J. Biochem. 2010;147(6):799–807 doi:10.1093/jb/mvq011

JB THE JOURNAL OF BIOCHEMISTRY

# Expression and characterization of PKL01, an Ndr kinase homolog in *Lotus japonicus*

*Received January 19, 2010; accepted January 22, 2010; published online February 4, 2010*

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**We isolated cDNA clones for novel protein kinases by expression cloning from *Lotus japonicus*. The LNZ001, one of the isolated clones, encodes a protein of 547 amino acids with a predicted molecular weight of 63,349. Since the protein contains 12 highly conserved subdomains specific to Ser/Thr protein kinases, we designated it PKL01. When homology searches based on the PKL01 sequence were carried out, the protein was found to show sequence homology with nuclear Dbf2-related kinases (Ndr kinases). When PKL01 was produced using an *Escherichia coli* expression system and purified to homogeneity, it underwent intermolecular autophosphorylation. The major autophosphorylation site was identified as Ser-317 by using various point mutants, and phosphorylation at this site was found to be critical for the kinase activity. PKL01 was found to be widely distributed in the leaves, stems, roots and root nodules by northern hybridization experiments. When endogenous substrates were screened using fractionated preparations from various parts of plants, PKL01 preferentially phosphorylated basic proteins in tissue extracts. These results suggest that PKL01 is an Ndr kinase homolog in *L. japonicus* and may be involved in the regulation of cellular functions through phosphorylation of basic protein substrates such as histones.**

*Keywords*: autophosphorylation/histone/*Lotus japonicus*/Ndr kinase/protein phosphorylation.

*Abbreviations*: GST, glutathione S-transferase; MBP, myelin basic protein; Ndr kinase, nuclear Dbf2-related kinase.

Protein kinases play pivotal roles in various signaling pathways in animals, plants and microorganisms. The eukaryotic protein kinases consist of 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 developed a unique expression cloning technique to isolate cDNA clones for various protein kinases using kinase-specific monoclonal antibodies (multi-PK antibodies) (*3*). Using this technique, we have isolated various known and novel protein kinases expressed in animals (*3*, *4*), plants (*5*) and mushrooms (*6*, *7*).

In a previous study, we isolated 15 different cDNA clones for novel protein kinases using a cDNA library from root nodules of *Lotus japonicus* (*5*). However, the characteristic properties and functional roles of these protein kinases remained unknown. In the present study, we attempted to characterize the protein product of LNZ001, one of the cDNA clones isolated from *L. japonicus*, and designated the protein PKL01. PKL01 showed sequence homology with the sequences of nuclear Dbf2-related kinases (Ndr kinases) in animals (*8*), yeast (*9*) and plants (*10*, *11*). These kinases are believed to be involved in regulating the cell growth, cell cycle and cell morphology in animals and yeast (*12*, *13*). However, the detailed properties and functional roles of Ndr kinases in plants have not yet been reported. Furthermore, the downstream targets for Ndr kinases and the effects of phosphorylation of their physiological substrates are still unclear.

The precise mechanisms of the regulation of Ndr kinases by phosphorylation and regulatory proteins have mainly been studied in mammalian systems (*12*, *14*). In the present study, we expressed PKL01 protein in *Escherichia coli*, purified it to homogeneity and characterized its molecular properties. We found that PKL01 undergoes intermolecular autophosphorylation and that this phosphorylation is important for its catalytic activity. We further found that PKL01 phosphorylates basic proteins including histones as possible endogenous substrates.

## Materials and Methods

### Materials

[$\gamma$-$^{32}$P]ATP (111 TBq/mmol) was purchased from PerkinElmer. ATP, myelin basic protein (MBP), bovine serum albumin and histone type IIA were obtained from Sigma Chemicals. pGEX-6P-1, HiTrap Chelating HP, glutathione-Sepharose 4B and PreScission Protease were obtained from GE Healthcare. pET-23a(+) and *E. coli* BL21(DE3) were purchased from Novagen. Goat anti-mouse IgA+IgG+IgM conjugated with horseradish peroxidase was obtained from ICN Pharmaceuticals. An anti-$His_6$ antibody was

purchased from Roche Diagnostics. A polyclonal antibody against histone H3 was obtained from active motif. Multi-PK antibodies, M1C and M8C, were prepared as described previously (*3*).

### *Plasmid construction, expression and purification of PKL01 and its mutants*

The sequence of the PKL01 cDNA (accession no. AB115547) was described previously (*5*). The cDNA was cloned into pET-23a(+) (Novagen) and the resulting plasmid was designated pET-PKL01(WT). To obtain glutathione S-transferase (GST)-PKL01 carrying a $His_6$ tag at the C-terminal end, PCR was performed with a sense primer (5′-AAAGAATTCATGGAAAGTACAAGG CGCTGG-3′) and an antisense primer (5′-TTTGTCGACTCAGT GGTGGTGGTGGTGGTG-3′) using Pyrobest DNA polymerase (TaKaRa Bio) and pET-PKL01(WT) as a template. The obtained PCR fragment was digested with EcoRI and SalI, subcloned into the EcoRI/SalI sites of pGEX-6P-1 (GE Healthcare) and designated pGEX-PKL01(WT)-$His_6$. Site-directed mutagenesis was performed on this vector by inverse PCR (*15*). The fidelities of the final expression constructs were confirmed by DNA sequencing.

cDNAs carrying GST-fused PKL01 and its point mutants were introduced into *E. coli* BL21(DE3), and expression of the recombinant proteins was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 6 h. The *E. coli* pellets containing GST fusion proteins were lysed with 20 mM Tris–HCl (pH 7.5) containing 150 mM NaCl and 0.05% Tween-40, followed by purification with a glutathione–Sepharose 4B column (1 ml) according to the manufacturer's protocol. Full-length PKL01 without GST was prepared by cleavage of GST-PKL01 with PreScission protease.

