

[a] Charge states for doubly and triply charged ions are shown in parentheses. [b] Abbreviations acet and ox designate acetylation and oxidation, respectively.

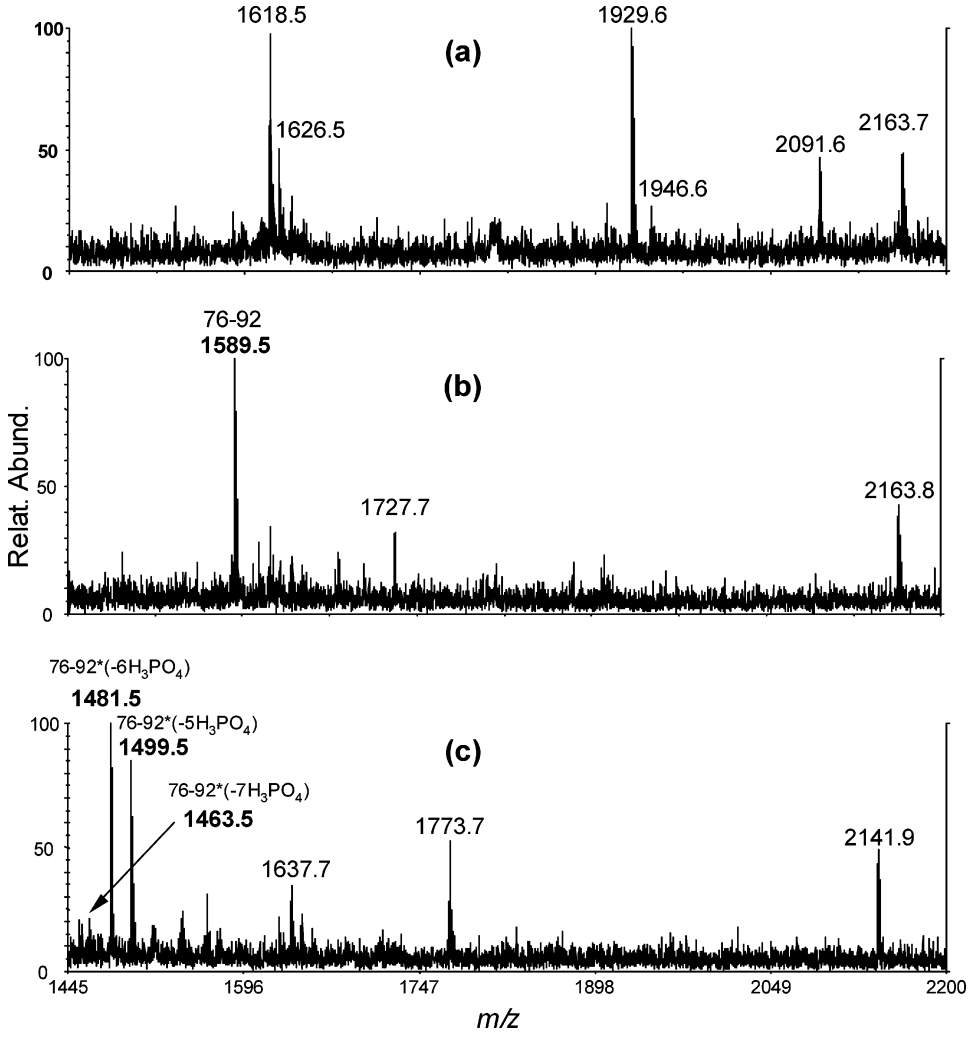
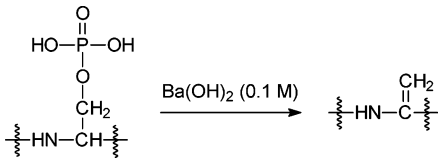


FIGURE 3: MALDI-MS of (a) tryptic digestion mixture of the dehydrin DHN1 protein purified from maize kernel and (b) tryptic digestion mixture of the CIP-treated DHN1 protein. Dephosphorylated tryptic peptide containing serine tract from residue 76 to 92 at *m/z* 1589.5 was observed. Panel c shows MALDI-MS of β-elimination products of tryptic digestion mixture of the DHN1 protein purified from maize kernel. Three β-eliminated tryptic peptides containing the serine stretch at *m/z* 1463.5 (7p), 1481.5 (6p), and 1499.5 (5p) were observed.

a phosphoserine that has undergone β-elimination with an unmodified serine, the former has a water loss (Scheme 2). Thus, the β-elimination reaction leaves a marker, that is, water loss, on the amino acid residues that are phosphorylated, which facilitates the determination of the site(s) of phosphorylation by tandem mass spectrometry. In this regard, it has been demonstrated in the past that such elimination procedure does not lead to water loss from the

Scheme 2 The β-elimination of H_3PO_4 from phosphoserine via $Ba(OH)_2$ Treatment



unmodified serine or threonine residues in proteins (*18*). After the β-elimination, three peaks were observed in MALDI-MS, that is, ions of *m*/*z* 1463.5, 1481.5, and 1499.5. These three ions are attributed to the protonated ions of the hepta-, hexa-, and pentaphosphorylated S-segment tryptic peptides (residues 76−92), respectively, after all their composing phosphorylated amino acid residues have undergone β-elimination (Figure 3c).

