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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*

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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 elicitins, 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. cinnamomi* [13], although the elicitin gene from *P. capsici* has not yet been cloned. All known *Phytophthora* elicitins 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 elicitins of *Phytophthora* have been divided into acidic (α)- and basic (*β*)-elicitins [14, 15]. The *β*-elicitins produce greater necrosis than the α-elicitins, 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 elicitins and elicitin-like proteins of *Phytophthora* and *Pythium* species into five classes based on phylogenic 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 elicitins are thought to be major determinants of non-host resistance against *Phytophthora* infection in *Nicotiana* species [5, 19], the role of elicitins in the biology of *Phytophthora* are not clear, and little is known about the molecular mechanisms involved in elicitin activity. Specific, high-affinity binding sites for β-cryptogein 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 elicitins is as sterol scavengers, because they are known to be sterol carrier proteins and *Phytophthora* species do not synthesize sterols [22].

Elicitins 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 elicitins interact with a host molecule in the plant plasma membrane to initiate plant cell responses following elicitin recognition. Despite the identification of many elicitin proteins, membrane receptors for elicitins 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 elicitin response in tobacco, but its expression in tomato or petunia did not result in gain of an elicitin response in those plants. Tyler suggested that additional membrane receptor proteins may be required for the elicitin response. Kanzaki et al. isolated and characterized NbLRK1, which is a *P. infestans* INF1 elictin-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 elicitin 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 autophosphorylated 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 elicitin 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_2PO_4, 0.25 g of $MgSO_4{\cdot}7H_2O$, 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 (Bio-Rad) 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

Table 1 Bacterial strains and plasmids

Strains/plasmids	Characteristics[a]	Sources/references
Strains		
Escherichia coli		
DH5α	F$^-$ Φ80d*lac*ZΔM15 Δ(*lacZYA-argF*)*U169 endA1 deoR recA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1gyrA96 relA1*	Gibco-BRL
BL21	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^+) *gal dcm*	Takara
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^+) *gal dcm* λ(DE3)	Novagen
Top10	F$^-$*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
Saccharomyces cerevisiae		
AH109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ*, gal80*Δ*, 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, RDA[b]
Plasmids		
pGEX-2T	tac promoter-based expression vector; Amp^r	GE Healthcare Bioscience
pGEX4T-1	tac promoter-based expression vector; Amp^r	GE Healthcare Bioscience
pCR4.1-TOPO	Cloning and sequencing vector; Amp^r	Invitrogen
pGEM-T easy	Cloning and sequencing vector; Amp^r	Promega
pGADT7-Rec	AD/library, *LEU2*, HA epitope; Amp^r	Clontech
pGADT7	AD/library, *LEU2*, HA epitope; Amp^r	Clontech
pGBKT7	DNA/bait, *TRP1*, c-Myc epitope; Km^r	Clontech
pGADT7-T	AD/T-antigen, control vector (yeast-two hybrid); Amp^r	Clontech
PGBKT7-53	DNA-BD/p58, control vector (yeast-two hybrid); Km^r	Clontech
pCold^{TM}II	*csp*A promoter-based cold shock expression vector; Amp^r	Takara
pGEMCAP1	*Cap-Pa28* in pGEM-T Easy; Amp^r	This study
pGEXCAP1	*Cap-Pa28* in pGEX-2T; Amp^r	This study
pBDCAP1	*Cap-Pa28* in pGBKT7; Km^r	This study
pADNG-238	2,749 bp cDNA fragment including entire *NgRLK1* from *N. glutinosa* cloned into pGADT7-Rec; Amp^r	This study
pAD-RLK1	*NgRLK1* in pGADT7; Amp^r	This study
pAD-RLK1out	*NgRLK1*$^{23-438}$ in pGADT7; Amp^r	This study
PAD-RLK11n	*NgRLK1*$^{522-789}$ in pGADT7; Amp^r	This study
pGX4-RLK1in	*NgRLK1*$^{522-789}$ in pGEX4T-1; Amp^r	This study
pCD-RLK1out	*NgRLK1*$^{23-438}$ in pCold^{TM}II; Amp^r	This study
pCD-RLK1in	*NgRLK1*$^{522-789}$ in pCold^{TM}II; Amp^r	This study

