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Molecular cloning of plant transcripts encoding protein kinase homologs

(gene family/homology probing/oligonucleotides)

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ABSTRACT **Oligonucleotides, corresponding to conserved regions of animal protein-serine/threonine kinases, were used to isolate cDNAs encoding plant homologs in the dicot bean (*Phaseolus vulgaris* L.) and the monocot rice (*Oryzae sativa* L.). The C-terminal regions of the deduced polypeptides encoded by the bean (PVPK-1) and rice (G11A) cDNAs, prepared from mRNAs of suspension cultures and leaves, respectively, contain features characteristic of the catalytic domains of eukaryotic protein-serine/threonine kinases, indicating that these cDNAs encode plant protein kinases. The putative catalytic domains are most closely related to cyclic nucleotide-dependent protein kinases and the protein kinase C family, suggesting the plant homologs may likewise transduce extracellular signals. However, outside these domains, PVPK-1 and G11A exhibit no homology either to each other or to regulatory domains of other protein kinases, indicating the plant homologs are modulated by other signals. PVPK-1 corresponds to a 2.4-kb transcript in suspension cultured bean cells. Southern blots of genomic DNA indicate that PVPK-1 and G11A correspond to single copy genes that form part of a family of related plant sequences.**

Phosphorylation of specific proteins is a major strategy for the regulation of protein and enzyme activity in the transduction of environmental, developmental, and metabolic signals in animals and simple eukaryotes (1). Over 100 different protein kinases have been described, the majority of which fall into two classes depending on their ability to phosphorylate either serine/threonine or tyrosine (2, 3). These kinases can be further grouped into families based on characteristic structural features, substrate specificity, regulatory ligand, and cellular function (4, 5).

This array of protein kinases provides a repertoire of specific signal transducers in eukaryotic cells. Signal input is determined by the regulatory properties of the kinase with respect to modulation by ligands such as cyclic nucleotides, Ca^{2+}, hormones, or growth factors. Signal output is determined by the catalytic properties of the kinase with respect to protein substrate specificity. In some cases protein kinase activity is itself modulated by phosphorylation mediated by a second protein kinase, thereby providing a mechanism for signal amplification. Detailed biochemical, molecular, and genetic studies have established that in animals as well as in *Dictyostelium* and yeast, specific protein kinases function as key elements in the regulation of cell division, transcription, translation, and metabolic flux or as receptors for hormones and growth factors in the cellular transduction of external signals (4).

By analogy, it is likely that in higher plants, protein kinases likewise act as signal transducers in the regulation of key cellular functions. However, although protein phosphorylation has been observed *in vivo* and protein kinase activity has been demonstrated *in vitro*, only in a few cases has reversible phosphorylation been shown to be associated with the regulation of plant proteins of known function (6). Thus, pyruvate dehydrogenase and several other enzymes of intermediary metabolism are regulated by phosphorylation in a similar manner to their mammalian counterparts. In addition, the distribution of photosynthetic excitation energy between photosystems I and II is governed by the phosphorylation of light-harvesting chlorophyll a/b-binding protein by a protein kinase sensitive to the redox state of plastoquinone (7), whereas NAD^+:quinate oxidoreductase is regulated by a Ca^{2+}/calmodulin-dependent protein kinase (8).

Overall, little information is available concerning the specific functions of individual plant protein kinases as signal transducers. Molecular cloning of plant protein kinase gene sequences would open up molecular genetic approaches for dissection of the function of specific protein kinases in plant signal transduction pathways and analysis of structure–function relationships. As a first step we report here the isolation and characterization of cDNAs from the dicot bean (*Phaseolus vulgaris* L.) and the monocot rice (*Oryzae sativa* L.) that encode protein-serine/threonine kinase homologs.‡

Identification of these cDNAs was based on the conservation of several characteristic amino acid sequences in the catalytic domains of eukaryotic protein kinases (5). Oligonucleotide probes corresponding to such conserved sequences have been used to identify different protein kinases in mammals (9) and yeast (10). The plant cDNAs were identified by a similar approach, which involved sequential probing with partially degenerate oligonucleotides corresponding to two discrete conserved regions characteristic of the catalytic domains of a group of mammalian protein-serine/threonine kinases. The deduced polypeptides contain many of the features characteristic of eukaryotic protein-serine kinases, and the putative catalytic domains most closely resemble those of the cyclic nucleotide-dependent protein kinases and the protein kinase C family, suggesting that the plant homologs may likewise function in the transduction of extracellular signals.