### *RNA extraction and northern hybridization*

Northern blotting was performed essentially as described previously (*16*). For RNA extraction, each tissue sample was frozen, ground to a fine powder under liquid nitrogen and subjected to total RNA isolation using an RNeasy Plant Mini Kit (Qiagen). The total RNA was separated by electrophoresis, transferred to a nylon membrane (Hybond $N^+$; GE Healthcare) and hybridized with a digoxigenin-labelled antisense RNA probe. As a template for RNA probe, a 529-bp fragment of PKL01 was amplified by PCR using a sense primer (5′-TGTTCCTTTCAAGATGAAGAGAATT-3′) and an antisense primer (5′-AATAAAGGGCGGATACCCGACA AG-3′) and subcloned into pGEM-T Easy (Promega). A digoxigenin-labeled antisense probe was synthesized using the DIG Northern Starter Kit (Roche).

### *Preparation of crude extracts and extraction of histones from L. japonicus*

Tissues from *L. japonicus* were separated into four parts: leaves, stems, roots and root nodules. Each tissue (fresh weight, 1 g) was homogenized by grinding with a mortar and pestle after adding a plant homogenization buffer [50 mM Hepes–KOH pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 5% polyvinylpolypyrrolidone (PVPP), 5 mM dithiothreitol, 0.01 mM leupeptin]. The homogenates were centrifuged at 20,000*g* at 2°C for 10 min, and the supernatants were used as crude extracts of *L. japonicus*. The crude extracts were aliquoted and stored at −30°C until use.

Mixed histones were prepared from *L. japonicus* by an acid extraction method. Briefly, each plant tissue (fresh weight, 0.25 g) was mixed with 1 ml of the plant homogenization buffer and homogenized by grinding with a mortar and pestle. The homogenate was centrifuged at 400*g* at 2°C for 10 min, and the supernatant was removed. Plant homogenization buffer without PVPP was then added to the precipitate. The precipitate was suspended by vortexing and centrifuged at 400*g* at 2°C for 10 min, followed by removal of the supernatant. These steps were repeated until the supernatant became clear. Next, 1 ml of 0.2 N HCl was added to the final precipitate and mixed overnight at 2°C to extract histones. The resulting mixture was centrifuged at 400*g* at 2°C for 10 min, and the supernatant was pooled. Next, six volumes of acetone were added to the pooled supernatants and incubated in a stationary manner at 2°C. The mixture was centrifuged at 20,000*g* at 2°C for 10 min, and the obtained precipitate was washed with acetone. The precipitate was dissolved in 200 µl of phosphate-buffered saline containing 0.05% Tween-20 by sonication, and the mixed histones thus obtained were stored at −30°C until use.

### *Fractionation of crude extracts using a MicroRotofor*

Isoelectric focusing with a MicroRotofor (Bio-Rad Laboratories) was carried out essentially as described previously (*17*). Crude extracts (2.5 mg) from *L. japonicus* were dissolved in 2.5 ml of isoelectric focusing buffer (7 M urea, 2 M thiourea, 5 mM dithiothreitol, 4% Chaps, 2% Pharmalyte, pH 3–10), applied to the MicroRotofor and electrophoresed for 2.5 h under a constant power of 1 W at 20°C. After the electrophoresis, the protein fractions from each compartment (200 µl) were harvested and an equal volume of 20% trichloroacetic acid was added. The precipitated proteins were collected by centrifugation at 20,000*g* for 10 min at room temperature. The precipitates were washed twice with cold acetone to remove any residual trichloroacetic acid. After air-drying, the precipitates were dissolved in 50 µl of phosphate-buffered saline containing 0.05% Tween-20 by sonication. The protein fractions thus obtained were used as substrates for PKL01.

### *Protein determination, SDS–PAGE and western blotting*

Protein concentrations were determined by the method of Bensadoun and Weinstein (*18*) using bovine serum albumin as a standard. SDS–PAGE was performed essentially according to the method of Laemmli (*19*) in slab gels consisting of a 12 or 15% acrylamide separation gel and a 3% acrylamide stacking gel. The resolved proteins were stained with Coomassie brilliant blue or analyzed by autoradiography or western blotting. Western blotting analysis was carried out essentially as described previously (*4*).

### *Protein kinase assay*

Autophosphorylation of PKL01 was carried out using a standard reaction mixture (10 µl) consisting of 40 mM Hepes–NaOH (pH 8.0), 5 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 2 mM dithiothreitol, 100 µM [$\gamma$-$^{32}$P]ATP and 200 ng of PKL01/GST-PKL01. For experiments involving phosphorylation of protein substrates, an appropriate amount of the protein substrate was incubated with 200 ng of PKL01/GST-PKL01 under the standard phosphorylation conditions described above. The reaction was started by the addition of PKL01 or its mutants and incubated at 30°C. After incubation for 30 min, an equal volume of 2× SDS sample buffer was added to the phosphorylation mixture to stop the reaction. The phosphorylated proteins were resolved by SDS–PAGE and analyzed by autoradiography.

## Results

### *Molecular cloning of PKL01*

In a previous study, we isolated 15 different cDNA clones for Ser/Thr protein kinases of *L. japonicus* by means of expression cloning using a root nodule cDNA library (*5*). Since LNZ001 appeared to contain the full-length sequence of an open reading frame of a novel Ser/Thr protein kinase, we focused on this gene and attempted to characterize its protein product. LNZ001 encodes a protein of 547 amino acids with a predicted molecular weight of 63,349 and contains 12 highly conserved subdomains specific to protein kinases. Since the amino acid sequence of this protein meets the criteria for a Ser/Thr protein kinase, we designated it PKL01. When homology searches based on the PKL01 sequence were carried out, Ndr kinases from various sources were found to show high homology with PKL01. An alignment of PKL01 with *Arabidopsis thaliana* Ndr kinase (accession no. AB047278), radish Ndr kinase (accession no. AB105045) (*10*) and human Ndr kinase NDR1 (accession no. NM_007271) (*8*) is shown in Fig. 1A. The amino acid sequence of PKL01 showed 56, 52 and 39% sequence identities with those of *A. thaliana* Ndr kinase, radish Ndr kinase and human Ndr kinase, respectively. Furthermore, PKL01 has a long insertion sequence between subdomains VII and VIII,

