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Two putative protein kinases from *Arabidopsis thaliana* contain highly acidic domains

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Abstract

Two cDNA clones (ASK1 and ASK2) for plant protein kinases were cloned from *Arabidopsis thaliana* by screening cDNA libraries with a degenerate oligonucleotide probe that corresponds to a highly conserved motif among protein kinases. Sequence analysis shows that the clones contain open reading frames that encode 41.2 kDa (ASK1) and 40.1 kDa (ASK2) proteins, respectively. These coding regions contain all the conserved motifs of protein kinases. Structural analysis of the coding regions revealed that the two protein kinase genes share high sequence similarity to each other (76.6% identity). The catalytic domain located in the amino terminal region is most similar to the calcium/calmodulin-dependent protein kinase subfamily (47.2% to 54.2% similarity) and the SNF1 kinase subfamily (48.1% to 53.3% similarity). However, the carboxy terminal regions contain distinctive stretches of 21 (ASK1) and 19 (ASK2) acidic amino acids. These clones are the first report of protein kinases with such acidic amino acid regions. The transcripts of both genes are most abundant in leaf but are also expressed in other organs. The expression of the two genes is highly affected by light regime.

Introduction

While cellular mechanisms of protein phosphorylation are largely unknown in higher plants, evidence that protein phosphorylation has important physiological regulatory functions in higher plants is rapidly accumulating. Phosphorylations of many enzymes in intermediary metabolism including phosphoenolpyruvate carboxylase, ATPase, sucrose phosphate synthase, pyruvate orthophosphate dikinase and pyruvate dehydrogenase affect catalytic activities [4, 15, 26, 33, 44].

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers M91548 (ASK1) and Z12120 (ASK2).

Photosynthetic energy distribution in photosynthetic system is regulated by phosphorylation of the light-harvesting complexes [1]. In many cases, the phosphorylation status of some plant proteins is changed according to environmental stimuli, including pathogen infection [31, 40]. Perception of the light signal in plants also seems to involve protein phosphorylation [40]. Recently, it has been shown that the DNA binding activity of a plant transcriptional factor, GBF1, is stimulated by phosphorylation [17]. All these observations indicate that, as in animals, protein phosphorylation has key regulatory functions in plants. To understand the roles of specific protein kinases in the regulatory pathways of cellular functions in plants, it will be necessary to molecularly clone the genes encoding protein kinases and conduct molecular genetic studies on the genes.

Here we report the molecular cloning and characterization of two novel putative protein kinase genes (designated as ASK1 and ASK2) from *Arabidopsis thaliana*, a model system for plant molecular genetic studies. The molecular cloning of the two cDNA clones was based on the conserved features of the catalytic domains of eukaryotic protein kinases [13]. The deduced amino acid sequences of both cDNA clones contain all the catalytic domains that are characteristics of the protein-serine/threonine kinase family. The N-terminal region is most similar to the calcium/calmodulin-dependent protein kinase subfamily and the SNF1 protein kinase subfamily, while the carboxy terminal region is enriched with acidic amino acids. The two genes appear to be regulated by light.

Materials and methods

Plant materials

The seeds of the *Arabidopsis* ecotypes La-O and Col-O were originally obtained from Dr M. Koorneef and Dr G.P. Redei, respectively. The plants were grown in an environmentally controlled growth chamber (Conviron Co.) under a 16/8 h light/dark cycle. For dark-treated samples, the plants were subjected to darkness in the exact middle of the light cycle and the dark treatment was continued for 24 h. For all the experiments described here, the ecotype Col-O was used except for the genomic Southern blot analysis where the ecotype La-O was included for comparison to the ecotype Col-O.

Other materials

[α-^{32}P]dCTP (110 TBq/mmol) and [γ-^{32}P]ATP (185 TBq/mmol) were purchased from Amersham. Various enzymes were from Boehringer Mannheim, New England Biolabs and Korea Steel Chemicals. The oligonucleotide was synthesized by the Milligen 7500 DNA synthesizer (Millipore).

Design of the oligonucleotide probe

Considering several factors for use of an oligonucleotide as a screening probe, such as probe length, G + C content and sequence complexity [19], the 32-mer oligonucleotide probe (5′-CGIACICCIGAITACITIGCICCIGAIATIAT-3′) was designed from the amino acid sequence of the conserved subdomain VIII (Gly-Thr-Pro-Glu/Asp-Tyr-Leu/Ile-Ala-Pro-Glu-Ile-Ile), which is one of the most conserved and longest motifs in the catalytic domain of the protein-serine/threonine kinase family [13]. To reduce the degeneracy of the oligonucleotide, codon preference in *A. thaliana* was considered and the nucleoside analog inosine was used where needed [24].

