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PK12, a Plant Dual-Specificity Protein Kinase of the LAMMER Family, Is Regulated by the Hormone Ethylene

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The ethylene signal is transduced in plant cells via phosphorylation events. To identify protein kinases whose levels of expression are modulated by the plant hormone ethylene, we utilized a differential reverse transcriptase–polymerase chain reaction approach using mRNA extracted from ethylene-treated and untreated tobacco leaves. An ethylene-induced cDNA clone, *PK12*, encoding a protein kinase, was isolated. PK12 is a new member of the recently defined LAMMER family of protein kinases, which has been identified in mammals, flies, yeasts, and plants. The LAMMER kinases are related to the cell cycle–dependent CDC2-type kinases and are characterized by their similarity at kinase subdomain X. The recombinant PK12 protein autophosphorylates in vitro on serine, threonine, and tyrosine residues, thereby making it a member of the dual-specificity protein kinases. Immunoprecipitation of PK12 from plant extracts and kinase assay revealed that the apparent PK12 activity is rapidly and transiently increased when plants are treated with ethylene. By using in situ hybridization, we detected accumulation of the *PK12* transcript in leaves after ethylene treatment and in the untreated flower abscission zone. The tissue in this zone is known to constitutively express ethylene-regulated genes.

INTRODUCTION

Protein phosphorylation regulates a myriad of processes in both the animal and plant kingdoms (Hunter, 1995). In plants, phosphorylation events participate in several physiological processes, including light responses (Allen, 1992; Elich et al., 1993), hormone signaling (Brzobohaty et al., 1994; Giraudat et al., 1994; Chang, 1996), leaf and flower development (Roe et al., 1993), pollen self-incompatibility reactions (Nasrallah et al., 1994), and plant–pathogen interactions (Raz and Fluhr, 1993; Staskawicz et al., 1995).

Protein phosphorylation plays a central role in the perception and signaling of the plant hormone ethylene (Ecker, 1995; Chang, 1996). Ethylene is involved in the regulation of many aspects of plant growth and development, including seed germination, senescence, abscission, and fruit ripening, as well as in the response of a plant to pathogens and environmental stresses (Abeles et al., 1992). During plant–pathogen interactions, the rate of ethylene biosynthesis increases rapidly (Yang and Hoffman, 1984), and ethylene subsequently induces transcription of pathogenesis-related protein genes (Ecker, 1995). In tobacco, pathogenesis-related protein accumulation in response to ethylene has been shown to be mediated by phosphorylation events (Raz and Fluhr, 1993) and to require the presence of calcium (Raz and Fluhr, 1992).

Ethylene response mutants have been isolated in Arabidopsis by using a genetic approach, defining several loci involved in ethylene signaling (Roman and Ecker, 1995). The *ETR1* gene, which corresponds to one of these loci, has been cloned by a map-based strategy, and its translation product encodes a putative transmembrane histidine protein kinase similar to bacterial two-component regulators (Chang et al., 1993). Furthermore, the ETR1 N terminus portion has been shown to bind ethylene in a yeast expression system (Schaller and Bleecker, 1995), suggesting that ETR1 acts as an ethylene receptor in Arabidopsis. Two *ETR1* homologous genes, *ERS* in Arabidopsis (Hua et al., 1995) and *NR* in tomato (Wilkinson et al., 1995), have been isolated. They encode proteins structurally similar to ETR1 in the N terminus and in the putative histidine kinase domain; however, they lack the response regulator domain found in ETR1, which is related to prokaryotic two-component signal transducers. Two additional genes, *CTR1* and *HLS1*, that act downstream of *ETR1* in ethylene signaling have been cloned by insertional mutagenesis (Kieber et al., 1993; Lehman et al., 1996). CTR1 is a Raf-like protein kinase and negatively regulates ethylene responses (Kieber et al., 1993). HLS1 is necessary for differential cell growth in the hypocotyl and shows similarity to a group of *N*-acetyltransferases (Lehman et al., 1996).

The genetic approach is successful when efficient screens of mutant plants can be devised for the isolation of components of signal transduction pathways. However, these screens may be unable to detect plants mutated in pleiotropic-type or

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essential genes that, if mutagenized, lead to lethality. To this end, alternative strategies have been considered. A differential hybridization approach was used to identify genes involved in wound signal transduction in tobacco (Seo et al., 1995). By using this strategy, Seo et al. (1995) isolated a mitogen-activated protein (MAP) kinase homolog shown to be part of the initial response of tobacco plants to mechanical wounding. A screen of an abscisic acid (ABA)–treated wheat embryo cDNA library with a probe developed by polymerase chain reaction (PCR) amplification of serine/threonine protein kinase–conserved domains allowed an ABA-induced protein kinase gene to be isolated (Anderberg and Walker-Simmons, 1992). In addition, molecular approaches based on in vitro protein–protein interactions also allowed signaling molecules, such as the tomato Pti1 protein kinase, which interacts with the Pto protein kinase and is involved in speck disease resistance (Zhou et al., 1995), and the Arabidopsis KAPP protein phosphatase, which interacts with a serine/threonine receptor kinase, to be isolated (Stone et al., 1994).

In an attempt to identify protein kinases regulated by ethylene induction and putatively involved in ethylene signal transduction, we used a differential reverse transcriptase (RT)–PCR approach, with mRNA extracted from ethylene-treated and untreated tobacco plants and degenerate oligonucleotides corresponding to conserved motifs of serine/threonine protein kinases. The rationale for using such an approach was based on the observation that mRNA levels of signal transduction components are enhanced by the signals that they transduce, as has been observed with the *ETR1*-homologous *NR* gene in tomato (Wilkinson et al., 1995) and with the *HLS1* gene in Arabidopsis (Lehman et al., 1996). This phenomenon may be a result of accelerated turnover of the transducer molecules after their activation in signaling pathways (Ullrich and Schlessinger, 1990). Their basal levels in the cell are then replenished by activating transcription. By using RT-PCR, we isolated clones of protein kinase genes induced by ethylene. Among them was the *PK12* cDNA clone, which encodes a member of the LAMMER family of protein kinases. The PK12 protein, when expressed in bacteria as a fusion protein, showed the properties of a dual-specificity protein kinase, autophosphorylating on serine, threonine, and tyrosine residues. Furthermore, the apparent activity of PK12 was regulated by ethylene in tobacco plants, suggesting that PK12 can transduce the ethylene signal.

