Effectors from Wheat Rust Fungi Suppress Multiple Plant Defense Responses

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ABSTRACT

Fungi that cause cereal rust diseases (genus *Puccinia*) are important pathogens of wheat globally. Upon infection, the fungus secretes a number of effector proteins. Although a large repository of putative effectors has been predicted using bioinformatic pipelines, the lack of available high-throughput effector screening systems has limited functional studies on these proteins. In this study, we mined the available transcriptomes of *Puccinia graminis* and *P. striiformis* to look for potential effectors that suppress host hypersensitive response (HR). Twenty small (<300 amino acids), secreted proteins, with no predicted functions were selected for the HR suppression assay using *Nicotiana benthamiana*, in which each of the proteins were transiently expressed and evaluated for their ability to suppress HR caused by four cytotoxic effector-R gene combinations (*Cp/Rx*, *ATR13/RPP13*, *Rpt2/RPS-2*, and *GPA/RBP-1*) and one mutated

R gene—*Pto(Y207D)*. Nine out of twenty proteins, designated *Shr1* to *Shr9* (suppressors of hypersensitive response), were found to suppress HR in *N. benthamiana*. These effectors varied in the effector-R gene defenses they suppressed, indicating these pathogens can interfere with a variety of host defense pathways. In addition to HR suppression, effector *Shr7* also suppressed PAMP-triggered immune response triggered by flg22. Finally, delivery of *Shr7* through *Pseudomonas fluorescens* EtHAn suppressed nonspecific HR induced by *Pseudomonas syringae* DC3000 in wheat, confirming its activity in a homologous system. Overall, this study provides the first evidence for the presence of effectors in *Puccinia* species suppressing multiple plant defense responses.

Additional keywords: reactive oxygen species.

The rust diseases, caused by basidiomycetous fungi of the genus *Puccinia*, are serious production constraints in all wheat production regions, leading to epidemic outbreaks on susceptible varieties under favorable climatic conditions. Three species of the fungus, *Puccinia graminis* f. sp. *tritici*, *P. triticina*, and *P. striiformis* f. sp. *tritici*, cause stem, leaf, and stripe rust, respectively. Despite best efforts to control the disease by fungicide application, use of resistant varieties and cultural practices like host free periods, the disease continues to affect production across the globe (Han et al. 2015; Hovmøller et al. 2008). Efficient management of rust becomes a challenge due to the ability of rust fungus populations to produce vast number of asexual spores throughout the cropping season that spread over long distances and to alter their repertoire of effectors that are detected by the resistance genes deployed in commercial cultivars (Hovmøller et al. 2008; Kolmer 2005).

Plant proteins that detect pathogens and trigger defense responses are typically grouped into two types (Jones and Dangl 2006; Thomma et al. 2011). Pattern recognition receptors (PRRs) detect conserved pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular patterns (MAMPs) and trigger responses conferring PAMP-triggered immunity (PTI). Common components of PTI include callose deposition, production of antimicrobial compounds such as reactive oxygen species (ROS), and accumulation of defense proteins. Effector-triggered immunity (ETI) constitutes the second, more robust line of defense of the host.

This is triggered in response to certain pathogen effector molecules secreted by pathogens during the infection process. Recognition of the effector molecules or their effects in the cell, by resistance proteins, leads to programmed cell death (PCD) along with other responses, some of which are more systemic. ETI-triggered PCD, known as the hypersensitive response (HR), can be particularly effective against biotrophic pathogens but can, in some cases, promote colonization by necrotrophic pathogens (Faris et al. 2010; Lorang et al. 2007).

Many plant pathogens secrete effectors that function to subvert host defense responses or enhance their success in pathogenicity in other ways. The best-characterized effectors are those from bacterial pathogens which deliver effectors to plant cells via their type III secretion system to suppress both PTI and ETI (Block and Alfano 2011; Lindeberg et al. 2012). Many have specific targets in host cells whose identification has revealed novel components of plant immune systems (Feng and Zhou 2012). Numerous effectors from oomycete pathogens have also been identified, which has been facilitated by their conserved protein motifs (Fabro et al. 2011; Whisson et al. 2007). A role in suppression of cell death has been demonstrated for many of these effectors by co-expression in *Nicotiana benthamiana* with proteins that trigger cell death (Bos et al. 2006; Dou et al. 2008; Kelley et al. 2010; Oh et al. 2009; Wang et al. 2011). A large-scale analysis of oomycete and bacterial effectors found they interacted with some of the same host proteins indicating they share targets for modulating defense (Mukhtar et al. 2011). Unlike bacterial and oomycete pathogens, large-scale functional analyses of fungal effectors have been hindered in part by a lack of conserved sequences to aid their identification although computational approaches coupled with expression analysis are addressing this challenge (Godfrey et al. 2010; Pedersen et al. 2012; Saunders et al. 2012; Sperschneider et al. 2015). A growing number of fungal effectors known to interact with plant R genes have been

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*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary tables are published online.

identified and found to be very diverse in their structures, evolutionary origins, proposed functions, and locations in plant cells (Giraldo and Valent 2013).

