

The *Pseudomonas syringae* Type III Effector HopAM1 Enhances Virulence on Water-Stressed Plants

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***Pseudomonas syringae* strains deliver diverse type III effector proteins into host cells, where they can act as virulence factors. Although the functions of the majority of type III effectors are unknown, several have been shown to interfere with plant basal defense mechanisms. Type III effectors also could contribute to bacterial virulence by enhancing nutrient uptake and pathogen adaptation to the environment of the host plant. We demonstrate that the type III effector HopAM1 (formerly known as AvrPpiB) enhances the virulence of a weak pathogen in plants that are grown under drought stress. This is the first report of a type III effector that aids pathogen adaptation to water availability in the host plant. Expression of HopAM1 makes transgenic Ws-0 *Arabidopsis* hypersensitive to abscisic acid (ABA) for stomatal closure and germination arrest. Conditional expression of HopAM1 in *Arabidopsis* also suppresses basal defenses. ABA responses overlap with defense responses and ABA has been shown to suppress defense against *P. syringae* pathogens. We propose that HopAM1 aids *P. syringae* virulence by manipulation of ABA responses that suppress defense responses. In addition, host ABA responses enhanced by type III delivery of HopAM1 protect developing bacterial colonies inside leaves from osmotic stress.**

Pseudomonas syringae relies on its type III secretion system (TTSS) to colonize plant hosts (Jakobek et al. 1993). Through the TTSS, each strain delivers a distinct collection of 15 to 30 type III effector proteins into host cells. These proteins collectively act as virulence factors promoting disease and pathogen growth (Chisholm et al. 2006; Grant et al. 2006; Jones and Dangl 2006; Mudgett 2005; Nomura et al. 2005). Although the function of the majority of *P. syringae* type III effectors is still poorly characterized, several have been shown to modify various

aspects of plant defense. For example, expression of the type III effector proteins AvrRpm1, AvrRpt2, AvrPto, or AvrPtoB in plants blocks deposition of callose-rich papillae in response to type III-defective bacteria (Hauck et al. 2003; Kim et al. 2005). Other potential functions of type III effectors include nutrient acquisition, enhanced dispersal (Badel et al. 2002), and adaptation to environmental stresses.

Plant hormones play a role in defense against pathogens. Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) all have well-defined roles in plant defense (Glazebrook 2005; Robert-Seilaniantz et al. 2007) and *P. syringae* type III effectors have been shown to modify responses to these hormones (Cohn and Martin 2005; DebRoy et al. 2004; He et al. 2004). Recently, infection with virulent *P. syringae* pv. *tomato* DC3000 has been shown to induce miRNA against auxin receptors inhibiting auxin responses important to defense (Navarro et al. 2006). Cytokinin, gibberellin brassinosteroid, and, most relevant to this work, abscisic acid (ABA) also have been implicated to affect host defense responses (Robert-Seilaniantz et al. 2007).

ABA is best known as the hormone responsible for seed dormancy and response to drought stress (Hirayama and Shinozaki 2007). However, it has long been associated with plant defense (Fujita et al. 2006; Mauch-Mani and Mauch 2005). In most cases, ABA is shown to suppress defense responses and ABA-deficient mutants are more resistant to pathogens (Anderson et al. 2004; Audenaert et al. 2002; Mohr and Cahill 2003). However, there are well-documented cases in which ABA has the opposite effect. For example, ABA promotes resistance to *Tobacco mosaic virus* (Whenham et al. 1986) and the necrotrophic pathogens *Pythium irregulare* and *Alternaria brassicola* (Adie et al. 2007).

The seemingly contradictory roles of ABA in defense suggest that it interferes with defense signaling indirectly, modifying targets that overlap in biotic and abiotic stress signaling (Mauch-Mani and Mauch 2005). Recently, de Torres-Zabala and associates (2007) showed that *Arabidopsis* ABA biosynthesis mutants and ABA-insensitive mutants were more resistant to infection by DC3000 and ABA hypersensitive mutants were more susceptible. They further demonstrated that application of ABA was sufficient to block basal defense responses such as deposition of callose-rich papillae in tissues infected with nonpathogenic type III secretion-defective *Pseudomonas syringae* pv. *tomato* mutant DC3000 *hrpA* (Roine et al. 1997). When they compared gene expression changes induced by challenge with virulent DC3000 or the DC3000 *hrpA* mutant, they found that DC3000 induced ABA responses but the non-pathogenic mutant did not. In fact, ABA measurements follow-

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ing infection by hand infiltration showed that DC3000 causes increased endogenous levels of ABA but *hrpA* mutants did not. Finally, they showed that in planta expression of one type III effector, AvrPtoB (renamed HopAB2) (Lindeberg et al. 2005) was sufficient to induce increased ABA levels when expressed from an inducible promoter in *Arabidopsis*. Thus, *P. syringae* manipulates ABA production and ABA responses in order to suppress defense responses.

If ABA synthesis and responses are manipulated by successful pathogens, one might expect to see that infection also can modify responses to drought stress. Drought stress leads to low water potential in leaves. Water potential in the host environment is important for *P. syringae* colony growth. DC3000 population size is reduced linearly as water potentials drop below optimal levels in infected leaves (Wright and Beattie 2004). Using a water-stress-responsive promoter from *Escherichia coli* (Axtell and Beattie 2002), Wright and Beattie (2004) demonstrated that DC3000 can sense the level of osmotic pressure in the host apoplast. In a successful infection, the water stress inside the host apoplast was measured to be similar to optimal conditions in growth medium. By contrast, a type III-defective mutant experienced lower, less-optimal water potentials as expected if the pathogen used its type III effectors to modify the water potential in the host.

In a search for type III effectors with a demonstrable virulence function, we found that expression of HopAM1 (formerly called AvrPpiB) (Cournoyer et al. 1995) enhanced growth of a weak pathogen. This effect was more pronounced if the plants were under slight drought stress, which led us to suspect that HopAM1 may affect ABA responses. *hopAM1*, found in a conserved pathogenicity island in diverse *P. syringae* isolates (Arnold et al. 2001), encodes a 31.3-kDa protein of unknown function (Cournoyer et al. 1995). It originally was identified as an avirulence gene in the pea pathogen *P. syringae* pv. *pisi* race 3. There are two identical *hopAM1* copies in DC3000, one in the chromosome and one on the endogenous A plasmid (Buell et al. 2003). Although expression of *hopAM1* on a plasmid can increase the virulence of the weak pathogen *Pma* M6CΔE (formerly called M6CKO) (Rohmer et al. 2003), deletion of the chromosomal copy of *hopAM1* from the highly virulent pathogen *P. syringae* pv. *tomato* DC3001 (a derivative of DC3000 with a 9.4-kb deletion of the A plasmid that removes *hopAM1-2*) (Landgraf et al. 2006) does not affect its virulence on either tomato or *Arabidopsis* (Boch et al. 2002). We demonstrate that expression of *hopAM1* enhances the growth of a weak *P. syringae* pathogen and, when expressed in transgenic *Arabidopsis*, enhances ABA responses and suppresses basal defense responses.

RESULTS

Because it often is difficult to measure loss of virulence after deletion of a type III effector from a virulent *P. syringae* strain (Collmer et al. 2002), we developed an assay to identify bacterial proteins that could enhance the growth of a weak *P. syringae* pathogen. Derived from a weakly virulent *Arabidopsis* pathogen, *Pma* M6 (LMG 5560) (Rohmer et al. 2003), *Pma* M6CΔE grows to 10-fold lower titers than the parental strain when hand inoculated into *Arabidopsis* leaves (Belkhadir et al. 2004). This derivative strain lost an endogenous plasmid that carried the type III effectors AvrRpm1 and HopX1 (formerly AvrPphE), and it also has a deletion in a second chromosomal copy of HopX1 (Rohmer et al. 2003). Addition of AvrRpm1 on a broad host range plasmid increases the virulence of *Pma* M6CΔE to parental levels (Rohmer et al. 2003).