[a] Amp^r, ampicillin resistance; Km^r, kanamycin resistance

[b] National Institute of Agricultural Science and Technology, Rural Development Administration

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′-GCRCABGTKGCYG ARAADCCRTT-3′), that were designed based on the alignment of the amino acid sequence determined for capsicein and the sequences of conserved domains of known elicitins taken from the ExPASy database (www.expasy.org). The synthesis of capsicein 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

1 min. The PCR product was gel purified and cloned into pGEM-T Easy vector (Promega) for sequencing by an ABI Prism 3100 automated DNA sequencer (Applied Biosystems). The nucleotide and amino acid sequences were analyzed using the BLASTX program (http://ncbi.nlm.nih.gov/BLAST, http://www.expasy.org/tools).

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′-CGGGATCCATGATTGCA GAACAAAGACTACAT-3′) and Cap-Pa28R (5′-CCG CTCGAGTTACCTTGGTCCAGATAAAACTGA-3′). The PCR product was digested with *Bam*HI and *Eco*RI, 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 (Sonics & 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 pGBKT7 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 pGBKT7-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-α-Gal and assayed for X-α-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 BD and pGBKT7-p53 (Clontech), expressing the SV40 large T-antigen fused to the GAL 4 AD. The cDNA fragments in pGADT7-Rec isolated from positive colonies of the X-α-galactosidase assay were sequenced, and the cDNA sequence data were analyzed using the BLAST X search of NCBI. Interactions of NgRLK1 and extracelluar and cellular domains of NgRLK1 with CAP-Pa28 were tested under the same experimental conditions as described above. $NgRLK1^{23–789}$, $NgRLK1^{23–438}$ and $NgRLK1^{522–789}$ were inserted into pGADT7 (BD Biosciences Clontech) as prey. Yeast transformants harboring bait and prey constructs were plated onto minimal medium containing X-α-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′-ATTCCATATGGCTATATCCCAAA TTTC-3′) and RLK1out-R (5′-CGGGATCCTTACACC TTAAGAAATACT-3′) for the extracellular domain, and RLK1in-F (5′-AATTCCATATGGATTTCAGCACAAAG CTT-3′) and RLK1in-R (5′-CGGGATCCTTACAGTGCC TTAACCACC-3′) for the cellular domain. The PCR products were cloned into the *Nde*I/*Bam*HI 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 bound to the Ni-NTA matrix by centrifugation at 1,000*g* for 2 min at room temperature. The protein binding matrix was washed two times with washing buffer (20 mM imidazole, 20 mM Tris–HCl, pH 8.0) to remove unbound protein. His-tagged protein was eluted with elution buffer (500 mM imidazole, 20 mM Tris–HCl, pH 8.0), and the eluted protein was dialyzed against 20 mM Tris–HCl (pH 8.0) to remove imidazole. The purified GST-CAP-Pa28 (bait protein) and parental GST (control) were immobilized on 50 µl of glutathione-Sepharose beads, which were equilibrated with the PBS buffer (pH 7.4) in the Handee™ Mini-Spin column (Pierce). Purified His_6–NgRLK1$^{23–438}$ and His_6–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 1,250*g* for 1 min at 25°C. Immunoblotting was used to detect His_6–NgRLK1$^{23–438}$ and His_6–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′-CGGGATCCGATTTCAGCACAAAGCTTGG GGAA-3′) and rlkD-R (5′-CCGCTCGAGCAGTGCCTTA ACCACCCATGTCAT-3′). The PCR product was digested with *Bam*HI and *Xho*I, 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 Hepes (pH 7.5), 10 mM $MnCl_2$ or $MgCl_2$, and 10 µCi [γ-^{32}P]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 NH_3-ATCTTTQQTAAYVALVSILSDSSFN-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 elicitins from *Phytophthora*, and sequenced the CAP-Pa28 clone (accession no. AF461432; Fig. 2). Similar to other *Phytophthora* elicitins, 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.