MATERIALS AND METHODS

Materials and General Methods. Maintenance of cell suspension cultures of bean (*P. vulgaris* L.) and construction of the bean plasmid cDNA library have been described elsewhere (11). The phage cDNA library from 5-day-old dark-grown rice (*O. sativa* L., IR36) leaves was a gift from N.-H. Chua. RNA was isolated as described (11) and DNA was isolated by the method of Schwartz-Sommer *et al.* (12). The oligonucleotides corresponding to the probes DLKPEN and GTPEYLAPE have been described (9). DNA manipulations,

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‡The sequences reported in this paper are being deposited in the EMBL/GenBank data base (accession nos. J04555 for *Phaseolus vulgaris* L. cDNA and J04556 for *Oryzae sativa* L. cDNA).

gel electrophoresis, radiolabeling of DNA fragments and oligonucleotides, and filter blot hybridizations were performed by standard methods (13, 14).

Bean cDNA Library Screening. Recombinants (2×10^5) from the plasmid cDNA library were plated at high density and replica filters were screened with oligonucleotide probes as described (9). The hybridization buffer contained a final concentration of 1 ng of each unique 5'-labeled oligonucleotide per ml (15). Plasmid DNA from hybridizing clones purified through several rounds was prepared, and the insert cDNAs were isolated on agarose gels following excision with *Pst* I.

Rice cDNA Library Screening. Recombinants (5×10^5) from the λgt11 cDNA library were plated out and replica lifts onto nitrocellulose filters were processed for hybridization as above. Filters were prehybridized for 4 hr at 37°C in 30% formamide/5× Denhardt's solution (Denhardt's solution: 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/5× SSC (SSC: 0.15 M NaCl/15 mM sodium citrate)/0.1% $NaDodSO_4$/100 μg of sheared salmon sperm DNA per ml and hybridized for 24 hr at 37°C in the same buffer containing nick-translated probe DNA. Filters were washed in 0.1× SSC/0.1% $NaDodSO_4$ at room temperature and exposed to x-ray film. Hybridizing plaques were picked into SM buffer (13) and purified as above. DNA was isolated using a small-scale procedure (13), and the cDNA inserts were excised with *Eco*RI and isolated from agarose gels.

Nucleotide Sequence Analysis. cDNA insert sequences were subcloned into pIBI24 and nested deletions were generated by exonuclease III digestion (16). Nucleotide sequence analysis was by the dideoxy chain-termination method (17) using Sequenase (United States Biochemical).

RESULTS

Homology Probing Strategy. To select against false positives, clones were sequentially probed with partially degenerate oligonucleotides corresponding to the amino acid sequences DLKPEN and GTPEYLAPE (single-letter code; ref. 18), which represent consensus sequences for discrete, highly conserved regions within the catalytic domains of protein-serine/threonine kinases (5, 9). Since cDNA synthesis proceeds 3' to 5', true homologs isolated using the primary DLKPEN probe should also contain sequences for GTPEYLAPE, assuming sequence conservation in these regions and complete second strand cDNA synthesis. Estimation of the degree of hybrid mismatch, and therefore the fidelity of the screening procedure, was aided by the use of tetramethylammonium chloride during the washing of filter blots (15).

cDNA Clones Encoding Plant Homologs. The oligonucleotides were used to screen a plasmid-based cDNA library corresponding to poly(A)$^+$ RNA from suspension cultured bean cells 38 hr after elicitor treatment (11). Of 100 colonies that hybridized with the DLKPEN probe, 33 continued to hybridize when purified. From a sample of 19, 4 hybridized to the secondary GTPEYLAPE probe. These clones were identical as judged by size of insert, restriction digestion pattern, and strength of hybridization, and clone PVPK-1 was chosen for further study.