We expressed C-terminally hemagglutinin (HA)-tagged versions of HopAM1, HopX1, and HopD1 (formerly AvrPphD) from the constitutive *nptII-lac* promoter on the broad host

range plasmid pBBR1-MCS2 (Kovach et al. 1995) in *Pma* M6CΔE. Only HopAM1 conferred a growth advantage on *Pma* M6CΔE after hand infiltration into *Arabidopsis* ecotype Ws-0 (Supplemental Figure 1). Expression of *hopAM-1* from its native promoter (*hopAMI(N)*) increased growth of *Pma* M6CΔE (*hopAMI(N)*) to levels 5- to 10-fold higher than *Pma* M6CΔE (vector) and *hopAM1* expression from the constitutive *nptII-lac* promoter (*hopAMI(C)*) led to slightly higher increases in growth (Fig. 1A). Because the stronger enhancement of bacterial growth was easier to measure, the *nptII-lac* promoter construct (*hopAMI(C)*) was used for further experiments. We confirmed that the *Pma* M6CΔE (*hopAMI(C)*) strains expressed HopAM1-HA by Western blots using anti-HA antibody. As expected, the constitutive promoter drove strong expression of HopAM1-HA in noninducing rich media, whereas the native promoter construct expressed HopAM1-HA only when grown in type III secretion-inducing minimal media. We confirmed that our constructs retained the ability to activate R3-dependent disease resistance response in pea, following mobilization of the plasmids into *P. syringae* pv. *pisi* race 2 strain (which lacks *hopAM1*) (Cournoyer et al. 1995). Bacteria expressing *hopAM1* from either promoter induced a specific hypersensitive response typical of resistance protein activation, when infiltrated into pea pods of cv. Belinda (R3).

HopAM1 increases the virulence of *P. syringae* on *Arabidopsis* grown under water-stressed conditions.

We initially noted that the HopAM1-mediated increase in bacterial growth was greater in plants that were grown in relatively dry soil. We conducted parallel experiments with plants grown consistently under wet (40% water content in soil by weight) or dry (22% water content in soil) conditions, as described below. The fresh weight of plants grown under wet and dry conditions was not significantly different (data not shown). Nevertheless, *Pma* M6CΔE (vector) did not grow as well in dry plants as in wet plants (Fig. 1B). In contrast, *Pma* M6CΔE(*hopAMI(C)*) inoculated into dry plants grew to levels comparable with *Pma* M6CΔE(vector) in wet plants (Fig. 1B). The HopAM1-mediated growth advantage could be seen consistently in the Ws-0 ecotype but not in other ecotypes tested (Supplemental Figure 2).

ABA response contributes to the virulence effect of HopAM1.

Because growth of bacteria expressing *hopAM1* was enhanced in water-stressed plants, we suspected that changes in ABA responses could be responsible for the HopAM1-mediated enhanced pathogen growth. We postulated that ABA nonresponsive mutants would not support increased growth of *Pma* M6CΔE(*hopAMI(C)*) in dry plants compared with *Pma* M6CΔE(vector). The only appropriate mutant available in the Ws-0 ecotype was *abi5-1* (Finkelstein 1994; Lopez-Molina and Chua 2000). ABI5 is a central regulator of ABA signaling in post-germinative growth. *abi5-1* mutants do not have obvious vegetative defects; however, *abi5* is expressed in vegetative tissues (Brocard et al. 2002) and overexpression of *abi5* in *Arabidopsis* causes rosette leaves to retain water better than wild-type plants (Lopez-Molina et al. 2001). *Pma* M6CΔE (*hopAM1-HA(C)*) grew better than the vector control in both Ws-0 and *abi5-1* plants (Fig. 1C). Importantly, the growth increase was higher in Ws-0 plants than in *abi5-1* plants in four out of four experiments (Fig. 1C). We used both a random and a fixed-effect analysis of variance to determine whether the difference between growth of M6CΔE(*hopAMI(C)*) and M6CΔE(vector) was significantly greater in the Ws-0 genotype than in the *abi5-1* genotype. The interaction was found to be significant using both tests with a *P* value of 0.014. We

conclude that ABI5 function is required for the full virulence effect of HopAM1 in relatively dry plants.

HopAM1 induces chlorosis in newly emerging *Arabidopsis* leaves.

When plants were infiltrated with *Pma* M6CΔE(*hopAM1(C)*) at densities commonly used for growth curve assays (below), the infiltrated leaves developed typical chlorotic disease symptoms 3 days after infiltration. They also developed a systemic chlorosis phenotype (i.e., newly emerging leaves became chlorotic 5 to 6 days after bacterial inoculation). The chlorotic new leaves did not contain any bacteria detectable by plating plant extract on selective media. This systemic chlorosis phenotype was ecotype dependent. Of nine ecotypes tested, Ws-0, Col-0, and Aa-0 could support strong systemic chlorosis in nearly all plants infiltrated (Fig. 2A) The ecotypes La-er and Bch-1 exhibited weak systemic chlorosis in some infiltrated plants and Ncl-0, No-0, Rld, and Mt-0 did not exhibit systemic chlorosis. Additionally, *abi5-1* mutant plants tested supported chlorosis after infiltration (data not shown). Thus, the systemic chlorosis phenotype does not depend on ABA signaling through ABI5.

HopAM1 enhances ABA sensitivity in *Arabidopsis*.

In order to further investigate the effect of HopAM1 on ABA and other drought-related abiotic stress pathways in *Arabidopsis*, we made two independent transgenic lines expressing *hopAM1-HA* from a dexamethasone-inducible promoter (below) in the Ws-0 ecotype. Dexamethasone-inducible *hopAM1-HA* expression could be detected in the transgenic lines by reverse-transcriptase polymerase chain reaction (RT-PCR) (Supplementary Figure 3). Accumulation of *hopAM1-HA* transcript was stronger and more rapid in the transgenic line, HopAM1-Tg35, than in the second line, HopAM1-Tg43.

Expression of *hopAM1* in the transgenic plants complemented the virulence function of HopAM1 *in trans*. *Pma* M6CΔE(vector) grew to 5 to 10-fold higher titers in the transgenic plants of both lines after they were treated with dexamethasone. Induction of *hopAM* expression in both of the transgenic lines also triggered a chlorotic phenotype in new leaves that emerged after dexamethasone spray. (Fig. 2B). The plants also were arrested in growth for several days after dexamethasone application; however, they eventually recovered to produce green leaves at normal rates. Systemic chlorosis was supported in plants that were homozygous for both the *hopAM1* transgene and the *abi5-1* allele after crossing HopAM1-Tg35 plants to *abi5-1* mutants (data not shown) as expected if ABI5 function is not necessary for HopAM1-mediated systemic chlorosis.

The type III effector HopAB2 in *Arabidopsis* leads to increased ABA synthesis detectable at 18 h after induction of expression from a dexamethasone-responsive promoter (de Torres-Zabala et al. 2007). In order to determine whether HopAM1 also could induce endogenous ABA production, we measured ABA levels in transgenic plants 18 h after induction of *hopAM1* expression by dexamethasone spray. Both transgenic lines were tested at 3 and 5 weeks after sowing.

