Protection Against Common Bean Rust Conferred by a Gene-Silencing Method

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Accepted for publication 19 April 2017.

ABSTRACT

Rust disease of the dry bean plant, *Phaseolus vulgaris*, is caused by the fungus *Uromyces appendiculatus*. The fungus acquires its nutrients and energy from bean leaves using a specialized cell structure, the haustorium, through which it secretes effector proteins that contribute to pathogenicity by defeating the plant immune system. Candidate effectors have been identified by DNA sequencing and motif analysis, and some candidates have been observed in infected leaves by mass spectrometry. To assess their roles in pathogenicity, we have inserted small fragments of genes for five candidates into *Bean pod mottle virus*. Plants were infected with recombinant virus and then challenged with *U. appendiculatus*.

Virus-infected plants expressing gene fragments for four of five candidate effectors accumulated lower amounts of rust and had dramatically less rust disease. By contrast, controls that included a fungal gene fragment for a septin protein not expressed in the haustorium died from a synergistic reaction between the virus and the fungus. The results imply that RNA generated in the plant moved across the fungal haustorium to silence effector genes important to fungal pathogenicity. This study shows that four bean rust fungal genes encode pathogenicity determinants and that the expression of fungal RNA in the plant can be an effective method for protecting bean plants from rust.

Millions of indigent people in Central America and Africa who practice subsistence agriculture depend on dry beans as a primary source of protein (Petty et al. 2015). Their food security is threatened by *Uromyces appendiculatus*, an obligate biotrophic fungus that causes rust disease on the common dry bean plant, *Phaseolus vulgaris*. Bean rust is one of the most devastating bean diseases in the world and can cause defoliation and severe yield loss on susceptible bean plants (Pastor-Corrales et al. 2001). Genetic hypersensitive resistance, the most cost-effective method of control, is conferred by dominant resistance (*R*) alleles that can be traced to two geographic regions of origin, Andes and Mesoamerica, where distinct bean rust isolates coevolved with their hosts (Pastor-Corrales 2004). Because of this regional evolutionary adaptation, Andean germplasm is a source of resistance to some Mesoamerican rust isolates and vice versa. International efforts are underway to breed new varieties of resistant beans by combining the geographic gene pools (Pastor-Corrales et al. 2001). Rusts, however, historically overcome *R* genes (Johnson 1961); therefore, alternative modes of control need to be considered and the mechanisms by which rusts infect plants need to be investigated.

The windborne asexual uredospores of *Uromyces* spp. germinate in dew on leaf surfaces, and the emergent germplings penetrate stomata to enter the leaf apoplast in search of suitable cells to infect (Hoch et al. 1987). Once inside the leaf, the fungal hypha swells to form a haustorium mother cell that degrades a mesophyll cell wall. The mother cell then forms a haustorium to interface with the plant cell membrane (Staples and Hoch 1988). The haustorium serves as a conduit of molecular exchange required to establish fungal biotrophy.

Proteins that enable the fungus to overcome the plant immune system or that program the plant to produce nutrients for the fungus are called effectors. It is likely that many effectors traverse the haustorial membrane (Garnica et al. 2014). There are a few effectors from fungal and oomycete plant pathogens for which the biochemical functions are known (Niks et al. 2015), but only recently have effectors from rust fungi been shown to disrupt immunity in host and surrogate plant systems (De Carvalho et al. 2016; Germain et al. in press; Kunjeti et al. 2016; Liu et al. in press; Qi et al. 2016; Ramachandran et al. 2017). For example, *PpEC23* from the soybean rust fungus likely interacts with a soybean transcription factor to suppress disease resistance (Qi et al. 2016). Meanwhile, there are two characterized *Uromyces* spp. proteins, RTP1 and a hexose transporter, but their defense suppression roles have yet to be demonstrated (Kemen et al. 2005; Voegelé et al. 2001). RTP1 may be a cysteine protease inhibitor or may provide structural support for the haustorium (Kemen et al. 2013; Pretsch et al. 2013) whereas the hexose transporter likely helps the fungus acquire carbohydrate nutrients from its host.

