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Expression Cloning of a Variety of Novel Protein Kinases in *Lotus japonicus*

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To investigate protein kinases expressed in *Lotus japonicus*, a cDNA expression library of the root-nodule of *L. japonicus* was immunologically screened with monoclonal antibodies directed to a highly conserved region in protein serine/threonine kinases (Ser/Thr kinases). Among 178 positive clones obtained from the λZAPII cDNA library, 164 clones were found to encode novel proteins possessing the subdomain VIB sequences characteristic of Ser/Thr kinases. By phylogenetic analysis on the basis of deduced amino acid sequences, the isolated clones could be classified into five different families of Ser/Thr kinases : the SnRK family, GSK-3 family, Ndr kinase family, Ark family, and receptor kinase family. These results suggest that this expression cloning using the kinase-specific antibodies will provide new clues for investigations of a wide variety of known and novel protein kinases in higher plants.

Key words: catalytic domain, expression cloning, immunoscreening, *Lotus japonicus*, protein kinase.

Abbreviations: CaM-kinase, Ca^{2+}/calmodulin-dependent protein kinase; GSK-3, glycogen synthase kinase-3, PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; Ser/Thr kinase, protein serine/threonine kinase; Tyr kinase, protein tyrosine kinase.

Protein kinases are known to play pivotal roles in various cellular functions through the regulation of diverse signaling pathways. The eukaryotic protein kinases encompass large families of homologous proteins, comprising 1.5–2.5% of all gene products (*1*). The catalytic domains of protein kinases are composed of 250–300 amino acid residues that can be divided into 12 subdomains essential for kinase activity (*2*). In order to detect a wide variety of protein kinases expressed in various cells and tissues, we produced unique antibodies directed to the highly conserved region (subdomain VIB) of protein kinases (*3*, *4*). By using an expression cloning technique involving these kinase-specific antibodies, we isolated various known and novel protein kinases expressed in mouse brain (*3*) and *Xenopus laevis* embryos (*5*).

In plants, protein kinases have been reported to be involved in the adaptation to changing environmental conditions such as drought, temperature, light, salt, hormones, pathogens, or nutrient conditions (*6*). Therefore, higher plants are known to possess unique Ser/Thr kinases distinct from those found in most eukaryotes, while no plant protein tyrosine kinases (Tyr kinase) have been reported so far (*7*). In order to detect and isolate protein kinases expressed in *Lotus japonicus*, we screened for various Ser/Thr kinases using a root-nodule cDNA library. In this study, we isolated 178 cDNA clones with the aid of kinase-specific antibodies. On the basis of the deduced amino acid sequences of these clones, 164 cDNA clones could be classified as 15 different clones encoding previously unreported putative Ser/Thr kinases.

MATERIALS AND METHODS

Materials—The kinase-specific antibodies (M8C and M1C) were obtained from two hybridoma cell lines established as described previously (*3*). Goat anti–mouse IgG conjugated with horseradish peroxidase was obtained from ICN Pharmaceuticals. Other reagents were obtained from Wako Chemicals.

SDS–Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting—L. japonicus was separated into three parts: leaf and stem, root, and nodule. These tissues were mashed, suspended in 3 volumes of SDS sample buffer, and homogenized by sonication. The homogenates were centrifuged at 20,000 × *g* for 10 min, and the supernatants thus obtained were used as crude samples. The crude extract of rat brain was prepared essentially according to the method described previously (*8*). Protein concentrations were determined by the method of Bensadoun and Weinstein using bovine serum albumin as a standard (*9*). SDS-PAGE was carried out essentially according to the method of Laemmli (*10*) on slab gels consisting of a 10% acrylamide separation gel and a 3% stacking gel. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Protran BA85, Schleicher & Schuell), and Western blotting was carried out using the M8C monoclonal antibody as described previously (*3*).