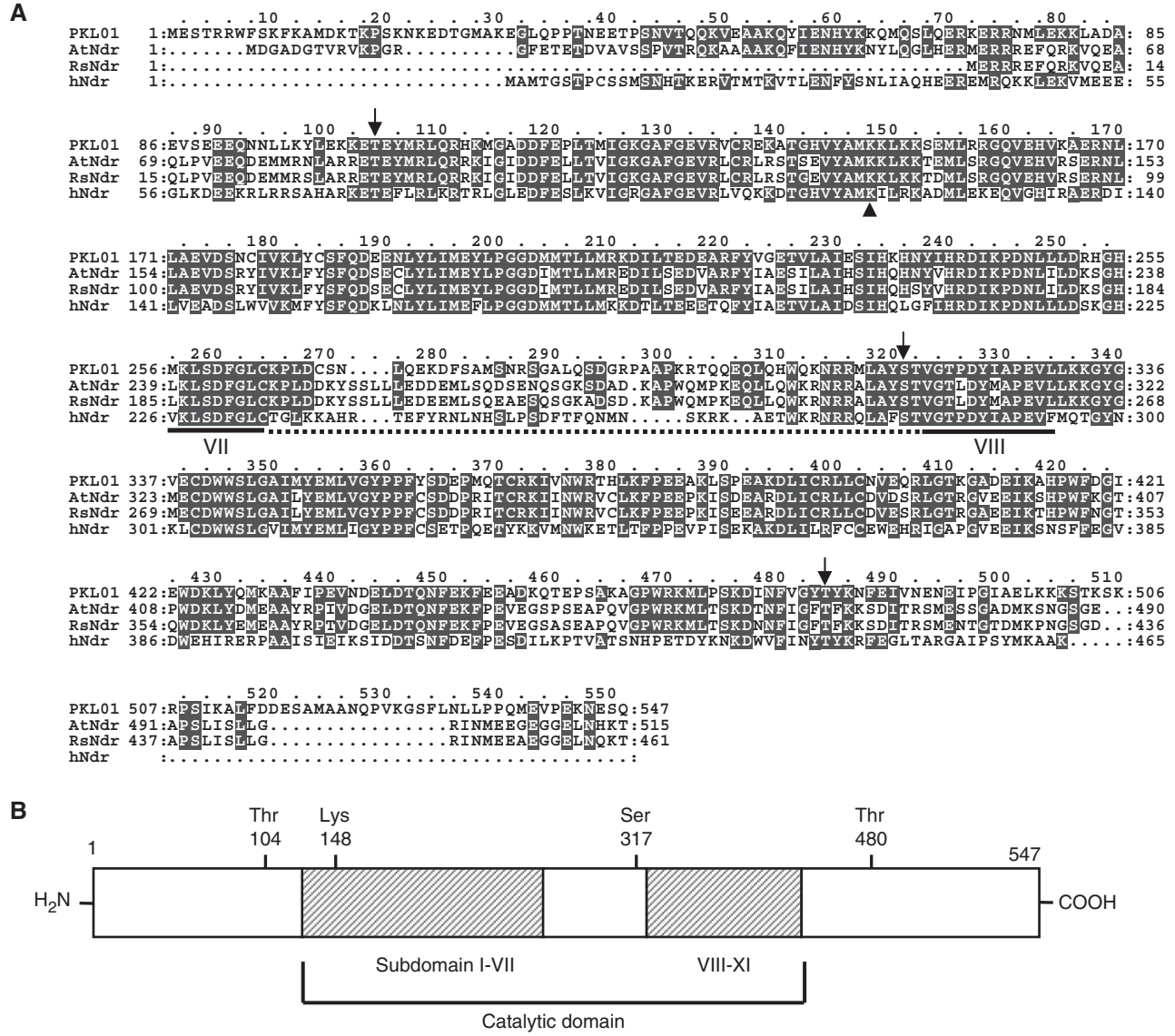


**Fig. 1 Primary structure of PKL01.** (A) The amino acid sequence of PKL01 was aligned with those of *A. thaliana* Ndr kinase (accession no. AB047278), radish Ndr kinase (accession no. AB105045) and human NDR1 (accession no. NM_007271) using CLUSTAL W. Identical amino acids are shaded in black, and gaps inserted into the sequences are indicated by dots. Putative phosphorylation sites are shown by arrows and the lysine residue at the ATP-binding site is indicated by an arrowhead. The long insertion sequence between subdomains VII and VIII is shown by broken underline. (B) Schematic illustration of the primary structure of PKL01. The conserved serine and threonine residues at the putative phosphorylation sites and the lysine residue at the ATP-binding site are shown.

which is characteristic of Ndr kinase family enzymes (Fig. 1A, broken underline). To date, three possible regulatory phosphorylation sites for human Ndr kinases have been reported, namely Thr-74, Ser-281 and Thr-444 in human NDR1 (*14*). All of these three phosphorylation sites are conserved in PKL01, as Thr-104, Ser-317 and Thr-480, respectively (Fig. 1A and B).

### *Expression of PKL01 in E. coli*

When PKL01 was expressed as a fusion protein with GST in an *E. coli* expression system, GST-PKL01 could be retrieved as a soluble form (Fig. 2A, lane 2). GST-PKL01 was loaded onto a glutathione–Sepharose column, washed with equilibration buffer and treated with PreScission protease to cleave the GST from PKL01. Purified PKL01 was recovered in the flow-through fractions from the glutathione–Sepharose (Fig. 2A, lane 4). Purified PKL01 was detected as a single band of ~70 kDa on SDS–PAGE and western blotting with a multi-PK antibody and anti-$His_6$ antibody (Fig. 2B, lanes 5 and 6).