The functional characterization of rust effectors has been slowed by the obligate biotrophic nature of rust fungi. No mutant lines of rust fungi exist, and it is difficult to distinguish between the biochemical functions of factors contributing to biotrophy and pathogenicity because they are intertwined for an obligate biotrophic pathogen. One possible mechanism for testing gene function in haustorium-forming fungi and oomycetes is gene silencing, also known as RNAi (Nunes and Dean 2012). Nowara et al. (2010) proved this concept by using *Barley stripe mosaic virus* (BSMV) to transiently express RNA fragments of powdery mildew fungus *Blumeria graminis* 1,3- β -glucanase genes in wheat and by using transformation to stably express RNA hairpin loops of the same in barley. They observed reduced amounts of powdery mildew on the plants. The results implied that the fungus was capable of receiving and processing an RNA-mediated gene-silencing signal from the plant. Panwar et al. (2013) subsequently showed that BSMV expressing RNA fragments of general pathogenicity genes of the wheat leaf rust fungus *Puccinia triticina* also led to a reduction of rust in wheat.

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary data set is published online.

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They observed in the BSMV-infected wheat the presence of small-interfering RNA (siRNA) for the targeted genes, an indicator of the activation of RNA-silencing pathways. The results from these and other studies have revealed that RNA produced by the plant can move to the fungus, possibly through the haustorium, to activate gene-silencing pathways in the fungus (Yin et al. 2011, 2015). This can compromise fungal gene expression and protein production, resulting in reduced fungal accumulation and pathogenicity. Consequently, plant-mediated gene silencing appears to be a useful method to test the function of fungal genes. At the same time, it also shows promise as a mechanism to protect plants from fungal disease (Cheng et al. 2015; Govindarajulu et al. 2015; Hu et al. 2015; Koch et al. 2013).

We are interested in characterizing the effectors of *U. appendiculatus* to understand their roles in pathology, and we are interested in improving bean cultivars and broadening their resistance to rust. To identify *U. appendiculatus* candidate effectors, we sequenced cDNA from *U. appendiculatus* germlings, purified haustoria, and infected bean leaves (Cooper et al. 2007; Link et al. 2014; Puthoff et al. 2008). Candidate effector genes were selected for the presence of sequences that could target their proteins for secretion from the fungus. Many of the candidates resembled genes found only in other rust fungi, and this implied that they were important to rust biology. We then used mass spectrometry to verify that 58 candidate effector genes were expressed as proteins in infected bean leaves (Cooper et al. 2016). Now, to verify whether these candidate effectors are important for fungal pathogenicity, we have expressed a small portion of their genes in plants using *Bean pod mottle virus* (BPMV), a virus that can initiate gene silencing (Zhang et al. 2013). We then challenged the virus-infected plants with *U. appendiculatus*. Here, we report that the common bean rust fungus genes are pathogenicity determinants and that host-induced RNA silencing can protect bean plants from rust infection.

MATERIALS AND METHODS

DNA cloning. DNA sequences for *U. appendiculatus* candidate effector genes *14349PIG15plike* (contig14349), *05723chit* (contig05732), *15794unkn* (contig15794), *05026fam26* (contig05026), *02CFH03sep* (Hyp_02C_F_H03), and *05556asppep* (contig05556) were previously described (Cooper et al. 2007; Link et al. 2014). Overlapping 80-mer oligonucleotides were used to synthesize a 258-bp DNA fragment proximal to the stop codon of each candidate gene (Cooper 2014; Cooper et al. 2013). BLAST was used to verify that the fragments did not have homology to DNA sequences in the *Phaseolus vulgaris* genome (Schmutz et al. 2014). The fragments were amplified with primers that contained unique *KpnI* and *BamHI* restriction endonuclease sites and that maintained the translational reading frame of the BPMV RNA2 polyprotein (Table 1). Enzymatically digested polymerase chain reaction (PCR) products were inserted in sense orientation between the *KpnI* and *BamHI* restriction sites of pIAD35, which encodes the second genomic segment of BPMV (Zhang et al. 2009, 2010). Plasmid inserts were validated by DNA sequencing.

