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Infection assays in *Arabidopsis* reveal candidate effectors from the poplar rust fungus that promote susceptibility to bacteria and oomycete pathogens

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SUMMARY

Fungi of the Pucciniales order cause rust diseases which, altogether, affect thousands of plant species worldwide and pose a major threat to several crops. How rust effectors—virulence proteins delivered into infected tissues to modulate host functions—contribute to pathogen virulence remains poorly understood. *Melampsora larici-populina* is a devastating and widespread rust pathogen of poplar, and its genome encodes 1184 identified small secreted proteins that could potentially act as effectors. Here, following specific criteria, we selected 16 candidate effector proteins and characterized their virulence activities and subcellular localizations in the leaf cells of *Arabidopsis thaliana*. Infection assays using bacterial (*Pseudomonas syringae*) and oomycete (*Hyaloperonospora arabidopsidis*) pathogens revealed subsets of candidate effectors that enhanced or decreased pathogen leaf colonization. Confocal imaging of green fluorescent protein-tagged candidate effectors constitutively expressed in stable transgenic plants revealed that some protein fusions specifically accumulate in nuclei, chloroplasts, plasmodesmata and punctate cytosolic structures. Altogether, our analysis suggests that rust fungal candidate effectors target distinct cellular components in host cells to promote parasitic growth.

Keywords: confocal microscopy, fungus, obligate biotroph, parasite, virulence assays.

INTRODUCTION

Plant-associated organisms secrete effectors that alter the host's cellular structure and function to promote colonization (Hogenhout *et al.*, 2009). The plethora of plant processes modulated by effectors indicates that effectors target most, if not all, cell structures (Giraldo and Valent, 2013; Petre *et al.*, 2015b, 2016), and that the study of effectors is useful to deepen our understanding of pathogenic processes (Win *et al.*, 2012). Specifically, effectors are used to probe plant functions that govern plant resistance (i.e. immune response-related pathways) or plant susceptibility (i.e. susceptibility genes). The identification and manipulation of such functions are key to the development of resistant crops (Dangl *et al.*, 2013). Using effectors as probes of the plant proteome to pinpoint the precise protein targeted by an effector may lead to the identification of susceptibility genes in the host. Alteration of these genes, which are critical for compatibility, could provide durable resistance.

Obligate biotrophic pathogens must evade and/or suppress host recognition for a lengthy period of time to complete their life cycle whilst their host remains alive. This leads to an intricate battle in which the pathogen rewires the host's cells to meet its needs and to thwart host defences. The suppression and evasion of innate immunity are undoubtedly important aspects of obligate biotrophy; they are, however, only a starting point in the establishment of a successful infection. Indeed, it has emerged recently that effectors also target cellular structures and processes that are not directly related to the plant immune system, thus suggesting that pathogens not only suppress immune responses, but also achieve a deep manipulation of their hosts (reviewed in Chaudhari *et al.*, 2014; Giraldo and Valent, 2013; Win *et al.*, 2012).

Rust fungi have a complex life cycle, cannot be cultured *in vitro* and are not easily amenable to transformation, three traits hindering functional investigations. Moreover, these fungi do not infect model plants such as *Arabidopsis thaliana* (Lawrence *et al.*, 2010), and consequently, very little is known about the molecular basis of their pathogenicity (Petre *et al.*, 2014). *Melampsora larici-populina* is the causative agent of poplar leaf rust, which has had devastating consequences on poplar plantations worldwide (Duplessis *et al.*, 2009, 2011b; Feau *et al.*, 2007; Hacquard *et al.*, 2011). In order to assess the full weaponry at the pathogen's disposal,

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thorough genomic information is required. The combination of transcriptomic and genomic analyses has provided a first glimpse into the putative effector arsenal of *M. larici-populina* (Duplessis *et al.*, 2011a; Hacquard *et al.*, 2010, 2012; Joly *et al.*, 2010). Hacquard *et al.* (2012) identified and annotated 1184 small secreted proteins (SSPs) which they highlighted as candidate secreted effector proteins (CSEPs). Most of these putative effectors show no sequence similarity to proteins from species outside of Pucciniales and are found in multigene families. A subset of this CSEP repertoire is expressed in haustoria (Hacquard *et al.*, 2010, 2012; Joly *et al.*, 2010), and is thus probably enriched in proteins which are effectively delivered inside poplar cells during infection.

Most of the progress made in effector biology during the last decade has relied on heterologous systems, enabling the study of effectors in plant cells (Fabro *et al.*, 2011; Rafiqi *et al.*, 2012; Sohn *et al.*, 2007). *Agrobacterium*-mediated heterologous expression or *Pseudomonas*-mediated delivery of tagged effectors in leaf cells are methods of choice for the study of effectors *in planta* (Torto *et al.*, 2003; Vleeshouwers *et al.*, 2008). Confocal imaging of green fluorescent protein (GFP) fusions has been widely used to determine the cellular compartments targeted by effectors (Caillaud *et al.*, 2012; Gaouar *et al.*, 2016; Schornack *et al.*, 2010; Takemoto *et al.*, 2012). More recently, *Nicotiana benthamiana* has been used to identify cellular compartments targeted by *M. larici-populina* candidate effectors as well as putative plant-interacting proteins (Petre *et al.*, 2015b). Effector delivery systems can be coupled with pathogen growth assays to identify effectors possessing virulence activities (Dou *et al.*, 2008; Fabro *et al.*, 2011). However, the use of stable *Arabidopsis* lines has several advantages over transient assays in *N. benthamiana*: it offers the possibility to use well-characterized genetic tools, such as knock-out lines of putative interactors, to search for phenotype similarities; it also enables the assessment of protein localization in guard cells, which are not transformed in transient assays; and it renders possible the generation of stable single insertion homozygous lines which can be used further for crosses, transcriptomic or metabolomic studies.

