Confocal Imaging of *Pseudomonas syringae* pv. *phaseolicola* Colony Development in Bean Reveals Reduced Multiplication of Strains Containing the Genomic Island PPHGI-1

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Pseudomonas syringae pv. *phaseolicola* is the seed borne causative agent of halo blight in the common bean *Phaseolus vulgaris*. *Pseudomonas syringae* pv. *phaseolicola* race 4 strain 1302A contains the avirulence gene *hopAR1* (located on a 106-kb genomic island, PPHGI-1, and earlier named *avrPphB*), which matches resistance gene *R3* in *P. vulgaris* cultivar Tendergreen (TG) and causes a rapid hypersensitive reaction (HR). Here, we have fluorescently labeled selected *Pseudomonas syringae* pv. *phaseolicola* 1302A and 1448A strains (with and without PPHGI-1) to enable confocal imaging of in-planta colony formation within the apoplast of resistant (TG) and susceptible (Canadian Wonder [CW]) *P. vulgaris* leaves. Temporal quantification of fluorescent *Pseudomonas syringae* pv. *phaseolicola* colony development correlated with in-planta bacterial multiplication (measured as CFU/ml) and is, therefore, an effective means of monitoring *Pseudomonas syringae* pv. *phaseolicola* endophytic colonization and survival in *P. vulgaris*. We present advances in the application of confocal microscopy for in-planta visualization of *Pseudomonas syringae* pv. *phaseolicola* colony development in the leaf mesophyll to show how the HR defense response greatly affects colony morphology and bacterial survival. Unexpectedly, the presence of PPHGI-1 was found to cause a reduction of colony development in susceptible *P. vulgaris* CW leaf tissue. We discuss the evolutionary consequences that the acquisition and retention of PPHGI-1 brings to *Pseudomonas syringae* pv. *phaseolicola* in planta.

Pseudomonas syringae strains are gram-negative bacteria that infect a wide variety of plants, causing necrotic symptoms in leaves, stems, and fruit. *Pseudomonas syringae* are divided into pathovars depending on plant-host range (Taylor et al. 1996). The primary mechanism of plant defense against *Pseudomonas syringae* is a basal defense response (Jones and Dangl 2006) that is induced upon detection of conserved microbe-associated molecular patterns (MAMPs), such as flagellin, lipopolysaccharide, or elongation factor Tu (Felix et al. 1999; Kunze et al. 2004; Zeidler et al. 2004). *Pseudomonas syringae* can manipulate their host by delivering an array of effector proteins into the host cytoplasm that inactivate plant surveillance mechanisms and signal transduction pathways, thereby allowing bacterial

growth. In the case of *Pseudomonas syringae* pv. *phaseolicola*, many plants have evolved a second mechanism of plant defense in which resistance (R) proteins recognize a subset of these bacterial effector proteins, the avirulence (Avr) proteins, and this interaction (R-Avr) triggers a hypersensitive reaction (HR) (Alfano and Collmer 2004; Jones and Dangl 2006). Analysis of the molecular genetics of the R-Avr interactions between the bean pathogen *Pseudomonas syringae* pv. *phaseolicola* and its host plant includes the identification and characterization of a number of *Pseudomonas syringae*-derived *avr* genes carried by the bacterium (Taylor et al. 1996). One of these *avr* genes, *hopAR1* (formerly *avrPphB*) (Lindeberg et al. 2005), leads to the activation of the HR in bean cultivar Tendergreen (TG), which carries the *R3* resistance gene (Jenner et al. 1991).

In *Pseudomonas syringae* pv. *phaseolicola* 1302A (race 4), *hopAR1* is located on a 106-kb genomic island (GI) designated PPHGI-1 (Pitman et al. 2005). PPHGI-1 has a number of classic features described in other bacterial GI; it is present in the genomes of some strains but not others and is flanked by specific DNA sequences that contain direct repeats or tRNA loci. It also carries genes coding for genetic mobility, such as phage genes, insertion sequence elements, integrases, and transposases (Hacker and Kaper 2000). We have previously shown that the complete episomal island of PPHGI-1 is able to transfer between strain 1302A (race 4) to strain 1448A (race 6) by transformation both in planta and in extracted *P. vulgaris* apoplastic fluids (Lovell et al. 2009). The significant phenotypic difference between strains 1448A and 1302A is that 1448A contains no known functional *avr* genes and therefore causes disease on all bean cultivars (Mansfield et al. 1994). Furthermore, it has been shown that, under *R3*-*HopAR1*-mediated HR conditions in TG, PPHGI-1 is lost from the genome of *Pseudomonas syringae* pv. *phaseolicola* 1302A during repeated infection of *P. vulgaris*, and this leads to the evolution of a virulent *Pseudomonas syringae* pv. *phaseolicola* strain (referred to as strain RJ3) that no longer triggers this HR (Pitman et al. 2005). Loss of PPHGI-1 is found in more than 70% of bacteria recovered after three passages in TG (equivalent to 21 days in planta); however, in the susceptible cultivar Canadian Wonder (CW), little or no PPHGI-1 loss occurs. Although PPHGI-1 has been sequenced (GenBank accession AJ870974), we know little about the possible function of the GI in *Pseudomonas syringae* pv. *phaseolicola*.