Pathogen inoculation. pBPMV-R1M (encoding genome segment 1 of a BPMV mutant that helps produce more visible mottle symptoms) and engineered pBPMV-R2 constructs were delivered by biolistics using a PDS-1000/He Gene Gun (Bio-Rad) to the primary leaves of 10-day-old *P. vulgaris* 'Black Valentine' seedlings. pIA1037, expressing the green fluorescent protein from BPMV genome segment 2 (1037), was used as a control. Bombarded plants developed symptoms on the first trifoliate leaf between 10 and 14 days later, at which point the first and second trifoliate leaves were sprayed with a liquid suspension of uredospores of *U. appendiculatus* race 41 (Cooper et al. 2007; Lee et al. 2009). The plants were placed in an 18°C dew chamber for 24 h, then moved to a 23°C growth room with fluorescent lighting. One virus-free, rust-inoculated plant was kept as an indicator to ensure successful rust infection, which was measured as two to

four pustules per square centimeter of leaf surface. Plants were examined for rust symptoms at 10 days after inoculation (DAI). Experiments in which the indicator plant did not develop rust symptoms at 10 DAI or did not display desired infection density were abandoned.

Reverse-transcription PCR. RNA was purified from virus-infected, uredospore-inoculated leaves when rust uredia appeared on indicator plants and controls. To verify that plants were infected with BPMV and that the virus retained the inserted gene fragment specific to each construct, 1 ng of RNA was amplified for 25 cycles using the Fseq primer for each specific rust gene insert, the IAD35CP655R primer specific to BPMV RNA2 cDNA, and the SuperScript III One-Step reverse-transcription (RT)-PCR kit (Table 1). The resulting approximately 1,240-bp products were separated on 1.0% agarose gels stained with ethidium bromide. To estimate the amount of *U. appendiculatus* present in inoculated leaves, 10 ng of DNase-treated RNA was tested with the QuantiFast Probe RT-PCR Plus kit (Qiagen) in an Mx3000P machine (Stratagene) according to the manufacturers' instructions. Primers and 6-FAM 5' end-labeled probes were designed to specifically amplify DNA 150 bp upstream from the segment of *14349PIG15plike* from which the silencing construct was derived (Table 1). Primers and a probe to detect the expression of the *P. vulgaris* elongation factor 1a gene were also used (Lee et al. 2009). All reactions were performed in duplicate. A standard curve consisting of serial 1:5 dilutions of total RNA starting with 250 ng was prepared from rust-infected, BPMV-1037-infected plants. RNA amounts from experimental plants were interpolated from standard curves with a correlation coefficient of 95% or greater. RNA amounts for *14349PIG15plike* were normalized to those for *P. vulgaris* elongation factor 1a.

Small RNA sequencing. Black Valentine leaves infected with BPMV-15794unkn were harvested at 10 DAI when virus symptoms began to appear. The plants were not inoculated with bean rust. RT-PCR (described above) confirmed virus infection and that the insert was retained. Small RNA were purified from the leaves using a miRNeasy purification kit (Qiagen). Small RNA were captured by 5'-phosphate groups and sequenced by Genewiz with an Illumina HiSeq2500, using 1 × 50-bp SR configuration (V4 Chemistry), producing 5 million siRNA sequences from 68 million reads.

RESULTS

In prior studies, we identified 58 *U. appendiculatus* effector protein candidates in rust-infected bean (Cooper et al. 2016; Link et al. 2014). Among these, 24 appeared to uniquely accumulate in infected bean leaves, while the others were found in both infected bean leaves and in germinated *U. appendiculatus* uredospores (germlings). These findings implied that the 24 proteins with specific accumulation in leaves, as opposed to germlings, could be secreted by a haustorium, the fungal cell structure that interfaces the plant cell during fungal biotrophy. Therefore, we initially chose to focus on these effector candidates found in infected plants and not germlings. Because *U. appendiculatus* expresses *DICER* and *AGO* genes needed to process siRNA (Cooper et al. 2007; Huang et al. 2016; Link et al. 2014) during infection, we hypothesized that we could use BPMV to express candidate effector RNA in the leaf and induce an RNA signal that could be transmitted to and processed in the haustorium, where effector gene expression and effector protein accumulation could be inhibited by RNAi. We predicted that this would lead to reduced fungal pathogenicity and growth (the two are tied together for an obligate biotroph).