Preparation of poly(A)$^+$ RNA

Total RNA was isolated from root, leaf, stem and floral organs of *Arabidopsis* ecotype Col-O by the guanidium isothiocyanate method [7]. The sample of the floral organ was a collection of the whole flower, including sepal, petal, stamen and pistil. Poly(A)$^+$ RNA was isolated by the oligo (dT)-cellulose chromatography [2, 7].

Construction and screening of Arabidopsis *cDNA library*

Arabidopsis leaf and flower cDNA libraries were constructed in the λgt11 vector using the cDNA synthesis kit from Pharmacia, following the procedures recommended by the manufacturer and the standard procedures [11]. For isolation of the cDNA clones for the *Arabidopsis* protein-serine/threonine kinases, ca. 100 000 plaques from each of the *Arabidopsis* leaf and flower cDNA libraries were transferred onto nylon membranes (ICN) and were screened by filter hybridization with the ^{32}P-labelled oligonucleotide. The filter hybridization reaction was carried out overnight at 42 °C in 0.5 M sodium phosphate pH 7.4, 1 mM EDTA, 1% BSA, 7% SDS [6]. The filters were washed at room temperature in 6 × SSC, 0.1% SDS twice [35]. The filters were exposed overnight with Kodak X-ray films. The insert DNA fragments of the isolated λ cDNA clones were subcloned into the *Eco* RI site of pUC19 for further analyses.

Southern and Northern blot hybridization

Genomic DNA was prepared from 5-week old *Arabidopsis* plants as described [32, 35]. Two μg of *Eco* RI- or *Hin*d III-digested genomic DNA of the *A. thaliana* ecotypes Col-O and La-O was electrophoresed on a 0.8% agarose gel and transferred onto a nylon membrane (ICN). The membrane was then pre-hybridized in 0.5 M sodium phosphate pH 7.4, 1 mM EDTA, 1% BSA, 7% SDS [6] for 4 h at 65 °C and hybridized in the same solution containing 2×10^6 cpm/ml of probes labelled by nick translation. The membrane was washed in 0.1 × SSC, 0.1% SDS at room temperature for 10 min and at 42 °C for 10 min twice. For Northern blot analysis, 10 μg of total RNA was denatured, fractionated on a 1.2% formaldehyde-agarose gel and blotted onto a nylon membrane filter. The membrane was pre-hybridized and hybridized as described above. After hybridization, the membrane was washed in 0.1 × SSC, 0.1% SDS at room temperature for 10 min twice and at 45 °C for 15 min twice. The *Arabidopsis cab2* gene used in the Northern blot analysis was kindly provided by Dr G. An.

DNA sequencing and analysis

Appropriate restriction fragments were subcloned into pUC19 for sequencing. The ordered sets of deleted clones were generated by the exonuclease III and S1 nuclease treatment (Promega) for the regions that were not covered by the restriction fragment subclones. Both strands were sequenced by dideoxynucleotide chain termination method [36] with the Sequenase kit from United States Biochemical. Analysis of the nucleotide and amino acid sequence data was carried out using the Micro-Genie program (Beckman). The deduced amino acid sequences were compared to the sequences in the GenBank or PIR 31 databases with the FASTDB search program (IntelliGenetics).

Results

cDNA isolation and sequencing

Since the amino acid sequences of the protein kinase family proteins share the characteristic features of the conserved subdomains in the catalytic region even between the evolutionarily distant organisms [13], a number of protein kinase genes have been successfully isolated using the oligonucleotide probes that correspond to these conserved subdomains [12, 20, 42]. To isolate the *Arabidopsis* cDNA clones that encode the putative protein kinase genes, we have employed a similar approach, using an oligonucleotide probe that corresponds to the conserved subdomain VI-II of the protein kinase family (see Materials and methods).

