

## Distinct *Pseudomonas* type-III effectors use a cleavable transit peptide to target chloroplasts

Guangyong Li<sup>1,2</sup>, John E. Froehlich<sup>3,4</sup>, Christian Elowsky<sup>5</sup>, Joseph Msanne<sup>1,6</sup>, Andrew C. Ostosh<sup>3,4</sup>, Chi Zhang<sup>1,7</sup>, Tala Awada<sup>6</sup> and James R. Alfano<sup>1,2,\*</sup>

<sup>1</sup>Center for Plant Science Innovation, University of Nebraska, Lincoln, NE 68588-0660, USA,

<sup>2</sup>Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583-0722, USA,

<sup>3</sup>Michigan State University-Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA,

<sup>4</sup>Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA,

<sup>5</sup>Center for Biotechnology, University of Nebraska, Lincoln, NE 68588-0660, USA,

<sup>6</sup>School of Natural Resources, University of Nebraska, Lincoln, NE 68583-0989, USA, and

<sup>7</sup>School of Biological Sciences, University of Nebraska, Lincoln, NE 68583-0118, USA

Received 12 April 2013; revised 13 November 2013; accepted 19 November 2013.

\*For correspondence (e-mail: jalfano2@unl.edu.)

### SUMMARY

The pathogen *Pseudomonas syringae* requires a type-III protein secretion system and the effector proteins it injects into plant cells for pathogenesis. The primary role for *P. syringae* type-III effectors is the suppression of plant immunity. The *P. syringae* pv. *tomato* DC3000 HopK1 type-III effector was known to suppress the hypersensitive response (HR), a programmed cell death response associated with effector-triggered immunity. Here we show that DC3000 *hopK1* mutants are reduced in their ability to grow in Arabidopsis, and produce reduced disease symptoms. Arabidopsis transgenically expressing HopK1 are reduced in PAMP-triggered immune responses compared with wild-type plants. An N-terminal region of HopK1 shares similarity with the corresponding region in the well-studied type-III effector AvrRps4; however, their C-terminal regions are dissimilar, indicating that they have different effector activities. HopK1 is processed *in planta* at the same processing site found in AvrRps4. The processed forms of HopK1 and AvrRps4 are chloroplast localized, indicating that the shared N-terminal regions of these type-III effectors represent a chloroplast transit peptide. The HopK1 contribution to virulence and the ability of HopK1 and AvrRps4 to suppress immunity required their respective transit peptides, but the AvrRps4-induced HR did not. Our results suggest that a primary virulence target of these type-III effectors resides in chloroplasts, and that the recognition of AvrRps4 by the plant immune system occurs elsewhere. Moreover, our results reveal that distinct type-III effectors use a cleavable transit peptide to localize to chloroplasts, and that targets within this organelle are important for immunity.

**Keywords:** bacterial pathogens, chloroplast biology, plant immunity, transit peptides, type-III effector.

### INTRODUCTION

The plant immune system can be considered as two overlapping branches, which are distinct based on which molecules are recognized (Jones and Dangl, 2006). The first branch involves the recognition of pathogen (microbe)-associated molecular patterns (PAMPs/MAMPs), molecules highly conserved in microorganisms (Ausubel, 2005; Boller and Felix, 2009). PAMPs include bacterial flagellin, translation factor EF-Tu, peptidoglycan and fungal chitin, and they are sensed at the plant cell surface through pattern-recognition receptors, leading to PAMP-triggered immunity (PTI); Dodds and Rathjen, 2010; Segonzac and Zipfel, 2011).

The other branch of the plant immune system recognizes pathogen effector proteins, and is called effector-triggered immunity (ETI). Resistant plants can recognize specific effectors such as those from bacteria [i.e. avirulence (Avr) proteins] using immune receptors called resistance (R) proteins. R proteins recognize effectors either directly or by recognizing the modification of target plant proteins by effectors (Van der Biezen and Jones, 1998; Jones and Dangl, 2006). Both PTI and ETI appear to activate similar signaling pathways and immune responses. These responses include the rapid production of reactive

oxygen species (ROS) and callose deposition at the cell wall, a late immune response (Gimenez-Ibanez and Rathjen, 2010; Tsuda and Katagiri, 2010); however, ETI generally activates these responses in a more prolonged and robust fashion than PTI, and usually includes the hypersensitive response (HR), an immunity-associated programmed cell death (Tsuda and Katagiri, 2010).

The Gram-negative bacterial plant pathogen *Pseudomonas syringae* requires a type-III protein secretion system (T3SS) to cause disease. The T3SS is a nanosyringe that allows many animal and plant pathogens to translocate, or inject, type-III effector proteins into eukaryotic cells (Buttner, 2012). Although the majority of the activities and/or plant targets of *P. syringae* type-III effectors are unknown, there has been significant recent progress (Hann *et al.*, 2010; Block and Alfano, 2011; Feng and Zhou, 2012). Type-III effectors can suppress plant immunity using a variety of strategies, including interference with immune receptor signaling, inactivating MAPK pathways, blocking RNA processes and vesicle trafficking, and altering organelle function (Block and Alfano, 2011; Feng and Zhou, 2012). Several *P. syringae* type-III effectors have been shown to localize to discrete subcellular plant compartments. A relatively common localization site for these within plant cells is the plasma membrane (Block and Alfano, 2011). For example, the well-studied type-III effectors AvrRpm1 and AvrPto are both localized to the plasma membrane (Nimchuk *et al.*, 2000; Shan *et al.*, 2000). Two *P. syringae* type-III effectors, Hopl1 and HopN1, localize to chloroplasts using uncharacterized non-cleavable transit peptides (Jelenska *et al.*, 2007, 2010; Rodriguez-Herva *et al.*, 2012). HopG1 has been shown to localize to plant mitochondria (Block *et al.*, 2010). Finally, HopM1 has been shown to localize to the *trans*-Golgi network/early endosome (Nomura *et al.*, 2011).

The type-III effectors from *P. syringae* pv. *tomato* DC3000 were separated into classes based upon their ability to suppress the HopA1-induced HR (Jamir *et al.*, 2004; Guo *et al.*, 2009). Eight DC3000 effectors, including an effector named HopK1, were identified as class-I suppressors because they possessed a robust ability to suppress HopA1-induced ETI. Little is known about HopK1 other than that DC3000 could inject HopK1 into the plant cells via the T3SS (Petnicki-Ocwieja *et al.*, 2002).

Here we show that DC3000 *hopK1* mutants are greatly reduced in virulence, and that HopK1 can suppress PTI. The N-terminal 147 amino acids of HopK1 are similar to the well-studied type-III effector AvrRps4. Surprisingly, the processed forms of HopK1 and AvrRps4 are found primarily in chloroplasts. Thus, the conserved N-termini of HopK1 and AvrRps4 contain a chloroplast transit peptide. The ability of HopK1 and AvrRps4 to suppress PTI-induced immune responses was dependent on their chloroplast transit peptides, indicating that their ability to suppress PTI

required chloroplast localization, and that chloroplast targets are important components of plant immunity.

## RESULTS

### HopK1 contributes to *P. syringae* virulence

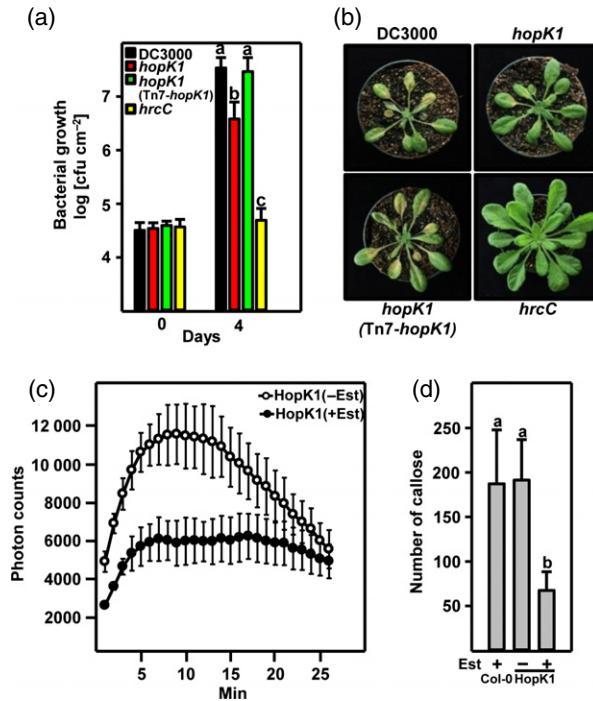
We became interested in HopK1 when we identified it as a strong suppressor of the HopA1-induced HR (Jamir *et al.*, 2004; Guo *et al.*, 2009). The *hopK1* gene is in a single gene operon predicted to encode a 338 amino acid long protein (Figure S1A). To determine the extent that HopK1 contributes to the virulence of *P. syringae*, we constructed independent DC3000 *hopK1* mutants (Figure S1B). A *hopK1* mutant was spray-inoculated onto *Arabidopsis* plants. The DC3000 *hopK1* mutant was significantly reduced in its ability to grow in plant tissue, compared with wild-type DC3000 (Figure 1a). The *hopK1* mutant also produced reduced disease symptoms compared with the wild-type strain at 4 days post-inoculation (Figure 1b). The reduced growth and the reduced disease symptom production exhibited by the *hopK1* mutant was complemented when *hopK1* was re-introduced (Figure 1a, b). Thus, HopK1 contributes significantly to DC3000 virulence.