In this study, we investigated a set of 16 CSEPs of the poplar rust fungus *M. larici-populina* by measuring their ability to promote bacterial and oomycete pathogen growth in a heterologous system. These CSEPs were stably transformed in *A. thaliana* and these lines were used for confocal imaging to assess the subcellular localization of candidate effectors *in planta*. Here, we demonstrate that CSEPs target various subcellular structures and promote pathogen colonization of leaves.

RESULTS

Selection of 16 *M. larici-populina* CSEPs

We selected 16 CSEPs from a set of 1184 SSPs described previously (Hacquard *et al.*, 2010, 2012) (Table S1, see Supporting Information). All selected CSEPs were less than 300 amino acids in length, had no sequence similarity with proteins from species outside the Pucciniales order, showed induction of the transcript expression during poplar leaf infection and reflected the diversity of CSEP families in *M. larici-populina*. Among the most informative sources of expression data were a haustoria-specific cDNA library and a transcriptomic analysis of laser-microdissected, rust-infected poplar leaves (Hacquard *et al.*, 2010; Joly *et al.*, 2010). Several of the selected CSEPs (MLP72983, MLP106078, MLP123218, MLP106078, MLP102036, MLP123531, MLP124305, MLP124518, MLP123531, MLP124305, MLP124466 and MLP102036) displayed a very high expression ratio in microdissected palisade mesophyll enriched in haustoria and infection hyphae when compared with microdissected uredinia, mostly composed of spores and sporogenous hyphae (Hacquard *et al.*, 2010). MLP124497, MLP124499 and MLP124518 [Mlp0032_0018] are different members of the same CSEP family, previously designated as CPGH1 (Hacquard *et al.*, 2012) which is composed of very small proteins (<100 amino acids) presenting signatures of positive selection and being over-represented in the haustorial library (Hacquard *et al.*, 2012; Joly *et al.*, 2010). MLP37347 was selected for its homology to AvrL567, a previously reported effector of the flax rust fungus *Melampsora lini* (Dodds *et al.*, 2004). MLP123227 (SSP15) shows a bimodal expression profile *in planta* and immunolocalization data indicate that it has an intriguing localization pattern *in planta*, in both haustoria and sporogenous hyphae (Hacquard *et al.*, 2012). The last two CSEPs (MLP123437 and MLP124111) belong to different classes of SSP conserved in *Puccinia* spp. (classes VI and III; Hacquard *et al.*, 2012) and display a conserved exon/intron structure and a characteristic spacing of cysteine residues (including the presence of Y/FxC motifs) (Hacquard *et al.*, 2012).

Three CSEPs promote the growth of *Pseudomonas syringae in planta* independently of the suppression of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI)

To test whether the 16 CSEPs can promote pathogen growth, we performed infection assays using the *P. syringae* pv. *tomato* DC3000–*A. thaliana* pathosystem (Sohn *et al.*, 2007). In this system, the sequences coding for the mature form of the CSEP, i.e. without the predicted signal peptide, are fused to the N-terminus of AvrRPM1 to drive their translocation inside host cells via the type III secretion system (T3SS) apparatus of *P. syringae*. Five of the 16 CSEPs significantly increased bacterial growth *in planta* (MLP124499, MLP102036, MLP124266, MLP124497, MLP106078) (Fig. 1A). Conversely, two candidate effectors decreased bacterial growth (MLP123227 and MLP37347), whereas the other nine did not affect bacterial growth when compared with the control. Standard growth assays confirmed that the CSEPs did not affect the rate of bacterial cell division (data not shown), thus suggesting

