Conserved Threonine Residues within the A-Loop of the Receptor NIK Differentially Regulate the Kinase Function Required for Antiviral Signaling

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Abstract

NIK-interacting kinase (NIK1) is a receptor-like kinase identified as a virulence target of the begomovirus nuclear shuttle protein (NSP). We found that NIK1 undergoes a stepwise pattern of phosphorylation within its activation-loop domain (A-loop) with distinct roles for different threonine residues. Mutations at Thr-474 or Thr-468 impaired autophosphorylation and were defective for kinase activation. In contrast, a mutation at Thr-469 did not impact autophosphorylation and increased substrate phosphorylation, suggesting an inhibitory role for Thr-469 in kinase function. To dissect the functional significance of these results, we used NSP-expressing virus infection as a mechanism to interfere with wild type and mutant NIK1 action in plants. The NIK1 knockout mutant shows enhanced susceptibility to virus infections, a phenotype that could be complemented with ectopic expression of a 35S-NIK1 or 35S-T469A NIK1 transgenes. However, ectopic expression of an inactive kinase or the 35S-T474A NIK1 mutant did not reverse the enhanced susceptibility phenotype of knockout lines, demonstrating that Thr-474 autophosphorylation was needed to transduce a defense response to geminiviruses. Furthermore, mutations at Thr-474 and Thr-469 residues antagonistically affected NIK-mediated nuclear relocation of the downstream effector rpl10. These results establish that NIK1 functions as an authentic defense receptor as it requires activation to elicit a defense response. Our data also suggest a model whereby phosphorylation-dependent activation of a plant receptor-like kinase enables the A-loop to control differentially aut- and substrate phosphorylation.

Introduction

The perception of external stimuli through cell surface receptors is a common mechanism of multicellular organisms that allows communication among cells and between cells and the external environment. In plants, an extensive battery of Ser/Thr receptor-like kinases (RLK) may transduce external signals into cells through the reversible phosphorylations that allow the cells to sense and respond to external signals in a precise, regulated and adaptive way [1].

In the Arabidopsis genome, the RLK family is represented by 417 sequences that are organized into a typical receptor configuration harboring an N-terminal extracellular domain followed by a transmembrane segment and a kinase domain at the C-terminus [1]. These receptors have been shown to be predominantly involved in developmental events and defense strategies. Characterized members of this family include BRI1 and BAK1 involved in brassinosteroid signaling [2,3,4], HAESA associated with floral abscission regulation [5], SERK1 associated with early embryogenesis [6], BONZAI responsible for growth capacity under different temperature conditions [7], ERECTA and GLAVATA1 that control size and shape of flowers [8,9], RLK2 that controls anther development [10] and typical defense proteins, such as FLS2 [11], BAK1 [12,13] and the rice Xa-21 protein [14].

The representatives of the Arabidopsis RLK superfamily have been phylogenetically organized into families based on the structural identity of extracellular domains and conservation of C-terminal kinase domains [1,15,16]. The major family comprises the leucine-rich repeats (LRR)-RLKs which contain three to 26 LRR motifs in their extracellular domains [1]. Further subdivision into 13 sub-families (LRR1-LRRXIII) is based on sequence identity and organization of the LRR domain. One of these, the LRRII-RLK subfamily, is constituted by 14 proteins harboring four complete LRRs (with 24 residues) and a fifth incomplete LRR (with 16 residues) arranged in a single continuous block within the extracellular domain [17]. Phylogenetic analysis based on sequence conservation and evolutionary structural features of LRRII-RLKs has demonstrated that the members of this subfamily are clustered into three distinct branches of functional relatedness: (i) antiviral defense proteins, (ii) developmental proteins and (iii) functionally unassigned proteins. The NSP-
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interacting kinase 1, NIK1 (At5g16000), NIK2 (At3g25560) and NIK3 (At1g65000) are inserted into the defense group I of the LRRRII-RLK sub-family [17] and they have been initially identified as virulence targets of the bipartite geminivirus nuclear shuttle protein, NSP [16]. The NSP-NIK interaction is conserved among geminivirus NSPs and NIK homologs from different hosts. Tomato and soybean NIK homologs also interact stably with NSP from CaLCuV (Cabbage leaf curl virus) and from the tomato-infecting geminiviruses TGMV (Tomato golden mosaic virus) and TCrLYV [Tomato crinkle leaf yellows virus; 18,19,20]. The assignment of NIK as a defense receptor gene was based primarily on the enhanced susceptibility phenotype to geminivirus infection [18,21]. Nevertheless, it remains to be determined whether the NIK kinase domain is involved in the antiviral defense responses based on our studies showing that loss of NIK function in Arabidopsis is linked to an enhanced susceptibility phenotype to CaLCuV infection [18,19,20].

