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Virology 318 (2004) 24–31
VIROLOGY
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Rapid Communication

Identification of a novel receptor-like protein kinase that interacts with a geminivirus nuclear shuttle protein

Andrea C. Mariano,[a] Maxuel O. Andrade,[a] Anésia A. Santos,[a] Sonia M.B. Carolino,[a,b] Marli L. Oliveira,[a] Maria Cristina Baracat-Pereira,[a,b] Sergio H. Brommonshenkel,[b] and Elizabeth P.B. Fontes[a,b,*]

[a] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, 36571.000 Viçosa, MG, Brazil*
[b] *BIOAGRO, Universidade Federal de Viçosa, 36571.000 Viçosa, MG, Brazil*

Received 1 September 2003; accepted 26 September 2003

Abstract

Despite extensive studies in plant virus–host interactions, the molecular mechanisms of geminivirus movement and interactions with host components remain largely unknown. A tomato kinase protein and its soybean homolog were found to interact specifically with the nuclear shuttle protein (NSP) of *Tomato golden mosaic virus* (TGMV) and Tomato crinkle leaf yellows virus (TCrLYV) through yeast two-hybrid screening and in vitro protein binding assays. These proteins, designated LeNIK (*Lycopersicon esculentum N*SP-*I*nteracting *K*inase) and GmNIK (*Glycine max* NIK), belong to the LRR-RLK (leucine rich-repeat receptor-like kinase) family that is involved in plant developmental processes and/or resistance response. As such, NIK is structurally organized into characteristic domains, including a serine/threonine kinase domain with a nucleotide binding site at the C-terminal region, an internal transmembrane segment and leucine-rich repeats (LRR) at the N-terminal portion. The potential significance of the NSP–NIK interaction is discussed.

Keywords: Tomato kinase protein; Geminivirus; Nuclear shuttle protein

Introduction

Geminiviruses constitute a large group of plant viruses whose genome is packed as single-stranded DNA circles in a small, twinned isometric particle and is converted to double-stranded forms in nuclei of differentiated plant cells (Hanley-Bowdoin et al., 1999). Members of the genus *Begomovirus*, such as *Tomato golden mosaic virus* (TGMV), possess two genomic components, DNA-A and DNA-B. DNA-A has the potential to code for five gene products (AV1, AC1, AC2, AC3, AC4) and is involved in replication, transcriptional activation of viral genes and encapsidation of the viral genome. DNA-B encodes two movement proteins, the movement protein MP (BC1) and the nuclear shuttle protein NSP (BV1), both required for systemic infection (Lazarowitz and Beachy, 1999). In fact, the nuclear localization of geminiviruses during replication requires two movement functions that act to move the viral genome intracellularly from the nucleus to the cytoplasm (NSP) and cell-to-cell across the wall (MP) (Gafni and Epel, 2002; Lazarowitz and Beachy, 1999). Current models for begomovirus cell-to-cell movement accommodate the argument that NSP and MP interact physically, as demonstrated by the relocation of NSP from the nucleus to the cell periphery by MP in *Nicotiana tabacum* protoplasts (Sanderfoot and Lazarowitz, 1995).

Research on plant virus–host interactions is currently providing considerable insights into the mechanisms by which viruses move within plants. Nevertheless, in the case of geminiviruses, the molecular basis for such interactions has not been determined and host proteins that interact with the movement proteins have yet to be identified. In this study, we used the two-hybrid system to provide evidence for interactions between NSP and MP from TGMV and to detect host proteins that interact with NSP from TGMV and from Tomato crinkle leaf yellows virus (TCrLYV).

* Corresponding author. Departamento de Bioquímica e Biologia Molecular, BIOAGRO/Universidade Federal de Viçosa, 36571.000 Viçosa, MG, Brazil. Fax: +55-31-3899-2864.
E-mail address: bbfontes@mail.ufv.br (E.P.B. Fontes).