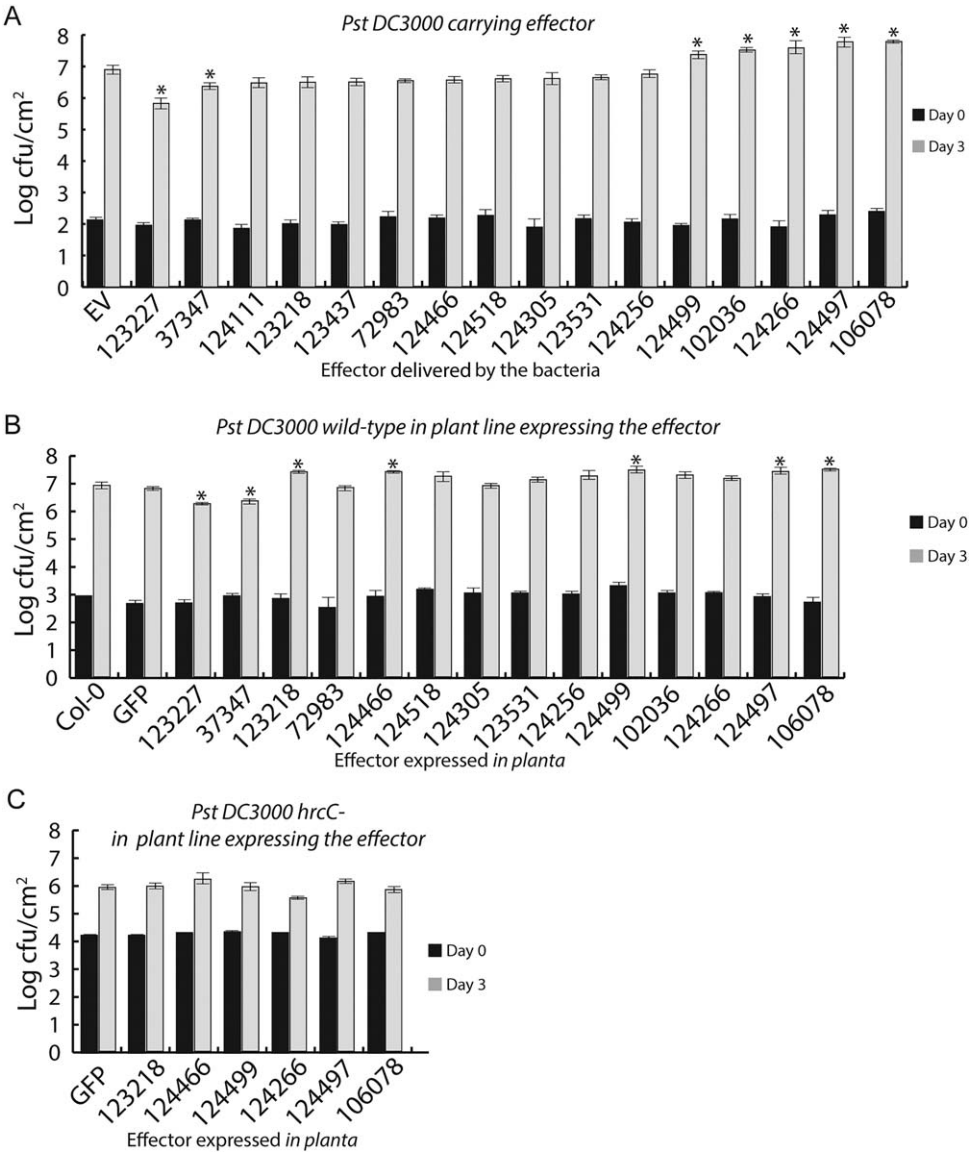

Fig. 1 Bacterial growth *in planta* differs when effectors are delivered by the bacterium or are constitutively expressed. (A) Growth of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 without (EV) or with candidate effectors (all other lanes) was measured on the day of infection and 3 days after infection of 4-week-old plants by leaf infiltration. A bacterial suspension with an optical density at 600 nm (OD_{600}) of 0.001 was used as inoculum. Statistical significance was evaluated using Student's *t*-test ($P < 0.05$); asterisks indicate statistically significant differences between the effector and empty vector. The experiment was repeated at least three times (a representative experiment is shown). (B) Growth of *Pst* DC3000 wild-type-in plants expressing an effector, Col-0 or Col-0 expressing green fluorescent protein (GFP) was measured on the day of infection and 3 days after infection of 4-week-old plants by leaf infiltration. A bacterial suspension with $OD_{600} = 0.001$ was used as inoculum. Statistical significance was evaluated using Student's *t*-test ($P < 0.05$); asterisk indicates statistically significant difference between plants carrying effector and Col-0 expressing GFP. Five replicates were included for each genotype. To facilitate the comparison with Fig. 1A, each transgenic line is presented in the same order. The experiment was repeated at least three times (a representative experiment is shown). (C) Growth of *Pst* DC3000 hrcC– strain in infected plants expressing an effector, Col-0 or Col-0 expressing GFP was measured on the day of infection and 3 days after infection of 4-week-old plants by leaf infiltration. A bacterial suspension with $OD_{600} = 0.001$ was used as inoculum. Statistical significance was evaluated using Student's *t*-test ($P < 0.05$); asterisk indicates statistically significant difference between plants carrying effector and Col-0 expressing GFP. Five replicates were included for each genotype. The experiment was repeated at least three times (a representative experiment is shown). Note that no transgenic lines were recovered for 123437 and 124111. cfu, colony-forming unit.

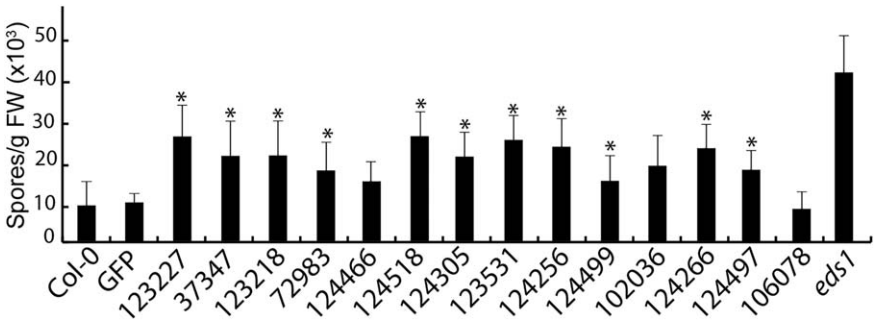

Fig. 2 Most candidate effectors affect the growth of *Hyaloperonospora arabidopsidis* Noco2 when constitutively expressed *in planta*. Two-week-old soil-grown plants were inoculated with *H. arabidopsidis* Noco2 at a concentration of 20 000 conidiospores/mL and the number of conidiospores was quantified 7 days after inoculation. Bars represent the mean of four replicates. Statistical significance was established using Student's *t*-test ($P < 0.05$), and statistically significant differences are represented by an asterisk between plants carrying an effector and Col-0 expressing green fluorescent protein (GFP). Each transgenic line is ordered as in Fig. 1 for easier comparison. The experiment was repeated five times (a representative experiment is shown). FW, fresh weight.

that the alteration of *in planta* bacterial growth was caused by the effect of the CSEPs on plant cells. We conclude that five CSEPs promote the growth of *P. syringae* when delivered by the bacterium, suggesting that they have a virulence activity in leaf cells.

To complement the effector delivery assays, we generated stable *A. thaliana* transgenic lines constitutively expressing CSEP–GFP fusions. Transgenic lines were generated for 14 CSEPs, whereas lines expressing MLP124111 and MLP123437 could not be retrieved. We then performed infection assays using wild-type *P. syringae* pv. *tomato* DC3000 on the aforementioned transgenic lines. Five of the 14 CSEPs promoted bacterial growth (MLP123218, MLP124466, MLP124497, MLP124499 and MLP106078), whereas two (MLP37347 and MLP123227) showed less growth compared with control plants (Fig. 1B). The combined results from these two experiments indicate that three CSEPs (MLP124499, MLP124497, MLP106078) promote bacterial growth, whereas two (MLP37347 and MLP123227) reduce bacterial growth in both assays.

