Isolation and characterization of NgRLK1, a receptor-like kinase of Nicotiana glutinosa that interacts with the elicitin of Phytophthora capsici

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Abstract Elicitins, extracellular proteins from Phytophthora fungi, elicit a hypersensitivity response (HR), including systemic acquired resistance, in some plants. The elicitin capsicein (~10 kDa) was purified by FPLC from culture filtrates of P. capsici. Purified native and recombinant capsicein induced a hypersensitive response in leaves of the non-host plants Nicotiana glutinosa and Brassica rapa subsp. pekinensis. To search for candidate capsicein-interacting proteins from N. glutinosa, a yeast two-hybrid assay was used. We identified a protein interactor that is homologous to a serine/threonine kinase of the plant receptor-like kinase (RLK) group and designated it NgRLK1. The ORF of NgRLK1 encodes a polypeptide of 832 amino acids (93,490 Da). A conserved domain analysis revealed that NgRLK1 has structural features typical of a plant RLK. NgRLK1 was autophosphorylated, with higher activity in the presence of Mn$^{2+}$ than Mg$^{2+}$.

Keywords Phytophthora capsici · Elicitin · Capsicein · HR · RLK · NgRLK1

Introduction

Fungi of the genus Phytophthora are a major cause of crop destruction. Root rot caused by Phytophthora capsici soil infection is a serious disease affecting the cultivation of red pepper [1], and Phytophthora blight of pepper caused by this pathogen is one of the most destructive soilborne diseases in Korea [2]. Many Phytophthora produce elicitors, members of a family of small extracellular proteins that cause a hypersensitive response associated with the defense response of Nicotiana and Brassica species [3]. Elicitins are conserved, 98-amino acid (~10 kDa) holoproteins that are abundantly secreted into the medium [4–7].

Elicitin genes and proteins have been cloned and purified from many species, including P. parasitica [8], P. cryptogea [9], P. sojae [10, 11], P. infestans [12], and P. cinnamomum [13], although the elicitin gene from P. capsici has not yet been cloned. All known Phytophthora elicitors show >60% sequence identity [4]. Elicitins from different fungal species cause necrotic lesions on tobacco, but plant defense reactions are induced at the same level [5]. The major 98-amino acid elicitors of Phytophthora have been divided into acidic (α)- and basic (β)-elicitors [14, 15]. The β-elicitors produce greater necrosis than the α-elicitors, and the difference in biological activity is correlated with differences in sequence and physico-chemical features among them [4, 16, 17]. Kamoun et al. have proposed dividing the elicitors and elicitor-like proteins of Phytophthora and Pythium species into five classes based on phylogenetic analysis and the overall structures of the proteins [12]. Jiang et al. searched for new elicitin and elicitin-like gene family members by making use of a Phytophthora expressed sequence tag (EST) database and classified all family members of elicitin based on sequence and protein motifs [18].

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Although elicitors are thought to be the major determinants of non-host resistance against Phytophthora infection in Nicotiana species [5, 19], the role of elicitors in the biology of Phytophthora are not clear, and little is known about the molecular mechanisms involved in elicitor activity. Specific, high-affinity binding sites for β-cryptogeen of P. cryptogea were observed in tobacco plasma membranes [20], but these proteins did not exhibit any protease, β-glucanase, or phospholipase activity, and no other enzymatic activity has been reported for them [3, 21]. One likely biological role for elicitors is as sterol scavengers, because they are known to be sterol carrier proteins and Phytophthora species do not synthesize sterols [22].

Elicitors are known to be secreted by Phytophthora species and have been assumed to localize in the extracellular space of plant tissue. Consequently, it is conceivable that elicitors interact with a host molecule in the plant plasma membrane to initiate plant cell responses following elicitor recognition. Despite the identification of many elicitor proteins, membrane receptors for elicitors have not been clearly identified to date. In a yeast two-hybrid assay, Tyler (unpublished data) found a tobacco cDNA encoding an intracellular elicitin-binding protein, which contained a nucleotide-binding site and leucine-rich repeat motifs [3]. The protein expressed from this cDNA was required for the elicitor response. Kanzaki et al. isolated and characterized NbLRK1, which is a P. infestans INF1 elicitin-interactor from N. benthamiana [23]. In their study, INF1 was found to bind to the intracellular domain (kinase domain) of NbLRK1. Therefore, they speculated that plant recognition of INF1 takes place inside the plant cells.

