

Water Deficit Rapidly Stimulates the Activity of a Protein Kinase in the Elongation Zone of the Maize Primary Root¹

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The mechanisms by which plants detect water deficit and transduce that signal into adaptive responses is unknown. In maize (*Zea mays* L.) seedlings, primary roots adapt to low water potentials such that substantial rates of elongation continue when shoot growth is completely inhibited. In this study, in-gel protein kinase assays were used to determine whether protein kinases in the elongation zone of the primary root undergo activation or inactivation in response to water deficit. Multiple differences were detected in the phospho-protein content of root tips of water-stressed compared with well-watered seedlings. Protein kinase assays identified water-deficit-activated protein kinases, including a 45-kD, Ca²⁺-independent serine/threonine protein kinase. Water-deficit activation of this kinase occurred within 30 min after transplanting seedlings to conditions of low water potential and was localized to the elongation zone, was independent of ABA accumulation, and was unaffected by cycloheximide-mediated inhibition of protein translation. These results provide evidence that the 45-kD protein kinase acts at an early step in the response of maize primary roots to water deficit and is possibly involved in regulating the adaptation of root growth to low water potential.

The accessibility of water is a critical environmental determinant of plant growth and distribution. Higher plants possess a variety of mechanisms that may enhance their ability to tolerate water limitation imposed by sporadic rainfall or temperature extremes, and the physiological basis of responses to water deficit has been studied extensively (reviewed by Smith and Griffiths, 1993). However, current understanding of the molecular mechanisms by which plants detect water deficit, transduce that signal to the intracellular machinery, and regulate adaptive responses is limited.

¹ This work was supported by National Science Foundation grants no. MCB-9417732 and no. MCB-9219075 to J.C.W. and no. IBN-9306935 to R.E.S. and Eric S. Ober and by the University of Missouri Food for the 21st Century Program (J.C.W. and R.E.S.). T.R.C. was supported by a postdoctoral fellowship from the University of Missouri Maize Biology Training Program, a unit of the Department of Energy/National Science Foundation/U.S. Department of Agriculture Collaborative Research in Plant Biology Program. This is a contribution from the Missouri Agricultural Experiment Station, Journal Series no. 12,536.

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Previous work with maize (*Zea mays* L.) seedlings has shown that shoot and root growth are differentially sensitive to water stress. Whereas shoot elongation decreases to 0 during moderate water deficit (ψ_w of -0.8 MPa), substantial rates of primary root elongation continue under more severe water deficit (ψ_w of -1.6 MPa; Sharp et al., 1988). Furthermore, it has been shown that ABA is involved in the regulation of these growth responses (Saab et al., 1990; Sharp et al., 1994). Because the effects of ABA may be mediated through changes in gene expression (reviewed by Chandler and Robertson, 1994), numerous molecular studies of water stress have focused on identifying gene transcripts that are positively or negatively regulated by ABA and/or water stress. These studies support a model in which multiple mechanisms, including ABA-dependent and -independent pathways, control gene expression in response to water deficit (reviewed by Skriver and Mundy, 1990; Yamaguchi-Shinozaki et al., 1995). However, other experimental approaches may be necessary to identify early-acting signaling molecules, since these are likely to be activated through posttranslational modifications such as reversible phosphorylation rather than at the step of gene transcription.

In plants reversible changes in protein phosphorylation are known to occur in response to extrinsic and intrinsic stimuli, including, for example, auxin (Reddy et al., 1987), light (Short and Briggs, 1990), and fungal elicitors (Suzuki and Shinshi, 1995). However, relatively little has been done to determine directly whether changes in protein phosphorylation occur in response to water stress and/or ABA. Koontz and Choi (1993) showed that changes in the extent of phosphorylation of three carrot embryo proteins occurred following treatment with ABA. Additional evidence that reversible changes in protein phosphorylation are involved in ABA-mediated responses comes from studies that identified protein phosphatases and protein kinases that function in ABA-mediated signaling pathways. These include an *Arabidopsis thaliana* protein phosphatase (ABI1) that may function in a variety of responses to ABA, such as seed dormancy, stomatal aperture regulation, and expression of ABA- or stress-inducible genes (Koornneef et al., 1984; Leung et al., 1994; Meyer et al., 1994; Gosti et al., 1995). An ABA- and water stress-inducible gene for a protein kinase (PKABA1) has been identified in wheat (Ander-

Abbreviations: CHX, cycloheximide; FLU, fluridone; Pi, orthophosphoric acid; ψ_w , water potential.

berg and Walker-Simmons, 1992; Holappa and Walker-Simmons, 1995).

We sought to identify early-acting signaling proteins that might function in regulating root growth responses to water stress in maize seedlings and to assess whether ABA accumulation is necessary for their activity. To address this problem we used a biochemical approach to detect signaling events that occur prior to the gene activation level that others have examined. In-gel protein kinase assays (Kameshita and Fujisawa, 1989) were used to determine whether protein kinases with increased or decreased activity could be identified in the elongation zone of water-stressed primary roots. A 45-kD protein kinase that localized to the elongation zone showed enhanced activity within 30 min after water stress imposition, and this response was independent of ABA accumulation and new protein synthesis. This protein may function at an early step in a signaling pathway involved in root growth adaptation to water stress.

