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The Cyst Nematode Effector Protein 10A07 Targets and Recruits Host Posttranslational Machinery to Mediate Its Nuclear Trafficking and to Promote Parasitism in Arabidopsis

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Plant-parasitic cyst nematodes synthesize and secrete effector proteins that are essential for parasitism. One such protein is the 10A07 effector from the sugar beet cyst nematode, Heterodera schachtii, which is exclusively expressed in the nematode dorsal gland cell during all nematode parasitic stages. Overexpression of H. schachtii 10A07 in Arabidopsis thaliana produced a hypersusceptible phenotype in response to H. schachtii infection along with developmental changes reminiscent of auxin effects. The 10A07 protein physically associates with a plant kinase and the IAA16 transcription factor in the cytoplasm and nucleus, respectively. The interacting plant kinase (IPK) phosphorylates 10A07 at Ser-144 and Ser-231 and mediates its trafficking from the cytoplasm to the nucleus. Translocation to the nucleus is phosphorylation dependent since substitution of Ser-144 and Ser-231 by alanine resulted in exclusive cytoplasmic accumulation of 10A07. IPK and IAA16 are highly upregulated in the nematode-induced syncytium (feeding cells), and deliberate manipulations of their expression significantly alter plant susceptibility to H. schachtii in an additive fashion. An inactive variant of IPK functioned antagonistically to the wild-type IPK and caused a dominant-negative phenotype of reduced plant susceptibility. Thus, exploitation of host processes to the advantage of the parasites is one mechanism by which cyst nematodes promote parasitism of host plants.

INTRODUCTION

Plant-parasitic cyst nematodes (Heterodera and Globodera spp) are sedentary endoparasites of roots of many crop plants. The sedentary endoparasitic lifestyles of cyst nematodes rely on close and sustained biotrophic interactions with their host plants where the formation and maintenance of feeding cells (syncytia), as the sole nutritive source for the nematodes, are essential for successful parasitism. Syncytium formation is mediated by effector proteins, which are produced in the nematode esophageal gland cells and delivered into plant cells through a hollow mouth spear termed the stylet (Hewezi and Baum, 2013). Despite the recent progress in the identification and functional characterization of effector proteins from a wide range of phytopathogens, including plant-parasitic cyst nematodes, little is known about the molecular mechanisms underlying effector trafficking inside host cells to reach their sites of action. Plant pathogens including bacteria, fungi, and oomycetes secrete effector molecules into the apoplast or cytoplasm of host cells to establish disease. Once secreted into plant cells, these effectors may be directed to specific cellular compartments where they may target cellular components to exert their virulence functions. In the case of nematode effectors, the initial site of delivery into host cells remains unclear, but several pieces of evidence point to the cytoplasm as the initial site of effector deposition (reviewed in Hewezi and Baum, 2013). After initial deposition into the cytoplasm, nematode effectors may translocate to specific cellular compartments. The trafficking of nematode effectors inside host cells, like all proteins, is a complex process that likely involves host factors. For example, a cell wall-modifying cellulose binding protein effector appears to translocate to the apoplast through its association with a host pectin methyltransferase protein (Hewezi et al., 2008). Similarly, a CLAVATA3/ENDOSPERM SURROUNDING REGION (CLE) peptide-like effector from cyst nematodes was shown to function in the apoplast although the initial localization was found to be the cytoplasm (Wang et al., 2010). The presence of nuclear localization signals (NLSs) in several nematode effectors suggests that nematode effectors co-opt the host nuclear transport machinery in order to localize to the nucleus. In addition, other nonclassical nuclear import machineries presumably have evolved in pathogens to enter the nucleus. In accordance with this premise, several oomycete effectors and certain bacterial type III effectors are targeted to the host nucleus independently of canonical nuclear localization signals (Canonne et al., 2011; Caillaud et al., 2012). Mounting evidence from a limited number of bacterial effectors demonstrates that pathogen effectors have evolved diverse mechanisms to guide their translocation not only to the nucleus...
but also to other cellular compartments. One such mechanism encompasses host-mediated posttranslational modifications of pathogen effectors. For example, host posttranslational modifications such as myristoylation, peptidy-l-prolyl isomerization, and phosphorylation have been shown to be exploited by pathogen effectors to mediate cellular translocation-associated functions (Nimchuk et al., 2000; Coaker et al., 2005; Giska et al., 2013). However, these biochemical modifications have been described only for a limited number of pathogen effectors; hence, the occurrence of these modifications in other pathosystems is largely elusive.

Nuclear translocation of pathogen effectors and other associated cellular factors during plant-pathogen interactions have emerged as new determinants of host cellular responses that render the plant resistant or susceptible to pathogen infection (Liu and Coaker, 2008; Rivas and Genin, 2011). However, the detailed molecular mechanisms underlying this process are mostly unknown. In this study, we report on a virulence strategy of the plant-parasitic cyst nematode *Heterodera schachtii* that requires posttranslational modification of a nematode effector to promote parasitism. We demonstrate that the *H. schachtii* 10A07 effector protein initially accumulates in the host (*Arabidopsis thaliana*) cytoplasm where it physically interacts with a host kinase and becomes phosphorylated. This posttranslational modification mediates effector trafficking from the cytoplasm to the nucleus where the effector then binds to the IAA16 transcription factor, presumably to interfere with auxin signaling and to enhance pathogenicity.

**RESULTS**

**Identification of the Sugar Beet Cyst Nematode Effector 10A07**

The 10A07 effector gene was initially identified in an esophageal gland cell cDNA library from the soybean cyst nematode (*Heterodera glycines*) (Gao et al., 2003). In order to use *Arabidopsis* as a model system to investigate the functional role of the 10A07 effector in cyst nematode parasitism, we isolated the orthologous sequence from a closely related species, the sugar beet cyst nematode (*H. schachtii*), which efficiently infects Arabidopsis plants. The *H. schachtii* 10A07 cDNA encodes a 246-amino acid protein with a predicted N-terminal signal peptide of 15 amino acids for secretion. The predicted Hs-10A07 protein shares 95% sequence identity with 10A07 from *H. glycines* (Hg-10A07) (Supplemental Figure 1). Database searches revealed that Hs-10A07 shares strong sequence identity with the other putative *H. glycines* effector proteins 27D09, 6E07, 20G04, and 13A06, suggesting that 10A07-related sequences constitute a gene family consisting of at least five members (Supplemental Figure 2). Genomic DNA gel blot hybridization further confirmed the presence of 10A07 homologous sequences both in *H. schachtii* and *H. glycines* (Supplemental Figure 3). No significant sequence similarity to 10A07 was identified from any other organisms, including the genome sequences of the potato cyst nematode *Globodera pallida* and the root-knot nematodes *Meloidogyne incognita* and *Meloidogyne hapla* (Abad et al., 2008; Opperman et al., 2008; Cotton et al., 2014).

**10A07 Is Expressed in the Dorsal Gland Cell during Parasitic Nematode Stages**

Nematode effector proteins originate from the esophageal gland secretory cells and are developmentally regulated with changing mRNA abundances during parasitic stages. In situ hybridization assays were used to localize 10A07 transcripts in different *H. schachtii* life stages and to visualize their abundance. The digoxigenin-labeled antisense cDNA probes of 10A07 revealed specific localization of 10A07 transcripts in the dorsal esophageal gland cell of all *H. schachtii* stages after plant penetration (Figure 1A). In addition, we quantified the 10A07 expression levels using quantitative RT-PCR (qPCR) through five *H. schachtii* developmental stages including eggs, preparasitic second-stage juveniles (pre-J2), parasitic J2 (par-J2), third-stage juveniles (J3), and fourth-stage juveniles (J4). The 10A07 expression levels showed an increase from eggs to par-J2 and then decreased at J3 and J4 stages (Supplemental Figure 4). This developmental expression profile is very similar to that previously reported in *H. glycines* (Elling et al., 2007) and further supports a role of this effector in plant parasitism.