### HopK1 suppresses PTI responses

To evaluate the effect of HopK1 on plant immunity, we made *Arabidopsis thaliana* Col-0 transgenic plants that express HopK1 fused to a hemagglutinin (HA) tag when induced with estradiol. These plants were confirmed to produce HopK1-HA upon induction with estradiol (Figure S2A). Wild-type Col-0 plants and HopK1-HA-expressing plants were treated with flg22 (a peptide derived from bacterial flagellin) and two PTI responses, ROS production (an early PTI response) and callose deposition (a late PTI response), were evaluated. HopK1-HA-expressing plants produced significantly less ROS (Figure 1c) and callose (Figure 1d) than uninduced HopK1-HA transgenic plants and wild-type Col-0 plants, clearly indicating that HopK1-HA can suppress flg22-induced PTI. Similar results were observed when PTI was induced with elf18 (a peptide derived from EF-Tu) or chitin (Figure S3). Therefore, HopK1 can suppress PTI.

### The N-terminal half of HopK1 is homologous to the Avr protein AvrRps4

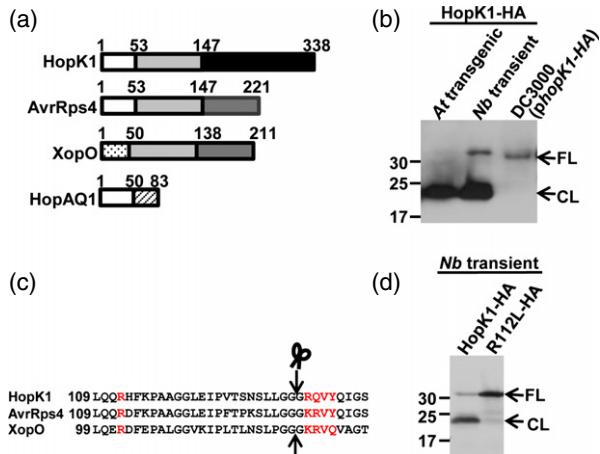
The N-terminal 147 amino acids of HopK1 have 75% identity to the corresponding amino acids of AvrRps4 (Figure 2a), a well-studied Avr protein from *P. syringae* pv. *pisi*, the causal agent of bacterial blight in *Pisum sativum* (pea; Hinsch and Staskawicz, 1996). A region of HopK1 encompassing amino acids 53–147 (HopK1<sub>53–147</sub>) is homologous to a similarly located region within the *Xanthomonas campestris* pv. *vesicatoria* type-III effector XopO. XopO is homologous to AvrRps4, beginning after the amino acid at position 53, and continuing throughout the remainder of



**Figure 1.** HopK1 contributes to the virulence of *Pseudomonas syringae*. (a) Arabidopsis plants were spray-inoculated with wild-type DC3000, the *hopK1* mutant, the complemented *hopK1* mutant *hopK1*(Tn7-*hopK1*) and the *hrcC* mutant defective in type-III secretion. The bacteria were enumerated at 0 and 4 days post-inoculation. Different letters indicate a statistically significant difference ( $P < 0.05$ ). (b) Representative disease symptoms of Arabidopsis plants inoculated with the bacterial strains in (a) are shown at 5 days post-inoculation. (c) Estradiol-inducible HopK1-HA transgenic plants were treated with 1  $\mu$ M flg22 and ROS was measured. (d) Wild-type and estradiol-inducible HopK1-HA transgenic plants were treated with 1  $\mu$ M flg22 and callose deposition was quantified. (c and d) Error bars represent standard errors ( $n = 20$ ). Different letters indicate a statistically significant difference ( $P < 0.05$ ). Plants were treated with 10  $\mu$ M estradiol (+Est) or without estradiol (-Est) for 16 h prior to flg22 treatment. (a-d) Each experiment was repeated at least three times with similar results.

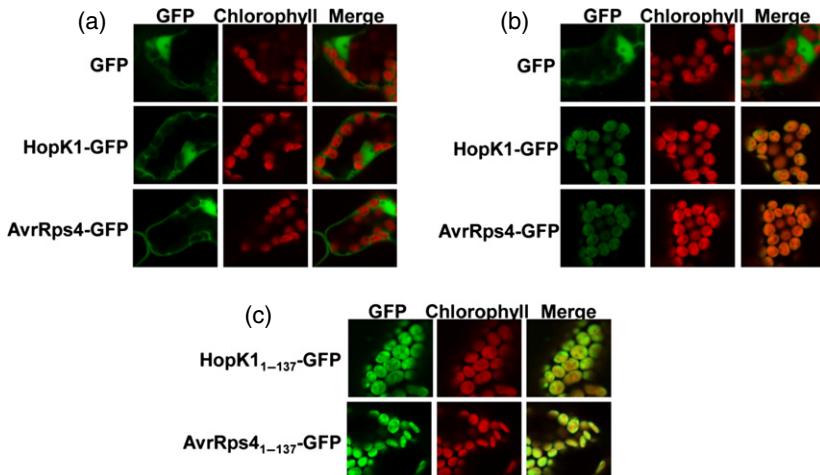
the 221 amino acid long AvrRps4 protein (Figure 2a; Sohn *et al.*, 2009), and therefore probably possess a similar effector domain. The HopK1 C-terminal effector domain is homologous to the *Xanthomonas* type-III effector XopAK (Potnis *et al.*, 2011). AvrRps4 and XopO have been shown to be processed *in planta* between two conserved glycine residues (residues 133 and 134 in AvrRps4) within this conserved region, but not when expressed in bacteria or yeast cells (Sohn *et al.*, 2009).

To determine if HopK1 is cleaved when expressed *in planta*, similar to AvrRps4, we transiently expressed HopK1-HA in *Nicotiana benthamiana* and subjected it to immunoblot analysis with anti-HA antibodies. We could clearly detect that the majority of the HopK1-HA protein was in a band at about a molecular mass of 22 kDa (Figure 2b), which is smaller than the predicted molecular



**Figure 2.** HopK1 is processed *in planta* identically as AvrRps4. (a) Schematic regions of HopK1 and the related proteins AvrRps4, XopO and HopAQ1. HopK1, AvrRps4 and HopAQ1 share the same type-III secretion signal at their N-termini (white; type-III secretion signal of XopO is stippled). HopK1, AvrRps4 and XopO have a conserved domain after the type-III secretion signal (light grey). AvrRps4 and XopO share a conserved effector domain (dark grey). The effector domain of HopK1 (black) is distinct from these proteins. (b) Immunoblot analysis of HopK1-HA produced transgenically from *Arabidopsis* (At transgenic), transiently in *Nicotiana benthamiana* (Nb transient), or in DC3000 grown in culture [DC3000(*phopK1-HA*)]. A processed form of HopK1-HA can only be detected in the plant samples. (c) Amino acid sequence alignment of HopK1, AvrRps4 and XopO in the region adjacent to the cleavage site (indicated by the black arrow and scissors). A conserved arginine (R112) found to be indispensable for cleavage and a conserved KRVY domain are indicated in red. (d) *Nicotiana benthamiana* was transiently transformed with *Agrobacterium* carrying pLN462-*hopK1-HA* or pLN462-*hopK1R112L-HA*. Immunoblot analyses of HopK1-HA with anti-HA antibodies indicate that the HopK1<sub>R112L</sub>-HA cannot be processed *in planta*. (b and d) Full-length HopK1 (FL); processed HopK1 (CL).

mass of the full-length HopK1-HA (~36 kDa). We made transgenic *Arabidopsis* plants that produced HopK1-HA, and immunoblot analysis of samples from these plants again showed the primary HopK1-HA band to be at about 22 kDa (Figure 2b). Soluble extracts from DC3000 expressing HopK1-HA contained only full-length HopK1-HA (Figure 2b), indicating that HopK1 is processed *in planta*, but not in bacteria, similar to the *in planta* processing of AvrRps4 (Sohn *et al.*, 2009). We isolated the processed form of HopK1-HA, subjected it to Edman degradation sequencing, and found that it was cleaved within the conserved region of HopK1 between Gly<sub>133</sub> and Gly<sub>134</sub> (Figure 2c), the identical location of the *in planta* cleavage sites identified in AvrRps4 and XopO (Sohn *et al.*, 2009). Additionally, the R112 residue, which was required for AvrRps4 processing, is also required for HopK1-HA processing, as transient expression of HopK1<sub>R112L</sub>-HA in *N. benthamiana* produced a HopK1-HA derivative that was no longer processed *in planta* (Figure 2d). Taken together, these data indicate that HopK1 is processed *in planta* within the conserved N-terminal region, identically to AvrRps4 and XopO.