In order to understand how the CSEPs MLP123218, MLP124466, MLP124266, MLP124497 and MLP106078 contribute to an increase in bacterial growth, we infected them with the T3SS-deficient bacterial strain *P. syringae* pv. DC3000 *hrcC*–. Although this strain is unable to produce a functional T3SS and inject effectors in host cells, it still carries PAMPs that will initiate PTI. Therefore, we monitored bacterial growth at days 0 and 3 in transgenic lines, Col-0 and a transgenic line expressing GFP without any effector (control). The results shown in Fig. 1C demonstrate that *P. syringae* pv. DC3000 *hrcC*– growth is not affected by the effectors when compared with the control. Taken together, these results suggest that PTI is not suppressed by the effectors that contribute to increased bacterial growth.

Eleven CSEPs promote the growth of *Hyaloperonospora arabidopsidis in planta*

As *M. larici-populina* is an obligate biotrophic filamentous pathogen, we sought to test whether the CSEPs would affect the growth of a pathogen with a similar lifestyle. As no rust infect *A. thaliana*, we used the oomycete *Hyaloperonospora arabidopsidis* to challenge the transgenic plants constitutively expressing the candidate effectors. In total, 11 of 14 transgenic lines expressing CSEPs supported greater *H. arabidopsidis* sporulation than control plants (mean ratio of two, 15 000–25 000 vs. 10 000 spores/g of leaf tissue) (Fig. 2). However, no CSEP-GFP line promoted the growth of *H. arabidopsidis* at a comparable level with the infection control line *eds1*, which is hypersensitive to *H. arabidopsidis*. We conclude that the majority (>75%) of the tested CSEPs promote *H. arabidopsidis* growth on *A. thaliana*.

Only two CSEPs (MLP124497 and MLP124499) promote pathogen growth in the three infection assays

In order to evaluate the consistency of the virulence activities detected for the CSEPs, we compared the results from the three sets of infection assays. This analysis revealed that all CSEPs promoted pathogen growth in at least one experiment, three did so in the two experiments involving *P. syringae*, whereas only two (MLP124497 and MLP124499) promoted pathogen growth in all three experiments (Figs 1–3). Conversely, although two candidate effectors consistently reduced the growth of *P. syringae* in both *in planta* experiments, they did not affect *H. arabidopsidis* growth. Some candidate effectors did not alter bacterial growth (MLP124256, MLP124518, MLP124266, MLP123531, MLP124305 and MLP72983), but did significantly promote *H. arabidopsidis* growth *in planta* (Figs 1–3). Interestingly, the CSEP that promoted the highest bacterial growth (MLP106078) did not significantly enhance *H. arabidopsidis* growth. Based on these results, we conclude that MLP124497 and MLP124499 promote the growth of unrelated pathogens in *A. thaliana*, whereas other CSEPs exhibit a pathogen-specific growth-promoting effect.

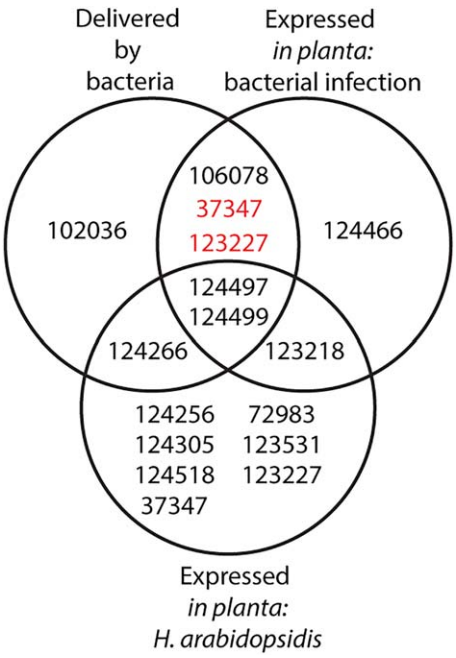

Fig. 3 Venn diagram showing the candidate effectors that induced increased virulence (black) or decreased virulence (red). Note that no transgenic lines were recovered for 123437 and 124111.

The transgenic line 124499 shows delayed senescence

We assessed the phenotypes of the transgenic lines to determine whether they displayed any growth or morphological alteration that could be caused by the expression of the fungal genes. To this end, the plants were grown at 20 °C as well as at 16 °C, and senescence was monitored as they aged. Figures S1 and S2 (see Supporting Information) show representative plants from each transgenic line when grown at 20 °C (Fig. S1) and 16 °C (Fig. S2). Most lines did not show any striking phenotype, except for MLP124499 plants, whose leaves remained green much longer than those of all the other transgenic and Col-0 plants. To ensure that this was not a positional effect in the flat, we started a new flat with alternating rows of Col-0 and MLP124499 plants (Fig. S3, see Supporting Information; tagged as H1), and again observed that the rows of MLP124499 remained green, when the Col-0 plants had dried completely. We conclude that, except for the transgenic line MLP124499, the phenotype of CSEP expressing plants was unaffected.

Three candidate effectors specifically accumulate in chloroplasts, cytosolic bodies and plasmodesmata