**10A07 Contains Functional Nuclear Localization Signals**

Sequence analysis of 10A07 revealed the presence of two putative NLSs, including bipartite (59KKLKPKNDTKGIKAKKA75) and SV40-like (77PAKKGKA83) motifs. Subcellular localization assays of the 10A07-sp:GFP:GUS reporter fusion (minus signal peptide-coding sequence) in *Allium cepa* epidermal cells showed nuclear-cytoplasmic accumulation (Figure 1B, top panel). However, when we tested the localization of a 42-amino acid fragment (10A0757-98) containing both NLS motifs, the green fluorescent protein (GFP) reporter was localized exclusively in the nucleus (Figure 1B, bottom panel).

Furthermore, we attached a wild-type and a mutated version of the protein kinase inhibitor PKI nuclear export signal (NES) (Wen et al., 1995) to the full-length 10A07 fused to the yellow fluorescent protein (YFP) (NES:10A07:YFP) as well as to 10A0757-98 (NES:10A0757-98:YFP) and assayed the localization of these four fusions in onion epidermal cells. As shown in Figure 2A, the NES:10A07:YFP fusion accumulated in the cytoplasm and was excluded from the nucleus, whereas a mutated version of NES (nes*) attached to 10A07:YFP (nes*:10A07:YFP) showed nuclear-cytoplasmic accumulation (Figure 2B). Similarly, NES:10A0757-98:YFP fusion was excluded from the nucleus and accumulated in the cytoplasm (Figure 2C), in contrast with the nuclear accumulation of nes*:10A0757-98:YFP (Figure 2D).

To test whether the predicted NLS59-75 and NLS77-83 act together or independently, the wild-type version as well as a mutated version of both motifs were fused to YFP and expressed in onion epidermal cells. The NLS59-75:YFP fusion localized to the nucleus (Figure 2E), whereas the mutated version (nes59-75:YFP) localized to the cytoplasm (Figure 2F). In contrast, both wild-type and mutated versions of NLS77-83 fusion localized to the cytoplasm (Figures 2G and 2H). Taken together, these results indicate that the predicted bipartite NLS59-75 motif is functional and likely responsible for the observed partial nuclear uptake of full-length 10A07.
Transgenic Arabidopsis Plants Expressing 10A07 Showed Stunted Growth and Hypersusceptibility to Nematode Infection

To determine the effect of 10A07 on plant development and nematode susceptibility, we generated multiple independent homozygous T3 lines expressing the 10A07 coding sequence without the signal peptide region and under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The N-terminal signal peptide directs effector proteins to the secretory pathway in the nematode and the exclusion of the signal peptide will allow a cytoplasmic version of 10A07 effector to be tested for its function in host cells. Three transgenic lines expressing high levels of the 10A07 transgene were selected for phenotypic analyses (Supplemental Figure 5). All such transgenic lines showed dramatically stunted shoots (Figure 3A) and roots (Figure 3B) relative to the wild type, suggesting that ectopic expression of 10A07 interferes with a spectrum of developmental processes both in shoots and roots. Importantly, these transgenic lines were hypersusceptible to the cyst nematode (Figure 3C). Specifically, the transgenic lines showed up to an 80% increase in the number of adult female nematodes. These latter observations demonstrate an important role for the 10A07 effector in mediating parasitic success of cyst nematodes.

10A07 Physically Interacts with a Protein Kinase and IAA16

The subcellular localization data mentioned above suggest that the 10A07 effector may directly target nuclear host factor(s) to facilitate parasitism. To search for Arabidopsis proteins that physically interact with 10A07, we conducted yeast two-hybrid (Y2H) screens with the full-length 10A07 coding sequence minus the signal peptide (10A07-sp) as bait. After screening more than 15 million yeast colonies from three prey libraries prepared from Arabidopsis roots inoculated with H. schachtii (Hewezi et al., 2008), we identified a protein kinase family protein (AT2G37840) and an auxin-responsive protein (INDOLE-3-ACETIC ACID INDOUCIBLE16 [IAA16]) (AT3G04730) as high fidelity interactors. Out of 21 positive clones identified in the initial screen, the interacting protein kinase (IPK) was represented by nine independent cDNA clones corresponding to the last 412 amino acids of the C terminus region. IAA16 was represented by one near-full length cDNA clone lacking only the first 30 amino acids. The remaining 11 prey clones showed nonspecific interactions with the control bait vector. We cloned the full-length coding sequences of these two genes for direct yeast cotransformation assays with 10A07. Yeast cells containing 10A07-sp as bait and either the full-length IPK or IAA16 coding sequence as prey were able to grow on synthetic defined (SD) medium lacking adenine and histidine (Figure 3D) and activated the MEL1 reporter gene in α-Gal quantitative assays (Figure 2E), indicating reproducible protein-protein interactions in the chosen Y2H system. To study the specificity of these interactions in yeast, we inserted IAA7 (AT3G23050) and IAA14 (AT4G14550), which are the Aux/IAA family members most closely related to IAA16, as well as the protein kinase family gene AT3G53930, which shares the strongest sequence homology with IPK, into the Y2H prey vector. The potential interactions between these preys and 10A07-sp as bait were tested in yeast cotransformation assays.
Figure 2. The Bipartite NLS of 10A07 Protein (NLS59-75) Mediates Nuclear Uptake.

Subcellular localization of the indicated YFP fusion in onion epidermal cells using biolistic bombardment. Bars = 200 μm.

(A) Cytoplasmic localization of NES:10A07:YFP.

(B) Nuclear-cytoplasmic localization of nes*:10A07:YFP.

(C) Cytoplasmic localization of NES:10A0757-98:YFP.

(D) Nuclear localization of nes*:10A0757-98:YFP.

(E) Nuclear localization of NLS59-75:YFP.

(F) Cytoplasmic localization of nls*59-75:YFP.

(G) Cytoplasmic localization of NLS77-83:YFP.

(H) Cytoplasmic localization of nls*77-83:YFP.
No interactions between 10A07-sp and these preys were detected, indicating that the interactions between 10A07 and IPK or IAA16 are specific (Supplemental Figure 6).

Protein-protein interactions within plant cells necessitate that these proteins are localized in the same cellular compartments. Therefore, we compared the subcellular localization of IPK, IAA16, and 10A07 when expressed in planta. To this end, GFP-GUS-tagged fusions of IPK (IPK:GFP:GUS) and IAA16 (IAA16:GFP:GUS) were generated and overexpressed in onion epidermal cells. IPK:GFP:GUS was localized throughout the plant cytoplasm (Supplemental Figure 7), whereas IAA16:GFP:GUS was localized exclusively in the nucleus (Supplemental Figure 7).

Figure 3. Functional Characterization of 10A07 Effector.

(A) to (C) Ectopic expression of 10A07 in Arabidopsis alters plant morphology and augments plant susceptibility to H. schachtii. Homozygous T3 plants expressing 10A07-sp produced stunted growth (A), shorter roots (B), and hypersusceptibility to H. schachtii infection (C) compared with the wild type (C24). Data are presented as means ± se (n = 20). Mean values significantly different from the wild-type plants were determined by unadjusted paired t tests (P < 0.05) and are indicated by an asterisk.

(D) to (F) 10A07 interacts with IPK and IAA16 both in yeast and in planta.

(D) and (E) Interactions between 10A07 (bait) and both IPK and IAA16 (preys) in yeast were visualized by differential growth on the selective synthetic dropout (SD/-Leu/-Trp/-Ade/-His) medium (D) and by activation of the MEL1 reporter gene in α-Gal quantitative assays (E). Yeast cells containing the IPK and IAA16 prey plasmids along with either the empty bait vector or bait vector containing the human Lamin C gene fail to grow on the selective medium or activate MEL1 reporter gene beyond the background level. Units of α-Gal activity are the average of 12 separate colonies per combination ± se.

