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Phosphorylation of a Renatured Protein from Etiolated Wheat Leaf Protoplasts Is Modulated by Blue and Red Light[1]

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Red-light irradiation of etiolated wheat (*Triticum aestivum* L.) leaf protoplasts rapidly increases calcium-dependent phosphorylation in vivo of 70- and 60-kD peptides, and the phosphorylation is attenuated by simultaneous far-red light (K.M. Fallon, P.S. Shacklock, A.J. Trewavas [1993] Plant Physiology 101:1039–1045). When these protoplasts were solubilized in sodium dodecyl sulfate and protein kinase was renatured in situ after gel electrophoresis, a single 60-kD protein kinase was detected. In situ phosphorylation was inhibited by prior exposure of etiolated protoplasts to 30 to 60 s of white, 1 to 2 min of blue, or 2 to 5 min of red light. The effect of red light was attenuated by concomitant far-red light. The inhibition of in situ phosphorylation by light was lost after a further prolonged incubation of protoplasts in darkness. In situ phosphorylation was calcium dependent, and the electrophoretic mobility of the protein kinase was increased in the presence of calcium ions. Although treatment of protoplasts with ionophores and channel blockers produced data consistent with in vivo regulation of phosphorylation by cytosol calcium, additional light-activated transduction pathways have to be invoked to explain all the observations.

The central role played by light in the coordination of plant growth and development has led to intense interest in the detection and transduction of light signals (Chory, 1993). The primary receptor of R is phytochrome (Hart, 1988) and multiple forms are known to exist, which may control different cellular processes. Increasing evidence suggests that one receptor for B may be a protein kinase (Gallagher et al., 1988). Since both R and B initiate separate but often interrelated physiological responses, it has been assumed that the transduction pathways may be distinct, but the identity of either remains unclear (Trewavas and Gilroy, 1991).

It has long been thought that $[Ca^{2+}_i]$ may act in transduction of photomorphogenetic signals resulting from R due to its important role in animal cells (Haupt and Weisenseel, 1976). Early studies involving $^{45}Ca^{2+}$ flux measurements and autoradiography (Weisenseel and Ruppert, 1977; Dreyer and Weisenseel, 1979; Hale and Roux, 1980) provided indirect evidence to support this hypothesis. Following the detection of a plasma membrane-located Ca-dependent protein kinase in plant cells (Hetherington and Trewavas, 1982), Roux (1984) proposed that phytochrome-dependent transduction involved elevation of $[Ca^{2+}]_i$ followed by modulation of Ca-dependent enzyme activity, e.g. in protein phosphorylation. Protein phosphorylation cascades allow efficient information transfer and provide considerable signal amplification and integration (Schachter et al., 1984).

Harper et al. (1991) and Schaller et al. (1992) have recently described the purification and sequence of a 61-kD Ca-dependent, plasma membrane-located protein kinase that contains a calmodulin-like domain and binds Ca directly. This enzyme has the potential to respond to R-mediated increases in $[Ca^{2+}]_i$. Transduction of R through protein phosphorylation is also suggested by observations made by Grimm et al. (1989), who showed that phytochrome often co-purifies with a 60-kD Ca-dependent protein kinase. Thummler et al. (1992) demonstrated that *Ceratodon* phytochrome, unlike other plant phytochromes, has a conserved protein kinase sequence.

When etiolated wheat (*Triticum aestivum* L.) leaf protoplasts are irradiated with R, their volume increases by up to 20% (Bossen et al., 1988). It is believed that R-induced protoplast swelling employs the same molecular mechanism as R-induced leaf unrolling (Zhou et al., 1990). Using Ca-sensitive fluorescent dyes and laser confocal scanning imaging, we have been able to show that R induces $[Ca^{2+}]_i$ transients lasting several minutes in single etiolated wheat leaf protoplasts (Shacklock et al., 1992). We mimicked these transients by photolysis of incorporated caged Ca (nitr-5) and incorporated caged inositol trisphosphate and showed that these artificially produced transients increased the volume of the protoplasts, thus mimicking the physiological effects of R. By incubating etiolated wheat leaf protoplasts in ^{32}Pi for time periods between 15 s and 15 min, we detected R-induced changes in the phosphorylation of two peptides with approximate electrophoretic mobilities of 60 and 70 kD (Fallon et al., 1993). Phosphorylation of these two peptides increased during 45 s of R irradiation and showed kinetics similar to those of the detected $[Ca^{2+}]_i$ transients. Again, we were able to mimic the R-increased phosphorylation of these two peptides by photolyzing incorporated caged Ca.