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doi:10.1016/j.virol.2003.09.038

Results

We have used the two-hybrid system to show that the movement protein MP from TGMV binds to the nuclear shuttle protein NSP (Fig. 1A). The DNA construct pBD-NSP contains the NSP coding sequence fused to the GAL4 DNA binding domain sequence, whereas in pAD-MP, the MP coding sequence was fused in-frame to the GAL4 activation domain sequence. Interaction between the viral proteins was detected when the *Saccharomyces cerevisiae* strain YRG-2 was co-transformed with pBD-NSP and pAD-MP and monitored for histidine prototrophy (Fig. 1A) and

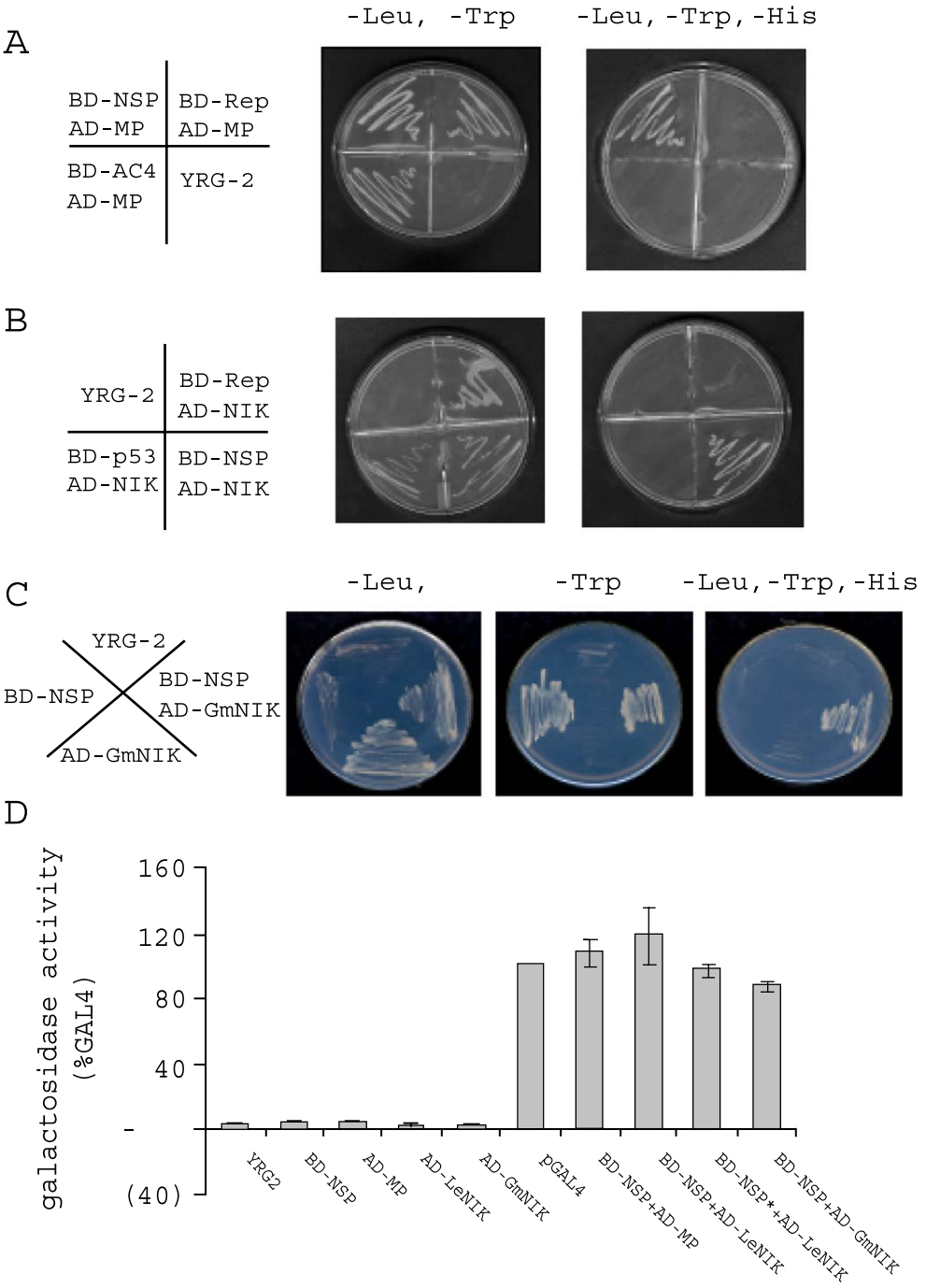

Fig. 1. Interactions of NSP with viral and host proteins. Yeast cells expressing the indicated recombinant proteins (alone or in pairs) were plated on selective medium lacking leucine, tryptophan and histidine (as indicated) and grown for 7 days at 30°C (A, B and C). In D, interactions between the indicated proteins were further confirmed by measuring the β-galactosidase activity in total protein extracts from yeast transformants grown on selective medium (lacking tryptophan, leucine and histidine). TGMV and TCrLYV proteins are denoted NSP and NSP*, respectively. Values for activity are the mean ± SD from four replicates.