In this paper, we report the molecular cloning and protein purification of the elicitor from P. capsici. In addition, we used a yeast two-hybrid assay to identify a capsicein-interacting protein from tobacco, N. glutinosa. Sequence analysis revealed that the capsicein-interacting protein, designated NgRLK1, has domains typical of plant receptor-like kinases (RLKs). The kinase domain of NgRLK1 was expressed in Escherichia coli, and this domain was auto-phosphorylated in the presence of Mn$^{2+}$ or Mg$^{2+}$.

Materials and methods

Strains, plasmids, and growth conditions

The Phytophthora, E. coli, and yeast strains and the plasmids used in this study are described in Table 1. The Phytophthora strain was grown at 24°C in cleared or uncleared 20% vegetable juice (V8) medium supplemented with 1.5% agar. For elicitor production, the strains were grown for 3 weeks at 28°C in still culture in a synthetic medium containing (per liter): 0.5 g of KH$_2$PO$_4$, 0.25 g of MgSO$_4$·7H$_2$O, 1 g of asparagine, 1 mg of thiamine, 0.5 g of yeast extract, and 25 g of glucose [17]. The E. coli strains were cultured in Luria-Bertani (LB) medium (USB) at 37°C, and the yeast cultures were grown at 30°C in either YPDA (1% yeast extract, 2% peptone, 2% glucose, and 0.2% adenine) or SD (minimal synthetic dropout) medium (Clontech) with dropout (DO) supplements (Clontech) according to the auxotrophies of the yeast strains. The antibiotics used in this study were ampicillin (100 μg/ml, Duchefa) and kanamycin (50 μg/ml, Sigma).

Isolation and purification of capsicein

Three weeks after inoculation, culture filtrates of P. capsici Pa28 were harvested through triple layers of Miracloth and centrifuged at 6,000 g for 30 min. The supernatant was filtered through a 0.8-μm filter and was brought to 95% saturation by adding ammonium sulfate with stirring at 4°C for 16 h. The precipitated proteins were collected by centrifugation (6,000 g for 30 min). The protein pellet was resuspended in 10 mM Tris–HCl (pH 7.0) and was dialyzed against the same buffer to remove the ammonium sulfate. After dialysis, capsicein was purified by Fast Protein Liquid Chromatography (FPLC) using a Superdex 200 HR 10/30 column (GE Healthcare Bioscience AB) in 10 mM Tris–HCl (pH 7.0). The concentration of the purified capsicein was determined by the Bradford method using BSA as a standard. The protein was visualized on 15% SDS–polyacrylamide gels stained with Coomassie Brilliant Blue (CBB) R-250 (Sigma).

N-terminal amino acid sequencing

After electrophoresis of the purified capsicein, the protein was transferred onto a PVDF membrane (Applied Biosystems) in 10 mM CAPS (Sigma) buffer under a constant current of 200 mA for 1 h. To confirm the transfer, the membrane was stained for a few seconds with 0.1% CBB R-250 (Sigma) in 1% acetic acid/40% methanol and then destained with 50% methanol. The capsicein on the membrane was sequenced by automated Edman degradation performed by an Applied Biosystem 476A sequencer (Applied Biosystems) using the reagents and methods supplied by the manufacturer.

Cloning of the capsicein gene

Standard methods were used for DNA/RNA manipulation, DNA cloning, and gel electrophoresis [24]. The capsicein
gene from *P. capsici* was cloned by a reverse transcription-polymerase chain reaction (RT-PCR). Total *P. capsici* RNA was obtained by phenol-chloroform extraction of 3-week-old mycelia in liquid culture [24], and 1 μg of total RNA was used as the template for RT-PCR with 100 pmol of degenerate primers, dCap-F (5'-ACVCARCARACVG CMGCMTA-3') and dCap-R (5'-GCRCABGTGKGCYG ARAADCCRTT-3'), that were designed based on the alignment of the amino acid sequence determined for capsidecin and the sequences of conserved domains of known elicinins taken from the ExPASy database (www.expasy.org). The synthesis of capsidecin cDNA was performed using an RNA PCR kit (AMV) Ver 2.1 (Takara) according to the manufacturer’s instructions. Amplification was performed for 30 cycles of 30 s each denaturation at 94°C, annealing at 55°C for 40 s, and extension at 72°C for