### *Mechanism of PKL01 autophosphorylation*

When PKL01 was incubated with [$\gamma$-$^{32}$P]ATP under the phosphorylation conditions without an exogenous substrate, time-dependent [$^{32}$P]phosphate incorporation into PKL01 was observed (Fig. 3A, upper panel). In contrast, PKL01(K148A), in which Lys-148 at the ATP-binding site was replaced with Ala, showed no [$^{32}$P]phosphate incorporation even after

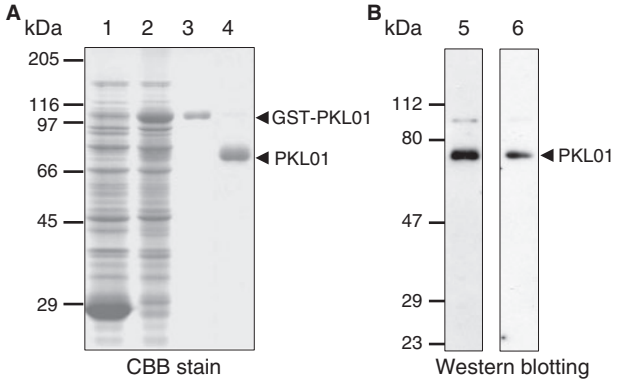


**Fig. 2 Expression and purification of recombinant PKL01 in *E. coli*.** PKL01 was expressed as a fusion protein with GST at the N-terminus and a $His_6$-tag at the C-terminus, and purified using an affinity column. (A) Coomassie brilliant blue staining pattern of PKL01. Lane 1, crude extract of *E. coli* transfected with pGEX-6P-1 (2.5 μl); lane 2, crude extract of *E. coli* transfected with pGEX-PKL01-His (2.5 μl); lane 3, glutathione–Sepharose-bound fraction (GST-PKL01) (1 μg); lane 4, flow-through fraction (1 μg) from glutathione–Sepharose after PreScission protease digestion. (B) Western blotting analysis of PKL01. Purified PKL01 (50 ng) was electrophoresed in an SDS–polyacrylamide gel and detected by western blotting with the multi-PK antibody M1C (lane 5) and an anti-$His_6$ antibody (lane 6).

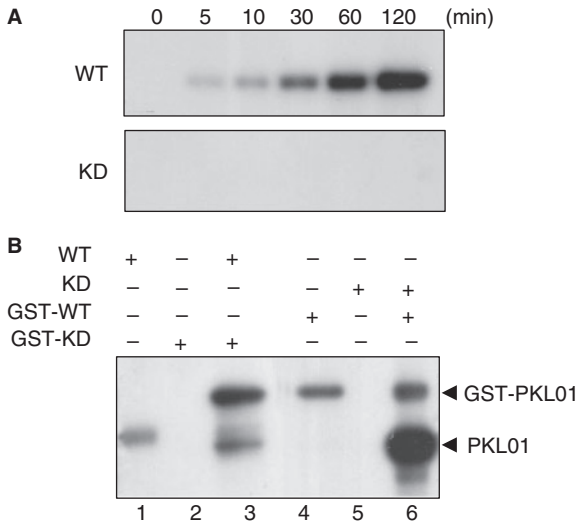


**Fig. 3 Analysis of PKL01 autophosphorylation.** (A) Purified PKL01 or PKL01(K148A) was phosphorylated with [γ-$^{32}$P]ATP at 30°C under the standard phosphorylation conditions. After incubation for the indicated times, aliquots were withdrawn and electrophoresed in a SDS–polyacrylamide gel for detection of their [$^{32}$P]phosphate incorporation by autoradiography. KD indicates the kinase-dead mutant, namely PKL01(K148A). (B) Mechanism of PKL01 autophosphorylation. Aliquots (200 ng) of active kinases PKL01 and GST-PKL01, and kinase-dead PKL01, PKL01(K148A) and GST-PKL01(K148A) mutants were incubated alone or in the indicated combinations with [γ-$^{32}$P]ATP under the standard phosphorylation conditions at 30°C for 60 min. [$^{32}$P]Phosphate incorporation into PKL01 or its mutants was detected by SDS–PAGE followed by autoradiography. The migration positions of GST-PKL01 and PKL01 are shown on the right.

120 min of incubation (Fig. 3A, lower panel), indicating that PKL01(K148A) is an inactive mutant (kinase-dead PKL01). There are two possible mechanisms for autophosphorylation of the protein kinase, namely an intermolecular reaction or an intramolecular reaction. To distinguish these two mechanisms, we produced both active and inactive (kinase-dead) forms of PKL01 and GST-PKL01 and carried out phosphorylation experiments for various combinations. PKL01 and GST-PKL01 each underwent autophosphorylation when they were individually incubated under the phosphorylation conditions (Fig. 3B, lanes 1 and 4). In contrast, PKL01(K148A) and GST-PKL01(K148A) showed no autophosphorylation activity (Fig. 3B, lanes 2 and 5), indicating that both of these mutants are inactive kinases. When kinase-dead GST-PKL01(K148A) was incubated with PKL01, [$^{32}$P]phosphate was incorporated into not only PKL01 but also GST-PKL01(K148A) (Fig. 3B, lane 3), suggesting that PKL01 efficiently phosphorylated kinase-dead GST-PKL01(K148A). Conversely, kinase-dead PKL01(K148A) was significantly phosphorylated by active GST-PKL01 (Fig. 3B, lane 6). These results suggest that autophosphorylation of PKL01 occurs through intermolecular phosphorylation.

