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Elicitin-responsive lectin-like receptor kinase genes in BY-2 cells

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Abstract (126 words)

The inhibition of elicitor-induced plant defense responses by the protein kinase inhibitors K252a and staurosporine indicates that defense responses require protein phosphorylation. We isolated a cDNA clone encoding lectin receptor-like kinase 1 (*NtlecRK1*), an elicitor-responsive gene, in tobacco bright yellow (BY-2) cells by a differential display method. *NtlecRK* forms a gene family with at least three members in tobacco. All three *NtlecRK* genes potentially encode the N-terminal legume lectin domain, transmembrane domain, and C-terminal Ser/Thr-type protein kinase domain. GFP fusion protein showed that the NtlecRK1 protein was located on the plasma membrane. In addition, *NtlecRK1* and *3* were responsive to INF1 elicitin and the bacterial elicitor harpin. These results indicate that NtlecRKs are membrane-located protein kinases that are induced during defense responses in BY-2 cells.

Introduction

Plants respond to various types of elicitors and defend themselves with varied array of gene expressions. Plant defense responses include oxidative burst, accumulation of phytoalexins, reinforcement of cell walls, production of antimicrobial proteins such as PR proteins, and the strongest response, hypersensitive reaction (HR), which accompanies rapid and localized plant cell death. Thus, plants induce a wide variety of gene expressions in response to elicitors (Ichinose et al., 2000). We previously investigated the HR signaling pathway triggered by INF1 elicitin, an HR elicitor produced by *Phytophthora infestans*, in suspension cell cultures of tobacco bright yellow (BY-2). Using protein kinase inhibitors, we found that phosphorylation is an indispensable step in elicitor-induced defense responses, including HR cell death, defense gene expression such as phenylalanine ammonia-lyase gene, and most generation of hydrogen peroxide (Sasabe et al., 2000). In contrast, application of protein phosphatase inhibitors enhanced the elicitor-induced defense responses described above (Sasabe et al., 2000). Similar observations were also reported for many combinations of plants and elicitors (Felix et al., 1994; Gerber and Dubery, 2004; Otte et al., 2001).

On the other hand, we attempted to isolate the genes that are induced by the HR elicitor INF1 elicitin in BY-2 cells because protein biosynthesis inhibitors also inhibit

elicitor-induced defense responses. We isolated a cDNA clone (*NtPDR1*) encoding a pleiotropic drug resistance (PDR)-type ABC transporter by the differential display method, and determined that treatment with various types of elicitors induced the expression of *NtPDR1* (Sasabe et al., 2002). In this differential display study, we also isolated the cDNA clone encoding lectin receptor-like protein kinase (lecRK).

Plants possess plant-specific families of receptor-like kinases (RLKs). RLKs comprise at least 610 members in *Arabidopsis thaliana*, and are involved in various cell-cell communication processes (Shiu and Bleecker, 2003; Shiu et al., 2004; Barre et al., 2002), such as the CLV1 receptor in meristem signaling, the SRK receptor in signaling from pollen to stigma in self-incompatible *Brassica* species, and the BRI1 receptor in brassinosteroid signaling (Barre et al., 2002). RLKs are also involved in plant defense signaling (Nürnberger et al., 2004; Zipfel et al., 2006). For example, Xa21 in rice confers cultivar-specific resistance on *Xanthomonas oryzae* pv. *oryzae* strains expressing AvrXa21. FLS2 and EFR are receptor molecules in *A. thaliana* that perceive the flg22 oligopeptide conserved in flagellin protein and the elf18 oligopeptide in the elongation factor EF-Tu, respectively. Furthermore, Eix2 in tomato is a receptor for a fungal elicitor, xylanase. Thus, FLS2, EFR, and Eix2 are receptors for pathogen-associated molecular patterns (PAMPs). These RLKs have been classified into

several classes on the basis of properties of extracellular domains.