The use of fluorescent reporter proteins in plant biology is well established, but such probes continue to be compromised by overlapping autofluorescence emission spectra from leaf and root tissue. In plant vascular tissues, autofluorescence from cell

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wall-associated phenolics occurs at wavelengths between 490 and 620 nm, whereas chlorophyll fluorescence is most prevalent between 650 and 770 nm (Chapman et al. 2005). In practice, this means that the imaging of green fluorescent protein (GFP) is most problematic in roots, whereas red fluorescent proteins (RFP) can be difficult to discriminate in tissues containing many chloroplasts (Chapman et al. 2005). Nonetheless, fluorescent proteins have been used extensively in studies of epiphytic and endophytic bacterial colonization (Bloemberg et al. 2000; Compant et al. 2005; Dulla and Lindow 2008; Elbeltagy et al. 2001; Han et al. 2008). Badel and associates (2002) used GFP for imaging and quantification of in-planta colony formation to examine differences in colonization between *Pseudomonas syringae* pv. *tomato* and *hopPtoA* mutants in *Arabidopsis*. This study measured GFP colony diameter (and assumed spherical colony formation) to show mutations in *hopPtoA* redundantly contributed to in-planta colony formation.

The plant apoplast consists of the intercellular space in the plant tissue (including the cell wall) that is outside the plasma membrane, through which nutrients and water can freely diffuse (Abramovitch et al. 2006). The apoplast is the primary site of *Pseudomonas syringae* pv. *phaseolicola* colonization and survival when nutrients are obtained directly from apoplastic fluid. It is also within this microenvironment that bacteria triggering defenses are exposed to antimicrobial factors (Rico and Preston 2008). The HR causes plant tissue collapse and the release of antimicrobial compounds that greatly affect the survival of invading *Pseudomonas syringae* pv. *phaseolicola* strains (Jambunathan et al. 2001; Whalen et al. 1991; Wright and Beattie 2004), and the timing and severity of the HR varies depending on the direct or indirect R-Avr interactions (Kamoun et al. 1999).

In addition to visual symptoms of HR being the distinct necrosis of plant tissue, the accumulation of autofluorescent compounds is also characteristic of HR lesions (Bennett et al. 1996; Yu et al. 1998). Such HR autofluorescence is primarily associated with accumulation of phenolic compounds (Bennett et al. 1996; Kamoun et al. 1999; Kombrink and Schmelzer 2001). This HR-associated autofluorescence, in addition to emission from lignin and chlorophyll (Chapman et al. 2005), severely limits the application of confocal microscopy for the in-planta imaging of selected fluorophores under HR-inducing conditions. To combat such problems, fluorophores with im-

proved biophysical properties have been developed (Chapman et al. 2005), and advances in confocal microscopic imaging technology continue to provide improvements of in-planta image acquisition. Newer confocal microscopes have optimized imaging capabilities, such as the acoustic optical tunable filters (AOTF) and acoustic optical beam splitters (AOBS) used to refine collected emission spectra and rapidly change excitation wavelengths, respectively (Saggau 2006). A valuable application for such confocal microscopic advances in plant biology is to eliminate or minimize detection of autofluorescence emitted by both healthy plant tissue and plants undergoing the HR.

Here, we have utilized fluorescent proteins and confocal microscopy with AOTF and AOBS to investigate *Pseudomonas syringae* pv. *phaseolicola* survival in the apoplast of *P. vulgaris*, gaining insight into the temporal development of in-planta colony formation within both susceptible and resistant cultivars of *P. vulgaris* (CW and TG, respectively). In particular, we examined one or more in-planta effects of the R3-HopAR1-mediated HR on colony establishment and distribution of *Pseudomonas syringae* pv. *phaseolicola* strains (with and without PPHGI-1) in the *P. vulgaris* leaf apoplast.

RESULTS

Development of fluorescently labeled strains.

Constitutively expressed fluorescent reporter genes (either eCFP, eGFP, eYFP, or dsRFP [enhanced cyan, green, yellow, and red fluorescent proteins, respectively]) were introduced into the chromosome of *Pseudomonas syringae* pv. *phaseolicola* strains 1302A and 1448A, using a Tn7 delivery system (Lambertsen et al. 2004). In addition to the brightest expressing constructs being selected, all strains with introduced fluorescent proteins (Table 1) were only used in this study if they retained progenitor phenotypes with respect to in vitro and in-planta growth rates, HR and disease phenotypes on TG and CW leaves and pods, plasmid profiles, in vitro PPHGI-1 excision, and the expected rates of PPHGI-1 loss via passaging through TG (Pitman et al. 2005).

In planta confocal microscopy of infiltrated *Pseudomonas syringae* pv. *phaseolicola* strains.

To assess the detectable levels of in-planta fluorescence of individual *Pseudomonas syringae* pv. *phaseolicola* strains (Ta-

Table 1. Plasmids and bacterial strains

Name	Genotype ^a	Reference
Plasmids		
pUXBF13	Helper plasmid, providing the Tn7 transposase proteins, Ap ^r	Bao et al. 1991
pRK2013	Conjugation helper plasmid, Km ^r	Figurski et al. 1979
AKN132	dsRFP, Ap ^r , Gm ^r	Lambertsen et al. 2004
AKN100	eGFP, Ap ^r , Gm ^r	Lambertsen et al. 2004
AKN069	eYFP, Ap ^r , Gm ^r	Lambertsen et al. 2004
AKN033	eCFP, Ap ^r , Gm ^r	Lambertsen et al. 2004
<i>Escherichia coli</i>		
39R861	Containing plasmids of known molecular weight for plasmid profiling	Threllfall et al. 1986
<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i> strains		
1302A	Wild-type strain, race 4	Taylor et al. 1996
1302A-eCFP	1302A::Tn7-eCFP, Gm ^r	This study
1302A-eGFP	1302A::Tn7-eGFP, Gm ^r	This study
1302A-eYFP	1302A::Tn7-eYFP, Gm ^r	This study
1302A-dsRFP	1302A::Tn7-dsRFP, Gm ^r	This study
RJ3	1302A with PPHGI-1 loss	Jackson et al. 2000
RJ3-eYFP	RJ3::Tn7-eYFP, Gm ^r	This study
1448A	Wild-type strain, race 6, Rif ^r	Mansfield et al. 1994
1448A-eYFP	1448A::Tn7-eYFP, Gm ^r	This study
1448A::PPHGI-1	1448ARif containing PPGHI-1, Km ^r	Lovell et al. 2009
1448A::PPHGI-1-eYFP	1448A::PPHGI-1-Tn7-eYFP, Km ^r , Gm ^r	This study