### *Effects of point mutations at the predicted phosphorylation sites in PKL01*

A previous study reported that there are three predicted phosphorylation sites in mammalian Ndr kinases (*14*). All of these three phosphorylation sites are conserved in PKL01, as Thr-104, Ser-317 and Thr-480 (Fig. 1B). We prepared point mutants with amino acid substitutions at these phosphorylation sites, and compared their catalytic properties with those of wild-type PKL01. We first prepared three point mutants, PKL01(T104A), PKL01(S317A) and PKL01(T480A), in which phosphorylatable Thr or Ser residues were replaced with the nonphosphorylatable amino acid Ala. Since we had preliminary results that MBP could be phosphorylated by the recombinant PKL01, the following experiments were carried out using MBP as an exogenous substrate for PKL01. Wild-type PKL01 significantly phosphorylated MBP concomitant with its autophosphorylation, whereas PKL01(K148A) showed neither MBP phosphorylation nor autophosphorylation (Fig. 4, lanes 1 and 2, respectively). PKL01(T104A) underwent autophosphorylation and showed MBP phosphorylation (Fig. 4, lane 3), similar to the case for PKL01(WT). In contrast, PKL01(S317A) showed no autophosphorylation (Fig. 4, lane 4), and PKL01(T480A) showed weak autophosphorylation (Fig. 4, lane 5). PKL01(S317A) and PKL01(T480A) exhibited no activity and very low activity against MBP, respectively.

Next, we prepared Asp mutants at the predicted phosphorylation sites that may mimic phosphorylated forms of PKL01. The autophosphorylation and kinase activities of the resulting point mutants, PKL01(T104D), PKL01(S317D) and PKL01(T480D), were compared with those of PKL01(WT). PKL01(T104D) showed essentially the same autophosphorylation activity and kinase activity toward MBP as PKL01(WT) and PKL01(T104A) (Fig. 4, compare lane 6 with 1 and 3). PKL01(S317D) showed neither autophosphorylation nor phosphorylation of MBP,

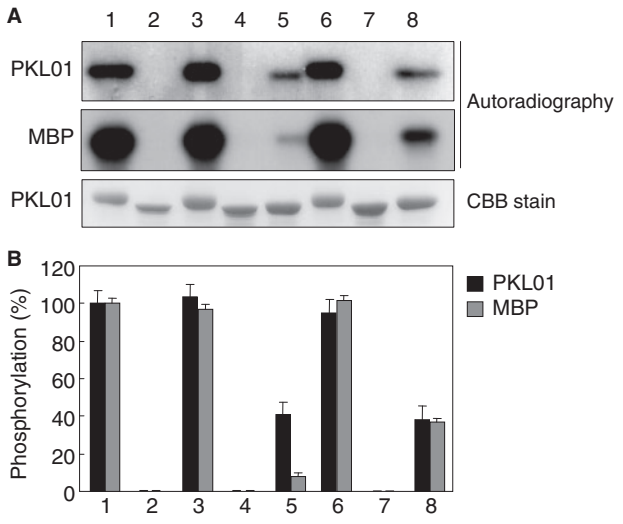


**Fig. 4 Autophosphorylation and MBP phosphorylation of PKL01 and its point mutants.** (A) Wild-type PKL01 (lane 1), kinase-dead PKL01(K148A) (lane 2), PKL01(T104A) (lane 3), PKL01(S317A) (lane 4), PKL01(T480A) (lane 5), PKL01(T104D) (lane 6), PKL01(S317D) (lane7) and PKL01(T480D) (lane 8) were prepared and examined for the effects of mutations at the predicted phosphorylation sites in PKL01. Purified PKL01 and its mutants (1 μg) were electrophoresed in an SDS–polyacrylamide gel and stained with Coomassie brilliant blue (lower panel). PKL01 and its point mutants (200 ng) were individually incubated in the presence of 100 μM [γ-$^{32}$P]ATP and MBP (50 ng) under the phosphorylation conditions at 30°C. After a 30-min incubation, the samples were resolved by SDS–PAGE and the [$^{32}$P]phosphate incorporations into the kinases (upper panel) and MBP (middle panel) were analyzed by autoradiography. Catalytically active forms of PKL01 were observed as upward-shifted protein bands, presumably due to autophosphorylation (lower panel). (B) [$^{32}$P]Phosphate incorporation into PKL01 and MBP shown in (A) was quantitated by Scion Image. Phosphorylation of each band was calculated taking the values of wild type PKL01 (lane 1) as 100%. Data are means ± SD values from three independent experiments.