Among the RLKs, one large family of putative receptor kinases with a legume lectin-like extracellular domain has been characterized (Herve et al., 1996; Herve et al., 1999). Forty-two lectin receptor-like kinase (lecRK)-related sequences and nine related soluble legume lectin sequences have been identified in *A. thaliana* (Riou et al., 2002). The expression and function of several members of the lecRK gene family were recently reported. The gene *PnLPK1*, a member of the lecRK family in lombardy poplar, is expressed in response to wounding, and it has been shown that PnLPK1 has phosphorylation activity (Nishiguchi et al. 2002). Riou et al. (2002) observed that the gene expression of one of the Arabidopsis *lecRK* genes, *lecRK-a1* (At3g59700), is induced during senescence, by wounding, and in response to oligogalacturonic acids. Expression of another *lecRK* gene (*AtlecRK2*, At3g45410) was induced by exposure to 1-aminocyclopropane-1-carboxylic acid, a precursor of ethylene (He et al., 2004). Further, Gouget et al. (2006) reported that lecRK (the At5g60300 gene product) participates in protein-protein interactions via the RGD (arginine-glycine-aspartic acid) tripeptide sequence.

In this paper, we report the structure and expression profile of *NtlecRKs*, and discuss their possible functions in tobacco-microbe interactions.

Materials and method

Plant cells and elicitors

Suspension cultures of tobacco Bright Yellow (BY-2) cells were maintained as described (Sasabe et al., 2000). INF1 elicitin (*inf1* gene product of *Phytophthora infestans*) was prepared as a recombinant protein using the method previously described (Sasabe et al., 2000). Preparation of harpin$_{Psg}$, an elicitor protein of *Pseudomonas syringae* pv. *glycinea*, followed the method of Taguchi et al. (2001). Yeast extract (Difco, Detroit, MI, USA) dissolved in water and filtered through a 0.45 μm pore filter (Kurabo, Osaka, Japan) was used as a general elicitor. The final concentration of yeast extract was 5 mg/ml; at this concentration, yeast extract did not induce cell death in BY-2 suspension cultures within 24 h. The treatment with elicitin, harpin^{Psg} (10 μg/ml as a BSA equivalent), or 0.5% yeast extract was performed 3 days after reinoculation, and the cells were collected at intervals by centrifugation.

Differential display PCR

Differential display PCR was carried out according to the method of Yoshida et al. (1994). The details were previously described by Sasabe et al. (2002). The nucleotide sequence of the

G27 primer, one of the arbitrary primers that were used to isolate the *NtlecRK1* gene fragment, was 5'-GGCGTGGAAGGA-3'.

Isolation of* NtlecRK *cDNAs and determination of nucleotide sequences

To obtain a full-length cDNA clone for *NtlecRK*, we screened a cDNA library that was constructed from INF1 elicitin-treated BY-2 cells for 6 h using a ZAP-cDNA® synthesis kit and a Gigapack® III Gold cloning kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. DNA sequences were determined by an ABI PRISM® 310 gene analyzer (PE Biosystems Japan, Chiba, Japan).

Subcellular localization of lecRK1-GFP

A chimeric fusion protein of lecRK1 and GFP was constructed to investigate the subcellular localization of lecRK1 in onion epidermal cells. The DNA fragment containing the coding region for the putative lecRK1 protein (positions 11 - 2038 of *lecRK1* cDNA) was fused to the 5' end of the GFP coding region in a CaMV35S Ω-sGFP(S65T)-nos3' plasmid (Niwa et al., 1999) to generate *plecRK1-GFP*. In order to transiently express the lecRK1-GFP fusion proteins, 0.2 μg of each plasmid DNA was coated with tungsten particles, then introduced into onion epidermal cells by bombardment (IDERA GIE-III; TANAKA, Hokkaido, Japan). The CaMV35S Ω-sGFP(S65T)-nos3' plasmid was used as a negative control. After

incubation for 48 h at room temperature, GFP fluorescence was observed using an Olympus IX70 microscope (Olympus, Tokyo, Japan).

Plasmid constructions and expression of recombinant protein

To produce the recombinant kinase domain of NtlecRK1 (PKD1), the corresponding DNA fragment was amplified with *Nde*I- and *Bam*HI-linked oligonucleotide primers, PKD1f (5'-CATATGGGTTTTGCTGATGAAAA-3') and PKD1r (5'-GGATCCCCTTTCAAAGAATTTCAC-3') for lecRK1 (italic letters indicate *Nde*I and *Bam*HI sites, respectively). After digestion with *Nde*I and *Bam*HI, the fragment was subcloned into a pET16b plasmid vector (Novagen, Madison, WI, USA), and the recombinant protein was expressed in *Escherichia coli* BL21(DE3) codon plus cells. After incubation, bacterial cells were harvested by centrifugation at 3,000 x g for 5 min according to the manufacturer's instructions, and the pellet was lysed in B-PER™ Reagent (Pierce, Rockford, IL, USA). Because recombinant PKD1 was accumulated in inclusion bodies, the inclusion bodies were purified by a lysozyme (200 μg/ml), and PKD1 was solubilized by a protein kinase buffer (50 mM Tris-HCl, pH 7.5, 2 mM DTT) containing 8 M urea and then regenerated by dialysis in the same buffer without urea. The 35 kDa PKD1 was detected by Western blotting analysis with anti His-tag antibody and goat anti-mouse second antibody

(Bio-Rad, Hercules, CA, USA) conjugated with alkaline phosphatase and its chemiluminescent substrate, CDP-*Star*™ (Boehringer Mannheim, Mannheim, Germany).