^a Ap^r, Gm^r, Rif^r, and Km^r indicate resistance to ampicillin, gentamicin, rifampicin, and kanamycin, respectively. dsRFP, eGFP, eYFP, and eCFP = red and enhanced green, yellow, and cyan fluorescent protein, respectively.

ble 1), various concentrations of inocula were syringe-infiltrated into the mesophyll layer of *P. vulgaris* cv. CW or TG, or both, and plants were maintained under glasshouse conditions for 24 to 144 h before sections of leaf tissue were removed for confocal observation.

Colony formation is reduced in TG.

The first observation during confocal microscopy of fluorescent *Pseudomonas syringae* pv. *phaseolicola* 1302A strains (containing avirulence gene *hopAR1*) infiltrated into TG leaf tissue (causing a R3-HopAR1-mediated HR) was the development of small, dense 1302A-eYFP colonies among the collapsed plant tissue (Fig. 1A) compared with the larger flourishing colonies in non-HR environments, e.g., strain RJ3 (with loss of *hopAR1*) in TG (Fig. 1B). These results are consistent with current knowledge that the HR causes reduced in-planta bacterial growth (recorded as CFU/ml).

Fluorophore detection in TG.

In addition to reduced colony morphology of *hopAR1*-containing strains in TG (Fig. 1A), results also showed the R3-HopAR1-mediated HR caused the accumulation of many autofluorescent compounds. The severity of TG autofluorescence increased with higher *Pseudomonas syringae* pv. *phaseolicola* 1302A infiltration concentrations (Fig. 1C and D). Both eGFP and eYFP could be effectively visualized within the HR autofluorescence; however, eYFP exhibited brighter fluorophore emission and thus enabled higher-quality confocal image acquisition of *Pseudomonas syringae* pv. *phaseolicola* colony formation. The *P. vulgaris* R3-HopAR1-mediated HR autofluorescence showed spectral overlap primarily with dsRFP, and therefore, dsRFP was very difficult to visualize. eCFP was also difficult to visualize because of its low quantum yield (3,000 compared with 24,300 in eYFP), as the increased parameters required to enhance eCFP detection also meant any HR auto-

Fig. 1. Confocal images showing various fluorescent *Pseudomonas syringae* pv. *phaseolicola* strains forming in-plant colonies in *Phaseolus vulgaris* leaf tissue. When *Pseudomonas syringae* pv. *phaseolicola* 1302A-eYFP is infiltrated into cv. Tendergreen (TG), the R3-HopAR1-mediated hypersensitive-response (HR) causes tissue collapse resulting in the accumulation of autofluorescent compounds that are visualized (as a pink discoloration) in confocal microscopy. **A**, The TG R3-HopAR1-mediated HR makes visualization of fluorophores difficult and only eYFP (shown here) and eGFP (enhanced yellow and green fluorescent protein, respectively) can be visualized efficiently. **B**, Strain RJ3 (lacking *hopAR1*) in TG infiltrated identically as **A** (note the flourishing colony development in a non-HR environment). **C**, The TG R3-HopAR1-mediated HR can be visualized as small localized regions (arrow) when infiltrated with low pathogen concentrations (8×10^4 CFU/ml, 72 h) or **D**, as total tissue collapse when infiltrated with a high pathogen concentration (8×10^7 , 72 h). **E**, At lower infiltration concentrations (8×10^4 CFU/ml), the HR in TG could be minimized (as in **C**) to permit imaging of individual *Pseudomonas syringae* pv. *phaseolicola* 1302A-eYFP colonies adjacent (arrow) to collapsing plant tissue undergoing the R3-HopAR1-mediated HR. **F**, Selected 1302A-eYFP colonies adjacent to TG HR tissue were observed to rupture (arrow) after approximately 144 h and **G**, release individual bacteria into the plant apoplast. Mixed *Pseudomonas syringae* pv. *phaseolicola* 1302A fluorescent strains (8×10^5 CFU/ml each) are readily visualized in non-HR cv. Canadian Wonder (CW) leaf tissue as either **H**, 1302A-eGFP and 1302A-dsRFP, **I**, 1302A-eCFP (enhanced cyan fluorescent protein) and 1302A-YFP, or **J**, 1302A-eYFP and 1302A-dsRFP. Although strains predominantly formed individual colonies emitting one fluorescence (e.g., **H** through **J**), mixed colonies expressing both fluorescent proteins were also observed: **K**, 1302A-eCFP and 1302A-eYFP; **L**, 1302A-eGFP and 1302A-dsRFP. All confocal images were assigned false color (eCFP, cyan; eGFP, green; eYFP, yellow; dsRFP, red; plant tissue, blue; and HR autofluorescence, an overlay of cyan, red, and blue channels to give pink). Size bars are in micrometers.

fluorescence was similarly enhanced. From these results, it was deemed that the optimal conditions for visualizing *Pseudomonas syringae* pv. *phaseolicola* colony development under *P. vulgaris* R3-HopAR1-mediated HR conditions was to infiltrate TG with strain 1302A-eYFP at 8×10^4 CFU/ml, which then enabled efficient in-planta confocal observation of colony formation adjacent to plant cells undergoing the HR (Fig. 1E).