Two candidates we first chose to study were 14349PIG15plike, a trehalose phosphatase family protein with 35% identity to *U. viciaefabae* PIG15, and 15794unkn, a protein of unknown function with 43% identity to PSTG_04761 from *Puccinia striiformis* f. sp. *tritici*

(Cooper et al. 2016). These candidate genes were first isolated from a haustorium-specific library and their proteins were among the most abundant candidate effectors found in infected leaves (Cooper et al. 2016; Link et al. 2014). Small DNA fragments of the 3' ends of the open reading frames for their genes were synthesized from oligonucleotides and inserted into a DNA plasmid of BPMV RNA2. As a control, we also tested 1037, a BPMV RNA2 that expresses the green fluorescent protein (as opposed to a gene fragment targeted for silencing). DNA plasmids for BPMV RNA1 and RNA2 were cobombarded onto *Phaseolus vulgaris* seedlings. 35S promoters drove the expression of BPMV RNA, and the virus propagated in the leaves and moved systemically as the plants developed. We confirmed by RT-PCR that the viruses retained the inserted gene fragments as they spread through the plants.

Virus-infected plants were then challenged with *U. appendiculatus* and monitored for rust pustules (uredia), the signs of rust infection. On many plants infected with BPMV-1037, the control virus, we observed rust pustules at 10 DAI (Fig. 1A). By and large, the BPMV-1037- and rust-infected plants had pustules covering the surfaces of the leaves. Quantitative RT-PCR analysis using primers that targeted a distinct region of *14349PIG15plike* confirmed the accumulation of rust RNA in BPMV-1037- and rust-infected plants (Fig. 2). In addition to rust pustules, we observed a synergistic effect in some BPMV-1037- and rust-infected plants, manifested as a spreading leaf necrosis that led to leaf death and drop for many

controls (Figs. 1B and E and 2). Consequently, it was not possible in all cases to extract RNA to test for fungal accumulation by RT-PCR (Fig. 2). Nevertheless, when leaves survived, ample amounts of fungal RNA were detected. Occasionally, however, smaller amounts of fungal RNA were detected on BPMV-1037-infected leaves, but we attributed this to the synergism that possibly limited fungal spread. Altogether, it was apparent that nearly all plants doubly infected with BPMV-1037 and *U. appendiculatus* suffered from the diseases. Synergistic effects between rust and virus infections have been previously documented (Yarwood 1951).

By contrast, plants infected with BPMV expressing the gene fragments for *14349PIG15plike* and *15794unkn* developed few rust pustules after being challenged with *U. appendiculatus* (Fig. 1C and D). Where occasional pustules did appear, it seemed that the leaf area had mild virus symptoms. Leaves infected with mosaic and mottle viruses can have green islands that are free of virus (Suzuki et al. 1989), and this might explain why parts of leaves with mild virus symptoms had one or two rust pustules. Variability in viral transgene expression also might have led to incomplete silencing. Such variability is expected and inherent to this well-established BPMV system and has been observed in other viral systems, too (Yin et al. 2015; Zhang et al. 2013). Nevertheless, the leaves appeared to be mostly free of fungal infection compared with controls. More notable was that none of the leaves infected with BPMV-*14349PIG15plike* and BPMV-*15794unkn* exhibited a