In vitro *phosphorylation assay*

The protein kinase activity of 1 μg of the purified PKD1 polypeptide was determined by an *in vitro* autophosphorylation assay. The reaction was performed at 30ºC for 30 min in a reaction buffer (30 mM Tris-HCl (pH 7.4), 10 μCi of [γ-^{32}P] ATP (6,000 Ci/mmol)). To examine the effect of cations, 0.5 mM Mg^{2+}, Mn^{2+}, or Ca^{2+} was added to the reaction buffer. The reaction was terminated after incubation at 95ºC for 5 min, and phosphorylated proteins were separated on SDS-PAGE. After washing the SDS-PAGE gel with wash buffer containing 5% trichloroacetic acid and 1.65% pyrophosphoric acid, autoradiography was carried out.

RT-PCR method

Reverse transcriptase polymerase chain reaction (RT-PCR) to analyze the expression of each *lecRK* gene was carried out using AMV reverse transcriptase (Takara, Kyoto, Japan) and Ampli Taq Gold DNA polymerase (PE Biosystems Japan). RT reaction solutions (20 μl in 1 X reaction buffer, 1 μM oligo-dT primer, 1 mM dNTPs, 20 U of RNase inhibitor, 5 U of reverse transcriptase, and 2 μg total RNAs) were incubated for 60 min at 42˚C. By using cDNA products as templates, amplification of *lecRK* genes was performed under the

following conditions, 30 cycles of 30 sec at 95°C, 30 sec at 58°C, and 1 min at 72°C. The sequences of specific primers were PK1f (5'-AATGCTGCTTCATGAACAA-3') and PK1r (5'-ACCTTAAGGGAACATTTGGT-3') for *lecRK1*, PK2f (5'-AATGCTGCTTCATGAACAA-3') and PK2r (5'-TCTAGCAGTGTTTGGTATTC-3') for *lecRK2*, and PK3f (5'-ATGGTTCACACCCAACATTT-3') and PK3r (5'-GTGGTAGAATTTGTTCTCT-3') for *lecRK3,* which were derived from the 3'-untranslated region of each *lecRK* cDNA.

Results

Isolation and sequence analysis of NtlecRK *genes*

To isolate differentially expressed genes during the defense response, we applied the random amplified polymorphic DNA/RT-PCR differential display method (Yoshida et al., 1994) to BY-2 cells treated with INF1 elicitin and Luria-Bertani medium (control) for 1 or 3 h. Among the 32 primers tested, we obtained 19 elicitin-responsive fragments (data not shown). One of them, the G27-E3-2 fragment, was specifically amplified with a G27 primer from mRNAs in BY-2 cells treated with elicitin for 3 h. Their nucleotide sequence showed high homology to serine/threonine type protein kinase. The result of Northern blot analysis, using the G27-E3-2

fragment as a probe, indicated that the expression of this gene was induced by INF1 elicitin (data not shown). For further analysis, cDNA clones corresponding to the G27-E3-2 fragment were isolated from an elicitin-treated cDNA library using the isolated fragment as a probe, and two other homologous genes were simultaneously identified. All three genes were predicted to encode RLKs, which consist of a lectin-like domain as an extracellular domain, a membrane-spanning domain, and a serine/threonine protein kinase domain (Fig. 1). The lectin domain has legume lectin α and β domains, which are conserved among legume lectins and a typical feature of glucose/mannose type sugar chain-binding lectins.