RESULTS

Isolation of Kinase Genes Differentially Expressed in Untreated and Ethylene-Treated Leaves

An RT-PCR approach was used to isolate genes encoding protein kinases whose level of expression increased after ethylene treatment. Degenerate oligonucleotides coding for conserved sequences from box VIb and box VIII of serine/threonine protein kinases (DLKPEN and YIRPEI, respectively; Hanks and Quinn, 1991) were synthesized, and RT-PCR was performed with these primers by using mRNA extracted from ethylene-treated or control leaves. The expected fragments' length range with these primers, based on analysis of known protein kinases, is between 150 and 250 bp. As shown in Figure 1 (compare lanes 1 and 2), PCR products appear or disappear upon ethylene treatment. Interestingly, major PCR-generated fragments found in ethylene-treated leaves also appeared as products of RT-PCR performed with mRNA isolated from the flower abscission zone (Figure 1, lane 3). In this tissue, constitutive expression of ethylene-induced genes, such as the pathogenesis-related gene *PRB-1b*, has been observed (Eyal et al., 1993).

When RT-PCR was performed with specific primers corresponding to sequences of the *PRB-1b* gene, the *PRB-1b* transcript was detected only in ethylene-treated leaves and in tissues from the flower abscission zone (Figure 1, bottom). This confirmed that both tissues expressed ethylene-regulated transcripts. The ethylene-induced fragments produced with the degenerate primers were eluted, cloned, and sequenced. In 80% of the clones examined, the decoded sequence revealed an internal DFG motif that is conserved among most protein kinases (box VII of the kinase catalytic domain; Hanks and Quinn, 1991). Analysis of one of these clones, *PK12*, is described below.

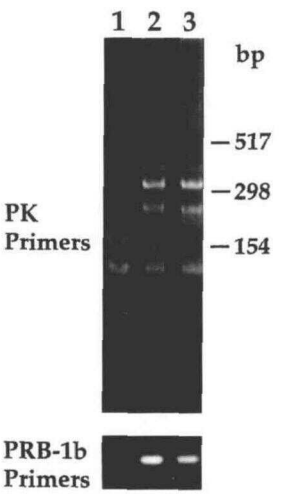

Figure 1. RT-PCR Products Obtained from mRNA of Untreated and Ethylene-Treated Plant Tissues.

Poly(A)$^+$ RNA (1 μg) from untreated leaves (lane 1), from leaves treated with ethylene for 24 hr (lane 2), and from the flower abscission zone (lane 3) was used as a template for RT-PCR. The PCR products were fractionated on a 4% acrylamide gel and stained with ethidium bromide.
(Top) Protein kinase (PK) primers were used for RT-PCR. Numbers at right indicate fragment length in base pairs (bp).
(Bottom) *PRB-1b* primers were used for RT-PCR.

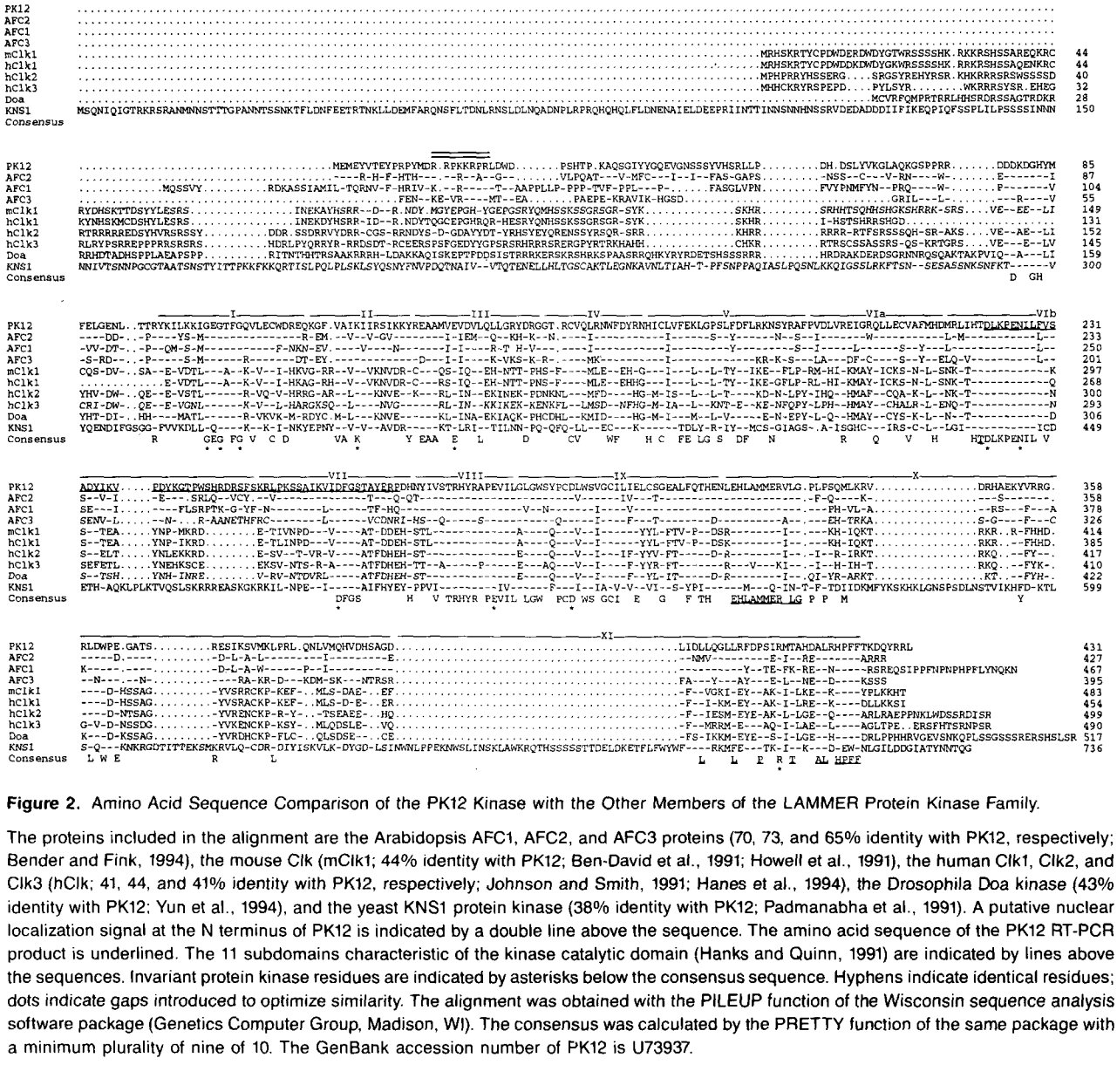

Figure 2. Amino Acid Sequence Comparison of the PK12 Kinase with the Other Members of the LAMMER Protein Kinase Family.