Results

Mutation at threonine residues within the A-loop of NIK1 impacts autophosphorylation

Activation of many kinases requires phosphorylation of the activation segment that is defined by the region delimited by two conserved tripeptide motifs, DFG and APE [31]; Figure 1A. Conserved secondary elements in this segment include the magnesium binding loop, b9, at the N-terminus, the centrally located activation loop (A-loop) and the P+1 loop at the C-terminus. The activation segment is highly conserved among members of the Arabidopsis LRRRII-RLK sub-family and NIK counterparts from other plant species, such as tomato (LeNIK) and soybean (GmNIK) (Figure 1A). In the case of SERK1, the conserved Thr-462 and Thr-468 residues within the A-loop have been shown to be intermolecular targets of SERK1 kinase activity in vitro [32]. Likewise, the conserved SERK3/BAK1 residues Thr-446, Thr-449 and Thr-455 have been shown to be phosphorylated in vitro as well as in vivo in response to brassinosteroid signaling [33]. Conservation of more distantly related kinases can also be seen by computer-assisted threading of the amino acid sequence of NIK1 onto the Ser/Thr kinase 2oidB (Interleukin 1 receptor associated kinase 4, IRAK4) as a template [34]. A striking feature is the almost perfect overlay of the activation segments of the two proteins which correspond to regions that have been shown to regulate kinase function. These observations prompted us to investigate the role of putative phosphorylation sites within NIK1 A-loop through site-directed mutagenesis (Figure 1B). The resulting mutant kinase domains were expressed as GST fusions (Figure S1) and examined for autophosphorylation activity (Figure 2). We have previously demonstrated that the kinase domain of NIK1 fused to GST exhibits Mg2+-dependent autophosphorylation activity that occurs intermolecularly [18].

Replacement of Thr-474 with alanine (T474A) strongly inhibited autophosphorylation as the T474A mutant exhibited only 23% residual activity (3.1 \times 10^3 \text{ cpm/\mu g/min}) as compared to NIK1 (15.7 \times 10^3 \text{ cpm/\mu g/min}; Figure 2). Very likely, this effect was due to the removal of Thr and not due to the nature of the newly introduced amino acid residue because glutamate in the same position (T474E) also promoted a similar inhibition of autophosphorylation from 13.7 \times 10^3 to 1.32 \times 10^3 \text{ cpm/\mu g/min} (lane 4). Nevertheless, replacement of Thr-474 with aspartate (T474D) did not promote an accentuated impact on autophosphorylation and the T474D mutant retained 72% activity (9.9 \times 10^3 \text{ cpm/\mu g/min}; Figure 2C, lane 3). This slight effect on the relative activity of the mutant kinase may be due to the loss of an incorporated radioactive phosphate rather than a decrease in the autophosphorylation activity. We confirmed that the Thr-474 residue can be in vitro phosphorylated by performing matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric analyses of tryptic digests of GST-KNIK1 and GST-KDT474A mutant (Figure 3); MALDI-TOF analyses of in vitro phosphorylated GST-KNIK1-derived tryptic fragments revealed a peak at m/z of 1960.854 corresponding to the peptide 473GTVGHIAPJEYLSSTGQSSEK 491 and a peak of m/z 2040.870 representing a shift of 80.02 that corresponds to the addition of a phosphate moiety on residues 473–491. To determine whether the Thr-474 was the site of phosphate incorporation, the tryptic peptides of in vitro phosphorylated GST-KDT474A mutant were
also analyzed by MALDI-TOF mass spectroscopy. The absence of the ion 2010.9504, corresponding to 1930.9504 Da (\textsuperscript{17}GAV-\textsuperscript{473}AVGHIAPEYLSTGQSEK\textsuperscript{491}) +80.00 Da (see arrow in Figure 3B), is indicative that the mutated peptide was not phosphorylated, confirming that the Thr-474 residue represents the phosphorylation site on wild type peptide\textsuperscript{473}G-K\textsuperscript{491}. Taken together, these results indicate that phosphorylation of the Thr-474 residue is crucial for NIK activity, as introduction of a correctly positioned carboxylate from aspartate at this position restored autophosphorylation activity. In addition, they are suggestive that regulation of kinase activity depends on a phosphorylation-induced conformational change of the A-loop, because a mutant with glutamate (T474E) had only 9.6% residual activity. We interpret this drop as reflecting antagonism of the activation process by imposition of steric constraints to a required conformational change. In support of this model, changing the

**Figure 1. Conservation in the LRRII-RLK family and sequence position of mutant NIKs.** (A) Sequence alignment of the 29 amino acid activation segment (boxed) of NIKs and other members of the LRRII-RLK family. The activation segment of NIK1 was compared to its counterparts from tomato (LeNIK) and soybean (GmNIK) and to others members of the Arabidopsis LRRII-RLK family, as indicated. Conserved residues as potential phosphorylation sites are shown in bold letters. The corresponding region of IRAK4 (30 amino acids) is shown below for comparison. (B) Schematic representation of the site-directed mutations within the NIK1 A-loop. The mutant kinases are indicated by their names and their respective mutations are indicated in bold letters. The arrows in the NIK1 sequence show mutated amino acid residues and the numbers refer to their respective positions. Boxed sequence delimits the activation segment. (C) Schematic representation of the kinase domain of two truncated forms of NIK1. The solid gray box represents the serine/threonine kinase domain; NBS represents the nucleotide binding site; AS, the active site; AL, the activation loop; NSP BS, the NSP binding site. The numbers correspond to amino acid positions on NIK1 primary structure and asterisks represent the relative position of serine residues.