One of the cDNA sequences obtained from the library was completely identical to the G27-E3-2 fragment, and we designated this clone *NtlecRK1* (*Nicotiana tabacum* lectin-like receptor protein kinase 1 protein). DNA sequences of the two other clones were also highly homologous to *NtlecRK1*, and we designated these clones *NtlecRK2* and *3*. In the sequence of *NtlecRK2*, there is frame shift mutation at the 438th amino acid from the N-terminus. This frame shift resulted in a non-homologous 14-amino acid C-terminal extension in the protein kinase domain. The amino acid sequences of NtlecRKs showed high homology to each other with about 80% identities when the frame-shifted region in NtlecRK2 was ignored. The complete sequences of *NtlecRK1* and *3* also showed high homology to various plant species

lecRK proteins, especially to *Arabidopsis thaliana* AtlecRK proteins encoded by At4g04960 and At4g28350 and *Medicago truncatula MtlecRK7;2* with about 60%, 50%, and 43% identities, respectively, at the amino acid level (Fig. 1).

LecRK1 protein may localize in plasma membrane

The deduced NtlecRK proteins have a membrane-spanning domain; consequently, they are expected to localize in certain membranes. In order to confirm the subcellular localization of lecRK1, we generated a plasmid with the *lecRK1-GFP* gene that expressed a chimeric protein consisting of full-length lecRK1 at the amino terminal end and green fluorescent protein (GFP) at the C-terminal end. The expression of the *lecRK1-GFP* gene was controlled by the CaMV 35S promoter in plant cells. Plasmid DNAs encoding GFP alone (Ω-sGFP(S65T)) and the lecRK1-GFP fusion protein were bombarded with tungsten particles into onion epidermal cells, and then transient expression of GFP fluorescence was observed. As a negative control, tungsten particles without DNA were also bombarded into onion cells (Fig. 2a, d, g and j). GFP fluorescence was localized to almost the whole cell including the nucleus (Fig. 2b and h). In contrast, the fluorescence was predominantly observed at the cell surface of the *lecRK1-GFP* gene-bombarded onion cells (Fig. 2c). In order to determine whether lecRK1-GFP was localized to the plasma membrane or the cell wall, the onion epidermal

cells were plasmolyzed by treatment with 2.5% KCl (Fig. 2g-l) after plasmid bombardment. As shown by the arrowheads in Fig. 2 (j-l), partial plasmolysis occurred, and lecRK1-GFP fluorescence was observed at the plasma membrane and/or in the area neighboring the plasma membrane (Fig. 2i).

In vitro *phosphorylation assay using recombinant protein kinase domain derived from* lecRK1

To characterize lecRK1 biochemically, the DNA sequence for the kinase domain (aa. 361 - 631) of *lecRK1* and a polyhistidine sequence were fused, and the recombinant polypeptide containing protein kinase 1 domain (PK1D) was expressed in *E. coli.* Then, purified PK1D was used in an *in vitro* autophosphorylation assay. The single band of PK1D detected by Coomassie brilliant blue (CBB) staining and Western blotting was about 33 kDa, which is consistent with the molecular mass predicted from the DNA sequence (Fig. 3A). Although PK1D showed nearly no autophosphorylation activity without any cation, the addition of Mg^{2+}, Mn^{2+}, or Ca^{2+} resulted in the activation of autophosphorylation by the PK1D recombinant polypeptide (Fig. 3B). Furthermore, the concomitant presence of Mg^{2+} and Mn^{2+} slightly increased the autophosphorylation activity.

Expression of* NtlecRK *genes

The *lecRK* gene was identified as an INF1 elicitin-inducible gene by the differential display experiment. Because it would be difficult to distinguish the expression of each *NtlecRK* gene by Northern blot analysis due to the high level of homology, we designed specific primers to detect each *NtlecRK* mRNA by the RT-PCR method and examined each expression in response to treatment with INF1 elicitin, harpin^{Psg}, and yeast extract as elicitors in tobacco BY-2 cells (Fig. 4). The expression of the *lecRK1* gene was weakly induced by treatment with elicitin, harpin^{Psg}, and yeast extract. The expression of *NtlecRK3* was also induced by treatment with the elicitors described above. Especially INF1 elicitin and yeast extract strongly induced *NtlecRK3* expression. On the other hand, the expression of *NtlecRK2* was not detected. *NtlecRK2* has a frameshift mutation in the coding region, indicating that *NtlecRK2* is a pseudogene.

Discussion

Plants are ordinarily exposed to environmental stresses such as changes in temperature, nutrient, and water conditions, and pathogen attacks. These changes are often perceived by receptors on the plant cell surface, and transmitted to cells and/or the whole plant via signal transduction pathways to induce adaptive responses in plants. Phosphorylation is a most

important step in the signal transduction pathways in plants stimulated by different environmental cues.