We next subjected these three ions to fragmentation and the resulting product-ion spectra confirmed that the sequences of the three peptides are amino acid residues 76−92, that is, SGSSSSSSSEDDGMGGR. Interestingly, each of the seven consecutive serine residues in the S-segment, that is, residues Ser78−Ser84, can be phosphorylated in the heptaphosphorylated peptide (Figure 4). In this respect, we observed that the mass of the y_8 ion is the same as the calculated mass of the y_8 ion of the unmodified peptide, demonstrating that none of the last eight amino acids at the C-terminal side of the peptide is modified. This result is consistent with there being no phosphorylatable residue in this segment. Cleavages of the amide bonds that are N-terminal to each of seven serines in the serine tract again give a series of y ions. Furthermore, the difference in *m*/*z* values of adjacent y ions (69 Da) is consistent with the presence of the β elimination product, that is, dehydroalanine, rather than an unmodified serine (87 Da), demonstrating clearly that each of the seven serines in the serine tract has undergone a phosphoric acid (H_3PO_4) loss during β elimination (Figure 4). Therefore, each of the seven serines is phosphorylated in the heptaphosphorylated peptide. Ser76 in this peptide segment, however, is not phosphorylated.

The hexaphosphorylated S-segment tryptic peptide was also found to be modified in the serine tract, that is, Ser79−Ser84. In MALDI-MS/MS (Figure 5), we observed that the y_1, y_2, y_3, y_4, y_5, y_6, y_7, and y_8 ions again have the same masses as those calculated from the unmodified peptide,