(F) BIFC visualization of the 10A07-IPK and 10A07-IAA16 interactions. Onion epidermal cells cotransformed with constructs expressing nEYFP-10A07 and cEYFP-IPK showed YFP fluorescence in the cytoplasm (top panel), while onion cells cotransformed with nEYFP-10A07 and cEYFP-IAA16 showed the YFP in the nucleus (bottom panel).

(G) Mapping the 10A07 region involved in the interaction with IPK and IAA16. Three bait constructs of 10A07 containing the N-terminal (10A0716-92), middle (10A0793-169), and C-terminal (10A07170-246) regions were generated and transformed into yeast cells in combination with various prey constructs of IPK and IAA16 indicated in the figure. Protein-protein interactions were visualized by differential growth on SD/-Leu/-Trp/-Ade/-His medium. Bars = 1 cm in (A) and (B) and 200 μm in (F).
These data demonstrate that the cellular localization of IPK and IAA16 overlaps with the nuclear-cytoplasmic localization of 10A07 (Figure 1B) and suggest that these proteins could physically associate with each other when present in the same plant cell.

The interaction of 10A07 with IPK and IAA16 was further tested in planta using bimolecular fluorescence complementation (BIFC) assays (Citovsky et al., 2006). In these experiments, 10A07 was fused to the N terminus of a nonfluorescent half of YFP to generate nEYFP-10A07 (Supplemental Figure 8A). On the other hand, IPK and IAA16 were fused to the N terminus of the other nonfluorescent half of YFP to generate cEYFP-IPK and cEYFP-IAA16, respectively. Coexpression of nEYFP-10A07 and cEYFP-IPK in onion epidermal cells reconstituted the activity of YFP in the cytoplasm of transformed cells (Figure 2F), while coexpression of nEYFP-10A07 and cEYFP-IAA16, respectively. Coexpression of nEYFP-10A07 and cEYFP-IAA16 reconstituted the activity of YFP in the nucleus (Figure 2F).

No YFP fluorescence was observed when the YFP plasmids containing 10A07/AT3G53930, IAA7/AT3G53930, and 10A07/AT3G53930/IAA16 were coexpressed in onion epidermal cells (Supplemental Figures 8A to 8D). In addition, no interactions were observed when BIFC constructs of IPK or IAA16 were coexpressed with BiFC construct of the cytoplasmic nematode effector 10A06 (Hewezi et al., 2010) (Supplemental Figures 8D and 8E). Also, we used BiFC assays to explore the potential interaction of IPK and IAA16 with another cyst nematode effector that we have shown to be nuclear localized (32E03, Supplemental Figure 8H). In both cases, no interaction was detected in the cotransformed onion epidermal cells (Supplemental Figures 8F and 8G), demonstrating the specificity of the interaction of 10A07 with IPK and IAA16. Furthermore, BIFC assays in agroinfiltrated Nicotiana benthamiana leaves confirmed the interaction of 10A07 with IPK and IAA16 in the cytoplasm and nucleus of another plant species, respectively (Supplemental Figure 9).

To identify the interacting regions of 10A07, IPK, and IAA16, a series of deletions was generated. Three 10A07 fragments containing the N-terminal (10A07170-246), middle (10A07170-246), and C-terminal (10A07170-246) regions were generated in the bait vectors. N-terminal deletions of IPK and C-terminal deletions of IAA16 were also generated in the prey vector (Figure 2G). Yeast cotransformation assays clearly revealed that the C-terminal region of 10A07 (10A07170-246) mediates the interaction with IAA16, while both the N-terminal (10A0716-92) and C-terminal (10A07170-246) regions mediate the interaction with IPK (Figure 2G). The deletion analysis also showed that the prey construct IPK(181-733), which lacks the kinase domain, gave rise to the strongest interaction with 10A07 (Figure 2G), suggesting that the kinase domain of IPK is not directly involved in the physical association between IPK and 10A07.

As a next assay, we determined if in fact both putative interacting proteins could be present in the developing syncytium, i.e., the site where the 10A07 effector could be found in planta. For this purpose, we analyzed the promoter activities of IPK and IAA16 at different time points after H. schachtii infection using transgenic Arabidopsis lines expressing pIPK:GUS or pIAA16: GUS constructs. The promoter activities of IPK and IAA16 in tissues of noninfected plants are provided in Supplemental Figure 10. Following nematode infection, the promoters of both IPK and IAA16 were strongly upregulated in the nematode-induced syncytia at 4 and 7 d after H. schachtii infection (dpi) (syncytia induced by parasitic J2 and J3 stages, respectively) (Figures 4A, 4B, 4D, and 4E). At later stages of infection (14 dpi), the promoters of both IPK and IAA16 became less active in the syncytia of J4 nematodes (Figures 4C and 4F). Furthermore, the mRNA levels of IPK and IAA16 were quantified using qPCR in the root tissues of wild-type plants at 4, 7, and 14 dpi with H. schachtii relative to noninfected plants. Data from three independent biological samples revealed that IPK and IAA16 expression levels were induced ~2- and 3-fold at 4 and 7 dpi, respectively whereas at the 14-d time point the induction level was ~1.5-fold (Figure 4G). Taken together, these expression profiles and the localization of IPK and IAA16 promoter activity in the syncytium, where the 10A07 effector presumably is delivered by the nematode, strongly suggest that the protein interactions postulated by our Y2H data in fact could be occurring in planta during nematode infection.

**IPK Phosphorylates 10A07 at Ser-144 and Ser-231**

The nuclear-cytoplasmic accumulation of 10A07 along with our Y2H data showing the interaction between 10A07 and both IPK and IAA16 prompted us to hypothesize that IPK phosphorylates 10A07 and mediates its trafficking from cytoplasm to the nucleus where it binds to the IAA16 transcription factor. To this end, we expressed 10A07 and wild-type IPK, as well as a kinase-inactive variant of IPK as His-tagged proteins in bacterial cells. The kinase-inactive variant of IPK was generated by mutating the conserved lysine residue in position 41 (ATP binding pocket) and 136 (substrate binding pocket) to arginine (IPK[K141R/K136R]). These His-tagged proteins were immunopurified and subjected to in vitro kinase assays. We first examined the potential autophosphorylation activity of IPK. This protein showed strong autophosphorylation activity, and this activity was completely inhibited by mutation of Lys-41 and Lys-136 (Figure 5A). The ability of IPK to phosphorylate the 10A07 protein was also assayed. When 10A07 was incubated with the wild-type IPK, a strong phosphorylation signal of 10A07 was detected (Figure 5A). In contrast, no phosphorylation signals were detected when 10A07 was used alone or in combination with IPK[K141R/K136R] in the kinase assays (Figure 5A). These data indicate that IPK directly interacts with and phosphorylates 10A07.

To identify phosphorylation sites in the Hs-10A07 protein, immunopurified 10A07 was incubated with wild-type IPK in vitro kinase assays using cold ATP. After SDS-PAGE separation, the 10A07 band was excised, trypsin-digested, and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Two phosphorylated serines in the Hs-10A07 protein at amino acid positions 144 and 231 were identified (Supplemental Figure 11). The Ser-144 is conserved in the amino acid positions 144 and 231 were identified in J2 and J3 stages, respectively (Figures 4A, 4B, 4D, and 4E). At later stages of infection (14 dpi), the promoters of both IPK and IAA16 became less active in the syncytia of J4 nematodes (Figures 4C and 4F). Furthermore, the mRNA levels of IPK and IAA16 were quantified using qPCR in the root tissues of wild-type plants at 4, 7, and 14 dpi with H. schachtii relative to noninfected plants. Data from three independent biological samples revealed that IPK and IAA16 expression levels were induced ~2- and 3-fold at 4 and 7 dpi, respectively whereas at the 14-d time point the induction level was ~1.5-fold (Figure 4G). Taken together, these expression profiles and the localization of IPK and IAA16 promoter activity in the syncytium, where the 10A07 effector presumably is delivered by the nematode, strongly suggest that the protein interactions postulated by our Y2H data in fact could be occurring in planta during nematode infection.