### Table 1  Bacterial strains and plasmids

| Strains/plasmids | Characteristicsa | Sources/references |
|------------------|------------------|--------------------|
| *Escherichia coli* |                 |                    |
| DH5z             | F- Φ80dlacZAM15 Δ(lacZYA-argF)U169 endA1 deoR recA1 hsdR17(rK- mK+) phoA supE44 thi-1 gyrA96 relA1 | Gibco-BRL |
| BL21            | F- ompT hsdS(dK)-mK+ gal dcm | Takara |
| BL21(DE3)       | F- ompT hsdS(dK)-mK+ gal dcm Δ(DE3) | Novagen |
| Top10           | F- mcrA Δ(mcr-hsdRMS-mcrBC) Φ80lacZAM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str8') endA1 mupG | Invitrogen |
| *Saccharomyces cerevisiae* |             |                    |
| AH109           | MATa, trp1-901, leu2-3, 112, ara3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1_UAS-GAL1_TATA-HIS3, GAL2_UAS-GAL2_TATA-ADE2, URA3::MEL1_UAS-MEL1_TATA-lacZ | Clontech |
| *Phytophthora capsici* |             |                    |
| Pa28            | Wild type (a Korean isolate) | NIAST, RDAb |
| pGEX-2T         | tac promoter-based expression vector; Amp' | GE Healthcare Bioscience |
| pGEX4T-1        | tac promoter-based expression vector; Amp' | GE Healthcare Bioscience |
| pCR4.1-TOPO     | Cloning and sequencing vector; Amp' | Invitrogen |
| pGEM-T easy     | Cloning and sequencing vector; Amp' | Promega |
| pGADT7-Rec      | AD/library, LEU2, HA epitope; Amp' | Clontech |
| pGADT7          | AD/library, LEU2, HA epitope; Amp' | Clontech |
| pGBK77         | DNA/bait, TRP1, c-Myc epitope; Km' | Clontech |
| pGADT7-T        | AD/T-antigen, control vector (yeast-two hybrid); Amp' | Clontech |
| pGBK77-53      | DNA-BD/p58, control vector (yeast-two hybrid); Km' | Clontech |
| pColdTMII      | cspA promoter-based cold shock expression vector; Amp' | Takara |
| pGEMCAP1       | Cap-Pa28 in pGEM-T Easy; Amp' | This study |
| pGEXCAP1        | Cap-Pa28 in pGEX-2T; Amp' | This study |
| pBDCAP1        | Cap-Pa28 in pGBK77; Km' | This study |
| pADNG-238       | 2,749 bp cDNA fragment including entire *NgRLK1* from *N. glutinosa* cloned into pGADT7-Rec; Amp' | This study |
| pAD-RLK1        | *NgRLK1* in pGADT7; Amp' | This study |
| pAD-RLK1out    | *NgRLK1*322-789 in pGADT7; Amp' | This study |
| pCD-RLK1        | *NgRLK1*322-789 in pColdTMII; Amp' | This study |
| pCD-RLK1out    | *NgRLK1*322-789 in pColdTMII; Amp' | This study |

**a**  Amp', ampicillin resistance; Km', kanamycin resistance  
**b**  National Institute of Agricultural Science and Technology, Rural Development Administration
Expression of capsicein in E. coli

For construction of the glutathione S-transferase (GST)-capsicein fusion protein expression plasmid, the capsicein gene was amplified from capsicein cDNA by PCR using the primers Cap-Pa28F (5′-CGGGATCCATGATGGCA GAACAGACTACAT-3′) and Cap-Pa28R (5′-CCG CTCGAGTTACCTTGTCAGATAAAACTGA-3′). The PCR product was digested with BamHI and EcoRI, followed by cloning into the corresponding sites of pGEX-2T vector containing the GST gene (GE Healthcare Bioscience AB). The E. coli BL21(DE3) strain harboring the cloned gene in pGEX-2T was grown in LB, and expression of the GST-capsicein fusion protein was induced with 0.5 mM IPTG at 37°C for 4 h. The cells were harvested by centrifugation and resuspended in 20 mM Tris–HCl (pH 7.5), 10 mM EDTA, and 1% Triton X-100. The bacterial suspension was sonicated with a VCX-400 sonicator (Sonic & Materials Inc.) to lyse the cells. The insoluble protein was collected by centrifugation (13,000×g for 10 min), solubilized using a CAPS buffer at alkaline pH in combination with N-lauroylsarcosine, and refolded using a protein refolding kit (Novagen). The refolded fusion protein was purified by glutathione Sepharose 4B affinity chromatography (GE Healthcare Bioscience AB) in PBS buffer, followed by GST cleavage (on-column) with thrombin (80 U) during 14 h at 24°C.