Cloning of Protein Kinases Expressed in Root-Nodule of L. japonicus—A cDNA library was constructed with mRNA isolated from the root-nodule of *L. japonicus* using

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the λZAPII cDNA Synthesis kit (Stratagene). The *Escherichia coli* strain XL1-Blue MRF' was infected with recombinant phage and then plated (5×10^3 plaques/dish) in LB top agar on 9-cm plates. When small plaques appeared, the proteins expressed in the plaques were blotted onto nitrocellulose membranes (Protran BA85, Schreicher & Schuell) that had been saturated with 10 mM isopropyl β-D-thiogalactoside. The blot membranes were blocked with 5% skim milk (Difco) in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) at room temperature for 2 h, and then washed with PBS-T for 20 min. The membranes were incubated with a mixture of M8C and M1C (1:500 dilution) at room temperature for 2 h and washed three times with PBS-T. Then, the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase (1:1,000 dilution) at room temperature for 1 h, washed twice with PBS-T, and then twice with PBS. The positive plaques were visualized by the peroxidase reaction with diaminobenzidine (Sigma Chemicals). The cDNA clones thus obtained after the third screening were subcloned into pBluescript II SK(+) phagemid vector (Stratagene) by *in vivo* excision procedures. The cDNA inserts of the isolated clones were sequenced on an automated ABI PRISM 3100 DNA sequencer using a BigDye Terminator ver.3.1 cycle sequencing kit (Applied Biosystems). Nucleotide and deduced amino acid sequences of the positive clones were analyzed by BLAST homology search. Phylogenetic analysis of the predicted amino acid sequences of the putative protein kinases was performed with CLUSTAL W by the neighbor-joining method (*11*).

RESULTS

Western Blotting Analysis Using a Kinase-Specific Antibody—In the previous study, we produced unique monoclonal antibodies directed to a highly conserved region of protein kinases, and used them to detect a variety of protein kinases (*3*). To investigate protein kinases expressed in *L. japonicus*, Western blotting experiments were carried out using a kinase-specific antibody. When the crude extracts from different organs of *L. japonicus* were resolved on SDS-PAGE and analyzed by Western blotting using the M8C antibody, various immunoreactive bands were observed as shown in Fig. 1. The crude extracts from leaf and stem, root, and nodule of *L. japonicus* exhibited different immunostaining patterns, suggesting that unique immunoreactive proteins are expressed in these tissues. At least 20 positive bands with different mobilities on SDS-PAGE could be detected when the root-nodule extract was analyzed by Western blotting (Fig. 1, lane 4). These results suggest that various protein kinases or kinase-like proteins are expressed in the different organs of *L. japonicus*.

Expression Screening of Protein Kinases in Root-Nodule of L. japonicus—Since a variety of protein kinases appeared to be expressed in the root-nodule of *L. japonicus*, expression cloning of protein kinases was carried out. When a cDNA expression library of the nodule from *L. japonicus* was screened with the kinase-specific monoclonal antibodies, 178 positive clones were obtained from 3.6×10^5 plaques after the third screening. All the positive clones thus obtained were sequenced and analyzed by BLAST homology search. Among the 178 positive clones isolated, 164 clones were found to encode a variety of protein kinase–like proteins. On the basis of deduced amino acid sequences, these clones were found to be derived from 15 different genes (Table 1). Since the predicted amino acid sequences of these clones are not found in the database, they may encode novel protein kinases or kinase-like proteins that have not been reported previously. Accession numbers of the cDNA clones obtained in this study and the numbers of the clones isolated are shown in Table 1. Single clones for LNZ001, LNZ109, LNZ149, LNZ293, LNZ344, and LNZ404 were isolated in the present screening. In the case of LNZ031, in contrast,