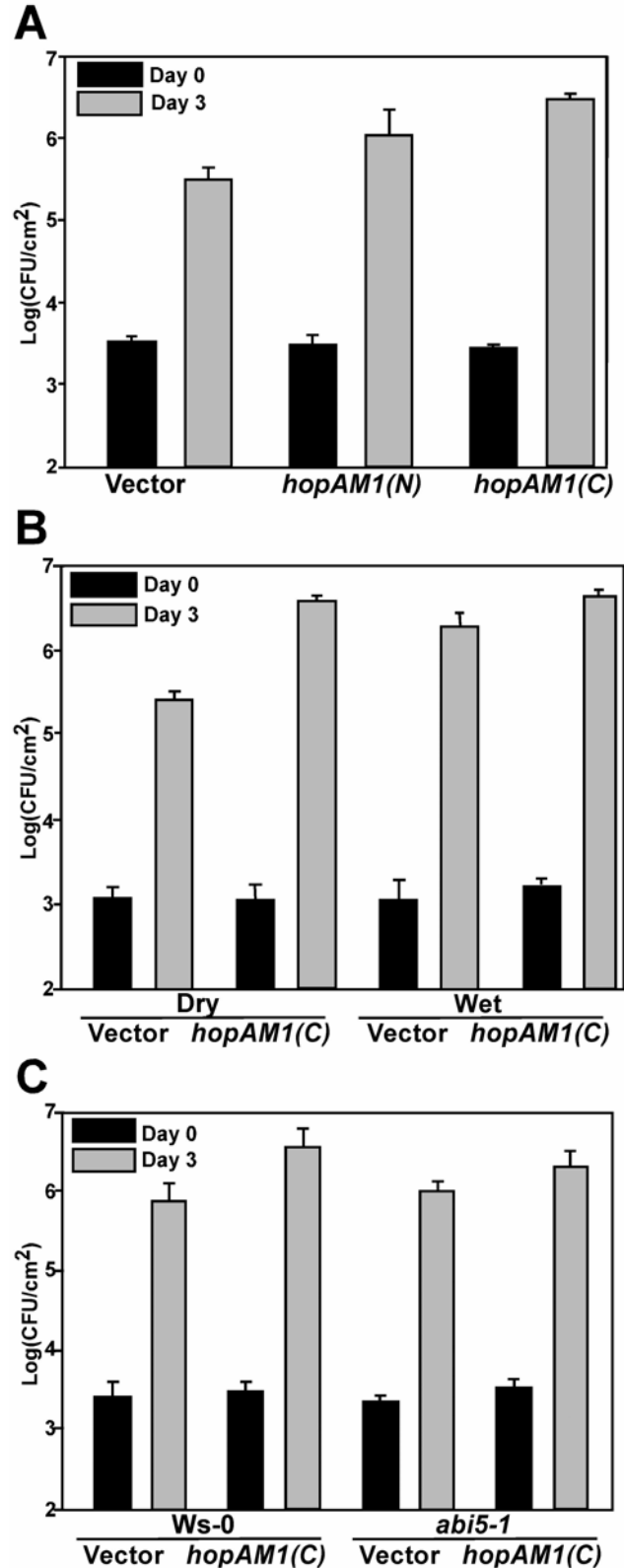


Fig. 1. HopAM1 increases virulence of a weak pathogen on *Arabidopsis*. **A**, Growth of *Pma* M6CΔE carrying the indicated type III constructs in plants grown in dry soil. The number of bacteria per area of leaf sampled is plotted on a log₁₀ scale. Error bars represent the standard deviation among three samples. The experiment is representative of two independent replicates. A Student's *t* test was applied to the difference in growth of the strain expressing *hopAM1* compared with growth of the vector-carrying strain. The *P* values were <0.06 for *hopAM1(N)* and *hopAM1(C)* in both experiments. **B**, Comparison of growth of *Pma* M6CΔE carrying the indicated plasmids on *Arabidopsis* Ws-0 plants grown under water-stressed (dry) and normal (wet) conditions. Error bars represent the standard deviation among three samples. The experiment is representative of three independent replicates. Student's *t* test *P* values were <0.04 for *hopAM1(C)* under dry conditions and <0.44 under wet conditions. **C**, Comparison of growth of *Pma* M6CΔE (vector) to *Pma* M6 E(*hopAM1(C)*) on *Arabidopsis* Ws-0 plants and isogenic *abi5-1* mutants grown in dry soil. Error bars represent the standard deviation among three samples.

ABA levels did not increase above the level in age-matched wild-type plants or mock-sprayed transgenic plants in any case (data not shown). Thus, it is unlikely that HopAM1 alters ABA biosynthesis.

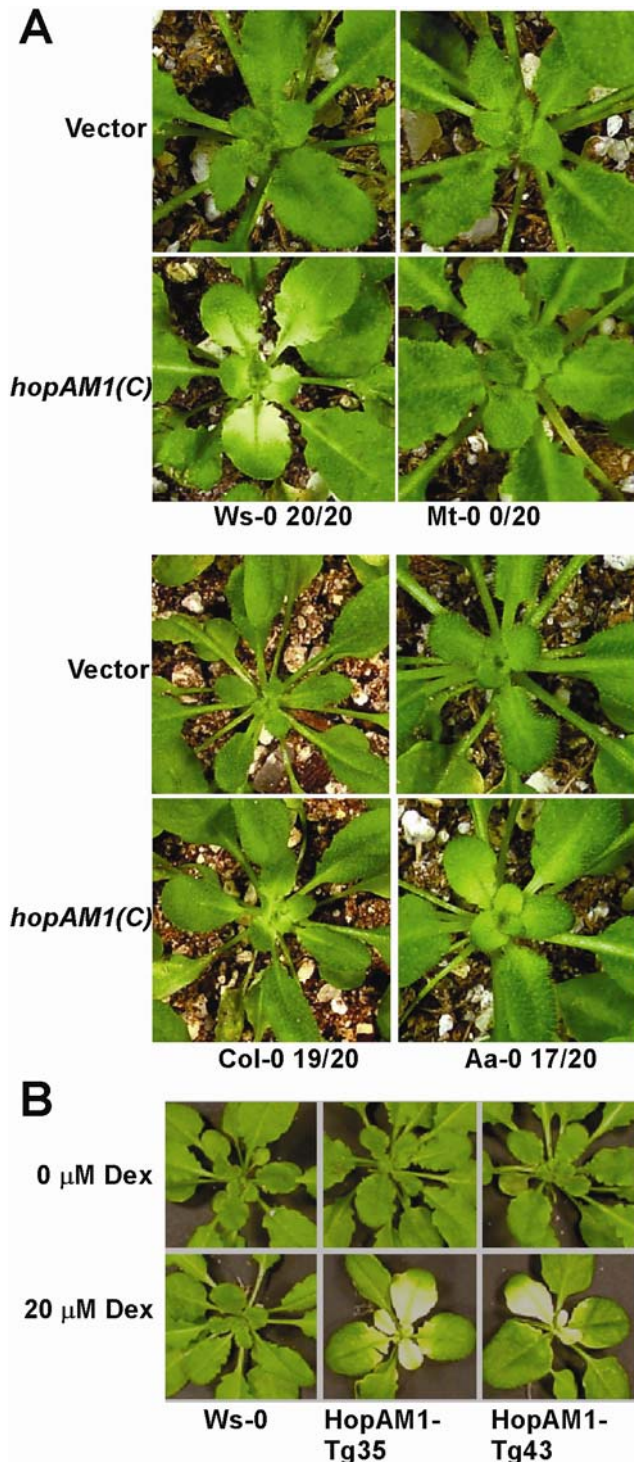


Fig. 2. HopAM1 induces chlorosis in newly emerging rosette leaves. **A**, Six-week-old plants of *Arabidopsis* of the indicated ecotypes were infiltrated with suspensions of *Pma* M6CAE with or without *hopAM1* at 5×10^5 CFU/ml. Six to eight leaves were inoculated per plant. Plants were photographed 5 to 7 days after infiltration. The number of plants exhibiting chlorosis out of 20 infiltrated is next to the ecotype name. **B**, Transgenic expression of *hopAM1* causes a similar chlorosis phenotype. Plants were sprayed with 20 μM dexamethasone and photographed 10 days later.

HopAM1 enhances ABA-mediated stomata closure.

ABA signaling induces stomata to close in response to drought stress (Schroeder et al. 2001). We detected HopAM1-dependent differences in the rate of stomatal closure in intact leaves of 3-week-old plants. Leaves of both *hopAM1* transgenic lines were sprayed with 20 μM dexamethasone and treated with ABA or water as described below. Thirty minutes after addition of either 50 or 100 μM ABA to intact leaves, the stomatal pores of the transgenic lines had a smaller width-to-length ratio on average than the stomatal pores of wild-type leaves (Fig. 3). At later time points (1 and 2 h after ABA addition), we found no difference in the average stomatal pore size between wild-type and transgenic lines. HopAM1 affects the kinetics of stomatal closure rather than the final level of closure (data not shown).

Expression of *hopAM1* enhances ABA-mediated inhibition of seed germination.