To gain further insight into the growth-promoting effect of CSEPs, we used confocal microscopy to determine the subcellular localization of the CSEP-GFP fusions in leaf epidermal cells (i.e. guard and pavement cells) obtained from *A. thaliana* stable transgenic lines. One candidate effector specifically accumulated in chloroplasts (MLP72983) and two in cytosolic puncta (MLP37347, MLP124305) (Fig. 4A). The fluorescence signal of MLP72983 was distributed homogeneously within the chloroplasts, suggesting that it accumulates in the stroma. The fluorescence signal of MLP37347 was restricted to small, bright and circular cytosolic structures of approximately 1 μm in diameter and of unknown nature, whereas the fluorescence signal of MLP124305 accumulated in small cytosolic dots that were excluded from nuclei (Fig. 4A). In order to investigate the nature of these cytosolic foci, we performed co-localization experiments of MLP37347 and MLP124305 with PDCB1-mCherry, a plasmodesmata marker, and RBP47b-CFP, a stress granule marker. MLP37347-GFP and PDCB1-mCherry fluorescent signals overlapped in punctate structures, indicating that this CSEP accumulates at plasmodesmata (Fig. 4B). MLP124305 did not localize to plasmodesmata or stress granules (not shown). To find the true localization of MLP124305-GFP, we crossed the transgenic line overexpressing MLP124305-GFP with DCP1-CFP (a processing body marker), but did not observe co-localization; therefore, we do not know what is the structure targeted by this effector. The 11 remaining CSEPs showed a non-informative distribution in the cytosol and in the nucleus, similar to the free GFP control (Fig. 4A). Anti-GFP western blots revealed a single band signal at the expected size for all but one CSEP-GFP, suggesting that the fusions were not cleaved or modified in leaf cells (Fig. S4, see Supporting Information). MLP124305 yielded multiple bands of lower molecular weight, suggesting protein degradation (data not shown). We conclude that, of the 16 CSEPs, three target specific cellular structures in leaf cells: certain undefined cytosolic puncta, chloroplasts and plasmodesmata.

DISCUSSION

In this study, we used *A. thaliana* as a heterologous system to perform the functional analysis of a subset of 16 CSEPs from the poplar leaf rust fungus *M. larici-populina*. Notably, we identified several CSEPs that promote bacterial or oomycete pathogen growth *in planta*, as well as some that accumulate in distinct plant cell compartments (Table 1). These proteins were flagged as putative virulence factors that needed to be investigated further.

Functional studies of rust fungi are challenging because of the obligate biotrophic status of these pathogens, and high-throughput 'effectoromics' approaches are needed (Petre *et al.*, 2014, 2015a, b). Such procedures are not fully available yet in most rust pathosystems. We sought to partially fulfil this need by the use of heterologous systems, a strategy increasingly being used in rust effector biology (Petre *et al.*, 2015a, b, 2016). To this end, we created a collection of *A. thaliana* transgenic lines to investigate the subcellular localization and virulence activities of rust CSEPs, complementing the efforts accomplished thus far

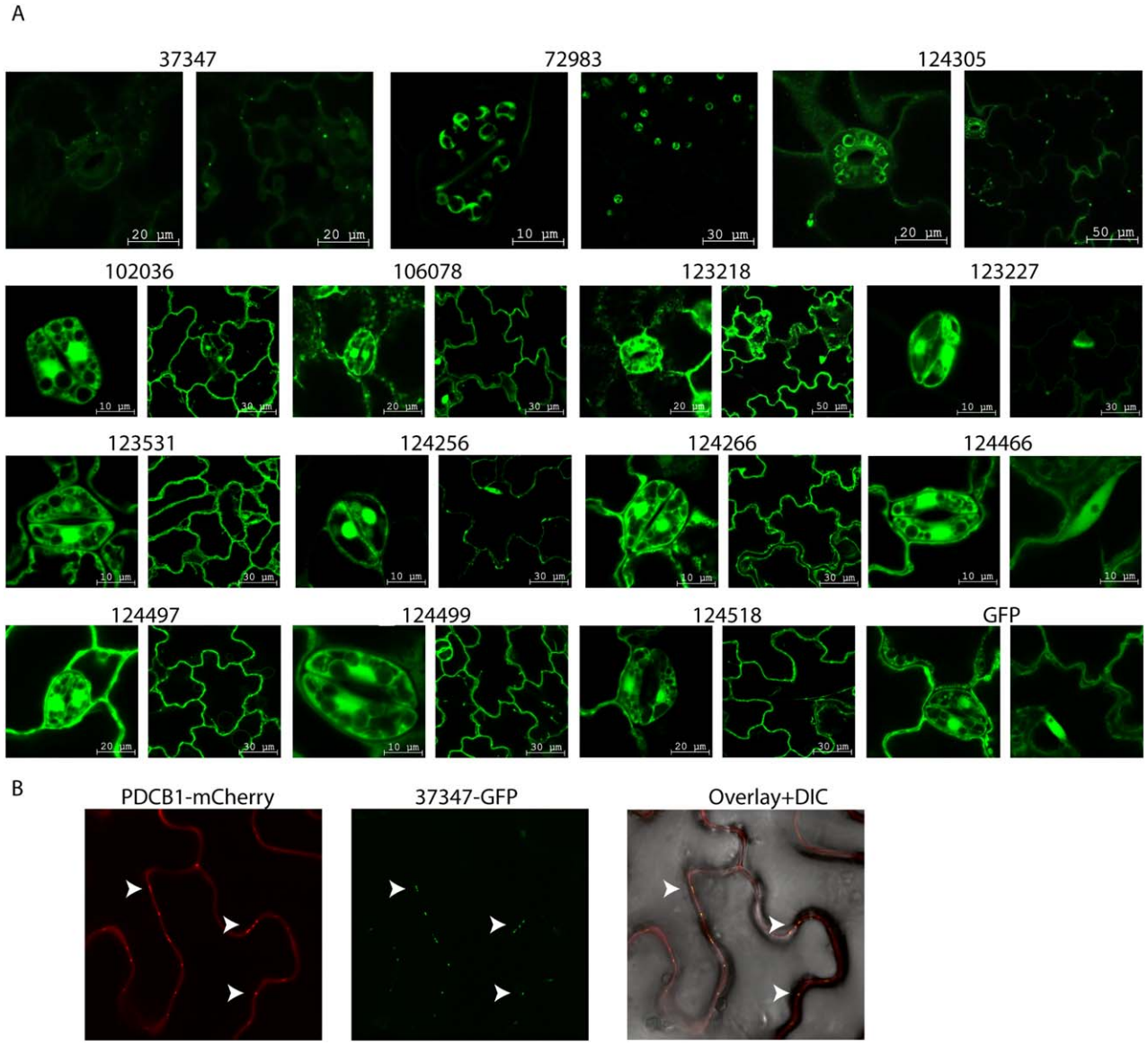

Fig. 4 Subcellular localization of the different candidate effectors. (A) Confocal images of leaf epidermal cells of 7-day-old transgenic plantlets expressing candidate effectors fused to green fluorescent protein (GFP) (SSP-GFP) in the Col-0 genetic background. The top three effectors are those displaying informative localization, whereas the bottom three rows display the effectors showing nucleocytoplasmic localization. (B) Co-localization between PDCB1-mCherry, a plasmodesmata marker, and 37347-GFP to infer its localization. Left panel, red channel; centre panel, green channel; right panel, overlay with differential interference contrast (DIC).