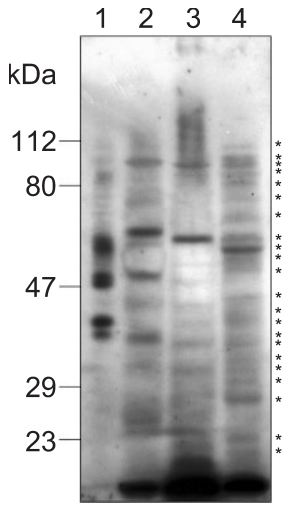

Fig. 1. **Western blotting analysis of crude extracts from *Lotus japonicus* by a kinase-specific antibody.** Crude extracts from rat brain (2 μg, lane 1), leaf and stem (30 μg, lane 2), root (30 μg, lane 3), and nodule (30 μg, lane 4) were electrophoresed in an SDS–polyacrylamide gel, transferred to a nitrocellulose membrane, and the proteins that immunoreacted with the M8C monoclonal antibody were detected as described in "MATERIALS AND METHODS." The immunoreactive protein bands observed in the nodule extract are indicated by asterisks on the right.

Table 1. **cDNA clones of putative protein kinases isolated from a *Lotus japonicus* nodule cDNA library.**

Clone	Number of clones[a]	Accession No.[b]
LNZ001	1	AB115547
LNZ002	23	AB113570
LNZ003	5	AB113571
LNZ016	17	AB113572
LNZ019	29	AB115548
LNZ020	23	AB113573
LNZ021	7	AB115549
LNZ024	8	AB115550
LNZ031	33	AB113574
LNZ109	1	AB184970
LNZ149	1	AB184971
LNZ215	12	AB184972
LNZ293	1	AB184973
LNZ344	1	AB184974
LNZ404	1	AB184975

[a]Total number of cDNA clones isolated in this study. [b]Accession numbers obtained in this study.

Fig. 2. **Sequence analysis of the LNZ016 gene.** (A) Nucleotide and deduced amino acid sequences of LNZ016. The predicted open reading frame of 448 amino acids is shown under the nucleotide sequence. The underlined sequence represents the possible recognition site for the kinase-specific antibodies used for the present immunoscreening. (B) Alignment of the deduced amino acid sequence of LNZ016 (Accession no. AB113572) with that of a putative Ser/Thr kinase from *Arabidopsis thaliana* (Accession no. BT000958) and the SOS2 kinase from *Arabidopsis thaliana* (Accession no. BT002138). Identical amino acids are shaded in black. Twelve subdomains specific to protein kinases (*2*) are underlined.