β-galactosidase activity (Fig. 1D). The integrity of the NSP sequences was confirmed by rescuing the plasmids from yeast and sequencing. These results further confirm the consensus model for cooperative intracellular and intercellular movement of the viral DNA. In addition, they demonstrated that the viral recombinant proteins are correctly expressed and properly folded in the yeast system and, therefore, provided the foundation for the screening of host interacting partners using the yeast two-hybrid system and pBD-NSP as bait.

To identify host proteins capable of interacting with the TGMV NSP, we generated a directionally cloned tomato leaf cDNA library in a pAD-GAL4 vector derivative. Independent double transformants (5×10^6) were plated on medium lacking leucine and tryptophan to select for the presence of both bait expression plasmid and cDNA library plasmid and were plated on medium lacking histidine to select for interactions between the bait BD-NSP and tomato cDNA library-encoded proteins. Five clones showed His protrophy and lacZ expression on X-gal indicator plates following secondary screening (data not shown). To determine whether the cDNA-encoded proteins specifically interacted with the BD-NSP, library plasmids were rescued from these five clones and used to transform the original selection strain as well as several negative control strains expressing either murine p53-, human lamin C-, TGMV Rep- or TGMV AC4-GAL4 fusion proteins as bait. Four identical clones (pAD-NIK) were found to interact specifically with BD-NSP, as neither the *HIS3* marker gene nor *lacZ* was activated in yeast cells co-transformed with AD-NIK plus BD-p53, BD-LaminC, BD-Rep or BD-AC4 (Fig. 1B and data not shown). These partial host cDNAs were found to be identical and encoded the C-terminal 211 amino acid residues of a kinase-like protein that was designated LeNIK (*Lycopersicon esculentum NSP Interacting Kinase*). It shares 85% sequence identity with a putative receptor protein kinase from *Arabidopsis thaliana* (GenBank accession number AY088040). LeNIK also interacted with NSP from TCrLYV, demonstrating that interaction with NIK is conserved among geminivirus NSPs. The interactions have been confirmed by monitoring β-galactosidase activity in yeast protein extracts (Fig. 1D). The remaining clone (host cDNA5), however, interacted with multiple fusion proteins encoded by the negative control strains and, thus, was not considered further.

An in vitro protein-binding assay was used to further confirm the interaction between NIK and NSP. *Escherichia coli*-produced and purified LeNIK and control proteins including bovine serum albumin (BSA), soybean BiP and a soybean cysteine protease were blotted onto a nitrocellulose membrane and probed with biotin-tagged NSP. The N-terminal biotin peptide-tagged NSP (BtT-NSP) showed a strong interaction with the kinase domain of LeNIK but not with the control proteins (Fig. 2A). The interaction was specific to NSP because the biotinylated tag peptide alone (BtT) did not interact with LeNIK. Together, these