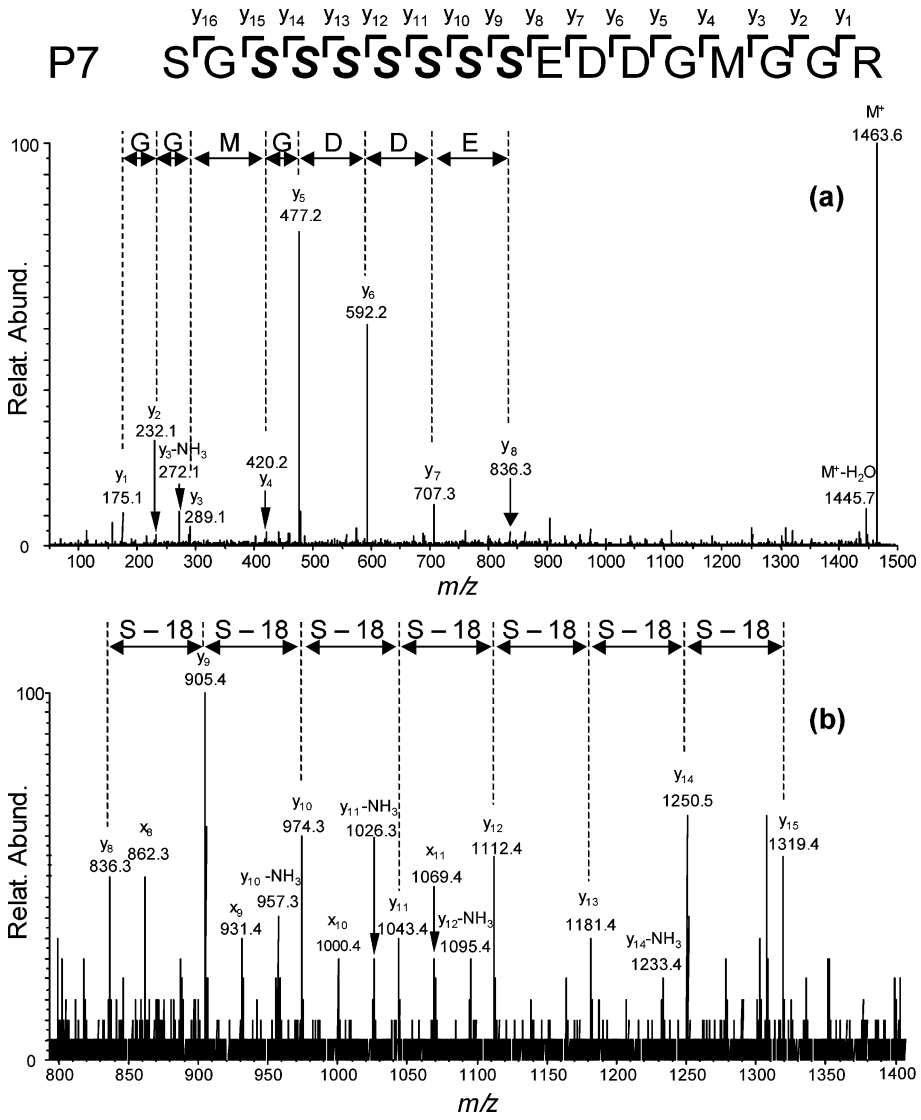


FIGURE 4: MALDI-MS/MS of the ion of *m*/*z* 1463.5 observed in Figure 3c. The spectrum in the mass range of *m*/*z* 800−1400 was enlarged and shown in panel b. The sequence of the peptide segment and the y-series fragment ions were shown above the spectrum. "***S***" in the peptide sequence and "S − 18" in panels a and b designate a dehydroalanine, which results from the β-elimination of phosphoserine.

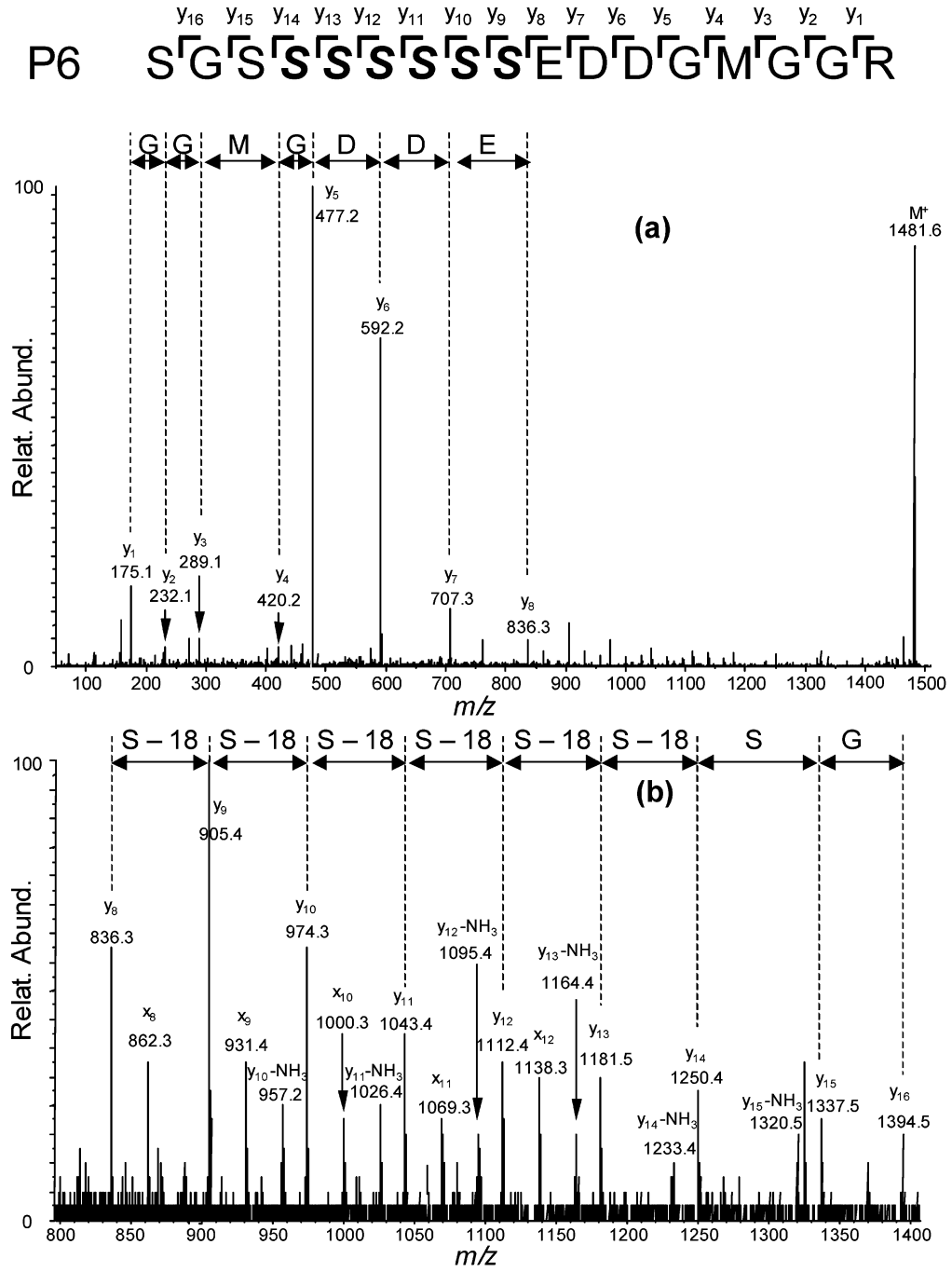


FIGURE 5: MALDI-MS/MS of the ion of *m*/*z* 1481.5 observed in Figure 3c. The spectrum in the mass range of *m*/*z* 800−1400 was enlarged and shown in panel b. The sequence of the peptide segment and the y-series fragment ions were shown above the spectrum. "***S***" in the peptide sequence and "S − 18" in panels a and b designate a dehydroalanine, which results from the β-elimination of phosphoserine.