**Figure 3.** HopK1 and AvrRps4 localize to plant chloroplasts.

(a) HopK1-GFP and AvrRps4-GFP were transiently (constitutively) expressed in *Nicotiana benthamiana*, and imaged 2 days after infiltration using confocal microscopy.

(b) Transgenic Arabidopsis plants expressing GFP, HopK1-GFP or AvrRps4-GFP were imaged 1 day after induction with 10 µM estradiol.

(c) Transgenic Arabidopsis that constitutively express HopK1<sub>1-137</sub>-GFP or AvrRps4<sub>1-137</sub>-GFP were imaged using confocal microscopy. The transgenic Arabidopsis expressing AvrRps4-GFP were in ecotype Col-0 rps4-2. All other transgenic Arabidopsis were made using wild-type Col-0. All confocal images are of single optical sections.

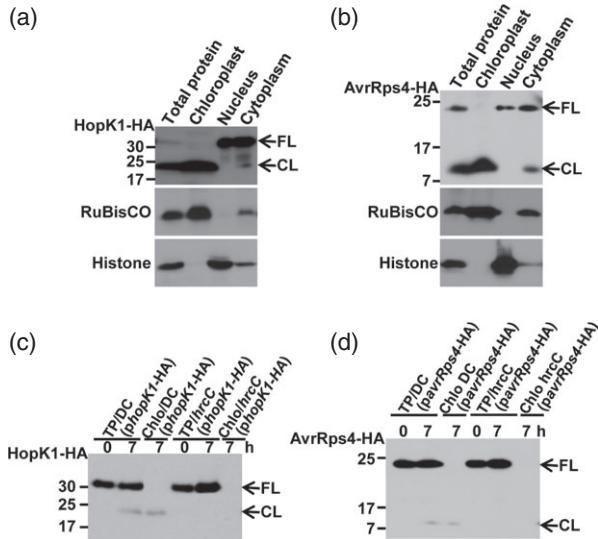
#### HopK1-GFP and AvrRps4-GFP localize to chloroplasts

To determine the subcellular location of HopK1 and AvrRps4, we transiently (constitutively) expressed HopK1-GFP and AvrRps4-GFP in *N. benthamiana* using agroinfiltration. The infiltrated plant leaf tissue was viewed using confocal microscopy, and the fluorescence was found in the cytoplasm and nucleus for both HopK1-GFP and AvrRps4-GFP (Figure 3a). To get uniformed expression we made transgenic Arabidopsis lines that conditionally expressed HopK1-GFP or AvrRps4-GFP after induction with estradiol. Interestingly, both HopK1-GFP and AvrRps4-GFP appeared to localize to chloroplasts in these plants when viewed with confocal microscopy (Figure 3b). We confirmed with immunoblots that both transiently and transgenically expressed HopK1-GFP and AvrRps4-GFP were expressed and processed (Figure S4). AvrRps4-GFP was recently reported to be localized to the cytoplasm and the nucleus in *Agrobacterium*-mediated transient assays (Sohn *et al.*, 2012). The localization of transgenically expressed full-length AvrRps4-GFP has not been previously reported. We do not know why there was a difference in the localization of these effector-GFP fusions when these were expressed transiently versus transgenically. It may result, at least in part, from the plant species used: transient expression was carried out in *N. benthamiana* and transgenic expression was carried out in Arabidopsis. We did find that less of the effector-GFP fusion is processed in transient than in transgenic expression (Figure S4), which is consistent with less of the effector fusion being localized to the chloroplast, and may make it more difficult to visualize.

We next sought to determine whether the N-terminal 137 amino acids of HopK1 or AvrRps4, which are within the conserved N-terminal region, are sufficient to target a GFP reporter to chloroplasts. This length was chosen because it extends several amino acids beyond the processing site of both of these proteins (Figure 2c). Transgenic Arabidopsis plants were made that constitutively express HopK1<sub>1-137</sub>-GFP or AvrRps4<sub>1-137</sub>-GFP. Both HopK1<sub>1-137</sub>-GFP and AvrRps4<sub>1-137</sub>-GFP were found to primarily localize to chloroplasts (Figure 3c).

#### Biochemical fractionation experiments support chloroplast localization for HopK1 and AvrRps4

To further address the possibility that HopK1 and AvrRps4 localize to chloroplasts, we used Arabidopsis plants that conditionally express either HopK1-HA or AvrRps4-HA and harvested plant tissue 24 h after estradiol induction. We separated these plant extracts into total soluble protein, cytoplasmic, nuclear, and chloroplastic fractions, and they were subjected to SDS-PAGE and immunoblot analyses using anti-HA antibodies. The chloroplast fraction was found to contain the processed form of both HopK1-HA and AvrRps4-HA, but not the full-length forms (Figure 4a, b). Conversely, the nuclear fraction was found to contain full-length HopK1-HA and AvrRps4-HA, but not the processed form of these proteins (Figure 4a, b). Additionally, we localized the effector derivatives that lacked their putative transit peptides (HopK1<sub>C</sub> and AvrRps4<sub>C</sub>) using confocal microscopy and biochemical fractionation, and these effector derivatives were not found in chloroplasts and remained mostly in the cytoplasmic fractions (Figure S5).



**Figure 4.** HopK1 and AvrRps4 localize to chloroplasts when expressed transgenically, and when delivered by bacteria.

(a and b) Transgenic *Arabidopsis* plants expressing HopK1-HA or AvrRps4-HA were induced with estradiol, and 16 h post-induction intact chloroplast and nuclear-enriched fractions were isolated. HopK1-HA (a) and AvrRps4-HA (b) were detected with anti-HA antibodies, anti-ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) antibodies were used to detect chloroplast-localized RuBisCO, and nuclear-localized histone H3 was detected with anti-histone H3 antibodies.

(c and d) DC3000 (DC) or the *hrcC* mutant expressing plasmid-encoded HopK1-HA (c) or plasmid-encoded AvrRps4-HA (d) were infiltrated into *Arabidopsis* leaves at a cell density of  $1 \times 10^9$  cells ml $^{-1}$ . Constructs pLN5252 and pLN5253 encoded HopK1-HA or AvrRps4-HA, respectively. Immediately after infiltration (0 h) and after 7 h, leaf disks were harvested. Chloroplasts were isolated from a subset of the leaf tissue and total protein (TP) and chloroplast (Chlo) fractions were subjected to SDS-PAGE and immunoblot analyses.

Importantly, antibodies that recognize a chloroplast protein (ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBisCO) and a nuclear protein (Histone H3) were used to assess the integrity of the chloroplast and nuclear fractions (Figure 4a, b). Taken together these data indicate that HopK1-HA and AvrRps4-HA localize to the chloroplast, and that these proteins are likely to be processed upon import into this organelle.

#### Bacterially delivered HopK1-HA and AvrRps4-HA are processed *in planta*, and only the processed forms are found in chloroplasts

We next asked if bacterially injected HopK1-HA and AvrRps4-HA were also localized to chloroplasts. To accomplish this, *A. thaliana* Col-0 leaves were infiltrated with DC3000 or a DC3000 *hrcC* mutant, both carrying a construct encoding HopK1-HA or AvrRps4-HA. At the time of infiltration and 7 h later leaf tissue was harvested and HopK1-HA and AvrRps4-HA levels were assessed with immunoblots. At 7 h, samples from plant tissue infiltrated with DC3000(*phopK1-HA*) or DC3000(*pavrRps4-HA*) were

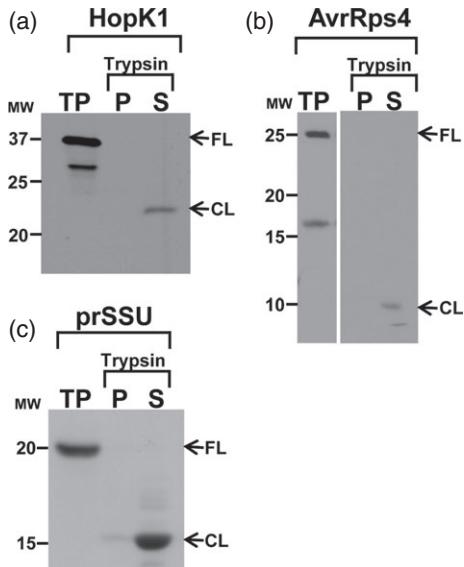
found to have the full-length and the processed forms of HopK1-HA and AvrRps4-HA, respectively, further confirming that these effectors are only processed *in planta* (Figure 4c, d). Moreover, processed HopK1-HA or AvrRps4-HA were not detected in plant tissue infiltrated with the *hrcC* mutant, indicating that processing is dependent on a functional TSS. Chloroplast fractions were isolated from a subset of these plant tissues, and only the processed forms of HopK1-HA and AvrRps4-HA were detected in the chloroplast fractions (Figure 4c, d). In contrast, we found that HopK1c-HA and AvrRps4c-HA were not present in chloroplast fractions in similar biochemical fractionation experiments (Figure S5). Taken together, these data further indicate that the N-terminus of HopK1-HA and AvrRps4-HA act as chloroplast transit peptides, and that these effectors are processed inside chloroplasts.