The 60- and 70-kD peptides were the primary species labeled in these short incubation periods; only after several hours of incubation in ^{32}Pi was the labeling of numerous other peptides detected. This suggested the possibility that the very rapid labeling of the 60- and 70-kD peptides was the result of autophosphorylation, which is often faster than

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Abbreviations: B, blue light; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; FR, far-red light; R, red light; W, white light.

the labeling of other protein kinase substrates (e.g. Blowers and Trewavas, 1987). Therefore, we decided to investigate the effects of light on putative phosphorylation in etiolated wheat leaf protoplasts by renaturing protein kinase activity in situ after SDS-PAGE (Geahlen et al., 1986; Blowers and Trewavas, 1987). Our initial investigations were limited to R but rapidly changed when we discovered a similar response to B.

MATERIALS AND METHODS

Chemicals

Cellulase "Onozuka" RS was obtained from Yakult Pharmaceutical (Tokyo, Japan), and [^{32}P]ATP (370 MBq/mL) was from ICN Flow (High Wycombe, Bucks, UK). All other chemicals were standard enzyme grade and were obtained from Sigma London Chemical Co. (Poole, Dorset, UK).

Plant Material and Protoplast Isolation

Wheat seedlings (*Triticum aestivum* L. cv Mercia) were grown in total darkness at 20°C and protoplasts were prepared under a dim (100 nmol $m^{-2}s^{-1}$ at bench surface) green (emission maximum 520 nm) safelight as described previously (Fallon et al., 1993). Each protoplast preparation used approximately 15 g of leaf tissue sliced into 1-mm long sections.

Light Sources and Irradiations

Light sources and fluences were as described previously (Fallon et al., 1993) except that W was provided by a mixture of fluorescent and tungsten bulbs in a controlled growth facility. B, R, and FR were provided by a Rank Aldis 2000 projector equipped with 150-W bulb and appropriate interference filters; B, 480 nM; R, 660 nM; FR, 720 nM, all with 10-nm half-band width (Glen Spectra, Stanmore, Middlesex, UK). Fluence rates at the protoplast suspension surface were: W, approximately 90 μmol m^{-2} s^{-1}; B, 77 μmol m^{-2} s^{-1}; R, 74.0 μmol m^{-2} s^{-1}; and FR, 61.8 μmol m^{-2} s^{-1} as determined using a digital quantum photometer (Skye Instruments, SKR100, R, FR) or analog quantum photometer (Lambda Instruments, LI-185, B, W).

Protoplast suspensions were irradiated at 20°C in a microcentrifuge tube before the reaction was terminated by addition of aliquots to an equal volume of SDS-PAGE sample buffer (0.125 M Tris-HCl, 5% SDS, 10% mercaptoethanol, 20% glycerol, 0.004% bromphenol blue). Samples were electrophoresed immediately or snap frozen in liquid N_2 and stored at −80°C.

Electrophoresis, Protein Kinase Reconstitution, and in Situ Phosphorylation

SDS-PAGE on minigels was performed as described previously (Blowers and Trewavas, 1987). Protein kinase activity was renatured and phosphorylated using [γ-^{32}P]ATP as described previously (Blowers and Trewavas, 1987), except that the phosphorylation buffer contained 25 mM Hepes (pH 7.2), 10 mM $MgCl_2$, 0.45 mM EGTA, and 0.55 mM $CaCl_2$, and gels were washed overnight with three changes in 40 mM Hepes, 50 g/L Dowex 2X8-50, 2.5 mM tetrasodium pyrophosphate, 1 mM disodium EDTA, before a further wash (1–16 h) in hot (70°C) 10% TCA to hydrolyze unreacted [^{32}P]ATP and remove residual noncovalently bound phosphate. After staining, destaining, and drying, gels were autoradiographed using preflashed Cronex-4 film and Lightning Plus intensifying screens (DuPont, Stevenage, Herts, UK).

Amino Acid Analysis

For amino acid analysis labeled bands were excised from several gels, pooled, and homogenized in 6 N HCl. After hydrolysis at 110°C for 6 h, the extracts were mixed with amino acid markers and separated by two-dimensional thin-layer electrophoresis at pH 1.9 (formic acid:glacial acetic acid:water, 5:15:180) and pH 3.5 (pyridine:glacial acetic acid water, 1:10:189). After drying, the plates were autoradiographed and then stained with 0.2% ninhydrin in ethanol to reveal the amino acid markers.