By screening the *Arabidopsis* flower and leaf cDNA library with the oligonucleotide probe, three cDNA clones from the leaf library and one cDNA clone from the flower library were initially isolated. Partial sequence analysis of these clones

revealed that two clones would potentially encode protein kinase-related proteins and the complete nucleotide sequences of these two cDNA clones, designated as ASK1 and ASK2, were further determined. The ASK1 cDNA clone is 1570 bp long with a 1089 bp open reading frame. The open reading frame of ASK1 starts from the first ATG codon at the nucleotide position 218 and extends to the stop codon at the nucleotide position 1307, encoding a protein of 363 amino acid residues with a molecular mass of 41.2 kDa. A polyadenylation signal (AATAAA) that is common to eukaryotic mRNAs is located 29 nucleotides upstream from the poly(A)$^+$ addition site [30]. The ASK2 cDNA clone is 1396 nucleotides in length and the most probable open reading frame encodes a protein of 353 amino acid residues with a molecular mass of 40.1 kDa. The initiation codon in this open reading frame is the second ATG codon placed at the nucleotide position 125 and the stop codon is found at the nucleotide position 1184. The ASK2 is not likely a full-length clone, since it does not contain poly(A)$^+$ tails.

Both cDNA clones seem to contain complete open reading frames for the two putative *Arabidopsis* protein kinases. The deduced open reading frames are the longest open reading frames possible in two clones and contain all of the conserved motifs of known protein kinases (see Fig. 1). In addition, the sequences surrounding the putative start codons in both clones match well with the plant consensus sequences [16]. In the ASK1 clone, this open reading frame starts from the first ATG codon found in the sequence and there exist four in-frame stop codons ahead of this putative initiation codon. In ASK2 clone, however, the putative initiation codon for the deduced open reading frame is the second ATG codon in the sequence as mentioned above. The first ATG codon located at the nucleotide position 11 in ASK2 clone is in frame with the second ATG codon but is followed by a stop codon TAG at the nucleotide position 56, after an open reading frame of only 15 amino acids. Indeed, three in-frame stop codons are present between the first and the second ATG codon. In addition, there exist stop codons in all of the three reading

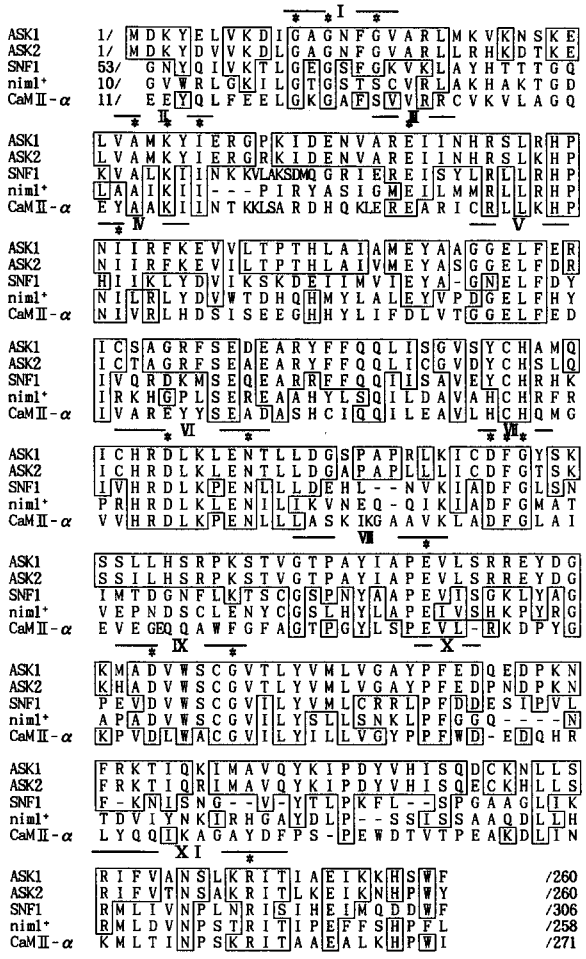

Fig. 1. Comparison of the amino acid sequences of the catalytic domains of ASK1 and ASK2 with those of the yeast SNF1 [5] nim1$^+$ [34] and the rat CamII-α [21]. The amino acid residues identical to either ASK1 or ASK2 are boxed. The invariant or almost invariant amino acid residues in the catalytic domains of protein kinases are indicated by asterisks. The conserved subdomains of the protein kinase family are indicated by the Roman numbers above the sequences. Numbers indicate the positions of the amino acid residues of each clone. Gaps are marked with dashes and are introduced to maximize the alignment.

frames upstream from the second ATG codon. These facts indicate that the open reading frame starting at the second ATG codon is the most probable open reading frame for the ASK2 clone. The presence of fortuitous ATG codons in the 5′-untranslated regions has been observed in other plant genes. Some plant genes like the ferritin of *Phaseolus vulgaris* [39] or the glutathione-

S-transferase gene of maize [37] has been found to contain the fortuitous ATG codons with a short open reading frame in the 5′-untranslated regions.