using *N. benthamiana* as a heterologous system. These lines represent a valuable resource for the community. For instance, they could be used in proteomic approaches to find interaction partners, confirming co-immunoprecipitation results from *N. benthamiana*, and/or to study the impact of effectors on the plant transcriptome and metabolome.

Ideally, the confirmation that a SSP is a bona fide effector requires the demonstration that it has the capacity to interfere with a host component to ultimately favour pathogenesis. Hence, we used pathogen growth as a proxy to measure the effect of these CSEPs on host cells. Three of the 16 candidate effectors tested (18%) robustly enhanced bacterial growth *in planta*. In contrast, when using a similar experimental set-up, Fabro *et al.* (2011) showed that 70% of *H. arabidopsidis* candidate effectors set caused an increase in bacterial growth. There are two possibilities that may explain the difference in number between this study and our results. First, our assays were performed in a non-host plant, in which *M. larici-populina* CSEPs might not be functional; second, Fabro *et al.* (2011) used RxLR candidate effectors that are all likely to be host translocated, whereas our portfolio may have

Table 1 Features of the Melampsora larici-populina candidate effectors investigated in this study.

Protein ID*	CPG or class (number of members)†	Protein (signal peptide) length‡	Cysteine residues§	Evidence of expression¶	Signature of positive selection**	Homology to *M. lini* HESPs††	Homology in *Pgt*††	Effect on pathogen growth‡‡	Subcellular localization
37347	–	152 (23)	2	OA	–	AvrL567	–	–, –, +	Plasmodesmata
72983	CPG332–CPG333 (13)	220 (26)	8	S, I, H, OA, A3	–	–	Yes	N, N, +	Chloroplast
102036	CPG2528 (5)	107 (25)	0	H, OA, A3	–	–	–	+, N, N	Nucleocytoplasmic
106078	–	137 (21)	10	I, OA, A3	–	–	–	+, +, N	Nucleocytoplasmic
123218	CPG543 (7)	209 (19)	6	I, OA, A3	–	–	Yes	N, +, +	Nucleocytoplasmic
123227 (SSP15)	CPG1059 (2)	124 (24)	3	I, OA	–	–	–	–, –, +	Nucleocytoplasmic
123437	Class VI (6)	180 (20)	11	I, H, OA	–	HESP-C49	Yes	N	Not available
123531	CPG4557 (2)	102 (21)	8	I, OA, A3	–	–	–	N, N, +	Nucleocytoplasmic
124111	Class III (8)	135 (21)	10	H	–	–	Yes	N	Not available
124256	CPG5464 (13)	89 (25)	6	I	Yes	AvrP4	–	N, N, +	Nucleocytoplasmic
124266	CPG5464 (13)	92 (25)	7	I, H	Yes	AvrP4	–	+, N, +	Nucleocytoplasmic
124305	CPG5184 (2)	147 (25)	12	OA, A3	–	–	–	N, N, +	Cytoplasmic foci
124466	–	76 (24)	0	H, OA, A3	–	–	–	N, +, N	Nucleocytoplasmic
124497	CPGH1 (33)	77 (21)	4	I, H, OA	Yes	–	–	+, +, +	Nucleocytoplasmic
124499	CPGH1 (33)	72 (21)	3	I, H	Yes	–	–	+. +, +	Nucleocytoplasmic
124518	CPGH1 (33)	76 (21)	3	I, H, OA, A3	Yes	–	–	N, N, +	Nucleocytoplasmic

*Best predicted gene model in the *Melampsora larici-populina* isolate 98AG31 JGI genome sequence.

†Families, clusters of paralogous genes (CPGs) and classes of small secreted proteins as described in Duplessis *et al.* (2011a) and Hacquard *et al.* (2011). Number in parentheses indicate the number of members in gene families.

‡Numbers of amino acids are indicated.

§Number of cysteine residues in the mature form of the protein (i.e. without signal peptide).

¶Evidence of expression among S (expressed sequence tags from resting spores; Duplessis *et al.*, 2011a), I (Roche 454 data from infected leaves; Hacquard *et al.*, 2012), H (expressed sequence tags from isolated haustoria; Joly *et al.*, 2010), OA (oligoarray data from infected leaves; Duplessis *et al.*, 2011a) and A3 (most highly up-regulated transcripts from oligoarray data in the palisade mesophyll versus sporogenous microdissected structures; Hacquard *et al.*, 2010).

**As described in Hacquard *et al.* (2012).

††Homology searches were carried out against the UNIPROT and *Puccinia* Group Database using BLASTP (E value $<$ 1e-6). *Pgt*, *Puccinia graminis* f. sp. *tritici*.

‡‡The first position indicates the effect on bacterial growth when the effector is delivered by *Pseudomonas syringae* pv. *tomato* (*Pst*); the second position denotes the effect on bacterial growth when the effector is expressed *in planta*; the third position denotes the effect on *Hyaloperonospora arabidopsidis* (*Hpa*) growth when the effector is expressed *in planta*. +, positive effect; N, no effect; –, negative effect.

included apoplastic candidate effectors or even non-effectors that are predicted secreted proteins and remain in the apoplastic space of the native poplar–poplar rust pathosystem.