In addition to drought responses, ABA mediates germination inhibition in seed (Finkelstein et al. 2002). In order to determine whether expression of *hopAM1* could enhance the sensitivity of transgenic *Arabidopsis* to a second ABA-mediated effect, we measured ABA-mediated inhibition of germination of seed from *hopAM1* transgenic lines. We collected age-matched seed of the Ws-0 parental line and both *hopAM1-HA* transgenic lines and germinated them on Murashige and Skoog (MS) media, with and without dexamethasone, in the presence of increasing concentrations of ABA. Germination inhibition was monitored as arrest of cotyledon opening and root radical extension. When expressing *hopAM1*, both transgenic lines were more severely inhibited in germination by ABA than the Ws-0 parent (Fig. 4A and B). Germination inhibition was similar in all three genotypes grown on ABA without dexamethasone (Fig. 4B). *abi5-1* mutants are resistant to ABA-mediated inhibition of germination (Finkelstein 1994). If the enhanced germination arrest seen in *hopAM1* transgenics was caused by enhanced ABA signaling, we expected that the *abi5-1* mutation would restore germination of *hopAM1* transgenics grown on ABA. We crossed *abi5-1* mutants with HopAM1-Tg35 transgenic plants and isolated F2 progeny homozygous for the transgene and either the *abi5-1* mutant allele or wild-

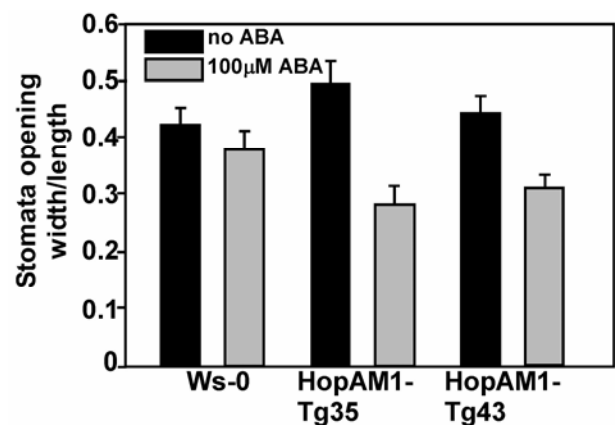


Fig. 3. HopAM1 enhances abscisic acid (ABA)-mediated stomata closure. Plants were treated with dexamethasone to induce *hopAM1* expression followed 18 h later by application of ABA as described in Methods. The ratio of length to width of the internal pore of 20 to 30 stomates from 6 to 10 leaves was measured. Error bars represent $2 \times$ standard error (approximately 95% confidence limits) among three samples. The experiment is representative of four independent replicates. A Student's *t* test applied to the difference between stomatal closure of Ws-0 and HopAM1-Tg35 was $P < 0.01$ in all four experiments. The *P* values were more variable for HopAM1-Tg43: $P < 0.01$, in two of four experiments.

type *ABI5*. Seed isolated from transgenic plants homozygous for both the transgene and *abi5-1* were insensitive to ABA-mediated germination inhibition (Fig. 4C). We conclude that the enhanced ABA-mediated germination inhibition seen in

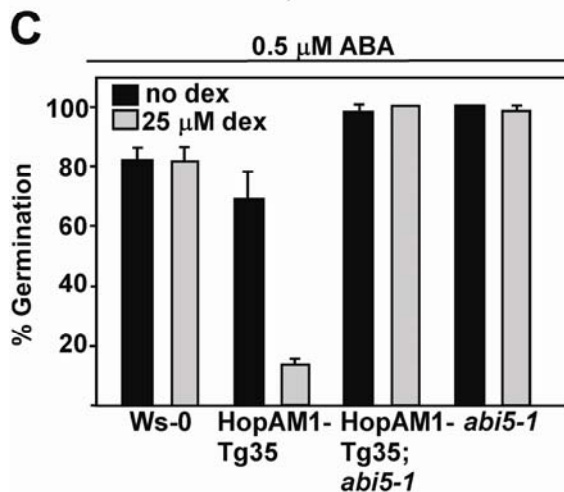
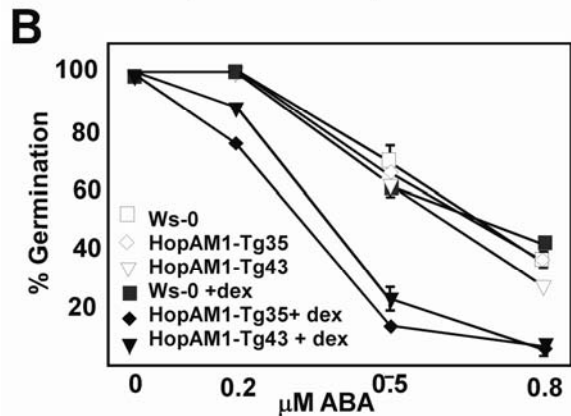
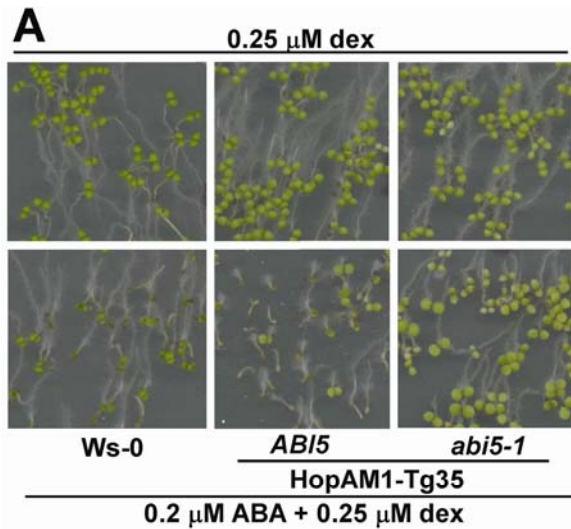


Fig. 4. HopAM1 enhances abscisic acid (ABA)-mediated inhibition of seed germination. **A**, Representative seedlings grown on 0.25 μ M dexamethasone with or without 0.2 μ M ABA. **B**, Seed of the indicated genotype germinated on medium with increasing concentrations of ABA with or without dexamethasone. Error bars represent 2 \times standard error among three samples. The experiment is representative of five independent replicates. **C**, Germination of seed of the indicated genotypes on medium with 0.5 μ M ABA with or without dexamethasone. Error bars represent standard deviation among three samples of 40 to 60 seeds each. The experiment is representative of four independent replicates. The Student's *t* test *P* values were <0.00 in all experiments.

plants expressing *hopAM1* depends on ABA signaling through *ABI5*.

Elevated ABA levels lead to accumulation of ABI5 protein (Lopez-Molina et al. 2001). We tested the possibility that HopAM1 affects the expression or stability of ABI5 protein by comparing ABI5 protein levels in transgenic plants grown in liquid culture with or without dexamethasone in either the presence of ABA or control media. As expected, ABI5 protein accumulated after ABA treatment. However, there was no difference in ABI5 protein levels when *hopAM1* was expressed compared with transgenic plants not treated with dexamethasone or wild-type plants grown under the same conditions (data not shown). Hence, HopAM1 does not alter ABA responses through changes in the level of ABI5 protein. In sum, our observations suggest that HopAM1 affects ABA responses that depend on ABI5.

HopAM1 also enhances sensitivity of transgenic plants to NaCl.

ABA and NaCl are known to elicit overlapping pathways in plants (Zhu 2002). Therefore, we tested the effect of *hopAM1* expression on salt sensitivity by growing wild-type and transgenic seedlings in the presence of different concentrations of NaCl. Seedling growth was quantified by measuring fresh weight after 10 days of growth. The transgenic lines exhibited

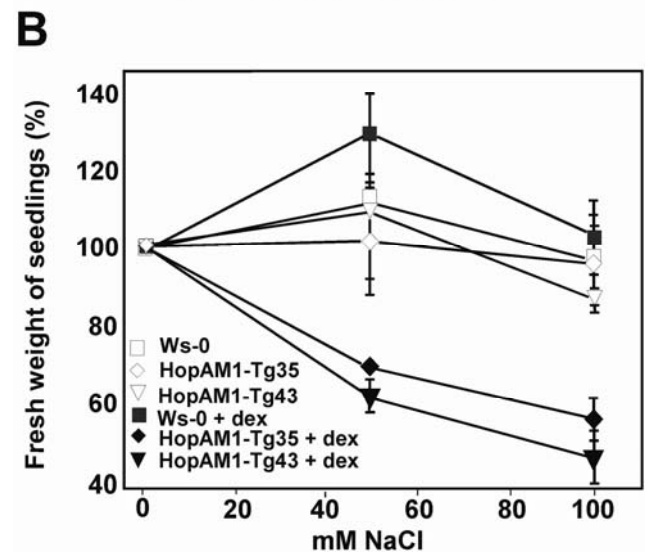
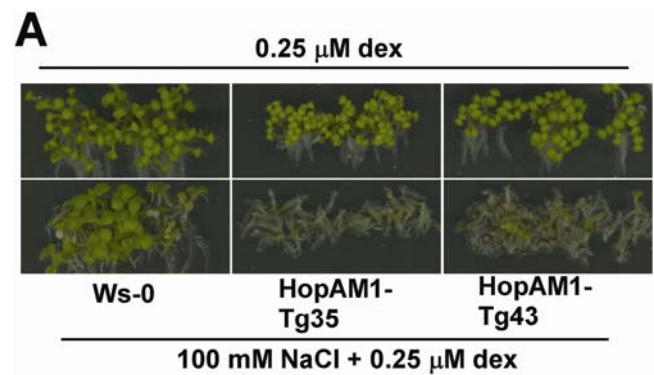