Hypersensitivity response (HR) assay

Induction of hypersensitivity by capsicein was determined by petiole dip assays [25]. Leaf petioles of freshly cut leaves of N. glutinosa and Brassica rapa subsp. pekinensis were dipped into a 50 ml sterile distilled water solution of capsicein. The solution was taken up after 2 h. Then leaves were transferred to sterile water. Necrotic lesions were visible after 24–48 h.

Yeast two-hybrid assay

Yeast two-hybrid screening was performed according to the manufacturer’s instructions (BD Matchmaker Library Construction & Screening kits, BD Biosciences Clontech). The PCR-amplified capsicein coding region was cloned into the binding-domain (BD) vector pGBK T7 as bait. A N. glutinosa activation domain (AD) fusion cDNA library was prepared from total RNA extracted from leaves of N. glutinosa. Competent AH109 yeast cells were co-transformed with combinations of the bait plasmid pGBK T7-capsicein, the AD fusion library cDNA, and the linearized activation-domain vector pGADT7-Rec using the LiAc/PEG method. The co-transformants were plated onto minimal medium lacking Ade, His, Leu and Trp and containing 10 mM 3-amino-1, 2, 4-triazole. The plates were incubated at 30°C for 3–5 days, and yeast colonies that grew on the dropout (DO) medium were sub-cultured onto the same medium containing X-a-Gal and assayed for X-a-galactosidase activity using a filter-lift assay. Positive control was produced by co-transformation with pGADT7-T (Clontech), which expresses murine p53 fused with the GAL4 DNA binding domain with N. glutinosa activation domain (AD) fusion cDNA library and sequence data were analyzed using the BLAST X search of NCBI. Interactions of NgRLK1 and extracellular and cellular domains of NgRLK1 with CAP-Pa28 were tested under the same experimental conditions as described above. NgRLK123–438, NgRLK123–438 and NgRLK1522–789 were inserted into pGADT7 (BD Biosciences Clontech) as prey. Yeast transformants harboring bait and prey constructs were plated onto minimal medium containing X-a-Gal without Ade, His, Leu and Trp.

Rapid amplification of cDNA ends (RACE) for full-length NgRLK1 cDNA, and sequence analysis

Total RNA (1 µg) from leaves of N. glutinosa was used as the template. Forward and reverse gene-specific primers (GSPs) were designed based on the sequence of the putative interactor cDNA identified in the yeast two-hybrid screening. RACE was carried out using a SMART RACE cDNA amplification kit (Clontech). PCR was performed on a PE GeneAmp system 9600 (Applied Biosystems) under the following conditions: denaturation at 94°C for 2 min; 30 cycles of 94°C for 5 s, 68°C for 10 s, and 72°C for 3 min; and a final extension at 72°C for 10 min. The PCR product was cloned into TOPO TA vector (Invitrogen) and sequenced. Sequence alignments and open-reading frame translations were performed on DNASTAR 5.05. BLAST analyses of the nucleotide and protein sequences were conducted using programs at the ExPASy (http://www.expasy.org) and NCBI (http://ncbi.nlm.nih.gov/BLAST) websites. The protein structure was predicted as described previously [26, 27] using programs on the SMART website (http://smart.embl-heidelberg.de). The protein-based phylogenetic analysis of NgRLK1 was conducted using
the PhyML program at Phylogeny.fr server (http://www.phylogeny.fr).