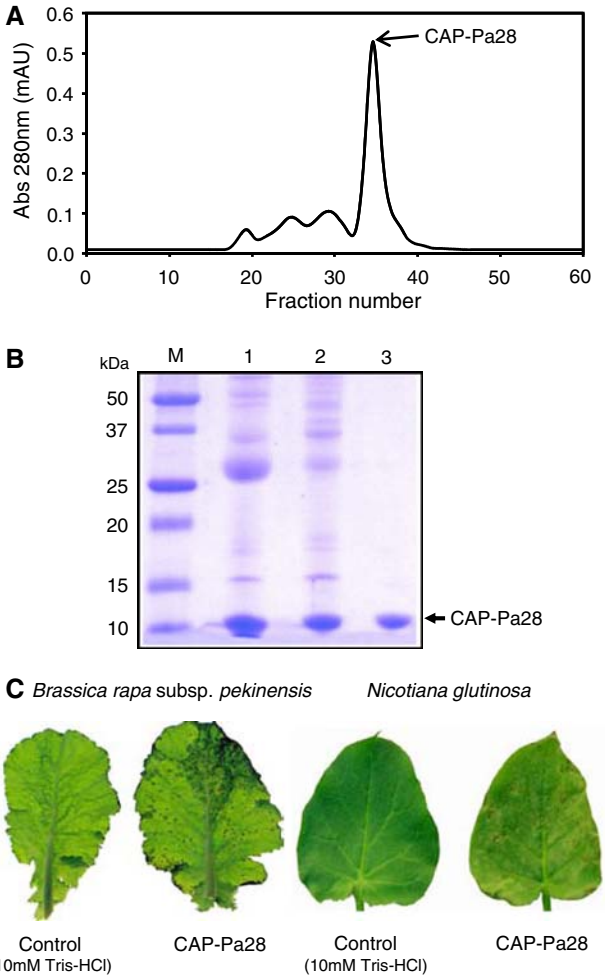

Fig. 1 Purification of capsicein from *P. capsici* Pa28 and the induction of a hypersensitive response by CAP-Pa28. **a** Elution of capsicein from a Superdex 200 HR gel filtration column. **b** SDS–PAGE of capsicein, purified from culture filtrate. M, molecular size markers; 1, dialyzed culture filtrate 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. *pekinensis* and *Nicotiana glutinosa* leaves by 50 nmol of purified CAP-Pa28

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  1 GCCACGTGTACGACTACCCAGCAAACGGCCGCCTACGTCGCCCTGGTG  48
  1 A  T  C  T  T   T  Q  Q  T  A  A  Y  V  A  L  V   16
 49 AGCATCCTCTCGGACTCTTCGTTCAACCAGTGCGCCACGGACTCTGGC  96
 17 S  I  L  S  D  S  S  F  N  Q  C  A  T  D  S  G   32
 97 TACTCGATGCTGACGGCCACGGCGCTGCCCACGACGGCGCAGTACAAG 144
 33 Y  S  M  L  T  A  T  A  L  P  T  T  A  Q  Y  K   48
145 CTCATGTGCGCGTCGACGGCGTGCAACACGATGATCACCAAGATCGTG 192
 49 L  M  C  A  S  T  A  C  N  T  M  I  T  K  I  V   64
193 TCGCTGAACCCCCCTGACTGCGAGCTGACGGTGCCCACGAGCGGTCTG 240
 65 S  L  N  P  P  D  C  E  L  T  V  P  T  S  G  L   80
241 GTGCTCAACGTTTACTCGTACGCGAATGGATTTTCAGCAACATGCGCA 288
 81 V  L  N  V  Y  S  Y  A  N  G  F  S  A  T  C  A   96
289 AGCCTT                                          294
 97 S  L                                             98
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Fig. 2 Nucleotide and amino acid sequences of CAP-Pa28. Underlined amino acid sequences were obtained from N-terminal amino acid sequencing

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 (pGBKT7). 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 5.7×10^5 clones. A total of 358 positive clones were obtained by selection with amino acid dropout medium and an X-α-galactosidase assay (data not shown).