Analysis of deduced amino acid sequence

The deduced amino acid sequences of the two cDNA clones, ASK1 and ASK2, contain all of the eleven conserved motifs found in the catalytic domain of protein kinase family [13], showing that these clones encode putative protein kinases of *Arabidopsis* (see Fig. 1). These conserved motifs include the motif GXGXXG in the subdomain I which functions in ATP binding, K in the subdomain II which is involved in the phosphotransfer reaction and D in the subdomain VI which appears to interact with the phosphate group of ATP through Mg^{2+} salt bridge [13]. The consensus triplet APE in the subdomain VIII is regarded as a key indicator of the protein kinase catalytic domain and is present in the open reading frames of both ASK1 and ASK2 clones. In addition, the invariant or almost invariant residues of subdomains III, IV, V, VI, VII, IX, X and XI are contained in these open reading frames of both clones. These conserved residues are located at the exactly same positions in both ASK1 and ASK2 clones. The catalytic domain portions of these putative protein kinases show 86.8% amino acid sequence identity and 93.8% amino acid sequence similarity to each other. Overall similarity of the deduced amino acid sequences of these two cDNA clones is 76.6%.

Sequence comparison of the catalytic domains (10–249 amino acid residues in ASK1 and ASK2) of the two cDNA clones to those of the other protein kinases revealed that these clones are most closely related to the protein kinases that fall into the calcium/calmodulin-dependent protein kinase subfamily (47.2% to 54.2% similarity to soybean CDPK [14], human PSK-HI [12] and rat Cam II-α [21] sequences) and the yeast SNF1 subfamily (48.1% to 53.3% similarity to the yeast SNF1 [5] and nim1$^+$ [34] sequences). Similarities to the catalytic domains of other plant protein kinases, such as PVPK1 [20], G11A [20] and ATPK64 [25], are considerably lower (less than 30% similarity). While long amino acid sequences (in excess of 80 amino acids) are present between subdomains VII and VIII of these plant protein kinases, both ASK1 and ASK2 clones have an insert of 15 amino acids in length, which is similar to the case of the calcium/calmodulin-dependent protein kinases (15, 17 and 19 amino acids in soybean CDPK [14], human PSK-HI [12] and rat Cam II-α [21], respectively) and the yeast SNF1 subfamily (15 amino acids in yeast SNF1 [5] and nim1$^+$ [34] kinases).

However, the sequences outside the catalytic domains, which are usually involved in the regulatory roles or in binding to ligands, possess no significant homology to any of the known protein kinases. Indeed, the distinctive feature of the ASK1 and ASK2 clones is found at the carboxy-terminal regions that contain stretches of acidic amino acid (aspartic or glutamic acid) residues. The carboxy terminal region of the ASK1 clone contains a stretch of 21 acidic amino acid residues (amino acid position 324–348). The carboxy terminal region of the ASK2 clone contains a stretch of 19 acidic amino acids (amino acid position 316–338). Two valines, one alanine and one tyrosine residues are interspersed in these acidic amino acid stretches. Interestingly, these acidic regions are well conserved between two clones (see Fig. 2). Computer search of the homologues of this acidic region in the GenBank and PIR 31 data bases revealed that similar sequences are present in hamster endoplasmin [38], human prothymosin [10], human ryanodine receptor [45], hamster nucleolin [18], rabbit calreticulin [8] and in some DNA-binding proteins [43].