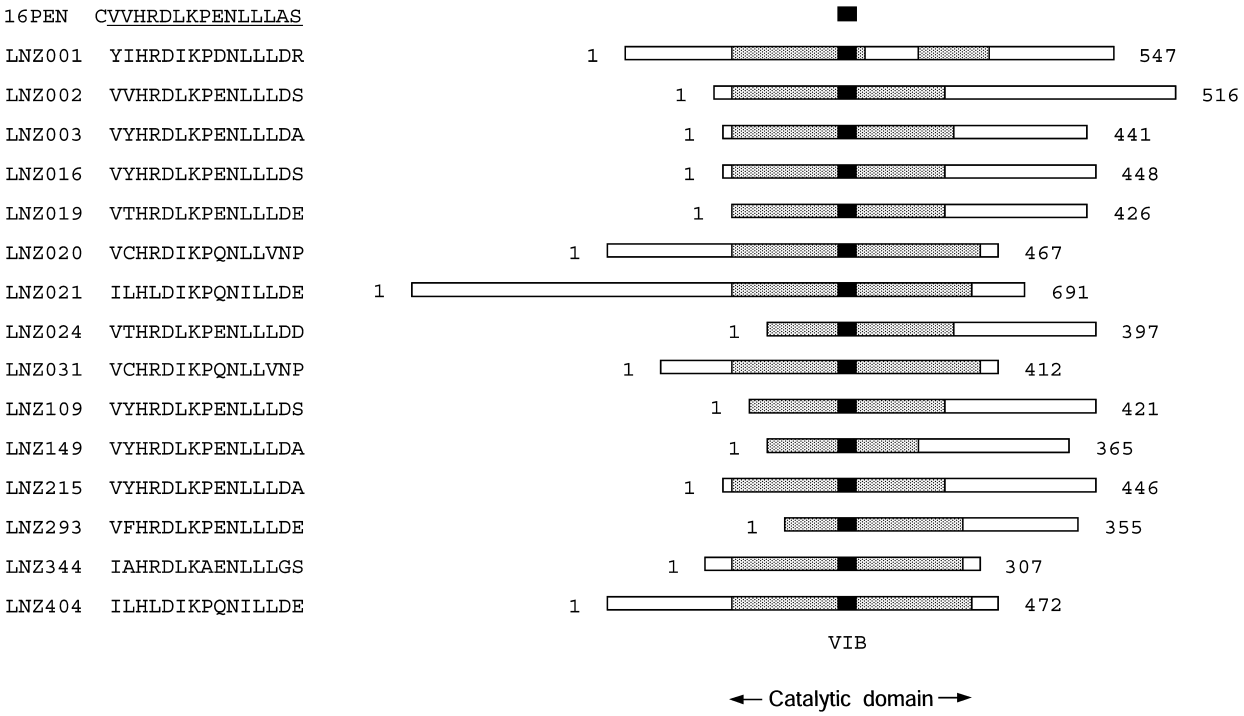

Fig. 3. **Amino acid sequences and positions of the subdomain VIB in the putative protein kinases.** 16PEN is the peptide used to generate the kinase-specific monoclonal antibodies (*3*). The amino acid sequences of the subdomains VIB of the putative kinases are shown on the left. The positions of subdomain VIB and the catalytic domain of the putative protein kinases are shown by the filled boxes and gray boxes, respectively.

as many as 33 clones with different insert sizes appeared to originate from the same mRNA. More than ten positive clones corresponding to LNZ002, LNZ016, LNZ019, LNZ020, and LNZ215 were also obtained in this study (Table 1). These differences in the number of positive clones isolated from the library may reflect the relative abundance of the transcripts of the putative kinases in the root-nodule of *L. japonicus*.

Sequence Analysis of LNZ016—In the case of LNZ016, 17 clones with cDNA inserts ranging from 0.8–1.6 kb were isolated (Table 1). The whole nucleotide sequence of this clone and its deduced amino acid sequence are shown in Fig. 2A. The open reading frame encodes a polypeptide consisting of 448 amino acid residues with a predicted molecular weight of 50,958. An antigenic recognition sequence, VYHRDLKPENLLLDS, which corresponds to the subdomain VIB of protein kinases, was found in the sequence (Fig. 2A, underlined). When BLAST homology search was carried out, the highest identity was found with a putative Ser/Thr kinase protein from *Arabidopsis thaliana* (80% identity, Accession no. BT000958). LNZ016 also showed relatively high homology with the SOS2 protein of *A. thaliana* (61% identity, Accession no. BT002138). An alignment of these three proteins is shown in Fig. 2B. All three proteins possess the 12 highly conserved subdomains characteristic of the protein kinase catalytic domain (*2*), suggesting that LNZ016 is also a member of the Ser/Thr kinase family.

Subdomain VIB of Protein Kinases—The monoclonal antibodies (M8C and M1C) used for the present immunoscreening were generated by immunizing a peptide, 16PEN (CVVHRDLKPENLLLAS), that corresponds to the subdomain VIB of rat Ca^{2+}/calmodulin-dependent protein kinase IIα (CaM-kinase IIα) (*3*). The deduced amino acid sequences of all the clones listed in Table 1 were found to possess the antigenic sequences corresponding to the subdomain VIB of protein kinases. The amino acid sequences of the possible antigenic sequences of these proteins are shown in Fig. 3. As shown in the left panel of Fig. 3, the core sequences of the subdomain VIB in these proteins are found to be composed of ten amino acid residues, H-R/L-D-L/I-K-P/A-E/D/Q-N-L/I-L. The positions of the subdomain VIB in the primary sequences of these proteins are also shown. The putative protein kinases shown in Fig. 3, however, exhibit a quite different molecular architecture; LNZ021 has a long N-terminal domain, while LNZ002 possesses a long C-terminal sequence as compared to other protein kinases. The N-terminal or C-terminal extra sequences outside the catalytic domain of putative kinases may be essential for substrate recognition, subcellular localization, and/or regulatory functions. These results indicate that various types of Ser/Thr kinases can be obtained by the present immunoscreening with the kinase-specific antibodies.

