

# NbLRK1, a lectin-like receptor kinase protein of *Nicotiana benthamiana*, interacts with *Phytophthora infestans* INF1 elicitor and mediates INF1-induced cell death

Hiroyuki Kanzaki · Hiromasa Saitoh ·  
Yoshihiro Takahashi · Thomas Berberich · Akiko Ito ·  
Sophien Kamoun · Ryohei Terauchi

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**Abstract** *Phytophthora infestans* INF1 elicitor causes the hypersensitive response (HR) in *Nicotiana benthamiana* (Kamoun et al. in Plant Cell 10:1413–1425, 1998). To identify *N. benthamiana* proteins that interact with INF1, we carried out a yeast two-hybrid screen. This screen resulted in the isolation of a gene *NbLRK1* coding for a novel lectin-like receptor kinase. NbLRK1 interacted with INF1 through its VIb kinase subdomain. Purified INF1 and NbLRK1 proteins also interacted in vitro. INF1 treatment of *N. benthamiana* leaves induced autophosphorylation of NbLRK1. Most importantly, virus-induced gene silencing (VIGS) of *NbLRK1* delayed INF1-mediated HR in *N. benthamiana*. These data suggest that NbLRK1 is a component of the *N. benthamiana* protein complex that recognizes INF1 elicitor and transduces the HR signal.

**Keywords** Autophosphorylation · Hypersensitive response · INF1 · NbLRK1 · *Nicotiana* · Virus-induced gene silencing · Y2H

## Abbreviations

HR	Hypersensitive response
LRK	Lectin-like receptor kinase
MBP	Myelin basic protein
VIGS	Virus-induced gene silencing
Y2H	Yeast two-hybrid assay

## Introduction

In the natural environment, plants are continuously exposed to attacks by a variety of microbial pathogens. Plants have therefore evolved diverse mechanisms to recognize invading pathogens and effectively protect themselves. Cultivars of the host species harboring a disease resistance (*R*-) gene exhibit resistance to a particular race of a pathogen that has a cognate avirulence (*Avr*-) gene. Molecular mechanisms of this gene-for-gene resistance have been extensively studied over the last decade (see Martin 1999; Ellis et al. 2000 for reviews). *R* gene products recognize pathogen invasion either by directly interacting with pathogen effector molecules or by interacting with host proteins modified by the pathogen effectors (Dangl and Jones 2001). Cloned *R* gene products are variable: the majority seem to be cytoplasmic proteins harboring the nucleotide binding site and leucine rich repeats (NBS–LRR) domains, but others include transmembrane proteins with the LRR domain or cytoplasmic proteins with kinase domain (Martin et al. 2003; Belkhadir et al. 2004). NBS–LRR proteins are believed to detect avirulence proteins that have entered host cytoplasm, whereas the latter detects avirulence proteins localized in host extracellular space (Ellis et al. 2006). Recent studies have shown that plants are, like animals, equipped with another layer of defense called innate immunity (reviewed in Zipfel and Felix 2005). Plants recognize pathogens

H. Kanzaki · H. Saitoh · Y. Takahashi · T. Berberich ·  
A. Ito · R. Terauchi (✉)  
Iwate Biotechnology Research Center,  
22-174-4 Narita, Kitakami, Iwate 024-0003, Japan  
e-mail: terauchi@ibrc.or.jp

R. Terauchi  
The United Graduate School of Agricultural Sciences,  
Iwate University, 3-18-8, Ueda, Morioka, Iwate 020-8550, Japan

S. Kamoun  
The Sainsbury Laboratory, John Innes Centre, Norwich, UK

through perception of pathogen-associated molecular patterns (PAMPs). For instance, flagellae of gram negative bacteria are PAMPs recognized by the FLS2 protein of *Arabidopsis*, which has a LRR-transmembrane (TM)-kinase domain (Felix et al. 1999). Recently, a rice plasma membrane protein CEBiP was shown to bind chitin, a fungal PAMP, and transduces defense signals downstream (Kaku et al. 2006).

*Phytophthora infestans*, the agent of potato and tomato late blight disease, produces a 10-kDa extracellular protein, INF1 elicitor (Kamoun et al. 1998). Elicitors are highly conserved 10-kDa proteins that are secreted in culture by all tested *Phytophthora* and *Pythium* species (Kamoun et al. 1993; Pernollet et al. 1993). They trigger a wide range of defense responses including the HR in most *Nicotiana* species (Kamoun et al. 1993; Bonnet et al. 1996), so that they can be conceived as oomycete PAMPs that are detected by *Nicotiana* species. However, their function in *Phytophthora* pathogenicity, if any, is not yet clear (reviewed in Kamoun et al. 1999a; Kamoun 2001; Tyler 2002). Wendehenne et al. (1995) demonstrated the existence of specific, high-affinity binding sites for cryptogein, an elicitor from *Phytophthora cryptogea*, in tobacco plasma membrane. However, the host molecule interacting with the elicitor has not been identified to date. Signaling cascades following INF1 recognition and leading to hypersensitive cell death have been studied in detail, resulting in the identification of a respiratory burst oxidase homolog Nbrboh (Yoshioka et al. 2003), heat shock proteins Hsp90 and Hsp70 (Kanzaki et al. 2003) and a ubiquitin ligase-associated protein NbSGT1 (Peart et al. 2002), required for INF1-mediated HR.

The lectin-like receptor kinases (LRKs) are a class of proteins originally described from *Arabidopsis* (Hervé et al. 1996). Their structure is similar to other plant receptor-like kinases (Cock et al. 2002) with an N-terminal targeting signal, an extracellular domain, a single transmembrane (TM)-spanning helix, and a highly conserved cytosolic kinase domain. The extracellular domain shows homology to lectin proteins known to bind carbohydrates (van Damme et al. 1998). The *Arabidopsis* genome contains at least 42 LRK sequences that have been grouped into three classes (classes A, B and C) (Barre et al. 2002). The C-terminal catalytic domain of *Populus nigra* LRK (PnLPK) showed high autophosphorylation and serine/threonine kinase activities (Nishiguchi et al. 2002). An *Arabidopsis* LRK (AtLecRK2; He et al. 2004) localized to plasma membrane and its transcripts were most prominently expressed in root. A recent work by Gouget et al. (2006) showed that a lectin receptor kinase of *Arabidopsis* (At5g60300) binds to RGD (arginin-glycine-aspartic acid) containing proteins including IPI-O, a secreted *in planta* induced protein of *P. infestans*, and mediates plasma membrane–cell wall adhesions,

suggesting the involvement of lectin receptor kinases in plant host–pathogen interaction.

In this paper, we aim at isolation and functional characterization NbLRK1, an INF1-interactor from *Nicotiana benthamiana*.