The proteins included in the alignment are the Arabidopsis AFC1, AFC2, and AFC3 proteins (70, 73, and 65% identity with PK12, respectively; Bender and Fink, 1994), the mouse Clk (mClk1; 44% identity with PK12; Ben-David et al., 1991; Howell et al., 1991), the human Clk1, Clk2, and Clk3 (hClk; 41, 44, and 41% identity with PK12, respectively; Johnson and Smith, 1991; Hanes et al., 1994), the Drosophila Doa kinase (43% identity with PK12; Yun et al., 1994), and the yeast KNS1 protein kinase (38% identity with PK12; Padmanabha et al., 1991). A putative nuclear localization signal at the N terminus of PK12 is indicated by a double line above the sequence. The amino acid sequence of the PK12 RT-PCR product is underlined. The 11 subdomains characteristic of the kinase catalytic domain (Hanks and Quinn, 1991) are indicated by lines above the sequences. Invariant protein kinase residues are indicated by asterisks below the consensus sequence. Hyphens indicate identical residues; dots indicate gaps introduced to optimize similarity. The alignment was obtained with the PILEUP function of the Wisconsin sequence analysis software package (Genetics Computer Group, Madison, WI). The consensus was calculated by the PRETTY function of the same package with a minimum plurality of nine of 10. The GenBank accession number of PK12 is U73937.

Isolation and Characterization of *PK12* cDNA Clones

A full-length cDNA clone of *PK12* was isolated by screening a cDNA library from ethylene-induced tobacco leaves by using the cloned *PK12* PCR product as a probe. The *PK12* cDNA clone is 1869 bp long, and its predicted product contains an open reading frame of 431 amino acids, with a calculated molecular mass of 50 kD. The deduced amino acid sequences of the *PK12* PCR product and of the full-length cDNA clone are shown in Figure 2. The translation product of *PK12* revealed high homology with the components of the recently defined LAMMER family of protein kinases (Figure 2; Yun et al., 1994). All of the components of the family contain the conserved residues and the 11 subdomains of the catalytic domains characteristic of serine/threonine protein kinases (Figure 2; Hanks and Quinn, 1991). In addition to a similarity in kinase-related motifs, LAMMER kinases share highly conserved motifs at the N terminus of kinase subdomain X (consensus EHLAMMERILG) and at the C terminus of subdomain XI (Figure 2). Similar to other members of the LAMMER family, the PK12 protein contains at the N terminus, from positions 16 to 23, a putative nuclear localization signal. This signal consists of a short stretch of basic amino acids (Figure 2; Raikhel, 1992).

To estimate the complexity of the *PK12* gene family in the tobacco genome, we hybridized a gel blot of genomic DNA with the *PK12* cDNA as a probe. As shown in Figure 3, two major hybridizing bands were detected in digests of restriction enzyme that either do not cut (EcoRI and EcoRV) or cut once inside the coding region of the gene (XbaI and BglII). We conclude that the *PK12* gene is present in the tobacco amphidiploid genome either as a single member or as part of a very small gene family. A few faint bands that may represent distant *PK12* homologs were also detected (Figure 3).

PK12 mRNA Accumulates in Response to Ethylene in Leaves, and It Is Constitutively Present in the Flower Abscission Zone

The effect of ethylene on the accumulation of *PK12* mRNA in tobacco leaves was tested on an RNA gel blot by hybridizing mRNA of ethylene-treated and untreated tobacco leaves with the *PK12* clone as a probe. As a control for poly(A)$^+$ RNA quality, blots were similarly hybridized with a probe from the β subunit of mitochondrial ATPase from *Nicotiana plumbaginifolia* (Boutry and Chua, 1985). As shown in Figure 4, the *PK12* transcript accumulated in poly(A)$^+$ RNA from ethylene-treated leaves at levels significantly higher than in control or ABA-treated leaves. The length of the *PK12* mRNA was estimated as 1.8 kb, in accordance with the length of the isolated cDNA clone. Figure 5 shows in situ hybridization studies performed on sections of the flower abscission zone and on sections of ethylene-treated and control leaves by using *PK12* as a probe. In leaves, accumulation of silver grains was observed only after ethylene treatment in all cell types, confirming the ethylene inducibility of *PK12* transcripts in leaves (compare Figures 5C and 5D). In contrast, the *PK12* transcript was found to accumulate specifically in cells of the flower abscission zone even without the application of exogenous ethylene (Figures 5A and 5B), in agreement with our observation of constitutive presence in this tissue of ethylene-regulated transcripts (Figure 1; Eyal et al., 1993). *PK12* transcript inducibility in leaves and its stable expression in the flower abscission zone are in accord with the results obtained in the RT-PCR experiments.

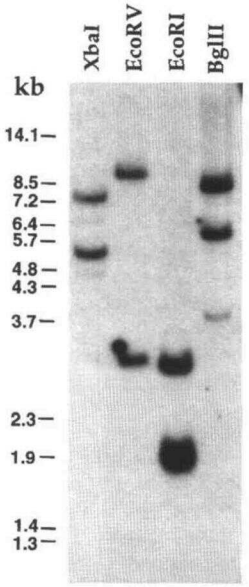

Figure 3. DNA Gel Blot Analysis of *PK12*-Related Genes in the *Nicotiana tabacum* Genome.

Tobacco genomic DNA (25 μg) was digested with XbaI, EcoRV, EcoRI, or BglII, blotted, and probed with a labeled *PK12* cDNA clone. EcoRV and EcoRI do not have restriction sites in the *PK12* cDNA clone, whereas XbaI and BglII have one site. Numbers at left show the positions of length markers indicated in kilobases.

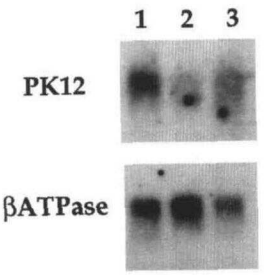

Figure 4. *PK12* Transcript Accumulation in Leaf Tissues of Tobacco Plants Exposed to Ethylene Treatment.