GST-pull-down assays

The CAP-Pa28 and NgRLK1 interaction was determined by GST-pull-down assay using the ProFound™ Pull-down GST Protein:Protein interaction kit (Pierce), as described by the manufacturer. For the preparation of prey proteins, pCold™II vectors (Takara) containing the cDNA coding sequence for the extracellular domain (NgRLK1 23–438) and the cellular domain (NgRLK1 522–789) of NgRLK1 were transformed into E. coli BL21. The coding sequences of the extracellular and cellular domains were amplified by PCR using NgRLK1 cDNA as the template and the PCR primer sets RLK1out-F (5'-ATTCCATATGGCTATATCCAAA TTTC-3') and RLK1out-R (5'-CGGGATCCTTACACC TTAAGAATTACT-3') for the extracellular domain, and RLK1in-F (5'-AATTCCATATGGATTTCCACAAAG CTT-3') and RLK1in-R (5'-CGGGATCCTTACAGTGCC TTAACCACC-3') for the cellular domain. The PCR products were cloned into the NdeI/BamHI sites of the pCold™II vector. The transformants were grown in LB, and expression of the His-tagged NgRLK1 23–438 and NgRLK1 522–789 was induced with 0.1 mM IPTG at 15°C for 24 h. The protein solubilization and refolding procedures were as described above. The refolded fusion protein was loaded onto a Ni-NTA spin column (Qiagen). His-tagged protein was eluted by incubating the beads with 100 mM glutathione-Sepharose beads, which were equilibrated with the PBS buffer (pH 7.4) in the Handee™ Mini-Spin column (Pierce). Purified His6–NgRLK1 23–438 and His6–NgRLK1 522–789 were used as prey proteins to detect interactions with the bait protein. The bait-prey proteins were eluted by incubating the beads with 100 mM glutathione for 10 min at 25°C and collected by centrifuging at 1,250g for 1 min at 25°C. Immunoblotting was used to detect His6–NgRLK1 23–438 and His6–NgRLK1 522–789 using anti-6×His tag antibody (Takara).

Autophosphorylation assay of the kinase domain of NgRLK1

For protein expression of the kinase domain of NgRLK1, the GST fusion protein expression vector pGEX4T-1 (GE Healthcare Bioscience) was used. The kinase domain of NgRLK1 (NgRLK1 522–789) was amplified by PCR using NgRLK1 cDNA as the template and specific PCR primers, rlkD-F (5'-CGGGATCCTTATCCACAAAGCGTGG GGAA-3') and rlkD-R (5'-CGGCTGAGCGTGCCTTA ACCACCATGTCAT-3'). The PCR product was digested with BamHI and Xhol, followed by cloning into the corresponding sites of pGEX4T-1 vector. The NgRLK1 522–789 DNA fragment in pGEX4T-1 was sequenced to confirm correct insertion, and the vector was introduced into E. coli BL21(DE3). The transformants were grown in LB containing ampicillin; when the culture reached an optical density of 0.6–0.8 at 600 nm, expression of the GST-capsicein fusion protein was induced with 1 mM IPTG at 37°C for 4 h. The protein solubilization, refolding, and purification procedures were as described above. A gel-based autophosphorylation assay [28] was performed in a 20-µl volume containing 1 µg of GST–NgRLK1 522–789 protein, 25 mM Heps (pH 7.5), 10 mM MnCl2 or MgCl2, and 10 µCi [γ-32P]ATP. After incubation at 30°C for 30 min, the reaction was quenched by the addition of SDS–PAGE electrode buffer. The phosphorylated protein was separated by SDS–PAGE, and the gel was exposed to a PhosphoImage screen (Fuji). The radioactivity incorporated into GST–NgRLK1 522–789 on the gel was detected using a Bio-Rad FX molecular imager system.

Results

Isolation and sequencing of capsicein CAP-Pa28

Capsicein was purified from P. capsici Pa28 culture medium by a one-step chromatographic procedure. We collected the fractions containing the single capsicein peak and confirmed the presence of a 10-kDa protein band by SDS–PAGE analysis (Fig. 1a, b). The N-terminal amino acid sequence derived from the purified SDS–PAGE protein band was NH3-ATCTTTQTAAYVALVSILSDSSFN-COOH, which matched the previously reported capsicein sequence [29]. This capsicein was named CAP-Pa28. Purified CAP-Pa28 induced a hypersensitive response (HR) in the leaves of N. glutinosa and B. rapa subsp. pekinensis (Fig. 1c) but not Capsicum annuum. We cloned the CAP-Pa28 gene by RT-PCR of total RNA extracted from P. capsici and degenerate primers based on conserved domains of known elicitors from Phytophthora, and sequenced the CAP-Pa28 clone (accession no. AF461432; Fig. 2). Similar to other Phytophthora elicitors, CAP-Pa28 comprises 98 amino acid residues, has a molecular weight of 10.16 kDa, and lacks Trp, His, and Arg. CAP-Pa28 has an acidic isoelectric point (pI = 4.23), characteristic of the α-elicitins.
Induction of HR by recombinant CAP-Pa28

GST-fused recombinant CAP-Pa28 (GST–CAP-Pa28) was overexpressed in *E. coli*, collected, solubilized, and refolded. The refolded protein was purified by chromatography on a GST-affinity column, and thrombin was used to remove the GST tag while on the column (Fig. 3a). Recombinant GST–CAP-Pa28 and CAP-Pa28 induced HR in the tobacco plant, *N. glutinosa* (Fig. 3b).