Like many other biotrophic fungi, rust fungi produce specialized feeding structures called haustoria, which penetrate plant cell walls and are separated from the cytoplasm by an extrahaustorial membrane. The haustorium forms an intimate association with the host plant, facilitating the uptake of nutrients and delivery of effector proteins (Garnica et al. 2014). Relatively small proteins expressed in haustorial cells with sequence motifs indicating secretion are considered to be good candidates for effector proteins (Ahmed et al. 2015; Liu et al. 2016; Petre et al. 2015; Whigham et al. 2015; Yin et al. 2015). Most, but not all (Nirmala et al. 2011), of the rust effectors identified, that function as avirulence proteins and interact with resistance proteins, fit the description of small, secreted proteins (Catanzariti et al. 2006, 2007; Dodds et al. 2004; Jia et al. 2000). Recent efforts have provided genome and transcriptome sequences along with gene expression and polymorphism analyses for the three *Puccinia* species that infect wheat (Bruce et al. 2014; Cantu et al. 2011, 2013; Garnica et al. 2013; Hubbard et al. 2015; Xu et al. 2011; Yin et al. 2015; Zheng et al. 2013). Candidate effector proteins are therefore very abundant, but few have been analyzed for function. In this study, we mined putative effector candidates from the available transcriptome of *Puccinia graminis* and *P. striiformis*. Twenty putative effector proteins were selected and expressed in *N. benthamiana* to test for their ability to suppress HR. Nine (*Shr1* to *Shr9*) out of the 20 proteins screened were found to suppress cell death caused by different elicitors indicating their possible role in suppressing ETI.

MATERIALS AND METHODS

Plant material, fungal strains, and growth conditions.

Plants used in this study included *Nicotiana benthamiana* and the wheat cultivars McNair701, Avocet, and Nugaines. *N. benthamiana* plants were grown from seed in the greenhouse at 24°C day and 20°C night. Four- to six-week-old *N. benthamiana* plants were used for cell death suppression assays. Wheat cultivar Avocet was infected with *P. striiformis* race PSTv37 (PST-100) for urediniospore increase and RT-qPCR analysis. Wheat leaves were infected by spreading a 10:1 mixture of talc/urediniospores on the leaves and incubating in a dew chamber for 24 h at 10°C with 100% relative humidity. The next day, plants were moved to a growth chamber with 16 h light at 16°C and 8 h dark at 8°C until the infected leaves began to sporulate. McNair 701 plants were infected with urediniospores of *P. graminis* CRL 75-36-700-3 race SCCL (Pgt7A) as previously described (Yin et al. 2011). Wheat leaves infected with fungal spores were harvested at different stages of infection (6 and 10 days postinoculation [dpi]) for RT-qPCR analysis. Fresh fungal spores were collected and stored at -80°C for RNA extraction or until further use. Germinated spores were obtained by floating fungal spores on sterile distilled water on a glass petri dish overnight in dark. Germinated spores were collected from the surface of the water and used for RNA extraction.

Effector selection and plasmid construction. Candidate effector genes were selected based on the following criteria for their predicted proteins: (i) size less than 300 amino acids; (ii) presence of a signal peptide (predicted using SignalP 4.0); and (iii) no significant homology to proteins with predicted functions. In addition, candidate *P. graminis* effectors were selected from transcripts that appeared to be enriched in haustoria cells from transcriptome data as described by Yin et al. (2015). *P. striiformis* were selected from PSTv37 (PST-100) based on their protein sequence homology (>80%) to effectors predicted by Cantu et al. (2011). Homology searches were conducted on the Broad Institute *Puccinia* Database using BLASTP ($P < 1.0E-10$). Genes were amplified from rust-infected wheat tissue cDNA using primers designed to amplify only the coding sequence of the mature protein

without the signal peptide (Supplementary Table S1). Effector genes were cloned into an *Agrobacterium* binary vector pBTEx between restriction sites *KpnI* and *XbaI* and expressed transiently in *N. benthamiana*. Effectors were cloned into pBTEx without a stop codon in fusion with a 6X FLAG tag at the 3' end. Primers were designed to generate truncations of gene *Shr7* which were also cloned into pBTEx (Supplementary Table S2). *P. graminis* f. sp. *tritici* *IaaM* was cloned into *Agrobacterium* binary vector pCHF3 (Yin et al. 2014). All constructs were verified by sequencing at Elim Biopharm, Hayward, CA.