In this study, we investigated tobacco *lecRK* genes. Expression of two *NtlecRKs* was induced by various elicitor treatments. Induction of some Arabidopsis *AtlecRK* genes by environmental signals from pathogens has also been reported. Microarray analysis indicated that two bacterial general elicitors, flg22 and elf18, rapidly induced many Arabidopsis genes in cell cultures and seedlings (Navarro et al., 2004; Zipfel et al., 2006). The expression of more than 100 *RLK* genes present in the Arabidopsis genome was rapidly induced by flg22 and elf18 (Zipfel et al., 2006). For example, four *lecRK* genes, including *At4g28350*, were rapidly induced by flg22 in Arabidopsis cell cultures (Navarro et al., 2004), and 11 *lecRK* genes were also rapidly induced by flg22 or elf18 in Arabidopsis seedlings (Zipfel et al., 2006). Based on these results, Navarro et al. (2004) suggested that flg22 may enhance the sensitivity of plant cells to many different PAMPs. Indeed, treatment of Arabidopsis cells with flg22 and elf18 increased the number of binding sites specific to elf18 and flg22, respectively, in extracts of seedlings (Zipfel et al., 2006). Interestingly, the deduced amino acid sequences of one of the flg22-induced genes encoded in At4g28350 and *NtlecRKs* are highly homologous to each other (Fig. 1). Proteasome analysis also revealed that

suspension-cultured Arabidopsis cells accumulated lectin receptor-like protein kinase protein (encoded by At1g78850) in response to a chitosan elicitor in the cell wall fraction (Ndimba et al., 2003). Thus, some members of the lecRK family seem to be involved in the signal transduction of the plant defense response or in interactions with plant-associated microbes. In related reports, four lecRKs in *Medicago truncatula* preferentially expressed in the root seemed to be involved in nodulation (Navarro-Gochicoa et al., 2003). As shown in Fig. 1, one *MtlecRK* product, MtlecRK7;2 also had high homology to NtlecRKs. This might indicate that NtlecRK is also involved in interaction with plant-associated microbes. These results indicate, as Navarro et al. (2004) suggested, that each PAMP may enhance the sensitivity of plant cells to many other PAMPs. The expression of *NtlecRKs* in BY-2 cells was increased by the application of elicitin, harpin, or yeast extract; therefore, NtlecRKs may increase the capability to recognize other PAMPs. Very recently it was also reported that a *B-lectin RLK* gene, *Pi-d2,* in rice conferred rice blast resistance to the *Magnaporthe grisea* strain ZB15 (Chen et al., 2006). Thus, *Pi-d2* was identified as a new class of plant resistance gene against *M. grisea*. Although *Pi-d2* is constitutively expressed, perception of the corresponding Avr protein may induce activation of receptor-related gene expression. In the case of NtlecRK proteins, the corresponding ligand molecules are not known yet. Biochemical and molecular

genetic approaches may elucidate the role of NtlecRK proteins in plant-microbe interactions.

***Footnote**:* The nucleotide sequences reported in this paper have been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB265221-AB265223.

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Figure legends

Fig. 1. Multiple alignment of the deduced amino acid sequences of NtlecRKs and related lecRKs. NtlecRK1 is aligned with NtlecRK2, 3, AtlecRKs encoded by At4g04960 and At4g28350, and *Medicago truncatula* MtlecRK7*;2* using the Malign program of Genetyx-Mac (Software Development, Tokyo, Japan). Below the amino acid sequences, identical and similar residues in all five or six sequences are marked by asterisks and dots, respectively. However, the amino acid sequence of the C-terminal extension (indicated by lower case) due to a frame-shift mutation in NtlecRK2 was ignored in this alignment. The putative sequences for signal peptides predicted by the iPSORT program (Bannai et al., 2002, http://hc.ims.u-tokyo.ac.jp/iPSORT) and the transmembrane domain predicted by the PSORT program (Nakai and Kanehisa, 1991, http://psort.ims.u-tokyo.ac.jp/form.html) are indicated as bold letters at the amino terminus and underlined at the middle of the sequence, respectively. Legume lectin α and β domains are represented above the sequences. Amino acid sequences for protein kinase domains are boxed.

Fig. 2. Subcellular localization of NtlecRK1.