## Materials and methods

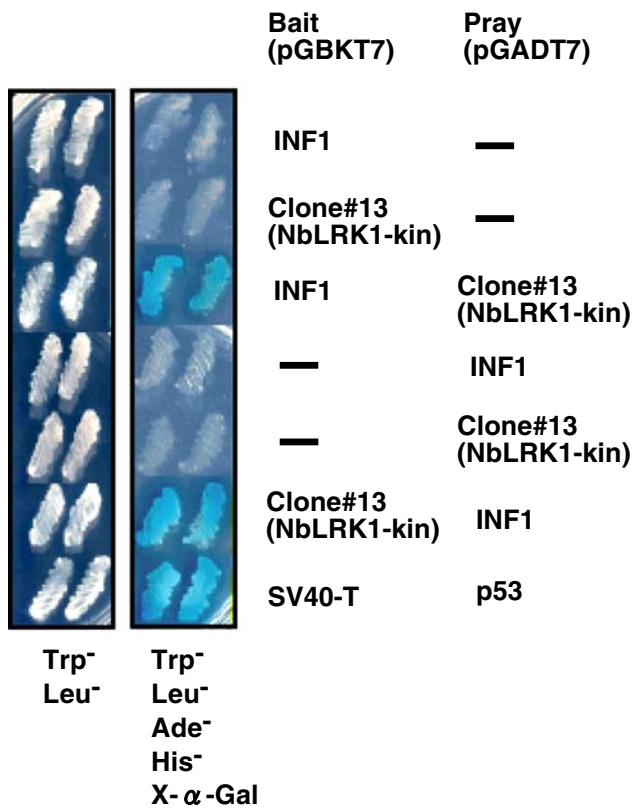
### Yeast two-hybrid screening and interaction assay

A cDNA fragment (designated as *INF1*) corresponding to the mature INF1 fragment without the signal peptide region was isolated from *pPVX::infl* plasmid (Kamoun et al. 1999b). The bait vector containing GAL4 DNA binding domain was prepared by cloning *INF1* into plasmid pGBKT7 (Clontech, Mountain View, CA, USA) resulting in pGBKT7-*INF1*. MATCHMAKER Library Construction & Screening Kit (Clontech) was used to construct a cDNA library from RNA extracted from *N. benthamiana* leaf blade 15 min after INF1-elicitor infiltration. Competent cells of a yeast strain AH109 were transformed with pGBKT7-*INF1*, pGADT7-Rec and the *N. benthamiana* cDNA library using the PEG/LiCl method, and plated on selective agar plates containing minimal medium without Leu, Trp, His and Ade supplemented with 10 mM 3-amino-1,2,4-triazole (3-AT) and 40 mg/L X- $\alpha$ -gal. cDNAs in the library were transferred to pGADT7-Rec vector harboring GAL4 activation domain (AD) by homologous recombination in yeast cells.

For confirmation of protein interaction of NbLRK1 with INF1 by yeast two-hybrid system (Fig. 1), yeast transformants harboring different combinations of bait and prey constructs were streaked onto minimal medium agar plates containing 40 mg/L X- $\alpha$ -Gal without Leu, Trp, His and Ade to assay the expression of *MEL1* and *ADE2* and *HIS3* genes. The combination of SV40-T and p53 was used as positive control. Interaction of truncated versions of NbLRK1 and INF1 were tested by the same experimental conditions as described here.

### Southern and Northern analysis

Ten micrograms each of genomic DNA of *N. benthamiana* isolated with DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) were digested with restriction endonuclease *KpnI* and *XbaI*, respectively, and loaded on 1% agarose gel for electrophoresis. The separated DNA fragments were blotted onto nylon membrane (Hybond N+, GE Healthcare, Piscataway, NJ, USA) and hybridized with a <sup>32</sup>P-labeled complementary DNA fragment (position no. 157–582 setting the first nucleotide of the start codon as no. 1) corresponding to the NbLRK1 lectin domain. Total RNA was



**Fig. 1** Protein interaction between INF1 and NbLRK1 as detected by yeast two-hybrid assay. Yeast cells harboring different combinations of bait and prey constructs were streaked on plates of minimal medium without Trp and Leu (*left*) or without Trp, Leu, Ade and His but containing X-α-Gal (*right*). Only when both INF1 and clone #13 (NbLRK1-kin) are simultaneously expressed, yeast cells survive on Trp<sup>-</sup> Leu<sup>-</sup> Ade<sup>-</sup> His<sup>-</sup> cells and exhibit blue color in the presence of X-α-Gal. The combination of SV40-T and p53 was used as positive control. Bars indicate that an empty vector of either pGBKT7 or pGADT7 was used for transformation

extracted from leaves, stems, roots or flowers with the RNeasy Plant Mini Kit (Qiagen), and ten micrograms were loaded on a formaldehyde-1.0% agarose gel for electrophoresis, blotted onto nylon membrane (Hybond N+, GE Healthcare) and used for hybridization analysis. DNA fragment used as probe is the same as that used for Southern analysis.

**Immunoprecipitation assay**

A complementary DNA fragment corresponding to triple c-Myc-tag was fused in-frame to the 5'-end of the full length cDNA of *NbLRK1*, and cloned into pASK-IBA44 vector (IBA, Goettingen, Germany) harboring Strep-tagII DNA sequence, resulting in pASK-IBA44-c-Myc-NbLRK1. This construct was transformed into *E. coli* strain BL21, and ST-c-Myc-NbLRK1 protein was produced and purified using a Strep-Tactin Column (IBA). FLAG-INF1 protein was produced from the plasmid pFB53 (Kamoun et al. 1997) and

purified by anti-FLAG antibody immuno-affinity column (anti-FLAG M2 Affinity Gel Freezer-Safe, Sigma–Aldrich, St Louis, MO, USA). Three micrograms of ST-c-Myc-NbLRK1 and 0.1 μg of FLAG-INF1 proteins were mixed in Tris-buffer, incubated and immunoprecipitated with an anti c-Myc antibody (Santa Cruz Biotech, Cat. No. A-14, Santa Cruz, CA, USA). Immunoprecipitated protein was separated by SDS-PAGE, and the presence of FLAG-INF1 was detected by Western-blot analysis using anti-Flag M2 antibody (Sigma–Aldrich) and the ECL system (GE Healthcare).