**Translocation of 10A07 to the Nucleus Is Phosphorylation Dependent**

We next tested whether IPK promotes the translocation of 10A07 to the nucleus. The subcellular localization of GFP-GUS-tagged 10A07 in presence of wild-type IPK was studied using an
onion epidermal cells cotransformation assay. An exclusive nuclear localization of 10A07:GFP:GUS in ~30% of the transformed cells was detected (Figure 5C). In contrast, onion epidermal cells expressing 10A07:GFP:GUS in combination with IPKK41R/K136R showed no exclusive nuclear localization in any transformed cells (Figure 5C). Furthermore, we tested whether the trafficking of 10A07 to the nucleus is phosphorylation dependent. To this end, the two phosphorylated serines in the 10A07 protein (Ser-144 and Ser-231) were substituted by alanine, thereby generating mutant protein 10A07S144A/S231A. This 10A07 mutant was fused to GFP:GUS and expressed in onion epidermal cells alone or in combination with wild-type IPK. Transformed cells expressing 10A07S144A/S231A alone or in combination with IPK showed only cytoplasmic distribution (Figure 5C). Furthermore, we generated a phosphomimic variant of 10A7 in which Ser-144 and Ser-231 were substituted by glutamic acid (10A07S144E/S231E). 10A07S144E/S231E was fused to GFP:GUS and expressed in onion epidermal cells. Interestingly, the phosphomimic mutations of 10A07 showed exclusive nuclear accumulation (Figure 5C), indicating that IPK promotes the trafficking of 10A07 to the nucleus and that this translocation is mediated through phosphorylation of residues Ser-144 and Ser-231.

IPK and IAA16 Promote Nematode Susceptibility

To examine the biological significance of IPK and IAA16 in mediating Arabidopsis susceptibility to H. schachtii, we manipulated the expression of IPK and IAA16 using overexpression and knockdown approaches. Homozygous T3 lines overexpressing IPK under the control of the 3SS promoter (Supplemental Figure 12) produced plants that were significantly more susceptible to H. schachtii (Figure 6A) relative to the wild type, whereas an IPK mutant (CS859240) (Supplemental Figure 13) exhibited the opposite phenotype of reduced susceptibility (Figure 6C). Similar to IPK, transgenic lines overexpressing IAA16 (Supplemental Figure 12) showed significantly increased plant susceptibility to
Figure 5. IPK Phosphorylates 10A07 and Promotes Its Nuclear Translocation.

(A) In vitro phosphorylation assay of 10A07 effector protein by Arabidopsis IPK. Bacterially expressed His-tagged wild-type IPK and inactive variant (IPK\textsuperscript{K41R/K136R}) were immunopurified and used in in vitro kinase assays to test their ability to phosphorylate 10A07 effector protein. Immunopurified
H. schachtii (Figure 6B), while IAA16 mutants (Salk_114809 and CS466686) (Supplemental Figure 13) showed low susceptibility phenotypes (Figure 6C). The contribution of IPK and IAA16 toward the nematode susceptibility was further investigated by RNA interference (RNAi)-mediated cosilencing of IPK and IAA16. Cosilencing of IPK/IAA16 in transgenic lines (Supplemental Figure 14) resulted in significant decrease in plant susceptibility compared with the wild-type plants (Figure 6D).

The Kinase-Dead Mutant of IPK Acts Antagonistically to the Wild-Type IPK

To investigate whether the kinase activity of IPK is associated with the increased nematode susceptibility phenotypes observed in IPK overexpression lines, we overexpressed the kinase-dead mutant of IPK (IPKK41R/K136R) in wild-type Arabidopsis (WT/IPK41R/K136R), and four transgenic lines were selected (Supplemental Figure 12) and assayed for nematode susceptibility. The kinase-dead mutant of IPK functioned antagonistically to the wild-type IPK and caused a dominant-negative phenotype of reduced plant susceptibility relative to the wild type (Figure 7A). The dominant-negative effect of the IPKK41R/K136R suggested that 10A07–IPK interaction is independent of successful 10A07 phosphorylation. To test this suggestion, the physical association between 10A07 and IPK41R/K136R and between 10A07S144A/S231A and IPK was investigated in planta through BiFC assays in agroinfiltrated N. benthamiana leaves. These assays confirmed the physical interaction of these two combinations in the cytoplasm (Supplemental Figure 15) despite the fact that no phosphorylation could be taking place.

10A07-Mediated Nematode Susceptibility Changes and Developmental Defects Are Abolished by Overexpressing the Kinase-Dead Mutant of IPK

The ability of IPKK41R/K136R to inhibit the hypersusceptibility phenotype observed in the 10A07 overexpression plants was investigated by overexpressing the IPKK41R/K136R in the 10A07-expressing transgenic plants (10A07/IPK41R/K136R) (Supplemental Figure 12). IPKK41R/K136R suppressed the 10A07-mediated nematode hypersusceptibility phenotype, and the 10A07/IPK41R/K136R transgenic plants showed reduced nematode susceptibility phenotype relative to wild-type plants (Figure 7B). Next, we tested whether the dominant-negative effects of the kinase-dead mutant will rescue the wild-type morphology phenotype in 10A07-expressing plants. To this end, the root length of 10A07, WT/IPK41R/K136R, and 10A07/IPK41R/K136R transgenic plants were measured in root-length assays. While IPKK41R/K136R had no effect on root length when overexpressed in the wild-type background, IPKK41R/K136R completely abrogated the 10A07-mediated short root phenotype when overexpressed in the 10A07-expressing background (Figure 7C). Similarly, overexpression of IPKK41R/K136R showed no visible effect on shoot development when overexpressed in the wild-type background (Figure 7D), but partially suppressed the stunted growth phenotype caused by 10A07 when overexpressed in 10A07-expressing transgenic plants (Figure 7E). Taken together, these data provide additional evidence that 10A07 mediates nematode susceptibility and stunted growth phenotypes primarily through its association with the IPK.

10A07 Interferes with the Expression of Auxin Response Factors

To test whether the 10A07 effector interferes with auxin signaling, as suggested by its interaction with IAA16, we quantified the expression of several auxin response factors (ARFs) in the transgenic plants overexpressing 10A07 or IAA16 under both noninfected and infected conditions (4 dpi) using qPCR. ARF5, 6, 7, 8, and 19 were selected because IAA16 was reported to interact with these proteins in Y2H (Vernoux et al., 2011; Piya et al., 2014). Under noninfected conditions, ARF6, 7, 8, and 19 showed gene downregulation in the transgenic plants overexpressing 10A07 or IAA16 relative to wild-type Columbia-0 (Col-0) plants (Figure 8A). In contrast, under infected conditions, these ARFs showed upregulation of ~1.5-fold in the transgenic plants overexpressing 10A07 or IAA16 relative to the infected Col-0 plants (Figure 8B). These data suggest that 10A07–IAA16 interaction interferes with auxin signaling.

DISCUSSION

Successful host-parasite interactions require tightly coordinated and interacting responses from both the host and pathogen.

Figure 5. (continued).

proteins were separated on 12% SDS-polyacrylamide gel and stained with Coomassie blue (bottom panel), and phosphorylation was detected by autoradiography (top panel).