Restriction endonuclease digestion and Southern analysis of PVPK-1 showed that, as expected, regions hybridizing to the DLKPEN and GTPEYLAPE probes were closely linked. Limited nucleotide sequence analysis of a 400-base-pair (bp) *Sca* I fragment that hybridized to the primary probe revealed an open reading frame that potentially encoded the amino acid sequence DLKPEN. In addition, adjacent unselected sequences resembled those surrounding this motif in eukaryotic protein kinases, prompting the determination of the nucleotide sequence of the entire 2.2-kilobase (kb) insert (Fig. 1*A*). In parallel, a 1.1-kb *Acc* I fragment of PVPK-1 containing the probe target regions was used to screen a monocot cDNA library made from leaves of dark-grown rice seedlings. One clone, G11A, hybridized strongly to the PVPK-1 probe and contained a 1.6-kb insert encoding a highly related polypeptide (Fig. 1*B*).

Deduced Amino Acid Sequences. PVPK-1 contains an open reading frame from nucleotides 217 to 2036 encoding a 609-amino acid polypeptide (Fig. 1*A*). The region from nucleotides 1 to 216 contains stop codons in all three reading frames. An *in vitro*-transcribed antisense RNA corresponding to nucleotides 1–340 was fully protected from RNase digestion in the presence of poly(A)$^+$ RNA from suspension cultured cells (data not shown) (19), indicating that these sequences are present in the mature PVPK-1 mRNA and represent a long 5'-untranslated leader rather than unprocessed intron sequences. Long untranslated regions have also been observed in the mRNAs encoding both the catalytic and regulatory subunits of cAMP-dependent protein kinase (20, 21). The 3' poly(A) sequence is likely to represent the poly(A)$^+$ tail added posttranscriptionally. No canonical poly(A) signal sequence is present, but in plants a variety of sequences appear to be recognized as signals (22). Overall, these data indicate that PVPK-1 represents a nearly full-length cDNA.

G11A contains an open reading frame encoding a 536-amino acid polypeptide (Fig. 1*B*). The reading frame extends throughout the cDNA, indicating that it is incomplete in both 5' and 3' sequences. Although PVPK-1 and G11A contain the target sequence DLKPEN, in both cDNAs the region homologous to the secondary probe corresponds to the sequence GTHEYLAPE rather than the target GTPEYLAPE. These sequences fall within a region of 410 amino acids that is highly conserved between PVPK-1 and G11A (PVPK-1 residues 177–586). The C terminus and N-flanking region are divergent.

Features Characteristic of Protein Kinases. Much of the region conserved between PVPK-1 and G11A is homologous to the catalytic domains of eukaryotic protein kinases and the deduced plant polypeptides contain all but one of the characteristic features of these enzymes (Fig. 2) (4, 5, 25). These include (relative to the residue numbers for the catalytic subunit of bovine cAMP-dependent protein kinase) amino acids corresponding to the motif $Gly^{50}XaaGly^{52}XaaXaaGly^{55}$, which forms part of the ATP binding region; Lys^{72}, which is implicated in ATP binding and the phosphotransfer reaction; Asp^{166} and Asn^{171}, which form part of the DLKPEN motif and are also implicated in ATP binding; Asp^{184}, whose chemical modification by carbodiimide destroys catalytic activity; Ala^{206} and Glu^{208}, which are diagnostic of the catalytic domain and are contained within the GTHEYLAPE motif.

Both cDNA sequences also contain the invariant or nearly invariant amino acids corresponding to Val^{57}, Ala^{70}, Glu^{91}, Asp^{220}, Gly^{225}, and Arg^{280}, whose functions have not been defined. The one invariant amino acid not conserved in the plant homologs is Gly^{186}, which in other protein kinases forms part of the nearly invariant sequence $Asp^{184}Phe^{185}Gly^{186}$. In PVPK-1 and G11A, Gly^{186} is replaced by an aspartate residue. Additionally, the residue corresponding to Thr^{197}, which in cAMP-dependent protein kinase is autophosphorylated (23), and whose mutation to a glycine leads to loss of cAMP regulation (26), is replaced by a serine, which could fulfill a similar function.