**Expression of NbLRK1-eGFP and its detection**

The C-terminal end of the *NbLRK1* ORF was fused in frame with a cDNA of enhanced GFP (eGFP; Clontech), and put downstream of CaMV35S promoter of a binary vector pCAMBIA1300S (Cambia, Canberra, Australia), resulting in pCAM-NbLRK1-eGFP. This vector was used for transformation of *Agrobacterium tumefaciens* GV3001. *Nicotiana benthamiana* was transiently transformed with *A. tumefaciens* harboring pCAM-NbLRK1-eGFP, and after 2 days, the epidermal cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

**Autophosphorylation assay**

A cDNA fragment corresponding to triple c-Myc-epitope tag was fused with that of the C-terminus of NbLRK1 and inserted into the GVG vector (Aoyama and Chua 1997) resulting in plasmid GVG-NbLRK1-cMyc. A kinase-dead mutant of NbLRK1 was made by replacing the catalytic essential Lys (K) 413 with Arg (R) using site-direct mutagenesis (NbLRK1<sup>KR</sup>), and was fused with the triple c-Myc-tag, and inserted into the GVG vector (pTA7001), resulting in GVG-NbLRK1<sup>KR</sup>-c-Myc. *Agrobacterium tumefaciens* strain GV3101 harboring GVG-NbLRK1-c-Myc, or GVG-NbLRK1<sup>KR</sup>-c-Myc, respectively, was infiltrated into leaf blades of *N. benthamiana*. Two days later, protein expression from GVG constructs was induced by infiltration with the glucocorticoid inducer dexamethasone (DEX, 30 μM). Twelve hours after DEX treatment, 100 nM INF1 protein was infiltrated to the leaves, and leaf tissue collected 10 and 60 min after the INF1 treatment. Proteins were isolated in extraction buffer (Berberich et al. 1999) in the ratio of 1.5 mL/g fresh weight and NbLRK1 proteins were immunoprecipitated from the extract using an anti-cMyc antibody. Kinase assay of the immune complex was carried out according to Berberich et al. (1999). The immunocomplexes were incubated at 25°C for 45 min with 50 μM ATP, 0.296 MBq [γ-<sup>32</sup>P]ATP. The reaction was stopped by adding the same volume of SDS-PAGE sample buffer. After fractionation in 12.5% polyacrylamide gel containing SDS,

radioactivity caused by autophosphorylation was detected by a BAS2000 phosphorimager (Fujifilm, Tokyo, Japan).

#### Myelin basic protein phosphorylation assay

A complementary DNA fragment corresponding to triple c-Myc-tag was fused *in-frame* to the 5'-end of the full length cDNA of *NbLRK1*, and cloned into pASK-IBA2 vector (IBA) harboring Strep-tagII DNA sequence, resulting in ST-c-Myc-NbLRK1. A kinase-dead mutant of NbLRK1 was made by replacing the catalytic essential Lys (K) 413 with Arg (R) using site-direct mutagenesis (NbLRK1<sup>KR</sup>), and was fused with the triple c-Myc-tag, and inserted into the pASK-IBA2 vector, resulting in ST-c-Myc-NbLRK1<sup>KR</sup>. These constructs were transformed into *E. coli* strain BL21, and ST-c-Myc-NbLRK1 or ST-c-Myc-NbLRK1<sup>KR</sup> proteins were produced and purified using a Strep-Tactin Column (IBA). Kinase assay of purified proteins were carried out according to Ouaked et al. (2003). The purified proteins were incubated in the kinase buffer containing 5 µg of Myelin Basic Protein (MBP), 0.1 mM ATP and 0.1 µCi of [ $\gamma$ -<sup>32</sup>P]ATP at room temperature for 30 min. The reaction was stopped by adding the same volume of SDS-PAGE sample buffer. After fractionation in 15% polyacrylamide gel containing SDS, radioactivity incorporated in MBP was detected by a BAS2000 phosphorimager (Fujifilm).

#### PVX constructs and PVX-mediated gene silencing in *N. benthamiana*

A partial cDNA fragment of *NbLRK1* (nucleotide position no. 1927–2144 setting the first nucleotide of the start codon as no. 1) was inserted into *EcoRV* and *Sall* sites of potato virus X (PVX) vector (pPC2S), resulting in pPVX.NbLRK1. A PVX vector harboring GFP (pTXS.GFP: Baulcombe et al. 1995) was used as control. pPVX.NbLRK1 as well as pTXS.GFP were linearized by restriction endonuclease *SpeI*, and *in vitro* runoff transcripts were synthesized by T7 RNA polymerase. The transcripts were inoculated onto leaves of *N. benthamiana* as described (Saitoh et al. 2001). Confirmation of gene silencing of *NbLRK1* transcripts was performed using reverse-transcription (RT)-PCR using *NbLRK1* gene specific primers (5'-GAC AACAACAGTGTTCAGTTAACACGTGATCTTGCC-3', 5'-CCAAGAAT-TGACTATATCACCCTTTGAGATCAACACC-3'). After the establishment of gene silencing, leaves of the control (PVX.GFP) and NbLRK1-silenced plants (PVX.NbLRK1) were infiltrated with 100 nM INF1 to induce HR. pTV.NbLRK1 (Fig. 7c) was prepared basically in the same manner as PVX.NbLRK1 except that we used TRV vector (Ratcliff et al. 2001; Coemans et al. 2008).

#### Hydrogen peroxide detection by DAB

*In vivo* H<sub>2</sub>O<sub>2</sub> generation in plants was detected by an endogenous peroxidase-dependent *in situ* histochemical staining procedure using 3,3'-diaminobenzidine (DAB; Sigma) as described (Thordal-Christensen et al. 1997).