Observation of in-planta colony dispersal in TG.

Within the mesophyll of TG undergoing the R3-HopAR1-mediated HR, single cells were observed to emerge from ruptured *Pseudomonas syringae* pv. *phaseolicola* 1302A colonies into the apoplast after approximately 144 h (Fig. 1F and G). Such bacterial dispersal was visualized in both samples mounted in water and in dry-mounted tissue samples. Dispersal was rarely observed in CW tissue, even after prolonged incubation.

Fluorophore detection in CW.

By contrast, confocal microscopy of fluorescent *Pseudomonas syringae* pv. *phaseolicola* 1302A strains infiltrated into CW leaf tissue (no R3-mediated HR) showed eGFP, dsRFP, eYFP, and eCFP emissions were readily distinguishable from plant tissue (individual data not shown; however, Figure 1H to L shows representative fluorescence recovery in CW).

In planta confocal microscopy of mixed infiltrations of *Pseudomonas syringae* pv. *phaseolicola* strains.

Mixtures of *Pseudomonas syringae* pv. *phaseolicola* strains were infiltrated as above to determine the efficacy of confocal visualization of multiple fluorescent bacteria within *P. vulgaris*, using the AOTF of the Leica microscope (Leica, Wetzlar, Germany).

TG. When mixtures of *Pseudomonas syringae* pv. *phaseolicola* strains were infiltrated into TG, similar imaging limitations as described above were encountered when the HR was initiated by one or both of the strains containing *hopAR1*. Minimizing the HR severity was achieved by lowering the infiltration concentration of strains containing *hopAR1* to 8×10^4 CFU/ml or less. Although this improved imaging of both eCFP and dsRFP strains, it also subsequently reduced the number of *Pseudomonas syringae* pv. *phaseolicola* colonies formed and reduced the HR intensity affecting *Pseudomonas syringae* pv. *phaseolicola* colony formation. These findings confirm that, although both eGFP and eYFP are optimal fluorescent proteins to use in the TG R3-HopAR1-mediated HR, they cannot be used together in planta, due to limitations in accurately differentiating fluorophore emission from close overlapping spectra (Zimmermann et al. 2003).

CW. Successful fluorescence recovery was achieved from mixed infiltrations of *Pseudomonas syringae* pv. *phaseolicola* 1302A fluorescent strains (8×10^5 CFU/ml each) in CW. Mixed *Pseudomonas syringae* pv. *phaseolicola* strains were visualized to either i) from individual colonies emitting predominantly one fluorescence (Fig. 1H to J), ii) from mixed colonies with cells expressing both fluorophores (Fig. 1K and L), or iii) both. In order to obtain clear images of mixtures, it was necessary to balance the sensitivity of detection of fluorophores. For example, as illustrated in Figure 1H, eGFP was detectable at low sensitivity, whereas the signals for dsRFP emission were maximized (thus simultaneously enhancing plant autofluorescence). The clearest consistent differential between *Pseudomonas syringae* pv. *phaseolicola* strains in CW was achieved by combining eYFP- and eCFP-labeled bacteria (Fig. 1I).

Quantification of confocal colony image data (CCID) and comparison with bacterial growth rate (CFU/ml).

Although both eGFP and eYFP could be effectively visualized within the HR autofluorescence, eYFP was chosen for routine use because of its higher quantum yield (eYFP of 24,300 compared with eGFP of 16,100). *Pseudomonas syringae* pv. *phaseolicola* colonies expressing eYFP provided high-quality confocal image acquisition, with good contrast between eYFP and plant tissue (Fig. 2A to C). This enabled ImageJ software (Abramoff et al. 2004) quantification of threshold data values to calculate the percentage of colony area within the imaged plant tissue. To determine the relationship between eYFP-CCID and the bacterial growth rate data of viable eYFP cells present, infiltrated tissue samples were collected immediately adjacent to each other—one for CCID and one for establishment of the bacterial growth rate. Serial dilutions of ground tissue extracts grown on agar supplemented with gentamicin (Gm, for selection of eYFP) showed 100% of gentamicin-resistant colonies recovered exhibited positive eYFP fluorescence. ImageJ CCID based on colony area showed equivalent trends to *Pseudomonas syringae* pv. *phaseolicola* bacterial growth rate recovered from leaves (Fig. 3), and therefore, CCID was considered a rapid and accurate way to generate statistical data for the comparison of *Pseudomonas syringae* pv. *phaseolicola* survival in *P. vulgaris* CW and TG.

Fig. 2. The R3-HopAR1-mediated hypersensitive response (HR) environment causes restricted colony development of *Pseudomonas syringae* pv. *phaseolicola* 1302A (containing *hopAR1*). Images subjected to threshold analysis using ImageJ show that **A**, strain 1302A-eYFP colony morphology is small and compact when infiltrated into Tendergreen (TG) compared with **B**, strain RJ3-eYFP (1302A with loss of PPHGI-1 and, therefore, *hopAR1*) grown in TG, which produces larger flourishing colonies. **C**, 1302A-eYFP exhibits flourishing colonies similar to strain RJ3-eYFP when grown in CW (no R3-HopAR1-mediated HR). eYFP colony expression was analyzed with ImageJ software to provide confocal colony image data (CCID) representing the percent eYFP colony area within the *Phaseolus vulgaris* leaf apoplast. **D**, Temporal CCID collection from single-strain infiltrations (1.6×10^6 CFU/ml) over 144 h. Data are means $\pm$ standard error of the mean ($n = 36$ for each timepoint).