(B) Phosphorylated Ser-144 and Ser-231 in Hs-10A07 are conserved in all 10A07 family members. Two unique peptides with two phosphorylated serines in 10A07 protein (10A07S144E/S231E) was localized exclusively to the nucleus when expressed in onion epidermal cells. pRUG34 vector containing GFP:GUS fusion was used as a cytoplasmic control, whereas pRJG23 vector containing NLS:GFP:GUS fusion was used as a nuclear control. Bars = 100 μm.
Plant-parasitic cyst nematodes evolved the ability to deliver effector proteins inside host cells, and many of these effectors associate with host proteins to subvert essential cellular functions for parasitic success. Cyst nematode effectors, like other pathogen effectors, need to be delivered to the correct subcellular compartments to exert their virulence function. Although 10A07 effector contains a functional bipartite NLS motif, the full-length protein showed a nuclear-cytoplasmic localization. Our studies showed that IPK binds to and phosphorylates 10A07 at Ser-144 and Ser-231. This phosphorylation mediates 10A07 trafficking from the cytoplasm to the nucleus where this effector associates with the IAA16 transcription factor to likely interfere with auxin signaling. While pathogen effectors have been shown to alter the function of target host proteins, our data show an example in which effectors can also utilize host cellular processes to regulate their own activity, stability, and subcellular localization. Host-mediated posttranslational modifications of pathogen effectors seem to be one of the main mechanisms that bring about effector translocation and function. For example, the bacterial effectors AvrPto, AvrRpm1, and AvrB are myristylated inside host cells. This modification stimulates their relocation to the plasma membrane, which is required for their avirulence function (Nimchuk et al., 2000). Also, peptidyl-prolyl isomerization of the AvrRpt2 effector by an Arabidopsis cyclophilin is essential for its N-terminal cleavage and localization to the membrane (Coaker et al., 2005). The nuclear trafficking of Hs-10A07 is phosphorylation-dependent because the kinase-inactive variant (IPK*K41R/K136R) failed to stimulate the trafficking of 10A07 into the nucleus in the onion epidermal cells co-transformation assay. These results were further confirmed by our data showing that substitution of the two phosphorylated serines in the 10A07 protein (10A07*5144A/5231A) abolished its nuclear localization in the presence of wild-type IPK and that the phosphomimic mutation of 10A07 (10A07*S144E/S231E) was localized exclusively in the nucleus. Collectively, these data demonstrate that 10A07 bears a nuclear targeting signal and utilizes host targeting proteins to localize to the nucleus. The nuclear translocalization of 10A07 is consistent with our finding that 10A07 specifically interacts with the nuclear-localized transcription factor IAA16. The human serine/threonine kinase ULK3, which shares strong sequence similarity with Arabidopsis IPK at the amino acid level (75% in the N-terminal region containing the kinase domain and 46% overall) also promotes the nuclear localization of its target GLI1 (glioma-associated oncogene homolog 1) transcription factor in a kinase-dependent manner (Maloverjan et al., 2010), indicating a mode of function similar to IPK. While the mechanisms by which phosphorylation mediates regulation of nuclear trafficking could be numerous, the most comprehensive mode of action is that phosphorylation could increase the binding affinity of the phosphorylated protein for a specific import factor (Nardozzi et al., 2010). Alternatively, phosphorylation can also induce conformational rearrangements that result in increasing the exposure of NLS to a particular importin factor. In this context, our data showing the

Figure 6. IPK and IAA16 Regulate Nematode Susceptibility.

(A) to (C) Nematode susceptibility assays of IPK or IAA16 overexpression and mutant lines. Transgenic plants overexpressing IPK (A) or IAA16 (B) exhibited increased susceptibility to H. schachtii relative to wild-type control, whereas the mutant alleles of IPK (CS859240) and IAA16 (Salk-144809 and CS466686) displayed increased resistance (C). (D) Cosilencing of IPK/IAA16 displayed an additive effect in increasing plant resistance to nematode susceptibility. Transgenic plants simultaneously silenced for both IPK and IAA16 showed higher increase in plant resistance to H. schachtii infection compared with the wild-type plants or the single IPK and IAA16 mutants. Data are presented as means ± se (n = 20). Mean values significantly different from the wild-type plants were determined by unadjusted paired t tests (P < 0.05) and are indicated by an asterisk. Similar results were obtained from three independent experiments.
exclusive nuclear localization of a 42-amino acids fragment of 10A07 (10A0757-98) that contains the NLS motifs but not the phosphorylated residues (Ser-144 and Ser-231) are consistent with the hypothesis that the phosphorylation status of 10A07 might induce conformational changes rather than increasing the binding affinity of the NLS for specific importin factors.

Several recent studies demonstrated that effector proteins can disrupt the localization and the function of host proteins. For example, the host protein NRIP1, which is normally localized to the chloroplasts, is recruited to the cytoplasm and the nucleus by the Tobacco mosaic virus helicase p50 effector (Caplan et al., 2008). And the Arabidopsis cysteine protease RD19 is translocated from mobile vacuole-associated compartments to the nucleus by the Rathsonia solanacearum PopP2 effector (Bernoux et al., 2008). In this context, we can’t rule out the possibility that the association of 10A07 with IPK may recruit IPK to the nucleus, taking into account the slight nuclear YFP fluorescence seen in BiFC assay between 10A07 and IPK in onion epidermal cells (Figure 3F).

Our data provide an intriguing example of host-dependent phosphorylation of a pathogen effector and suggest that phosphorylation is a common posttranslational modification of pathogen effectors. The P. syringae type III effectors AvrPto, AvrPtoB, and AvrB were found to be phosphorylated in the presence of plant extracts independently of the corresponding plant disease resistance proteins (Anderson et al., 2006; Desveaux et al., 2007; Xiao et al., 2007). The NopL and NopP effectors from Rhizobium sp strain NGR234 are also phosphorylated by plant extracts (Bartsev et al., 2003; Skorpil et al., 2005). While the phosphorylation of these effectors is evidently associated with their function, the host factors mediating these modifications are unknown. Our results show that activation of the 10A07 effector

Figure 7. The Kinase-Dead Mutant of IPK Acts Antagonistically to the Wild-Type IPK.

(A) Reduced nematode susceptibility in transgenic plants overexpressing a kinase-dead mutant of IPK in Arabidopsis Col-0 background. Four independent transgenic lines overexpressing the kinase-dead mutant IPK\(^{K441R/K136R}\) showed a statistically significant increase in plant resistance to H. schachtii compared with the wild-type control.

(B) The kinase-dead mutant IPK\(^{K441R/K136R}\) suppressed 10A07-mediated nematode hypersusceptibility phenotype. Four independent transgenic lines overexpressing the kinase-dead mutant IPK\(^{K441R/K136R}\) in the 10A07 transgenic plants background (10A07/IPK\(^{K441R/K136R}\)) showed a statistically significant increase in plant resistance to H. schachtii compared with the wild-type control. Data are presented as means ± se (n = 20). Mean values significantly different from the wild-type plants were determined by unadjusted paired t tests (P < 0.05) and are indicated by an asterisk. Similar results were obtained from three independent experiments.

(C) The short-root phenotype caused by 10A07 is completely suppressed by overexpressing the kinase-dead mutant IPK\(^{K441R/K136R}\). Overexpression of IPK\(^{K441R/K136R}\) showed no effect on root length when overexpressed in the wild-type background but completely abolished the 10A07-mediated short-root phenotype when overexpressed in the 10A07 background. Transgenic and wild-type seeds were planted on four-well plates containing modified Knop’s medium, and root lengths of 10-d-old seedlings were measured and photographed. Data are presented as means ± se (n = 40).