The panels show onion epidermal cells expressing *GFP* (b, e, h, k), *NtlecRK1-GFP* (c, f, i, l),

or bombarded tungsten particles with no plasmid as a negative control (a, d, g, j). Green fluorescence images of the cells are shown in panels a-c and g-i. Transmission light images of the cells are shown in panels d-f and j-l. The cells in the panels (g-l) were plasmolyzed by treatment with 2.5% KCl. White bars indicate 50 μm. W and M indicate cell wall and plasma membrane, respectively.

Fig. 3. Generation of PK1D polypeptide and its autophosphorylation.

A, Generation of recombinant PK1D polypeptide. Lane M: Molecular size marker. Lane 1: CBB staining of purified PK1D. Lane 2: Western blotting to detect recombinant polypeptide with anti-His antibody. **B**, *In vitro* autophosphorylation assay of PK1D. Recombinant PK1D was autophosphorylated by [γ-^{32}P]ATP with Mg^{2+} plus Mn^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, or no cation. Cations indicated were added to the reaction buffer at a final concentration of 0.5 mM.

Fig. 4. Effects of different elicitors on the expression of *NtlecRK* genes in BY-2 cells.

RNAs used for RT-PCR were successively prepared from BY-2 cells treated with INF1 elicitin (10 μg/ml), harpin^{Psg} (100 μg/ml), and yeast extract (YE; 5 mg/ml).