RESULTS

Phosphorylation in Situ of a 60-kD Peptide Is Inhibited by Prior Irradiation of Etiolated Wheat Leaf Protoplasts with W

Protoplasts were prepared in dim green light from chopped etiolated wheat leaves. Protoplast viability using fluorescein diacetate was estimated at >85%, and routine yields were on the order of 10^6 protoplasts. Protoplasts were solubilized in SDS sample buffer, and the extract was separated by SDS-PAGE. Protein kinase activity was renatured by SDS removal. After incubation of the gel in [γ-^{32}P]ATP and hydrolysis of residual ATP with hot 10% TCA at 70°C for 16 h, autoradiography revealed the presence of a single renatured protein kinase with an electrophoretic mobility equivalent to 60 kD, as shown in Figure 1. The band could represent autophosphorylation of a protein kinase or the phosphorylation of a coincident protein substrate. By overloading the gel with protein extract (150 μg/slot), traces of other renatured protein kinases could be detected (data not shown), including one band at 18 kD (Blowers and Trewavas, 1987). A detectable phosphorylated protein band required a protein-loading equivalent to about 2×10^5 protoplasts and could easily be detected with 1 week of exposure with intensifying screens. Figure 1C shows an autoradiograph of a two-dimensional separation of an acid hydrolysate of the radiolabeled 60-kD band. Phosphoserine was the only detected radioactive amino acid.

When the protoplasts were irradiated with W for periods up to 10 min before SDS solubilization and PAGE, subsequent in situ phosphorylation was strongly inhibited after only 30 to 60 s of irradiation (Fig. 1A). Variation in the rate of W-induced loss of in situ phosphorylation between different experiments was slight, and in situ phosphorylation was usually no longer detectable by 90 s. Phosphorylation was presumably occurring on unoccupied sites of either a protein kinase or a coincident substrate and a decrease of in situ phosphorylation might reflect the prior phosphorylation of such sites in the irradiated protoplasts. Alternatively, an

induced change in protein conformation might explain the observations. The effect of W irradiation is not permanent. Figure 1B shows that protoplasts exposed to W for 5 min before returning to darkness recover detectable in situ autophosphorylation some 60 to 100 min later.

In Situ Phosphorylation of the 60-kD Protein Is Modified by Prior Irradiation of the Protoplasts with Both R and B

Figure 2A illustrates the effect of B irradiation of etiolated wheat leaf protoplasts on subsequent in situ phosphorylation. The effect of B is definitely slower than that of W, and in separate experiments up to 2 min of irradiation was required for detectable in situ phosphorylation to disappear. The effect of R is slower than that of W or B (Fig. 2B), and in separate experiments 3 to 4 min of irradiation of the protoplasts was required to accomplish the same result. With some protoplast preparations traces of in situ phosphorylation could still be detected after 5 min of irradiation (e.g. Fig. 2D). Concomitant irradiation with FR attenuates the R response, suggesting phytochrome involvement (Fig. 2B). Complete reversal of R by concomitant FR normally requires a much higher fluence rate of FR, which at present we are unable to provide. Thus, we cannot exclude Pchlide as a possible contributor to the R response. FR on its own has no influence on in situ phosphorylation (Fig. 2C).

Phosphorylation of the 60-kD Peptide Is, in Part, Ca Dependent

The W-induced loss of in situ protein phosphorylation is attenuated if Ca is entirely omitted from the protoplast irradiation medium (Fig. 3A). However, addition of even a very low concentration of $CaCl_2$ (1 μM) is sufficient to completely recover the response to W irradiation (Fig. 3A). To further investigate the relationship with Ca, we have incubated protoplasts in: A23187, a Ca ionophore, to increase cytosol Ca; TMB8, an endomembrane Ca channel blocker, to inhibit internal release; and verapamil, a plasma membrane channel blocker, to inhibit extracellular Ca entry. All three inhibitors have been reported to affect Ca homeostasis in plant cells in the indicated manner (Graziana et al., 1987; Brosnan and Saunders, 1990; Gilroy et al., 1991). As shown in Figure 3B, the dark level of in situ phosphorylation was increased by TMB8 and decreased by A23187, i.e. in the expected direction if dark in vivo phosphorylation was Ca dependent. We were unable to detect effects of verapamil even at high concentrations. However, the subsequent W-induced loss of in situ phosphorylation was not detectably altered by preincubation of protoplasts in any of these inhibitors (Fig. 3B).