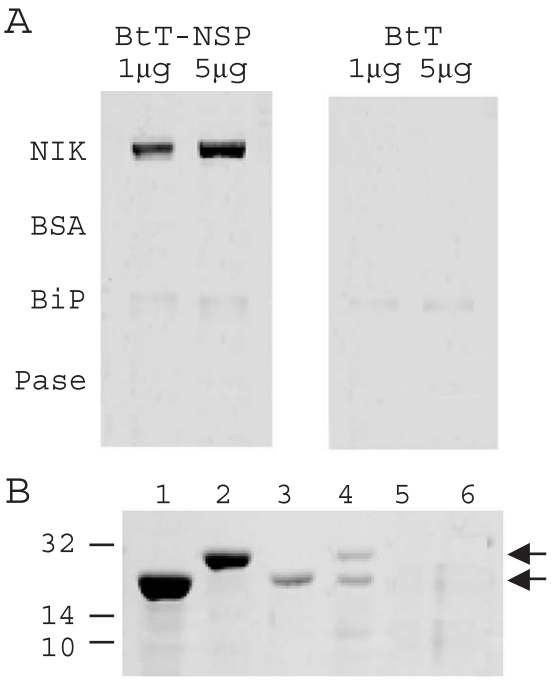

Fig. 2. In vitro interaction of NSP and LeNIK. (A) Increasing amounts (1 and 5 µg) of *E. coli*-produced His-LeNIK recombinant protein (NIK), purified soybean BiP recombinant protein (BiP), a soybean cysteine protease (Pase) and BSA were blotted onto nitrocellulose and probed with either biotinylated NSP fusion protein (BtT-NSP) or biotinylated tag peptide (BtT). (B) Co-immunoprecipitation assays. Protein extracts of *E. coli* expressing NSP (5), truncated LeNIK (3) and both NSP and truncated LeNIK (4) were immunoprecipitated with an anti-LeNIK serum. The immunocomplexes were fractionated by SDS-PAGE and Coomassie blue stained. In lane 6, the combined protein extracts were immunoprecipitated with an antibody prepared against soybean cysteine protease (Pase). Lane 1 corresponds to truncated His-LeNIK and lane 2, to affinity-purified His-NSP.

results demonstrated that NSP interacts specifically with LeNIK in vitro.

Specific interaction between NSP and LeNIK was also assayed by immunoprecipitation of combined protein extracts from *E. coli* expressing NIK and NSP with an anti-NIK serum (Fig. 2B). Although the anti-NIK serum did not cross-react with NSP (lane 5), this polypeptide was co-immunoprecipitated by the NIK-specific antibody from combined extracts (lane 4). The precipitation of the NSP polypeptide by anti-NIK serum was not due to non-specific interactions because antibodies to an unrelated protein failed to precipitate NSP (lane 6). Collectively, these in vitro results further substantiated the interaction in yeast cells.

In a similar approach, we screened a soybean cDNA library for interaction with the TGMV BD-NSP bait construct (Figs. 1C and D). A clone (pGmNIK) was identified that interacted specifically with NSP and was closely related to LeNIK (88% identity at amino acid level) (Fig. 3). The full-length soybean ortholog cDNA sequence, designated *GmNIK* (*Glycine max* NIK), is 1800-bp long and encodes a protein with an estimated M_r of 68585.88 and p*I* 6.97. Except for the viral MP, which showed weak interaction