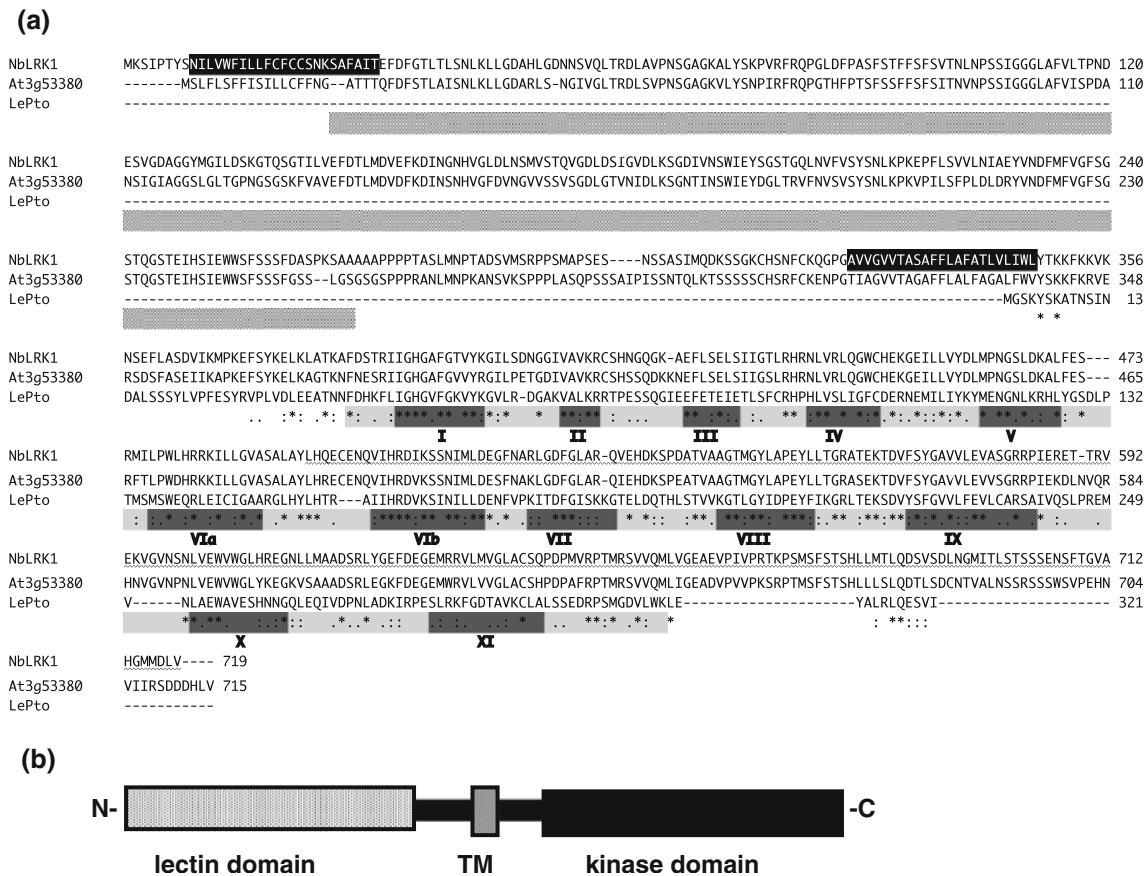
## Results

#### Yeast two-hybrid screening of INF1-interactors identifies NbLRK1

To search for *N. benthamiana* proteins that interact with INF1, we carried out yeast two-hybrid assay (Fields and Song 1989) using INF1, for which the signal peptide necessary for its extracellular secretion was removed, as the bait. This screen identified 29 cDNA clones whose protein products showed interaction with INF1, among which one clone #13 exhibited by far a stronger interaction than the other clones (Fig. 1). Interaction between INF1 and the clone #13 product could also be observed when cDNA #13 was used as bait and INF1 as the prey in the yeast two-hybrid assay (Fig. 1). The cDNA clone #13 contained a 573 bp fragment showing high sequence similarity to the kinase domain of an *Arabidopsis* lectine-like receptor-like kinase protein At3g53380. A 2,154 bp full length cDNA corresponding to the clone #13 was isolated by 5'-RACE. The largest open reading frame (ORF) in this cDNA corresponded to a protein of 717 amino acids. Judging from the overall similarity to known lectin-like receptor kinases, we named this gene as *NbLRK1* (Fig. 2; Genbank accession no. AB247455).

#### NbLRK1 belongs to the class B lectin-like receptor kinases

Analysis of predicted protein sequences of NbLRK1 with TMHMM (Krogh et al. 2001) suggests that the protein has two putative transmembrane regions. The first one, located near the N-terminus, comprises 24 amino acids (Asn9 to Thr31) and probably represents a secretion signal. The second one containing 23 amino acids (Ala325 to Leu347) corresponds to a transmembrane domain (Fig. 2). The C-terminal region of the protein shows homology to the catalytic domain of protein kinases, consisting of the 12 subdomains characteristic of the eukaryotic kinase superfamily (Hanks and Quinn 1991). The protein domain C-terminal to the kinase subdomain VIa interacted with INF1 in the yeast two-hybrid assay (Fig. 2). A phylogenetic analysis of lectin receptor-like kinases placed NbLRK1 in the class B of LRKs (Fig. 3; Barre et al. 2002). The protein showing the highest similarity to NbLRK1 was At3g53380 (GeneBank Accession no. CAB67645) of *Arabidopsis*.



**Fig. 2** NbLRK1 amino acid sequence, and its predicted domain structure. **a** Amino acid sequence alignment of NbLRK1 with an Arabidopsis lectin-like receptor kinase, At3g53380, and tomato Pto kinase, LePto. Putative transmembrane regions are indicated by white letters in black boxes. Lectin-like domain near the N-terminus is indicated by

a dotted shadow below the sequence. Roman numerals (I–XI) in the C-terminal region show conserved Ser/Thr kinase subdomains. Underlined region corresponds to the fragment isolated by the yeast two-hybrid screen. **b** Predicted domain structure of mature NbLRK1. TM indicates transmembrane region

**Genomic organization and gene expression of NbLRK1**

In order to determine the copy number of NbLRK1 gene in the *N. benthamiana* genome, Southern hybridization experiment was carried out using a DNA fragment corresponding to the N-terminal lectin domain of NbLRK1 as probe (Fig. 4a). Under stringent washing conditions only one strong and a second rather weak signal were detected, indicating that NbLRK1 has one or two copies in the *N. benthamiana* genome.

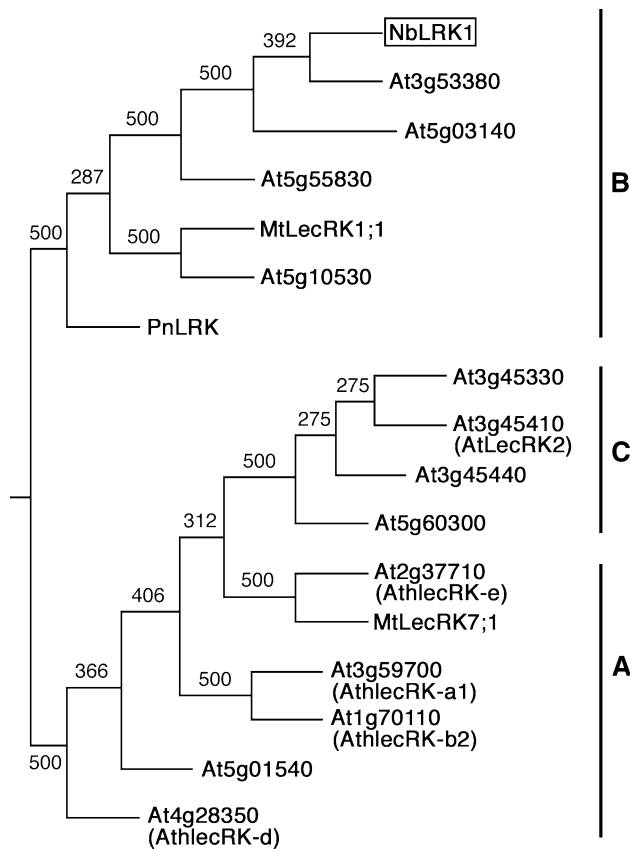
NbLRK1 gene expression was studied by Northern hybridization experiment using the same hybridization probe as used in the Southern-blot analysis. In normal conditions, NbLRK1 expression was observed in the entire *N. benthamiana* plant with a stronger expression in root and stem compared to leaf and flower (Fig. 4b). Treatment of *N. benthamiana* with INF1 did not significantly alter the expression of NbLRK1 (data not shown).