***Pseudomonas syringae* pv. *phaseolicola* 1302A shows reduced in-plant growth compared with strain RJ3 in both TG and CW leaves.**

We examined the effect of PPHGI-1 on colony development in susceptible and resistant plants. The temporal CCID collected for strains 1302A-eYFP (containing PPHGI-1 with *hopAR1*) and RJ3-eYFP (a 1302A strain that has lost PPHGI-1) within TG (containing R3) and CW (no R3) are shown in Figure 2D. Results confirmed that, after 144 h, *Pseudomonas syringae* pv. *phaseolicola* 1302A colonies have a 76% decrease in colony formation in TG undergoing the R3-HopAR1-mediated HR compared with strain RJ3. In CW interestingly, strain RJ3 also had a 34% increase in colony development compared with *Pseudomonas syringae* pv. *phaseolicola* 1302A, even in the absence of the R3-HopAR1-mediated HR. Recovered bacterial growth rate results supported CCID (Fig. 3) in which i)

Fig. 3. Comparison of in-plant colony-forming units recovered (measured in CFU/ml) and confocal colony image data (CCID) from infiltrated *Pseudomonas syringae* pv. *phaseolicola* strains (1.6×10^6 CFU/ml) after 120 h. **A**, Bacterial growth found in 1302A-eYFP (13) and RJ3-eYFP (RJ) from Tendergreen (TG) and Canadian Wonder (CW) leaf tissue. **B**, Data represents the percent eYFP expression from 1302A-eYFP and RJ3-eYFP in TG and CW. The similar trends between A and B suggests CCID is a good representation of in-planta bacterial growth. Data are means $\pm$ standard error of the mean ($n = 10$ for in-planta bacterial growth and $n = 36$ for CCID). For both graphs, analysis of variance showed significant differences between strains and between cultivars ($P < 0.0001$ and $df = 1$ for both tests); significant differences among sample means were revealed by Student's *t*-test ($P = 0.05$) and are indicated by different letters above bars.

growth was better in CW than TG for both strain RJ3-eYFP and 1302A-eYFP, and ii) strain RJ3-eYFP displayed better growth than 1302A-eYFP in either TG or CW. Significantly, no growth rate differences were observed between the strains under varying in vitro growth conditions (data not shown).

Comparison of different *Pseudomonas syringae* pv. *phaseolicola* strains containing PPHGI-1.

Results presented in Figure 3 suggest PPHGI-1 (containing *hopAR1*) negatively affects both in-plant growth rate (CFU/ml) and colony formation (CCID) in both resistant (TG) and susceptible (CW) tissue. To confirm the effect of PPHGI-1, we compared different *Pseudomonas syringae* pv. *phaseolicola* strains, strain 1448A (race 6) and strain 1448A::PPHGI-1 (containing a copy of PPHGI-1 transferred from 1302A [Lovell et al. 2009]). Strains 1448A and 1448A::PPHGI-1 were chromosomally labeled with Tn7 eYFP and were infiltrated into TG and CW. Data presented in Figure 4 show that the presence of PPHGI-1 in strain 1448A reduced colony size (by 72% in TG and 31% in CW). By contrast, no differences in multiplication between strains 1448A and 1448A::PPHGI-1 were observed in vitro using various growth media (data not shown).

***Pseudomonas syringae* pv. *phaseolicola* strains do not migrate significantly from infiltration sites.**

To determine movement of fluorescent *Pseudomonas syringae* pv. *phaseolicola* strains within inoculated TG and CW plants, we examined sections of plants outside the infiltration site from i) immediately adjacent to infiltration, ii) secondary leaf tissue, iii) leaf stems, and iv) root tissue. All samples were imaged and no fluorescent colony formation was detectable. Furthermore, the same samples were homogenized, and analysis failed to recover bacteria. These results show that infiltrated *Pseudomonas syringae* pv. *phaseolicola* strains remain localized to the area of inoculation and, if single cell dispersal and re-establishment of colonies occurs in planta, such movement is restricted within the microenvironment of the infiltrated apoplast.

Fig. 4. Comparison of in-plant confocal colony image data (CCID) between *Pseudomonas syringae* pv. *phaseolicola* 1448A strains (with and without PPHGI-1) after 120 h. CCID represents percent *Pseudomonas syringae* pv. *phaseolicola* eYFP expression in *Phaseolus vulgaris* leaf apoplast from single-strain infiltrations (1.6×10^6 CFU/ml). Data are means $\pm$ standard error of the mean ($n = 36$ each), and numbers above bars represent percent decrease as a result of the presence of PPHGI-1 in 1448A. Analysis of variance showed significant differences between strains and between cultivars ($P < 0.0001$ and $df = 1$ for both tests); significant differences among sample means were revealed by Student's *t*-test ($P = 0.05$) and are indicated by different letters above bars. CW = Canadian Wonder; TG = Tendergreen; 14 = 1448A-eYFP; 14+ = 1448A::PPHGI-1-eYFP (a 1448A strain that has acquired PPHGI-1 by horizontal transfer).

Infiltration concentration significantly affects *Pseudomonas syringae* pv. *phaseolicola* colony formation during the HR.

Initial infiltration concentration in TG affected colony development by *Pseudomonas syringae* pv. *phaseolicola* strains harboring *hopAR1* (Fig. 5). When high inoculum concentrations (1.6×10^8 CFU/ml) of strain 1302A-eYFP were infiltrated into TG tissue, large colony-area development occurred in the mesophyll after 120 h, whereas low inoculum concentrations resulted in much smaller colony-area development, a 30% decrease at 1.6×10^6 CFU/ml and 59.3% decrease at 1.6×10^4 CFU/ml (Fig. 5). No significant differences were observed between infiltration concentration and colony development of strain RJ3 (no *hopAR1*) in TG.