Phylogenetic Analysis of the Putative Ser / Thr Kinases—The phylogenetic relationships of the putative Ser/Thr kinases obtained in the present study were analyzed. As shown in Fig. 4, the predicted kinases can be classified into five major groups on the basis of the neighbor-joining tree. The SnRK family is a group of plant protein kinases with a catalytic domain similar to that of SNF1 of yeast and AMP-dependent protein kinase of animals (*12*). The predicted kinases for LNZ003, LNZ215, and LNZ293 are homologues of SNFL1 (Accession no. Y12464), SOS2 pro-

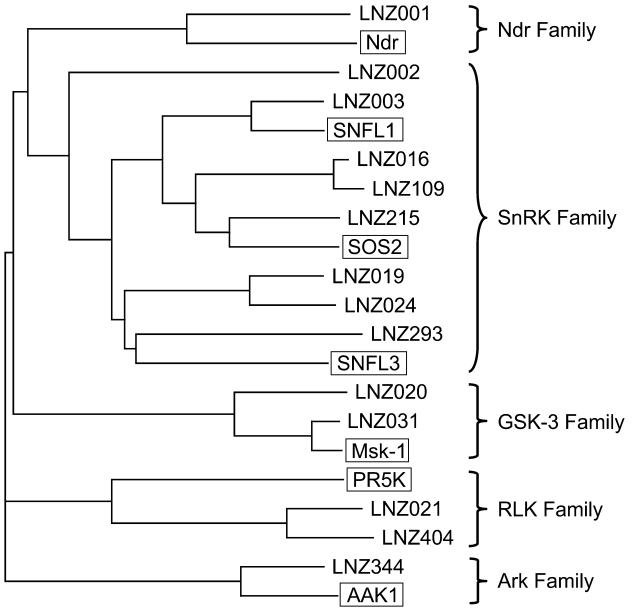

Fig. 4. **Phylogenetic analysis of putative Ser/Thr kinases.** The phylogenetic relationships between the putative protein kinases are represented by a neighbor-joining tree on the basis of the deduced amino acid sequences corresponding to the entire coding regions of the corresponding cDNA clones. Closely related kinases from other plants are also shown in the phylogenetic tree in boxes: Ndr, the Ndr kinase from *Arabidopsis thaliana* (Accession no. AB047278); SNFL1, the SNFL1 protein kinase from *Sorghum bicolor* (Accession no. Y12464); SOS2, the SOS2 protein from *Arabidopsis thaliana* (Accession no. BT002138); SNFL3, a probable Ser/Thr kinase from *Sorghum bicolor* (Accession no. Y14274); Msk-1, a GSK-3 like protein from *Medicago sativa* (Accession no. X68411); PR5K, the receptor Ser/Thr kinase PR5K from *Arabidopsis thaliana* (Accession no. U48698); AAK1, a putative AAK1 protein from *Oryza sativa* (Accession no. AP005874). The putative protein kinases can be grouped into five families as shown on the right.