In situ Ca dependence of the phosphorylation was further demonstrated by cutting individual strips from gels containing similar separated aliquots of dark-incubated protoplasts. These gel strips were then incubated in [^{32}P]ATP in the presence of different concentrations of Ca ions. Figure 3C shows a substantial increase with added Ca in the extent of in situ phosphorylation, suggesting that the protein kinase (or coincident substrate) may be Ca dependent.

In Figure 3D, EGTA was added to protoplasts at two different concentrations and samples of the protoplasts were W-irradiated. Exogenous EGTA in excess of added Ca has been reported to lower $[Ca^{2+}]_i$ in guard cells (Gilroy et al., 1991) and should remove Ca bound to the outer plasma membrane lipid bilayer when added in excess. Incubation of protoplasts in an excess (10 mM) of EGTA did not prevent the W-induced loss of in situ phosphorylation, but did reduce the subsequent mobility of the phosphorylated protein on SDS-PAGE. Since a similar mobility shift can be produced by adding an excess of EGTA to the electrophoresis gel slot (data not shown), this is not the result of some alteration of the protein kinase structure in the protoplast. An electrophoretic mobility shift induced by EGTA is considered diagnostic of a Ca-binding protein (Anderson et al., 1980; Watterson et al., 1980).

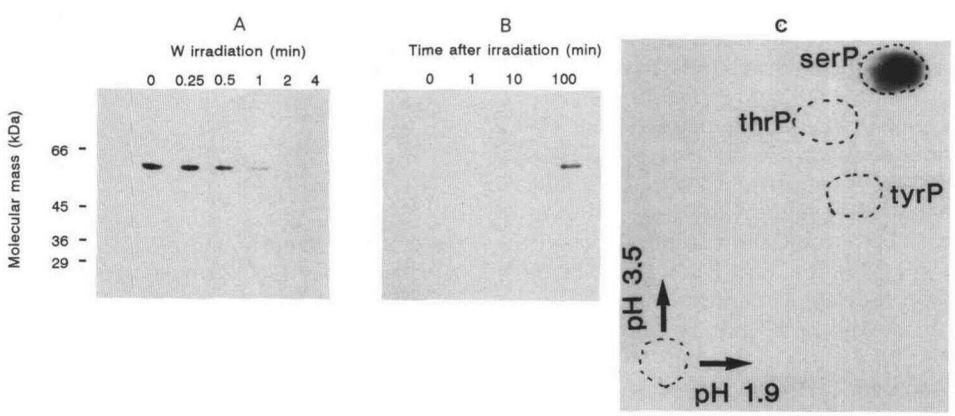

Figure 1. The effect of W irradiation of etiolated wheat leaf protoplasts on protein kinase activity subsequently renatured after SDS-PAGE. Etiolated wheat leaf protoplasts were irradiated with W before mixing with SDS sample buffer, electrophoresis, and renaturation of protein kinase activity on the gel. A, Protoplasts were W irradiated for time periods up to 4 min. B, Protoplasts were W irradiated for 5 min and then placed in darkness for periods up to 100 min before separation and protein kinase renaturation. C, Two-dimensional electrophoretic separation of amino acid hydrolysate of the labeled band in A. Markers were serine P (ser P), threonine P (thr P), and tyrosine P (tyr P).