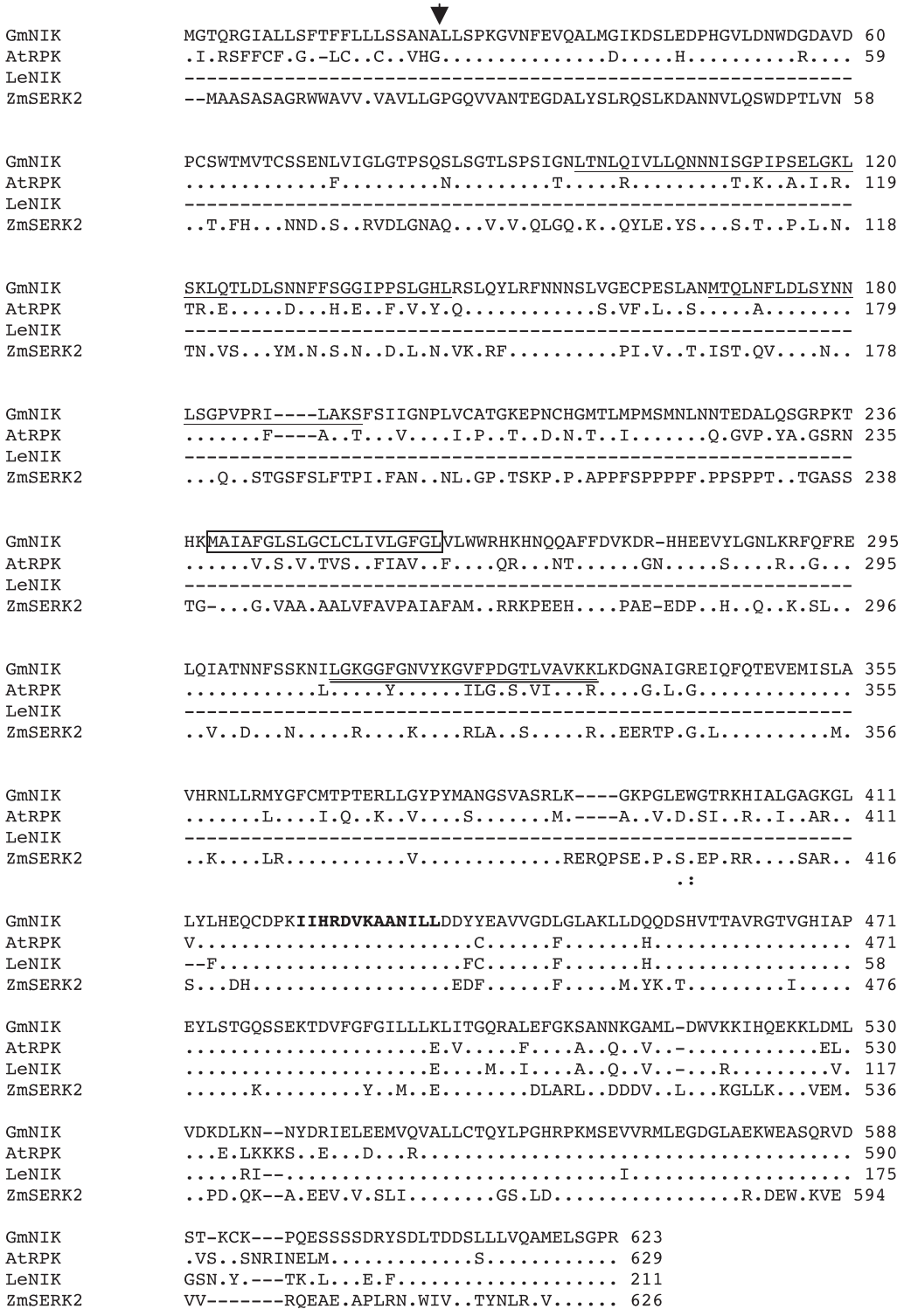

Fig. 3. Comparison of the amino acid sequence of LeNIK and GmNIK with LRR-RLKs from other organisms. A multiple sequence alignment of the deduced amino acid sequence of LeNIK, GmNIK, an *Arabidopsis thaliana* receptor protein kinase-like (AtRPK, GenBank accession number AY088040), and a *Zea mays* somatic embryogenesis receptor kinase (ZmSERK2, GenBank accession number AJ277703) was obtained with the CLUSTAL-W program. The amino acid sequences are in the one-letter code and have been aligned by introducing gaps (shown as dashes) to maximize identity. Dots represent identity to GmNIK sequence. The arrow indicates the putative signal peptide cleavage site of GmNIK. The leucine-rich repeats are underlined, the transmembrane segment is boxed, the conserved nucleotide binding site is double underlined and the putative serine/threonine kinase active site is in bold.

with GmNIK as judged by the low level of β-galactosidase activity of the double transformant, GmNIK did not interact with any other unrelated protein tested (data not shown).

GmNIK is most closely related to an *A. thaliana* protein (AtRPK, GenBank accession number AY088040, 76% sequence identity) (Fig. 3). It also shares significant conservation of primary structure with the maize ortholog (>60% identity). These proteins correspond to a class of putative receptor-like serine/threonine protein kinases of unknown function. Analysis of the deduced amino acid sequence of GmNIK allowed us to predict a transmembrane segment, a signal peptide and its processing site, which suggests that the protein is targeted to the secretory pathway. In fact, NIK was detected in microsomal fractions of soybean and tomato leaves, using antibodies prepared against *E. coli*-produced N-terminal his-tagged LeNIK (data not shown). The modular organization of GmNIK includes three domains characteristic of leucine-rich repeat receptor-like kinases (LRR-RLKs): (1) a serine/threonine protein kinase domain at the C-terminal region, including the active site ^{419}IIHRDVKAANILL^{431} and a conserved nucleotide binding site ^{309}LGKGFGNVYKKGVFPDGTLVAVKK^{332}, (2) an internal transmembrane helix ^{239}MAIAFGLSLGCLCLI VLGFGLVL^{261}, and (3) at the N terminus, leucine-rich repeats ^{96}LTNQIVLLQNNNISGPIPSELGKLK LTQLDLSNNFFSGGIPPSLGHL^{144} and ^{167}MTQLNFLDLSYNNLSGPVPRILAKS^{192}. The LRR-RLK proteins represent a complex family of protein kinases with diverse functions in signaling found in many different tissues.