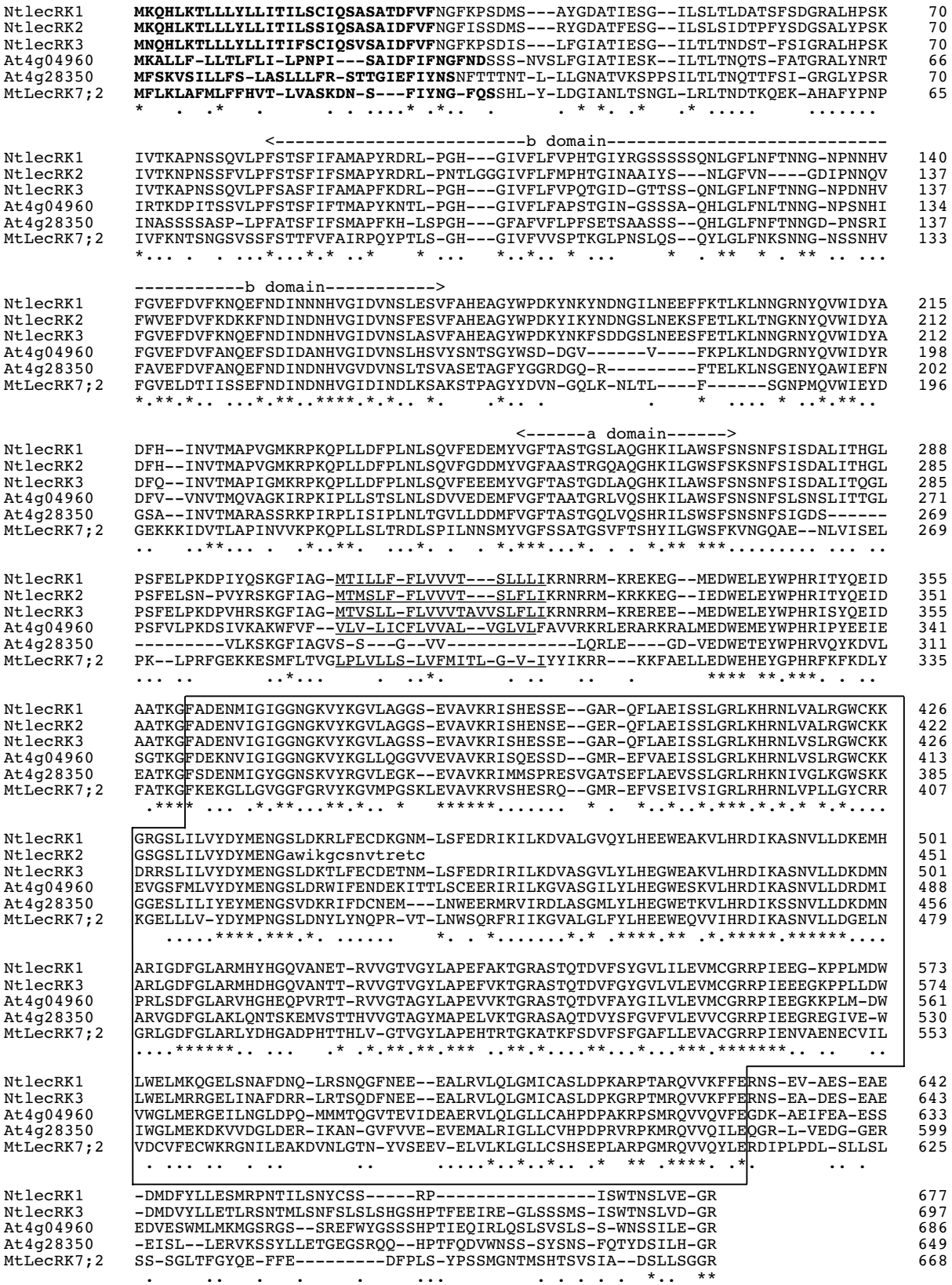

NtlecRK1
NtlecRK2
NtlecRK3
At4g04960
At4g28350
MtLecRK7;2
<-------------------------b domain----------------------------
-----------b domain----------->
<------a domain------>
a domain
b domain

Fig. 2

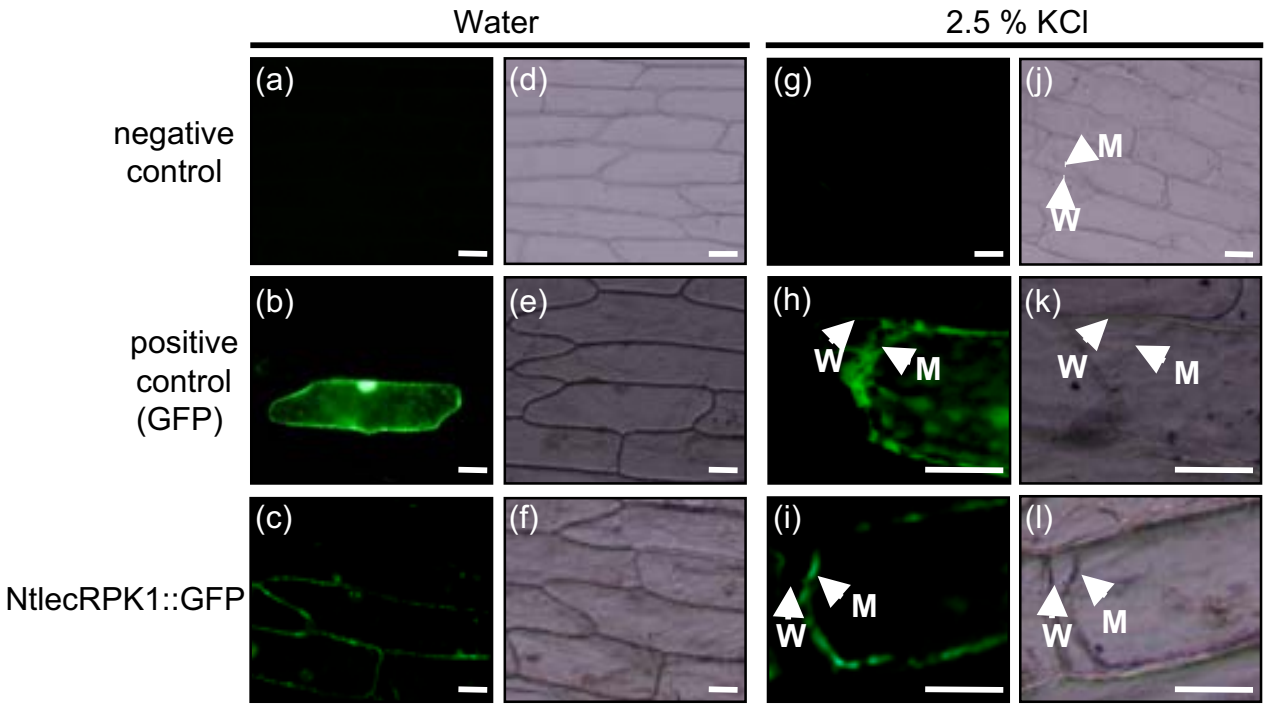

Water
2.5 % KCl
negative control
positive control (GFP)
NtlecRPK1::GFP
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
W
M