TABLE 1. Oligonucleotides used in this study

Name ^a	Sequence
14349PIG15plike 1F	TTTGCGGCGCTTACGCACAATGCATGTAACAAGGGAAGACTTGCTTCCACTTTACTTGATAGGTATAGTGGTAATCATTT
14349PIG15plike 2R	TGTTGCTCCAGCGCTTCGAACATTTGGAACGCTACTTGTAGTATCTCCAGGGACATGACAAAATGATTACCACTATACCT
14349PIG15plike 3F	TTTGAAGCGCTGGAGCAACATTTCCACCTAGTCGGGGCGCGCAAAACATTATCTGTGAGGGTTGGCTCTGCAGATATTTTC
14349PIG15plike 4R	AAGTAGTGCAATTGAAAAATTTGTAAACGTCGTGATGACTTCGTAAACTTGCGTCTGCTGAAATATCTGCAGAGCCAAC
14349PIG15plike Fseq	aaag GGA TCC CAC GTG TTTGCGGCGCTTACGCACAA
14349PIG15plike Rseq	ttg G GTA CCt AAGTAGTGCAATTGAAAAAT
14349PIG15plike RT-qPCR F	GAAAACGTGGAGCGTCAGTA
14349PIG15plike RT-qPCR R	GCAATGGGTTGGCTAGAAGT
14349PIG15plike RT-qPCR T	CCTCACCGTATCTTCGACTCGTTGC
05732chit 1F	AAAAGCGGTCTCAATGGATACAAACGTTACTTCGATGAGTGCAGTGGTGTCCCTTCTTAACCAACGGTAAATATTTTCAT
05732chit 2R	AAATGTTTCGCTTTTCGCAATTTGGGCTTTTGACACTGTGCTTTGTTGTATCGTATGAAATGAAATATTTACCGTTGGT
05732chit 3F	TTCCGAAAGCGGAAACATTTAGGCCGGAATCTACTTCTTTGACACACAAGGCCCGTAGATGCCACGGTCCAAGCAGCACG
05732chit 4R	AGTATTGCAGTGATCTCGGGACCTGTATGAAGACGTTCCGCGTGGGTTTATGACTTGGCGTGTGCTTGGACCGTGGC
05732chit Fseq	aaag GGA TCC CAC GTG AAAAGCGGTCTCAATGGATA
05732chit Rseq	ttg G GTA CCt AGTATTGCAGTGATCTCGGG
15794unkn 1F	ACTTTTCGTGAATCTGCGCAATGGATCAAAATGCCGAGCTAATAACCGATCCATCCCTTAGAGCCTCGGTGCAAGAGGT
15794unkn 2R	TTGGGAACGACCTCTACTTCCATGGGATGATTCTGGGACGTTTTCACCTGAACCTTCCAACACCTCTTGCACCGAGGCTCT
15794unkn 3F	GAAGTAGAGGTGCTTCCCAAGAAAGGTGGAACACTTTTATTGTTAGTCAAATTGATTCTGATGGGCTTGAAAACAAGAT
15794unkn 4R	TCTGCTCAATTTGCGCCGTTTGGGTTCAACCCCTTGCCATCCAATCGATTAAAGTTTGGATCTTGTTTTCAAGCCCATC
15794unkn Fseq	aaag GGA TCC CAC GTG ACTTTTCTGTAATTTCTGCGC
15794unkn Rseq	ttg G GTA CCt TCTGCTCAATTTGCGCCGTT
05026fam26 1F	TCTGGTGACTTTGTCAAGCGTATGAAAGAGTTTACGACACTTTCGCAATCAATGGACGCAAAATTCGCTCTTGGAGAAGC
05026fam26 2R	GAAAGCTATTTAGTGAGCCAGTCCGACACGATCTTTGTAGTACGTTTCAATGCAGCCCTGCTTCTCCAAGAGCGAATTT
05026fam26 3F	TGGCTCACTCAAAATAGCTTCGTCCCGAGATCAGCTTCTTCAACTGGTATACGCCGTTTGGTACAACAGTAAGAAAGACATA
05026fam26 4R	TTCCAGCTTGGCCGTCAGAAGGGCTGTTGATAAGCCGTTTCCAGCTAAGTTGATGTTGTTCTTTCTTACTGTTGTA
05026fam26 Fseq	aaag GGA TCC CAC GTG TCTGGTGACTTTGTCAAGCG
05026fam26 Rseq	ttg G GTA CCt TTCCAGCTTGGCCGTCAGAA
02CFH03sep 1F	TATAGAACTCAAACCTTTAAGTCAAGGTAATGCTGATCAAATTGGTCAATCCATTCAACCTGAAGATATGGCTACTCAATC
02CFH03sep 2R	AATTCAAATTTCTTCAATTTTCTTCTTCTTCTTCTTCAATTTGTTCTTCTTCAACCTGAATGATTGAGTAGCCATATCTTC
02CFH03sep 3F	AAATTGAAAGAAATGAAATACGTGTGCAACGTGAGATTAGTGAAGAAACGACAAGAACTCTTGGTTAAAGAAAGAAAGTCT
02CFH03sep 4R	AATGTATTCATCGATAGAAAGCAATGGACGCGTGCGCCAAGCGAGCCTCCATGTTTCGAAGACTTTCTTCTTAAACCAA
02CFH03sep Fseq	aaag GGA TCC CAC GTG TATAGAACTCAAACCTTTAAG
02CFH03sep Rseq	ttg G GTA CCt AATGTATTCATCGATAGAAAG
05556asppp 1F	GAGGCTTCCGAAACCTACAAGGCCAATGGTACAGAGTTCCAAATTCGATATGGTTCCGGTAGCTTGGAAAGGTGTTATTAG
05556asppp 2R	GTGCTTTTCGGCAAAATCAACGCCAGGAATGACCAAGTCAACCAATTTCTGAGCAGCTCATTTGCTAATAACACCTTCCAAGCT
05556asppp 3F	GTTGATTTTGGCGAAAGCACTAAAGAGCCTGGACTTGCGTTTGTCTTTTGGCAAGTTTGACGGAATCTTTGGTCTCGGTTA
05556asppp 4R	ACGAAGCACTttgTTaAGTaAAGGtGGGGACGGTGTGAAGGACTGAAATAGTATGGTAACCGAGACCAAGATTCC
05556asppp Fseq	aaag GGA TCC CAC GTG GAGGCTTCCGAAACCTACAA
05556asppp Rseq	ttg G GTA CCt ACGAAGCACTttgTTaAGT
IAD35CP655R	TGAGTTGACCAAGCTGTAAGA