Identification of proteins from *N. glutinosa* that interact with CAP-Pa28

To identify proteins that potentially interact with CAP-Pa28, we performed a yeast two-hybrid assay. First, the CAP-Pa28 cDNA was fused to the GAL4 DNA-binding domain in the bait vector (pGBK7). Next, cDNA made by reverse transcription of *N. glutinosa* RNA was used to construct an activation domain (AD) fusion library. Co-transformation of yeast cells with the bait vector, the AD fusion library, and linearized pGADT7-Rec allowed the screening of $5.7 \times 10^5$ clones. A total of 358 positive clones were obtained by selection with amino acid dropout medium and an Xα-galactosidase assay (data not shown).

**Fig. 1** Purification of capsicein from *P. capsici* Pa28 and the induction of a hypersensitive response by CAP-Pa28. a Elution of capsicein from a Superdex 200 HR gel filtration column. b SDS-PAGE of capsicein, purified from culture filtrate. M, molecular size markers; 1, dialyzed culture filtrate after 95% ammonium sulfate precipitation; 2, after filtering through 0.2 μm pore size membrane (Whatman); 3, CAP-Pa28 fraction from FPLC gel filtration. c The hypersensitive response induced in *Brassica rapa* subsp. *pekinesis* and *Nicotiana glutinosa* leaves by 50 nmol of purified CAP-Pa28.

**Fig. 2** Nucleotide and amino acid sequences of CAP-Pa28. Underlined amino acid sequences were obtained from N-terminal amino acid sequencing.

**Fig. 3** Purification and the hypersensitive-response activity of recombinant capsicein GST–Cap-Pa28. a SDS–PAGE analysis of GST–Cap-Pa28. M: molecular size markers, 1: solubilized and refolded GST–Cap-Pa28; 2: Purified GST–Cap-Pa28; 3: Cleaved Cap-Pa28, after thrombin treatment. b Induction of the hypersensitive response by GST–Cap-Pa28 in tobacco (*Nicotiana glutinosa*) leaves. CAP, purified Cap-Pa28 from *Phytophthora capsici* Pa28; GST–CAP, GST–Cap-Pa28. The hypersensitive response was detected 18 h after the treatment of 10–50 nmol of protein.
The positive clones were individually isolated and sequenced, and one (pADNG-238) of these clones showed high similarity to the Ser/Thr kinase domain of many plant receptor-like kinases (RLKs). To obtain the full length of this gene for sequencing, RACE was performed.

Isolation of the NgRLK1 and its sequence analysis

The nucleotide sequence of the clone obtained from the yeast-two hybrid assay was determined, revealing a 2,749-bp in length with a 2,499-bp ORF encoding a polypeptide of 832 amino acids. This gene was designated NgRLK1. The amino acid sequence of NgRLK1 is 33% and 27.6% identical to sequences of RLKs from Arabidopsis thaliana and Oryza sativa, respectively. In addition, NgRLK1 shares sequence similarity with RLKs of Brassica oleracea (26.7% identity) and Zea mays (24.9% identity) (Fig. 4a). The calculated molecular mass of NgRLK1 is 93,490 Da, and the pI is 6.67. The structural features of NgRLK1 are typical of a receptor-like kinase. It is composed of three main domains: a putative extracellular domain, a single transmembrane domain, and a C-terminal cytoplasmic Ser/Thr protein kinase domain. The putative extracellular domain contains an N-terminal signal sequence, a B-lectin domain, an S-locus glycoprotein domain, and a PAN AP domain (Fig. 4b). The transmembrane segment consists primarily of hydrophobic amino acids, and the kinase domain has about 40% sequence similarity to other plant RLKs. The putative extracellular domain showed a low level of sequence identity (20–25%), which is considered a determinant factor of ligand specificity [30]. The NgRLK1 sequence has been deposited in GenBank (accession no. FJ217819). In phylogenetic analysis of NgRLK1, NgRLK1 is most closely related to PR5K (PR5-like receptor kinase) of A. thaliana [31] (Fig. 5).