Gel blot analysis of poly(A)$^+$ RNA (3 μg in each lane) extracted from leaves of plants exposed to 50 ppm ethylene for 24 hr (lane 1), from untreated leaves (lane 2), and from detached leaves imbibed for 24 hr in a 50 μM ABA solution (lane 3) is shown.
(Top) The RNA gel blot was probed with the *PK12* cDNA clone.
(Bottom) The RNA gel blot was probed with the β subunit of the mitochondrial ATP synthase gene from *N. plumbaginifolia*.

PK12 Encodes a Functional Dual-Specificity Protein Kinase

To determine whether *PK12* encodes a functional protein kinase, we fused the *PK12* coding region in frame to the C terminus of the glutathione *S*-transferase (GST) protein and expressed it in bacteria. The resulting fusion protein was affinity purified using glutathione–agarose beads, and, as shown in Figure 6A, it had the expected molecular mass of ~75 kD. Additional degradation products of lower molecular mass were also observed. In an in vitro kinase assay, the PK12–GST recombinant protein showed autophosphorylation activity (Figure 6B) and was competent in phosphorylating the myelin basic protein (MBP) as substrate (Figure 6C). As shown in Figure 7, phosphoamino acid analysis revealed that the autophos-

phorylated PK12–GST protein was phosphorylated on serine and threonine as well as on tyrosine residues (Figures 7A and 7C), whereas the MBP substrate was phosphorylated only on serine and threonine residues (Figures 7B and 7C). The amount of phosphorylated tyrosine originating from autophosphorylated PK12 was significantly lower than that of phosphorylated serine and threonine. This can be the result of different phosphorylation kinetics or a reflection of the relative number of available tyrosine phosphorylation sites.

Ethylene Modulation of Kinase Activity in Vivo

The ability of PK12 to phosphorylate the MBP substrate prompted us to analyze the general ethylene-induced activation of MBP-phosphorylating protein kinases from tobacco leaves. This was accomplished by fractionating protein extracts of ethylene-treated leaves by using a denaturing PAGE system preloaded with MBP as substrate, followed by renaturation and an in-gel kinase assay. As shown in Figure 8A, at least two major protein kinases of ~70 and 50 kD, capable of MBP phosphorylation, were detected. Fainter bands representing weaker activities were observed in prolonged exposures (data not shown). If MBP was not included in the PAGE system, the same two major bands were detected but their activities were 50-fold lower (data not shown), probably representing autophosphorylation activity. Interestingly, the activity of the 50-kD band was modulated by ethylene, whereas the 70-kD migrating band remained unchanged (Figure 8A). The time for maximal MBP-phosphorylating activity varied from 5 to 15

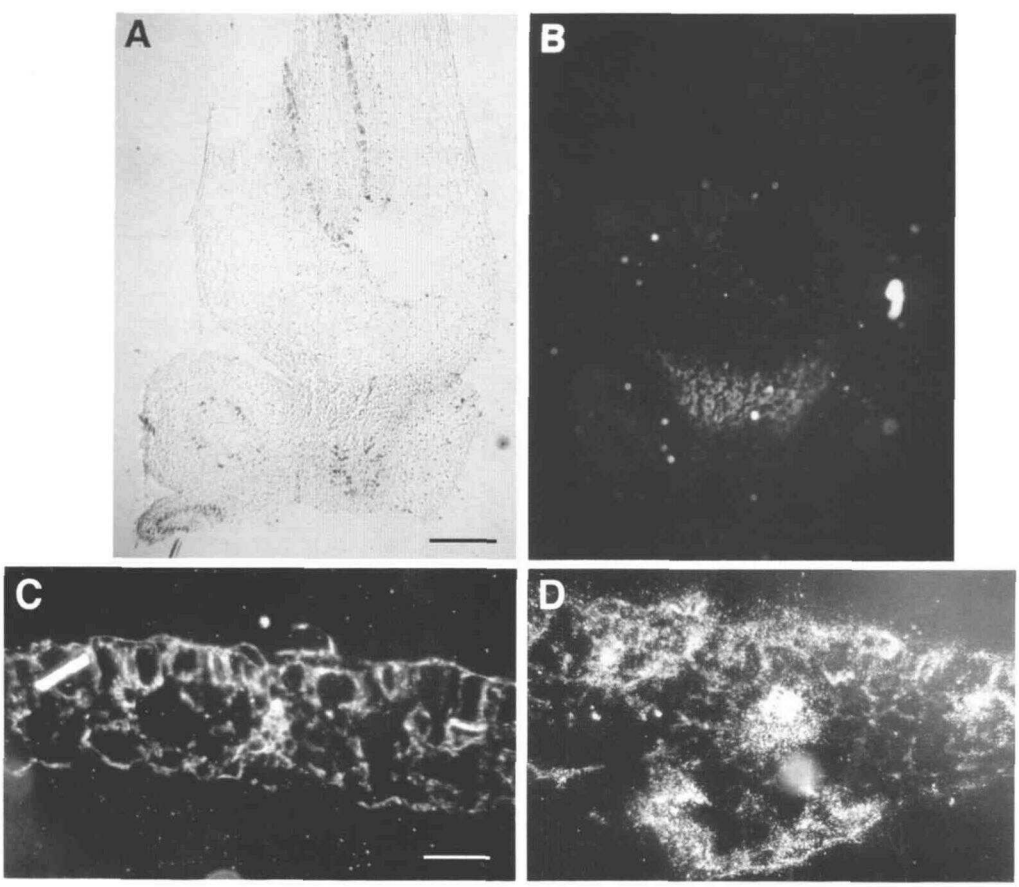

Figure 5. In Situ Hybridization of the *PK12* Transcript in Plant Tissues.

(A) Stained section of a flower bud visualized by bright-field illumination.

(B) Section of a flower bud hybridized with the *PK12* riboprobe and visualized by dark-field illumination. The hybridization signal is present mainly in the smaller, more compact cells of the abscission zone.

(C) Section of a control leaf hybridized with the *PK12* riboprobe and visualized by dark-field illumination.

(D) Section of an ethylene-treated leaf hybridized with the *PK12* riboprobe and visualized by dark-field illumination. The hybridization signal is present in all of the leaf cell types.

In **(C)** and **(D)**, cell walls are highlighted by reflected light. Bar in **(A)** = 600 μm; bar in **(C)** = 50 μm.

min and decreased after 30 min (Figure 8A). At the maximal activity level, the average magnitude of ethylene induction in different experiments was ~3.5-fold that of the basal level (Figure 8B). The molecular mass and MBP phosphorylation detected are consistent with the signal originating from a PK12 kinase–like activity.