Fig. 3

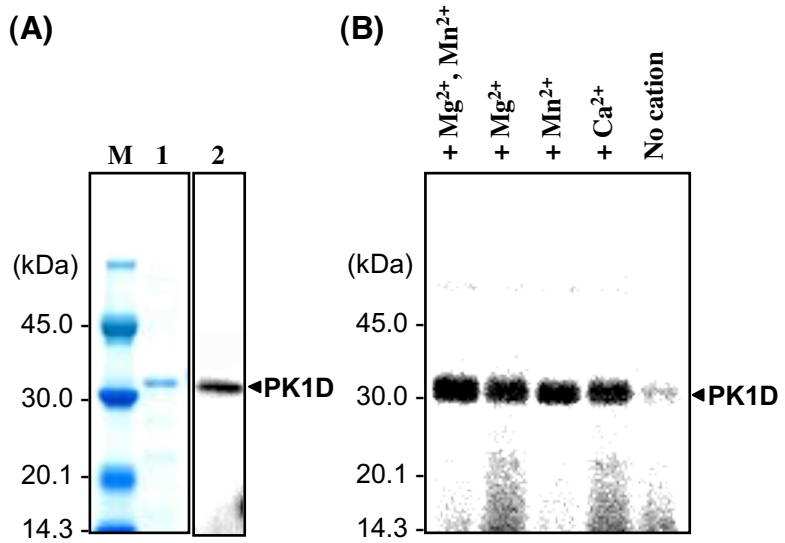
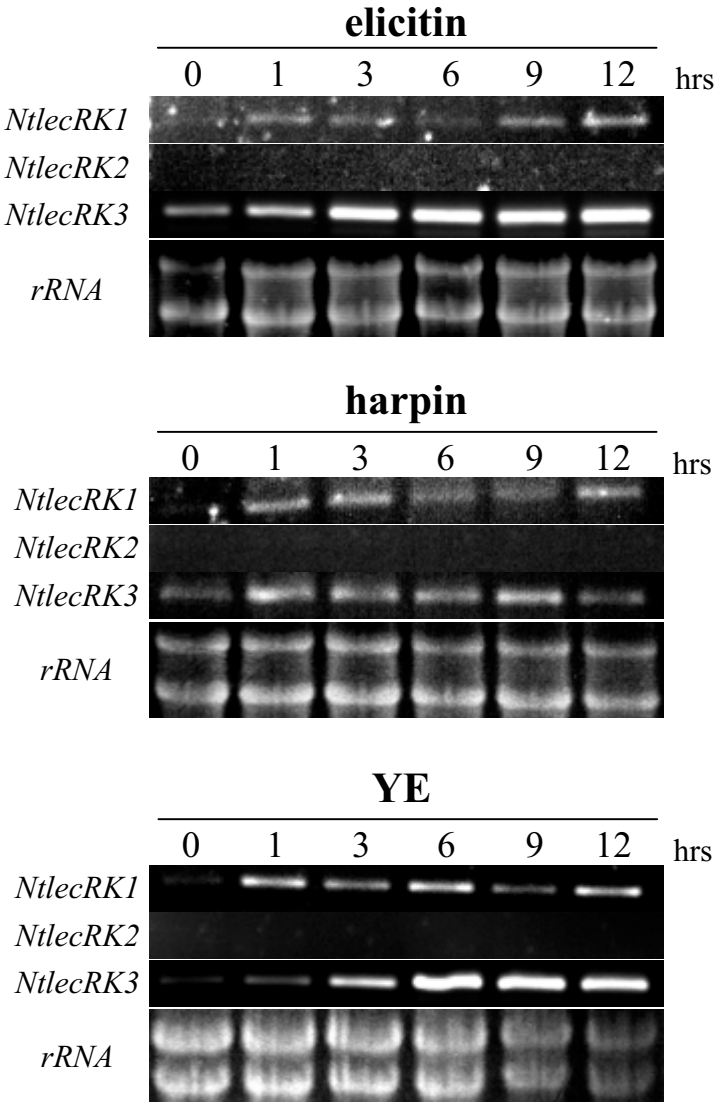

(A)
M
1
2
(kDa)
45.0
30.0
20.1
14.3
PK1D
(B)
+ Mg2+, Mn2+
+ Mg2+
+ Mn2+
+ Ca2+
No cation
(kDa)
45.0
30.0
20.1
14.3
PK1D

Fig. 4


elicitin
0 1 3 6 9 12 hrs
NtlecRK1
NtlecRK2
NtlecRK3
rRNA
harpin
0 1 3 6 9 12 hrs
NtlecRK1
NtlecRK2
NtlecRK3
rRNA
YE
0 1 3 6 9 12 hrs
NtlecRK1
NtlecRK2
NtlecRK3
rRNA