***Agrobacterium*-mediated transient assay.** For transient expression of proteins, all constructs were electroporated into *Agrobacterium* GV2260. Six- to eight-week-old *N. benthamiana* plant leaves were infiltrated with the bacterial suspensions as described previously by Sessa et al. (2000). Only the second to fourth leaves of the plants were chosen for inoculations. All gene constructs and cell death elicitors were diluted to an optical density (OD₆₀₀) of 0.4 to 0.6 and infiltrated into leaves using a needle-less syringe. For zero hour infiltrations, effector gene constructs and HR constructs were mixed in a 1:1 ratio before infiltration. For 24 h staggered inoculations, the *Agrobacterium* strain containing the putative effector genes were infiltrated 24 h prior to the cell death causing constructs. Effector constructs *ATR13*, *Cp*, *AvrRpt2*, and *RBP-1* were mixed with their corresponding R genes; *RPP13*, *Rx*, *RPS-2*, and *GPA*, respectively, in a 1:1 ratio before infiltration. Each putative effector gene was tested on four leaves and the experiment was repeated three separate times. Development of cell death was monitored over a period of 5 to 7 days and leaves were photographed at 5 dpi.

Quantification of HR. HR was quantified using LeafQuant, a matlab-based image processing package. Briefly, images were loaded onto LeafQuant-T3S and the inoculated area was selected using the elliptical selection tool. ExposureVal was set at 1.2 for all images. Twelve inoculated spots were analyzed for each gene. Average percent reduction in HR was calculated by the formula (percent nongreen area for green fluorescent protein (GFP) – percent nongreen area for effector) × 100/percent nongreen area for GFP. Analysis of variance was performed to test for statistical significance using JMP software (SAS Institute, Inc., Cary, NC) and Microsoft Excel.

Quantitative PCR analysis. The expression of the nine identified effector proteins was determined using quantitative PCR. Total RNA was isolated from infected wheat leaves, urediniospores, and germinated urediniospores using TRIzol reagent (Thermo Fischer Scientific, Waltham, MA) according to manufacturer's protocol. cDNA was synthesized using iScript reverse transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA). Real-time quantitative PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). PCR was performed on the My iQ Single Color Real-Time PCR detection system (Bio-Rad) under the following conditions: 95°C for 3 min, 95°C for 15 s, 60°C for 45 s; followed by a dissociation program to obtain the melting curves. Actin genes specific for *P. striiformis* and *P. graminis* were used as internal reference controls to calculate relative gene expression for *P. striiformis* and *P. graminis* effectors, respectively. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ approach (Schmittgen and Livak 2008) with three biological replicates for each effector gene.

ROS measurement. Measurement of ROS was performed using a luminol-based chemiluminescence assay as described previously (Gimenez-Ibanez et al. 2009). Briefly, leaf discs (0.5 cm in diameter) from pre-infiltrated *N. benthamiana* leaves were floated overnight on 500 µl of water in a 24-well plate. The following day, water was carefully aspirated out of the wells and replaced with 300 µl of oxidative burst solution containing horseradish peroxidase at 20 µl/ml (Sigma, St. Louis, MO), luminol at 34 µg/ml (Sigma), and 100 nM flg22. Luminescence was measured every 2 min for a period of 30 min total using a multimode

plate reader (Enspire 2300; Perkin Elmer, Turku, Finland), and results were analyzed using JMP software (SAS Institute, Inc.) and Microsoft Excel.

Plasmid construction for expression via type III secretion system. PCR products for GFP, and PSTG_14695 were cloned into Gateway entry vector pCR8 using TA cloning. Plasmids containing the genes were then transferred to destination vector pEDV6 using LR reaction and transformed into electrocompetent *Pseudomonas fluorescens* strain EtHAn (effector to host analyzer) provided by Jeff Chang (Oregon State University). pEDV6 contains the AvrRps4 promoter along with the first 137 amino acids of AvrRps4 at its N terminal. The N terminal region of AvrRps4 provides the necessary signal for secretion of the cloned protein through the type III secretion system of *Pseudomonas*.

Wheat assay. *Pseudomonas* cells were grown in King's B media for 48 h at 28°C using appropriate antibiotics, washed once, and resuspended in sterile double-distilled water. *Pseudomonas fluorescens* and *Pseudomonas syringae* DC3000 suspensions were diluted to an OD₆₀₀ of 1.0 and *Pseudomonas fluorescens* strain containing GFP and the candidate suppressors was grown for 48 h at 28°C in KB media supplemented with chloramphenicol, tetracycline, and gentamycin at 25 µg/ml. *Pseudomonas syringae* DC3000 was grown in KB media containing rifampicin at 25 µg/ml and ampicillin at 100 µg/ml. Bacterial cells were washed once and resuspended in sterile double-distilled water. *Pseudomonas fluorescens* cells were diluted to an OD₆₀₀ of 0.6 and *Pseudomonas syringae* was diluted to an OD₆₀₀ of 0.3. The two bacterial solutions were mixed in a 1:1 ratio and infiltrated into 10- to 14-day-old wheat leaves (cultivar Nugaines). Results were repeated at least three times. Photographs of treated leaves were taken 3 dpi and used for quantification. A total of 11 leaves were selected for statistical analysis.