Expression of the ASK1 and ASK2 clones

To investigate the expression patterns of the ASK1 and ASK2 genes, northern blot analyses were performed with total RNA prepared from leaf, flower, stem and root organs of *A. thaliana*. The northern blot analysis revealed that the transcript of the ASK1 gene is most abundant in leaf

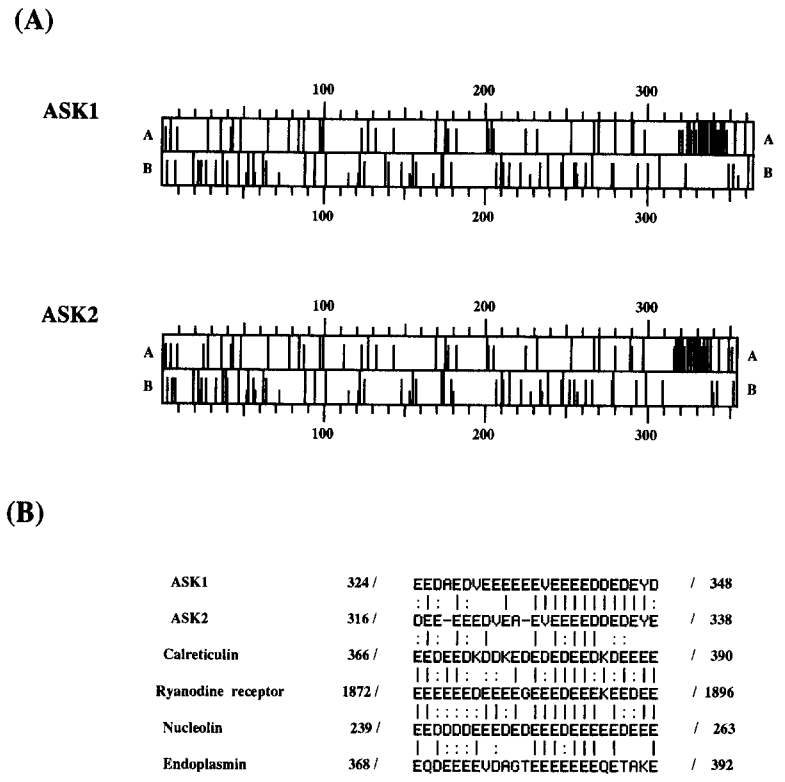

Fig. 2. A. Acidic (A) and basic (B) maps of the predicted amino acid sequences of ASK1 and ASK2. B. Alignment of the acidic amino acid domains of ASK1 and ASK2 with the acidic regions of the rabbit calreticulin [8], the human ryanodine receptor [45], and the hamster nucleolin [18] and endoplasmin [38]. The identical amino acids are marked with vertical lines (|) and the similar amino acids are marked with two dots (:). Numbers refer to the amino acid positions in each clone. Dashes were introduced to optmize the alignment.

organ and less abundant in other organs, the order of transcript abundance being leaf, stem, root and then flower (Fig. 3A). The expression of the ASK2 gene is highest in leaf as that of the ASK1 gene, but the expression patterns in other organs are different from those of the ASK1 gene. The order of transcript abundance of the ASK2 gene is leaf, flower, root and then stem (Fig. 3A). Since expression of many genes that are expressed in leaf is affected by light regime, we have tested if the expression of these clones responds to light regime. Six-week old *Arabidopsis* plants were subjected to dark treatment for 24 h as described in Materials and methods. The expression of both clones is completely turned off after 24 h of dark treatment (Fig. 3B). The same blot was washed and probed with the *Arabidopsis cab2* gene. As shown in Fig. 3B, the response of both ASK1 and ASK2 genes to dark treatment is more drastic than the response of the *Arabidopsis cab* gene family that is one of the typically light-regulated genes. (Note that, even though we have used the *Arabidopsis cab2* gene as a probe, *Arabidopsis* possesses three highly homologous *cab* genes and the signals detected in the northern blot analysis are a mixture of transcripts from all of the three cab genes.)

Southern blot analysis

To examine the genomic complexity of the two genes, the filters containing the *Arabidopsis* genomic DNA digested with *Eco* RI or *Hin*d III