Fig. 5. HopAM1 enhances sodium chloride-mediated growth inhibition of *Arabidopsis* seedlings. **A**, Representative samples of seedlings grown on 0 or 100 mM NaCl with 0.25 μ M dexamethasone. **B**, Seed were plated on MS agar plates with 0, 50, and 100 mM NaCl with or without 0.25 μ M dexamethasone. Fresh weight of 20 seedlings was measured from each sample in triplicate and presented as percent of the control sample (0 mM NaCl). Error bars represent 2 \times standard error among three samples. The experiment is representative of two independent replicates.

stronger hypersensitivity to NaCl than wild-type only when grown on dexamethasone (Fig. 5) *hopAM1* transgenic lines were not hypersensitive to growth inhibition by high concentrations of sucrose (1, 2, and 4%) or mannitol (100, 200, and

400 mM) (data not shown), suggesting that HopAM1 affects a pathway shared by ABA and salt signaling.

HopAM1 suppresses basal defense in *Arabidopsis*.

Several type III effector proteins have been shown to suppress basal defense in *Arabidopsis*. The *P. syringae* type III secretion mutant DC3000 *hrcC* (formerly *hrpH*) (Yuan and He 1996) is virulence deficient and induces deposition of autofluorescent callose-rich papillae when infiltrated at high doses in plant leaves (DebRoy et al. 2004; Hauck et al. 2003; Kim et al. 2005). We found that DC3000 *hrcC* bacteria grow approximately $1.0 \log_{10}$ CFU/cm² more on the *hopAM1* transgenic lines compared with wild-type Ws-0 plants (Fig. 6A). Moreover, expression of *hopAM1* in HopAM1-Tg35 and HopAM1-Tg43 suppresses papilla induction by DC3000 *hrcC* (Fig. 6B and C).

DISCUSSION

HopAM1 suppresses defense responses and enhances ABA responses.

Type III effectors are delivered into host cells upon bacterial infection, where they reduce plant basal defenses against pathogens (Chisholm et al. 2006; Grant et al. 2006; Jones and Dangl 2006). We have shown that HopAM1 enhances the virulence of the weak pathogen *Pma* M6CΔE. Furthermore, expression of *hopAM1* in planta reduces basal defenses, allowing both *Pma* M6CΔE and DC3000 *hrcC* to grow to 10-fold higher levels in *hopAM1* transgenics than in wild-type plants. Like many other type III effectors, including *avrRpm1*, *avrRpt2*, *hopM1*, *avrE*, and *avrPto* (DebRoy et al. 2004; Hauck et al. 2003; Kim et al. 2005), expression of *hopAM1-HA* in transgenic *Arabidopsis* inhibits the deposition of fluorescent papillae in leaves infected with type III-defective *P. syringae*. However *P. syringae* does not strictly require the *hopAM1* gene to be virulent. It is found only sporadically in *P. syringae* strains (Hwang et al. 2004), and DC3000 mutants lacking *hopAM1* do not lose virulence (Boch et al. 2002). We predict that HopAM1 protein contributes to defense in combination with other type III effectors and its function may be advantageous only under particular environmental circumstances, such as during infection of water-stressed plants.

Expression of *hopAM1* in *Pma* M6CΔE bacteria was responsible for a more obvious growth enhancement in slightly water-stressed plants of the Ws-0 ecotype than in properly watered Ws-0 plants (Fig. 1B). In addition, conditional expression of *hopAM1* in transgenic plants enhanced two different ABA-mediated responses: stomatal closure and ABA-dependent germination inhibition of seed (Figs. 3 and 4). Therefore, we conclude that HopAM1 is sufficient to enhance particular ABA responses in infected plants.

P. syringae pathogens modify ABA responses at multiple stages of infection.

Type III-defective DC3000 or individual pathogen- or molecular-associated molecular patterns such as flag22 induce stomatal closure within 1 h of application to *Arabidopsis* leaves (Melotto et al. 2006). This ABA- and SA-dependent stomatal closure appears to be part of the basal defense response that blocks access of *P. syringae* to the leaf apoplast via open stomata. The virulent DC3000 strain counters this defense mechanism by producing the coronatine phytotoxin. Coronatine stimulates reopening of stomata (Melotto et al. 2006), facilitating entry of *P. syringae* into the host apoplast. Once inside the apoplast, the local osmotic pressure can affect the establishment and viability of *P. syringae* colonies (Wright and Beattie 2004). Wright and Beattie demonstrated that DC3000 requires a functional TTSS to optimize osmotic pressures in the leaf

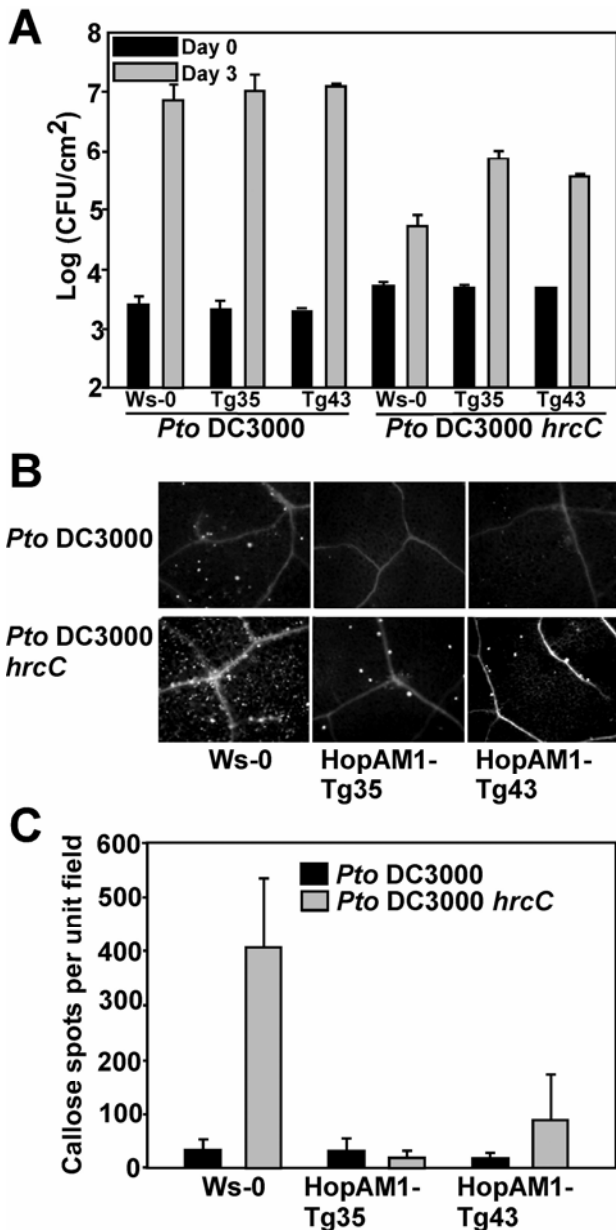


Fig. 6. HopAM1 suppresses basal defense in *Arabidopsis*. **A**, Conditional expression of *hopAM1* increases the growth of *P. syringae* pv. *tomato* (*Pto*) DC3000 *hrcC* on *Arabidopsis*. Plants were sprayed with 20 μ M dexamethasone and infiltrated 12 h later with suspensions of either *Pto* DC3000 or *Pto* DC3000 *hrcC* at 10^5 CFU/ml. Error bars represent standard deviation among three samples. The experiment is representative of four independent replicates. The Student's *t* test *P* values were <0.04 in all experiments. **B**, *hopAM1* expression suppresses *Pto* DC3000 *hrcC*-induced autofluorescent papilla deposition in *Arabidopsis*. Wild-type Ws-0 and transgenic plants were sprayed with 20 μ M dexamethasone and infiltrated 12 h later with suspensions of either *Pto* DC3000 or *Pto* DC3000 *hrcC* at 5×10^5 CFU/ml. Leaves were cleared and stained with aniline blue and analyzed by fluorescence microscopy 12 h after infiltration as explained in Materials and Methods. **C**, Quantification of autofluorescent spots analyzed per unit field of view (one field at $\times 200$ magnification). Error bars represent $2 \times$ standard error between five fields counted in one experiment. The experiment is representative of three independent replicates. Student's *t* test *P* values for the difference between Ws-0 and transgenic plants was <0.01 in all experiments.

apoplast. We propose that, once bacteria reach the apoplast, HopAM1 that is translocated into host cells could enhance ABA-mediated host responses in order to adjust the internal osmotic pressure to levels optimal for pathogen growth.