showing that the eight amino acids on the C-terminal side of the peptide are not modified. The y-series product ions emanating from the rupture of the amide bonds on the N-terminal side of the serine residues in the serine tract demonstrate that all serines except Ser78 had undergone β elimination (Figure 5). This allows us to conclude that each of the six serines from Ser79 to Ser84 is phosphorylated in the hexaphosphorylated peptide. Pentaphosphorylated peptide was also found to be modified in the serine tract region; the sites of phosphorylation, however, were heterogeneous, that is, Ser79, 81, and 82 were phosphorylated, whereas Ser80 and Ser83 were partially phosphorylated (data not shown).

It is interesting to note that, in addition to the y ions, we also found a number of x and [y − NH_3] ions in both Figure 4 and Figure 5. The latter two types of ions are rarely observed in the product-ion spectra of protonated ions of peptides, though z ions, which are analogous to the [y − NH_3] ions observed here but present as odd-electron species, are frequently observed in the electron-capture dissociation spectra of proteins (*20*). From Figures 4 and 5, we found that the x and [y − NH_3] ions only form from cleavages at the sites where there is a dehydroalanine residue. The mechanisms for their formation remain unclear. We, however, reason that the introduction of a C═C bond to the C_α

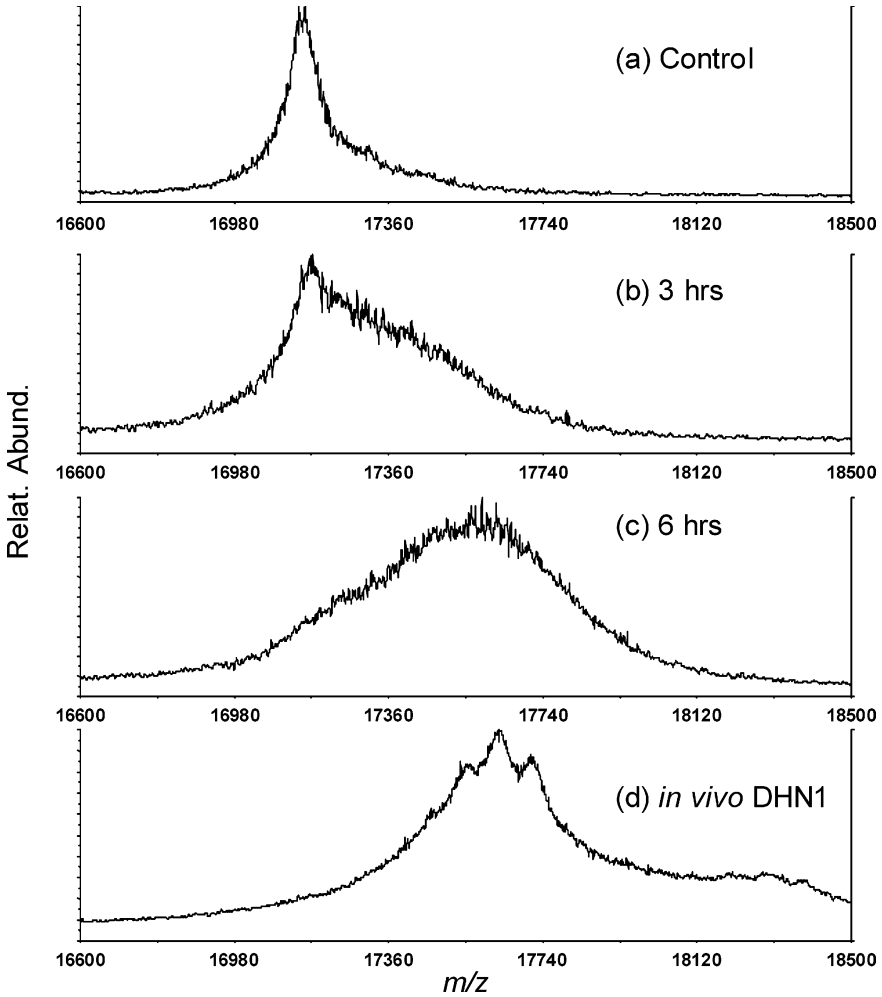


FIGURE 6: MALDI-MS of DHN1 protein that has been treated with CIP (a), CIP-treated DHN1 that has been rephosphorylated for 3 h with 20 units of CK2 per microgram of protein (b), DHN1 treated for 6 h with 200 units of CK2 per microgram of protein (c), and DHN1 isolated from dry maize kernel (d).

carbon atom from the β elimination reaction weakens the N−C_α and C_α−C bonds and makes them more susceptible to cleavage upon collisional activation.