#### HopK1 and AvrRps4 localized to chloroplasts in chloroplast import assays

To independently confirm that HopK1 and AvrRps4 localized to chloroplasts, we performed *in vitro* chloroplast import assays using radiolabeled HopK1 or AvrRps4, produced by *in vitro* transcription and translation of their corresponding genes. Radiolabeled full-length HopK1 and AvrRps4 were incubated with isolated pea chloroplasts, followed by treatment with the protease trypsin. After the trypsin treatment the chloroplasts were recovered, lysed, and separated into membrane and soluble fractions. Our results indicate that the processed forms of HopK1 and AvrRps4 were protected from trypsin degradation, indicating that they are present inside chloroplasts (Figure 5). Moreover, each protein was found in the soluble chloroplast fraction (Figure 5a, b), indicating that they localize to the chloroplast stroma. These results are consistent with our confocal microscopy and our biochemical fractionation experiments (Figures 3 and 4). Thus, we have independent lines of evidence that indicate that HopK1 and AvrRps4 are localized to the chloroplast, and that they possess N-terminal transit peptides.

#### The virulence activities of HopK1 and AvrRps4 require their chloroplast transit peptides

To test whether the transit peptide of these effectors were needed for their activities inside plant cells, we made transgenic *Arabidopsis* plants that expressed the processed form of HopK1-HA (HopK1c-HA) or the processed form of AvrRps4-HA (AvrRps4c-HA) (Figure 2). We confirmed that these plants expressed proteins of the predicted molecular mass after induction with estradiol (Figure S2). The *Arabidopsis* plants expressing HopK1c-HA were unable to complement the reduced-growth phenotype exhibited by the *hopK1* mutant (Figure 6a), indicating that full-length HopK1 is required to restore virulence to the *hopK1* mutant. Likewise, the ability of HopK1 and Av-



**Figure 5.** HopK1 and AvrRps4 are imported into isolated chloroplasts and are localized to the chloroplast soluble fraction.  
(a) [ $^{35}\text{S}$ ]HopK1, (b) [ $^3\text{H}$ ]AvrRps4 and (c) [ $^{35}\text{S}$ ]small subunit of RUBISCO (prSSU) were incubated with isolated intact *Pisum sativum* (pea) chloroplasts for 40 min at room temperature ( $23^\circ\text{C}$ ) in the presence of 3.0 mM Mg-ATP. After completion of the import, all reactions were treated with trypsin for 30 min on ice. Intact chloroplasts were recovered, lysed, and fractionated into pellet (P) or soluble (S) fractions. All fractions were then analyzed by a 14% tricine gel and fluorography. prSSU was used as a chloroplastic stromal marker. The labeled translation products (TP) are shown after the *in vitro* transcription/translation reaction, prior to mixing with isolated chloroplasts. Full-length (FL) proteins and processed (CL) proteins are indicated. This experiment was repeated three times with similar results.

rRps4 to suppress ROS production and callose deposition after PAMP treatment required their transit peptides (Figures 6 and S3). The presence of AvrRps4 inside plant cells is recognized by the R protein RPS4 inducing ETI. AvrRps4<sub>C</sub> retained the ability to elicit an RPS4-induced HR (Figure 6d), indicating that AvrRps4 is recognized by the RPS4 R protein outside of the chloroplast.

As a matter of course, we also evaluated two other HopK1 derivatives for their ability to restore growth *in planta* to a *hopK1* mutant, and whether they retained the ability to suppress PTI responses. Transgenic Arabidopsis plants expressing the N-terminal portion of HopK1 fused to GFP (HopK1<sub>1-137</sub>-GFP) after estradiol treatment were unable to restore the growth of the DC3000 *hopK1* mutant to wild-type levels (Figure S6A). Additionally, plants expressing HopK1<sub>1-137</sub>-GFP were not reduced in their PTI responses after induction with flg22 (Figure S6B and C). This HopK1-GFP fusion is localized to chloroplasts (Figure 3c), but lacks the C-terminal portion of the protein that is likely to contain its effector domain. Transgenic Arabidopsis plants conditionally expressing HopK1<sub>R112L-HA</sub> after induction with estradiol restored growth to the DC3000 *hopK1* mutant, and were capable of suppressing PTI responses (Figure S7). This HopK1 mutant is no longer

cleaved *in planta*; however, it retains the ability to localize to chloroplasts (Figure S7). Together, these data indicate that the processed form of HopK1 carries its virulence activity, and that cleavage of the HopK1 transit peptide is not required for its virulence activity.

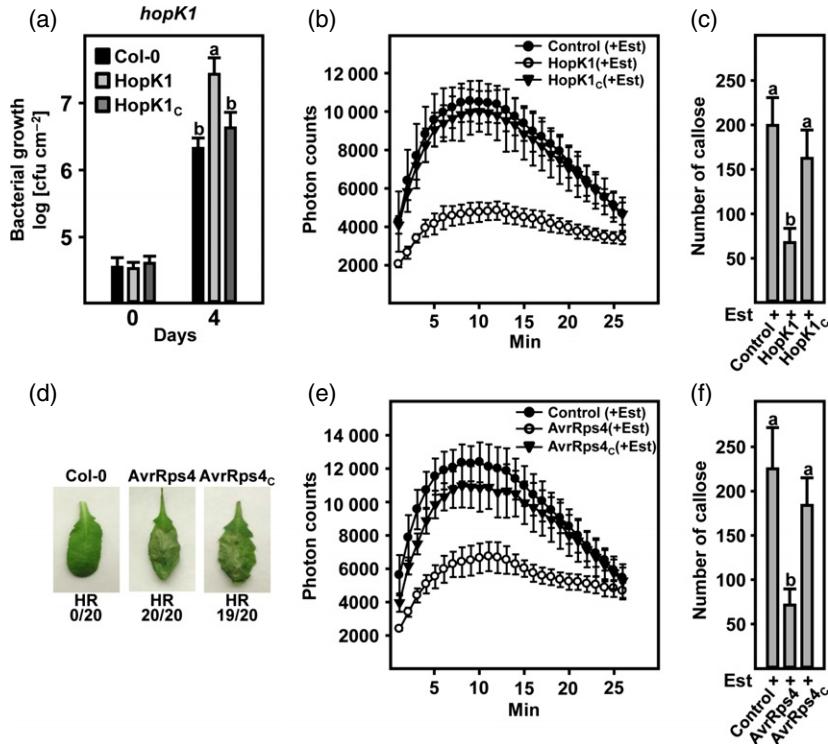
To directly determine whether the virulence activity of HopK1 resides inside chloroplasts, we made transgenic Arabidopsis plants expressing a HopK1 derivative (TP-K1<sub>C-HA</sub>) that substituted the HopK1 native transit peptide with the transit peptide from pea ribulose 1,5-bisphosphate carboxylase (Nawrath *et al.*, 1994; Zhong *et al.*, 2003). We confirmed that a protein of the approximate molecular mass was made and processed when these transgenic plants were treated with estradiol (Figure 7a). Plants expressing TP-K1<sub>C-HA</sub> restored *in planta* growth to the DC3000 *hopK1* mutant, and were reduced in their PTI responses, indicating that TP-K1<sub>C-HA</sub> retained the ability to suppress PTI (Figure 7). These data clearly indicate that the primary virulence activity of HopK1 occurs inside chloroplasts, and that the N-terminal portion does not carry detectable virulence activity.

#### The AvrRps4 transit peptide directs an oomycete effector to chloroplasts

To test whether the transit peptide of AvrRps4 could direct a heterologous effector to chloroplasts, we made transgenic Arabidopsis plants expressing the effector domain of the *Hyaloperonospora arabidopsidis* effector ATR13 (ATR1<sub>42-154</sub>) fused to the AvrRps4 transit peptide at its N-terminus and GFP at its C-terminus. Leaf tissue from Arabidopsis plants expressing this fusion protein was confirmed to produce this fusion protein when induced with estradiol (Figure 8a). Estradiol-induced leaf tissue was viewed with confocal microscopy, and the majority of the GFP signal was found in chloroplasts, indicating that the AvrRps4 transit peptide (AvrRps4<sub>1-136</sub>) directed ATR13<sub>42-154</sub> to chloroplasts (Figure 8b). These data provide additional evidence that the N-terminal portion of AvrRps4 can act as a chloroplast transit peptide.