**INF1 interacts with the subdomain VIb of NbLRK1 kinase domain**

To narrow down the site of NbLRK1 involved in its interaction with INF1, we tested the binding of a series of truncated versions of NbLRK1 kinase domain with INF1 using yeast two-hybrid assay (Fig. 5). While full-size NbLRK1 kinase domain and the fragments C-terminal to the V and VIa sub-domains of NbLRK1 kinase, respectively, interacted with INF1, the fragments C-terminal to the subdomain VIb did not. This result suggests that NbLRK1 interacts with INF1 via its 31 amino acid region of VIb sub-domain of kinase domain.

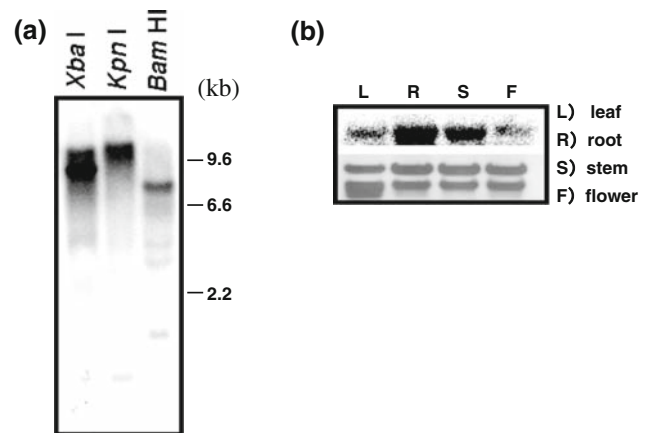
**INF1 and NbLRK1 interact in vitro**

To further confirm the interaction between INF1 and NbLRK1, we carried out a pull-down assay by immunoprecipitation. INF1 protein fused with the FLAG tag in its



**Fig. 3** A phylogenetic tree of lectin-like receptor kinases including NbLRK1. Neighbor-Joining (NJ) tree (Saitoh and Nei 1987) derived from a distance matrix calculated by Dayhoff's PAM matrix (Dayhoff 1978) based on aligned amino acid sequences generated by CLUSTALW. Numbers on the branches indicate the number of times these clustering were supported in 500 bootstrapping resamplings. MtLecRK1 and 7 are as described in Navarro-Gochicoa et al. (2003) and the proteins indicated by AthlecRK are from Herve et al. (1996)

N-terminus (FLAG-INF1) as well as NbLRK1 protein fused with triple c-Myc and Strep-tagII tags in its N-terminus (ST-c-Myc-NbLRK1) were produced in *E. coli* cells, and purified by anti-FLAG antibody immunoaffinity column and Strep-Tactin column, respectively (Fig. 6a). Purified FLAG-INF1 and ST-c-Myc-NbLRK1 proteins were mixed, and immunoprecipitated with an anti c-Myc antibody. Western-blot analysis of the immunoprecipitant using an anti-FLAG antibody detected the INF1 protein (Fig. 6b), suggesting that NbLRK1 can interact in vitro with purified and biologically active INF1. This experiment provides an independent confirmation of the interaction obtained by the yeast two-hybrid assay. We further tried to see in planta interaction between INF1 and NbLRK1 by transient co-expression of the proteins in *N. benthamiana* followed by immunoprecipitation. However, because of instability of INF1 protein in plant, we have not been successful in detecting the interaction in planta (data not shown).



**Fig. 4** Genomic organization and expression of NbLRK1. **a** Southern-blot analysis of NbLRK1 in *N. benthamiana*. Genomic DNA was digested with restriction endonucleases BamHI, KpnI or XbaI, respectively, and separated by agarose gel electrophoresis. A cDNA fragment corresponding to the lectin domain of NbLRK1 was used as hybridization probe. **b** Northern-blot analysis of NbLRK1 in *N. benthamiana*. Total RNA extracted from leaves (L), roots (R), stems (S) or flowers (F) was loaded for electrophoresis. Agarose gel image of ribosomal RNAs is given below to show equal loading of RNAs. The DNA probe used is the same as in Southern analysis

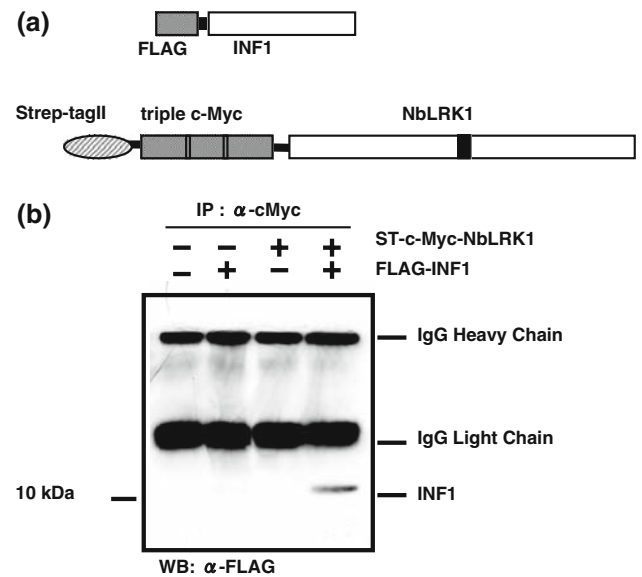
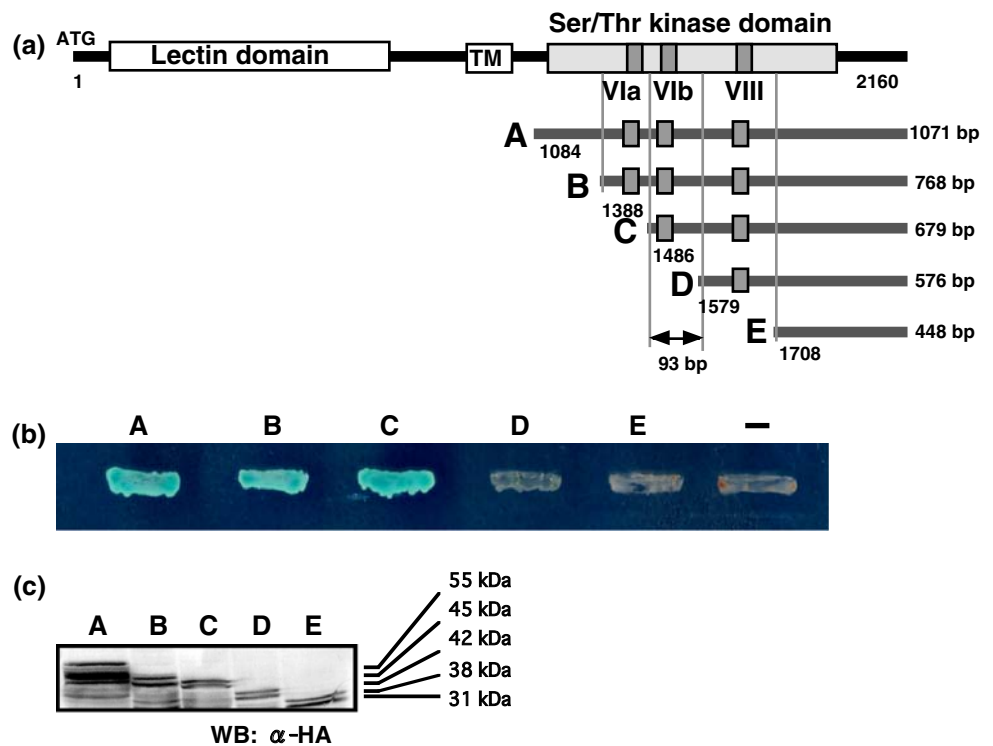
NbLRK1 is localized at plasma membranes