**Fig. 4** NgRLK1 amino acid sequence and its predicted domain structure. **a** Amino acid sequence alignment of NgRLK1 with members of the receptor-like kinase family. Identical amino acids are represented by solid black lines. Spaces indicate gaps introduced to maximize the alignment. The other RLKs are indicated by their EMBL accession numbers: NP_198644 (Arabidopsis thaliana), O49974 (Zea mays), P93068 (Brassica oleracea), and Q9LLN5 (Oryza sativa). **b** Schematic representation of NgRLK1 based on a conserved domain analysis. SP, signal peptide; TM, transmembrane...
Interaction of CAP-Pa28 and NgRLK1

Interaction between CAP-Pa28 and NgRLK1 could be observed when NgRLK1 was used as bait and CAP-Pa28 as prey in a yeast-two hybrid assay (Fig. 6). To identify the domain of NgRLK1 interacting with CAP-Pa28, a binding test of the extracellular and cellular domains of NgRLK1 with CAP-Pa28 was conducted using the yeast-two hybrid assay. Interestingly, the extracellular and cellular domains, respectively, interacted with CAP-Pa28 (Fig. 6). To further confirm the interaction between CAP-Pa28 and NgRLK1, we performed a pull-down assay using purified His\textsubscript{6}–NgRLK\textsubscript{123–438} and His\textsubscript{6}–NgRLK\textsubscript{1522–789} incubated with immobilized GST or GST–CAP-Pa28. In this assay, His\textsubscript{6}–NgRLK\textsubscript{123–438} and His\textsubscript{6}–NgRLK\textsubscript{1522–789} interacted with GST–CAP-Pa28 but not with GST alone (Fig. 7). These results indicated that CAP-Pa28 can bind directly to the extracellular and cellular domains of NgRLK1, which is consistent with the results of the yeast two-hybrid assay.

Autophosphorylation of the kinase domain of NgRLK1

For biochemical studies investigating kinase activity, the kinase domain of NgRLK1 (NgRLK\textsubscript{1522–789}) was expressed as a fusion protein with GST in E. coli (Fig. 8a). In the presence of ATP and Mn\textsuperscript{2+} or Mg\textsuperscript{2+}, GST–NgRLK\textsubscript{1522–789} was autophosphorylated, with higher activity in the presence of Mn\textsuperscript{2+} (Fig. 8b). GST–NgRLK\textsubscript{1522–789} activated by Mn\textsuperscript{2+} showed a diffuse band and lower mobility than GST–NgRLK\textsubscript{1522–789} activated by Mg\textsuperscript{2+} in SDS–PAGE with CBB staining (Fig. 8b).

Discussion

Although many elicitors from Phytophthora species have been reported, it is not clear whether elicitors contribute directly to Phytophthora pathogenicity. Elicitins trigger a plant defense response in most Nicotiana species, and this response is sufficient to protect against infection by not only Phytophthora but also bacteria, fungi, and viruses [3]. A prominent symptom of the defense response is the hypersensitive response (HR), which results from incompatible interactions between a plant and a pathogen. The HR involves rapid cell death of infected plant tissues and the elaboration of inducible defense proteins. The way in which elicitors induce the HR is puzzling. Elicitins are extracellular proteins, and thus it would seem likely that an elicitin receptor would be located in the plant plasma membrane. Identifying the receptor responsible for the elicitin response is of considerable interest, as most Phytophthora species produce elicittins and therefore an elicitin receptor should confer broad-spectrum Phytophthora resistance [32–34].