To examine directly PK12 kinase activity in plant tissues, we raised PK12 antibodies against a PK12–GST fusion. As shown in Figure 9A, their ability to cross-react with the PK12 protein was assessed on immunoblots in which a total extract of bacteria expressing the PK12 protein in a pRSET vector was fractionated. Extracts of ethylene-treated and untreated leaves were immunoprecipitated with anti–PK12–GST antibodies, and the kinase activity of the immunocomplex was examined in vitro in the presence of the MBP substrate. The kinase activity of the immunocomplex was rapidly (within minutes) and transiently induced by ethylene treatment (Figure 9B). At the maximal activity level, the average induction in different experiments was approximately fourfold that of the basal activity level (Figure 9C). The specificity of PK12 immunoprecipitation was tested in a competition experiment using a mutated recombinant PK12–GST fusion protein, which was devoid of kinase activity by a site-specific mutation in the LAMMER sequence (S. Savaldi, unpublished data). As shown in Figure 9D, the inactive PK12–GST fusion protein competed efficiently with the endogenous PK12 from extracts of ethylene-treated plants and caused a significant decrease in the kinase activity of the immunocomplex.

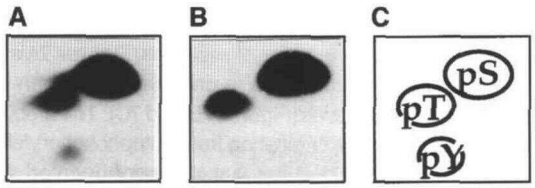

Figure 7. Phosphoamino Acid Analysis by Two-Dimensional Thin-Layer Electrophoresis of the Autophosphorylated PK12–GST Fusion Protein and of the Phosphorylated MBP Substrate.

(A) Phosphoamino acid analysis of the PK12–GST fusion protein autophosphorylated in vitro.
(B) Phosphoamino acid analysis of the MBP substrate phosphorylated in vitro by the PK12–GST fusion.
(C) Positions of the stained standards phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) on the thin-layer chromatography plates.
Thin-layer chromatography plates in **(A)** and **(B)** were exposed for 3 days and 1 day, respectively.

DISCUSSION

A differential RT-PCR approach with ethylene-treated and untreated tobacco leaves enabled the isolation of a novel protein kinase gene, *PK12*. The mRNA level of *PK12* increases in leaves after ethylene induction, and it is constitutively elevated in abscission zone tissues, in which ethylene-regulated genes are stably expressed (Eyal et al., 1993). The *PK12* gene encodes a novel member of the LAMMER family of protein kinases (Yun et al., 1994). Members of this family are the Arabidopsis AFC1, AFC2, and AFC3 proteins (Bender and Fink, 1994), the mouse and human Clk homologs (Ben-David et al., 1991; Howell et al., 1991; Johnson and Smith, 1991; Hanes et al., 1994), the Drosophila Doa kinase (Yun et al., 1994), and the yeast KNS1 protein kinase (Padmanabha et al., 1991). At subdomain X of the kinase catalytic domain, the PK12 protein contains a LAMMER motif, which differs from the consensus sequence only by a conservative substitution of a valine for an isoleucine

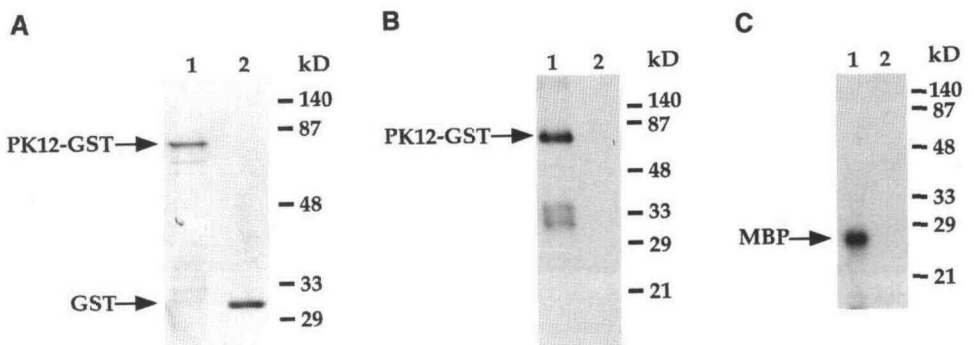

Figure 6. Kinase Assay of the Recombinant PK12–GST Protein.

(A) Coomassie blue staining of affinity-purified PK12–GST (lane 1) and GST (lane 2) proteins (1 μg) expressed in *Escherichia coli*.
(B) Autophosphorylation in vitro of recombinant PK12–GST (lane 1) and GST (lane 2) proteins (1 μg).
(C) Phosphorylation in vitro of MBP substrate by recombinant PK12–GST (lane 1) and GST (lane 2) proteins (30 ng).
Numbers at right indicate molecular masses of standards in kilodaltons.

amino acid residue (EHLAMMERV/ILG; Figure 2). By analogy to the three-dimensional structure of crystallized protein kinases (Debondt et al., 1993; Zhang et al., 1994), the LAMMER motif lies below the substrate binding cleft, where it may have contact with substrates and be involved in the determination of substrate specificity. Indeed, site-directed mutagenesis of the LAMMER motif leads to the in vitro abrogation of PK12 kinase activity toward the MBP substrate (S. Savaldi, unpublished data).

The finding that the PK12 protein autophosphorylates in vitro on serine, threonine, as well as tyrosine residues defines it as a component of the dual-specificity class of protein kinases (Lindberg et al., 1992). Other LAMMER kinases display a similar type of specificity, suggesting that all of the LAMMER family members are dual-specificity protein kinases (Ben-David et al., 1991; Howell et al., 1991; Duncan et al., 1995). Dual-specificity protein kinases are currently considered to be indistinguishable from serine/threonine kinases on the basis of their primary amino acid sequence. Interestingly, LAMMER kinases, in contrast with most of the serine/threonine kinases, contain in the catalytic loop a threonine instead of an arginine (HTDLKPEN, box VIb; Figure 1). This substitution lies in a residue preceding an aspartate, which is considered to activate the incoming substrate hydroxyl and thus may have significance in terms of amino acid selectivity. Currently, it is not known on which residues PK12 and most of the dual-specificity class components phosphorylate their endogenous substrates. In fact, a MAP kinase kinase is the only protein kinase that has been shown thus far to autophosphorylate and to phosphorylate its endogenous substrate on both threonine and tyrosine residues (Haystead et al., 1992). In contrast, the mammalian LAMMER Clk has been shown to autophosphorylate on serine threonine and tyrosine residues but to phosphorylate in vitro the putative substrate ASF/SF2 only on serine residues (Duncan et al., 1995; Colwill et al., 1996). In plants,

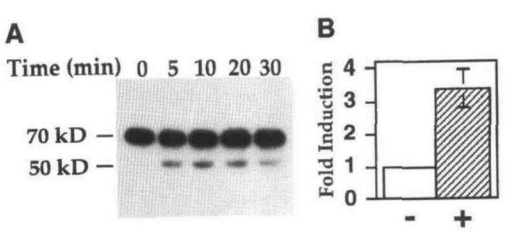

Figure 8. Activation of MBP-Phosphorylating Protein Kinases in Ethylene-Treated Tobacco Leaves.