#### DISCUSSION

*Pseudomonas syringae* type-III effectors generally contribute weakly to virulence, as illustrated by the weak reduction in virulence of most *P. syringae* mutants defective in single effector genes (Lindeberg *et al.*, 2012). In contrast, HopK1 makes a significant contribution to virulence, based on the reduced growth *in planta* and disease symptom production of DC3000 *hopK1* mutants (Figure 1a, b). HopK1 is probably not a widely distributed effector within *P. syringae*, because of the 35 *P. syringae* genomes that are available online, the *hopK1* gene is found only in DC3000. This may suggest that HopK1 is a more recently acquired effector, or that it may be specific for a narrow range of host plants. The virulence contribution of HopK1



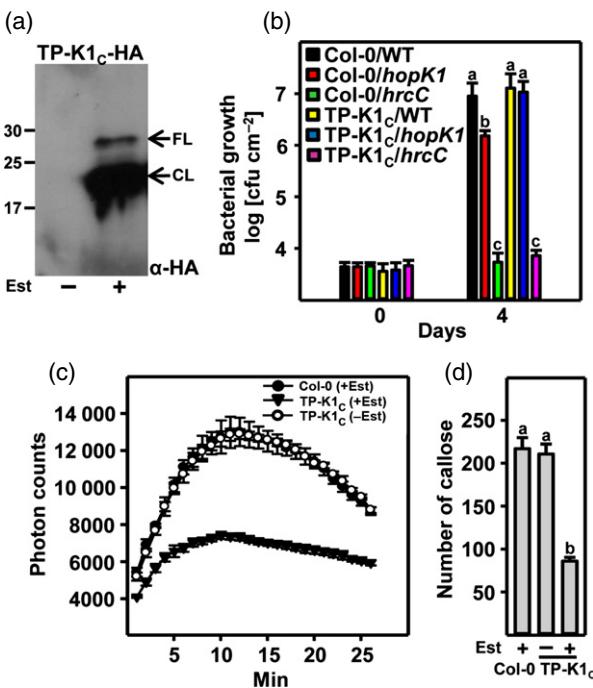
**Figure 6.** Chloroplast localization is required for the virulence role of HopK1 and AvrRps4, but is not required for AvrRps4 to be recognized by RPS4.

- (a) *In planta* bacterial growth assays of the *hopK1* mutant were performed in wild-type Arabidopsis, and in transgenic plants expressing the full-length HopK1-HA or expressing the processed HopK1 (HopK1<sub>c</sub>-HA).
- (b) Transgenic Arabidopsis plants expressing HopK1<sub>c</sub>-HA were treated with 1  $\mu$ M flg22 and reactive oxygen species (ROS) were measured.
- (c) Transgenic Arabidopsis expressing HopK1<sub>c</sub>-HA were treated with 1  $\mu$ M flg22 and callose deposition was quantified.
- (d) Estradiol-inducible transgenic wild-type Arabidopsis (Col-0) expressing AvrRps4-HA or AvrRps4<sub>c</sub>-HA were induced with 10  $\mu$ M estradiol, and evaluated for HR production. Representative leaves are shown 3 days post-induction. The ratio of the number of leaves exhibiting an HR for the total number of leaves infiltrated is shown below each leaf picture.
- (e) Estradiol-inducible transgenic Arabidopsis *rps4-2* mutant plants expressing AvrRps4-HA or AvrRps4<sub>c</sub>-HA were treated with 1  $\mu$ M flg22 and ROS were measured.
- (f) Estradiol-inducible transgenic Arabidopsis *rps4-2* mutant plants expressing AvrRps4-HA or AvrRps4<sub>c</sub>-HA were treated with 1  $\mu$ M flg22 and callose deposition was quantified.
- (a, c, f) Different letters indicate significant differences ( $P < 0.05$ ), as calculated with a Student's t-test.
- (b, c, e, f) Plants were treated with 10  $\mu$ M estradiol (+Est) or without estradiol (-Est) 16 h prior to flg22 treatment. Error bars represent standard errors ( $n = 24$ ).
- (e and f) Control plants were untransformed *rps4-2* mutant plants.

is likely to arise from its ability to suppress ETI (Jamir *et al.*, 2004; Guo *et al.*, 2009) and PTI (Figure 1c, d). Because of its significant virulence contribution, HopK1 is likely to target immunity components inside plant cells that are not targeted by the majority of the other *P. syringae* effectors.

The N-terminal sequence similarity of HopK1 to the type-III effectors AvrRps4 and the *X. campestris* XopO led us to investigate the function of this conserved N-terminal region. Hypothetically, this region could represent a second effector domain; however, the N-terminal region of HopK1 did not make a detectable contribution to virulence, nor did it possess the ability to suppress PTI (Figure S6). Additionally, the processed forms of both HopK1 and AvrRps4 are found only in chloroplasts, based on biochemical fractionation and *in vitro* import assays (Figures 4 and 5). When GFP is fused to this N-

terminal region, it is sufficient to direct GFP to the chloroplast (Figure 3c). Thus, the conserved N-terminal region acts as a chloroplast transit peptide. Moreover, the virulence activity of HopK1 requires that it be localized to the chloroplast, based on the pathogenicity phenotypes of DC3000 *hopK1* mutants (Figure 6a). The ability of HopK1 and AvrRps4 to suppress PTI-induced immune responses also requires that plants expressed full-length effector proteins: their processed forms did not have this activity (Figure 6). Perhaps most importantly, we were able to restore the virulence activity of HopK1 by providing a heterologous transit peptide (Figure 7), clearly illustrating that HopK1 needs to be localized to the chloroplast to contribute to virulence. However, we cannot exclude the possibility that the full-length forms of these effectors act in other subcellular compartments within the plant cell.



**Figure 7.** A heterologous chloroplast transit peptide restores the virulence activities to the processed HopK1 form.

(a) An immunoblot using anti-HA antibodies ( $\alpha$ -HA) of protein extracts from transgenic Arabidopsis plants expressing a HopK1 derivative that contain the transit peptide (TP) from the pea ribulose 1,5-bisphosphate carboxylase enzyme fused to the processed form of HopK1 (TP-K1c-HA), with and without induction by 10  $\mu$ M estradiol (Est). Molecular mass markers and the full-length (FL) and processed (CL) TP-K1c-HA are indicated.

(b) Wild-type Arabidopsis (Col-0) and transgenic Arabidopsis expressing TP-K1c-HA (after induction with 10  $\mu$ M estradiol) were spray-inoculated with wild-type DC3000 (WT), the DC3000 *hopK1* mutant and the DC3000 *hrcC* mutant defective in type-III secretion. The bacteria were enumerated at 0 and 4 days post-inoculation. Different letters indicate statistically significant differences ( $P < 0.05$ ).

(c) Wild-type Arabidopsis (Col-0) and estradiol-inducible TP-K1c-HA transgenic plants were treated with 1  $\mu$ M flg22 and reactive oxygen species (ROS) were measured.

(d) Wild-type and estradiol-inducible HopK1-HA transgenic plants were treated with 1  $\mu$ M flg22 and callose deposition was quantified.

(c and d) Error bars represent standard errors ( $n = 20$ ). Different letters indicate statistically significant differences ( $P < 0.05$ ). Plants were treated with 10  $\mu$ M estradiol (+Est) or without estradiol (-Est) 16 h prior to flg22 treatment.

(a-d) Each experiment was repeated at least three times with similar results.

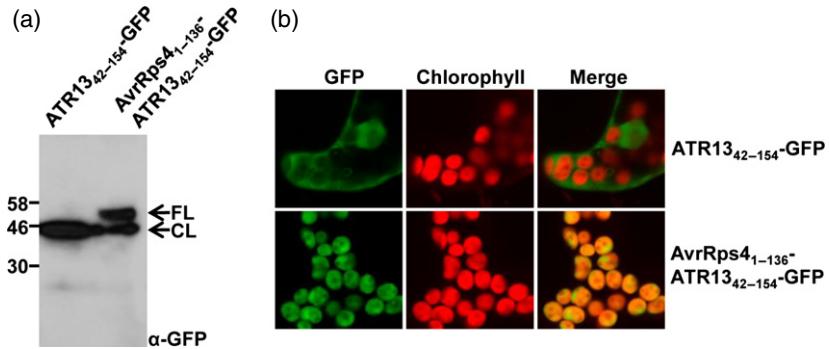
The transit peptide of HopK1 and AvrRps4 does not share any obvious homology with transit peptides of other proteins targeted to chloroplasts. Predicting the localization of *P. syringae* effectors has been confounded by the fact that the biochemical properties of *P. syringae* type-III secretion signals resemble those of chloroplast targeting signals (Guttmann *et al.*, 2002), even though these effectors do not localize to chloroplasts. However, PSORT and WOLF PSORT predict that the transit peptide regions of HopK1 and AvrRps4 (amino acids 53–160) are localized to chloroplasts (Nakai and Horton, 1999; Horton *et al.*, 2007).