Phosphorylation of DHN1 by CK2 in Vitro. We next examined the level and the sites of phosphorylation of DHN1 catalyzed by protein kinase CK2. To this end, we obtained the dephosphorylated DHN1 protein by treating the above DHN1 protein with CIP. MALDI-MS analysis of the resulting CIP-treated DHN1 protein showed that the dephosphorylation was complete (Figure 6a). The dephoshorylated DHN1 was then treated with human recombinant CK2 for different time periods, and the products were monitored by MALDI-MS. The results showed that the level of phosphorylation in DHN1 rises with increasing CK2 incubation time. The extent of in vitro phosphorylation could be as high as that of in vivo phosphorylation when more enzyme was added and incubation time was increased to 6 h (Figure 6a−c).

We next digested the in vitro phosphorylated DHN1 (overnight reaction, details shown in Experiment Procedures) with trypsin and again removed the phosphoric acid groups from the resulting phosphopeptides by β-elimination. MALDI-MS of β-eliminated DHN1 tryptic peptides showed two peaks at *m*/*z* 1497.5 and 1515.5 (Figure 7b), which are attributed to the hexa- and pentaphosphorylated S-segment tryptic peptides with all their composing phosphoric acid groups being eliminated. In addition, Met89 in both peptides has been oxidized to methionine sulfoxide. In this regard, the product-ion spectra of the ions *m*/*z* 1497.5 and 1515.5 showed that the sequence of these two peptides was amino acid residues 76−92. Moreover, the product-ion spectrum of the former ion showed that all the six serines from Ser79 to Ser84 are phosphorylated (Figure 8). The pentaphospho-

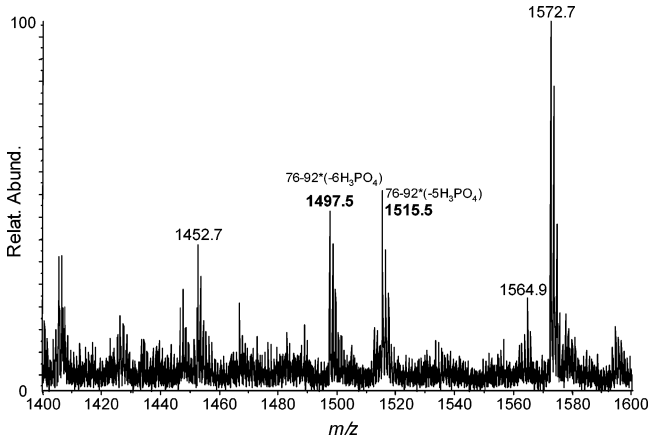


FIGURE 7: A portion of MALDI-MS of the β-elimination products of trypsin digestion mixture of CK2-rephosphorylated dehydrin DHN1 protein. Ions of *m*/*z* 1497.5 and 1515.5 corresponded to products formed from the β-elimination of hexa- and pentaphosphorylated S-segment peptides.