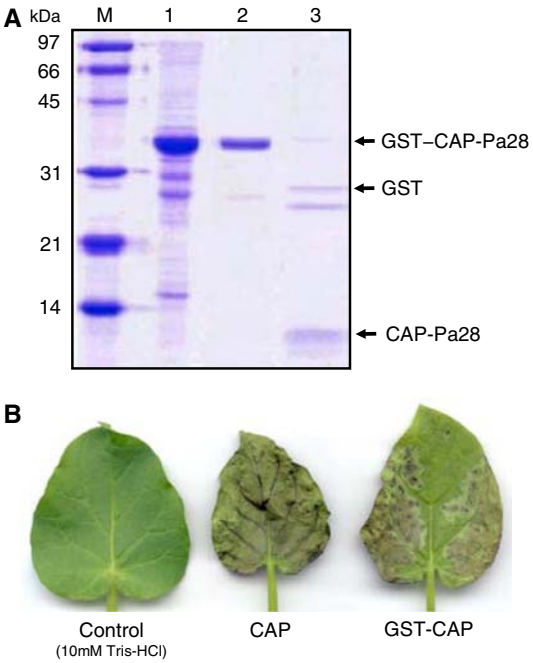

Fig. 3 Purification and the hypersensitive-response activity of recombinant capsicein GST–Cap-Pa28. **a** SDS–PAGE analysis of GST–Cap-Pa28. M: molecular size markers, 1: solubilized and refolded GST–Cap-Pa28; 2: Purified GST–Cap-Pa28; 3: Cleaved Cap-Pa28, after thrombin treatment. **b** Induction of 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).