MATERIALS AND METHODS

Seedling Culture

Seeds of maize (*Zea mays* L., cv FR27 × FRMo17) were germinated in moist vermiculite and after 32 to 36 h seedlings with primary roots 10 to 20 mm in length were transplanted directly into vermiculite that was moistened with sufficient water to yield a ψ_w of approximately -0.03 or -1.6 MPa (Sharp et al., 1988), or the seedlings were first labeled as described below. At various times after transplanting, whole seedlings were harvested directly into liquid nitrogen and stored frozen at -70°C until analysis. Seedlings were maintained in darkness at 29°C and near-saturation humidity in all experiments. Illumination during seedling manipulation, transplanting, and harvesting was provided by a green safelight (Saab et al., 1990). Vermiculite ψ_w was measured by isopiestic thermocouple psychrometry (Boyer and Knipling, 1965). Unless stated otherwise, a minimum of 25 seedlings was sampled for each treatment condition.

In Vivo Pi and [^{35}S]Met Labeling and CHX Treatment

Intact seedlings with primary roots 10 to 15 mm in length were labeled for 1 h with [^{32}P] Pi (DuPont NEN). The seedlings were placed upright into individual wells of a polypropylene microtube rack (Fisher Scientific). Each well contained 1 mL of labeling solution and a 15-mm-long piece of 10-mm o.d. Tygon tubing (Norton Performance Plastics, Akron, OH) that suspended the root in the labeling solution and prevented the root tip from contacting the bottom of the well. The volume of labeling solution was sufficient to submerge the entire primary root and the kernel. The [^{32}P] Pi labeling solution consisted of 25 mM Mes (pH 6.7) and 0.04% Tween 20 as described by Friedmann and Poovaiah (1991), with 50 μCi of [^{32}P] Pi (8500 Ci mmol^{-1}). Ten seedlings were used for each treatment con-

dition. After labeling, seedlings were transplanted into vermiculite at a ψ_w of -0.03 or -1.6 MPa for 1 h.

Using a similar procedure, we labeled the seedlings for 1 h with [^{35}S]Met (DuPont NEN). The wells contained 1 mL of [^{35}S]Met labeling solution, consisting of 20 μCi of [^{35}S]Met (>1000 Ci mmol^{-1}) in 25 mM Mes (pH 6.7) and 0.04% Tween 20. For seedlings that were also treated with CHX, the [^{35}S]Met labeling solution was supplemented with 60 $\mu\text{g mL}^{-1}$ CHX (Sigma). Fifteen seedlings were used for each treatment condition. Following the 1-h labeling and/or CHX treatment, seedlings were transplanted into vermiculite at a ψ_w of -0.03 or -1.6 MPa for 30 min.

Protein Extraction and in-Gel Protein Kinase Assays

Protein extraction and in-gel protein kinase assays were performed essentially as described by Kameshita and Fujisawa (1989). The apical 10 mm of the primary root (encompassing the elongation zone; Sharp et al., 1988) from frozen seedlings was homogenized in a mortar containing a small volume of buffer (50 mM Hepes, pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 10 mM sodium vanadate, 10 mM NaF, 50 mM DL- α -glycerophosphate, 20 mM PMSF, 0.2 $\mu\text{g mL}^{-1}$ leupeptin, and 0.2 $\mu\text{g mL}^{-1}$ aprotinin). Protein extracts were applied to a column containing Sephadex G-25 equilibrated in a buffer consisting of 20 mM Hepes (pH 7.5), 1 mM MgCl_2 , 1 mM DTT, 1 mM sodium vanadate, 5 mM NaF, 10 mM DL- α -glycerophosphate, 20 mM PMSF, 0.2 $\mu\text{g mL}^{-1}$ leupeptin, and 0.2 $\mu\text{g mL}^{-1}$ aprotinin. Proteins were eluted from the columns with 1.2 void volumes of the same buffer. Protein concentrations were determined by the method of Bradford (1976).