Bacterial infection assays revealed dissimilarities depending on whether CSEPs were delivered by the bacterium or constitutively expressed *in planta*. For example, MLP123218 and MLP124466 had a positive effect on pathogen growth when expressed *in planta*, but not when they were bacterially delivered. Conversely, MLP102036 and MLP124266 had a positive effect on pathogen growth when they were bacterially delivered, but not when they were constitutively expressed. Two factors may explain the differences. First, bacterially produced CSEPs may be mistargeted or misfolded as a result of their passage through the T3SS to reach the plant cell. Second, GFP tags may generate steric interference, causing a mistargeting or misfolding of the CSEPs, especially if they are small.

The demonstrated function of many effectors is to suppress PTI (Gohre and Robatzek, 2008; Zhang *et al.*, 2007). To study this possibility, we used the *P. syringae* pv. *tomato* DC3000 *hrcC*– strain, which is deficient in the production of the T3SS and which triggers PTI but not effector-triggered immunity (ETI). Our infection results with bacteria and oomycetes suggest that the increased pathogen growth observed with MLP106078, MLP124497 and MLP124499 was not caused by PTI suppression. Remarkably, it suggests that the CSEPs assayed favour pathogen infection without suppressing plant immunity. Other aspects of plant immunity, such as reactive oxygen species (ROS) production and signalling pathways, are currently being investigated as part of a more detailed analysis of these CSEPs. As obligate biotrophs are extremely sophisticated parasites that often establish long-standing associations with host tissues, we speculate that many of their effectors not only suppress immune responses, as do many hemibiotrophs, but deeply modulate host functions.

Our results show that infection assays using different pathogens having various lifestyles, i.e. bacteria and oomycetes, can yield strikingly different results. *Hyaloperonospora arabidopsidis* and *M. larici-populina* are both obligate biotrophic filamentous pathogens of dicot plants and share some similarities in their

propagation modes in leaf tissues. Therefore, we would expect that *H. arabidopsidis*' pathogenicity would be more likely than *P. syringae*'s bacterial pathogenicity to be affected by rust effectors. Indeed, our results showed that 11 CSEPs promoted the growth of *H. arabidopsidis*, which is more than twice the number of CSEPs promoting the growth of *P. syringae*. It is possible that *M. larici-populina* CSEPs target mechanisms that specifically assist in leaf infection by biotrophic pathogens.

We were able to identify rust CSEPs affecting pathogen growth in *A. thaliana*, a non-host plant. As many effectors exert their activities by associating with plant proteins, this observation suggests that some CSEPs may have the ability to associate with *A. thaliana* proteins despite the fact that they do not under natural conditions. In line with this hypothesis, Petre *et al.* (2015b, 2016) identified rust CSEPs that associate with specific *N. benthamiana* proteins which have conserved homologues in the natural host plant poplar. As our present report includes candidate effectors that were also investigated by Petre *et al.* (2015b), we assessed whether some of the putative targets identified by co-immunoprecipitation/mass spectrometry could explain the pathogen assay results observed. Only two effectors common between the two studies had specific interactors: MLP37347 and MLP124111. MLP124111 did not show any effect on pathogen growth when the effector was delivered by *P. syringae* and could unfortunately not be assayed with the effector constitutively expressed *in planta* as it did not produce a viable transgenic line. MLP37347, however, displayed an interesting effect: it decreased bacterial growth (in both systems), but increased oomycete growth. In the co-immunoprecipitation assay, MLP37347 had a single specific interactor: glutamate decarboxylase 1 (GAD1). It should be noted that MLP37347 is the effector localized to the plasmodesmata. An evaluation of the potential role of GAD1 as a putative virulence target of MLP37347 will require further investigation.

Two CSEPs (MLP37347, MLP123227) had a negative effect on bacterial growth, but a positive effect on *H. arabidopsidis* growth. One option for the decreased bacterial growth is possible recognition by *A. thaliana*'s immune receptors and triggering of the defence response. *Arabidopsis* is a non-host for *M. larici-populina* or any rust fungus and, for this reason, it seems surprising that *A. thaliana* would recognize these candidate effectors. We rather hypothesize that *Arabidopsis* does not directly recognize these CSEPs, but instead they may target proteins guarded by an R-protein in *A. thaliana* (Dangl and Jones, 2001; Van der Biezen and Jones, 1998). Thus, the identification of the target of these CSEPs could provide information with regard to decoy or bait, and ultimately, by homology, allow the identification of important components of the plant immune system (Khan *et al.*, 2016). We must also keep in mind that the expression of a given effector in *P. syringae* may affect the secretion of other endogenous effectors and may thus decrease its virulence.

Confocal microscopy assays revealed that some candidate effectors accumulate in chloroplasts, plasmodesmata and cytosolic foci in leaf cells, whereas most displayed a non-informative nucleocytosolic distribution. It is important to consider that these localizations were obtained using expression driven by the 35S promoter, and so may be different from those observed when CSEPs are delivered by the pathogen. Petre *et al.* (2015a) studied the localization and interaction partners of some *M. larici-populina* CSEPs. In both studies, the subcellular localization of MLP102036, MLP123227, MLP124266, MLP124497, MLP124499 and MLP37347 was identical. Interestingly, several rust candidate effectors accumulate in chloroplasts (Petre *et al.*, 2015a, b, 2016). Despite intensive cell biology screens, no other effectors of filamentous pathogens have been reported to target chloroplasts. Whether chloroplasts are important organelles for rust fungi to manipulate remains to be investigated. The similar localization of the common CSEPs in both studies strengthens the use of heterologous systems to infer CSEP localization, putative function and interaction partners.