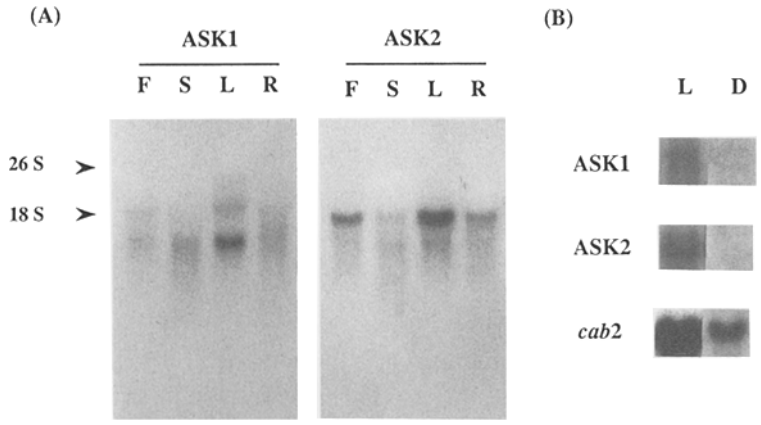

Fig. 3. Northern blot analyses of the ASK1 and ASK2 genes. A. Ten μg of total RNA was prepared from root (R), leaf (L), stem (S) and flower (F). The RNA samples were electrophoresed under denaturing condition and blotted onto a nylon membrane. The blots were probed with the ASK1 or the ASK2 clone. B. Total RNA was prepared from *Arabidopsis* leaf tissues grown under light (L) or after 24 h dark treatment (D). The nylon filters with the gel-electrophoresed RNA samples were probed with the ASK1 or the ASK2 clone. As a control, the same blot was also hybridized with the *Arabidopsis cab2* gene.

were hybridized with the ASK1 or the ASK2 probe. The results displayed in Fig. 4A show relatively simple genomic patterns for both cDNA clones. The ASK1 clone shows a strong hybridization signal only to a single 6.2 kb fragment when the genomic DNA was digested with *Hin*d III. The ASK2 clone shows a strong hybridization signal only to a single 4.1 kb fragment when the genomic DNA was digested with *Eco* RI. These results indicate that both clones are probably encoded by single-copy genes. In addition to the fragments that show strong hybridization signals, there are also weakly hybridizing fragments. The presence of these weakly hybridizing bands was more evident when the filters were overexposed (data not shown). Some of these weakly hybridizing fragments do not correspond to the sizes of any fragments hybridized strongly to the ASK1 or the ASK2 clone, indicating that there exist a few other related genes in the genome of *Arabidopsis thaliana*. There is a restriction fragment length polymorphism (RFLP) between the two *Arabidopsis* ecotypes (La-O and Col-O), when the *Eco* RI-digested genomic DNAs were probed with the ASK1 clone (Fig. 4B). This RFLP pattern found in the ASK1 gene may be used to identify the genomic location of this gene by RFLP mapping.

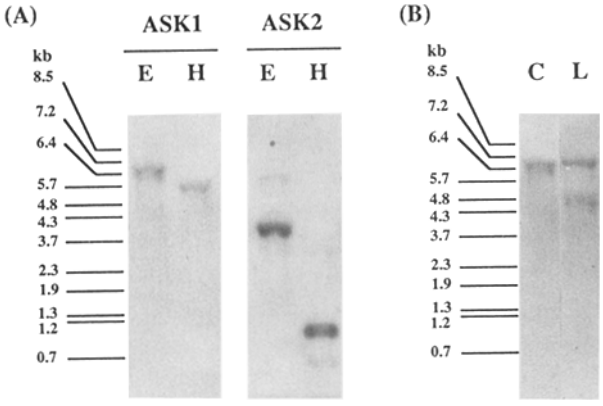

Fig. 4. Southern blot analyses of the ASK1 and ASK2 clones. A. Two μg of the *Arabidopsis* genomic DNA digested with *Eco* RI (E) or *Hin*d III (H) was separated on a 0.8% agarose gel. The DNA fragments were blotted onto a nylon filter and hybridized with the ASK1 or the ASK2 probe. B. The genomic DNA samples (2 μg) from the two *Arabidopsis* ecotypes, Col-O (C) and La-O (L), were digested with *Eco* RI (E). The filter was hybridized with the ASK1 probe. Note the restriction fragment length polymorphism (RFLP) between the two ecotypes.

Discussion

As a part of our effort to elucidate the possible roles of protein phosphorylation in plants, we

have isolated two novel protein kinase cDNA clones from *Arabidopsis thaliana* and have shown that these cDNA clones would encode two distinct plant protein-serine/threonine kinases. Although the true identity of these clones as that of protein kinase genes should be addressed by the biochemical approaches like expression of these clones in *Escherichia coli* and characterization of the protein products, the clones are likely to encode true protein kinases in *Arabidopsis*, when judged from the highly conserved structural features specific to the protein kinase family proteins.