ABA suppresses defense responses.

Previous studies have shown that ABA is sufficient to suppress defense responses such as callose accumulation in plants infected with type III-deficient *P. syringae* (de Torres-Zabala et al. 2007) and that genes altered by infection with various pathogens overlap with genes regulated by ABA and drought stress (Adie et al. 2007; de Torres-Zabala et al. 2007). ABA could interfere with defense signaling pathways at several common points in their respective signal transduction pathways. First, the initial responses to ABA in stomatal closure include increases in reactive oxygen species and nitric oxide, both of which are important signaling molecules in defense (Desikan et al. 2004; Garcia-Mata and Lamattina 2003). Second, both pathways require signaling through Ca²⁺ fluxes, calcium-dependent kinases, and mitogen-activated protein kinases (Fujita et al. 2006). ABA-regulated transcription factors affect transcription of genes responsive to the hormones JA, ET (Anderson et al. 2004; Mengiste et al. 2003), gibberellin (Achard et al. 2006), and SA (Delessert et al. 2005). All four of these hormones have been associated with responses to pathogens (Glazebrook 2005; Robert-Seilantantz et al. 2007). Because integrated hormone signaling is involved in basal defense, type III effectors such as HopAM1 could alter hormone signaling pathways by interaction with a number of target proteins in order to suppress basal defenses.

HopAM1 is not sufficient to induce ABA production when expressed in transgenic plants but it does lead to enhanced ABA responses, as expected if HopAM1 modifies ABA signaling downstream of ABA perception. HopAM1 can enhance ABA-mediated germination inhibition in wild-type but not in *abi5-1* mutant plants (Fig. 4C) as expected if HopAM1 modifies ABI5-dependent ABA signaling. This is supported by the observation that *Pma M6CΔE(hopAMI(C))* do not show the full growth enhancement seen in wild-type Ws-0 plants when grown in *abi5-1* mutant plants (Fig. 1C).

ABI5 is one of several ABA-responsive transcription factors that each control diverse but overlapping ABA responses (Finkelstein and Lynch 2000). *ABI5* expression is induced in *Arabidopsis* seed 48 to 60 h after stratification (Lopez-Molina et al. 2001, 2002). Application of ABA to germinating seed leads to accumulation of ABI5 protein because of increased transcription and reduced proteasome-mediated degradation (Lopez-Molina et al. 2001, 2002; Stone et al. 2006). ABI5 is a member of an *Arabidopsis* gene family of 14 related bZIP proteins (Finkelstein and Gibson 2002; Jakoby et al. 2002), many of which are ABA inducible (Choi et al. 2000; Uno et al. 2000). Several, including ABI5, have been shown to bind ABRE elements in the promoters of ABA-responsive genes (Finkelstein et al. 2005; Kim et al. 2002). The water-stress-dependent virulence effect of HopAM1 that we observed (Fig. 1C) could be explained if HopAM1 modifies an ABI5-dependent ABA response.

The *abi5-1* mutation did not relieve the chlorosis induced in new leaves by either delivery of HopAM1 from *Pma M6CΔE* or dexamethasone-induced expression of HopAM1 in transgenic plants (Fig. 2). This is most easily explained if HopAM1 has multiple targets, some functioning independently of ABI5. Other type III effectors are likely to have multiple host targets, including AvrRpm1 and AvrRpt2 (Belkadir et al. 2004; Chisholm et al. 2005).

Effects of HopAM1 are ecotype dependent.

The effects of HopAM1 on virulence of *Pma M6CΔE* were most pronounced in the Ws-0 ecotype. It is noteworthy that Ws-0 carries a defective allele of the gene for the flg22 receptor *FLS2* (Gomez-Gomez and Boller 2002). This defective allele is responsible for enhanced virulence of *P. syringae* pv. *phaseolicola* RW60 carrying HopAB2 (AvrPtoB) on *Arabidopsis* (de Torres et al. 2006). *Pph* RW60 is not typically virulent on *Arabidopsis*, yet the addition of *avrPtoB* to this strain enhanced growth by approximately 10-fold on Ws-0 but not on Nd-0. The difference in response depended on lack of a functional allele of *FLS2* in Ws-0. We observed no growth differences of either *Pma M6CΔE(hopAMI(C))* or *Pma M6CΔE(vector)* on Col-0 compared with the *FLS2*-deficient mutant line Col-*fls2* (Zipfel et al. 2004) (data not shown). In the same experiment, we inoculated Ws-0 and Ws-0 carrying a *35S:FLS2* transgene (Gomez-Gomez et al. 2001) with both strains. Both Ws-0 genotypes allowed 10-fold more growth of *Pma M6CΔE(hopAMI(C))* than of *Pma M6CΔE(vector)*. Finally, the *Pma M6CΔE(vector)* grew to similar levels in both Col-0 and Ws-0 backgrounds. Therefore, the *FLS2* gene is not responsible for the ecotype differences in HopAM1 sensitivity that we document above.

Arabidopsis ecotypes express different responses to drought stress (Leon-Kloosterziel et al. 1996; North et al. 2007; Tian et al. 2005). For example, North and associates (2007) have measured higher ABA levels in untreated Ws-0 plants than in Col-0 plants and higher levels of ABA induction upon drought stress in Ws-0 than in Col-0. We expect that Ws-0 is more sensitive than other ecotypes to an as-yet-unknown aspect of ABA signaling that is modified by HopAM1.

In conclusion, we propose that HopAM1 suppresses defense responses by enhancing ABA responses that at least partially overlap with defense responses. Because ABA responses are involved, HopAM1 also facilitates disease in plants that are slightly drought stressed. This could represent a useful evolutionary adaptation for phytopathogenic bacteria under certain circumstances, such as infection of plants during mild drought stress.

MATERIALS AND METHODS

Bacterial strains and plasmids.

In order to express *hopAMI* in *P. syringae*, the *hopAMI-1* open reading frame was amplified from DC3000 genomic DNA using the primers AG1 and AG2 (Table 1) and cloned into pCR2.1 (Invitrogen, Carlsbad, CA, U.S.A.). The resulting *hopAMI-HA* fragment was excised by cutting *pCR2.1::hopAMI-HA* with *Nde1* and *BamH1* and cloned downstream