Proteins (25–30 μg , as noted in figure legends) were separated by SDS-PAGE in 10% (w/v) polyacrylamide gels (Laemmli, 1970). For in-gel protein kinase assays, the separating gel contained a protein kinase substrate such as partially dephosphorylated casein (casein concentrations are stated in the text and figure legends), 0.100 mg mL^{-1} myelin basic protein, or 0.100 mg mL^{-1} histone type III-S. All substrates were obtained from Sigma. After electrophoresis, the gels were washed for 1 h at room temperature in two changes of 50 mM Tris-HCl (pH 8.0), and 20% (v/v) 2-propanol and then for 1 h in two changes of 50 mM Tris-HCl (pH 8.0) and 5 mM 2-mercaptoethanol. The proteins were then denatured for 1 h at room temperature in 6 M guanidine-HCl, 50 mM Tris-HCl (pH 8.0), and 5 mM 2-mercaptoethanol and then renatured overnight at 4°C in 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, and 0.04% (v/v) Tween 20. The gels were equilibrated for 30 min at room temperature in 40 mM Hepes (pH 7.5), either 0.1 mM or 1.0 mM EGTA, 20 mM MgCl_2 , and 2 mM DTT. To examine the effects of different divalent cations on phosphorylation, the buffer was supplemented with 2.0 mM MnCl_2 and/or 2.0 mM CaCl_2 in the presence or absence of 1 mM EGTA as noted in the figure legends and text. Phosphorylation was performed for 1 h at room temperature in the same buffer supplemented with 50 μM ATP and 50 μCi [γ - ^{32}P]ATP (DuPont NEN). The gels were washed for 2.5 h at room temperature in five changes of 5% (w/v) TCA and 1% (w/v) sodium PPI. Finally, the gels were dried on filter

paper prior to autoradiography. Dried gels were exposed to X-Omat AR film (Kodak) with one Cronex Lightning Plus intensifying screen (Du Pont) at -70°C .

Phosphoamino Acid Analysis

Analysis of phosphorylated amino acids was performed essentially as described by Cooper et al. (1983). The region of a gel containing phosphorylated protein was excised and ground to a slurry in 50 mM ammonium carbonate. The solution was brought to 0.1% (w/v) SDS, 0.7 M 2-mercaptoethanol and boiled for 5 min, incubated for 3 h at 37°C , and then centrifuged for 10 min at $10,000g$. After 20 μg of carrier protein (boiled RNase A) was added to the supernatant, proteins were precipitated with TCA, pelleted by centrifugation, washed with cold acetone, and air-dried. The pellet was resuspended in 100 μL of 6 N HCl (Pierce), incubated for 1 h at 110°C , dried under vacuum, and resuspended in buffer (2.5% [v/v] formic acid, 7.8% [v/v] acetic acid, final pH 1.9) containing phosphoserine, phosphotyrosine, and phosphothreonine standards. Samples were applied to a $20 \times 20\text{-cm}$ precoated cellulose TLC plate without a fluorescent indicator (Merck, Darmstadt, Germany) and separated by two-dimensional electrophoresis (Hunter model 7000 Thin Layer Peptide Mapping System, CBS Scientific, Del Mar, CA). In the first dimension the electrophoresis buffer used was 2.5% (v/v) formic acid, 7.8% (v/v) acetic acid (final pH 1.9); the second-dimension electrophoresis buffer was 5% (v/v) acetic acid, 0.5% (v/v) pyridine (final pH 3.5). The positions of the phosphoamino acid standards were visualized by spraying the plates with 0.25% (w/v) ninhydrin dissolved in acetone. TLC plates were exposed to imaging plates (BAS-IIIIS, Fuji Photo Film, Tokyo, Japan), which were then analyzed with a bioimaging analyzer (Fujix BAS 1000 MacBas, Fuji).

Figure Preparation

Autoradiographic images were digitized at a resolution of 324 pixels inch^{-1} using a flat-bed scanner (Silverscanner II, LaCie, Beaverton, OR). Image files were assembled, sized, and labeled, and brightness and contrast were adjusted when necessary using Adobe Photoshop 2.0.1 (Adobe Systems, Mountain View, CA). Figures were printed at a resolution of 162 pixels inch^{-1} with a dye sublimation printer (Codonics, Mitsubishi, Cleveland, OH) on manufacturer-provided paper.

FLU Treatment and ABA Quantification

To reduce ABA accumulation after transplanting to low ψ_w , in some experiments FLU (Dow Chemical Co., Midland, MI) at a final concentration of 10 μM was added to the water-vermiculite mixture in which the seeds were germinated (Saab et al., 1990). FLU inhibits carotenoid (and ABA) biosynthesis (Fong et al., 1983; Moore and Smith, 1985). One hour after seedlings were transplanted to either high or low ψ_w , they were frozen and the apical 10 mm of the primary root was excised. Root tips were analyzed for protein kinase activity as described above or were freeze-dried and extracted with cold (4°C) water, and their ABA

content was measured by radioimmunoassay, as described by Quarrie et al. (1988) and Saab et al. (1990). Each ABA sample comprised 10 root tips. Data reported are the means of duplicate measurements on two or three samples.

Relative Water Content

The relative water content of the apical 10 mm of the primary root was determined at 0.5, 1, and 2 h after the seedlings were transplanted in vermiculite at a ψ_w of -0.03 or -1.6 MPa. Root tips were weighed before and after being submerged for 3 h in an ice/distilled water mixture (to inhibit growth but allow hydration; Sharp et al., 1990), as well as after oven drying. Relative water content was calculated as (fresh weight - dry weight)/(fully turgid fresh weight - dry weight).