Predicted structure of NbLRK1 with a transmembrane domain (Fig. 2) suggests that this protein is localized to the plasma membranes. To confirm this prediction, we constructed a binary vector harboring 35S::NbLRK1-eGFP coding for NbLRK1 fused with an enhanced jelly fish green fluorescent protein (eGFP) in its C-terminus, and transiently expressed the protein in *N. benthamiana* leaves by Agrobacterium infiltration. The transformed leaf epidermal cells were observed under UV microscope (Fig. 7). Strong green fluorescence was localized at cellular membranes, validating our prediction that NbLRK1 is plasma membrane localized protein.

INF1 treatment of *N. benthamiana* results in NbLRK1 autophosphorylation

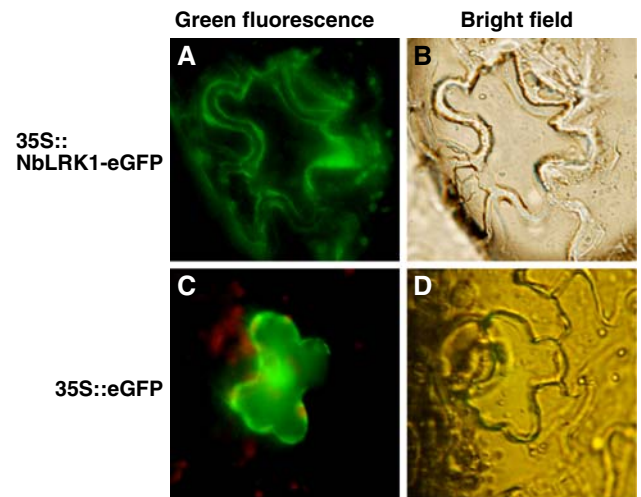
Before activation, protein kinases are frequently autophosphorylated. To see the changes in autophosphorylation potency of NbLRK1 after INF1 treatment of *N. benthamiana*, we carried out an autophosphorylation assay of transiently overexpressed NbLRK1 that was extracted from *N. benthamiana* leaves. For this purpose, a full length cDNA of NbLRK1-c-Myc was introduced into the GVG vector (Aoyama and Chua 1998). GVG-NbLRK1-c-Myc plasmid was introduced into *Agrobacterium tumefaciens* GV3101 which was then used for transient transformation of *N. benthamiana*. Two days after the transformation,

**Fig. 5** INF1 interacts with the subdomain VIb of NbLRK1 kinase domain. **a** A series of truncated versions (A–E) of NbLRK1 kinase domain were cloned into yeast two-hybrid pray vector and tested for interaction with INF1 as bait. The number below each line indicates the first nucleotide position of each protein with reference to the start codon (A of the first codon was set 1). **b** Results of yeast two-hybrid assay (alphabet corresponds to that in **a**). The fragments A–C interacted with INF1, but the fragments D and E did not. **c** Western-blot analysis shows that all truncated versions (A–E) of NbLRK1 proteins are expressed in yeast as detected by anti-HA antibody that recognizes HA-epitope at the N-terminus of each protein



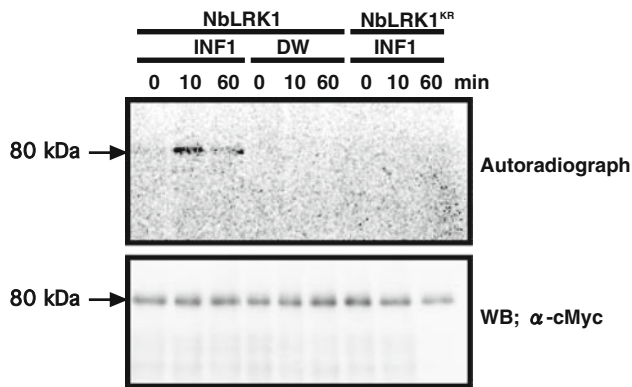
**Fig. 6** NbLRK1 and INF1 interact in vitro. **a** Schematic representation of FLAG-INF1 and ST-c-Myc-NbLRK1. **b** Western-blot analysis of immunoprecipitated proteins shows binding of NbLRK1 to INF1. ST-c-Myc-NbLRK1 and FLAG-INF1 proteins were together or separately added to the mixture, and immunoprecipitated by an anti-c-Myc antibody. The resulting immunoprecipitants were separated by SDS-PAGE, blotted, and analyzed by Western analysis using an anti-FLAG antibody to detect FLAG-INF1 protein

expression of NbLRK1-c-Myc was induced by application of dexamethason (DEX). Twelve hours after DEX treatment, INF1 or water was further infiltrated to the leaves, and leaf tissue collected after 0, 10 and 60 min. NbLRK1-c-Myc



**Fig. 7** NbLRK1 is localized to plasma membranes. NbLRK1-eGFP fusion protein (**a, b**) or eGFP (**c, d**) was transiently expressed in epidermal cells of *N. benthamiana* leaves by Agroinfiltration, and observed by microscope under UV (**a, c**) or under bright light (**b, d**)

protein was immunoprecipitated from the leaf extract by an anti c-Myc antibody, and was incubated with [ $\gamma$ - $^{32}$ P]ATP before separation by SDS-PAGE. As seen in Fig. 8, whereas a ~80 kDa protein corresponding to NbLRK1-c-Myc was equally expressed and immunoprecipitated at all the time points for INF1 and water treated samples as shown by Western-blot analysis, radioactivity was only incorporated to NbLRK1-c-Myc when the plant was infiltrated with INF1. This result suggests that NbLRK1-c-Myc protein became specifically autophosphorylated after treatment of