(A) Tobacco leaves were treated with 20 ppm ethylene for the indicated times, and total extracts (40 μg) were fractionated on a 12% SDS–polyacrylamide gel containing 0.5 mg/mL MBP. The proteins were then denatured, renatured, and incubated in an in-gel kinase assay.
(B) Shown is fold induction of labeling intensity of the 50-kD polypeptide at the time of maximal activity. The average and standard deviation of four different experiments are shown. (−) and (+) represent the basal and induced levels of activity, respectively.

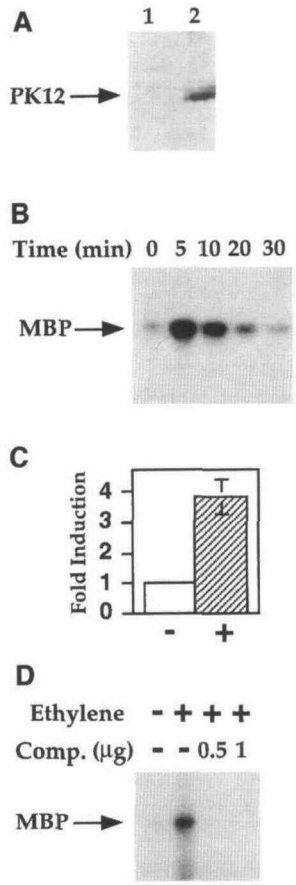

Figure 9. Ethylene Induction of PK12 Kinase Activity.

(A) Antibodies were used to detect the recombinant PK12 protein on immunoblots. Lane 1 contains 40 μg of total protein extract from *E. coli* containing an empty pRSETB vector; lane 2, 40 μg of total protein extract from *E. coli* expressing the PK12 protein in a pRSETB vector.
(B) Extracts of leaves (500 μg) treated with ethylene for the indicated times were immunoprecipitated with anti-PK12 antibodies, and kinase activity on MBP was tested. The labeled MBP product was fractionated on a 12% SDS–polyacrylamide gel, dried, and exposed for autoradiography.
(C) Fold induction of the labeling intensity of the MBP substrate at the time of maximal activity is shown. The average and standard deviation of five different experiments are shown. (−) and (+) represent the basal and induced levels of activity, respectively.
(D) Competition of the immunoprecipitation process by the addition of inactive PK12–GST is shown. The PK12 protein was immunoprecipitated from protein extracts of untreated leaves or leaves treated with ethylene for 15 min in the presence of the indicated amounts of inactive PK12–GST as a competitor (Comp.). The immunocomplex was then tested for kinase activity on the MBP substrate. The labeled MBP was fractionated on a 12% SDS–polyacrylamide gel, dried, and exposed for autoradiography.

the characterization of dual-specificity protein kinase in vitro is restricted to the Arabidopsis ADK1 protein (Ali et al., 1994) and to a pollen-expressed receptor-like kinase (Mu et al., 1994). Tyrosine phosphorylation has been implicated in the transduction of the ABA signal in barley aleurone protoplasts (Knetsch et al., 1996) and in the response of tobacco cell cultures to a fungal elicitor (Suzuki and Shinshi, 1995).

The PK12 protein contains at its N terminus a stretch of basic amino acids that represents a putative nuclear localization signal. Similar motifs are present in the sequence of the other LAMMER kinases, suggesting that they all may be targeted to the cell nucleus. Indeed, the mouse Clk has been shown to localize in the nucleus and to regulate the distribution of serine/arginine splicing factors within the nucleus (Colwill et al., 1996). The precise biological significance of the different LAMMER kinases is not known, but they appear to be involved in signal transduction pathways that control cellular growth and differentiation. The *Doa* gene is essential in Drosophila for a variety of functions, including nervous system and eye development and normal segmentation and development to larval stages (Yun et al., 1994). The mammalian Sty/Clk homolog is involved in the control of PC12 cell differentiation (Myers et al., 1994) and interacts in a yeast two-hybrid system with members of the serine/arginine–rich family of splicing factors (Colwill et al., 1996). Arabidopsis AFC1 suppresses mating-pheromone signal transduction defects in yeasts by constitutively stimulating the transcription factor STE12 to express mating-specific genes (Bender and Fink, 1994). It remains to be elucidated if the function of LAMMER protein kinases from different organisms is required in similar molecular mechanisms that have been conserved throughout evolution.

A 50-kD MBP-phosphorylating activity from tobacco leaf extracts was rapidly and transiently induced by ethylene, as shown by in-gel kinase assay. The similarity in molecular mass, ethylene inducibility, and activity on the MBP substrate make the PK12 kinase a good candidate for being the source of this activity. The detection in tobacco leaf extracts of a 46-kD MBP-phosphorylating activity was recently reported (Seo et al., 1995; Usami et al., 1995). The 46-kD activity was induced either by cutting (Usami et al., 1995) or by wounding (Seo et al., 1995). Activities of similar molecular masses were also observed in extracts of suspension cultures of tobacco cells and were found to be induced by a fungal elicitor (Suzuki and Shinshi, 1995) or by auxin treatment (Mizoguchi et al., 1994). Because molecular masses of most MBP-phosphorylating protein kinases tend to be within the 50-kD range, direct examination via antibodies or transgene analysis is necessary in each case. Recently, by using anti-ERK1 antibodies that recognize MAP kinases in different species, Knetsch et al. (1996) have shown that in barley aleurone protoplasts, ABA rapidly induces transient activation of a MAP kinase, measured as MBP phosphorylation by MAP kinase immunoprecipitates.