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conserved Gly-473 residue in the T474A mutant to valine (G473V/T474A) totally abolished autophosphorylation activity and indicated that removing a strong structural constraint by substituting the flexible Gly-473 prevented the A-loop from adopting an active conformation. In contrast, mutation at Thr-469 caused a much lower impact on autophosphorylation activity of the resulting mutant kinase (10.9×10^3 cpm/mg/min compared to 13.7×10^3; Figure 2C, lane 6) and like T474D, may reflect reduction of the incorporated radioactivity without alteration of the autophosphorylation activity of the protein. In fact, using MALDI-TOF mass spectrometric analyses we detected a monophosphorylated form (peak at m/z of 1671.74) of the peptide ^459_LLDHQDSHVTTAVR^472 (peak of m/z 1591.75, Figure 4A). MALDI-TOF/TOF analysis of the respective peptides was further used to examine the phosphorylation site on the monophosphorylated form. In Figure 4B, the MS/MS spectrum of the non-phosphorylated ^459_L-LDHQDHSVTTAVR^472 peptide is shown. Phosphorylated peptides were confirmed by identification of a strong signal ([M+H] 1573,74) corresponding to the mass difference of -98.0 Da from the neutral loss of phosphoric acid (H3PO4), as indicated in Figure 4C. Phosphorylation at Thr-469 was confirmed by the presence of the ion b11 [M+H] 1328.10 that corresponds to the fragment LLDHQDSVTT-PO3.

To confirm the regulatory role of the A-loop phosphorylation, we made double mutations at T474A and the conserved putative phosphorylation sites, Ser-465, Thr-468 and Thr-469, as indicated in Figure 1B. All double mutants exhibited reduced autophosphorylation activity which varied from 34% to 40% of the normal NIK1 activity (Figure 2C). Although we did not obtain a Thr-468 single mutation, the substitution of this residue to alanine in the T469A mutant (T468A/T469A) caused a strong reduction in autophosphorylation down to 34% activity (4.7×10^3 cpm/min/mg, lane 7) as compared to the normal kinase, suggesting that Thr-468 also plays a relevant role in the kinase activation.

**NIK1 kinase inhibition by the geminivirus NSP**

Previous work had indicated that the NSP binding site on NIK overlaps two kinase sub domains VIb and VIII [18]. This observation raised the possibility that NSP binding to NIK might prevent A-loop transphosphorylation and hence kinase activation (Figure 1C). Inclusion of GST-NSP fusion in phosphorylation assays in vitro promoted a 50% reduction on NIK1 autophosphorylation (Figure 5). Except for the T474D mutant, NSP effectively caused a further 50–70% reduction in the residual activity of the mutant kinases independently of the mutation in the conserved residues of NIK1. The capacity of NSP to inhibit the mutant proteins indicates that any effect of the single and double amino acid replacements on NIK structure was too small to impair NSP binding (Figure 1C). Thus, the reduction in kinase activity by Thr replacements within the A-loop may be due to the lack of phosphorylation sites rather than a global misfolding of the kinase domain upon introduction of a different amino acid residue.

In contrast to the alanine-substituted NIK A-loop mutants, the T474D mutant showed an attenuated effect of NSP on inhibition of autophosphorylation with only a 20% reduction in kinase activity in the presence of NSP. The lesser effect of NSP on inhibition of the activity of the T474D mutant could be due to a conformational change leading to destabilization of the NSP-NIK complex when

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**Figure 2. Autophosphorylation properties of mutant NIK1 proteins.** GST-fusion proteins (as indicated) were incubated with [γ-32P]ATP, separated by SDS-PAGE and stained with Coomassie-brilliant blue. Phosphorylated proteins were visualized by autoradiography (A). The coomassie-stained gel (B) was used to normalize protein loading with Multi Gauge V3.0 (Fujifilm) software. The radioactivity incorporated into proteins was quantified by phosphoimaging (C). The relative kinase activity was expressed as Vunits/min/μg protein. Values are given as mean±S.D. of three determinations.

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in silico

Peptide mass was calculated using FlexAnalisys package (Bruker Daltonics). Only the regions of the spectra containing the peptides of interest are shown.

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These results are consistent with the current model for Ser/Thr kinase to phosphorylate the substrate rpL10 (Figures 7B and 7C).

G473V/T474A, T474A/T468A, also reduced the capacity of the kinase protein, such as T474A, T474E, (Figure 7). In general, mutations that negatively affect the autophosphorylation activity of NIK1 (Fontes et al., 2004). We also monitored the effect of the point mutations on the substrate phosphorylation activity of NIK1.

Conserved threonine residues within the A-loop play distinct roles in kinase function

The ribosomal protein L10 has been shown to be a substrate of NIK1 that functions as the immediate downstream component of the NIK-mediated antiviral signaling [21,22]. Inclusion of the recombinant NIK-L10 fusion in the kinase assay demonstrated that the ribosomal protein was efficiently phosphorylated by NIK1 (Figure 7). There was no measurable 32P incorporation into GST when GST alone was incubated with KDNIK1 and [γ-32P]ATP (data not shown; Fontes et al., 2004). We also monitored the effect of the point mutations on the substrate phosphorylation activity of NIK1 (Figure 7). In general, mutations that negatively affect the autophosphorylation activity of the kinase protein, such as T474A, T474E, G473V/T474A, T474A/T468A, also reduced the capacity of the kinase to phosphorylate the substrate rpL10 (Figures 7B and 7C). These results are consistent with the current model for Ser/Thr kinase regulation in which autophosphorylation promotes kinase activation and precedes substrate phosphorylation [35,36]. In contrast, replacing the essential Thr-474 residue with aspartate caused a 28% reduction in autophosphorylation activity (Figures 2 and 7B, lane 3), but promoted a 50% increase (up to 150% activity) in the substrate phosphorylation activity (Figure 7C, compare lane 1 (0.6 x 10^4 cpm/μg substrate) with lane 3 (0.9 x 10^4 cpm/μg substrate)). This observation suggests that introduction of a correctly positioned carboxylate at position 474 causes constitutive activation of the protein by mimicking the induced phosphorylation of Thr-474. In addition, it indicates that phosphorylation of Thr-474 is a determinant of kinase activation. Likewise, the T469A mutant caused about 40% decrease in autophosphorylation, but exhibited a 3-fold higher substrate phosphorylation (1.83 x 10^4 cpm/μg substrate) than that of the wild type (Figure 7C, compare lanes 8 and 1). Very likely phosphorylation at Thr-469 inhibits substrate phosphorylation, as mutation of this residue to alanine relieves repression and enhances the capacity of the enzyme to phosphorylate rpL10. This inhibitory effect of Thr-469 was further evidenced in T468A/T469A (lane 9) and T474A/T469A (lane 11) double mutants, which exhibited enhanced substrate phosphorylation in comparison with NIK1, in spite of harboring mutations in the relevant Thr-474 or Thr-468 residues for autophosphorylation but enhanced substrate phosphorylation activity (Figure 7, lane 12).