DISCUSSION

It is well-established that HR greatly affects endophytic bacterial survival and that a higher inoculum of avirulent bacteria infiltrated into a resistant host, such as *Pseudomonas syringae* pv. *phaseolicola* 1302A into TG, will cause a more severe host HR response. Here, we utilized fluorescent proteins and confocal microscopy to investigate phenotypic variation in colony formation by avirulent bacteria within the hostile environment of the plant mesophyll undergoing the HR.

Constitutive fluorescent reporter genes (either eCFP, eGFP, eYFP, or dsRFP) were chromosomally introduced into *Pseudomonas syringae* pv. *phaseolicola* strains 1302A and 1448A, using a Tn7 delivery system (Lambertsen et al. 2004) that inserts site-specifically downstream of the terminal part of *glmS* encoding glucosamine synthetase, required for cell-wall synthesis (Vogler et al. 1989). This Tn7 insertion does not disrupt the *glmS* gene (Gringauz et al. 1988) and, indeed, no *Pseudomonas syringae* pv. *phaseolicola* fluorescent constructs created in this study showed any differences in phenotype compared with their progenitors.

We utilized technological advancements in confocal microscopy of AOTF and AOBs to improve further understanding of *Pseudomonas syringae* pv. *phaseolicola* bacterial colonization in planta. The standard band-pass filters available on the Zeiss Axiovert did not provide sufficient discrimination of the spectral overlap of HR autofluorescence and fluorescent proteins. Therefore, the high quality in-planta resolution obtained in this study depended greatly on the AOTF of the Leica TCS-SP2-DM IRE2, which enabled real-time adjustment of detection filter parameters to minimize the unwanted HR autofluorescence while maximizing fluorescent protein detection. The rapid change of excitation wavelengths using AOBs of the Zeiss Axiovert during CCID data collection enabled efficient high-throughput scanning of live plant tissue samples with minimal damage to either bacterial colonies or plant cells due to over-exposure of laser intensity. Such results confirm that the advances in confocal imaging technology continue to improve in-planta image acquisition.

Autofluorescence accumulating in TG as a result of the R3-HopAR1-mediated HR complicated detection of dsRFP and eCFP emission from bacteria. Although eGFP proved effective for visualization within the HR autofluorescence, eYFP was chosen for routine use because of its higher quantum yield, which enabled high-quality confocal image acquisition. Furthermore, the higher quantum yield of eYFP was beneficial because it allowed colony detection at lower laser excitation intensities, which resulted in less plant-tissue damage. *Pseudomonas syringae* pv. *phaseolicola* colonies expressing eYFP provided good contrast (Fig. 2A to C) for obtaining quantitative CCID on the percentage of the area of *Pseudomonas syringae* pv. *phaseolicola* colony development within the im-

aged mesophyll. Because CCID is dependent on aggregation and growth of fluorescent *Pseudomonas syringae* pv. *phaseolicola* cells into colonies, it is possible that individual cells were not fully represented by the confocal detection methods used in this study. However, because the CCID obtained in this study showed equivalent trends to *Pseudomonas syringae* pv. *phaseolicola* bacterial growth rate isolated in planta (Fig. 3), CCID was considered a rapid and accurate way to record and compare *Pseudomonas syringae* pv. *phaseolicola* survival in planta.

In addition to providing quantitative information, imaging allowed the shape of colonies and bacterial dispersal to be examined. During the HR, *Pseudomonas syringae* pv. *phaseolicola* colonies were generally compact and observed to be restricted in size compared with non-HR colony development (Fig. 2A to C). However, such colonies were often observed to become fragmented and release bacterial cells after prolonged incubation in TG (Fig. 1G). The breakdown of colony structure would expose individual bacteria directly to the adverse conditions within the apoplast. The dynamics of colony dispersal as indicated from this work may influence the evolution of new virulent strains in planta, as has been suggested previously (Arnold et al. 2007). Such exposure may kill off the avirulent bacteria and is likely to enhance the deletion of PPHGI-1 and, therefore, increase selection for cells lacking avirulence genes such as *hopAR1*. The detection of differentially labeled strains was also optimized using eYFP and dsRFP, but using mixtures required a compromise on excitation and emission parameters. We are currently developing strains suitably tagged in both PPHGI-1 and the chromosome to attempt to examine loss of the GI within plant tissues.

Imaging data consistently showed that *Pseudomonas syringae* pv. *phaseolicola* 1302A containing PPHGI-1 exhibited reduced colony formation compared with strain RJ3 without PPHGI-1 (Figs. 2D and 3). This result was expected in TG with 1302A harboring PPHGI-1 (and therefore *hopAR1*), in which induc-

Fig. 5. Comparison of in-planta confocal colony image data (CCID) from varying infiltration concentrations after 120 h. Results are standardized to an equivalent starting infiltration concentration (1.6×10^6 CFU/ml) to allow direct comparison after 120 h. *Pseudomonas syringae* pv. *phaseolicola* 1302A (enhanced yellow fluorescent protein [eYFP]-labeled and containing *hopAR1*) shows greatly reduced colony formation at low infiltration concentrations compared with strain RJ3 (eYFP-labeled and lacking *hopAR1*) in Tendergreen (TG). Data are means $\pm$ standard error of the mean ($n = 12$ each). Analysis of variance showed a significant effect of infiltration concentration on 1302A-eYFP compared with strain RJ3-eYFP ($P = 0.0277$, $df = 2$); significant differences among sample means were revealed by Student's *t*-test ($P = 0.05$) and are indicated by different letters above bars.