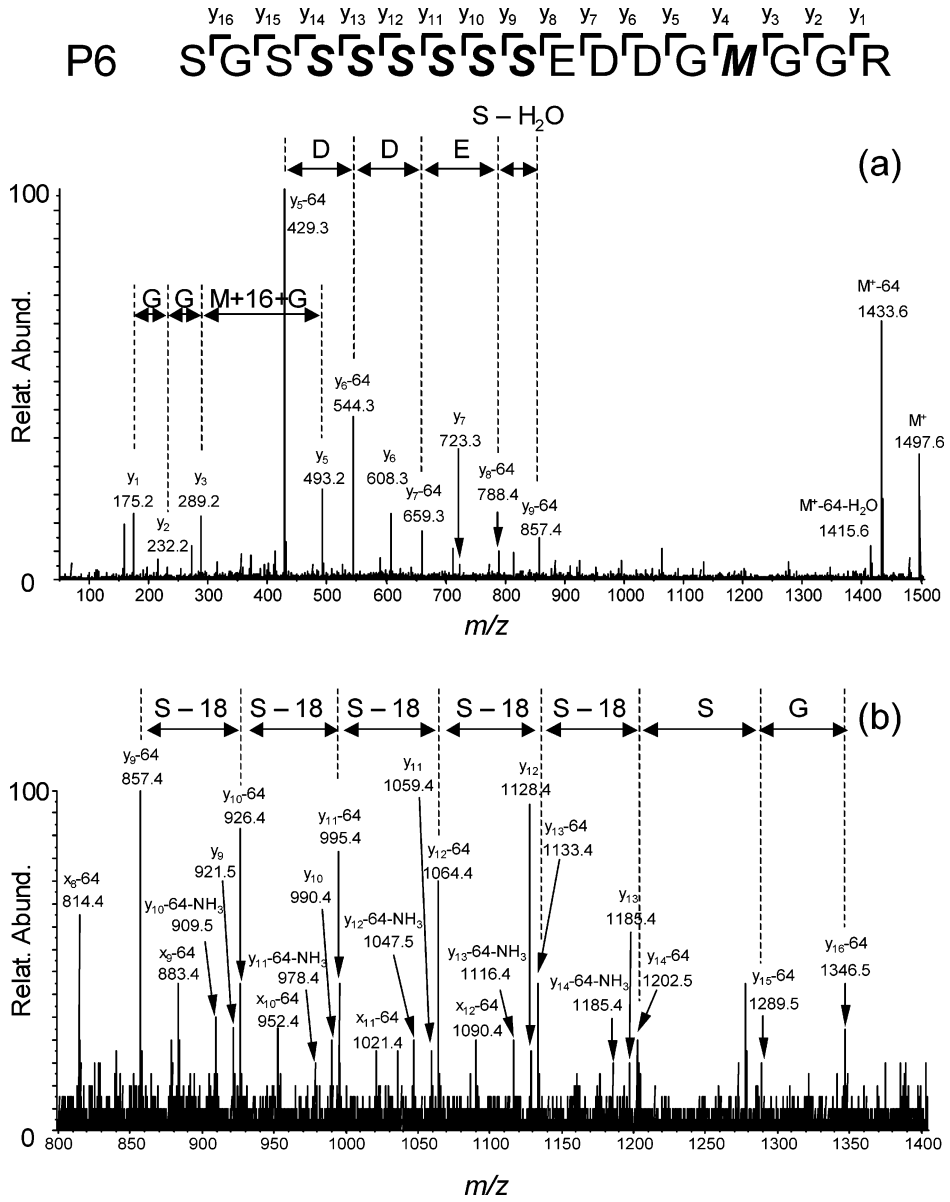


FIGURE 8: MALDI-MS/MS of the ion of *m*/*z* 1497.5 observed in Figure 7. Shown on the top is the sequence of the peptide segment with the modified serines and methionine highlighted. The *m*/*z* 800−1400 portion of the spectrum was enlarged and shown in panel b.

rylated S-segment peptide was again found to be heterogeneous in sites of phosphorylation (data not shown).

It is worth noting that we observed a ubiquitous neutral loss of a 64-Da fragment for y-series fragment ions bearing the oxidized methionine (Figure 8), which is attributed to the elimination of methane sulfenic acid (CH_3SOH) from the side chain of methionine sulfoxide (*21*−*23*). Oxidation of Met89 might have occurred during various stages of sample handling.

DISCUSSION

Dehydrin proteins are among the "intrinsically unstructured" proteins as they show good solubility in boiling water (*14*). By taking advantage of this property, dehydrin proteins can be enriched from plant tissue extract by using a heat-and-cool procedure (*14*). Serendipitously, we found that DHN1 protein can be obtained in approximately 90% purity by extraction with 2% trichloroacetic acid followed by a single-step HPLC purification. In this respect, acid extraction has been commonly used for the facile isolation of HMG proteins (*24*, *25*). It is interesting to note that both the HMG proteins (*26*) and dehydrin proteins (*14*) are intrinsically unstructured. In addition, these two groups of proteins are rich in both acidic (phosphorylated S-segment in dehydrin) and basic amino acids (the K-segment in dehydrin). This unique structure feature may facilitate the high solubility of dehydrin DHN1 in 2% TCA. When the acid extraction and the heat-and-cool methods were compared, the DHN1 protein is less susceptible to phosphate group loss during the acid extraction procedure.

The MALDI-MS of the purified DHN1 protein isolated from dry maize kernel showed that 5−7 mol of phosphate groups can be incorporated to 1 mol of protein. We, however, failed to detect any phosphopeptides from trypsin or Glu-C digestion mixtures of DHN1 by LC-ESI-MS/MS and MALDI-MS/MS, which was attributed to the low ionization efficiency of the phosphopeptides. To determine the sites of phosphorylation of this protein, we adopted a β-elimination procedure, which removes phosphoric acid groups from phosphoserines and phosphothreonines (*18*). It turned out that the

products formed from the β-elimination are amenable to detection by MALDI-MS. Furthermore, the sites of phosphorylation can be readily determined from the product-ion spectra of the MALDI-produced ions of the β-elimination products of those phosphorylated S-segment tryptic peptides. Since phosphorylated serine and threonine residues, but not phosphotyrosine, can undergo β-elimination very efficiently (*18*), we reason that the combination of β-elimination with MALDI MS/MS will also be useful for the site mapping of other proteins that are highly phosphorylated on serine or threonine residues or both.

Interestingly, each of the seven contiguous serine residues in the S-segment tryptic peptide SGSSSSSSSEDDGMGGR of the dehydrin DHN1 protein isolated from maize kernels can be phosphorylated in maize seeds. Although ERD-14 from *Arabidopsis*, which is also a dehydrin protein, was reported to be phosphorylated on serine or threonine residues outside of the S-segment (*9*), we did not observe phosphorylation of any residues other than the serine residues in the serine track of the DHN1 protein.