^a RT-qPCR = quantitative reverse-transcription polymerase chain reaction.

synergistic symptom effect after infection with bean rust. In other words, in contrast to many BPMV-1037- and rust-infected plants, BPMV-14349PIG15plike and BPMV-15794unkn plants challenged with rust survived (Fig. 1E). Quantitative RT-PCR analysis using primers specific to a distinct region of 14349PIG15p-like revealed that most plants inoculated with the silencing viruses and challenged with rust accumulated much less 14349PIG15plike RNA (Fig. 2). Hence, the symptoms and the RT-PCR analysis with respect to the controls revealed that BPMV-14349PIG15plike and BPMV-15794unkn protected the plants from rust infection.

These results imply that the plant produced a silencing signal against the virus and that the signal or RNA migrated across the haustorium, leading to a reduction in fungal gene expression. To determine whether siRNA were potential signals, we inoculated bean with BPMV-15794unkn (but not rust) and sequenced small RNA from leaves at the time we would normally challenge with rust. In all, 1,000 small RNA (approximately 180,000 reads) of 20 to 25 bp and spanning the entire 258-bp length of the inserted 15794unkn fragment were detected (Supplementary Data S1). These data, along with the prior RT-PCR results of reduced RNA expression of 14349PIG15p-like in protected plants (Fig. 2), support our hypothesis that an RNA-silencing signal produced by the plant against a rust gene fragment in BPMV induced RNAi in the rust fungus.