RESULTS

Selection of candidate effectors. A diverse collection of 20 candidate effector proteins were selected based on their small size (105 to 270 amino acids), predicted signal peptide, and lack of homology to proteins with known functions. Nine were selected

from *P. striiformis* sequences and 11 from *P. graminis* (Table 1). In addition to small size and the presence of a signal peptide, many effectors are known to have high cysteine content (Kamoun 2007). In this study, all genes except for PGTG_07550, PGTG_08644, PGTG_10751, and PGTG_13212 contained more than 2% cysteine residues in their predicted proteins. *P. graminis* genes were selected from the list of 1,182 haustorium-enriched genes sequences provided by Yin et al. (2015). For *P. striiformis* genes, a list of effectors predicted by Cantu et al. (2011) was used as a reference and transcripts coding for homologous proteins were chosen from the PST-78 sequence database (Broad Institute *Puccinia* Group Database). When used in homology searches (BLASTP) against the Broad Database, 15 of the 20 effectors were found to belong to gene families with 2 to 37 family members ($P < 1.0E-10$). Most of the genes, especially those belonging to gene families, had conserved homologs in two or more of the three *Puccinia* species with sequenced genomes; *P. striiformis*, *P. graminis*, and *P. tritici*. The largest gene families, with 15 or more members, had homologs in all three *Puccinia* species (Table 1).

Suppression of HR. To test whether the effector genes could suppress PCD, candidate effector genes were either co-infiltrated or infiltrated 24 h before the HR constructs into 4- to 6-week-old *N. benthamiana* plants. When inoculated 24 h prior to the HR-causing constructs, two *P. graminis* (*Shr8* and *Shr9*) and seven *P. striiformis* effectors (*Shr1* to *Shr7*) were able to suppress cell death induced by at least one of the six HR elicitors used (Table 2). However, suppression was not observed with any of the effectors when infiltrated simultaneously with the HR construct. *Shr1*, *Shr2*, *Shr4*, *Shr5*, and *Shr7* suppressed cell death caused by *Pto*(Y207D) when inoculated 24 h prior to the *Agrobacterium* strain carrying *Pto*(Y207D) (Fig. 1A; Table 2). When compared with the GFP control, most proteins that suppressed HR inhibited cell death in more than half of the leaf area infiltrated with the cell death construct (Table 2). *Shr4*, *Shr5*, and *Shr7* showed nearly complete suppression, resulting in 74.4, 74.9, and 81% reduction in HR, respectively. All other effectors showed less complete suppression of PCD (Table 2; Supplementary Table S3). The representative phenotype observed for less complete suppression by *Shr8* is shown in Figure 1B. *Shr1* and *Shr7* suppressed cell death caused by *ATR13/RPP13*, while

TABLE 1. Summary of candidate effector genes tested for suppression of cell death

Candidate effector gene	Copy number ^a	Protein length (aa)	Position of signal peptide	Cysteine (%)	Conservation in <i>Puccinia</i> sp. ^b	Tribe ^c	Protein expression ^d
<i>P. striiformis</i>							
PSTG_00494	15	199	23	3.09	PSTG,PGTG,PTTG	19	–
PSTG_01062	3	182	22	5.92	PSTG,PGTG,PTTG	148	–
PSTG_01724	4	114	24	5.26	PSTG, PGTG	149	+
PSTG_09266	8	191	19	4.28	PSTG, PGTG	31	–
PSTG_10812	2	105	20	4.76	PSTG	619	+
PSTG_12529	1	173	26	3.9	PSTG	361	+
PSTG_14250	6	199	19	6.09	PSTG, PGTG	31	–
PSTG_14695	1	151	19	3.52	PSTG,PGTG,PTTG	696	+
PSTG_16009	6	158	20	6.33	PSTG,PGTG,PTTG	11	+
<i>P. graminis</i>							
PGTG_00639	20	217	20	2.3	PGTG,PSTG,PTTG	10	–
PGTG_03101	37	251	21	3.9	PGTG,PSTG,PTTG	1	+
PGTG_04606	15	214	19	3.7	PGTG,PSTG,PTTG	5	+
PGTG_07550	1	128	19	0.7	PGTG	–	–
PGTG_08644	2	255	19	0.3	PGTG,PSTG,PTTG	54	+
PGTG_10268	1	123	19	4.8	PGTG	–	–
PGTG_10642	5	150	19	4.0	PGTG,PSTG,PTTG	125	+
PGTG_10751	1	235	18	1.7	PGTG, PTTG	1339	–
PGTG_13212	4	270	21	1.1	PGTG,PSTG,PTTG	276	–
PGTG_15486	2	191	23	3.1	PGTG,PSTG,PTTG	–	+
PGTG_17549	6	210	20	6.19	PGTG,PSTG,PTTG	63	+
<i>P. graminis</i> f. sp. <i>tritici</i> IaaM	1	588	–	1.4	PGTG,PSTG,PTTG	–	Not tested