Additionally, the experimentally defined transit peptides of HopK1 and AvrRps4 both have biochemical characteristics of chloroplast transit peptides, including high serine content and basic and hydrophobic amino acids. The KRVY motif reported to be required for the virulence activity of AvrRps4 (Sohn *et al.*, 2009) and conserved in HopK1 (Figure 2c) is within a subsequence of these transit peptides with these characteristics.

Two other *P. syringae* type-III effectors localize to the chloroplast: Hopl1 and HopN1 (Jelenska *et al.*, 2007, 2010; Rodriguez-Herva *et al.*, 2012). Both of these proteins do not use a cleavable transit peptide. Other than HopK1 and AvrRps4, we are unaware of other type-III effectors that use a cleavable organelle targeting sequence including animal and plant pathogen type-III effectors that target mitochondria (Kenny and Jepson, 2000; Papatheodorou *et al.*, 2006; Block *et al.*, 2010). Both HopK1 and AvrRps4 appear to localize to the chloroplast stroma, as their processed forms were found in the soluble portion of the chloroplast in *in vitro* import assay experiments (Figure 5). Consistent with this finding is that transgenic Arabidopsis and tobacco (*N. tabacum* cv. Xanthi) plants expressing HopK1-HA were reduced in their photosynthesis efficiency (Figure S8). Future research will be focused on identifying the targets of HopK1 and AvrRps4 inside chloroplasts.

Recently, it was reported by two independent research groups that AvrRps4 targets the immunity-associated EDS1 protein (Bhattacharjee *et al.*, 2011; Heidrich *et al.*, 2011). Heidrich *et al.* found that AvrRps4 targets EDS1 in the cytoplasm and the nucleus, and that AvrRps4-induced ETI requires nucleocytoplastic coordination (Heidrich *et al.*, 2011). Bhattacharjee *et al.* found that AvrRps4 and another *P. syringae* effector HopA1 targets EDS1 at the cytoplasmic membrane, and that their corresponding R proteins (RPS4 and RPS6, respectively) are part of an R protein complex with EDS1 (Bhattacharjee *et al.*, 2011). Our finding that AvrRps4 localizes to the chloroplast is not in conflict with AvrRps4 being recognized at the cytoplasmic membrane. Indeed, we found that the transgenic expression of the processed form of AvrRps4, which cannot be imported into chloroplasts, retained the ability to induce an HR, indicating that the recognition of AvrRps4 does not require that it be localized to the chloroplast (Figure 6d). However, the ability of AvrRps4 to suppress PTI-induced immune responses did require that it be localized to the chloroplast, which suggests that the virulence target (or targets) that are required for this suppression reside in chloroplasts.

An important molecular tool, known as the effector detector vector (EDV; Sohn *et al.*, 2007; Fabro *et al.*, 2011), produces protein fusions where the N-terminal region of AvrRps4 is fused to candidate plant pathogen effectors (including those from fungal and oomycete pathogens), allowing them to be delivered into plant cells by *P. syringae*. This tool allows the delivery of a test effector



**Figure 8.** The AvrRps4 transit peptide can direct an oomycete effector to chloroplasts.

(a) An immunoblot using anti-GFP antibodies of crude extracts from transgenic Arabidopsis plants expressing the effector domain of the *Hyaloperonospora arabidopsidis* effector ATR13 fused to GFP (ATR13<sub>42–154</sub>-GFP) or expressing the AvrRps4 transit peptide fused to ATR13 at its N-terminus and GFP fused to its C-terminus minus (AvrRps4<sub>1–136</sub>-ATR13<sub>42–154</sub>-GFP) after 16 h of treatment with 10 μM estradiol.

(b) Single optical sections of Arabidopsis leaf tissue expressing ATR13<sub>42–154</sub>-GFP or AvrRps4<sub>1–136</sub>-ATR13<sub>42–154</sub>-GFP after 24 h of induction with 10 μM estradiol.

inside plant cells via the *P. syringae* T3SS, and once inside the AvrRps4 moiety of the fusion is cleaved away, allowing the effector to function in its intended subcellular location. Our results indicating the processed form of AvrRps4 is localized to chloroplasts has important practical implications regarding the utility of the EDV, because it suggests that the effector of interest introduced into plant cells using the EDV system may be mis-localized to chloroplasts. Indeed, we show that the AvrRps4 transit peptide can direct a heterologous effector to chloroplasts in transgenic Arabidopsis expressing the effector domain of the *H. arabidopsidis* effector ATR13 fused to the AvrRps4 transit peptide at its N-terminus, and to GFP at its C-terminus (Figure 8). This fusion protein is identical to the ATR13 fusion protein used in the first description of the EDV (Sohn *et al.*, 2007).

It is important to note that it remains possible that some of the AvrRps4-effector fusion remains outside of the chloroplast, and that localization signals (if there are any) on a test effector may override the AvrRps4 transit peptide, allowing it to localize to the correct subcellular location. However, the processed form of AvrRps4 or any processed AvrRps4 effector fusion is likely to exist only in the chloroplast, because we only detect the processed forms of HopK1 and AvrRps4 inside chloroplasts, where the stromal processing peptidase that cleaves transit peptides has been shown to be exclusively localized and is quite specific (Li and Chiu, 2010). Thus, any effector screen using the EDV system should use additional controls to confirm that the effector of interest is not mis-localized to chloroplasts, as well as independent assays to confirm any phenotypes identified using it.

Which chloroplast targets of HopK1 and AvrRps4 can result in the suppression of plant immunity? The chloroplast-localized Hop1 appears to suppress immunity by reducing salicylic acid (SA) levels (Jelenska *et al.*, 2007, 2010). Transgenic Arabidopsis plants expressing

HopK1-HA or AvrRps4-HA did not suppress SA levels (Figure S9), and therefore appear to function differently than Hop1. It is important to note that HopK1 and AvrRps4 contain distinct effector domains, and these are predicted to have different targets within chloroplasts. Because these effectors suppress early and late immune responses, their targets may be components of retrograde signaling: signaling that transmit signals that originate in the organelles to regulate nuclear gene expression (Pogson *et al.*, 2008; Woodson and Chory, 2008). The signals that mediate retrograde signaling from the chloroplasts are likely to be metabolites; however, they and their production are not well understood (Woodson and Chory, 2012). Recently, a calcium sensor in the chloroplast was found to be required for PTI and ETI, suggesting retrograde signaling via calcium fluxes (Nomura *et al.*, 2012). It seems plausible that chloroplast-localized effectors such as HopK1 and AvrRps4 may suppress plant immunity by disrupting retrograde signaling. In any case, understanding how these effectors function to suppress plant immunity will shed important light on the role that the chloroplast plays in plant-microbe interactions.

## EXPERIMENTAL PROCEDURES

### Bacterial strains and media

The bacterial strains and plasmids used in this work are listed in Table S1. Additional methods are described in the Appendix S1.

### General DNA manipulation

A list of the primers is shown in Table S2. For cloning we used Gateway technology (Invitrogen, now Life Technologies, <http://www.lifetechnologies.com>).

### Construction of DC3000 *hopK1* mutants

An unmarked mutagenesis strategy was used to make DC3000 *hopK1* mutants (House *et al.*, 2004; Crabill *et al.*, 2010). For the complementation of the *hopK1* mutants, *hopK1* was recombined

into the Tn7 vector pLN2992 (Crabill *et al.*, 2012). pLN2992 is a Gateway destination derivative of pUC18T mini-Tn7. The resulting Tn7 constructs were co-electroporated into *hopK1* mutant strains. The gentamicin-resistant colonies were selected for Tn7 transposition events.

### Transgenic plant production

The transgenic Arabidopsis plants were transformed by the floral-dip method (Bechtold *et al.*, 1993). The transgenic Arabidopsis plants that constitutively expressed GFP fusions were made with T-DNA binary vector pK7FWG2 (Karimi *et al.*, 2002), carrying either *hopK1* or *avrRps4*. The HopK1-GFP and AvrRps4-GFP inducible Arabidopsis lines were made by PCR cloning the respective gene fusions from the constitutive T-DNA binary constructs into the Gateway destination vector pLN604. This is a pER8 (Zuo *et al.*, 2000) derivative containing an estradiol-inducible promoter and nucleotides that encode a C-terminal HA tag. The transgenic Arabidopsis plants that conditionally express effector-HA fusions were also made with pLN604. The DNA of interest was first cloned into a Gateway pENTR vector, and then recombined into pLN604 using LR Clonase. Transgenic tobacco plants expressing HopK1 derivatives were made using leaf-disc transformation (Horsch *et al.*, 1985). For AvrRps4-expressing transgenic Arabidopsis, Col-0 *rps4-2* mutant plants were used (Wirthmueller *et al.*, 2007), with the exception of the Arabidopsis plants shown in Figure 6d, which were wild-type Col-0. The full list of entry clones and destination constructs used to make transgenic plants are listed in Table S1.