Familial Relationships. The sequences of the putative catalytic domains of PVPK-1 (nucleotides 895–1920, amino acids 239–568) and G11A (nucleotides 417–1448, amino acids 139–482) were compared with the catalytic domains of other protein kinases and homologs, using the tree-building concept described previously (5). This analysis (not shown) revealed that PVPK-1 and G11A fall into a branch cluster of the protein-serine/threonine kinases that contains the cyclic

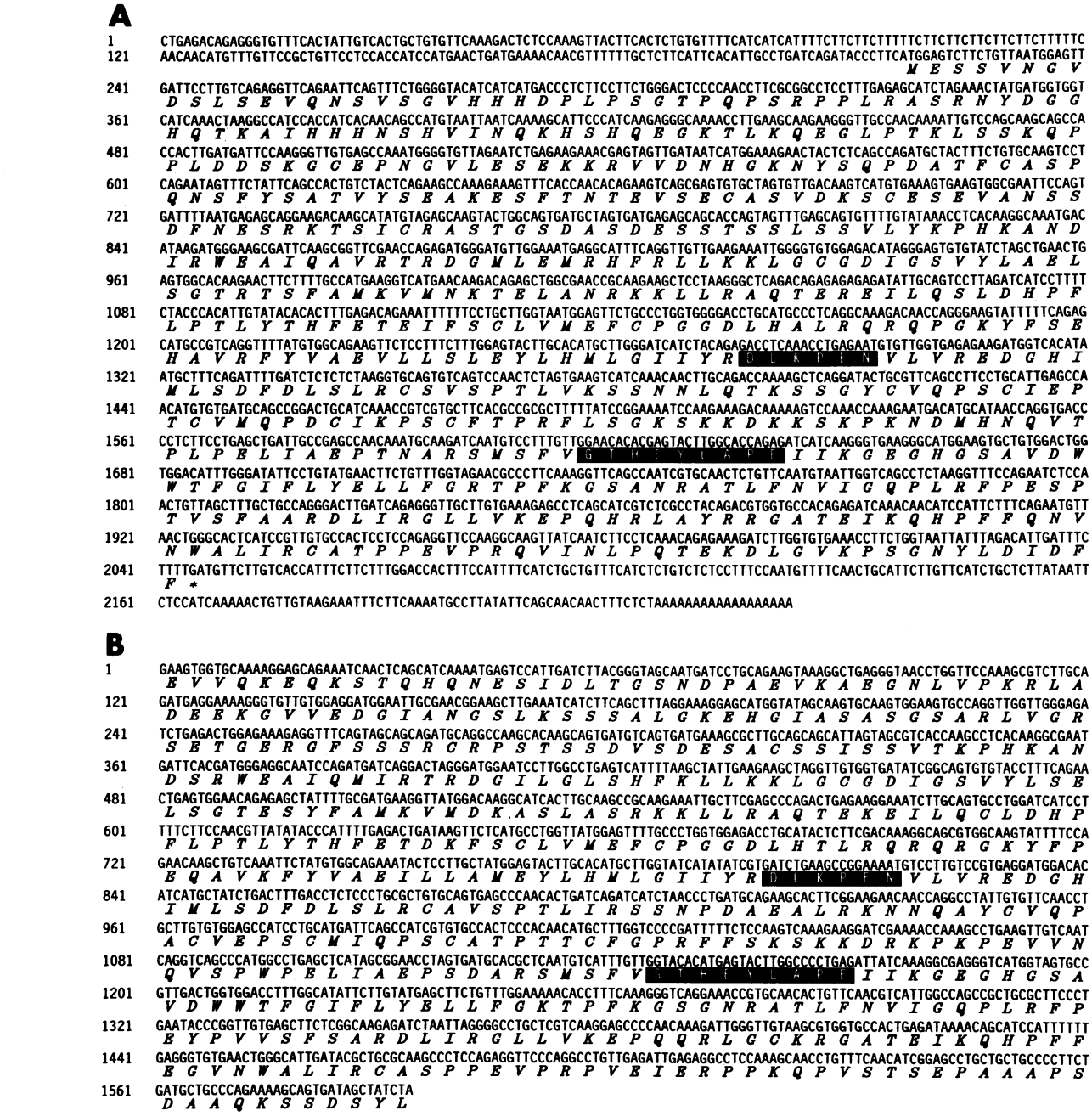

FIG. 1. Nucleotide sequence and derived amino acid sequence of PVPK-1 (*A*) and G11A (*B*). Sequences targeted by the probes are highlighted. The deduced amino acid sequence is shown only for the long open reading frame.

nucleotide-dependent protein kinases and the protein kinase C family (5).