In this study, we isolated a gene encoding capsicein CAP-Pa28 from P. capsici Pa28 and identified a capsicein-interacting protein, which is homologous to plant RLKs, from N. glutinosa using a yeast two-hybrid assay. RLKs are unusual membrane-associated plant protein kinases, some of which have important roles in pathogen resistance [35]. An RLK comprises a putative extracellular domain, a transmembrane domain, and a protein kinase domain. All RLKs identified in plants thus far have a Ser/Thr kinase domain, in contrast to the tyrosine-specific kinase domain common in animals. NgRLK1 has a domain structure similar to that of plant RLKs. Based on the structure of the putative extracellular domain, Satterlee et al. divided plant receptor kinases into several classes [35]: those with an extracellular domain homologous to S-locus glycoproteins [36], homologous to putative carbohydrate-binding lectins.
or homologous to pathogenesis-related (PR5) proteins [31]; some RLKs have extracellular domains with a variable number of leucine-rich repeats (LRRs) [38]. Interestingly, the extracellular domain of NgRLK1 contains both lectin-like and S-locus glycoprotein domains, in addition to a PAN AP domain, which is known to mediate protein–protein or protein–carbohydrate interactions [39]. The domain structure of NgRLK1 is similar to that of the S receptor kinase (SRK) from B. oleracea [40]. It has been shown that SRK mediates the self-incompatibility response in Brassicaceae [41]. Recently, Kanzaki et al. reported on NbLRK1, which is a lectin-like receptor kinase protein of N. benthamiana that interacts with INF1 elicitin of P. infestans [23]. NbLRK1 plays an important role in triggering the INF1-mediated HR signal downstream. Kanzaki et al. also demonstrated that INF1 binds to the intracellular kinase domain of NbLRK1 in a yeast-two hybrid assay. This result suggested that plant recognition of INF1 takes place inside the plant cells. Unlike NbLRK1, extracellular NgRLK1 was found to interact with CAP-Pa28 in yeast-two hybrid and GST-pull-down assays, and the structure of the NgRLK1 extracellular domain suggests possible interactions with proteins or carbohydrates. This evidence supported a direct interaction of the extracellular domain of NgRLK1 with CAP-Pa28. Interestingly, CAP-Pa28 was also found to bind to the intracellular kinase domain of NgRLK1. Kanzaki et al. proposed that the INF1 protein could be transported inside plant cells by endocytosis or an unknown mechanism to interact with the kinase domain of NbLRK1 [23]. Based on that report and our findings, we speculate that plant recognition of CAP-Pa28 occurs in the extracellular and intracellular spaces. To support this hypothesis, plant cell localization of CAP-Pa28 should be confirmed.

In protein-based phylogenetic analysis, NgRLK1 was more closely related to PR5K from A. thaliana than to the lectin-like receptor kinases, which include NbLRK1, and other plant RLKs. Wang et al. suggested that PR5K receptor is involved in the perception of microbial signals [31]. The analysis of phylogenetic and domain-structure of NgRLK1 implies that NgRLK1 might play a role as receptor for pathogen-derived signal molecules like...
elicits, and we propose that NgRLK1 is a new type of plant RLK that recognize Cap-Pa28.

The recombinant Ser/Thr kinase domain of NgRLK1 had autophosphorylation activity, and this activity was higher with Mn\(^{2+}\) than with Mg\(^{2+}\), as previously reported for other plant RLKs [42–44]. Compared with GST–NgRLK1\(^{522–789}\) activated by Mg\(^{2+}\), GST–NgRLK1\(^{522–789}\) activated by Mn\(^{2+}\) gave a more diffuse band of lower mobility on SDS-PAGE, suggesting that GST–NgRLK1\(^{522–789}\) undergoes a conformational change upon enzymatic activation. This result shows that NgRLK1 encodes an active protein kinase and raises the possibility that NgRLK1 is involved in the HR signaling pathway in *N. glutinosa*.

Southern blot analysis indicated that there is more than one copy of NgRLK1 in *N. glutinosa* (Y. T. Kim, unpublished data), whereas it has been reported that RLKs from *Petunia inflata*, *B. napus*, and *Catharanthus roseus* exist only as single copies [42, 45, 46].

Here, we report using a yeast-two hybrid assay to isolate a plant RLK based on its interaction with capsicein. We propose that NgRLK1 represents a new kind of plant RLK and is a potential capsicein receptor. To verify this, it will be necessary to determine whether NgRLK1 functions as a capsicein receptor in vivo. The data presented in this study showing that NgRLK1 is activated by Mg\(^{2+}\) but not with Mn\(^{2+}\), and gives a more diffuse band of lower mobility on SDS–PAGE, suggesting that GST–NgRLK1 522–789 undergoes a conformational change upon enzymatic activation. This result shows that NgRLK1 encodes an active protein kinase and raises the possibility that NgRLK1 is involved in the HR signaling pathway in *N. glutinosa*.

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