### Pathogenicity assays

Pathogenicity assays were performed as described by Block *et al.* (2010). For the pathogenicity assays with estradiol-inducible transgenic Arabidopsis lines, plants were first sprayed with 10 µM estradiol in 0.01% (v/v) Silwet L77.

### Callose deposition assays

Callose deposition assays were performed as described by Jeong *et al.* (2011).

### ROS assays

HopK1-HA or AvrRps4-expressing transgenic lines were confirmed as making the protein of interest after 16 h of induction with 10 µM estradiol. Leaf discs of 4 mm in diameter were taken from expressed plants and kept in water in a 96-well plate overnight. After 16 h the water was removed. ROS assays were performed as previously described (Jeong *et al.*, 2011). Luminescence was measured with a Synergy 2 luminometer (BioTek Instruments, <http://www.bioteck.com>).

### Edman degradation sequencing

The processed form of HopK1-HA was purified from estradiol-inducible transgenic lines, which were induced with 10 µM estradiol for 16 h. Co-immunoprecipitation was performed with anti-HA Affinity Matrix (Roche Applied Science, <http://www.roche-applied-science.com>) according to the manufacturer's instructions. After SDS-PAGE the proteins were transferred to polyvinylidene difluoride (PVDF) membrane. The band corresponding to processed HopK1-HA was cut and N-terminal sequenced by the Edman degradation method. The sequencing was performed by the protein structure core facility at the University of Nebraska Medical Center.

### Confocal microscopy

The localization of GFP fusions was visualized by sequential laser scanning confocal microscopy, using a Nikon A1 microscope, with sequential imaging at 488 nm excitation and 505–525 nm emission, and at 633 nm excitation and 660 nm emission.

### Protein manipulation and immunoblots

Leaf disks of transgenic and wild-type plants were harvested with a 0.64-cm<sup>2</sup> cork borer, and were ground in liquid nitrogen. The ground tissues were resuspended with 100 µl of 1.5× SDS sample buffer, and centrifuged for 5 min at 20000 g. The decanted supernatant was subjected to SDS-PAGE and immunoblot analyses. Anti-HA antibodies (Roche), anti-RuBisCO antibody (Abcam, <http://www.abcam.com>) and anti-Histone H3 antibody (Abcam) were used to detect HA, RuBisCO, and histone proteins bound on PVDF membranes. Proteins on immunoblots were visualized using the CDP-Star chemiluminescence detection kit (Roche), followed by autoradiography.

### Biochemical fractionation

Estradiol-inducible Arabidopsis lines expressing HopK1-HA or AvrRps4-HA were induced with 10 µM estradiol for 16 h. The intact chloroplasts were isolated using a continuous Percoll gradient centrifugation, as previously described by Seigneurin-Berny *et al.* (2008). Nuclear fractionation was performed as previously described by Xia *et al.* (1997). Nuclear and chloroplast fractions were evaluated with immunoblot analyses using anti-HA antibodies.

### Bacterial effector injection assay

DC3000 or *hrcC* carrying plasmids that express HopK1-HA or AvrRps4-HA were infiltrated into Arabidopsis leaves at a cell density of  $1 \times 10^9$  cells ml<sup>-1</sup> ( $OD_{600} = 1.0$ ). After 7 h, leaf disks were harvested and subjected to immunoblot analyses. To determine the extent that these proteins localized to chloroplasts, chloroplasts were isolated as described above and subjected to immunoblot analyses using anti-HA antibodies.

### Chloroplast import assays

The DNA encoding HopK1 and AvrRps4 were PCR cloned into pDEST14 according to the manufacturer's instructions (Invitrogen). Intact chloroplasts were isolated from 8–12-day-old pea seedlings and purified over a Percoll gradient, as previously described (Bruce *et al.*, 1994). Intact pea chloroplasts were re-isolated and resuspended in import buffer (330 mM sorbitol, 50 mM Hepes/KOH, pH 8.0) at a concentration of 1 mg chlorophyll ml<sup>-1</sup>. HopK1 was radiolabeled using [<sup>35</sup>S]methionine and translated with TNT® Coupled Reticulocyte Lysate System (Promega, <http://www.promega.com>), whereas AvrRps4 was radiolabeled with [<sup>3</sup>H] Leucine and translated with the TNT® Coupled Wheat-germ Lysate System (Promega). Import assays were performed as described by Tranel *et al.* (1995).

### ACKNOWLEDGEMENTS

This research was supported by grants to JRA from the US Department of Agriculture (award no. 2007-35319-18336) and the Institutes of Health (award no. 1R01AI069146-01A2). The Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the US Department of Energy (award no. DE-FG02-91ER20021) funded the chloroplast import assays.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** A schematic map of the *hopK1* gene locus and confirmation of the *hopK1* mutant.

**Figure S2.** Immunoblots show that estradiol-inducible transgenic Arabidopsis plants express HopK1-HA, HopK1c-HA, AvrRps4-HA or AvrRps4c-HA.

**Figure S3.** The ability of HopK1 to suppress PTI was dependent on its chloroplast localization.

**Figure S4.** Analysis of transient and transgenic expression of effector-GFP fusions with immunoblots.

**Figure S5.** Processed HopK1 and AvrRps4 do not localize to chloroplasts.

**Figure S6.** HopK1<sub>1–137</sub> does not contribute to the virulence of *Pseudomonas syringae*.

**Figure S7.** A HopK1 derivative that is not processed retains its virulence activity and its ability to localize to chloroplasts.