In addition to analyzing the A-loop, we analyzed the C-terminal region by introducing a stop codon at position 609 or 588...
Figure 1C. Each deletion decreased autophosphorylation activity to the same extent and totally abolished substrate phosphorylation (Figure 7, lanes 6 and 7). These results indicate that the C-terminal fragment, delimited by positions 609 to 638, may exhibit a regulatory role. Inspection of this region revealed the presence of three serine residues at positions 613, 615 and 619 with the potential to be substrates for serine/threonine kinases. Like other receptor-like serine/threonine kinases, NIK1 may possess several other autophosphorylation sites that represent docking sites for substrate recruitment or play distinct regulatory function. Although the biological relevance of this region on kinase function remains to be determined as it extends beyond the scope of the present investigation, these preliminary experiments suggest that NIK1 shares with receptor-like serine/threonine kinases the same complex mechanism of autophosphorylation-dependent activation of kinase function.

Thr-474 and Thr-469 exhibit antagonistic roles in regulation of NIK-mediated antiviral signaling

Assignment of NIK as a transmembrane signaling receptor that mediates an antiviral defense response has been based on the biochemical properties of the kinase in vitro, its inhibition by the geminivirus NSP and the enhanced susceptibility phenotype to geminivirus infection of knockout lines [18]. To determine whether the NIK-mediated antiviral signaling is indeed transduced through the intracellular kinase domain in vivo, functional complementation assays were performed by expressing the NIK1 wild type protein or a mutant NIK1 harboring an inactive kinase domain (G473A/T474A) in nik1 null alleles. We also included the mutants T474A and T469A in the complementation assays to dissect the functional significance of these mutations in vivo. The transformed plants were confirmed by PCR (data not shown) and the transgene expression was monitored by real time RT-PCR for a transgenic line ectopically expressing the wild type protein (NIK*), two independently transformed KO lines.
expressing the G473V/T474A mutant protein (mNIK-A and mNIK-B), and transformed lines expressing T469A or T474A mutant proteins (Figure 8A). In addition, we also demonstrated by transient expression in tobacco leaves that the mutant receptors fused to GFP accumulated to detectable levels in transfected plants, as they displayed the same fluorescence pattern as a GFP-fused wild type kinase (Figure 8B). Immunoblots showed that NIK1 and mutant proteins fused to GFP accumulated to similar levels when transiently expressed in tobacco leaves [data not shown, 21]. These results confirmed that the mutations in the kinase domain of NIK1 did not affect the capacity of the protein to be associated with the cell surface and to accumulate stably in transformed plants. The transgenic lines expressing the intact NIK1 (NIK*), the double mutant protein (mNIK-A and mNIK-B), T469A and T474A mutants were biolistically inoculated with an attenuated form of CaLCuV [18]. All inoculated lines developed typical symptoms of CaLCuV infection although with different intensity (Figure 9A and data not shown). The accumulation of viral DNA was detected in all symptomatic plants by PCR with transgene-specific and viral DNA-specific multiplex primers (Figure 9B). Ectopic expression of wild type NIK1 in n1k1 KO lines restored the wild type phenotype with respect to susceptibility to geminivirus infection, as the transgenic lines developed attenuated symptoms (mild stunting with epinasty and moderate chlorosis) and displayed similar infection rates as Col-0 (Figure 9C). In other independent experiments, the infectivity data, expressed as DPI50% (days post-inoculation to reach 50% of infected plants), further confirmed that NIK* and Col-0 displayed the same

![Figure 7. Substrate phosphorylation activity of NIK1 and mutants on rpl10.](image-url)

Purified GST fusions (as indicated) were incubated with equal amounts of GST-L10 in the presence of [γ-32P]ATP and separated by SDS-PAGE (A). The gels were stained with Coomassie-brilliant blue (bottom) and visualized by autoradiography using a phosphoimager (top). Protein loading was normalized by densitometric scanning of the Coomassie-stained gel. The relative activity of autophosphorylation (B) and phosphorylation of the substrate (C) were quantified and expressed as Vunits/µg enzyme/min and Vunits/µg enzyme/µg substrate/min, respectively.