^a Number of genes encoding highly homologous proteins ($P < 1.0E-10$) predicted in the PST-78 or PGT genome (Broad Institute Database).

^b *Puccinia* species with genes encoding highly homologous proteins ($P < 1.0E-10$; Broad Institute Database).

^c Tribes numbers (assigned by Cantu et al. 2013) to which the proteins belong to based on their BLASTP percentage sequence identity of >80%.

^d +, protein expression detected 24 to 36 h postinoculation; –, protein not detected on western blot.

Shr2, *Shr3*, *Shr6*, *Shr8*, and *Shr9* suppressed cell death caused by *GPA/RBP-1* (Table 2). Two effector genes, *Shr1* and *Shr7*, were able to suppress PCD caused by both *Pto(Y207D)* and *ATR13/RPP13*, while *Shr2* suppressed cell death induced by *Pto(Y207D)* and *RBP-1/GPA*. The ability of some genes to suppress more than one PCD elicitor but not others, suggests that these effectors employ different mechanisms to interfere with host cell death. Interestingly, a majority of the effectors suppressed cell death caused by either *Pto(Y207D)* or *RBP-1/GPA* suggesting that there might be at least two modes of actions involved in inhibition of cell death by these effectors. Taken together, these results indicate that some effectors from *P. graminis* and *P. striiformis* play a role in suppression of host HR. The similarity in suppression activities between some of the effectors suggests a functional redundancy in the effector repertoire of *Puccinia*. The *P. graminis* f. sp. *tritici IaaM* gene was used as a control in the study. While important for pathogenicity, its protein did not fit the description of a small, secreted effector protein, but rather an enzyme expressed in *Puccinia* haustoria (Yin et al. 2014). As expected, *P. graminis* f. sp. *tritici IaaM* did not show suppression with any of the R genes tested.

Truncations of *Shr7*. To validate the results obtained from suppression assays, we tested two truncations of effector protein *Shr7* for their ability to suppress *Pto(Y207D)* and *ATR13/RPP13* induced PCD. Truncations of *Shr7*, designated *Shr7-T1* and *Shr7-T2* were generated where 44 and 88 amino acids, respectively, were deleted from the C-terminal of the protein. When tested using the

suppression assay, both truncations lost their ability to suppress cell death caused by *Pto(Y207D)* (Fig. 2) and *ATR13/RPP13* (data not shown). This confirmed the activity of the mature protein (without the signal peptide) to suppress PCD. However, further nested deletions may be required to pin-point the region responsible for suppression.

Transcription pattern of the identified effector genes. In order to assess the pattern of expression of the identified effectors, we examined the relative abundance of their corresponding transcripts during different stages of fungal development. Total RNA was extracted from urediniospores, urediniospores germinated in vitro, and infected wheat leaves 6 dpi for *P. graminis* and 10 dpi for *P. striiformis*. *P. graminis* and *P. striiformis* transcript levels for each tissue type were normalized using the *P. graminis* and *P. striiformis* actin genes, respectively. Relative gene expression for all tissue types was calculated with respect to transcript levels in urediniospores. The results from RT-qPCR results indicated that six out of nine effector genes were induced upon infection, although the level of induction varied considerably (Fig. 3). All the effectors had minimal to no expression in fungal urediniospores, indicating they are indeed effector proteins involved in pathogen colonization. Accumulation of transcripts for six genes (*Shr1*, *Shr2*, *Shr4*, *Shr5*, *Shr6*, and *Shr9*) was highest at 10 dpi, for two genes (*Shr7* and *Shr8*) at 6 dpi and for one (*Shr3*) in the germinated urediniospores (Fig. 3). A similar ‘sequential wave’ in effector expression has been reported previously in *P. striiformis* and *Phytophthora sojae* (Cantu et al. 2013; Wang et al. 2011). Transcription patterns for two genes (*Shr2* and *Shr4*), had higher expression levels in germinated urediniospores and at 10 dpi but lower expression levels in spores and at 6 dpi, suggesting their involvement in very early and late stages of plant colonization (Fig. 3B and D). *Shr1* and *Shr7*, which suppressed HR induced by *Pto(Y207D)* and *ATR13/RPP13*, and *Shr6* that suppressed HR induced by *GPA/RBP-1*, accumulated to levels roughly 1,000 fold or more in infected tissue as compared with urediniospores and germinated urediniospores (Fig. 3A, F, and G). In contrast, *Shr3*, accumulated to more than 800-fold ($P = 0.01$) in germinated urediniospores compared with other developmental stages of the fungus (Fig. 3C). Overall, there was no clear relationship between the developmental timing of expression and ability to suppress HR caused by specific inducers. The differences in expression patterns suggest that different effectors may be involved at different stages of fungal development within the host.