**Fig. 8** NbLRK1 autophosphorylates after INF1 treatment of *N. benthamiana* plant. NbLRK1-c-Myc and its kinase-dead mutant NbLRK1<sup>KR</sup>-c-Myc were transiently expressed in *N. benthamiana* leaves by agroinfiltration. Twelve hours after induction of the protein expression, INF1 or distilled water (DW) were infiltrated to the leaves, and leaf tissue harvested 0, 10 or 60 min after the treatment. NbLRK1-c-Myc and NbLRK1<sup>KR</sup>-c-Myc proteins were immunoprecipitated using an anti c-Myc antibody. The immunoprecipitants were incubated with [ $\gamma$ -<sup>32</sup>P]ATP, separated by SDS-PAGE, and the radioactivity detected by autoradiography (top). Immunoprecipitated protein was detected by western blot analysis using anti c-Myc antibody (bottom)

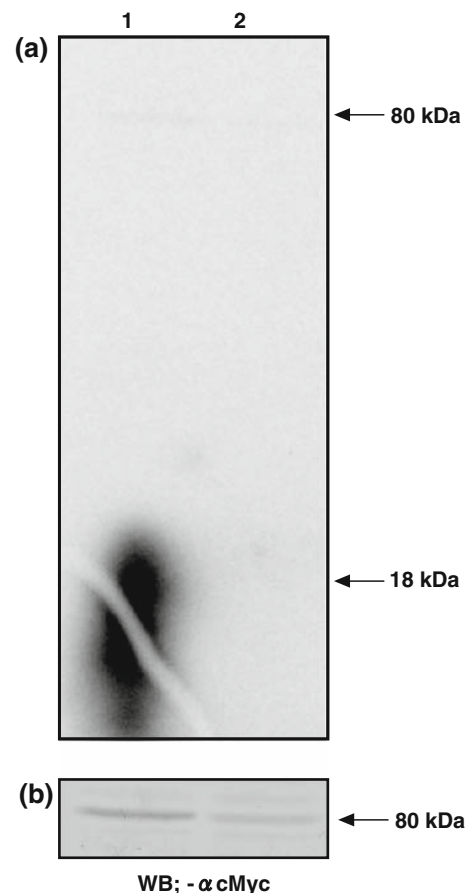
the plant with INF1. The autophosphorylation signal is stronger at 10 min after INF1 treatment than at 60 min after the treatment. To prove this result, we made NbLRK1<sup>KR</sup>-c-Myc, a kinase-dead mutant of NbLRK1, by changing the lysine residue at the position 413, required for ATP binding, into arginine. When NbLRK1<sup>KR</sup>-c-Myc was expressed instead of NbLRK1-c-Myc, INF1-induced phosphorylation could not be observed, suggesting that NbLRK1-c-Myc phosphorylation is indeed autophosphorylation.

#### NbLRK1 phosphorylates myelin basic protein in vitro

To test whether NbLRK1 can phosphorylate other proteins than itself, we used myelin basic protein (MBP) as substrate (Fig. 9). After incubation of purified NbLRK1 or NbLRK1<sup>KR</sup> with MBP and [ $\gamma$ -<sup>32</sup>P]ATP, the reaction was separated in SDS-PAGE and subjected to autoradiography. 18 kDa MBP was phosphorylated by NbLRK1, but not by NbLRK1<sup>KR</sup>, demonstrating that NbLRK1 has kinase activity to substrates. This result suggests that NbLRK1 may participate in signaling by phosphorylation of target proteins.

#### VIGS of *NbLRK1* delays INF1-mediated HR in *N. benthamiana*

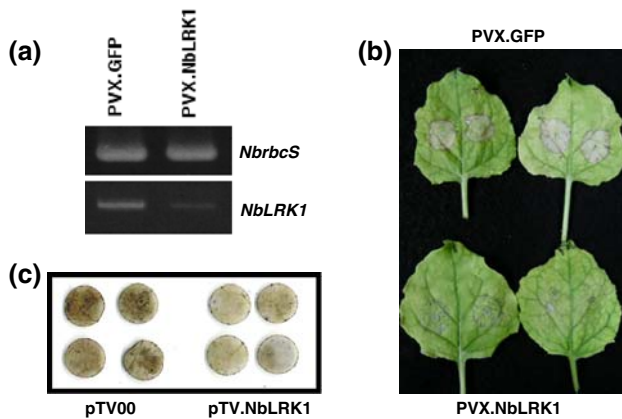
To evaluate the function of NbLRK1 in HR signaling emanating from INF1 perception, we carried out virus-induced gene silencing (VIGS) of *NbLRK1* using the potato virus X (PVX) system (Baulcombe 1999). A partial cDNA of *NbLRK1* was cloned into the gene silencing vector pPC2S



**Fig. 9** NbLRK1 phosphorylates MBP. **a** MBP was incubated with cMyc-NbLRK1 (lane 1) or cMyc-NbLRK1<sup>KR</sup> (lane 2) in the presence of [ $\gamma$ -<sup>32</sup>P]ATP, followed by separation in SDS-PAGE and autoradiography. [ $\gamma$ -<sup>32</sup>P]ATP was incorporated to MBP (18 kDa) only by cMyc-NbLRK1. **b** Western-blot analysis of cMyc-NbLRK1 (lane 1) and cMyc-NbLRK1<sup>KR</sup> (lane 2)

(Baulcombe et al. 1995) resulting in PVX.NbLRK1, and its in-vitro run-off transcripts were inoculated to the leaves of *N. benthamiana*. Three weeks later, using the third and fourth leaves above the inoculated one, levels of *NbLRK1* transcripts were determined by RT-PCR using PCR primers located outside of the cDNA region that was used for insertion into the PVX vector. Transcript level of *NbLRK1* was not affected in PVX.GFP-inoculated plants (control), whereas it was reduced in the plants inoculated with PVX.NbLRK1 (Fig. 10a). In leaves where VIGS of *NbLRK1* was established, INF1 elicitor was infiltrated. Three days after INF1-infiltration, a typical HR response occurred in PVX.GFP-inoculated plants. On the other hand, PVX.NbLRK1-inoculated plants showed a limited HR at the same time point (Fig. 10b). One week after INF1 treatment, HR equally developed in the control and *NbLRK1*-silenced plants. The same result was obtained in three independent experiments. To evaluate the function of *NbLRK1* in H<sub>2</sub>O<sub>2</sub> generation that accompanies INF1-mediated HR (Yoshioka