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efficiency of virus infection (Figure 9D). In contrast, ectopic expression of the G473A/T474A inactive kinase in KO lines (mNIK-A and mNIK-B) did not complement the niki loss-of-function defect and the independent transgenic lines kept a similarly enhanced susceptibility phenotype as the niki line (Figures 9C and 9D). Conversely, the mutant T469A with a high substrate phosphorylation activity complemented the niki loss-of-function defect as efficiently as the NIK1 transgene (NIK*) and the T469A transformed line displayed an infection rate similar to NIK* (Figure 9C), with DPI50% similar to Col-0 and NIK* (Figure 9D). Ectopic expression of T474A defective kinase did not fully complement the niki loss-of-function phenotype and an enhanced susceptibility phenotype in T474A lines was apparent from 12 days post-inoculation as compared to that of NIK* and T469A lines (Figure 9D). These results establish that NIK1 functions as an authentic defense receptor as it requires activation to mediate an antiviral signaling. Furthermore, they confirmed in vivo that phosphorylation at position Thr-474 is essential for full NIK1 kinase activation and defense signaling as opposed to phosphorylation at position Thr-469 that is likely to repress substrate phosphorylation activity.

Regulation of NIK1 Activation

Thr-474 and Thr-469 residues sustain their antagonistic roles in NIK-mediated nuclear relocalization of rpl10

The T474D mutant was not included in our complementation assay because we could not recover T474D expressing transformed lines. However, the relevance of this mutation has been recently addressed in vivo by the demonstration that the T474D mutant exhibits an enhanced capacity to phosphorylate and relocate rpl10 from the cytoplasm to the nucleus when transiently expressed in tobacco infiltrated leaves [21]. NIK-mediated nuclear relocalization of rpl10 is thought to trigger a defense response that impairs virus proliferation or spread. We used the antagonistic
mutants T469A and T474A to examine whether redirection of rpL10 to the nucleus would couple NIK activation to the defense response. The subcellular localization of rpL10 in co-transfected leaf cells was assayed by confocal microscopy (Figure S2, merged field) and the frequency of cells with nuclear-localized rpL10 was recorded (Figure 10). Ectopically expressed rpL10 in transfected cells is predominantly localized in the cytoplasm and only a small fraction (5%) of transfected cells contains nuclear rpL10. Co-expression with NIK1, however, altered the nucleocytoplasmic shuttling of rpL10 because the cell frequency with nucleus-localized YFP-rpL10 was significantly increased to 50.6±4% of co-transfected leaf cells. As expected [21], the hyperactive T474D mutant enhanced translocation of rpL10 to the nucleus (68±8% of transfected cells with nuclear rpL10). While the inactive G473V/T474A double mutant kinase gene (mNIK-A and mNIK-B), T469A or T474A transgenes. The values represent the percentages of systemically infected plants at different DPI50% and are given as mean of three determinations from independent experiments. (D). Infection rates in nik1 transgenic lines. The infection rate is expressed as number of DPI required to get 50% infected plants (DPI 50%). The data are means of four independent experiments.

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Discussion

The NIK receptor has been hypothesized to mediate an antiviral defense response through a reversible phosphorylation strategy that both initiates a signaling pathway as well as
modulates the consequent adaptive response. Here we separated auto- and substrate phosphorylation and identified Thr-474 as targets for intermolecular autophosphorylation and full kinase activity. Conversely, a Thr-469 residue within the A-loop seems to play an inhibitory role in kinase function as its individual replacement with alanine did not impair autophosphorylation but enhanced substrate phosphorylation.

The Thr-468 and Thr-474 residues aligned at the same position as Thr-462 and Thr-468 on SERK1 as well as Thr-449 and Thr-455 on SERK3/BAK1, other members of the LRRII-RLK sub-family. Both threonine residues within SERK1 A-loop and BAK1 A-loop have been shown to play relevant roles in kinase function. Thr-468 on SERK1 is absolutely essential for in vitro kinase activity [33] and the equivalent residue on BAK1, Thr-455, has been shown to play a critical role in BAK1 signaling [34]. Our results implicate Thr-468 and Thr-474 on NIK1 as functional analogs of Thr-462 and Thr-468 on SERK1, as well as of Thr-449 and Thr-455 on SERK3/BAK1, respectively. Individual mutations in NIK1 respective residues promote an 80% reduction in kinase autophosphorylation that in turn leads to equivalent decrease in the substrate phosphorylation activity. Phosphorylation of residues within the A-loop constitutes one of the key regulatory mechanisms not only of Ser/Thr kinases but also of Tyr kinases [35,36,37,38,39].