RESULTS AND DISCUSSION

Rapid Changes in Phosphoproteins Occur in Response to Water Deficit

Figure 1 shows that multiple changes in phosphoprotein content, including increases and decreases in the phosphorylation of individual proteins, were evident in the apical 10 mm of the primary root 1 h after maize seedlings were transplanted into low- ψ_w (-1.6 MPa) compared with high- ψ_w (-0.03 MPa) vermiculite. Although the identity of these proteins is unknown, these results support the hypothesis that changes in protein phosphorylation function in regulating root growth responses to low ψ_w . Although equal amounts of protein for each sample were assayed, the sample from the well-watered seedlings (lane WW) had a lower background radioactivity than the sample from water-stressed seedlings (lane WS). This lower background radioactivity in the well-watered samples was observed each time the experiment was performed.

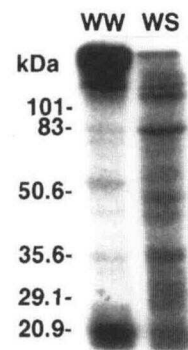


Figure 1. Changes in phosphoprotein content occur in response to water deficit in the apical 10 mm of the primary root. Seedlings were labeled with 50 μCi of buffered [^{32}P]Pi solution for 1 h and then transplanted into vermiculite at high ψ_w (-0.03 MPa; lane WW [well-watered]) or low ψ_w (-1.6 MPa; lane WS [water-stressed]) for 1 h. Proteins were separated by SDS-PAGE (30 $\mu\text{g}/\text{lane}$) and visualized by autoradiography. The experiment was performed twice, with consistent results.

The vermiculite system used to impose low- ψ_w conditions was chosen because the physiology of maize seedling growth responses to this environment has been characterized in considerable detail (Sharp et al., 1988, 1990, 1994). In addition, use of the vermiculite system avoided confounding variables such as O_2 availability, which may occur when low ψ_w is imposed using osmotica in solution culture environments (Verslues et al., 1995). To quantify the water deficit of the root tips during the short-duration experiments in this study, the time course of their relative water content was measured. After the seedlings were transplanted to low- ψ_w vermiculite, the relative water content of the apical 10 mm decreased 10.5% after 0.5 h, 12.8% after 1 h, and 13.5% after 2 h, relative to well-watered roots. The root tip ψ_w was not measured because psychrometric measurements of ψ_w using growing tissues may be inaccurate (Cosgrove, 1986), and the short length of the root (10–20 mm) precluded ψ_w measurements of mature tissue.

Activity of a 45-kD Protein Kinase Is Rapidly Stimulated in Response to Water Deficit

Since multiple differences in phosphoprotein content were observed between protein samples from root tips at high and low ψ_w , a more specific assay was sought to identify protein kinases that could be involved in mediating these differences. The technique of in-gel protein kinase

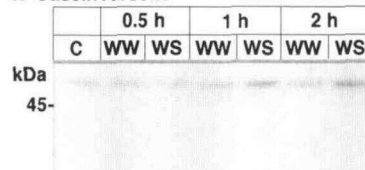
assay (Kameshita and Fujisawa, 1989) was used to determine whether water stress-activated protein kinases associated with the root elongation zone could be identified. Assays were made on samples harvested at 0.5, 1, and 2 h after transplanting.

In the absence of protein kinase substrate (Fig. 2, A-1), low-level autophosphorylation activity, mostly confined to a single protein band of approximately 60 kD, was detected in the pretransplant control (lane C), high- ψ_w (lanes WW), and low- ψ_w (lanes WS) treatments. The extent of phosphorylation of this band did not differ reproducibly between the high- and low- ψ_w treatments at any time. In contrast, in the presence of increasing concentrations of the protein kinase substrate casein, at least three phosphorylated bands were detected (Fig. 2A, 2–4). One, located at approximately 45 kD, had casein phosphorylation activity (as estimated by densitometry) that was in repeated experiments stimulated 3- to 5-fold in the low- ψ_w treatment at all three sampling times. In addition, the extent of phosphorylation detected at 45 kD increased proportionately in magnitude as the amount of casein was increased. Therefore, this activity was most likely due to phosphorylation of casein by a 45-kD protein kinase. In addition to the relatively rapid stimulation of its activity in response to low ψ_w , activity of the 45-kD protein kinase decreased to the control level within 2 h when the vermiculite ψ_w was increased from

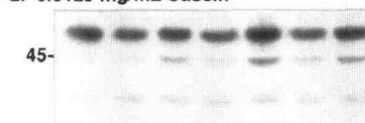
Figure 2. Water deficit stimulates activity of a casein-phosphorylating 45-kD protein kinase in the apical 10 mm of the primary root. Seedlings were transplanted to vermiculite at high ψ_w (-0.03 MPa; lanes WW [well-watered]) or low ψ_w (-1.6 MPa; lanes WS [water-stressed]) for the times indicated. Proteins were separated by SDS-PAGE ($30 \mu\text{g}$ protein/lane), and results of in-gel protein kinase assays were visualized by autoradiography. A, In-gel protein kinase assays performed without added substrate (1) and with varying concentrations of casein (2–4). A duplicate gel prepared without added substrate was stained with Coomassie blue (5). B, In-gel protein kinase assays performed on gels containing 0.100 mg mL^{-1} myelin basic protein (1) or 0.100 mg mL^{-1} histone type II-S (2) as substrates. Experiments were repeated three times, with consistent results.