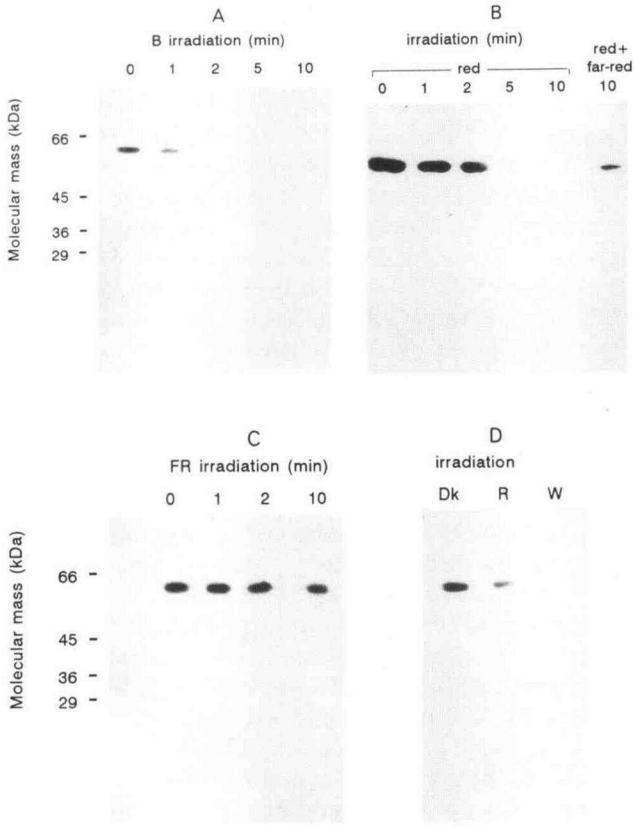

Figure 2. The effect of B, R, and FR irradiation of etiolated wheat leaf protoplasts on protein kinase activity subsequently renatured after SDS-PAGE. Etiolated wheat leaf protoplasts were irradiated with B, R, coincident R + FR, FR, or W for varying time periods before mixing with SDS sample buffer, gel electrophoresis, and renaturation of protein kinase activity in the gel. Light treatments and time periods of irradiation are indicated above the autoradiographs. In D, the periods of R and W irradiation are 5 min.

DISCUSSION

The 60-kD ^{32}P-labeled peptide described in this paper could represent the coincidental electrophoretic mobility of a protein kinase with a substrate or could represent the autophosphorylation of a protein kinase. The mobility shift observed in Figure 3D that occurs in the presence of EGTA is indicative of a Ca-binding protein (Anderson et al., 1980). Since it is unlikely that a kinase and substrate are both Ca-binding proteins and that both would show identical mobility shifts in the presence of EGTA, the more likely possibility is protein kinase autophosphorylation. Purification of the appropriate kinase will enable this problem to be resolved.

The data described in this paper suggest that W irradiation of protoplasts inhibits subsequent in situ phosphorylation of a 60-kD protein after renaturation from SDS-PAGE. There are at least three ways in which this can occur. First, W irradiation could increase the autophosphorylation of a protein kinase in vivo, thus reducing the capabilities for autophosphorylation in situ. W could either induce the phosphorylation of more amino acid sites within the same molecule or a much greater number of protein kinase molecules could be autophosphorylated on the same site. Second, some other W-induced protein kinase modification or phosphorylation itself could alter the renatured conformation so that amino acid sites are no longer available for phosphorylation. Partial proteolytic digestion with an electrophoretic resolution of the labeled fragments could help differentiate between these hypotheses. Third, W could increase the phosphorylation of a protein kinase substrate in vivo, thus reducing the number of available amino acids for phosphorylation in situ. Since either the kinase or substrate is Ca^{2+} dependent, an increase in cytosol Ca^{2+} might increase in vivo phosphorylation, thus modifying the potential for in situ phosphorylation. (Short et al. [1992] concluded that B increased the in vivo level of autophosphorylation of a plasma membrane-bound 120-kD protein, thus reducing subsequent detectable in vitro autophosphorylation.)

Our data show that the phosphorylation of the 60-kD protein can be influenced by B and R as well as W. Assuming that the rate of loss of in situ phosphorylation represents responsiveness to light, the protoplasts may be more sensitive to W than B or R on their own when the three forms of light are applied at about equal fluence rates (Fig. 2). It is possible that B and R act synergistically in the form of W. The data