**Figure S8.** HopK1 can inhibit photosynthesis, and the inhibition is dependent on its chloroplast localization.

**Figure S9.** HopK1 and AvrRps4 cannot inhibit salicylic acid biosynthesis.

**Table S1.** Strains and plasmids used in this study.

**Table S2.** Primers used in this study.

**Appendix S1.** Materials and methods.

## REFERENCES

- Ausubel, F.M. (2005) Are innate immune signaling pathways in plants and animals conserved? *Nat. Immunol.* **6**, 973–979.
- Bechtold, N., Ellis, J. and Pelletier, G. (1993) In planta Agrobacterium mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *CR Acad. Sci. Paris*, **316**, 1194–1199.
- Bhattacharjee, S., Halane, M.K., Kim, S.H. and Gassmann, W. (2011) Pathogen effectors target Arabidopsis EDS1 and alter its interactions with immune regulators. *Science*, **334**, 1405–1408.
- Block, A. and Alfano, J.R. (2011) Plant targets for *Pseudomonas syringae* type III effectors: virulence targets or guarded decoys? *Curr. Opin. Microbiol.* **14**, 39–46.
- Block, A., Guo, M., Li, G., Elowsky, C., Clemente, T.E. and Alfano, J.R. (2010) The *Pseudomonas syringae* type III effector HopG1 targets mitochondria, alters plant development and suppresses plant innate immunity. *Cell. Microbiol.* **12**, 318–330.
- Boller, T. and Felix, G. (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* **60**, 379–406.
- Bruce, B.D., Perry, S., Froehlich, J. and Keegstra, K. (1994) *In vitro* import of protein into chloroplasts. In *Plant Mol. Biol. Manual* (Gelvin, S.B. and Schilperoort, R.B., eds). Boston: Kluwer Academic Publishers, pp. 1–15.
- Buttner, D. (2012) Protein export according to schedule: architecture, assembly, and regulation of type III secretion systems from plant- and animal-pathogenic bacteria. *Microbiol. Mol. Biol. Rev.* **76**, 262–310.
- Crabill, E., Joe, A., Block, A., van Rooyen, J.M. and Alfano, J.R. (2010) Plant immunity directly or indirectly restricts the injection of type III effectors by the *Pseudomonas syringae* type III secretion system. *Plant Physiol.* **154**, 233–244.
- Crabill, E., Karpisek, A. and Alfano, J.R. (2012) The *Pseudomonas syringae* HrpJ protein controls the secretion of type III translocator proteins and has a virulence role inside plant cells. *Mol. Microbiol.* **85**, 225–238.
- Dodds, P.N. and Rathjen, J.P. (2010) Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* **11**, 539–548.
- Fabro, G., Steinbrenner, J., Coates, M. et al. (2011) Multiple candidate effectors from the oomycete pathogen *Hyaloperonospora arabidopsisidis* suppress host plant immunity. *PLoS Pathog.* **7**, e1002348.
- Feng, F. and Zhou, J.M. (2012) Plant-bacterial pathogen interactions mediated by type III effectors. *Curr. Opin. Plant Biol.* **15**, 469–476.
- Gimenez-Ibanez, S. and Rathjen, J.P. (2010) The case for the defense: plants versus *Pseudomonas syringae*. *Microbes Infect.* **12**, 428–437.
- Guo, M., Tian, F., Wamboldt, Y. and Alfano, J.R. (2009) The majority of the type III effector inventory of *Pseudomonas syringae* pv. *tomato* DC3000 can suppress plant immunity. *Mol. Plant Microbe Interact.* **22**, 1069–1080.
- Guttman, D.S., Vinatzer, B.A., Sarkar, S.F., Ranall, M.V., Kettler, G. and Greenberg, J.T. (2002) A functional screen for the type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. *Science*, **295**, 1722–1726.
- Hann, D.R., Gimenez-Ibanez, S. and Rathjen, J.P. (2010) Bacterial virulence effectors and their activities. *Curr. Opin. Plant Biol.* **13**, 388–393.
- Heidrich, K., Wirthmueller, L., Tasset, C., Pouzet, C., Deslandes, L. and Parker, J.E. (2011) Arabidopsis EDS1 connects pathogen effector recognition to cell compartment-specific immune responses. *Science*, **334**, 1401–1404.
- Hinsch, M. and Staskawicz, B. (1996) Identification of a new Arabidopsis disease resistance locus, Rps4, and cloning of the corresponding avirulence gene, *avrRps4*, from *Pseudomonas syringae* pv. *pisi*. *Mol. Plant Microbe Interact.* **9**, 55–61.
- Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985) A simple and general method for transferring genes into plants. *Science*, **227**, 1229–1231.
- Horton, P., Park, K.J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C.J. and Nakai, K. (2007) WoLF PSORT: protein localization predictor. *Nucleic Acids Res.* **35**, W585–W587.
- House, B.L., Mortimer, M.W. and Kahn, M.L. (2004) New Recombination Methods for *Sinorhizobium meliloti* Genetics. *Appl. Environ. Microbiol.* **70**, 2806–2815.
- Jamir, Y., Guo, M., Oh, H.-S., Petnicki-Ocwieja, T., Chen, S., Tang, X., Dickman, M.B., Collmer, A. and Alfano, J.R. (2004) Identification of *Pseudomonas syringae* type III effectors that suppress programmed cell death in plants and yeast. *Plant J.* **37**, 554–565.
- Jelinska, J., Yao, N., Vinatzer, B.A., Wright, C.M., Brodsky, J.L. and Greenberg, J.T. (2007) A J domain virulence effector of *Pseudomonas syringae* remodels host chloroplasts and suppresses defenses. *Curr. Biol.* **17**, 499–508.
- Jelinska, J., van Hal, J.A. and Greenberg, J.T. (2010) *Pseudomonas syringae* hijacks plant stress chaperone machinery for virulence. *Proc. Natl. Acad. Sci. USA*, **107**, 13177–13182.
- Jeong, B.R., Lin, Y., Joe, A. et al. (2011) Structure function analysis of an ADP-ribosyltransferase type III effector and its RNA-binding target in plant immunity. *J. Biol. Chem.* **286**, 43272–43281.
- Jones, J.D. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323–329.
- Karimi, M., Inze, D. and Depicker, A. (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sci.* **7**, 193–195.
- Kenny, B. and Jepson, M. (2000) Targeting of an enteropathogenic *Escherichia coli* (EPEC) effector protein to host mitochondria. *Cell. Microbiol.* **2**, 579–590.
- Li, H.M. and Chiu, C.C. (2010) Protein transport into chloroplasts. *Annu. Rev. Plant Biol.* **61**, 157–180.
- Lindeberg, M., Cunnac, S. and Collmer, A. (2012) *Pseudomonas syringae* type III effector repertoires: last words in endless arguments. *Trends Microbiol.* **20**, 199–208.
- Nakai, K. and Horton, P. (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem. Sci.* **24**, 34–36.
- Nawrath, C., Poirier, Y. and Somerville, C. (1994) Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. *Proc. Natl. Acad. Sci. USA*, **91**, 12760–12764.
- Nimchuk, Z., Marois, E., Kjemtrup, S., Leister, R.T., Katagiri, F. and Dangl, J.L. (2000) Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*. *Cell*, **101**, 353–363.
- Nomura, K., Mecey, C., Lee, Y.N., Imboden, L.A., Chang, J.H. and He, S.Y. (2011) Effector-triggered immunity blocks pathogen degradation of an immunity-associated vesicle traffic regulator in Arabidopsis. *Proc. Natl. Acad. Sci. USA*, **108**, 10774–10779.
- Nomura, H., Komori, T., Uemura, S. et al. (2012) Chloroplast-mediated activation of plant immune signalling in Arabidopsis. *Nat. Commun.* **3**, 926.

- Papatheodorou, P., Domanska, G., Oxle, M., Mathieu, J., Selchow, O., Kenny, B. and Rassow, J.** (2006) The enteropathogenic *Escherichia coli* (EPEC) Map effector is imported into the mitochondrial matrix by the TOM/Hsp70 system and alters organelle morphology. *Cell. Microbiol.* **8**, 677–689.
- Petnicki-Ocwieja, T., Schneider, D.J., Tam, V.C. et al.** (2002) Genomewide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl Acad. Sci. USA* **99**, 7652–7657.
- Pogson, B.J., Woo, N.S., Forster, B. and Small, I.D.** (2008) Plastid signalling to the nucleus and beyond. *Trends Plant Sci.* **13**, 602–609.
- Potnis, N., Krasileva, K., Chow, V. et al.** (2011) Comparative genomics reveals diversity among xanthomonads infecting tomato and pepper. *BMC Genomics*, **12**, 146.
- Rodríguez-Hervá, J.J., González-Melendi, P., Cuartas-Lanza, R. et al.** (2012) A bacterial cysteine protease effector protein interferes with photosynthesis to suppress plant innate immune responses. *Cell. Microbiol.* **14**, 669–681.
- Segonzac, C. and Zipfel, C.** (2011) Activation of plant pattern-recognition receptors by bacteria. *Curr. Opin. Microbiol.* **14**, 1–8.
- Seigneurin-Berny, D., Salvi, D., Dorne, A.J., Joyard, J. and Rolland, N.** (2008) Percoll-purified and photosynthetically active chloroplasts from *Arabidopsis thaliana* leaves. *Plant Physiol. Biochem.* **46**, 951–955.
- Shan, L., Thara, V.K., Martin, G.B., Zhou, J.M. and Tang, X.** (2000) The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. *Plant Cell*, **12**, 2323–2338.
- Sohn, K.H., Lei, R., Nemri, A. and Jones, J.D.** (2007) The downy mildew effector proteins ATR1 and ATR13 promote disease susceptibility in *Arabidopsis thaliana*. *Plant Cell*, **19**, 4077–4090.
- Sohn, K.H., Zhang, Y. and Jones, J.D.** (2009) The *Pseudomonas syringae* effector protein, AvrRPS4, requires *in planta* processing and the KRVY domain to function. *Plant J.* **57**, 1079–1091.
- Sohn, K.H., Hughes, R.K., Piquerez, S.J., Jones, J.D. and Banfield, M.J.** (2012) Distinct regions of the *Pseudomonas syringae* coiled-coil effector AvrRps4 are required for activation of immunity. *Proc. Natl Acad. Sci. USA* **109**, 16371–16376.
- Tranel, P.J., Froehlich, J., Goyal, A. and Keegstra, K.** (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. *EMBO J.* **14**, 2436–2446.
- Tsuda, K. and Katagiri, F.** (2010) Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* **13**, 459–465.
- Van der Biezen, E.A. and Jones, J.D.** (1998) Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem. Sci.* **23**, 454–456.
- Wirthmueller, L., Zhang, Y., Jones, J.D. and Parker, J.E.** (2007) Nuclear accumulation of the *Arabidopsis* immune receptor RPS4 is necessary for triggering EDS1-dependent defense. *Curr. Biol.* **17**, 2023–2029.
- Woodson, J.D. and Chory, J.** (2008) Coordination of gene expression between organellar and nuclear genomes. *Nat. Rev. Genet.* **9**, 383–395.
- Woodson, J.D. and Chory, J.** (2012) Organelle signaling: how stressed chloroplasts communicate with the nucleus. *Curr. Biol.* **22**, R690–R692.
- Xia, Y., Nikolau, B.J. and Schnable, P.S.** (1997) Developmental and hormonal regulation of the *Arabidopsis* CER2 gene that codes for a nuclear-localized protein required for the normal accumulation of cuticular waxes. *Plant Physiol.* **115**, 925–937.
- Zhong, H., Teymour, F., Chapman, B., Maqbool, S.B., Sabzikar, R., El-Maghriby, Y., Dale, B. and Sticklen, M.B.** (2003) The pea (*Pisum sativum* L.) rbcS transit peptide directs the *Alcaligenes eutrophus* polyhydroxybutyrate enzymes into the maize (*Zea mays* L.) chloroplasts. *Plant Sci.* **165**, 455–462.
- Zuo, J., Niu, Q. and Chua, N.** (2000) An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J.* **24**, 265–273.