A Casein Phosphorylation

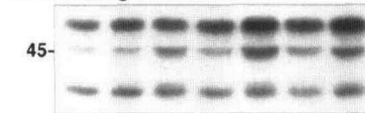
1. Casein Absent



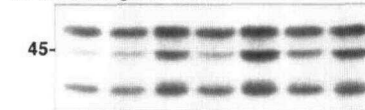
2. 0.0125 mg/mL Casein



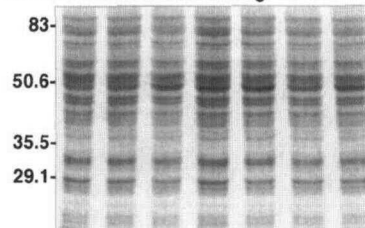
3. 0.0375 mg/mL Casein



4. 0.1225 mg/mL Casein

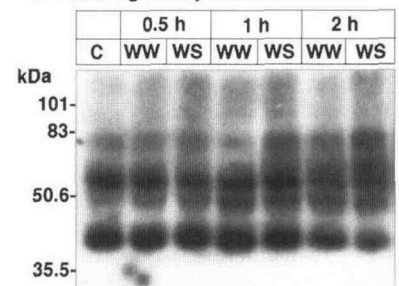


5. Coomassie Blue Staining

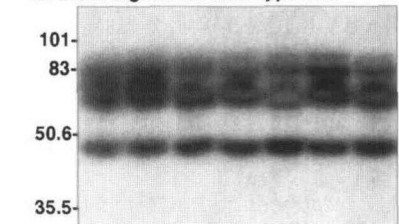


B Other Substrates

1. 0.100 mg/mL Myelin Basic Protein



2. 0.100 mg/mL Histone Type III-S



–1.6 MPa to approximately –0.03 MPa by adding water to the vermiculite (data not shown). Other kinase activities detected when casein was used as a substrate, located at approximately the 60- and 32-kD positions (Fig. 2B), did not change reproducibly between the high- and low- ψ_w treatments.

Myelin basic protein and histone (type III-S) were also tested as substrates for in-gel protein kinase assays with these samples. As shown in Figure 2, multiple protein kinases phosphorylated myelin basic protein (B-1) or histone (B-2). Myelin basic protein-phosphorylating protein kinase activities were detected at approximately the 42- and 49-kD positions, in addition to at least two other high-molecular-mass activities (>52 kD). With histone present, protein kinase activity was detected at approximately 48 kD, and at least three other high-molecular-mass (>60 kD) activities were observed. None of these protein kinases reproducibly exhibited higher (or lower) activity in low- ψ_w samples, compared with the high- ψ_w treatment, at any of the sampling points. In addition, the 45-kD protein kinase activity detected in assays performed with casein present was not detected when myelin basic protein or histone were tested as substrates.

Activity of the 45-kD Water-Deficit-Activated Protein Kinase Is Localized to the Root Elongation Zone

In-gel protein kinase assays were performed on sections of the primary root taken serially from the root apex to determine whether the 45-kD kinase activity was localized to the elongation zone. Figure 3A shows that 2 h after transplanting to both the high- ψ_w and low- ψ_w treatments the activity of the 45-kD protein kinase was greatest in the apical 5 mm (lane 0–5), decreased progressively in root sections taken farther from the apex, and was barely detectable in the 15- to 20-mm section. These results show that the activity was associated with the elongation zone, which, at high ψ_w , encompasses the apical 10 to 12 mm (Sharp et al., 1988; Saab et al., 1992). Relative to the well-watered treatment (Fig. 3, lanes WW), the activity at low ψ_w (Fig. 3, lanes WS) was elevated throughout the apical 15 mm.

Figure 2 shows that activity of the 45-kD protein kinase had increased within 0.5 h after transplanting to low ψ_w and remained activated after 2 h. To determine whether the increase in activity continued during longer exposures to water stress, in-gel protein kinase assays were also performed 48 h after transplanting. Relative to the high- ψ_w treatment, activity of the 45-kD protein kinase remained increased in the apical 5 mm but, interestingly, was decreased in the 5- to 10- and 10- to 15-mm regions (Fig. 3B). It is known that by 48 h after seedlings are transplanted to vermiculite at a ψ_w of –1.6 MPa the elongation zone shortens to encompass only the apical 6 mm (Sharp et al., 1988; Saab et al., 1992). Thus, the activity profile of the 45-kD protein kinase exhibited a spatial correlation to the shortening of the elongation zone that occurs in response to low ψ_w .