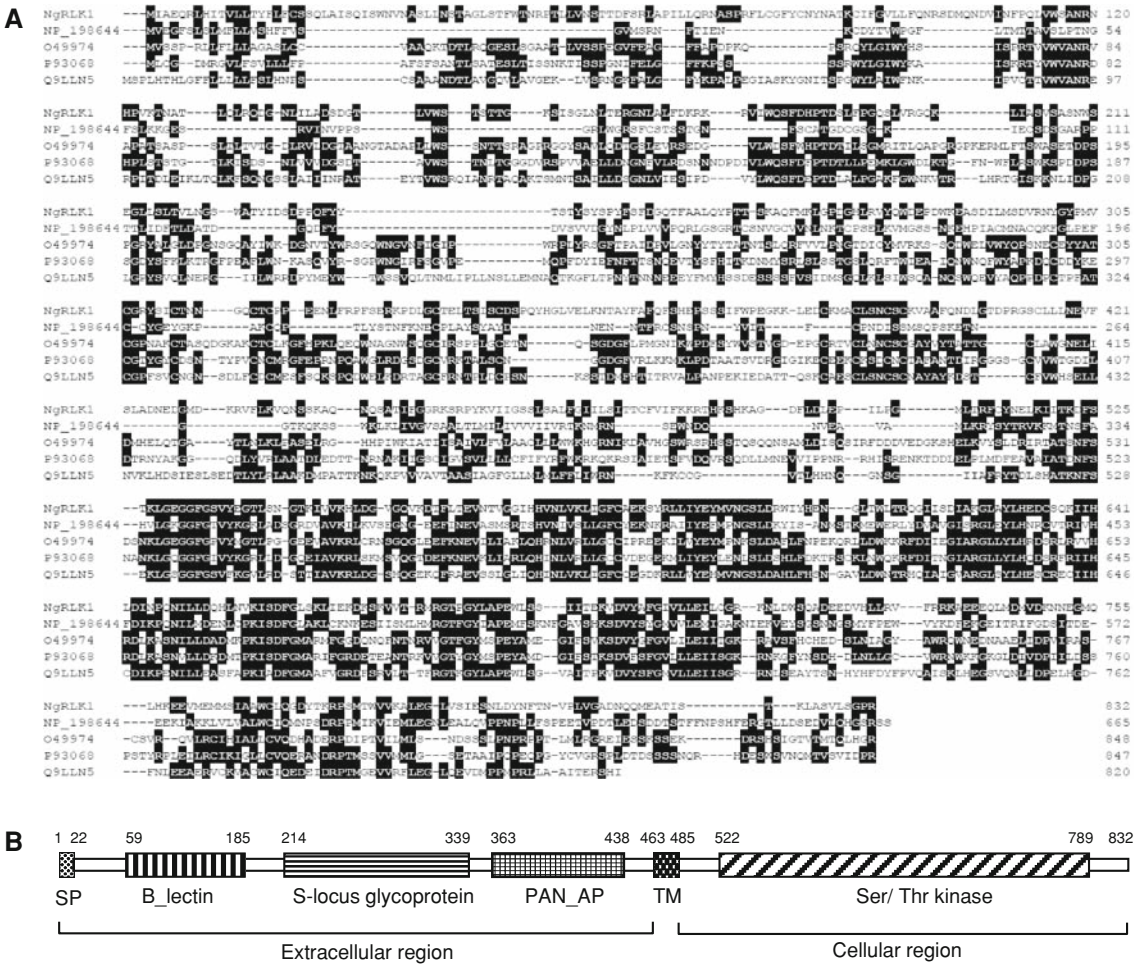

Fig. 4 NgRLK1 amino acid sequence and its predicted domain structure. **a** Amino acid sequence alignment of *NgRLK1* with members of the receptor-like kinase family. Identical amino acids are represented by solid black lines. Spaces indicate gaps introduced to maximize the alignment. The other RLKs 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

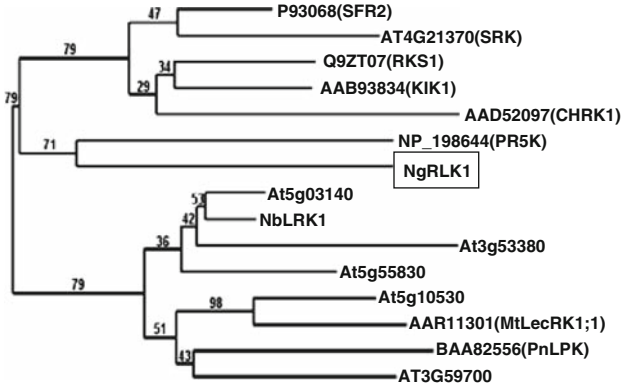

Fig. 5 Phylogenetic tree for the known plant receptor-like kinases (RLKs) including NgRLK1. Maximum likelihood analysis was performed using PhyML program on the Phylogeny.fr server (http://www.phylogeny.fr). The amino acid sequences of plant RLKs were obtained from Genbank. Bootstrap values from 500 bootstrap replicates are shown next to the branches

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_6–$NgRLK1^{23–438}$ and His_6–$NgRLK1^{522–789}$ incubated with immobilized GST or GST–CAP-Pa28. In this assay, His_6–$NgRLK1^{23–438}$ and His_6–$NgRLK1^{522–789}$ 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.

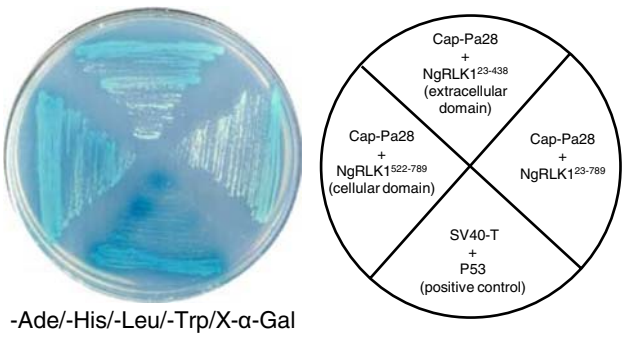

Fig. 6 Protein-protein interaction of the CAP-Pa28 and NgRLK1 domains (yeast-two hybrid assay). Yeast cells harboring the bait and prey plasmid pairs were grown and showed blue color on selective medium lacking Ade, His, Leu, and Trp with X-α-Gal at 30°C. pGBKT7-53 and pGADT7-T were co-transformed into AH109 as positive control

Autophosphorylation of the kinase domain of NgRLK1

For biochemical studies investigating kinase activity, the kinase domain of NgRLK1 ($NgRLK1^{522–789}$) was expressed as a fusion protein with GST in *E. coli* (Fig. 8a). In the presence of ATP and Mn^{2+} or Mg^{2+}, GST–$NgRLK1^{522–789}$ was autophosphorylated, with higher activity in the presence of Mn^{2+} (Fig. 8b). GST–$NgRLK1^{522–789}$ activated by Mn^{2+} showed a diffuse band and lower mobility than GST–$NgRLK1^{522–789}$ activated by Mg^{2+} in SDS–PAGE with CBB staining (Fig. 8b).