Discussion

NSP (BV1) from begomoviruses has been shown to facilitate the intracellular transport of viral DNA from the nucleus to the cytoplasm and to act in concert with MP (BC1) to promote the cell-to-cell spread of the viral DNA (Gafni and Epel, 2002; Lazarowitz and Beachy, 1999). The identification of cellular proteins capable of interacting with NSP corresponds to the first step toward understanding the mechanism for NSP-mediated intracellular movement of viral DNA or other functions of this protein. We report here the identification of NIK, a cellular interaction partner for NSP, by screening tomato and soybean cDNA libraries using NSP as bait. On the basis of structural homology of the deduced amino acid sequence, NIK can be classified as a member of the LRR-RLK family of proteins. Although the function of NIK remains to be determined, several lines of evidence indicate that the NSP–NIK interaction may be biologically relevant during virus infection. First, NIK specifically interacts with NSP, as judged by the complete failure of its serine/threonine kinase domain to interact with a series of unrelated, control proteins in the two-hybrid assay. The inclusion of TGMV Rep in the control experiments, which has been previously demonstrated to interact with GRIK, a serine/threonine kinase protein from *A. thaliana* (Kong and Hanley-Bowdoin, 2002), further substantiates that the interaction between NIK and NSP is highly specific. Second, recombinant LeNIK and NSP were found to interact in vitro using two independent approaches for monitoring protein/protein interactions (Fig. 3). Third, the two-hybrid screening of tomato and soybean cDNA libraries for NSP interacting partners resulted in the independent isolation of the ortholog kinase genes from these plant species. Fourth, both LeNIK and GmNIK also interacted with NSP from TCrLCV, demonstrating that interaction with NIK was conserved among geminivirus NSPs. Finally, specific interaction between NSP and a kinase activity is expected to occur in vivo since NSP from *Squash leaf curl virus* (SLCV) has been demonstrated to be modified by phosphorylation (Pascal et al., 1994).

Although the subcellular localization of NIK was not precisely determined, NIK fractionated with leaf microsomal preparations composed primarily of endomembrane vesicles and plasma membranes (data not shown). Consistent with this observation, GmNIK possesses an internal transmembrane helix and a signal peptide that probably target the protein to the secretory apparatus. As a membrane-associated protein, NIK is expected to be transiently co-localized with NSP, since SLCV nuclear-localized NSP is redirected to the plasma membrane in insect Sf9 cells and in *N. tabacum* protoplasts upon expression of MP (Sanderfoot and Lazarowitz, 1995). Even though the NIK–NSP complex is stable enough to be detected by the two-hybrid system, we have not yet been able to demonstrate an interaction in vivo by a classical co-immunoprecipitation assay, as both proteins accumulate to extremely low levels in infected tomato plants.

Sequence comparison analysis revealed that GmNIK is most closely related to a group of putative LRR receptor serine/threonine protein kinases, whose modular structure allows signal transduction through membranes. In fact, GmNIK possesses conserved domains arranged into a modular organization that resembles a subclass of plant resistance genes and a group of structurally related proteins that are involved in various aspects of plant development. The resistance genes are represented by *Xa-21* from *Oryza sativa*, which confers resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Song et al., 1995). Examples of the developmental *LRR-RLK* genes include the *Arabidopsis ERSACTRA* and *CLAVATA1* genes that determine floral organ shape and size (Clark et al., 1997) and the *Arabidopsis BRI1* gene, which encodes a brassinosteroid receptor (He et al., 2000). Evidence that homology-defined domains of the LRR-RLK class of proteins represent functional modules was provided by domain swapping experiments in which a chimeric protein, composed of the BRL1 LRR and the Xa21 kinase domain, activated pathogen response upon brassinosteroid treatment (He et al., 2000). Thus, based on structural homology with RPP-RLKs, NIK may be involved in resistance and/or developmental signal transduction pathways.

Two possibilities, which are consistent with the function of GmNIK as a LRR-RLK protein, may be considered to explain the biological significance of the NSP–NIK interaction. In the first one, *NIK* would function as the R gene and *NSP* as the avirulence (*Avr*) gene for the induction of resistance to the geminivirus, as would be predicted in the elicitor-receptor model. Even though BDMV *NSP* has been demonstrated to function as an *Avr* gene in resistant bean cultivars (Garrido-Ramirez et al., 1998), we consider it unlikely that a resistance response to geminivirus infection is triggered by the NSP–NIK interaction because *NIK* was in fact isolated from the tomato cultivar Santa Clara that is susceptible to TGMV and TCrYLV infection. However, the observation that upstream components of the pathogen resistance response are present in both susceptible and resistant plants is not unprecedented (Kooman-Gersmann et al., 1996, 1998; Ren et al., 2000). In these reported systems, the identified components of the resistance pathway exhibit properties that are consistent with the guard hypothesis of plant resistance proposed by Van der Biezen and Jones (1998). According to this model, NSP would function as a virulence factor by binding to NIK to prevent the induction of a resistance response.