**Fig. 10** INF1-mediated HR is delayed in *NbLRK1*-silenced plants of *N. benthamiana*. **a** Three weeks after inoculation of *N. benthamiana* plants with PVX.GFP and PVX.NbLRK1, *NbLRK1* gene silencing was confirmed by RT-PCR. RT-PCR of *rbcS* gene (*NbrbcS*) served as control. **b** To the *N. benthamiana* leaves which were inoculated with PVX.GFP (top) or PVX.NbLRK1 (bottom), 100 nM INF1 elicitor was infiltrated. A complete HR develops in the control, but not in *NbLRK1*-gene silenced leaves. The picture was taken 3 days after INF1 treatment. **c** H<sub>2</sub>O<sub>2</sub> generation detected in leaf discs of *N. benthamiana* by DAB staining 24 h after INF1 infiltration to leaves which were inoculated with pTV00 (left, control) or pTV.NbLRK1 (right). *NbLRK1* gene silencing was confirmed by RT-PCR (data not shown)

et al. 2003), we monitored the H<sub>2</sub>O<sub>2</sub> generation by diaminebenzidine hydrochloride (DAB) stain. For this experiment we used TRV vector (Ratcliff et al. 2001) for gene silencing. Twenty-four hours after INF1 infiltration, H<sub>2</sub>O<sub>2</sub> generation was clearly detected in pTV00-inoculated control plants as monitored by DAB precipitate, whereas only a limited amount of H<sub>2</sub>O<sub>2</sub> generated in pTV. *NbLRK1*-inoculated *NbLRK1* gene silenced plants (Fig. 10c). These results suggest that *NbLRK1* is involved in the H<sub>2</sub>O<sub>2</sub> generation and HR induced by treatment of *N. benthamiana* with INF1.

**Discussion**

In order to identify plant factors that interact with the *P. infestans* INF1 elicitor, we carried out a yeast two-hybrid screen using INF1 as the bait, and isolated a cDNA coding for lectin like receptor kinase gene *NbLRK1* from *N. benthamiana*.

The yeast two-hybrid finding was validated with two independent experiments. *NbLRK1* directly interacts with INF1 in vitro, and INF1 treatment of *N. benthamiana* triggers autophosphorylation of *NbLRK1* in vivo. Furthermore, VIGS of *NbLRK1* delayed INF1-mediated HR. This biochemical and genetic evidence strongly suggest that *NbLRK1* is an important component of a receptor complex that recognizes INF1 protein, and triggers the HR signal downstream.

INF1 is known to be secreted by *P. infestans* through its N-terminal signal peptide and was assumed to localize at the extracellular space of plant tissue (Kamoun et al. 1993). Nevertheless, in the present study, INF1 was found to bind to the intracellular kinase domain of *NbLRK1* (Figs. 1, 2). Therefore, the interaction detected between INF1 and *NbLRK1* seems enigmatic. However, Tyler (2002) suggested that plant recognition of elicitors takes place inside the plant cells. He proposed that elicitors could be transported inside plant cells by receptor-mediated endocytosis (Tyler 2002). Support for this model was provided by Brummer et al. (2002), which showed that the elicitor quercinin of *Phytophthora quercina* was localized inside the cells of host oak plants by immunocytology. Based on these papers and our own findings, we speculate that INF1 protein initially localizes in the apoplast but then trafficks inside plant cells by endocytosis or other unknown mechanisms, where it interacts with the kinase domain of *NbLRK1*. Recently, Catanzariti et al. (2006) proposed that small secreted cysteine-rich proteins of the flax rust, which are similar in size and overall structure to INF1, are able to enter plant cells in the absence of the pathogen. Therefore, it is possible that a diversity of small cysteine-rich proteins from oomycete and fungal pathogen can cross plant plasma membranes. To validate this hypothesis, precise localization of INF1 after *P. infestans* infection should be confirmed in the future studies.

Yeast two-hybrid assay showed that the 31 amino acids fragment of *NbLRK1* kinase domain within VIb subdomain interacted with INF1 (Fig. 5). The VIb subdomain of Ser/Thr kinase is known to contain the catalytic loop with an invariant Asp serving as the catalytic base necessary for the kinase function (Hanks and Hunter 1995). This site is close to the VII and VIII domains where the activation loop is located, which is necessary for autophosphorylation of kinases (Adams 2003; Dardick and Ronald 2006). There is a previous report indicating that VIb subdomain of receptor kinases interacts with pathogen molecules (Fontes et al. 2004; Florentino et al. 2006). For instance, VIb subdomain of leucine-rich-repeat (LRR) receptor-like-kinases (RLK), NIK1, NIK2 and NIK3 of Arabidopsis, are bound by a nuclear shuttle protein (NSP) of a geminivirus, *Cabbage leaf curl virus*, which, after binding, acts to reduce the kinase activity of target kinases (Fontes et al. 2004). Thus it is possible that INF1 binding to the VIb subdomain of *NbLRK1* alters its kinase activity presumably by triggering autophosphorylation. *NbLRK1* contains a conserved arginine (R) at immediately preceding the invariant aspartate (D) in subdomain VIb, so is a typical RD kinase. It does not belong to the non-RD kinases known to harbor many kinases involved in pathogen recognition receptors signaling (Dardick and Ronald 2006).

*NbLRK1* belongs to the class B lectin-like receptor kinases (Barre et al. 2002) and is most closely related to

*Arabidopsis* At3g533800 (Fig. 3). Class B lectin-like receptor kinases are not well characterized in contrast to those in class A (Ath.LecRK-a1 in *A. thaliana*; Hervé et al. 1996) and class C (At5g60300; Gouget et al. 2006). Only PnLPK1 in *Populus nigra* (Nishiguchi et al. 2002) was studied among class B lectin-like receptor kinases for its biochemical nature. Strong expression of *NbLRK1* in roots is similar to that of *PnLRK*, but in both cases biological meaning of their high expression in roots is not clear. Like other proteins in the family, *NbLRK1* has a lectin-like extracellular domain. Lectins are known to bind saccharides. We speculate that *NbLRK1* functions in binding to such interactors, probably saccharides, and transduces signals upon binding, although our initial trial to detect binding of *NbLRK1* with saccharides was not successful (data not shown). Binding of INF1 to the intracellular domain of *NbLRK1* may change its kinase activity, and transduces the HR signal. Alternatively, binding of INF1 to *NbLRK1* may alter the protein conformation of *NbLRK1*, and this change is recognized by another *R*-gene product like protein that “guards” protein condition of *NbLRK1* and transduces the HR signal (Dangl and Jones 2001). In order to substantiate such hypotheses, future works in search of the ligands and protein interactors of *NbLRK1* is necessary to understand the function of this protein in plant–pathogen interaction.

VIGS of *NbLRK1* did not completely abrogate INF1-mediated HR. This may be because our VIGS assay did not completely shut down *NbLRK1* expression so that a residual amount of *NbLRK1* functioned in HR signaling and/or because there is another yet unidentified receptor protein besides *NbLRK1* that functions in INF1 mediated HR signaling.

In conclusion, we showed that *NbLRK1* of *N. benthamiana* is an interactor of INF1 and an important component of INF1-mediated HR signal transduction. We hypothesize that similar receptor molecules might be involved in the perception of the diverse range of elicitors produced by oomycete plant pathogens.

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