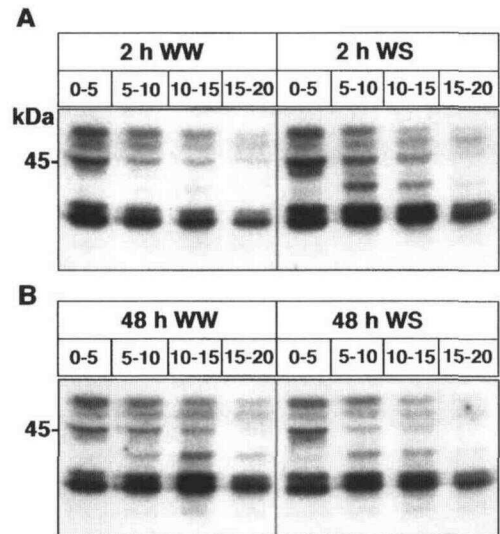


Figure 3. Activity of the 45-kD protein kinase is localized to the elongation zone of the primary root (see text for details). Seedlings were transplanted to vermiculite at high ψ_w (–0.03 MPa; lanes WW [well-watered]) or low ψ_w (–1.6 MPa; lanes WS [water-stressed]) for 2 or 48 h. Seedlings were frozen and their primary roots sectioned into serial 5-mm-long segments 20 mm from the apex (lanes 0–5, 5–10, 10–15, and 15–20). Twenty-five micrograms of total protein extract (from 30 sections) was loaded per lane and separated by electrophoresis in casein (0.1225 mg mL^{–1}) gels, and in-gel protein kinase assays were performed.

Casein Phosphorylation by the 45-kD Protein Kinase Is Ca²⁺-Independent

The standard reaction conditions used for in-gel kinase assays, including those shown in Figure 2 and elsewhere in this paper, included 20 mM Mg²⁺, 2 mM Mn²⁺, and 0.1 mM EGTA. Protein kinases require Mg²⁺ for their function but may also require other cations such as Ca²⁺ or Mn²⁺. When kinase assays were performed in the absence of Mg²⁺ (with 0.1 mM EGTA present), activity of the 45-kD protein kinase was not detected (data not shown). To further characterize the 45-kD protein kinase activity, divalent cation requirements were analyzed by performing in-gel protein kinase assays in the presence of different combinations or concentrations of Mg²⁺, Ca²⁺, Mn²⁺, and EGTA (0.100 mg mL^{–1} casein present).

Figure 4A shows results obtained when the standard assay conditions were used as a control to measure the activity of the 45-kD protein kinase in roots subjected to high- (lane WW) or low- ψ_w treatments (lane WS; 2 h after transplanting). Comparable results were obtained when both Mn²⁺ and EGTA were omitted from the reaction buffer (Fig. 4B; 20 mM Mg²⁺ present). To evaluate any Ca²⁺ dependence of the 45-kD protein kinase, the assay was then performed in the presence of a higher concentration of EGTA to chelate Ca²⁺ derived from the casein or present as a trace contaminant in other reagents. With 20 mM Mg²⁺ and 1 mM EGTA present, the 45-kD protein kinase activity was comparable to that detected in other assays, in both the high- and low- ψ_w treatments (In Fig. 4, compare C with A

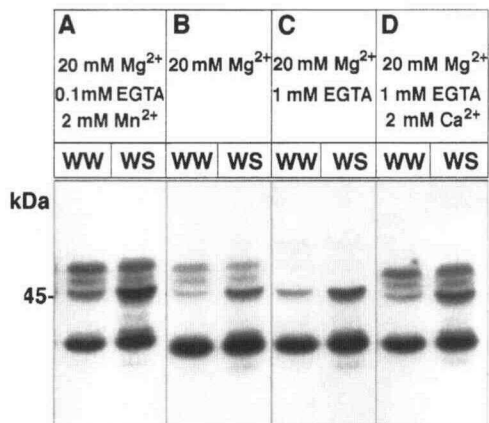


Figure 4. Casein phosphorylation by the water-deficit-activated, 45-kD protein kinase is independent of Ca²⁺. Seedlings were transplanted to vermiculite at high (−0.03 MPa; lanes WW [well-watered]) or low (−1.6 MPa; lanes WS [water-stressed]) ψ_w for 2 h. Proteins were extracted from the apical 10 mm of the primary roots and separated by SDS-PAGE (30 μ g protein/lane). In-gel protein kinase assays were performed with 0.100 mg mL^{−1} casein and the results were visualized by autoradiography. These assays were repeated three times; the results shown here are representative.

and B), whereas the activities of several other protein kinases were greatly reduced. Adding back an excess of Ca²⁺ to the reaction restored those activities but had no effect on the 45-kD protein kinase (Fig. 4D).

We conclude from these results that the water-deficit-stimulated casein phosphorylation observed at the 45-kD position was due to activity of a Mg²⁺-dependent, Ca²⁺-independent protein kinase. Additional water-deficit-stimulated kinase activities were detected by these assays (Fig. 4). The weak activities at approximately 36 and 30 kD were repeatedly detected but have not yet been extensively analyzed. It is not known whether these kinase activities were due to distinct proteins or to incomplete sample denaturation or sample proteolysis.