Several protein kinases have been isolated and related to specific physiological responses in plants, for example, ETR1 and CTR1 (Chang, 1996), involved in ethylene signal transduction; Pto and Pti1, involved in speck disease resistance (Martin et al., 1993; Zhou et al., 1995); Xa21, conferring resistance to *Xanthomonas oryzae* race 6 (Song et al., 1995); and SRK (Nasrallah et al., 1994), involved in self-incompatibility. However, their activities have not been examined in vivo. Here, we show that the apparent PK12 kinase activity is enhanced by ethylene in vivo. The effect of ethylene is rapid (within minutes), transient, and well in accord with the kinetics of ethylene-induced phosphorylation observed in total plant extracts (Raz and Fluhr, 1993). The possibility that the observed changes in PK12 activity in response to ethylene are the result of a rapid protein synthesis followed by degradation are unlikely; however, this mechanism should also be considered.

Similar transient activation of protein kinases in the presence of a continuous stimulating signal has been observed both in animals and in plants (Seger and Krebs, 1994; Suzuki and Shinshi, 1995; Knetsch et al., 1996). Therefore, ethylene may exert on the PK12 protein a short-term effect, via activation of its kinase activity, and a long-term effect, via elevation of its transcription level, which is consistent with our rationale that activation of components in a signal transduction pathway may lead to a heightened turnover. The activation characteristics of PK12 suggest its involvement in the ethylene signal transduction pathway, possibly coordinating general transcriptional or splicing mechanisms, as suggested for other members of the LAMMER family (Yun et al., 1994; Colwill et al., 1996). The role and hierarchical position of PK12 in ethylene responses in the plant remain to be examined in relation to identified components of the ethylene signal transduction pathway.

METHODS

Plants and Ethylene Treatment

Tobacco (*Nicotiana tabacum* cv Samsun NN) plants were grown in a greenhouse with 18-hr-day (26°C) and 6-hr-night (22°C) cycles. Experiments were performed in the greenhouse or under Grolux (Sylvania) fluorescent lamps (2.5 to 3.0 nE cm^{-2} sec^{-1}), using young potted plants with three to five leaves at least 10 cm in length. For ethylene treatment, a constant air stream of 20 ppm of ethylene was applied in a sealed glass box containing the potted plants or excised leaves acclimated for 2 hr before treatment.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) and Subcloning of PCR Fragments

RNA was isolated from untreated tobacco leaves, from ethylene-treated leaves, and from flower abscission tissues, as previously described (Logemann et al., 1987). Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose chromatography (Boehringer Mannheim) and treated with DNase I, as described by Dilworth and McCarrey (1992). cDNA was synthesized from 1 μg of poly(A)$^+$ RNA by using as a primer a degenerate oligonucleotide (5051) corresponding to the amino acid sequence DLKPEN, conserved at domain VIb of serine/threonine protein kinases. In addition, oligonucleotide 5051 contains an EcoRI site,

and its sequence is as follows: 5′-CCGGAATTCGAC(T)C(T)TA(G,C,T)AAA(G)CCA(C,G,T)GAA(G)AA-3′. The resulting cDNA was amplified by PCR, using oligonucleotides 5051 and 4817. Oligonucleotide 4817 contains an EcoRI restriction site and codes in the reverse orientation for the YIRPEI amino acid sequence conserved at domain VIII of serine/threonine protein kinases. Its sequence is as follows: 5′-CCGGAATTCCGGATC(T)TCA(G,T)GGA(G,T)CGA(G,T)ATA(G)TA-3′. For RT-PCR of the *PRB-1b* transcript, oligonucleotide 4567, from positions 1059 to 1080 in the *PRB-1b* genomic nucleotide sequence (Eyal et al., 1993; GenBank accession number X66942) (5′-GACTGCAGGATGCAGCATTCCG-3′), was used in the sense orientation, and oligonucleotide 4568, from positions 1522 to 1542 (5′-CCACCCCTTGCTACACCATTG-3′), was used in the antisense orientation. The conditions for PCR were as follows: 40 sec at 95°C, 40 sec at 37°C, 60 sec at 72°C for 10 cycles and 40 sec at 95°C, 40 sec at 47°C, 60 sec at 72°C for 20 cycles. PCR products were fractionated on a 4% acrylamide gel, excised from the gel, digested with the restriction enzyme EcoRI, and ligated to the pBluescript SK+ plasmid (Stratagene, La Jolla, CA). Clones hybridizing with both oligonucleotides 4817 and 5051 were sequenced using Sequenase version 2 (United States Biochemical).

Isolation of cDNA Clones

A cDNA library from ethylene-treated tobacco leaf, constructed in the λZAPII phage vector (Clontech, Palo Alto, CA), was screened with a *PK12* PCR fragment as probe. The screening procedure and excision of the pBluescript SK+ plasmid, containing the cloned insert, from the λZAPII vector were performed according to Clontech protocols. Positive clones were isolated, excised, and sequenced with the thermocycling automatic sequencer (model 373A; Applied Biosystems, Foster City, CA).

Expression of Recombinant Proteins in Bacteria and Antibody Preparation

For PK12 expression in bacteria, the PK12 coding region, from amino acids 27 to 431 (Figure 2), was subcloned in frame into the BamHI restriction site of a pGEX1 expression vector (Pharmacia; Smith and Johnson, 1988), fused to the C terminus of the GST protein.

The inactive PK12 mutant used in the experiments shown in Figure 9D was generated by site-directed mutagenesis, using the *PK12* cDNA as a template and oligonucleotide 13822 (5′-GAGCACCTGGCGAGGGCGCAAAGAGTATTGGG-3′). The substitution mutations inserted in the PK12 coding region were as follows: methionine 332 was substituted by arginine, methionine 333 by alanine, and glutamic acid 334 by glutamine. The mutant *PK12* coding region, from amino acids 27 to 431 (Figure 2), was subcloned in the pGEX1 vector at the BamHI restriction site. PK12–GST fusion proteins were expressed in *Escherichia coli* and purified as described by Smith and Johnson (1988).

For antibody production, a PK12 fragment, from amino acids 222 to 276 (Figure 2), was subcloned into the EcoRI restriction site of a pGEX1 vector and used for expression. The PK12–GST fusion proteins were isolated from bacteria by using glutathione–agarose beads, as described by Smith and Johnson (1988). Guinea pigs were injected three times in 10-day intervals with 100 μg of protein each time. Anti–PK12–GST antibodies were tested for cross-reaction with PK12 protein on immunoblots containing fractionated total protein of *E. coli* (40 μg), which expressed the PK12 protein in a pRSETB vector (Invitrogen, San Diego, CA; Figure 9A). In the pRSETB vector used, the PK12 protein region from position 27 to 431 was subcloned at the BamHI and XhoI restriction sites.