Alternatively, the interaction between NSP and NIK would serve as a regulatory mechanism to control the biochemical activities of the viral protein by phosphorylation. The movement functions of a plant virus movement protein have been demonstrated to be regulated by the phosphorylation state of the protein (Waigmann et al., 2000). In this case, NSP would recruit in a specific manner the kinase activity of the transmembrane receptor NIK for regulation of viral DNA movement. Consistent with this hypothesis, NSP from SLCV has been shown to be post-translationally modified by phosphorylation (Pascal et al., 1994). The biochemical confirmation of the NIK kinase activity using model substrates, NSP as substrate and in autophosphorylation assays will allow us to distinguish between these possibilities.

The localization of NSP and its proposed role in cell-to-cell movement of the viral DNA predict that interactions with host factors may occur in both cytoplasm and nucleus. Thus, the interaction of NSP with host factors could involve an active recognition in the nuclear pore, plasma membrane or plasmodesmata. Thus, the determination of the precise localization of GmNIK will provide valuable information about the functional significance of the interaction between NIK and NSP.

Materials and methods

Plasmid constructions

The binding domain vector pBDGAL4 and the activation domain vector pADGAL4 were purchased from Stratagene. The NSP and MP coding regions were amplified by PCR from TGMV DNA-B or TCrLYV DNA-B (Galvão et al., 2003) with Pfu DNA polymerase (Stratagene). The AC4 and Rep coding regions were amplified from TGMV DNA-A.

A 5′ *Eco*RI site and a 3′ *Pst*I site were introduced into the amplified MP fragment by PCR, and the MP coding region was cloned into the same sites of pADGAL4 to generate pAD-MP, which contained the GAL4 activation domain fused to MP sequences. The creation of a 3′ *Pst*I site in the amplified NSP fragment facilitated the cloning of the NSP coding into the *Sma*I/*Pst*I sites of pBDGAL4. The resulting clone, pBD-NSP, contained the GAL4 DNA binding domain fused to NSP sequences. The amplified Rep coding region was cloned into the *Sma*I site of pBD-GAL4 to give pBD-Rep. *Eco*RI sites was generated by PCR at the 5′ and 3′ ends of the AC4 coding region that was transferred as an *Eco*RI fragment to pBDGAL4, yielding pBD-AC4. The sequences of all inserts were determined to confirm the proper fusion of the constructs.

Yeast strain and two-hybrid assays

The yeast (*S. cerevisiae*) strain YRG-2 (Matα ura3 his3-200 ade1-1 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-553 LYS::$UAS_{GAL1}TATA_{GAL1}$TATA-HIS3 URA3::UAS_{GAL4}-$TATA_{GCYC}$-lacZz) is deficient in producing both tryptophan and leucine (Trp^-, Leu^-). The activation domain vector (pADGAL4) is Leu^+ and Trp^-, and the DNA binding domain vector pBDGAL4 is Leu^- and Trp^+.

Competent cells of strain YRG-2 were co-transformed with combinations of recombinant plasmids, plated onto synthetic dropout medium lacking leucine, tryptophan and histidine to investigate interactions of the chimeric proteins. Interactions of two partners were confirmed by a 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) filter staining assay. Untransformed yeast cells and yeast transformed with empty vectors (pAD-GAL4 and pBD-GAL4) were used as negative control, whereas yeast transformed with pGAL4 that expresses a complete GAL4 transactivator protein was used as positive control. Yeast cells were also transformed with the DNA constructs alone to assay for independent transactivation or DNA binding complementation by the recombinant proteins.

Yeast two-hybrid screening

The tomato cDNA library was prepared from mRNA isolated from young leaves using HybriZAP vectors (Stratagene). Total cDNAs of a phagemid form were obtained by the mass library in vivo excision method, using the ExAssist helper phage (Stratagene). The YRG-2 strain, containing pBD-NSP, was transformed with 100 μg of plasmid DNA of the HybriZAP excised cDNA library, along with 3 mg of salmon sperm carrier DNA, using the lithium acetate/polyethylene glycol method. The transformants were plated on synthetic dropout (SD) medium lacking Trp, Leu and His, but supplemented with 1 mM 3-aminotriazole and cultured

for 5–7 days at 30°C. Approximately 5×10^6 transformants were obtained, as estimated based on the number of transformants growing on the SD-Trp-Leu plate. Plasmid DNA was recovered from yeast and transformed into *E. coli* strain XL-1-Blue (Stratagene) by electroporation.