The longest contiguous regions homologous with other eukaryotic protein kinases are those surrounding the probe target sequences. These regions are indicators of substrate amino acid specificity (5, 9) and confirm PVPK-1 and G11A as protein-serine/threonine kinases. Sequences in and around these regions are also characteristic of subfamilies, and on this basis PVPK-1 and G11A (DLKPEN, GTHEYLAPE) more closely resemble the cyclic nucleotide-dependent protein kinases (DLKPEN, GTPEYLAPE) than the protein kinase C family (DLKLDN, GTPDYIAPE).

The most striking difference between the putative catalytic domains of PVPK-1 and G11A compared to those of other protein kinases is the presence between the regions encoding DLKPEN and GTHEYLAPE of an additional 79 or 81 nucleotides, respectively. This region is rich in charged hydrophilic residues, particularly lysine, and contains five tandem copies of a sequence that can be described in general by the motif $C(X)_{2-3}PX$ and for most of the repeats by the sequence C(V/I)(Q/E)P(T/S). The protein kinase encoded by the *Saccharomyces cerevisiae CDC7* gene (27) contains a similar size insert, indicating that additional sequences can be accommodated in this region without destroying catalytic activity. Presumably, these sequences do not alter the configuration of the bordering conserved regions implicated in catalysis.

A polypeptide initiated from the methionine corresponding to nucleotides 217–219 of PVPK-1 would contain 238 amino acids upstream of the putative catalytic domain. By analogy with other protein kinases, this region could represent a regulatory domain involved in the binding of effectors. This region is smaller than the equivalent regions present in the cGMP-dependent protein kinase (28) and the protein kinase