The four lines of evidence presented here demonstrate that the underlying mechanism for Thr-474-dependent kinase activation is due to phosphorylation. Firstly, MALDI-TOF mass spectrometric analyses of tryptic digests of in vitro phosphorylated GST-KDNK1 and GST-KDT474A revealed that Thr-474 was the phosphorylated residue on the tryptic phosphopeptide $^{57}$GTpVGHIA-PEYLSTGQSEK [40]. Secondly, replacing Thr-474 with alanine or glutamate reduced kinase activity to similar extent, eliminating the possibility that the variation observed might has been due to a possible effect of the newly introduced amino acid residue on structure. Thirdly, the T474A mutant keeps the capacity to be further inhibited by NSP, indicating that replacement of Thr-474 with alanine did not cause a global misfolding of the kinase domain that would impair NSP binding and affect kinase activity. Finally, the introduction of a negatively charged aspartate residue at position 474 did not alter the autophosphorylation activity but enhanced the efficiency of substrate phosphorylation. The findings extend genetic studies linking inactivation of NIK genes to enhanced susceptibility to geminiviruses and protein structural analysis of residues required for kinase activity. Very likely the presence of a correctly positioned carboxylate mimicked the phosphorylation state of Thr-474 resulting in constitutive activation of the receptor kinase. SNF-1 kinases and plant SOS2 (Salt Overlay Sensitive2)-like protein kinases – PSKs have been shown to be highly activated by substitution of phosphorylated residues with aspartate within the activation loop [40,41,42]. However, the extent by which A-loop phosphorylation-induced kinases are activated by mutations of phosphorylation residues to aspartate or glutamate has been shown to vary considerably among Tyr kinases and Ser/Thr kinases [reviewed in 43]. In the case of NIK1, we further demonstrated that the correct position of the introduced carboxylate within the A-loop was relevant for activity because there was almost no detectable activity with the T474E mutant. The contrasting results of T474D and T47E on NIK1 activity resemble those found for the A-loop mutants of Protein Kinase C, in which the replacement of its critical Thr-500 residue with glutamate increases kinase activity, whereas aspartate in same position inactivates kinase [44]. While the extra methylene in glutamate may be necessary to position the carboxyl group in the correct orientation for electrostatic interactions that could align catalytic residues in the protein kinase C activation loop, it clearly imposes a conformational constraint for NIK1 activation. Similar results have been reported for the SERK1 protein in which the substitution of Thr-468 residue, which is analogous to the NIK1 Thr-474 residue, with glutamate abolishes autophosphorylation activity [33]. Taken together, our results with the Thr-474 mutants of NIK1 are consistent with the current activation model of kinases in which phosphorylation of the A-loop induces conformational changes to position correctly the residues that interact directly with the substrate and the catalytic domain [reviewed in 31]. In addition they implicate the NIK1 residue Thr-474 as the critical phosphorylation site for kinase activation.

We also demonstrated that although Thr-469 within the A-loop is not important for autophosphorylation it functionally antagonizes Thr-474 by playing an inhibitory role in the substrate phosphorylation activity. In fact, replacing Thr-469 with an alanine residue relieves repression and enhances considerably substrate phosphorylation. One possible explanation is that phosphorylation of these residues would antagonize phosphorylation of the relevant Thr-474 residue by blocking the conformational change that permits unrestricted access of ATP and protein substrates to the kinase active site, as that deduced from crystal structures of phosphorylated active kinases [35,36]. The slight decrease in $^{32}$P incorporation into T469A upon autophosphorylation may account for loss of a phosphorylation site in the mutant proteins, favoring the argument that Thr-469 is a target of NIK1.
kinase activity. In support of this, MALDI-TOF/TOF experiments conducted on the phosphorylated form of the tryptic peptide 459-474 demonstrated that Thr-469 can be phosphorylated. However, our data do not rule out the possibility that the putative relief of structural constraints was due to the introduction of alamime at the structurally restrictive position. The confirmation that this conserved threonine residue is phosphorylated in vivo is necessary to distinguish between these possibilities.

The previous assignment of NIK as a defense gene was based on circumstantial evidence that linked the enhanced susceptibility phenotype to geminivirus infection to the loss of NIK function. Here we performed complementation assays in nkl null alleles, which demonstrated that NIK is an authentic defense signal transducer as it we performed complementation assays in phenotype to geminivirus infection to the loss of circumstantial evidence that linked the enhanced susceptibility possibilities.

The NIK1 activation loop mutations were obtained through the Gene Tailor™ Site-directed Mutagenesis system (Invitrogen Life Technologies, Inc.) using the recombinant plasmid pDON-AtNIK1 [18] as template and partially overlapped primers (Table S1) that generated the nucleotide changes: Thr474 to Ala474 (pDON-AtNIK1T474A), or Glu474 (pDON-AtNIK1T474E) or Asp474 (pDON-AtNIK1T474D), Thr469 to Ala469 (pDON-AtNIK1T469A) as well as Gly473/Thr474 to Val473/Ala474 (pDON-AtNIK1G473TV474A). The resulting clones harboring single mutations were used to generate the double mutants: pDON-AtNIK1D468A/T469A, pDON-AtNIK1T474A/T468A, pDON-AtNIK1T474A/T469A, and pDON-AtNIK1T474A/S465A with the primers shown in Table S1. To create plasmids for E. coli expression, the mutant NIK1 C-terminal kinase domains (KD), encoding amino acids 297–638 were amplified from the appropriate mutated clone with the primers NBSNIK1-GA (5'-aaaaggttaatacagagagagagagagagagagagtggatgagtc-3') and KDAAtNIK1RG (5'-agaaagggattctgctgagagagagagagagagagagtgtgagtc-3'), introduced by recombinase into the entry vector pDONR201 and then transferred to the bacterial expression vector pDEST15, resulting in GST fused to mutant kinase domains, such as pGST-KDN1K1T474A, pGST-KDN1K1T474D, pGST-KDN1K1G473V/T474A, pGST-KDN1K1G473V/T474A, pGST-KDN1K1G473V/T474A, pGST-KDN1K1G473V/T474A, pGST-KDN1K1G473V/T474A, pGST-KDN1K1G473V/T474a and pGST-KDN1K1T474A/S465A. The double mutant NIK1G473V/T474A cDNA, as well as T469A, T474A and T474D mutant cDNAs were also amplified from the respective mutated clones with the primers AtNIK1FG (5'-aaatggtcttaaaagagagagagagagagagagagtattac-3') and AtNIK1RGNS (5'-aaatggtcttaaaagagagagagagagagagagagtattac-3').
agaagagtgggtttcagaccagagactccatt-3'), re-introduced by recombination into the entry vector pDONR201 and then transferred to the binary vector pK7Wg2 [46] to yield pK7F-NIK1G473V/F474A, pK7F-NIK1T469A, pK7F-NIK1T474A and pK7F-NIK1T474D. These resulting clones harbor a GFP gene fused in-frame after the last codon of the respective mutant cDNAs under the control of the CaMV 35S promoter.