The open reading frames of these two putative protein kinase genes are composed of a modular structure with the two distinctively identifiable domains, the catalytic domain and the flanking acidic domain. This modular feature is similar to the cases of many other protein kinase family proteins. Many of the protein kinases are composed of a divergent regulatory module attached to an evolutionary conserved catalytic domain and it is the divergent regulatory domain that binds to the effectors specific to a protein kinase [20]. Thus, the distinctive acidic module found in the C-terminus of the ASK1 and ASK2 genes may provide these protein kinase genes with unique functions and properties. In fact, among more than 200 protein kinase genes isolated so far mostly from organisms other than plants [23], there is no report on the protein kinases with such a distinctive acidic module in the regulatory region. The distinctive modular structure of these genes also suggests that these genes have evolved by the fusion of the gene fragments that encode the respective domains. It would be interesting to examine whether the two modular domains are interrupted by an intron in the genomic sequences to obtain insight into the evolution of these genes [9].

No simple clues regarding the function or regulation of the two putative protein kinases can be obtained from the similarity of the catalytic domain sequences of these protein kinases to those of the calcium/calmodulin-dependent protein kinases or the yeast SNF1 kinase subfamily. However, the homology of the acidic domains in these putative protein kinases to the acidic domain sequences of the rabbit calreticulin and the human ryanodine receptor is of a particular interest, since the acidic regions in these proteins have been suggested as a potential low affinity calcium-binding domain [8, 45] and since calcium has been known to play important roles in many plant cellular processes including activation of several protein kinases [3, 29]. Recently, similar motifs found in the 70 kDa heat shock protein of bean mitochondria have also been suggested as a low-affinity calcium-binding domain [41]. Interestingly, phosphorylation of this heat shock protein is strictly regulated in a calcium-dependent manner and *in vitro* phosphorylation of this protein requires at least 200 μM Ca^{2+}. It would be tempting to speculate that the acidic domain in these putative protein kinase genes we have isolated from *Arabidopsis* is also a low-affinity calcium-binding region and these protein kinases are modulated by cellular calcium at a relatively high concentration. This situation would be an interesting contrast to the soybean calcium-dependent protein kinase in which the catalytic domain is attached to a high affinity calcium-binding regulatory domain related to calmodulin and the protein kinase activity is regulated by a low concentration of Ca^{2+}. In any event, it is likely that this acidic region plays important roles in the function or regulation of these protein kinases, either by binding to specific ligands or by interacting with other proteins. The functional importance of this acidic module is further supported by the fact that this region is highly conserved between these two clones, while the region connecting the catalytic domain and this acidic region is diverged. We have recently isolated two protein kinase genes that are homologous to the *Arabidopsis* ASK1 gene from *Brassica napus* and have found that the acidic regions are also present in the open reading frames of these *B. napus* genes (unpublished results). Further studies on the gene product of the two *Arabidopsis* protein kinase genes will be needed to examine the functional identity of this distinctive acidic region.

Although the clues to the function or regulation of these genes may be obtained by a combination

of several approaches, one of the immediate approaches is to examine the expression patterns of these genes. Our Northern blot hybridization analysis shows that, although both genes are most highly expressed in leaf, the expression patterns of these two isoform protein kinase genes in each plant organ show some differences. The differential accumulation of the isoforms of the rabbit protein kinase C and of several pea protein kinase clones led to the suggestion that the differential accumulation of protein kinase isoforms may provide the protein kinases with specific functions in different cells or in different developmental stages [22, 27, 28]. The differences in the accumulation of the two transcripts encoding the two isoforms of the *Arabidopsis* protein kinase together with the differences in the primary structures of these isoform genes might also suggest different roles of these genes for plant cellular functions. As many leaf-expressed genes, expression of these genes is highly affected by the light regime. This observation suggests that these protein kinases might have a certain function related to the photoregulation of plant cells. Further detailed analyses on the regulation of these genes, including the kinetics of expression of these genes upon light treatment or plant hormone treatment, etc., will be necessary to gain more insight into the regulation and the function of these genes.

Molecular cloning of the genes that encode the plant protein kinases as described here provides the opportunities to study the specific functions and the properties of plant protein kinases. The transient or stable gene transfer experiments along with functional identification of the *in vivo* substrates of these protein kinases should help our understanding of the *in vivo* roles of the protein phosphorylation in plant cellular processes. We are in the process of generating transgenic *Arabidopsis* plants that express the sense or antisense RNA of these genes.

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