(D) and (E) The stunted growth phenotype caused by 10A07 is partially suppressed by overexpressing the kinase-dead mutant IPK\(^{K441R/K136R}\). Overexpression of IPK\(^{K441R/K136R}\) showed no visible effect on shoot development when overexpressed in the wild-type background (D) but partially suppressed 10A07-mediated stunted growth phenotype when overexpressed in 10A07 background (E).
by the host IPK is a defined mechanism for directing 10A07 to the nucleus and may contribute to its function. Overexpression of 10A07 produced a hypersusceptible phenotype in response to H. schachtii infection, consistent with its function as a virulence determinant. Interestingly, the virulence activity of 10A07 was completely abolished by overexpressing the kinase-dead mutant of IPK (IPKKK41R/K136R) in 10A07 plants. These results indicate that IPKK41R/K136R functioned antagonistically to the wild-type IPK and caused a dominant-negative phenotype of reduced plant susceptibility by blocking 10A07 nuclear import and, therefore, 10A07-mediated susceptibility responses. This is consistent with our data showing that K41R and K136R substitutions in IPK did not impede the binding ability of IPK to associate with 10A07 in planta (Supplemental Figure 15).

The significant reduction in nematode susceptibility phenotype of 10A07/IPKKK41R/K136R plants when compared with 10A07 plants indicates an additional role of IPK in mediating plant susceptibility. In support of this hypothesis, IPK was found to be predominantly upregulated in nematode-induced feeding sites at 4 and 7 dpi. In addition, IPK overexpression produced plants that were more susceptible to H. schachtii than the wild type, whereas an insertional mutagenesis event produced the opposite phenotype of reduced susceptibility. Perturbation of host protein activities is a common mechanism employed by various pathogens to mediate disease (Salomon and Orth, 2013). In this context, we cannot rule out the possibility that 10A07 manipulates the activity of IPK and associated processes in favor of nematode parasitism. Several mammalian and plant pathogens target various kinase family proteins to inhibit innate immune systems (Krachler et al., 2011; Dou and Zhou, 2012).

The ability of plant pathogens to evolve effector proteins that exploit the host posttranslational machinery to impose such modifications into their structures or activities is a compelling mechanism of pathogenesis. This raises the question of how pathogen effectors can engage host proteins for these functions. One efficient way is for pathogen effectors to mimic host factors structurally or enzymatically (Stebbins and Galán, 2001; Drayman et al., 2013). Thus, it is worthwhile to suggest that the 10A07 effector may mimic a host substrate for IPK to facilitate direct interaction with the host and hence promote virulence activity. Target mimicry of host factors is a well-known mechanism evolved by various pathogens to prevent detection by host immune systems (Stebbins and Galán, 2001; Drayman et al., 2013). Because amino acid sequence similarity between 10A07 and Arabidopsis proteins is insignificant, it is likely that 10A07 convergently evolved to structurally mimic a host protein with minimal or no sequence similarity.

The biological significance of the nuclear trafficking of 10A07 was further elucidated by our identification of IAA16 as a nuclear target of 10A07. IAA16 belongs to the 29-gene family members

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**Figure 8.** 10A07 Interferes with the Expression of Auxin Response Factors.

The expression level of ARF5, 6, 7, 8, and 19 was quantified in the transgenic plants overexpressing 10A07 or IAA16 under noninfected conditions (A) and at 4 dpi (B) using qPCR. The fold-change values represent changes of mRNA levels in the transgenic plants relative to wild-type Col-0 plants. Arabidopsis Actin8 was used as an endogenous reference gene to normalize gene expression levels. Data are the average of three independent biological samples, each consisting of three technical replicates ± se.

**Figure 9.** Phosphorylation of the 10A07 Effector by Host IPK Mediates Its Nuclear Trafficking Presumably to Perturb Auxin Signaling.

10A07 is initially delivered into the cytoplasm of infected host cells. Once delivered, 10A07 physically associates with the host IPK and is thereby phosphorylated. This phosphorylation mediates 10A07 trafficking to the nucleus where this effector binds to the IAA16 transcription factor to interfere with auxin signaling through manipulating the expression of various ARFs including ARF5, 6, 7, 8, and 19.
of Aux/IAA transcription factors (Liscum and Reed, 2002). Members of this gene family encode short-lived nuclear proteins that regulate auxin-dependent gene expression (Abel et al., 1994). Molecular and genetic evidence indicates that auxin plays key roles in triggering the initiation of the syncytium (reviewed in Grunewald et al., 2009b; Goverse and Bird, 2011). Mutants deficient in auxin signaling have been frequently shown to exhibit increased resistance to nematode parasitism (Goverse et al., 2000; Karczmarek et al., 2004; Grunewald et al., 2009a). Because auxin mediates the integration of external stimuli and synchronizes rapid response of plant cells, manipulating auxin signaling by cyst nematode effectors in nematode feeding cells is expected to yield immediate responses. However, the mechanisms by which cyst nematodes regulate auxin accumulation, transport, and signaling are not fully understood. Recently, we found that *H. schachtii* 19C07 effector protein interacts with an auxin influx transporter, LAX3 (Auxin transporter-like protein 3), probably to maintain auxin balance during the early stages of syncytium formation (Lee et al., 2011). In this context, our finding that the 10A07 effector directly targets IAA16 provides additional insights into how cyst nematode effectors potentially manipulate auxin response. Aux/IAA proteins negatively regulate the abundance of ARFs and subsequently the expression of auxin-responsive genes (Chapman and Estelle, 2009).

Our data also point to an essential role of IAA16 during cyst nematode parasitism. The IAA16 gene was transcriptionally up-regulated in the developing syncytium at 4 dpi, and surprisingly this upregulation was sustained in the syncytium up to the J4 female stage. This expression pattern indicates a role of Aux/IAA proteins, and more specifically IAA16, not only during the early stage of syncytium formation as previously speculated but also during later stages of nematode parasitism. The IAA16 overexpression lines showed a significant increase in plant susceptibility to cyst nematodes, while *iaa16* insertion mutants resulted in the opposite phenotypes of reduced susceptibility. The physical association between 10A07 and IAA16 could impair the DNA or protein binding activities of IAA16, resulting in specific alteration in auxin-dependent transcription programs. Taking into account the ability of IAA16 to form heterodimers with six ARF proteins (ARF5-9 and 19) (Vernoux et al., 2011; Piya et al., 2014), such modification could provide numerous levels of regulation of auxin-responsive gene expression leading to various physiological and developmental changes. In support of this hypothesis, we found the expression of *ARF6-8* and 19 to be downregulated under non-infected conditions in the transgenic plants overexpressing 10A07 or IAA16 relative to Col-0 and upregulated under *H. schachtii*-infected conditions (Figure 8). Interestingly, these ARFs are highly induced in the *H. schachtii*-induced syncytium (Hewezi et al., 2014) and have similar temporal and spatial expression patterns as IAA16. The upregulation of *ARF5-8* and 19 in the transgenic plants overexpressing the 10A07 effector gene under infected conditions suggests that the cyst nematode targets IAA16 to sequester its activity as a negative regulator of ARFs, thereby activating the expression of specific ARFs to alter the transcriptional program of specific auxin response genes in the developing syncytium.

Like other Aux/IAA proteins, IAA16 contains four conserved domains that are involved in transcription repression (domain I), protein degradation (domain II), and homo- and heterodimerization (domains III and IV) (Dreher et al., 2006; Guilfoyle and Hagen, 2007). Our deletion analysis of IAA16 to map a specific region involved in the interaction with 10A07 indicates that IAA16<sup>1-116</sup>, which contains domains I and II, is required for recognition and association with the C terminus of 10A07. Thus, by binding to IAA16, 10A07 may mask domains I and II, which have been shown to bind to the TOPLESS corepressor (Szemenyi et al., 2008) and TIR1 (TRANSPORT INHIBITOR RESPONSE1), the F-box subunit of the ubiquitin ligase complex SCF<sup>TIR1</sup> (Panry and Estelle, 2006; Mockaitis and Estelle, 2008), respectively.