We also demonstrated that dehydrin DHN1 protein can be phosphorylated in vitro by recombinant human protein kinase CK2. Moreover, both the level and the sites of in vitro phosphorylation are very similar to those observed for DHN1 isolated from maize seeds. A recent analysis of 308 sites that are phosphorylated by protein kinase CK2 highlights the paramount importance of amino acids with negatively charged side chains that are at positions $n + 3$ (the most crucial one), $n + 1$, and $n + 2$, where n is the position of phosphorylation (*27*). Therefore, the DHN1 protein has a consensus CK2 substrate recognition sequence at the C-terminal side of the S-segment, which comprises of three acidic amino acid residues, that is, Glu85, Asp86, and Asp87. As a result, Ser84 is the first site that can be phosphorylated. Because the phosphorylated serine largely resembles an acidic amino acid, CK2 can phosphorylate the rest of the serine residues down the chain from Ser84 to Ser78. In this regard, dehydrin ERD-14 from *Arabidopsis* (*9*) and a dehydrin-like protein, VCaB45, from celery (*11*) were also found to be phosphorylated by CK2. The extent of phosphorylation reported in this study was higher than those reported previously for DHN1 (*10*) and ERD-14 (*9*), which might be due to different enzyme-to-substrate ratios employed, different sample handling processes used, or both. The above results showed that protein kinase CK2 might play an important role in the phosphorylation of DHN1 in vivo. Recent work by Riera et al. (*28*), however, reported that, in the presence of a CK2 inhibitor, recombinant DHN1 protein can still be phosphorylated by maize embryo extract, indicating that other kinase(s) might also be involved in the phosphorylation of this protein in vivo. In this respect, a tomato DHN1 homologue protein, TAS14, was shown to be phosphorylated by both CK2 and cyclic-AMP-dependent protein kinase (*29*), though the sites of phosphorylation were not examined.

The phosphorylation of DHN1 may play an important role in the function of this protein. Recently Riera et al. (*28*) showed that CK2 can specifically associate with DHN1 through the regulatory subunit of the enzyme and the phosphorylation of the DHN1 protein by CK2 is important in growth process under stress conditions. It has also been demonstrated previously that the ERD-14 and VCaB45 proteins have calcium binding properties upon their phosphorylation (*9*, *11*). Because DHN1, ERD-14, and VCaB45 share common structure features, the phosphorylation of the DHN1 protein may also facilitate its binding to Ca^{2+} ion.

ACKNOWLEDGMENT

The authors want to thank Dr. Ing-Feng (Ira) Chang and Prof. Julia Bailey-Serres for supplying maize seeds and Prof. Timothy J. Close for helpful discussion. We also appreciate Dr. Songqin Pan at the W. M. Keck Proteomics Laboratory, Center of Plant Cell Biology, University of California at Riverside, for help with MALDI-MS/MS measurements.

REFERENCES

1. Nylander, M., Svensson, J., Palva, E. T., and Welin, B. V. (2001) Stress-induced accumulation and tissue-specific localization of dehydrins in *Arabidopsis thaliana*, *Plant Mol. Biol. 45*, 263−279.
2. Lang, V., Mantyla, E., Welin, B., Sundberg, B., and Palva, E. T. (1994) Alterations in water status, endogenous abscisic acid content, and expression of rab18 gene during the development of freezing tolerance in *Arabidopsis thaliana*, *Plant Physiol. 104*, 1341−1349.
3. Welin, B. V., Olson, A., Nylander, M., and Palva, E. T. (1994) Characterization and differential expression of dhn/lea/rab-like genes during cold acclimation and drought stress in *Arabidopsis thaliana*, *Plant Mol. Biol. 26*, 131−144.
4. Lang, V., and Palva, E. T. (1992) The expression of a rab-related gene, rab18, is induced by abscisic acid during the cold acclimation process of *Arabidopsis thaliana* (L.) Heynh, *Plant Mol. Biol. 20*, 951−962.
5. Vilardell, J., Goday, A., Freire, M. A., Torrent, M., Martinez, M. C., Torne, J. M., and Pages, M. (1990) Gene sequence, developmental expression, and protein phosphorylation of RAB-17 in maize, *Plant Mol. Biol. 14*, 423−432.
6. Pla, M., Goday, A., Vilardell, J., Gomez, J., and Pages, M. (1989) Differential regulation of ABA-induced 23−25 kDa proteins in embryo and vegetative tissues of the viviparous mutants of maize, *Plant Mol. Biol. 13*, 385−394.
7. Close, T. J. (1997) Dehydrins: a commonalty in the response of plants to dehydration and low temperature, *Physiol. Plant. 100*, 291−296.
8. Koag, M. C., Fenton, R. D., Wilkens, S., and Close, T. J. (2003) The binding of maize DHN1 to lipid vesicles. Gain of structure and lipid specificity, *Plant Physiol. 131*, 309−316.
9. Alsheikh, M. K., Heyen, B. J., and Randall, S. K. (2003) Ion binding properties of the dehydrin ERD14 are dependent upon phosphorylation, *J. Biol. Chem. 278*, 40882−40889.
10. Plana, M., Itarte, E., Eritja, R., Goday, A., Pages, M., and Martinez, M. C. (1991) Phosphorylation of maize RAB-17 protein by casein kinase 2, *J. Biol. Chem. 266*, 22510−22514.
11. Heyen, B. J., Alsheikh, M. K., Smith, E. A., Torvik, C. F., Seals, D. F., and Randall, S. K. (2002) The calcium-binding activity of a vacuole-associated, dehydrin-like protein is regulated by phosphorylation, *Plant Physiol. 130*, 675−687.
12. Jensen, A. B., Goday, A., Figueras, M., Jessop, A. C., and Pages, M. (1998) Phosphorylation mediates the nuclear targeting of the maize Rab17 protein, *Plant J. 13*, 691−697.
13. Goday, A., Jensen, A. B., Culianez-Macia, F. A., Mar Alba, M., Figueras, M., Serratosa, J., Torrent, M., and Pages, M. (1994) The maize abscisic acid-responsive protein Rab17 is located in the nucleus and interacts with nuclear localization signals, *Plant Cell 6*, 351−360.
14. Ceccardi, T. L., Meyer, N. C., and Close, T. J. (1994) Purification of a maize dehydrin, *Protein Expression Purif. 5*, 266−269.
15. Ismail, A. M., Hall, A. E., and Close, T. J. (1999) Purification and partial characterization of a dehydrin involved in chilling tolerance during seedling emergence of cowpea, *Plant Physiol. 120*, 237−244.
16. Grasser, K. D., Grimm, R., and Ritt, C. (1996) Maize chromosomal HMGc. Two closely related structure-specific DNA-binding proteins specify a second type of plant high mobility group box protein, *J. Biol. Chem. 271*, 32900−32906.