Discussion

Although many elicitins from *Phytophthora* species have been reported, it is not clear whether elicitins 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 elicitins 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 elicitins 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

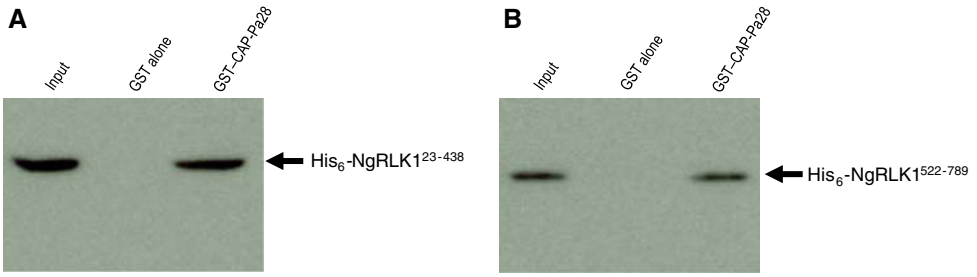

Fig. 7 Direct interaction between CAP-Pa28 and the extracellular and cellular domains of NgRLK1 *in vitro* (GST-pull-down assay). **a** GST-pull-down assay to test the binding of His_6–$NgRLK1^{23–438}$ with GST and GST–CAP-Pa28. The arrow indicates the positions of His_6–$NgRLK1^{23–438}$ (48.2 kDa). **b** GST-pull-down assay to test the binding of His_6–$NgRLK1^{522–789}$ with GST and GST–CAP-Pa28. The arrow indicates the positions of His_6–$NgRLK1^{522–789}$ (32.6 kDa). The bound proteins were eluted, separated by SDS–PAGE, and immunoblotted with anti-6×His tagged antibody (Takara)

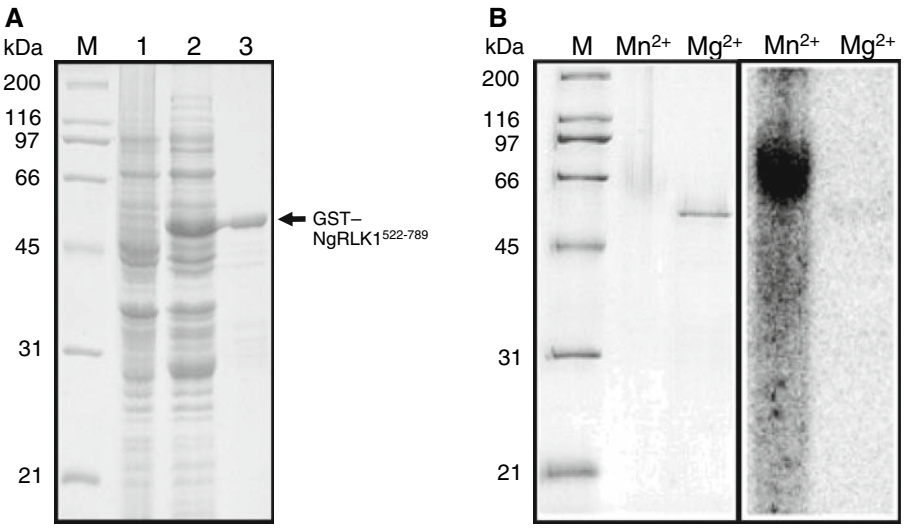

Fig. 8 Purification and autophosphorylation assay of GST–$NgRLK1^{522–789}$. **a** SDS–PAGE analysis of GST–$NgRLK1^{522–789}$. M, molecular size markers; 1, Non-induced; 2, IPTG-induced; 3, Purified GST–$NgRLK1^{522–789}$. GST–$NgRLK1^{522–789}$ was detected by CBB staining. **b** autophosphorylation activity of GST–$NgRLK1^{522–789}$. Autoradiogram (*right*) and stained gel (*left*) are shown

[37], 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

elicitins, 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 contribute to an understanding of the biochemical and physiological functions of plant RLKs and the mechanism of the elicitin-induced defense response. These findings may have important implications for the molecular breeding of disease-resistance in crops.

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