tein (Accession no. BT002138), and SNFL3 (Accession no. Y14274), respectively, and all belong to the SnRK family. These protein kinases are closely related to the Ca^{2+}-dependent protein kinases (CDPK) (*13*). LNZ020 and LNZ031 are related to a GSK-3–like putative protein (Accession no. AJ295939) and GSK-3 homologue Msk-1 (Accession no. X68411), respectively, and are members of the GSK-3 family. The predicted proteins for LNZ021 and LNZ404 appear to be closely related to receptor Ser/Thr kinase PR5K (Accession no. U48698) (*14*, *15*). Although LNZ021 and LNZ404 lack the initiation codon of the open reading frame, the predicted protein possesses a long N-terminal extension for ligand binding, which is a characteristic feature for RLK family proteins. Predicted protein kinases for LNZ001 and LNZ344 belong to the Ndr family and Ark family, respectively.

Lotus Ser/Thr Kinases with Unique Catalytic Domains—LNZ001 encodes a protein exhibiting 62% amino acid identity to the Ndr kinase of *A. thaliana* (Accession no. AB047278). The Ndr family of Ser/Thr kinases is widely distributed from human to plants, and an alignment of these family proteins is shown in Fig. 5A. The LNZ001 kinase contains a long insert between subdomain VII and subdomain VIII as a unique structural feature (Fig. 5A). A characteristic feature of members of the Ndr kinase family is an insert of about 30–60 amino acids located in this region, but the function of this long insert is not well understood. Therefore, LNZ001 appears to encode a homologue of Ndr kinase in *L. japonicus*.

LNZ344 encodes a putative Ser/Thr kinase with 307 amino acids including 12 subdomains. In general, Ser/Thr kinases possess the typical consensus sequence G-x-G-x-x-G/S/A as the ATP-binding motif in subdomain I (*2*). On the other hand, Ark family members have their own consensus sequence, S/E-G-G-F-S-x-V-Y, in the subdomain I region (*16*). As shown in Fig. 5B, the deduced amino acid sequence of the subdomain I in the LNZ344 was found to be E-G-G-F-S-C-V-Y, suggesting that the putative kinase is a member of the Ark family. The predicted amino acid sequence of LNZ344 shows 76% identity with the putative Ser/Thr kinase AAK1 of *Oryza sativa* (Accession no. AP005874).

DISCUSSION

Western blotting analysis with the kinase-specific antibodies was used to detect various positive bands in crude extracts from *L. japonicus* (Fig. 1). It is not clear, however, whether the immunoreactive bands observed by Western blotting are protein kinases and/or their degradation products. To validate the specificity of our monoclonal antibodies, we attempted to immunoscreen protein kinases expressed in *L. japonicus*. In this study, 178 positive clones were isolated from a *L. japonicus* λ phage cDNA library by means of expression screening with the monoclonal antibodies (M8C and M1C) directed to a highly conserved region in protein kinases. Among the positive clones obtained, 164 were found to encode putative Ser/Thr kinases or kinase-like proteins, and these cDNAs were attributed to 15 different genes that have not been reported previously (Table 1). These results indicate that more than 92% of the immunoreactive proteins detected with our antibodies were identified as protein kinases or kinase-like proteins.

Phylogenetic analysis suggests that the putative kinases obtained in this study can be classified into five different families of Ser/Thr kinases (Fig. 4): RLK family kinases are membrane-bound receptor kinases that transduce extracellular stimuli to intracellular signals (*15*). SnRK family kinases appear to function in cellular responses to environmental conditions via the regulation of key metabolic pathways (*12*). The Ndr family of Ser/Thr kinases is believed to be involved in the control of cell division and morphogenesis (*17*). GSK-3 family kinases are multifunctional Ser/Thr kinases that are involved in the regulation of cell cycle, development, stress and hormone signaling (*18*). The characteristic properties and physiological functions of plant AAK1, which belongs to the Ark family, are not known yet. Taken together, the present expression cloning was found to be a very useful technique for isolating a wide variety of known and novel Ser/Thr kinases with different physiological functions.