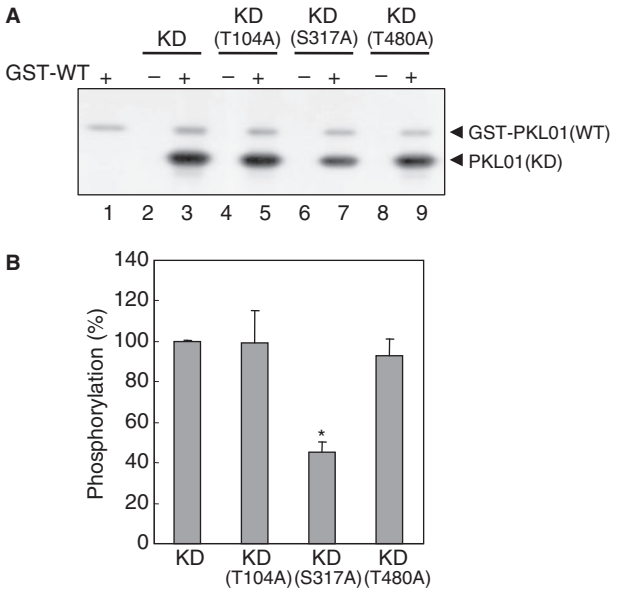


**Fig. 5 Intermolecular autophosphorylation sites in PKL01.** (A) Kinase-active GST-PKL01 (200 ng) was incubated alone (lane 1) or in combination with 200 ng of the kinase-dead mutant PKL01(K148A) (lane 3), PKL01(K148A/T104A) (lane 5), PKL01(K148A/S317A) (lane 7) or PKL01(K148A/T480A) (lane 9) in the presence of 100 μM [γ-$^{32}$P]ATP under the phosphorylation conditions. Each mutant was also incubated in the absence of GST-PKL01 as a negative control (lanes 2, 4, 6 and 8, respectively). After incubation at 30°C for 60 min, the samples were resolved by SDS–PAGE and the [$^{32}$P]phosphate incorporations into the PKL01 mutants were detected by autoradiography. (B) Radioactive bands corresponding to the PKL01(KD) mutants were excised and their radioactivities were counted using a liquid scintillation spectrophotometer. Data are the means ± SD obtained from three independent experiments. Asterisk indicates that there is significant difference ($P$<0.005) between PKL01(K148A) and PKL01 (K148A/S317A). KD and PKL01(KD) indicate the kinase-dead mutant, namely PKL01(K148A).

similar to the case for PKL01(S317A) (Fig. 4, compare lane 7 with 4). PKL01(T480D) exhibited moderate phosphorylation of MBP along with weak autophosphorylation (Fig. 4, lane 8). Taken together, these results suggest that Thr-104 is not an autophosphorylation site essential for activation of PKL01. In contrast, Ser-317 and Thr-480 appear to be possible regulatory phosphorylation sites in PKL01.

In the next set of experiments, the mechanism of the autophosphorylation was examined using four kinase-dead mutants of PKL01, namely PKL01(K148A), PKL01(K148A/T104A), PKL01(K148A/S317A) and PKL01(K148A/T480A). These mutants did not show any autophosphorylation activity (Fig. 5A, lanes 2, 4, 6 and 8, respectively), indicating that all of these mutants were kinase-dead. As shown in Fig. 5, PKL01(K148A/T104A) and PKL01(K148A/T480A) were significantly phosphorylated by GST-PKL01, similar to the case for PKL01(K148A), a kinase-dead PKL01 (Fig. 5A and B). On the other hand, phosphate incorporation into PKL01(K148A/S317A) was reduced to <50% of that into PKL01(K148A) (Fig. 5A, compare lane 7 with 3). These results indicate that autophosphorylation of PKL01 at Ser-317 occurs through an intermolecular reaction.

### *Tissue distribution of PKL01 in L. japonicus*

In a previous study, we obtained 164 clones encoding putative Ser/Thr kinases, and classified these cDNAs into 15 different genes (*5*). Among the 164 clones, there was only one clone for PKL01, suggesting that the expression level of PKL01 in *L. japonicus* would be very low. Indeed, although we prepared a PKL01-specific antibody, we were unable to detect endogenous PKL01 in crude extracts from *L. japonicus* by western blotting (data not shown). We roughly estimated that the amount of PKL01 was <0.001% of the total protein in tissue extracts of *L. japonicus* based on the reactivity of the PKL01-specific antibody (data not shown). Since it was difficult to examine the tissue distribution of PKL01 by western blotting, we performed northern hybridization of LNZ001. As shown in Fig. 6, mRNA for PKL01 was detected as a single band of ~2.0 kb in the leaf, stem, root and nodule extracts. These results indicate that the expression levels of PKL01 are very low but it is widely distributed throughout the plant tissues.

### *Endogenous substrates of PKL01*

To elucidate the physiological functions of Ndr kinases, their downstream targets in cells need to be

clarified. However, the physiological substrates of Ndr kinases have not yet been fully characterized. To investigate the endogenous substrates for PKL01, crude extracts from plant tissues were used for phosphorylation by PKL01. We were unable to detect clearly phosphorylated protein bands when a total crude extract was used for phosphorylation by PKL01, probably due to the presence of interfering substances. Therefore, we used fractionated proteins after isoelectric focusing in a MicroRotofor for detection of endogenous substrates. When 10 separated fractions from a root extract were incubated with PKL01 and [$\gamma$-$^{32}$P]ATP under the standard phosphorylation conditions, significant incorporation of [$^{32}$P]phosphate into multiple proteins with high pI values was observed (Fig. 7B). Although the majority of resolved proteins were highly concentrated in fractions containing proteins with a neutral pI (Fig. 7A, fractions 4–6), significant phosphorylation of proteins in these fractions was not detected (Fig. 7B). Essentially the same results were obtained for the leaf, stem and root nodule extracts (data not shown). Autoradiography of the phosphorylated proteins in fraction 10 from the leaf, stem, root and nodule extracts is shown in Fig. 7C. Although each tissue showed slightly different patterns, they mostly involved common proteins with molecular masses

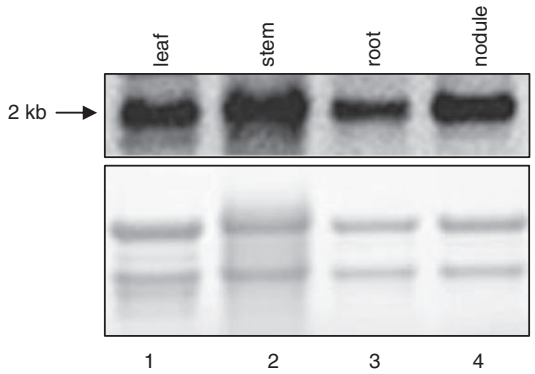


**Fig. 6 Expression of the PKL01 gene in various tissues of *L. japonicus*.** Equal amounts of total RNA (3 μg) from leaf (lane 1), stem (lane 2), root (lane 3) and root nodule (lane 4) samples were electrophoresed in individual lanes and hybridized with a digoxigenin-labeled antisense RNA probe (upper panel). The arrow indicates the 2-kb mRNA for PKL01. The rRNAs were stained with methylene blue as a control (lower panel).