Immunoprecipitation

Excised leaves were treated with ethylene for the indicated times, and samples were taken and frozen in liquid nitrogen. Samples were homogenized in extraction buffer (50 mM Hepes, pH 7.5, 1 mM EDTA, 1.5 mM EGTA, 10 mM NaF, 100 μM $NaVO_3$, 50 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) and centrifuged at 10,000*g*; the pellet was then discarded. Components that interfere with the assays were removed by sequential passage through P-6 (Bio-Rad) and Sepharose CL-6B columns (Pharmacia) prewashed with extraction buffer. Protein concentration was then determined with a protein assay reagent (Bio-Rad), and Nonidet P-40 and deoxycholic acid were added to a final concentration of 1 and 0.5%, respectively. Protein extracts (0.5 mg) were incubated for 2 hr at 4°C with anti–GST–PK12 antibodies prebound to Sepharose–protein A (Pharmacia). In experiments performed in the presence of a competitor, the GST–PK12 antibodies bound to Sepharose–protein A were preincubated for 30 min with the indicated amount of competitor. Immunoprecipitates were washed with washing buffers (1 mL) containing 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 0.5% deoxycholic acid, 1% Nonidet P-40, and decreasing concentrations of NaCl (1 M, 0.5 M, and final wash without NaCl). Two additional washes were then performed with 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100. Bound proteins were directly assayed for kinase activity.

Kinase Activity Assays

For the in vitro kinase activity assay, immunoprecipitates or purified recombinant proteins (1 μg for autophosphorylation and 30 ng for phosphorylation of myelin basic protein [MBP] substrate) were incubated with kinase buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 0.2 μL γ-^{32}P-ATP (3000 μCi/mL; Amersham Corp.) in a final volume of 50 μL. Substrate phosphorylation included 0.2 mg/mL MBP in the kinase buffer. Reactions were incubated for 20 min at 30°C, precipitated with 10% trichloroacetic acid, neutralized with 100 μL of 0.1 M NaOH and 1 mL of acetone, resuspended in sample buffer (Laemmli, 1970), and fractionated on an SDS–polyacrylamide gel.

For the kinase assay in MBP-containing polyacrylamide gels, total protein extracts were prepared as described for immunoprecipitation experiments, and aliquots of 40 μg were subjected to electrophoresis on 12% SDS–polyacrylamide gels that were polymerized in the presence of 0.5 mg/mL MBP. The protocol used for the in-gel kinase assay was essentially as described by Tobe et al. (1991) with some modifications. SDS was removed from the gel by washing twice for 30 min with 100 mL of 20% isopropanol and 50 mM Hepes, pH 7.6, followed by two washes of 30 min each with buffer A (5 mM β-mercaptoethanol in 50 mM Hepes, pH 7.6).

Protein denaturation was then performed by incubating the gel for 15 min in buffer A containing 6 M urea. This step was repeated, and the gel was subjected to renaturation by washing at 4°C in decreasing concentrations of urea (3, 1.5, and 0.75 M for 15 min each) in buffer A supplemented with 0.05% Tween 20.

Finally, the gel was washed three times in buffer A supplemented with 0.05% Tween 20 for 15 min and left overnight at 4°C in the last washing solution. The gel was then incubated for 30 min at 30°C in 30 mL of kinase buffer (20 mM Hepes, pH 7.6, containing 20 mM

$MgCl_2$). This buffer was replaced with kinase buffer supplemented with 2 mM DTT, 20 μM ATP, and 100 μCi γ-^{32}P-ATP. After 2 hr at 30°C, the gel was washed in washing solution (100 mL of 5% trichloroacetic acid and 1% Na-pyrophosphate) until the radioactivity of the solution was negligible. The washed gel was stained with Coomassie Brilliant Blue R 250 to confirm loading of equal amounts of protein in each lane and then dried and subjected to autoradiography.

Phosphoamino Acid Analysis

Products of kinase reactions were fractionated by SDS–PAGE and transferred to polyvinyldifluoride membranes (Immobilon; Millipore Corporation), and γ-^{32}P–labeled polypeptides were visualized by autoradiography. Pertinent regions of the polyvinyldifluoride membrane were excised, and the γ-^{32}P–labeled protein was hydrolyzed in 6 M HCl for 60 min at 110°C, as described by Kamps and Sefton (1989). Supernatants were dried in a speedvac concentrator (Savant, Holbrook, NY), mixed with nonradioactive phosphoamino acid standards (phosphoserine, phosphothreonine, and phosphotyrosine; 2 μg each), and analyzed by two-dimensional electrophoresis on thin-layer cellulose plates (pH 1.9 and 3.5, for the first and second dimension, respectively), as described by Boyle et al. (1991). Labeled phosphoamino acids were detected by autoradiography, and standard phosphoamino acids were visualized by spraying the thin-layer chromatography plate with an acetone solution containing 0.2% ninhydrin.

DNA and RNA Gel Blot Analysis

Genomic DNA (25 μg) was digested by restriction enzymes overnight, fractionated on a 1% agarose gel, and blotted on Hybond N^+ nylon membranes (Amersham), according to the manufacturer's protocols. Blots were hybridized with probes at 65°C for at least 6 hr in hybridization solution containing 25 mM phosphate buffer, pH 7.0, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 1% SDS, and 100 μg/mL salmon sperm DNA. Blots were washed twice for 15 min at 55°C with 1 × SSC, 0.1% SDS and exposed for autoradiography. For RNA gel blots, total RNA was extracted according to Logemann et al. (1987), and poly(A)$^+$ RNA was isolated by using Dynabeads oligo(dT)$_{25}$ (Dynal, Oslo, Norway), according to manufacturer protocols. Poly(A)$^+$ RNA was then fractionated in a formaldehyde denaturing gel and transferred to a Hybond N^+ nylon membrane (Amersham). Probes were hybridized with blots at 42°C for at least 6 hr in a hybridization solution containing 5 × Denhardt's solution, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 1% SDS, 50% formamide, and 100 μg/mL salmon sperm DNA. Blots were then washed twice for 15 min at room temperature with 1 × SSC, 0.1% SDS and exposed for autoradiography.

In Situ Hybridization

Control leaves, ethylene-treated leaves (24-hr treatment), and flower abscission tissues were sliced and fixed in 10% formaldeyde. The in situ hybridization procedure was performed according to Cox and Goldberg (1988). Riboprobes were synthesized with ^{32}P-UTP, using the *PK12* cDNA as a probe.

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