tion of the R3-HopAR1-mediated HR would occur and thus create a hostile environment inhibiting *Pseudomonas syringae* pv. *phaseolicola* growth. However, reduced colony formation by 1302A in CW was unexpected, given the lack of any known race-specific *R* genes in this cultivar. Similar reduction in colony development was observed with 1448A harboring PPHGI-1. These observations suggest that PPHGI-1 may harbor one or more additional genes responsible for eliciting plant basal defenses in *P. vulgaris*. Although basal and *R* gene-mediated defenses (Abramovitch et al. 2006) share some common molecular mechanisms (Sun et al. 2006), the most characteristic feature of *R* gene-mediated defenses is induction of the HR (Lam 2004). There was no evidence of *Pseudomonas syringae* pv. *phaseolicola* strains 1302A or 1448A::PPHGI-1 showing HR-like symptoms, including accumulation of autofluorescent compounds in CW, so reduced fitness was unlikely to be a result of a R-Avr interaction. It is possible that PPHGI-1 contains one or more genes that encode MAMPs activated in both TG and CW basal defenses and, therefore, may account for the reduced fitness of *Pseudomonas syringae* pv. *phaseolicola* strains harboring PPHGI-1. With the exception of *hopAR1*, bioinformatic analysis of PPHGI-1 reveals no obvious candidates that would elicit known MAMP-activated basal resistance; however, there are many transmembrane pattern recognition receptors in plants that are not yet fully characterized (Jones and Dangl 2006).

Unless newly acquired GI encode their own specific regulators or are integrating into pre-existing regulatory networks (Gal-Mor and Finlay 2006), such foreign DNA acquisition may lead to an initial fitness loss of the bacterial host due to the host bacterium needing time to adapt to the acquisition of a new GI (Dobrindt et al. 2004). Because there is no evidence to show that *Pseudomonas syringae* pv. *phaseolicola* 1448A has harbored PPHGI-1 (or similar GI) previously, the effects of PPHGI-1 integration in 1448A::PPHGI-1 was regarded as de novo behavior and not the result of a previous adaptation to a long-term interaction between GI and host. Interestingly, 1448A::PPHGI-1 shared similar trends to strain 1302A (in both CW and TG), with reduced colony formation as a result of harboring PPHGI-1, suggesting that 1302A has only recently acquired PPHGI-1 and, therefore, PPHGI-1 may be a nonadapted integrative nucleotide sequence causing an in-plant fitness compromise. It is interesting that no in vitro difference is observed between 1302A and strain RJ3 or 1448A and 1448A::PPHGI-1, which would suggest the reduced growth is not due to the metabolic burden of harboring PPHGI-1 but, rather, a direct result of one or more plant responses.

Many of the unidentified open reading frames (ORF) in PPHGI-1 may be associated with fitness gain in the exploitation of new environmental niches (Pitman et al. 2005). The growth and survival of *Pseudomonas syringae* pv. *phaseolicola* 1302A under field conditions will involve its ability to either tolerate or escape, or both, the internal and external leaf environmental stresses, including desiccation, nutrient deprivation, exposure to ultraviolet radiation, and oxidative stress encountered both from exposure to UVA radiation and from the plant defense response. Predicted ORF identified in PPHGI-1 (Pitman et al. 2005) that could aid *Pseudomonas syringae* pv. *phaseolicola* under such situations include those encoding i) type IV pilus, known for their importance in host colonization by aiding translocation over moist surfaces by the so-called twitching motility (Mattick 2002); ii) bacteriophytochrome, belonging to a class of light-responsive proteins involved with signaling pathways in changing light levels (Davis et al. 1999); iii) SOS response and DNA repair proteins, potential candidates for UV tolerance and survival in direct sunlight (Nohmi 2006); iv) chemotaxis proteins, known to be important in plant

colonization for regulating bacterial motility in response to environmental signals (Danhorn and Fuqua 2007); and v) histidine kinase and response regulators, involved in two-component systems that induce a multitude of gene-regulatory systems in response to environmental changes (Beier and Gross 2006). In addition, many novel genes are identified in PPHGI-1 that have no detectable homologs and therefore currently have unknown function. Such novel GI genes may be considered possible candidates for conferring selective advantage to the host organism (Hsiao et al. 2005) and warrant future investigation.

In conclusion, this work provides a significant contribution to the understanding of endophytic colonization by establishing efficient methodology for temporal studies of *Pseudomonas syringae* pv. *phaseolicola* colony development within the apoplast of both healthy *P. vulgaris* leaf tissue and tissue undergoing the HR. Our preliminary experiments with *Arabidopsis* and tomato indicate that the same imaging methods will be applicable to other bacterium-plant interactions. Furthermore, we have shown that, while GI may prove to be of great evolutionary benefits under certain environmental conditions, under others, they may be disadvantageous.

MATERIALS AND METHODS

Bacterial strains and techniques.

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria Bertani (LB) media (Sambrook et al. 1989) and *Pseudomonas* strains at 25°C in LB or King's B (KB) (King et al. 1954). Antibiotics were used at the following concentrations (µg/ml): Gm, 10; kanamycin, 50; ampicillin, 100; nitrofurantoin (NF), 100; cycloheximide (CHX), 100; and rifampicin, 100. Polymerase chain reaction of PPHGI-1 circular intermediate and plasmid profiling of *Pseudomonas syringae* pv. *phaseolicola* strains in comparison with *E. coli* 39R861 were performed as described by Lovell and associates (2009). Determination of PPHGI-1 evolutionary loss by passaging through TG and termination of HR disease phenotypes in TG and CW leaves and pods was performed as by Pitman and associates (2005).