Disrupting the function or the stability of Aux/IAA proteins results in dramatic developmental changes (Mockaitis and Estelle, 2008). Because of the structure and functional similarity between the 29 predicted Aux/IAA proteins, they exhibit redundant functions and several Aux/IAA mutants show weak or no visible phenotypes (Overvoorde et al., 2005). However, gain-of-function mutants of various Aux/IAA genes exhibit dominant auxin resistance, resulting in increased protein stability and prominent repression of ARFs (Calderon-Villalobos et al., 2010). Recently, a gain-of-function mutant of IAA16 displayed stunted growth and several developmental defects both in roots and shoots that mimic those of 10A07 overexpression plants (Rinaldi et al., 2012), indicating a connection between IAA16 protein stability and the developmental defects observed in the transgenic plants expressing 10A07 effector gene. This phenotype seems to be specific to IAA16-mediated auxin signaling and not due to overall disruption of auxin sensing, as 10A07 failed to interact with IAA7 and IAA14, the closest homologs of IAA16. Importantly, IPK and IAA16 seem to act in an additive manner to mediate nematode susceptibility. IPK (CS859240) and IAA16 (Salk_114809) mutants express only 15 and 29% of the wild-type expression levels, respectively (Supplemental Figure 13) and exhibited up to 25% reduction in nematode susceptibility (Figure 6C). While cosilencing of IPK and IAA16 in transgenic lines to the exact level of the single mutants is experimentally challenging, we selected and tested several RNAi lines in which the silencing levels were determined to be between 17 and 42% for IPK and between 23 and 57% for IAA16 relative to wild-type plants (Supplemental Figure 14). IPK/IAA16 cosilencing lines showed ~50% reduction in nematode susceptibility (Figure 6D), suggesting that IPK and IAA16 function in a synergistic fashion to promote nematode susceptibility.

On the basis of our current data, we hypothesize that during parasitism, the cyst nematode sequesters 10A07 into the cytoplasm of infected host cells. In the cytoplasm, 10A07 physically associates with the host IPK and is phosphorylated. This phosphorylation mediates 10A07 trafficking to the nucleus where it binds to the IAA16 transcription factor most likely to interfere with auxin signaling (Figure 9). Co-opting the host posttranslational machinery by the cyst nematode effector 10A07 to localize to the nucleus represents a critical mechanism for cyst nematode parasitism.

**METHODS**

**Plant Materials and Growth Conditions**

*Arabidopsis thaliana* wild-type Col-0 was used in all experiments except for the 10A07 and IPK<sup>KK136R</sup> overexpression lines, which are in the...
C24 background. Plants were grown in long days (16 h light/8 h dark) at 24°C.

**Plasmid Construction and Generation of Transgenic Arabidopsis Plants**

For overexpression constructs, the coding sequence of the 10A07-40, IPK, and IAA16 were cloned into the binary vector pBI121, while IPK(Δ136R) was cloned into pCB302-3 vector containing the BASTA selectable marker (Xiang et al., 1999). For IPK-IAA16 cosilencing construct, the IPKCS8-899 region was fused to the IAA161-800 region by PCR and inserted into phanllarial vector (Wesley et al., 2001) in sense and antisensc orientations. Then, a 4922-bp fragment containing the RNAi construct, 3SS promoter, and octopine esynthase (ocs) terminator was excised and inserted into the binary vector pART27 (Gleave, 1992). Agrobacterium tumefaciens strain C58 was transformed with the binary plasmids by the freeze-thaw method and used to transform Arabidopsis wild-type C24, Col-0, or 10A07 overexpression plants as described by Clough and Bent (1998). Transformed T1 plants were screened on MS medium containing 50 mg/L kanamycin or by the herbicide BASTA (ammonium glufosinate) treatment to select for transgenic plants. Homozygous T3 seeds were collected from T2 lines after segregation analysis. Primer sequences used for cloning are provided in Supplemental Data Set 1.

**Identification of T-DNA Mutants of IPK and IAA16**

T-DNA insertional alleles of IAA16 (Salk-114809 and CS466686) or IPK (CS859240) in the Col-0 background were obtained from the ABRC. See Supplemental Figure F for details.

**DNA Gel Blot Analysis**

Total genomic DNA was isolated from *Heteroderaschachtii*, *Heteroderaglycines*, and *Meloidogyne incognita* as previously described (Blin and Stafford, 1976) and from soybean (*Glycine max*) according to Fulton et al. (1995). Ten micrograms of DNA was digested overnight at 37°C with the HindIII and EcoRI restriction enzymes (Invitrogen). DNA transfer, probe hybridization, and signal detection were performed as described by Hewezi et al. (2006).

**In Situ Hybridization**

In situ hybridizations were performed using parasitic and parasitic stages of *H. schachtii* nematodes isolated from inoculated Arabidopsis plants as previously described by de Boer et al. (1998). Hybridization signals within the nematodes were detected using alkaline phosphatase-conjugated antidigoxigenin antibody (diluted 1:100) and substrate, and examples were observed and photographed with a Zeiss Axiovert 100 inverted light microscope.

**Histochemical Analysis of GUS Activities**

The histochemical staining of GUS enzyme activity was performed according to Jefferson et al. (1987). Tissue samples were viewed using a Zeiss SV-11 microscope, and the images were captured using a Zeiss AxioCam MRc5 digital camera and then processed using Zeiss Axiovision software (release 4.8).

**Nematode Infection Assay**

Arabidopsis seeds were surface sterilized and randomly distributed on 12-well plates (BD Biosciences). Ten-day-old seedlings were inoculated with ~200 surface-sterilized J2 *H. schachtii* nematodes per plant as previously described (Hewezi et al., 2010). Three weeks after inoculation, the number of J4 females was counted for both the transgenic lines and the wild-type control and used to quantify plant susceptibility. Each line was replicated 20 times, and at least three independent experiments were performed. The statistically significant differences were determined in a modified t test using the statistical software package SAS.

**Root Length Measurements**

Arabidopsis plants were grown vertically on modified Knop’s medium for 10 d and then the root length of at least 40 plants per line was measured as the distance between the crown and the tip of the main root in four independent experiments. Statistically significant differences between lines were determined by unadjusted paired t test (*P* < 0.01).

**RNA Isolation and qPCR**

Total RNA was extracted from 200 mg frozen ground plant tissue using the method described by Veroord et al. (1989) or from 50 mg nematode tissue at various developmental stages using the Versagene RNA tissue kit (Genta Systems) following the manufacturer’s instructions. DNase treatment of total RNA was performed using DNase I (Invitrogen). Ten nanograms of DNase-treated RNA was used as a template in quantitative RT-PCR reactions to quantify expression levels using the One-Step RT-PCR kit (Bio-Rad) following the manufacturer’s protocol. PCR conditions and quantification were performed as previously described by Hewezi et al. (2010). Primer sequences are provided in Supplemental Data Set 1.

**Yeast Two-Hybrid Screen**

Yeast two-hybrid screening was performed as described in the BD Matchmaker Library Construction and Screening Kits user manual (Clontech). The coding sequence of 10A07 without the signal peptide coding sequence was amplified and fused to the GAL4 DNA binding domain of pGBK7 vector to generate pGBK7-10A07 and then introduced into *Saccharomyces cerevisiae* strain Y187 to generate the bait strain. Three Arabidopsis cDNA libraries prepared from RNA isolated from roots of ecotype C24 at 3, 7, and 10 d after *H. schachtii* infection were generated in *S. cerevisiae* strain AH109 as a fusion to the GAL4 activation domain of pGAD77-Rec2 vector (Hewezi et al., 2008). Screens for interacting proteins were conducted using the mating method and subsequent analyses were performed according to Clontech protocols.