The monoclonal antibodies used for immunoscreening were raised against a peptide antigen corresponding to the amino acid sequence of the subdomain VIB of rat CaM-kinase IIα (*3*). The amino acid sequences of the subdomain VIB in the putative kinases isolated in this study are aligned and shown in Fig. 6B. The amino acid

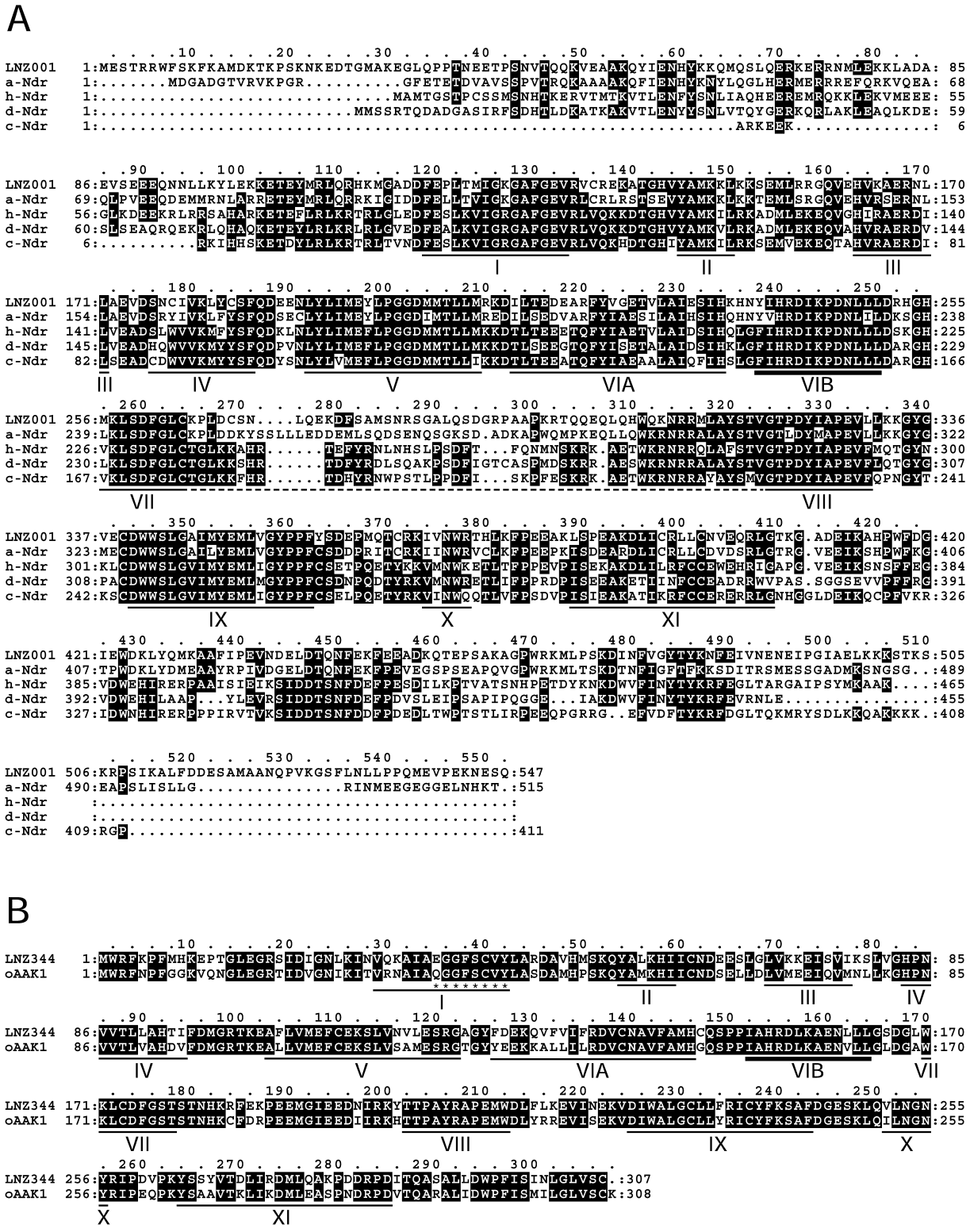

Fig. 5. **Lotus protein kinases with unique catalytic domains.** (A) Alignment of the deduced amino acid sequence of LNZ001 with other Ndr family members. The deduced amino acid sequence of LNZ001 was aligned with the sequences of Ndr kinases from *Arabidopsis thaliana* (a-Ndr), human (h-Ndr), Drosophila (d-Ndr), and *C. elegans* (c-Ndr). The broken underline shows the long insert between the subdomain VII and the subdomain VIII. (B) Alignment of the deduced amino acid sequence of LNZ344 with AAK1 from *Oryza sativa*. Asterisks represent the unique amino acid sequence of subdomain I that is characteristic to Ark family. Twelve subdomains characteristic of protein kinases are underlined. Identical amino acids are shaded in black.

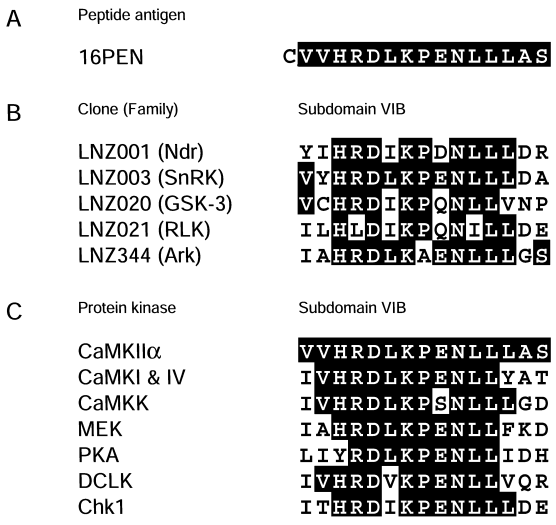

Fig. 6. **Alignment of the subdomain VIB region of Ser/Thr kinases detected by kinase-specific antibodies.** (A) Amino acid sequence of 16PEN, an antigenic peptide used for the production of the kinase-specific antibodies. (B) Subdomain VIB of the putative Ser/Thr kinases isolated in the present study. Representative protein kinases of the five families are shown. Protein kinase homologues from other plants are shown in parentheses. (C) Subdomain VIB of