A ribosomal L10 cDNA was isolated from an Arabidopsis cDNA two-hybrid library in the context of its capacity with interacting with the kinase domain of NIK1 [22]. The L10 cDNA was amplified by PCR with appropriate extensions provided by the primers rpL10Fwd (5’-aaagaagctgttcaatggagagct-3’) and rpL10Rvs (3’-agaagctgttcaatgtggaggctgaaaa-3’), introduced by recombination into the entry vector pDONR201 and then transferred to pDEST15 to generate GST-fused L10 (pGST-L10).

To obtain the YFP gene fused before the first coding of L10, the respective cDNA was transferred from pDONR207 to 35S-YFP-cassette-A-Nos-pCAMBIA1300, yielding pYFP-L10 [21]. The plasmid pGST-NSP, harboring the CaLCuV NSP sequence fused to GST, has been described previously [18].

Expression in E. coli and purification of GST-fused proteins

E. coli, strain BL21::DE3 pLysS, was transformed with plasmids containing different fusions and the synthesis of the recombinant proteins was induced with 0.4 mM isopropyl-d-thiogalactopyranoside (IPTG) for 16 h at 20°C and 200 rpm. The accumulation of recombinant proteins was monitored by SDS-PAGE in whole cell extracts, as well as in soluble and insoluble fractions. Cells were pelleted by centrifugation, resuspended in lysis buffer [140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4, 0.4% (v/v) Triton X-100 supplemented with 0.15 mg/mL of lysozyme, 0.8 mM PMSF, 1 mM benzamidine, 1 mM thiorurea], incubated at 4°C for 30 min, disrupted by sonication, and centrifuged at 14,000 g for 20 min at 4°C. The GST fusions were affinity-purified using GST-Sepharose beads (GE healthcare), according to manufacturer’s instructions. The efficiency of protein purification was monitored by SDS-PAGE.

Protein kinase assay

The purified GST-fused proteins (KDNIK1T474A, KDNIK1T474D, KDNIK1T474E, KDNIK1G473V/F474A, KDNIK1T469A, KDNIK1T468A/T469A, KDNIK1T474A/T468A, KDNIK1T474A/T469A, KDNIK1T474A/S465A) were incubated alone or with the substrate GST-L10 at 25°C for 45 min in 20 μL of kinase buffer containing 18 mM HEPES pH 7.4, 10 mM MgCl2, 10 mM MnSO4, 1 mM DTT, 200 μM ATP. Phosphoproteins were resolved by SDS-PAGE. GST-KDNIK1 or GST-KDT474A spots were excised from SDS-PAGE gel, rinsed with distilled water and destained twice with 30-min washes in 50 mM NH4HCO3/50% acetonitrile. After dehydration in 100% acetonitrile, the gel pieces were incubated with 30 μL of 12.5 ng/μL sequencing grade trypsin (Promega, Madison, WI) for 12 h at 37°C. Tryptic peptides were extracted from the gel pieces twice with 15 μL of 1% formic acid/50% acetonitrile for 20 min. The pooled extracts were dried in speed-vac and tryptic digests were resuspended in 50% acetonitrile/1% phosphoric acid/1% TFA.

MALDI-TOF/TOF experiments were conducted on an Ultraflex MALDI-TOF/TOF Analyzer (Bruker Daltonics). Peptides from each spot were mixed 1:1 with matrix solution [2,5-dihydroxybenzoic acid (2,5-DHB) in 50% acetonitrile/0.1% trifluoroacetic acid/1% phosphoric acid] and 1 μL were applied to wells of a sample target plate. Peptide mass fingerprints were obtained using the reflector and positive ion mode. Mass spectra were collected from the sum of 200–800 laser shots, and monoisotopic peaks were obtained. LIFT mass spectra were acquired and metastable fragmentation was induced without the further use of collision gas.

Peptides mass spectra were matched in silico against the GST-KDNIK1 theoretical tryptic mass using the PIUMS software with mass tolerance of 100 ppm, one missed trypsin cleavage, fixed modification of carbamidomethyl cysteine, and variable modifications of methionine oxidation and threonine, tyrosine and serine phosphorylation. Peptides sequencing was made by visual inspection of mass spectrum using FlexAnalysis software. Phosphorylated peptides were confirmed by an increase in mass of 80 Da and a decrease of 98.0 Da corresponding to the addition of PO3 and neutral loss of H3PO4, respectively.