**BiFC**

The coding sequences of 10A07-40, 10A07-Δ34, and 32E03-Δ40 were PCR amplified and cloned into pSAT4-nEYFP-C1 to generate nEYFP-10A07-40, nEYFP-10A07-Δ34, and nEYFP-32E03-Δ40 fusions, respectively. The full-length IPK and IAA16 coding sequences were amplified and cloned into pSAT4-cEYFP-C1(B) to generate cEYFP-IPK and cEYFP-IAA16, respectively. The IPK homolog AT3G53930 and IAA7 were also cloned in pSAT4-cEYFP-C1(B) and used as negative controls. For BiFC in onion (*Allium cepa*) epidermal cells, particle bombardment was performed as described by Hewezi et al. (2010). Bombarded onion epidermal cells were incubated at 25°C in darkness for ~24 h before being assayed for YFP activity. The bright-field and fluorescent images were taken using the Zeiss Axiovert 100 microscope with appropriate YFP filters. For BiFC in *Nicotiana benthamiana*, the nEYFP-10A07, nEYFP-10A07-Δ34, cEYFP-IPK, cEYFP-IAA16, cEYFP-AT3G53930, and cEYFP-IAA7 fusions were digested from pSAT plasmids using I-SceI restriction enzyme and ligated to a I-SceI-digested binary vector pZP-RCS2-ocs-bar-RIF. Also, IPKΔ136R and 10A07Δ136R were cloned in the pZP-RCS2-ocs-bar-RIF vector. The binary vectors were transformed into Agrobacterium strain GV3101. For infiltration, overnight Agrobacterium cultures harboring individual constructs were generated and mixed in equal proportion to the
final OD of 0.2 at 600 nm using infiltration medium (10 mM MES and 10 mM MgCl2). These cultures were infiltrated into the leaves of 5- to 7-week-old N. benthamiana plants using a blunt-ended 1-mL syringe. Approximately 40 h postinfiltration, leaf sections from the infiltration zones were tested for YFP signal using a Zeiss Axiosvert 100 fluorescent microscope.

Subcellular Localization and Cotransformation Assays

For subcellular localization assays, 10A07-SP, 10A07-676-68, 10A07-75-78A/S231A, 10A07-75-78A/S231E, IPK, and IAA16 were amplified and cloned into a modified pRJG23 vector (Grebenok et al., 1997) before the start codon of GFP fused into the GUS reporter gene and under the control of a double CaMV 35S promoter. Wild-type and mutated versions of NLS59-75 and NLS77-83 were cloned into pSAT6-EYFP-N1 vector and under the control of double CaMV 35S promoter. A wild-type (MNLALKLGLDLN) and a mutated version (MNEAALKAAGADAN) of the NES from the protein kinase inhibitor PKI (Wen et al., 1995) were attached to 10A07-SP and 10A07-676-68 and fused to the N-terminal of EYFP in the pSAT6-EYFP-N1 vector. These constructs were delivered into onion epidermal cells by biolistic bombardment using standard procedures. For cotransformation assays, 10A07-676-68-GFP-GUS, 10A07-75-78A/S231A-GFP-GUS, and 10A07-75-78A/S231E were delivered to onion epidermal cells alone or in combination with the wild-type IPK or its inactive variant (IPK41R/K136R). After bombardment, epidermal peels were incubated at 26°C for 24 h in the dark. The subcellular localization of the fused proteins was visualized using a Zeiss Axiosvert 100 microscope. Both subcellular localization and co-transformation assays were repeated at least four times independently.

Protein Expression and Purification

The coding sequences of 10A07-SP and IPK were cloned into pET28a (+) (Novagen) to generate N- and C-terminal 6xHis-tag fusion proteins. IPK41R/K136R was cloned into Champion pET302/NT-His (Invitrogen) to generate N- and C-terminal 6xHis-tag fusion proteins. The coding sequences of 10A07-SP, IPK, and IPK41R/K136R were cloned into pET28a(+) containing 25 mM MgCl2. These cultures were incubated at 26°C for 24 h in the dark. The subcellular localization of the fused proteins was visualized using a Zeiss Axiosvert 100 microscope. Both subcellular localization and co-transformation assays were repeated at least four times independently.

In Vitro Kinase Assay

The in vitro phosphorylation assays were performed in 1× kinase buffer (Cell Signaling) in a total volume of 40 μL containing 25 mM ATP, 5 μCi [γ-32P]ATP, and 5 μL of bacterially expressed and purified His-tagged proteins. Kinase reactions were incubated at 30°C for 30 min and terminated by adding concentrated SDS-PAGE loading buffer and heating for 5 min at 95°C. Proteins were resolved by SDS-PAGE and analyzed by autoradiography and Coomassie Brilliant Blue staining using standard procedures.

LC-MS/MS Analysis

A Coomassie Brilliant Blue-stained 10A07 protein band was excised from the SDS-PAGE gel and submitted to the Genome Center Proteomics Core at the University of California, Davis, for LC-MS/MS-based protein phosphorylation analysis. Proteins were reduced with DTT and alkylated with iodoacetamide and then digested with sequencing grade trypsin (Promega). Digested peptides were analyzed by LC-MS/MS on a Thermo Scientific Q Exactive Orbitrap Mass spectrometer in conjunction with a Paradigm M34 HPLC and CTC PAL autosampler (Michrom Bio Resources). The peptides were separated using a Michrom 200 μm × 150 mm Magic C18Q reverse phase column at flow rate of 2 μL/min. Tandem mass spectra were extracted and charge state deconvoluted by MM File Conversion Version 3. Database searching and criteria for protein identification and validation were performed as described by Rigor et al. (2011).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: Hs-10A07 (KP728937), Hg-10A07 (AAP30760), 27D09 (AAM50938), 32E03 (AAP30775), 1AAO3476), 20004 (AAP30761), 13A06 (AAP30759), IPK (AT2G37840), IPK homolog (AT3G53930), IAA16 (AT3G04730), IAA7 (AT3G23050), IAA14 (AT4G14550), and Actin8 (AT1G49240).

Supplemental Data

Supplemental Figure 1. Sequence Alignment of 10A07 Effector Protein Isolated from the Beet Cyst Nematode, Heterodera schachtii, and Its Ortholog from the Soybean Cyst Nematode, H. glycines.

Supplemental Figure 2. Protein Sequence Alignment of Hs-10A07 and Four Putative Effector Proteins Identified in the Soybean Cyst Nematode, H. glycines.

Supplemental Figure 3. DNA Gel Blot Analysis of 10A07 Sequences.

Supplemental Figure 4. Developmental Expression Levels of 10A07.

Supplemental Figure 5. Quantification of 10A07 Levels in Transgenic Arabidopsis Lines Using qPCR.

Supplemental Figure 6. 10A07 Does Not Interact with IPK Homolog or with IAA7 and IAA14.

Supplemental Figure 7. Subcellular Localization of IPK and IAA16.

Supplemental Figure 8. BIFC of Onion Epidermal Cells Showing the Specificity of the Interaction of 10A07 with IPK and IAA16.

Supplemental Figure 9. BIFC of Agroinfiltrated Nicotiana benthamiana Leaves Confirming the Interaction of 10A07 with IPK and IAA16.

Supplemental Figure 10. Spatial Expression Patterns of IPK and IAA16 under Noninfected Conditions.

Supplemental Figure 11. Peptide Spectra of Two Phosphorylated Serines Identified in the 10A07 Protein by Mass Spectrometry.

Supplemental Figure 12. qPCR Quantification of IPK and IAA16 Overexpression Levels in IPK, IAA16, IPK41R/K136R, and 10A07/ IPK41R/K136R Transgenic Plants.

Supplemental Figure 13. Characterization of Arabidopsis IPK and IAA16 Mutants.

Supplemental Figure 14. Cosilencing of IPK/IAA16 Using RNAi.

Supplemental Figure 15. 10A07-IPK Interaction Is Independent of 10A07 Phosphorylation.

Supplemental Data Set 1. Primer Sequences Used in This Study.

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