Chromosomal introduction and visualization of fluorescent proteins.

Fluorescent protein variants (eYFP, AKN069 [Table 1]; eGFP, AKN100; eCFP, AKN033; and dsRFP, AKN132) were chromosomally introduced into 1302A and 1448A strains via the Tn7 transposon delivery system, as described previously by Lambertson and associates (2004). Gentamicin-resistant *Pseudomonas syringae* pv. *phaseolicola* strains (Table 1) had respective fluorophore expression in colonies confirmed using a Leica TCS4D dissecting microscope (Leica Microsystems Wetzlar GmbH).

In planta *Pseudomonas syringae* pv. *phaseolicola* infiltrations.

P. vulgaris bean cultivars TG and CW were grown at 23°C 95% humidity, and artificial light was maintained for 16-h periods within the 24-h cycle. In preparation for plant inoculations, overnight bacterial cultures were washed twice and were resuspended in one-quarter Ringers solution. Single or mixed bacterial inoculums were diluted in one-quarter Ringers to one of the following final concentrations: i) 0.6 optical density at 600 nm (OD₆₀₀) (approximately 5×10^8 CFU/ml), ii) 0.1 OD₆₀₀ (8×10^7), iii) 1×10^{-2} OD₆₀₀ (8×10^6), iv) 1×10^{-3} OD₆₀₀ (8×10^5), v) 1×10^{-4} OD₆₀₀ (8×10^4), or vi) 1×10^{-5} OD₆₀₀ (8×10^3). Desired inocula were syringe-infiltrated into the mesophyll of the primary leaf and were incubated for up to 144 h as required. Infiltrated leaf tissue was excised immediately prior to analysis.

In planta confocal imaging.

Sections of infiltrated *P. vulgaris* leaves (approximately 5 mm²) were removed, and the slides were mounted in double-distilled H₂O (lower epidermis toward objective) under a 0.17-mm coverslip. The leaf mesophyll was imaged using one of two confocal microscopes. i) For high-quality image acquisition, the Leica TCS-SP2-DM IRE2 confocal laser scanning microscope (Leica Microsystems Wetzlar GmbH) with AOTF was used at 25× or 40× (objective magnification) for colony morphology and 100× for visualization of individual cell dispersal. Variable AOTF filters were used for the following fluorophores (excitation/emission): eYFP (514 nm/525 to 600 nm), eCFP (440/465 to 495 nm), eGFP (488/516 to 539 nm); dsRFP (568/600 to 644 nm), and for plant autofluorescence (440/650 to 785 nm). Z-series imaging was performed at intervals of between 0.3 (individual cells) and 1 μm (colonies). ii) For CCID collection, the Zeiss Axiovert 200 was used in conjunction with the AOBs Ultraview FRET H rapid confocal imaging system (Perkin Elmer Instruments Ltd., Beaconsfield, Buckinghamshire, England) with z-series imaging at 1-μm intervals. Band-pass filter sets were used for respective fluorophore detection (excitation/filter/emission): eCFP (440 nm/CFP_Cy5/450 to 470 and 660 to 750 nm); eGFP (488 nm/GFP/495 to 562 nm); eYFP (514 nm/YFP/520 to 650 nm); dsRFP (568 nm/DAPI_mRFP/412 to 480 nm and 660 to ≥750 nm); and plant autofluorescence (440 nm/DAPI_mRFP/412 to 480 nm and 660 to ≥750 nm). For consistency, all CCID were obtained at 10×, using identical confocal parameters between infiltration variables.

CCID.

CCID was used as a means of quantifying confocal observations to allow objective and statistical comparison between infiltration variables. Three randomly selected 5-mm² sections of *Pseudomonas syringae* pv. *phaseolicola* eYFP-infiltrated tissue from the same leaf were examined at 10× (objective magnification), using the Zeiss confocal as above. random areas were imaged 12 times (50× z-sections at 1-μm intervals) from each leaf section and were combined (extended depth of field view) to represent a two-dimensional confocal image of all fluorescent *Pseudomonas syringae* pv. *phaseolicola* cells from each focal plane through the leaf mesophyll. Using ImageJ software (Abramoff et al. 2004), threshold algorithms converted confocal images into pixels and assigned eYFP emission pixels as white and non-eYFP emission pixels (plant material) as black (Fig. 2A to C). Quantification of pixel data enabled calculation of the percentage of white pixels (eYFP), which was expressed as the percent eYFP colony formation area within the mesophyll. The CCID percentage data presented was analyzed using a single factor analysis of variance test ($P < 0.05$) and standard error of the mean, using JMP IN 7.0 analysis software.

Pseudomonas syringae pv. *phaseolicola*

bacterial growth rate determined from plant tissue extract.

To compare CCID with viable eYFP-expressing bacterial cells present, a 5-mm diameter cork borer was used to extract infiltrated tissue adjacent to tissue samples analyzed by confocal imaging. Samples were pestle-ground in 500 μl of one-quarter Ringers solution, and serial dilutions were placed onto KB agar supplemented with CHX (to prevent fungal contamination) and NF (to select for *Pseudomonas syringae* pv. *phaseolicola*). Dilutions were also placed on KB agar supplemented with CHX, NF, and Gm (select for Tn7 eYFP inserts). All colonies were observed for correct colony morphology (compared with *Pseudomonas syringae* pv. *phaseolicola* 1302A and strain RJ3), and random colonies were pod-stab assayed to

ensure correct *P. vulgaris* disease and HR phenotypes. Furthermore, all gentamicin-resistant colonies were assessed for eYFP expression using fluorescent stereo microscopy. Quantification of total colonies and gentamicin-resistant colonies provided percent eYFP survival.

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