The 45-kD Water-Deficit-Stimulated Protein Kinase Phosphorylates Casein on Serinyl and Threonyl Residues

Amino acid specificity of the 45-kD protein kinase was determined by performing a two-dimensional thin-layer electrophoretic analysis of phosphoamino acid content (Cooper et al., 1983). Figure 5 shows that both phosphoserine (pSer) and phosphothreonine (pThr) were present on casein that had been phosphorylated by the 45-kD protein kinase (2 h of low- ψ_w treatment). The location of the radioactive amino acids corresponded exactly to the locations of the nonradioactive phosphoserine and phosphothreonine standards (circled in Fig. 5). Ser was phosphorylated to a greater extent (9- to 10-fold) than Thr. Radioactive phosphotyrosine was not detected. The nonradioactive phosphotyrosine standard migrated to a position that was located below that of the phosphothreonine standard and was readily distinguished from phosphothreonine when the two-dimensional separation technique was used. The phosphorylated products lying above and

slightly to the right of the sample origin are probably due to incomplete hydrolysis of protein; such products are routinely detected in phosphoamino acid assays.

Water-Deficit Stimulation of the 45-kD Protein Kinase Is Not Affected by the Protein Translation Inhibitor CHX

Figure 2 shows that activity of the 45-kD protein kinase was rapidly stimulated (within 0.5 h) following the transfer of seedlings to low- ψ_w vermiculite. It is likely that this elevated activity was due to posttranslational activation of an existing protein kinase, but it could have been due to the accumulation of a newly synthesized protein. To distinguish between these possibilities, intact seedlings were treated with the eukaryotic protein translation inhibitor CHX before transplanting to high- or low- ψ_w vermiculite (samples harvested 0.5 h after transplanting).

To determine the effectiveness of the CHX treatment, seedlings were labeled with [³⁵S]Met to permit quantification of protein synthesis. Figure 6A shows that [³⁵S]Met incorporation into proteins in CHX-treated seedlings was greatly reduced (by 88%, as estimated by densitometry) compared with untreated seedlings. Figure 6B shows that water-deficit stimulation of casein phosphorylation by the 45-kD protein kinase was unaffected by CHX treatment. This result indicates that stimulation of activity of the 45-kD protein kinase at low ψ_w was not due to new protein synthesis but involved a rapid posttranslational activation.

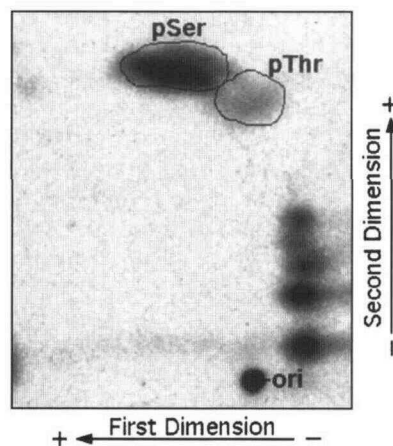
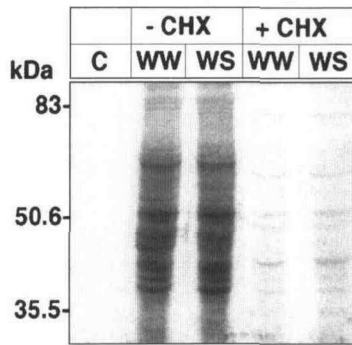


Figure 5. The water-deficit-activated, 45-kD protein kinase phosphorylates casein on serinyl and threonyl residues. The casein-phosphorylating activity of the 45-kD protein kinase was detected by an in-gel protein kinase assay. Protein samples were from the apical 10 mm of roots harvested 2 h after seedlings were transplanted to low- ψ_w (−1.6 MPa) vermiculite. The region of the gel containing this activity was excised, eluted, and acid-hydrolyzed. Samples (1000 cpm) were applied to cellulose-coated plates and separated by two-dimensional thin-layer electrophoresis (pH 1.9 buffer in the first dimension; pH 3.5 buffer in the second dimension). The positions of nonradioactive standards (phosphoserine [pSer] and phosphothreonine [pThr]) detected with ninhydrin are marked. The analysis was performed twice, using independently isolated protein samples, with the same results.

A S-35 Met Incorporation



B Protein Kinase p45 Activation

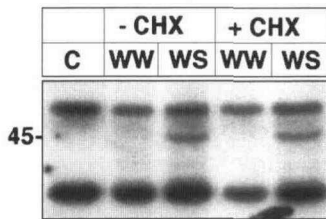


Figure 6. Inhibition of protein translation does not affect water-deficit activation of the 45-kD protein kinase. Intact seedlings were labeled for 1 h with [³⁵S]Met in the presence (+CHX) or absence (-CHX) of 60 $\mu\text{g mL}^{-1}$ CHX and then transplanted to high- ψ_w (-0.03 MPa; lanes WW [well-watered]) or low- ψ_w (-1.6 MPa; lanes WS [water-stressed]) vermiculite for 0.5 h. A, Proteins (30 μg) extracted from the apical 10 mm of the primary roots were separated by SDS-PAGE. Lane C, Unlabeled control seedlings. B, In-gel protein kinase assays (0.100 mg mL^{-1} casein) performed on protein samples from the above treatments. The experiment was performed twice, with consistent results.