We could not recover stable transgenic lines expressing MLP124111 and MLP123437, which suggests that their accumulation in cells could be toxic to plants. MLP123437 was not investigated by Petre *et al.* (2015b); however, in *N. benthamiana*, Mlp124111 localized in chloroplasts as well as in large, bright and irregular cytosolic structures that were probably aggregates. It is possible that these aggregates, as they may be toxic to the cell, were the reason why a stable transgenic line for MLP124111 could not be recovered, although no signs of necrosis were reported by Petre *et al.* (2015b). Alternatively, a chloroplast localization could trigger the chlorosis observed in the unique transgenic T1 line which did not reach maturity. Our results demonstrate that advances in the biology of rust fungi, as well as in the biology of other obligate biotrophs that cannot be easily and/or routinely transformed, rely on a combination of parallel approaches (e.g. *in vitro* and heterologous assays). Our approach is valuable because of the large pool of genetic knowledge and resources that are available for *Arabidopsis* comparatively to *Nicotiana*.

EXPERIMENTAL PROCEDURES

Plasmid constructs

DNA sequences encoding all mature SSPs were ordered from GenScript in a lyophilized form (the stop codon was removed to enable GFP fusion). All constructs were transferred by BP recombination to the Gateway pDONR Zeo vector (Invitrogen, Carlsbad, CA, USA). From pDONR^{TM}/Zeo, constructs were either sent to pB7FWG2 (Karimi *et al.*, 2002) to generate stable C-terminally GFP-tagged transgenic *Arabidopsis* plants or pVSP-PsSPdes (courtesy of Guus Bakkeren's laboratory) for *Pseudomonas* infection assays. pVSP-PsSPdes harbours the AvrRPM1 secretion signal and a C-terminal haemagglutinin (HA) tag (Rentel *et al.*, 2008).

Transformation of *A. thaliana*, pathogen assay and western blotting

Five-week-old soil-grown *A. thaliana* plants were transformed using the floral dip method with minor modifications (Clough and Bent, 1998). Modifications included substitution of Silwet L-77 by OFX-0309 (Norac Concept Inc., Guelph, ON, Canada) (Mireault *et al.*, 2014) and dipping the plants a second time 1 week later to increase the number of transformants. T1 plants were selected using ammonium glufosinate (Basta) at 60 mg/L. T2 plants grown in Petri dishes (with Basta at 25 mg/L) and lines displaying a 3 : 1 ratio were selected to obtain single insertion lines. T3 plants were also grown in Petri dishes, and lines showing 100% germination were selected as homozygotes. An average of 12 transgenic lines for each construct was selected and screened for GFP expression by western blot (Fig. S1), and the strongest expressing line was kept for each construct. This primary screen was necessary to ensure that GFP was not cleaved from SSPs, in which case we would have observed a fluorescence signal from free GFP in confocal microscopy. It should also be noted that two constructs did not yield any transgenic lines despite many transformation attempts; these were SSPs MLP124111 and MLP123437. Indeed, one line was recovered from MLP124111, but it showed important chlorosis, stunted morphology, did not grow higher than 2 cm in 7 weeks, never produced any viable seeds and died. Additional attempts to produce more transgenic lines overexpressing MLP124111 failed. We conclude that constitutive *in planta* expression of MLP124111 and, perhaps, MLP123437 is most probably toxic to the plant. Infections were carried out on one line per construct. Western blots and infection assays were performed as described previously (Germain *et al.*, 2007, 2010). Briefly, bacterial infections were performed with 4-week-old *Arabidopsis* plants grown at 22 °C in a 16-h/8-h light/dark cycle. *Pseudomonas syringae* pv. *tomato* diluted at an optical density at 600 nm (OD_{600}) of 0.001 was leaf inoculated on the abaxial side of the leaf. A minimum of 36 leaves was infected for each effector or transgenic line. Leaf punches were taken from 12 leaves at day 0 and 24 from leaves at day 3 to assess bacterial quantity. *Hyaloperonospora arabidopsidis* infection was performed as described by Dong *et al.* (2016) with no modification to the protocol. For western blots, two leaf punches (0.384 cm^2) were taken from 3-week-old T3 plants and stored at −80 °C. Leaf punches were disrupted using a TissueLyser (Qiagen, Toronto, ON, Canada) and a 5-mm stainless steel bead agitated at 26 cycles/s for 10 s. Laemmli sample buffer (100 μL) was added and the tube was heated at 95 °C for 5 min and spun at 13 000 ***g*** for 1 min. Supernatant (10 μL) was loaded on a 10% acrylamide gel. Western blot was carried out as described previously (Germain *et al.*, 2007); mouse anti-GFP antibody used to detect the recombinant protein was purchased from Cedarlane (Burlington, ON, Canada) and used at 1 : 5000.

Confocal imaging

T3 seedlings (6–10 days old), collected from Petri dishes containing half-strength Murashige and Skoog (MS) medium + ammonium glufosinate (25 mg/L), were placed in a water drop under a coverslip and imaged immediately. Before each imaging session, wild-type plants were visualized to adjust the settings in order to exclude autofluorescence of the chloroplasts. Images were captured with a Carl Zeiss LSM700 or Nikon A1 confocal microscope; 40× oil PlanApo immersion objectives were used. An excitation laser (488 nm) was used and photons were collected between 510 and 520 nm. Image analysis was performed with ImageJ (http://imagej.nih.gov/ij/).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Photographs of plants after 4 weeks of growth at 20 °C.

Fig. S2 Photographs of plants after 4 weeks of growth at 16 °C.

Fig. S3 Photographs of 124499 and Col-0 plants after 6 weeks of growth at 20 °C to visualize senescence.

Fig. S4 Western blot showing the molecular weights of the different candidate effectors tagged with green fluorescent protein (GFP). Immunoblot analysis was performed using an anti-GFP antibody. The molecular weight was calculated using the Bio-Rad low-molecular-weight protein standard. Only the transgenic line that was selected for infection and confocal imaging is shown.

Table S1 Full DNA and peptide sequences of the candidate effectors. '—' indicates where the candidate effector is predicted to be cleaved. The C-terminal section was used as the mature protein.