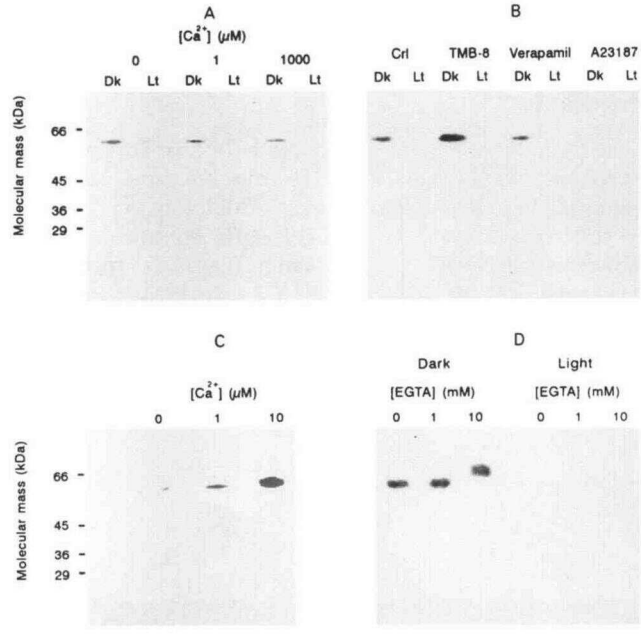

Figure 3. The effect of different preincubation conditions on the W-induced loss of protein kinase activity renatured after SDS-PAGE. In A, B, and D, protoplasts were preincubated for 15 min in Ca-free incubation medium supplemented with 0. 1 μM or 1 mM $Ca^{2+}Cl_2$ (A); incubation medium supplemented with TMB-8 (200 μM), verapamil (100 μM), or A23187 (50 μM) (B); or incubation medium containing 1 mM $CaCl_2$ supplemented with 0, 1, or 10 mM EGTA (D). A further 5-min incubation in the presence or absence of W was then carried out. Protein kinase activity was then renatured after SDS-PAGE. In C, equivalent samples of dark-incubated protoplasts were subjected to SDS-PAGE and protein kinase activity was renatured. The individual gel strips were then excised and the protein kinase reaction was carried out in Ca-free medium supplemented with 0, 1, or 10 μM $CaCl_2$.

are also consistent with the conclusion, based on a large body of evidence, that R and B pathways share common elements. For example, Chory (1993) concluded from her genetic analyses of photomorphogenic mutants of *Arabidopsis* that the transduction pathways of B and R are interlinked. The protein kinase detected here may be one such link. Short et al. (1992) detected the effects of B on the autophosphorylation of a 120-kD protein kinase but could not detect any significant effects of R. They were also able to detect effects of B on the 120-kD protein kinase with only 10 s of irradiation. The effects of B detected here are very much slower and might suggest that the 60-kD protein kinase is further downstream in the transduction sequence. Alternatively, the use of protoplasts (compared with intact plants in Gallagher et al., 1988) might explain the difference. It is unlikely that the protein kinase reported here is similar to that reported by Short et al. (1992). The proteins (protein kinases) have different mol wts, are found in different tissues, have different kinetics of labeling, and have different light sensitivities and Ca requirements.

The protein kinase or kinase substrate described is Ca dependent (Fig. 3). Since we have previously shown that R increases cytoplasmic Ca levels in these protoplasts (Shacklock, et al., 1992) and that in vivo Ca-dependent phosphorylation can also be increased by R (Fallon et al., 1993), the altered level of 60-kD protein phosphorylation can be explained as resulting from R-induced changes in cytoplasmic Ca. However, A23187, verapamil, TMB-8, or incubation of protoplasts in an excess of EGTA was unable to prevent W-induced loss of in situ phosphorylation. We believe the implication of that observation is that the transduction sequence of B, the other active component we detected in W, does not directly involve cytoplasmic Ca. The transduction sequence of B is clearly a major unresolved problem. The observations of Short et al. (1992) suggest a possible direct effect of B on protein kinase activity. When we used B at the same fluence rate as R, the loss of in situ phosphorylation did appear to be faster, suggesting a more direct effect by B. If the transduction sequence of R involves cytoplasmic Ca, then the longer time required with R irradiation to eliminate in situ autophosphorylation compared with B may be related to the time required to alter channel activity and increase cytoplasmic Ca concentrations.

We know little of the identity of this wheat protein kinase, but there are two reports that may present clues. Grimm et al. (1989) purified phytochrome from etiolated wheat leaves and were able to separate (with difficulty) phytochrome from a co-purifying 60-kD protein kinase, which they claimed was Ca dependent. These data suggest an intimate involvement between protein kinase and phytochrome. Schaller et al. (1992) isolated an oat plasma membrane-located protein kinase that is also Ca dependent and binds Ca through a calmodulin-like domain. This 61-kD protein kinase undergoes a mobility shift when it is electrophoresed in the absence of Ca, much as the protein kinase detected here. However, it must be emphasized that we have used whole protoplast extracts, and that the plasma membrane enzymes may be only a very small component of these. Schaller et al. (1992) also detected a soluble version of the plasma membrane kinase (Harper et al., 1991) that may be a degradation product. The relationship of the 60-kD protein kinase to these two other kinases must await purification.

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