protein kinases isolated by the expression screening of mouse brain (3) and *Xenopus* embryo (4) cDNA libraries. Identical amino acids are shaded in black. CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; MEK, MAP kinase and ERK kinase; PKA, cAMP-dependent protein kinase; DCLK, doublecortin-like kinase; Chk, checkpoint kinase.

sequences of this region could be classified into five groups coinciding with the Ser/Thr kinase families shown by the phylogenetic analysis in Fig. 4. Five amino acids in the subdomain VIB, H-x-D-x-K-x-x-N-x-L, were conserved in all the proteins. In our previous expression screening using cDNA libraries from mouse brain (*3*) and *Xenopus* embryos (*5*), we isolated cDNA clones for various Ser/Thr kinases including CaM-kinase I, II, IV, CaM-kinase kinase, MEK, cAMP-dependent protein kinase, and checkpoint kinases. The amino acid sequences of the subdomain VIB in these kinases are also shown in Fig. 6C. Consensus sequence for recognition by the kinase-specific antibodies was found to be H/Y-R/L-D-L/V/I-K-P/A-E/D/Q/S-N-L/I-L. These results, taken together, suggest that a variety of Ser/Thr kinases, but not Tyr kinases, can be detected with these antibodies. Although many animal receptor kinases have been identified as Tyr kinases, all the receptor kinases from plants reported so far are Ser/Thr kinases (*15*). Therefore, kinase-specific monoclonal antibodies can serve as a powerful tool for the investigation of a wide range of protein kinases involved in cellular signaling pathways in higher plants.

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