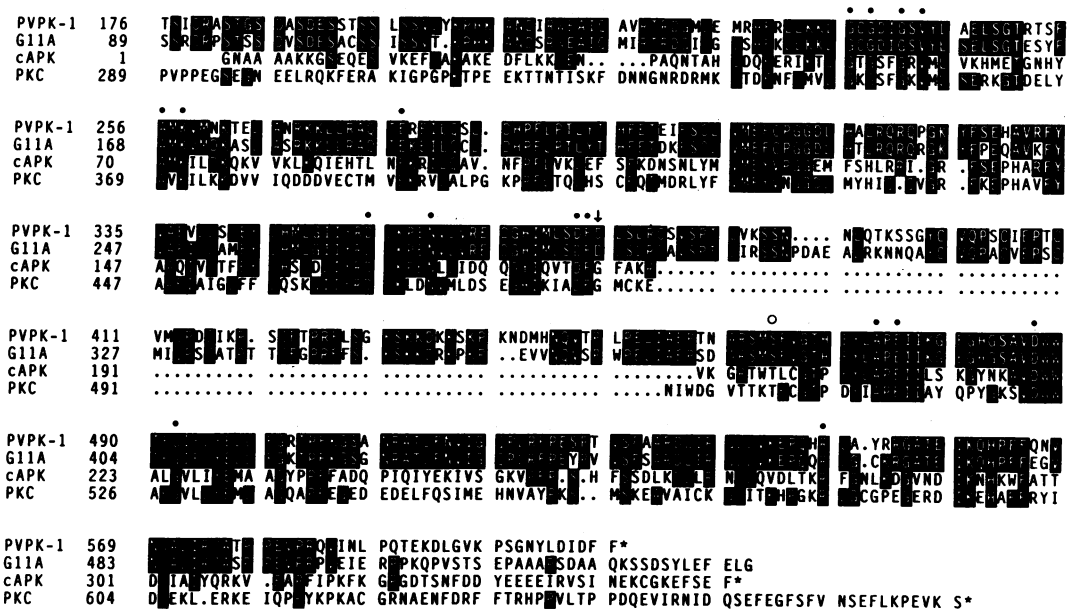

FIG. 2. Comparison of the deduced amino acid sequences of PVPK-1, G11A, the catalytic subunit of bovine cAMP-dependent protein kinase, α-form (cAPK; ref. 23), and bovine brain protein kinase C, β-form (PKC; ref. 24). The single-letter amino acid code is used. Numbers indicate the position relative to the N terminus or, in the case of PVPK-1 and G11A, the position of the residue relative to the start of the open reading frame. Positions of identity with either plant sequence are highlighted. Positions that are invariant or nearly invariant among known protein kinases are indicated by dots. The arrow indicates the position of the conserved glycine not present in either plant sequence. The autophosphorylated threonine of cAPK is indicated by the open circle. Gaps introduced in the alignment are indicated by dotted lines.

C family (29), which contain 330–356 amino acids, but of a similar size to the N-terminal regions of the myosin light-chain kinase and the *src* family of protein-tyrosine kinases (3, 30). However, no significant sequence similarities were found using the FASTA program (31) between the putative regulatory regions of PVPK-1 or G11A and the regulatory domains of other protein kinases, or indeed other amino acid sequences within the GenBank database.

Organization of Genomic Sequences. Southern blots of restriction enzyme-digested genomic DNA from bean and rice were probed, respectively, with the cDNA inserts isolated from PVPK-1 and G11A. Hybridization at high stringencies generally revealed only a single strongly hybridizing band and one or two weakly hybridizing bands, depending upon the particular restriction enzyme used (Fig. 3*A*). At low stringencies, additional bands hybridized to the PVPK-1 probe, suggesting that there are related genomic sequences that may encode other protein kinases.

Expression of PVPK-1. PVPK-1 hybridized to a 2.4-kb RNA present in poly(A)$^+$ RNA from both unstimulated and