Table 1. Polymerase chain reaction primers used

Primer	Sequence
AG1	5'-GCGCTCGAGCATATGCACGCAAATCCTTTA
AG2	5'-CCGGATCCACTAGTTCATGCGTAATCAGGAACATCGTAAGGGTA GTCGCCTAGGAAATTATTTAGTT
AG3	5'-CAGAACCAGCCACGCTGGCGTTATGAAG
abi5-1F	5'-GGTTATTGTTGTATATGATGCAGTTG
abi5-1R	5'-CCACTACTCTTTTCCTTCCCC
AvrPpiB-F:	5'-CAAAAAAGCAGGCTCCGGCGGCGTTTATGTGGAATG
AvrPpiB-R:	5'-GAAAGCTGGGTGGTGCCTAGGAAATTATTTAGTTCC

of a hybrid *nptII-lacZ* promoter followed by an idealized Shine-Delgarno sequence from *gene10* of phage T7 in the pCR2.1:*GFP2* plasmid (L. Rohmer and J. L. Dangl, unpublished). The resultant promoter-*hopAMI-HA* fragment was excised with *SspI* and *BamHI* and cloned into pBBR1-MCS2 (Kovach et al. 1995) digested with *EcoRV* and *BamHI* to obtain pBBR1-MCS2::*hopAMI-HA(C)*. In order to make pBBR1-MCS2::*hopAMI-HA(N)* in which *hopAMI-HA* is driven by its native promoter, the *hopAMI-1* gene (150 bp upstream from the *hrp* box to the last translated codon) was amplified from genomic DNA using primers AvrPpiB-F and AvrPpiB-R (Table 1) and recombined into the Gateway-ready DFI vector 1 (based on pBBR1-MCS2) described in the supporting text of (Chang et al. 2005). These constructs were transformed into either *Pma* M6CAE (Belkhadir et al. 2004; Rohmer et al. 2003) or *P. syringae* pv. *pisi* race 2 (Bevan et al. 1995). In order to express *hopAMI-HA* in *Arabidopsis*, pCR2.1::*hopAMI-HA* was cut with *XhoI-SpeI* and ligated with *XhoI-SpeI*-digested vector pBUD1. pBUD1 is a modified version of pTA7002 (Aoyama and Chua 1997) in which the GVG receptor is driven by the *AtUBQ3* promoter instead of the CaMV 35S promoter (H. Kaminaka and J. L. Dangl, unpublished results). The plasmid pBUD1::*hopAMI-HA* was transformed into the *Agrobacterium* strain GV3101 for plant infiltrations.

Plant growth conditions for bacterial infiltrations.

Seedlings were grown in 3-in. pots for 3 weeks and then transferred to soil trays (25.5 by 25.5 by 10.5 cm in length, width, and height, respectively). Soil trays were watered with 250 ml/week for dry (water-stressed) trays and 500 ml/week for wet trays. Plants were grown for 2 to 3 weeks in these trays and, on the day of bacterial infiltration, soil samples were taken for measuring soil water content. Percent water (wt/wt of soil) was calculated by the formula $[(\text{fresh weight} - \text{dry weight})/\text{fresh weight} \times 100]$, where fresh weight is the initial weight of soil and the dry weight is measured after drying the soil in an oven at 80°C for 24 h. All bacterial infiltrations on plants were done under dry conditions unless mentioned otherwise. Bacterial growth assays were done as described (Nimchuk et al. 2000). Briefly, bacteria were grown on King's B agar plates containing appropriate antibiotics for 1 day and then resuspended in 10 mM MgCl₂ solution at a concentration of 2.5×10^5 CFU/ml before infiltrating leaves of 5- to 6-week-old plants with a needleless syringe. Four discs of 3 mm each were ground in 1 ml of 10 mM MgCl₂. Appropriate serial dilutions were made and plated. The experiment was done in triplicate each time. Bacterial inoculations for assaying the novel chlorosis phenotype were done with bacteria at 5×10^5 CFU/ml in MgCl₂ and chlorosis was scored 5 to 6 days after inoculation.

Transgenic and mutant plants.

Agrobacterium strain GV3101 carrying pBUD1::*hopAMI-HA* was transformed into *Ws-0 Arabidopsis* and transgenic plants were selected using BASTA as described (Holt et al. 2005). The *abi5-1* mutant was obtained from The Arabidopsis Biological Resource Center, Ohio State University (Columbus, U.S.A.). Col-0 *fls2* was a gift of Cyril Zipfel and *Ws-0 35S:FLS2* was a gift from T. Boller. The primers *abi5-1F* and *abi5-1R* (Bensmihen et al. 2002) and the enzyme *AvaII* were used for testing homozygosity of the *abi5-1* mutants after crossing to the *HopAMI-Tg35* transgenic line.

Expression analysis.

RNA was isolated using Trizole Reagent (Invitrogen). Northern blots involved 10 µg of total RNA probed with radioactive gene-specific PCR products. RT-PCR was done using the Retroscript kit (Ambion, Bath, U.K.) according to manu-

facturer's instructions. RNA (2 µg) from 5- to 6-week-old leaf samples with and without induction with 20 µM dexamethasone were used for the RT reaction and primers AG1 and AG3 were used for PCR. Protein extractions and Western blots with anti-HA antibody were performed as described (Nimchuk et al. 2000).

ABI5 westerns were performed as follows. Sterilized seed was stratified for 3 days, then grown for 7 days under continuous light in liquid media (in 24-well plates) on a rotating shaker. On the seventh day, one-half of the seedlings were treated overnight with 20 µM dexamethasone for 12 h. The next morning, indicated seedlings were treated with 10 µM ABA, harvested, and frozen in liquid nitrogen. Samples were ground in 2× protein sample buffer + β-mercaptoethanol and 20 µl per sample was run on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The blot was probed with Anti-ABI5 antiserum at a concentration of 1:2000. The same blot was stripped and re-probed with antiserum against the proteasome subunit PBA1 as a loading control.

Stomata closure assays.

Seed were sown in 3-in. pots and stratified at 4°C in the dark for 2 days. The seedlings then were grown in a growth chamber with 9 h of light per day and approximately 22°C for 3 weeks. Plants were sprayed with dexamethasone just before 6:00 p.m. and kept covered to optimize dexamethasone uptake. Then, 16 h later, leaves were harvested from the seedlings and left in 100 mM morpholineethanesulfonic acid (MES), pH 6.15, and 50 mM KCl buffer for 2 h and the samples were induced with ABA at noon. Experiments all were done at the same time of day to prevent deviations caused by circadian rhythms. Leaves were ground with a polytron to release epidermal fragments for microscopy and rapidly photographed (Pei et al. 1997, 2000). The ratio of width to length of the inside of the stomate pore was measured.

Germination assays.

Age-matched seed were sown on 0.5× MS agar (1%) plates containing MES at 0.5 g/liter, 1% sucrose, and various concentrations of ABA or NaCl (Kang et al. 2002; Smalle et al. 2003) with or without 0.25 µM dexamethasone. Plates were kept in the dark at 4°C for 4 days and then transferred to growth chambers maintaining 22°C and 16 and 8 h of light and dark, respectively. ABA germination assays were scored after 7 days. Fresh weights of seedlings (20 × 3 for each treatment) were measured for NaCl growth assays 10 days after transferring the plates to growth chambers.

Callose assays.

DC3000 *hrcC* was a gift from S.-Y. He, Michigan State University. Callose assays were done as described previously (Kim et al. 2005). Briefly, 5- to 6-week-old plants were sprayed with 20 µM dexamethasone and, 16 h later, were infiltrated with bacteria at 5×10^7 CFU/ml. Then, 12 h later, four to six leaves from independent plants were cleared, washed, and stained with aniline blue dye and autofluorescent callose spots were analyzed by fluorescence microscope (Nikon, Melville, NY, U.S.A.) at ×200 magnification. Six fields were counted per experiment.

ABA assays.

In the assays, 3-week-old plants were grown as for stomatal closure assays or 5-week-old plants were grown as for bacterial infiltration assays. Plants were sprayed with 20 µM dexamethasone 18 h before harvesting leaves. Hormone assays were done according to supplemental text from de Torres-Zabala and associates (2007).