17. Close, T. J., Fenton, R. D., Yang, A., Asghar, R., DeMason, D. A., Crone, D. E., Meyer, N. C., and Moonan, F. (1993) Dehydrin: the protein, *Curr. Top. Plant Physiol. 10*, 104−118.
18. Byford, M. F. (1991) Rapid and selective modification of phosphoserine residues catalyzed by barium(2+) ions for their detection during peptide microsequencing, *Biochem. J. 280*, 261−265.
19. Resing, K. A., Johnson, R. S., and Walsh, K. A. (1995) Mass spectrometric analysis of 21 phosphorylation sites in the internal repeat of rat profilaggrin, precursor of an intermediate filament associated protein, *Biochemistry 34*, 9477−9487.
20. Zubarev, R. A., Kelleher, N. L., and McLafferty, F. W. (1998) Electron capture dissociation of multiply charged protein cations. A nonergodic process, *J. Am. Chem. Soc. 120*, 3265−3266.
21. Jiang, X., Smith, J. B., and Abraham, E. C. (1996) Identification of MS-MS fragment diagnostic for methionine sulfoxide, *J. Mass Spectrom. 31*, 1309−1310.
22. O'Hair, R. A. J., and Reid, G. E. (1999) Neighboring group versus cis-elimination mechanisms for side chain loss from protonated methionine, methionine sulfoxide and their peptides gas-phase ion chemistry of biomolecules, *Eur. Mass Spectrom. 5*, 325−334.
23. Guan, Z., Yates, N. A., and Bakhtiar, R. (2003) Detection and characterization of methionine oxidation in peptides by collision-induced dissociation and electron capture dissociation, *J. Am. Soc. Mass Spectrom. 14*, 605−613.
24. Lim, J. H., Catez, F., Birger, Y., Postnikov, Y. V., and Bustin, M. (2004) Preparation and functional analysis of HMGN proteins, *Methods Enzymol. 375*, 323−342.
25. Reeves, R. (2004) HMGA proteins: isolation, biochemical modifications, and nucleosome interactions, *Methods Enzymol. 375*, 297−322.
26. Reeves, R. (2001) Molecular biology of HMGA proteins: hubs of nuclear function, *Gene 277*, 63−81.
27. Meggio, F., and Pinna, L. A. (2003) One-thousand-and-one substrates of protein kinase CK2, *FASEB J. 17*, 349−368.
28. Riera, M., Figueras, M., Lopez, C., Goday, A., and Pages, M. (2004) Protein kinase CK2 modulates developmental functions of the abscisic acid responsive protein Rab17 from maize, *Proc. Natl. Acad. Sci. U.S.A. 101*, 9879−9884.
29. Godoy, J. A., Lunar, R., Torres-Schumann, S., Moreno, J., Rodrigo, R. M., and Pintor-Toro, J. A. (1994) Expression, tissue distribution and subcellular localization of dehydrin TAS14 in salt-stressed tomato plants, *Plant Mol. Biol. 26*, 1921−1934.

BI0483965