Suppression of ROS. In addition to suppressing PCD, we tested the ability of the identified effectors to suppress PTI responses.

TABLE 2. Suppression of programmed cell death caused by candidate effector genes from the stripe and stem rust fungi

Effector designations	Broad institute designations	Suppression of effector-triggered immunity-induced HR ^a		
		<i>Pto(Y207D)</i>	<i>ATR13/RPP13</i>	<i>GPA/RBP-1</i>
<i>Shr1</i>	PSTG_00494	58.4	48.2	–
<i>Shr2</i>	PSTG_01062	63.2	–	42.3
<i>Shr3</i>	PSTG_01724	–	–	42.2
<i>Shr4</i>	PSTG_09266	74.4	–	–
<i>Shr5</i>	PSTG_10812	80.0	–	–
<i>Shr6</i>	PSTG_14250	–	–	59.6
<i>Shr7</i>	PSTG_14695	74.9	43.1	–
<i>Shr8</i>	PGTG_03101	–	–	66.6
<i>Shr9</i>	PGTG_10642	–	–	50.2

^a Percent reduction in hypersensitive response (HR) when *Agrobacterium* carrying candidate effector gene was infiltrated 24 h prior to *Agrobacterium* carrying the cell death elicitor; – indicates no noticeable suppression.

Fig. 1. Suppression assay for cell death. **A**, *Shr7* and **B**, *Shr8*. Leaves of *Nicotiana benthamiana* were infiltrated with *Agrobacterium tumefaciens* cells carrying effector gene (*Shr7* or *Shr8*) 24 h prior to *A. tumefaciens* carrying the cell death elicitor, *Pto(Y207D)*. **A**, Near complete suppression: 1, *Pto(Y207D)*; 2, effector protein (*Shr7*); 3, *Shr7* + *Pto(Y207D)*; and 4, GFP + *Pto(Y207D)*. **B**, Partial suppression of cell death caused by *GPA + RBP-1*: 1, effector protein (*Shr8*); 2, *Shr8* + *GPA+RBP-1*; 3, GFP + *GPA+RBP-1*; and 4, *AvrPtoB* + *GPA+RBP-1*.

Leaf punches of transiently infiltrated *N. benthamiana* plants were treated with flg22 (100 nM) and ROS production was followed for 30 min immediately after elicitation. Of the nine effectors tested, consistent suppression in ROS production was observed only for *Shr7*. Significant reduction in ROS accumulation was observed in most cases when the experiment was repeated ($P < 0.01$) (Fig. 4). Since it is known that accumulation of ROS accompanies PCD (Zurbriggen et al. 2010), it may be possible that *Shr7* interferes with PCD by down-regulating the production of ROS in the plant.

Suppression of HR in wheat. Having found a suppressive effect for *Puccinia* effectors in a heterologous system, we sought to translate these results in a homologous system. For this, we used an approach similar to that of Whigham et al. (2015) where a bacterial strain delivering a putative defense suppressor by the type III secretion system is infiltrated into leaves along with another strain delivering defense-eliciting effectors. For our purpose, we used *Pseudomonas fluorescens* EtHAN to deliver a putative suppressor and *Pseudomonas syringae* pv. *syringae* DC3000, the causative agent of bacterial speck of tomato, to deliver defense-inducing effectors. The *Pseudomonas fluorescens* effector to host analyzer strain (EtHAN) has been previously shown to effectively deliver Avr proteins in wheat (Upadhyaya et al. 2014). *Pseudomonas syringae* DC3000, on the other hand, elicits a strong HR response when syringe-infiltrated into wheat (Yin and Hulbert 2010). The HR is likely caused by multiple effectors since a DC3000 variant with several effectors deleted also elicited HR, although it was significantly weaker. Since both *Pseudomonas fluorescens* strain EtHAN and *Pseudomonas syringae* DC3000 are able to deliver effectors to wheat, their codelivery provides a suppressor assay in a homologous system.