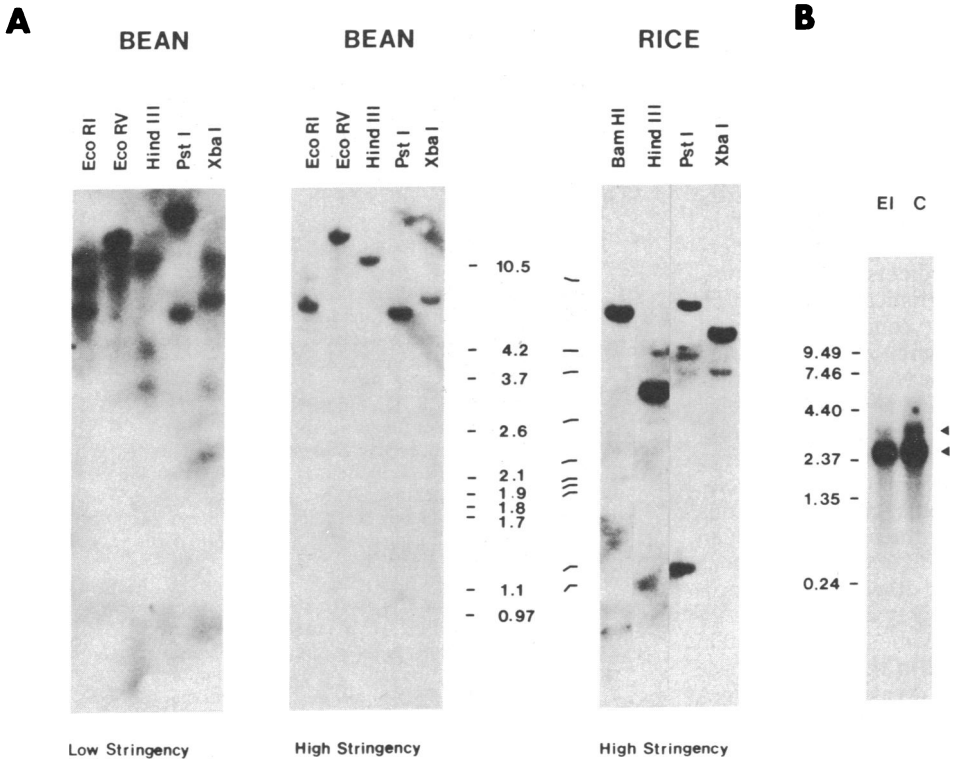

FIG. 3. (*A*) Southern blot hybridization of PVPK-1 and G11A genomic sequences. PVPK-1 and G11A cDNA sequences were used as probes for genomic sequences present in bean and rice, respectively. Genomic DNA was digested with the indicated restriction enzymes, fractionated through agarose, and transferred to nitrocellulose. Filters were hybridized as described (32) at 50°C and washed in 0.5× SSC/0.1% NaDodSO$_4$ at either 50°C (low stringency) or 65°C (high stringency). Sizes of DNA molecular weight markers are indicated in kbp. (*B*) Northern blot analysis of PVPK-1 transcripts. Poly(A)$^+$ RNA from unstimulated bean cells and cells 38 hr after elicitor treatment was fractionated by electrophoresis through agarose, transferred to nitrocellulose filters, and probed with the PVPK-1 cDNA insert. Sizes of RNA molecular weight markers are indicated in kb. The arrowheads indicate the positions of the major and minor hybridizing species.

elicited bean cells (Fig. 3*B*). This is ≈200 nucleotides larger than the PVPK-1 cDNA and indicates that the mature mRNA contains additional 5′- or 3′-untranslated sequences. A weak signal corresponding to a larger RNA species was also detected. No additional signals were detected at low stringencies (data not shown).

DISCUSSION

We have used degenerate oligonucleotides corresponding to conserved regions of the catalytic domain of protein-serine/threonine kinases to isolate homologous sequences from bean and rice cDNA libraries. Many features of the deduced amino acid sequences indicate that PVPK-1 and G11A encode true protein kinases. In particular, the C-terminal regions closely resemble the catalytic domains of eukaryotic protein kinases and contain the highly conserved residues involved in ATP binding, phosphotransfer, and target amino acid specificity (5). Conservation of these features suggests that the development of protein kinases as regulatory proteins predated the evolutionary separation of animals from plants. By analogy with other eukaryotic protein kinases, the regions flanking the catalytic domains may be involved in effector binding or interaction with other macromolecules. Since there is no obvious sequence similarity with the regulatory regions of other protein kinases, the plant homologs may be modulated by other signals. The amino acid region spanning the DLKPEN and GTHEYLAPE sequences may similarly be involved in interactions with regulatory molecules or, alternatively, may play a role in defining substrate specificity.

A clue to the possible functions of PVPK-1 and G11A comes from the close similarity of their catalytic domains to those of the cyclic nucleotide-dependent protein kinases, which are involved in the transduction of extracellular signals. Hence, PVPK-1 and G11A may have equivalent functions in plant cells, with similar signal outputs in terms of target substrate specificities. However, physiological studies to date indicate that cAMP and cGMP do not have major regulatory roles in higher plants (6), in line with the conservation of the catalytic but not the regulatory domains compared to the animal enzymes.

The great similarity between the catalytic domains of PVPK-1 and G11A indicates a strong selection pressure for conservation of this structure and highlights the likely importance of these protein kinases in plant cells. A surprising feature is the divergence of the putative regulatory regions of PVPK-1 compared to G11A, and hence these enzymes may be distinct members of a family of protein kinases. Alternatively, PVPK-1 and G11A may be functionally equivalent, in which case the modular structure would imply major differences in the effector ligands between dicots and monocots.

Eukaryotic protein kinases characteristically exhibit such a modular construction, in which a conserved catalytic domain is attached to divergent regulatory domains for binding specific effector ligands, and it was this feature that allowed the isolation of plant genes encoding protein-serine/threonine kinase homologs independent of their regulatory properties, using hybridization probes that span large evolutionary distances. Sequential probing with oligonucleotides corresponding to conserved motifs, or variants of this approach based on amplification using the polymerase chain reaction (33), should be useful for isolation of plant genes encoding additional protein kinases or other regulatory proteins in which specific peptides are highly conserved—e.g., G proteins (34). The existence of additional plant protein kinases is suggested by the presence in the bean and rice genomes of fragments that hybridize weakly to the cDNA probes. The isolation of molecular clones encoding plant protein kinase homologs will provide the basis for examination of the specific functional properties of the encoded enzymes by a combination of gene transfer experiments and biochemical analysis using antibodies generated from proteins expressed in heterologous systems.

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