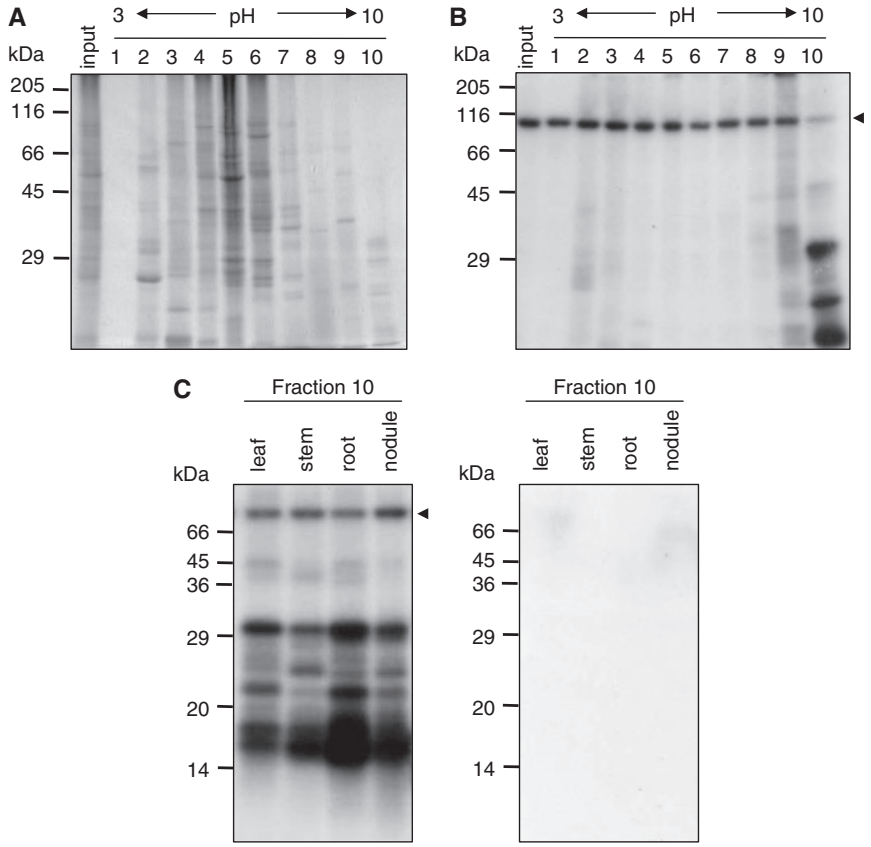


**Fig. 7 Detection of endogenous substrates for PKL01 in tissue extracts from *L. japonicus*.** A crude extract (25 μg) prepared from the root of *L. japonicus* was applied to a MicroRotofor and separated into 10 fractions on the basis of the pI values of the endogenous proteins. Each fraction (5 μl) was separated by SDS–PAGE (12% acrylamide gel) and stained with Coomassie brilliant blue (A). (B) The resolved proteins in each fraction (5 μl) were incubated with GST-PKL01 in the presence of 100 μM [$\gamma$-$^{32}$P]ATP at 30°C for 30 min. Each sample was separated by SDS–PAGE (12% acrylamide gel) and [$^{32}$P]phosphate incorporation into the endogenous proteins was detected by autoradiography. The arrowhead indicates the autophosphorylated protein bands of GST-PKL01. (C) Phosphorylation of proteins in fraction 10 from leaf, stem, root and root nodule extracts. Protein samples of fraction 10 (5 μl) from each plant tissue extract were phosphorylated in the presence (left panel) or absence (right panel) of PKL01 as described for (B). Each sample was separated by SDS–PAGE (15% acrylamide gel) and analyzed by autoradiography. The arrowhead indicates the autophosphorylated protein bands of GST-PKL01.

between 15 and 30 kDa that were only phosphorylated in the presence of PKL01.

PKL01 appeared to phosphorylate basic proteins more efficiently than neutral proteins as its substrates (Fig. 7B). However, since soluble tissue extracts from *L. japonicus* were used for separation by MicroRotofor in Fig. 7, histones are not included in fraction 10, though they are highly basic proteins (Fig. 8A). Next, we examined whether PKL01 phosphorylate basic nuclear proteins such as histones. To examine this possibility, a mixture of histones was prepared from *L. japonicus* by an HCl extraction method. When histone preparations were incubated with PKL01, not only mixed histones from *L. japonicus* but also histone type IIA from calf thymus were efficiently phosphorylated by PKL01 (Fig. 8B). Taken together, these results suggest that PKL01 preferentially phosphorylated highly basic proteins including histones in *L. japonicus*.

## Discussion

In a previous study, we obtained 164 clones encoding putative Ser/Thr kinases in *L. japonicus* by expression screening with Multi-PK antibodies (*5*). Among these clones, LNZ001 contained the full-length open reading frame of a novel Ser/Thr protein kinase gene, and we therefore attempted to characterize its protein product in the present study. We designated the protein PKL01, because the gene contains 12 subdomain sequences specific to Ser/Thr protein kinases. The amino acid sequence of PKL01 is also highly homologous to Ndr kinases in plants and animals, and has a long insertion sequence of more than 50 amino acids between subdomains VII and VIII, which is a characteristic of the Ndr protein kinase family (*14*). Taken together, these facts suggest that PKL01 is an Ndr kinase homolog of *L. japonicus*. Although Ndr kinases in animals have been reported to be closely correlated with cell division and morphogenesis, the functional roles of Ndr kinases in plants have not been reported to date. In the present study, we characterized PKL01, an Ndr kinase homolog in *L. japonicus*, and compared its catalytic properties with those of mammalian Ndr kinases.