The coding sequence of effector *Shr7* lacking the signal peptide was cloned into vector pEDV6 in fusion with the N-terminal signal

peptide of AvrRps4 under the control of the AvrRps4 promoter. When infiltrated into wheat leaves, neither *Pseudomonas fluorescens* nor GFP alone cause any visible symptoms. When *Pseudomonas syringae* DC3000 was co-infiltrated with *Pseudomonas fluorescens*, a slight reduction in HR was observed, however this was not significantly different from the HR elicited by *Pseudomonas syringae* DC3000 alone (Fig. 5). On co-infiltrating *Pseudomonas syringae* DC3000 and *Pseudomonas fluorescens* EtHAN carrying *Shr7*, a visible suppression of HR was observed (Fig. 5).

DISCUSSION

Recent advances in bioinformatic prediction of putative effectors have provided a large repository of small secreted proteins that require functional validation. In the present study, we screened 20 effector candidates for their ability to suppress plant HR using a heterologous expression system. Our results indicate that much like many other obligate plant pathogens, *Puccinia* also deploys effectors to manipulate the host immune system and promote host susceptibility by suppression of defense responses. One effector, *Shr7*, when delivered in wheat using a *Pseudomonas* delivery system confirmed its ability to suppress defense responses in a homologous system.

Although a number of transient expression systems like biolistics and delivery of proteins via bacterial type III secretion system have been reported for monocots, they have not been routinely used for high-throughput screening of effector proteins (Panwar et al. 2013). Transient expression systems using biolistic or *Agrobacterium* transformation are inefficient in cereals and generally require analysis of individual cells. Bacterial type III secretion systems using *Pseudomonas* or *Xanthomonas* species have been used to deliver specific fungal effectors (Upadhyaya et al. 2014; Whigham et al. 2015; Yin and Hulbert 2010) but the constraints

Fig. 2. Truncations of gene *Shr7* showing the loss of suppression. Two truncations of *Shr7*-T1 and *Shr7*-T2 were tested using suppression assay. Leaves of *Nicotiana benthamiana* were infiltrated with *Agrobacterium* strain containing truncations *Shr7*-T1 and *Shr7*-T2 24 h prior to infiltration with *Pto*(Y207D). **A**, *Shr7* full-length protein; **B**, GFP- control; **C**, *Shr7*-T1; and **D**, *Shr7*-T2.

on delivering diverse eukaryotic effector proteins are not well understood. Considering these limitations of transient gene expression systems in wheat, we used a heterologous plant species for screening effectors from *Puccinia*. *Agrobacterium* delivery into *N. benthamiana* has become a popular system for expression and co-expression of proteins, analysis of effector function (Chen et al. 2013; Song et al. 2015; Wang et al. 2011) and has been used to examine cellular localization of effectors from the rust fungus *Melampsora larici-populina* (Petre et al. 2015). Plant defense responses are known to be largely conserved between monocots and dicots. Recent studies show that PRRs can be transferred from taxonomically divergent plants providing durable broad-spectrum resistance to different plant species (Holton et al. 2015). In some cases, resistance genes also function after transfer from monocot species to dicots (Mendes et al. 2010). It has also been shown that pathogen effectors tend to interact with proteins that are well connected and play a central role in host cellular defense (Mukhtar et al. 2011). Given this conservation of host defense responses across plant species and the ability of *Puccinia* species to infect both monocots and dicots to complete their life cycle, we hypothesized that *N. benthamiana* should prove useful for screening effectors involved in defense suppression. Delivery of *Shr7* in wheat using a *Pseudomonas* delivery system confirmed its ability to suppress defense responses in wheat. Thus, this study demonstrates the usefulness of *N. benthamiana* as an easy and effective system for phenotypic examination of effectors from rust fungi.

Nine of twenty putative effector proteins inhibited plant PCD in our transient expression assays. The nine likely represent a larger number of effectors since all but one are encoded by members of gene families with 2 to 37 predicted family members in their

respective species. Effector proteins with no known function were selected since most fungal effectors characterized to date share little similarity to already known proteins (Stergiopoulos and de Wit 2009). Since the assay was performed using a nonhost plant, these effectors must supposedly target conserved components of the plant defense machinery. It is possible that some of the *Puccinia* effectors examined also function to suppress defense related cell death but do not function in this heterologous system. Alternatively, these other

Fig. 4. Suppression of reactive oxygen species (ROS) production by *Puccinia striiformis* suppressor *Shr7*. ROS response was measured over a period of 30 min in relative light units (RLUs) in response to 100 nM flg22. Significance was compared with the GFP control using one-way analysis of variance followed by Tukey's honest significant difference test ($P < 0.01$). Bars represent the average of technical replicates $\pm$ standard error ($n = 12$).