RT-PCR and qRT-PCR analyses

Total RNA was extracted from Arabidopsis seedlings using TRizol (Invitrogen). Reverse transcription (RT)-PCR assays were performed with 2 μg of total RNA, 0.5 μM of poly-dT and 1U of M-MLV reverse transcriptase (Invitrogen Life Technologies, Inc.), as previously described [47,48]. PCR was carried out with NIK1-specific primers (AtNIK1-Fwd, 5’-acccggatctcaagagcagcg-3’ and AtNIK1-Rvs, 5’-atgtcgtgagccatcctgtc-3’) and with actin-specific primers (AtACTIN-Fwd, 5’-atgtcgtgagccatcctgtc-3’ and AtAC-TIN-Rvs, 5’-acccggatctcaagagcagcg-3’) to assess the quantity and primer specificity, we verified the quality of the cDNA. The PCR comprised 30 cycles of 45 s at 94°C, 30 s at 55°C, and 2 min at 72°C.

Real-time RT-PCR reactions were performed on an ABI7500 instrument (Applied Biosystems, Foster City, CA), using SYBR® Green PCR Master Mix (Applied Biosystems), as described [49,50]. The amplification reactions were performed as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 94°C for 15 sec and 60°C for 1 min. To confirm quality and primer specificity, we verified the size of amplification products after electrophoresis through a 1.5% agarose gel, and analyzed the Tm (melting temperature) of amplification products in a dissociation curve, performed by the ABI7500 instrument. The RNA actin was used as endogenous control to normalize all values in the real-time RT-PCR assays [49]. Gene expression was quantified using the 2-ΔΔCt method.

Plant material, growth conditions, and plant transformation

The Columbia (Col-0) ecotype of Arabidopsis thaliana was used as the wild type control and nik1 knockout line [18] was used for complementation experiments. Seeds were surface sterilized and

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cold treated at 4°C for 2 days in the dark and then exposed to white light. Seedlings were grown at 22°C on plates containing Murashige-Skoog medium for 3 weeks and then transferred to soil. Plants were grown in a growth chamber at 22°C under long-day conditions (16 h light/8 h dark). nkl KO lines were transformed with pK7F-NIK1 [18], pK7F-NIK1G473V, pK7F-NIK1T474A, pK7F-NIK1T469A or pK7F-NIK1T474A using the floral dip method [51]. Transgene incorporation was monitored by PCR using a forward 35S promoter-specific primer, MC36 (5′-tcttgctg-gacgcccttc-3′), and a reverse nkl-1-specific primer, LRAt-NIK1RG (5′-aggaagtttcggagctataagatggtt-3′). Transgene expression was confirmed by real time PCR with the primers AtNIK1-Fwd and AtNIK1-Rvs, as described above. For quantitation of gene expression, we used actin as a control gene and the primers AtACTIN-Fwd and AtACTIN-Rvs as described above. We selected two independently transformed lines expressing the NIK1G473VT474A transgene (mNIK-A and mNIK-B), one nkl line expressing NIK1 (NIK1*), T474A or T469A transgene for the infection assays.

**Accumulation of NIK mutant proteins in planta**

*Nicotiana tabacum* leaves were agroinoculated with pK7F-NIK1 [18], pK7F-NIK1G473V/T474A, pK7F-NIK1T469A, pK7F-NIK1T474A and pK7F-NIK1T474D using *Agrobacterium tumefaciens* strain GV3101, as previously described [28]. About 72 hours post-agroinfiltration, 1-cm² leaf explants were excised and GFP and YFP fluorescence patterns were examined in epidermal cells using a Zeiss inverted LSM510 META laser scanning microscope equipped with an argon laser and a helium laser as excitation source. For imaging GFP, the 488 nm excitation line and the 500 to 530 nm band pass filter were used. Excitation of YFP was at 514–560 nm and YFP emission was detected by using a 560–600 nm filter. Controls were performed to ensure clear separation of GFP and YFP signals. The pinhole was usually set to give a 1 to 1.5 µm optical slice. In each independent experiment, a total of 100 to 150 cells were observed and the number of cells with nucleus-localized rpL10 was recorded.

**Supporting Information**

**Figure S1** SDS-PAGE of E. coli- produced GST fusions. GST-fused to the C-terminal kinase domain of normal NIK1 (GST-KDNIK1) or to mutant NIK1s, as indicated, were produced in E. coli, affinity-purified, separated by SDS/PAGE and stained with coomassie brilliant blue. GST-L10 corresponds to a ribosomal protein L10 (rpL10) fused to GST and GST-NSP is a CaLCuV NSP fusion. Molecular mass markers (kDa) are shown on the left.

**Figure S2** Effect of ectopic expression of NIK1 and A-loop mutants on nucleocytoplasmic shuttling of rpL10A. NIK1- GFP+YFP-L10, T469A-GFP+YFP-L10, T474D-GFP+YFP-L10 or T474A-GFP+YFP-L10 were co-expressed in tobacco leaf epidermal cells and the subcellular localization of the fluorescent fusion proteins was monitored by confocal microscopy. The frequency of co-transfected cells (merged field) with rpL10A localized within the nuclei was obtained. In each experiment, a total of 100 to 150 cells were observed. Full arrows indicate fluorescent nuclei. Scale bars are 10 µm.

**Table S1** Primers for mutagenesis within the kinase domain of NIK1

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**Author Contributions**

Conceived and designed the experiments: AAS CMC HJOR EPBF. Performed the experiments: AAS CMC LHF HJOR. Analyzed the data: AAS CMC HJOR EPBF. Contributed reagents/materials/analysis tools: EPBF. Wrote the paper: AAS EPBF.
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