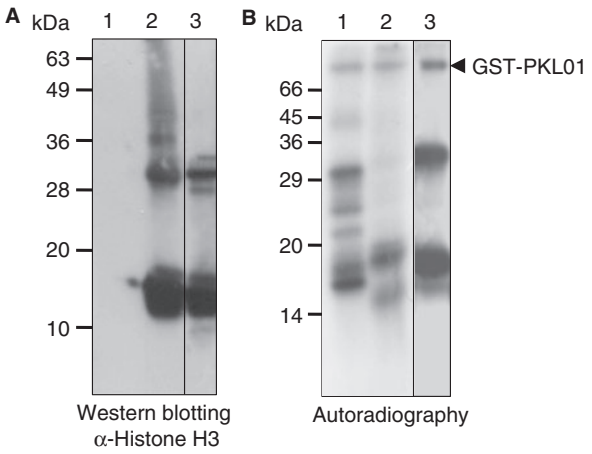


**Fig. 8 Phosphorylation of histones from *L. japonicus* by PKL01.** (A) Western blotting of histones from *L. japonicus*. MicroRotofor fraction 10 from a leaf extract (5 µl, lane 1), a histone preparation from *L. japonicus* (3.5 µg, lane 2) and type IIA histone from calf thymus (500 ng, lane 3) were separated by SDS–PAGE (15% acrylamide gel) and detected with an anti-histone H3 antibody. (B) Fraction 10 from a leaf extract (5 µl, lane 1), a histone fraction from *L. japonicus* (3.5 µg, lane 2) and histone type IIA (100 ng, lane 3) were incubated with GST-PKL01 in the presence of 100 µM [$\gamma$-$^{32}$P]ATP at 30°C for 30 min. Protein samples were resolved by SDS–PAGE (15% acrylamide gel) and [$^{32}$P]phosphate incorporation was detected by autoradiography. The arrowhead indicates autophosphorylated GST-PKL01.

The precise mechanisms of the regulation of Ndr kinases by phosphorylation and modulator proteins such as S100B and MOB proteins have mainly been investigated using mammalian systems (*14*). The human Ndr kinases NDR1 and NDR2 are potently activated in cells following treatment with the protein phosphatase 2A inhibitor okadaic acid, suggesting that these kinases are regulated by phosphorylation/dephosphorylation (*20*, *21*). The regulatory phosphorylation sites were identified as Ser-281 and Thr-444 in NDR1 (*20*). In addition, Thr-74 was reported to be another autophosphorylation site in NDR1, which corresponds to the N-terminal S100B-binding region (*22*). These three putative phosphorylation sites are conserved in PKL01, as Thr-104, Ser-317 and Thr-480, respectively. In the present study, various point mutants of PKL01 at the predicted phosphorylation sites were prepared and examined for their catalytic properties. The point mutants of PKL01 at Thr-104, namely PKL01(T104A) and PKL01(T104D), exhibited similar autophosphorylation rates to that of wild-type PKL01 and had kinase activity against the exogenous substrate MBP. These results indicate that Thr-104 in PKL01 is not an autophosphorylation site *in vitro* and is not a critical residue for the regulation of PKL01. The point mutants of Thr-480, namely PKL01(T480A) and PKL01(T480D), underwent weak autophosphorylation, and PKL01(T480D) exhibited significant kinase activity toward MBP. These results suggest that Thr-480 is a possible phosphorylation site in PKL01 and that PKL01(T480D) partly mimics the phosphorylated form of PKL01. The point mutants of PKL01 at Ser-317 showed neither autophosphorylation nor substrate phosphorylation activity. Although autophosphorylation at Ser-317 was essential for PKL01 activation, PKL01(S317D) could not mimic the phosphorylated form of PKL01. Autophosphorylation at Ser-317 in PKL01 occurred via an intermolecular reaction but not through phosphorylation by an upstream kinase. Taken together, these regulatory mechanisms by phosphorylation at Ser-317 and Thr-480 appear to be consistent with previous reports regarding mammalian Ndr kinase (*14*, *20*). However, it remains unclear whether PKL01 requires an upstream kinase for full activation of the enzyme activity.

Human Ndr kinases were reported to be activated by S100B, an EF-hand $Ca^{2+}$-binding protein, or MOB proteins, which interact with the N-terminal region of Ndr kinases (*12*, *14*). Unlike human Ndr kinases, PKL01 was not activated by the addition of S100B protein (data not shown). Thr-104, which corresponds to Thr-74 of human NDR1, is located in the

N-terminal S100B-binding region of NDR1. Mutation of Thr-74 in NDR1 results in a significant decrease in the kinase activity (*23*), whereas mutation of Thr-104 to Ala or Asp had essentially no effects on PKL01 activity in the present study. Therefore, although PKL01 is an Ndr kinase homolog in *L. japonicus*, the regulatory mechanisms of PKL01 appear to be somewhat different from those of mammalian Ndr kinases.

Although there have been several articles regarding the physiological functions and regulatory mechanisms of mammalian Ndr kinases, the characteristic features of Ndr kinases in plants have not been documented to date. Furthermore, the physiological targets of Ndr kinases are still unclear. In the present study, we found that PKL01 tended to phosphorylate basic proteins and preferentially phosphorylated histones extracted from plant tissues. Human NDR1 has been reported to have a non-consensus nuclear localization signal in the insertion sequence in between subdomains VII and VIII (*8*). In contrast, NDR2, another human Ndr kinase, was reported to show a cytoplasmic localization (*21*). It is important to know subcelluar localization of PKL01 in *L. japonicus*. However, the localization of endogenous PKL01 could not be determined by histochemical approaches because of its low content in plants. We, therefore, examined subcellular localization of PKL01 by transfecting PKL01 subcloned into plasmid CaMV35S-sGFP(S65T)-nos3′ (*24*) in *Arabidopsis* cells. Under the conditions, however, GFP-PKL01 was detected not only in the nucleus but also in the cytosol (data not shown). Therefore, PKL01 could phosphorylate highly basic proteins, possibly including nuclear proteins such as histones, as endogenous substrates. The identification of other physiological targets of PKL01 and the consequence of their phosphorylation are the next issues to be addressed.

**Funding**

Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**Conflict of interest**

None declared.

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