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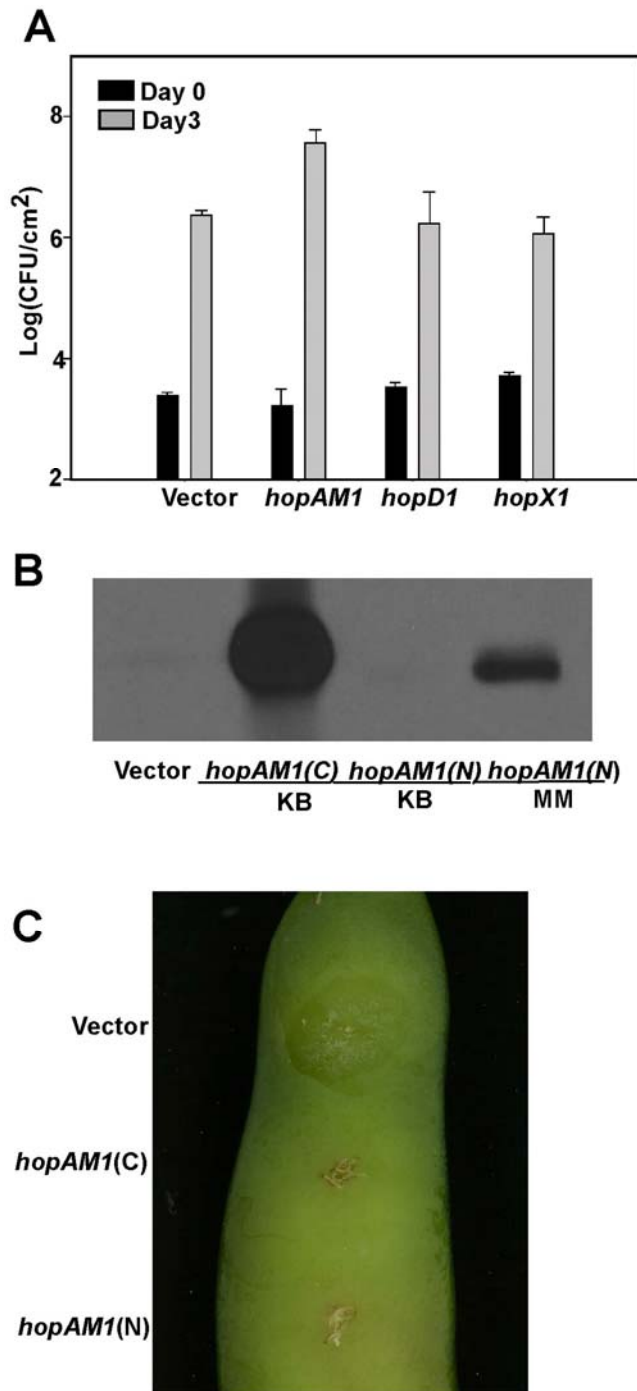
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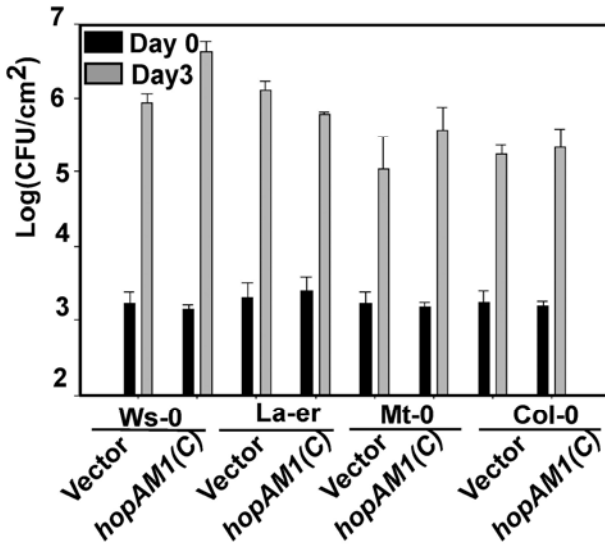
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AUTHOR-RECOMMENDED INTERNET RESOURCES

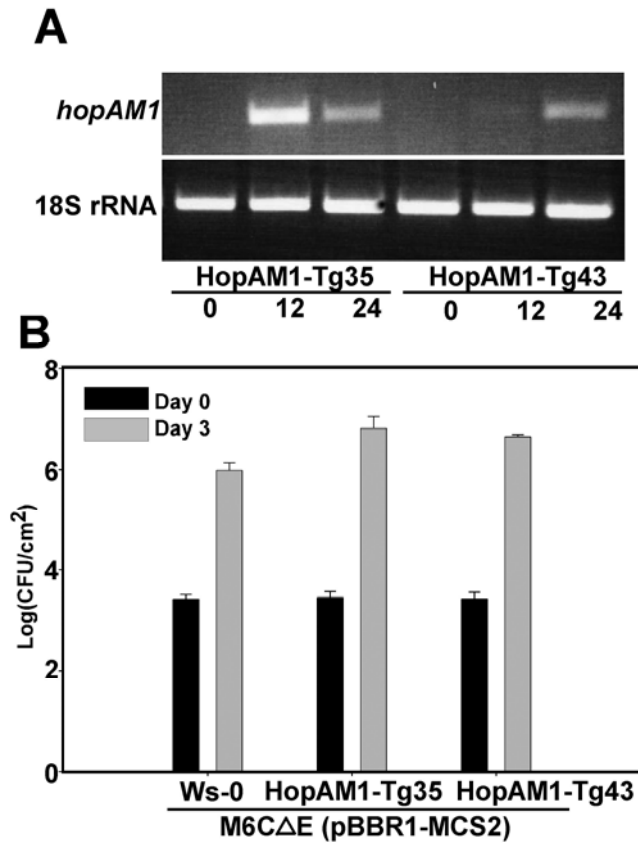
Pseudomonas syringae Genome Resources website:
pseudomonas-syringae.org/



Supplemental Fig. 1. A. Expression of other type III effectors does not mimic the growth effect of HopAM1 on *Pma* M6CΔE, Comparison of growth of *Pma* M6CΔE carrying pBBR1-MCS2 plasmid (vector) or pBBR1-MCS2 designed to express the full length ORF of *hopAM1*, *hopD1* or *hopX1* from the *lac-ntpII* promoter in Ws-0 plants grown under water-stressed conditions. The number of bacteria per area of leaf sampled directly after infiltration (day 0) or after three days (day 3) is plotted on a log₁₀ scale. Error bars represent standard deviation among three samples. The experiment is representative of two independent replicates. The Student's *t*-test *P*-value for the difference between vector and *hopAM1(C)* was *P* < 0.01, *hopD1* was *P* < 0.77, *hopX1* *P* < 0.22. **B.** Western blot of HopAM1-HA protein from *Pma* M6CΔE carrying pBBR1-MCS2 (Vector), pBBR1-MCS2::*hopAM1*-HA in which *hopAM1* is expressed from the constitutive *ntpII-lac* promoter (*hopAM1(C)*) in non-inducing, rich KB medium, or pBBR1-MCS2::*hopAM1*-HA in which *hopAM1* is expressed from its native promoter (*hopAM1(N)*) in KB or *hrp*-expression-inducing medium (MM). A 31.3 kD protein is visualized with anti-HA antiserum. **C.** HopAM1-HA is recognized by the pea *R3* disease resistance gene product to stimulate an HR. *P. syringae* pv. *psii* race 2 strain (lacking *hopAM1*) carrying pBBR1-MCS2 (Vector) was inoculated into a pod of pea cultivar Belinda (*R3*) at 5 × 10⁷ CFU/ml and the pod was photographed three days after infiltration. A water-soaked lesion typical of disease is visible (top inoculation site). The pod was also inoculated with *Psp* race 2 (*hopAM1(C)*), or (*hopAM1(N)*). In each case, the contained, light brown lesions observed are typical of HR.



Supplemental Fig. 2. HopAM1 enhances growth of *Pma* M6CΔE on Ws-0 but not on other ecotypes. Comparison of growth of *Pma* M6CΔE (vector) with *Pma* M6CΔE (*hopAM1(C)*) on Arabidopsis plants of different ecotypes grown under water-stressed conditions. Error bars represent standard deviation among three samples. The experiment is representative of two independent replicates. Student's *t*-test *P*-values comparing vector to *hopAM1(C)* in Ws-0 were $P < 0.01$, La-er $P < 0.03$, Mt-0 $P < 0.17$, Col-0 $P < 0.59$.



Supplemental Fig. 3. *hopAM1* transcripts are expressed in transgenic plants and restore virulence to *Pma* M6CΔE. **A**, RT-PCR analysis of *hopAM1-HA* transcripts from leaves of 3 week old *hopAM1* expressing transgenic lines 0, 12 and 24 h after induction of *hopAM1* expression by dexamethasone. **B**, Conditional expression of *hopAM1* increases the growth of *Pma* M6CΔE on Arabidopsis. Wild-type Ws-0 and transgenic plants were sprayed with 20 μM dexamethasone and were infiltrated 12 h later with *Pma* M6CΔE (vector) at 10⁵ CFU/ml. Error bars represent standard deviation among three samples. The experiment is representative of two independent replicates. Student's t-test values comparing growth in Ws-0 to growth in both transgenic lines were $P < 0.02$.