Quantitative assay of β-galactosidase activity

Mid to late exponential-phase yeast cells were collected and resuspended in Z buffer (100 mM sodium phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, 0.05% sodium dodecyl sulphate (SDS)) and β-galactosidase activity was assayed with *o*-nitrophenyl β-D-galactopyranoside (ONPG; Sigma/Aldrich), as described by Uhrig et al. (1999). All tests were repeated four times. Negative controls with untransformed yeast and transformed YRG-2 cells with the DNA constructs alone and a standard positive control containing yeast cells transformed with pGAL4 (that expresses the complete GAL4 protein upon yeast transformation) were included in the assays.

Recombinant protein expression and purification

Both NSP and LeNIK were expressed as fusion proteins using the pET-16b vector (Novagen), which provides an N-terminal His tag. For this purpose, LeNIK sequence was released from pAD-LeNIK with *Eco*RI and *Pst*I, treated with Klenow fragment and inserted into the blunt-ended *Bam*HI site of pET16b to create pHis-LeNIK. This construction was transformed into *E. coli* strain BL21 (DE3) and the synthesis of the recombinant protein was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). The induced protein was affinity-purified using Ni-chelating sepharose resin (Amersham Pharmacia Biotech.) and used as an antigen to raise polyclonal antisera in rabbits, which were immunized through subcutaneous injections during 2-week intervals.

A *Xho*I site at 5′ and 3′ ends of the NSP coding region was created by PCR using Pfu DNA polymerase and specific primers and the amplified fragment was then inserted as a *Xho*I fragment into pET16b to yield pHis-NSP. N-terminal His-tagged NSP fusion proteins were expressed in *E. coli* strain BL21 (DE3) following induction by IPTG and purified according to manufacturer's instructions (Novagen).

The PinPoint Xa protein purification system (Promega) was used to produce a NSP protein fused to a biotinylated tag polypeptide. The biotinylated fusion protein was produced in *E. coli* JM109 and was affinity-purified using SoftLink Soft Release Avidin Resin (Promega).

In vitro protein/protein binding assay

The in vitro binding assay was performed as described (Lin and Heaton, 2001). Briefly, *E. coli*-expressed His-NSP fusion protein was affinity-purified and blotted onto nitrocellulose membrane using a Slot-blot manifold (Bio-Rad). The membranes were probed with either the biotinylated NSP fusion protein or the 13-kDa biotinylated tag polypeptide, which were biotin-labeled in vivo using the PinPoint Xa protein purification system (Promega), as described above. The membranes were incubated with biotinylated proteins in TBST (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.05% Tween20) for 4 h, followed by streptavidin–alkaline phosphatase conjugate. Alkaline phosphatase activity was assayed using 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitroblue tetrazolium (Life Technologies, Inc.).

Co-immunoprecipitation assay

For co-immunoprecipitation assays, we used soluble protein extracts from *E. coli* cells, which were transformed with pHis-NSP or pHis-LeNIK. For this purpose, transformed cells were grown at 30°C and the synthesis of the recombinant proteins was induced by IPTG for 4 h. All of the subsequent procedures were conducted at 4°C. Soluble protein extracts from *E. coli* induced cells were prepared by homogenization of pellet cells with 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.5% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF). After incubation of protein extracts for 10 min, insoluble protein fractions were removed by centrifugation at $13{,}000 \times g$ for 15 min. Equal amounts of soluble protein extracts from *E. coli* expressing NSP and LeNIK were combined and incubated under gentle agitation for 12 h. Co-immunoprecipitation assays were performed as described by Cascardo et al. (2000).

Acknowledgments

We are grateful to Dr. Márcia Rogéria de Almeida for helpful discussions and critical reading of the manuscript. This research was supported by the Brazilian Government Agency, PADCT/CNPq Grant 62.0272/97.0 (to E.P.B.F.), FAPEMIG Grant CBB 333/01 (to E.P.B.F.) and a post-doctoral fellowship from the Brazilian Government Agency CNPq to S.M.B.C. A.C.M. and A.A.S. were supported by CNPq graduate fellowships. M.O.A. was a recipient of a CNPq undergraduate scholarship from the Brazilian Government.

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