Fig. 3. Quantitative real-time PCR showing transcript level patterns for nine suppressor genes during different stages of *Puccinia graminis* f. sp. *tritici* or *P. striiformis* development and infection. **A to G**, Transcript levels of genes from *P. striiformis*. **H and I**, Transcript levels of genes from *P. graminis*. Ured, urediniospores; GerUred, germinated urediniospores; 6 and 10 Dpi, 6 and 10 days postinoculation of wheat leaves. Standard deviation was calculated from values obtained for three biological replicates.

protein may function to suppress defense responses that are not associated with cell death or in other processes like nutrient acquisition.

Of the nine effectors identified, *Shr3*, 4, 5, 8, and 9 suppressed cell death induced by only one R protein, while *Shr1*, 2, and 7 suppressed HR caused by two distinct R proteins. These results suggest that some effectors might be specific in their mode of action, while others may have multiple functions or interact with components of R-protein-mediated cell death that are common to multiple pathways. Similar effectors with broad-spectrum activities have been previously reported from *Pseudomonas* (Abramovitch et al. 2003). In general, most effectors suppressed either *Pto*(Y207D) or *RBP-1/GPA* induced HR suggesting that there might be at least two different mechanisms by which these effectors operate. Previous studies aimed at understanding the mechanism of effector action have shown that most effectors work downstream of the R gene effector interaction (Gimenez-Ibanez et al. 2009). Our results also suggest a similar mechanism of action for effectors from *Puccinia*, as five of the effectors tested were able to suppress *Pto*(Y207D)—an auto-active mutant, capable of inducing cell death independent of effector recognition. Overall, our studies clearly demonstrate that *P. graminis* and *P. striiformis* secrete a number of effector proteins that act in different ways to suppress host immune response. Transcription profiles of the nine effectors showed differences in their expression profiles through infection and colonization. A similar pattern was observed for other putative effectors predicted from *P. striiformis* (Cantu et al. 2013). This variation in expression suggests that these proteins may be involved in suppression of defense responses that are activated at different time points during infection.

In addition to the HR, PAMP-triggered immunity also plays an important role during host–pathogen interactions. A hallmark of

PTI is the rapid and transient production of ROS (Torres 2010). Consequently, many plant pathogens have evolved to suppress ROS as a strategy to overcome plant innate immunity (Guo et al. 2009; Wang et al. 2011). In this study, we identified one effector, *Shr7*, that suppressed production of ROS in addition to cell death. Previous studies with *Avr3a* from *Phytophthora infestans*, demonstrated its ability to suppress both R gene and flg22 induced ROS (Chaparro-Garcia et al. 2015). It is possible that *Shr7*, like *Avr3a*, acts via two different pathways. Alternatively, since ROS production is common to both ETI and PTI, it is possible *Shr7* interferes with in a single step leading to suppression of both PCD and flg22 induced innate immunity.

Previous studies with *Pseudomonas fluorescens* EtHAN have utilized the bacterium for screening Avr genes from wheat rusts (Upadhyaya et al. 2014). In this study, we adapted it for the purpose of screening for HR suppressors. Results from expression of *Shr7* in wheat indicate that it can suppress nonspecific HR induced by *Pseudomonas syringae* DC3000. However, *Shr7* only resulted in partial suppression of HR in wheat. This effect could be due to the ability of *Shr7* to suppress only one or a few defense responses elicited by DC3000. Further, it remains to be tested if *Shr7* renders wheat susceptible to infection by *Pseudomonas syringae* DC3000 or interferes exclusively with pathways leading to HR. Since this effector also interferes with ROS production, we hypothesize that suppression of ROS might be central to its activity as a suppressor. In addition to validating the activity of *Shr7*, the wheat assay results provide further evidence that *Nicotiana* is a good system for examining functions of effectors from rust fungi.

Understanding the functions of genes associated with fungal pathogenesis can facilitate development of rust resistant transgenic wheat. Elucidating functions of effector proteins, along with functions of their interacting partners, should help dissect the various players involved in host defense processes. Knowledge as to which

Fig. 5. *Shr7* delivered via *Pseudomonas fluorescens* EtHAN into 10- to 14-day-old wheat leaves suppresses hypersensitive response (HR) triggered by *Pseudomonas syringae* pv. *syringae* DC3000. **A**, Bar graph showing percent reduction in HR caused by *Shr7* (P value was <0.001 for both treatments). **B**, Syringe-infiltrated wheat leaves of cultivar Nugaines. GFP:pEDV6 served as control for co-infiltration experiments. Photographs were taken 3 days after infiltration. Experiments were repeated at least three times. a, GFP:pEDV6 + *Pseudomonas syringae* DC3000; b, *Pseudomonas syringae* DC3000; and c, *Shr7*:pEDV6 + *Pseudomonas syringae* DC3000.

defense pathways are targeted by the fungus may help breeders select R genes or gene combinations that are more resistant to breakdown by the fungus. Furthermore, as some of these effectors are conserved in all three species of *Puccinia* that infect wheat, they may prove to be valuable candidates for engineering rust resistance.

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