Water-Deficit Stimulation of the 45-kD Protein Kinase Is Independent of ABA Accumulation

To evaluate whether water-deficit stimulation of the 45-kD protein kinase activity was dependent on ABA accumulation, seedlings were treated (during imbibition) with FLU to inhibit carotenoid (and ABA) biosynthesis. This treatment has been shown to largely prevent ABA accumulation in maize seedlings after transplantation to low- ψ_w vermiculite (Saab et al., 1990). Figure 7 shows that the FLU treatment had no effect on the activation of the 45-kD protein kinase at low (lanes WS) compared with high ψ_w (lanes WW; samples harvested 1 h after transplanting).

To verify that ABA accumulation actually occurred and was inhibited by FLU in this short-time-course experiment, root tip ABA levels were quantified by radioimmunoassay (Quarrie et al., 1988; Saab et al., 1990). One hour after the seedlings were transplanted to high- ψ_w vermiculite, the ABA contents of FLU-treated and -untreated root tips had not changed appreciably from pretransplant levels (Table I). In the absence of FLU, ABA levels had increased approximately 3-fold 1 h after the seedlings were transplanted to the low- ψ_w vermiculite, and this increase was completely prevented by the FLU treatment (Table I). Thus,

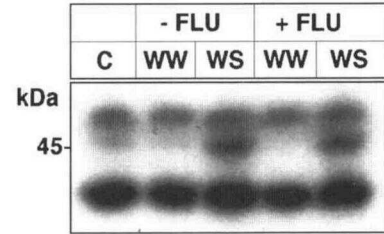


Figure 7. Water-deficit activation of the 45-kD protein kinase is independent of ABA accumulation. Seedlings were germinated either with or without FLU (10 μM) and were then transplanted to high- ψ_w (-0.03 MPa; lanes WW [well-watered]) or low- ψ_w (-1.6 MPa; lanes WS, [water-stressed]) vermiculite for 1 h. Proteins were extracted from the apical 10 mm of the primary roots and separated by SDS-PAGE (30 μg protein/lane). In-gel protein kinase assays were performed on gels prepared with 0.100 mg mL^{-1} casein as the substrate, and the results were visualized by autoradiography. The experiment was performed twice, with consistent results.

we conclude that water-deficit stimulation of the 45-kD protein kinase activity was independent of ABA accumulation.

SUMMARY

Our results show that the activity of a 45-kD protein kinase is stimulated in the elongation zone of the primary root of maize seedlings subjected to water deficit. The 45-kD protein kinase phosphorylated casein, but not myelin basic protein or histone (type III-S), and exhibited an absolute requirement for Mg^{2+} ions but not Ca^{2+} ions. Phosphoamino acid analysis showed that the 45-kD protein kinase specifically phosphorylated serinyl and threonyl residues on casein. Thus, the 45-kD protein kinase is a Mg^{2+} -dependent, Ca^{2+} -independent Ser/Thr protein kinase.

The 45-kD, water-deficit-stimulated protein kinase likely functions at an early step in a signaling pathway that is activated in response to water deficit, based on the following evidence. First, casein phosphorylation by the 45-kD protein kinase was activated very rapidly (within 0.5 h) after low- ψ_w conditions were imposed. Second, the activity

Table I. Effect of FLU on the ABA content in the apical 10 mm of the primary root after transplanting to high- or low- ψ_w vermiculite

Treatment	ψ_w MPa	ABA Content $\text{ng g}^{-1} \text{H}_2\text{O}$
-FLU		
Pretransplant	-0.03	34.6 \pm 2.1
1 h after transplanting	-0.03	27.8 \pm 1.2
1 h after transplanting	-1.6	101.9 \pm 12.7
+FLU		
Pretransplant	-0.03	38.7 \pm 3.8
1 h after transplanting	-0.03	32.3 \pm 4.4
1 h after transplanting	-1.6	35.4 \pm 1.0

of this protein kinase was unaffected when protein synthesis was blocked, indicating that its activation is regulated primarily by posttranslational modification. Activity of the 45-kD protein kinase was independent of ABA accumulation. The localization of the activity of the 45-kD protein kinase in the elongation zone suggests that it may be involved in regulating growth responses of the primary root to water stress. Future research efforts will focus on identification of the 45-kD, water-deficit-stimulated protein kinase and on characterizing other activities identified in these assays.

ACKNOWLEDGMENTS

We thank Dr. Mary E. LeNoble and Dr. Julie M. Stone for technical assistance.

Received July 25, 1996; accepted September 25, 1996.

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