After these successful trials, we examined genes for four other *U. appendiculatus* proteins. A chitinase-like protein, 05732chit, with 47% identity to a *Phakopsora pachyrhizi* (soybean rust) homolog, and 05026fam26, a family 26 glycoside hydrolase with 43% identity to a *P. pachyrhizi* homolog, were also among the highly abundant proteins found in rust-infected leaves (Cooper et al. 2016). By contrast, 05556aspppep, an aspartic peptidase with 77% identity to a *Puccinia striiformis* homolog, accumulated to lower levels in plant leaves (Cooper et al. 2016). In addition, 05556aspppep was also different than the others because it accumulated in germlings as well as infected leaves (Cooper et al. 2016). Finally, we examined 02CFH03sep, a septin protein that was not observed in infected plants but was found to accumulate to high levels in ungerminated asexual uredospores (Cooper et al. 2007).

Plants infected with BPMV-05732chit were protected from rust similarly to plants infected with BPMV-14349PIG15plike and BPMV-15794unkn (7 of 10 plants) (Fig. 3A) but BPMV-05732chit elicited much stronger virus symptoms on some plants, and this phenotype was associated with leaf stunting and rust accumulation (3 of 10 plants) (Fig. 3B). It is possible that there was off-target silencing that was detrimental to the plant or that the inserted gene fragment produced a read-through protein that increased symptomology in some cases. On the other hand, BPMV-05026fam26 strongly protected some plants (4 of 10 plants) and weakly protected others (4 of 10 plants) (Fig. 3C and D); synergistic necrosis was observed in just 2 of the 10 plants tested. By contrast, there was no apparent protection conferred by BPMV-05556aspppep (0 of 10 plants) (Fig. 3E) or BPMV-02CFH03sep (1 of 10 plants) (Fig. 3F). These plants suffered from rust infection or died from the synergistic reactions.

DISCUSSION

Few rust fungus effectors have been characterized at a biochemical level. The best described are the avirulence (Avr) proteins from flax rust that elicit hypersensitive resistance in plants with cognate *R* genes (Barrett et al. 2009; Catanzariti et al. 2006, 2010; Dodds et al. 2004, 2006). Their interactions with *R* proteins have been characterized in some cases but their primary functions in fungal virulence are not known. Some non-Avr effectors also have been described biochemically but their roles in virulence have been shown in only a few cases. For example, the non-Avr *U. fabae* RTP1 may support haustorium formation or act as a protease (Kemen et al. 2013; Pretsch et al. 2013). Effector candidates from other rust fungi such as poplar rust have been shown to have distinct subcellular localizations in plant cells, and it is hoped that their cellular

Fig. 1. *Phaseolus vulgaris* leaves infected with *Bean pod mottle virus* (BPMV) constructs and subsequently inoculated with *Uromyces appendiculatus*. **A**, Fungal uredia (pustules) and spores on a leaf infected with BPMV-1037 (nontargeted silencing control); **B**, beginnings of synergistic necrosis on a leaf infected with BPMV-1037; **C**, leaf infected with BPMV-14349PIG15plike showing few pustules; **D**, leaf infected with BPMV-15794unkn showing few pustules; and **E**, plants infected with BPMV-1037 (left) and BPMV-14349PIG15plike (right).

activities may betray their roles in pathogenicity or virulence (Germain et al. 2017; Hacquard et al. 2012; Petre et al. 2015). Meanwhile, some soybean rust proteins have been shown to promote virulence but how they function biochemically remains unknown (De Carvalho et al. 2016; Kunjeti et al. 2016; Qi et al. 2016). Hence, it can be said that scientists have made great strides in identifying rust fungal effector proteins but that the quest to characterize the intriguing properties of these effectors goes on.

Our participation in this quest has led to the identification of 58 *U. appendiculatus* candidate effectors in infected common bean leaves (Cooper et al. 2016). Here, we have tested whether five of these candidates are important for pathogenicity by trying to reduce their expressions by BPMV to activate in a bean plant gene-specific silencing

signals that could be transmitted back to the fungus. Hypothetically, if the candidate fungal genes are important for pathogenicity, then silencing them could lead to inhibition of pathogenicity and fungal growth. Our results show that the plants infected with BPMV expressing RNA for fragments of four of five candidate effectors developed fewer rust disease symptoms and accumulated less haustorium marker RNA compared with controls (Figs. 1, 2, and 3). In fact, some plants appeared to be free of rust disease, and this distinction was more pronounced compared with some of the controls that died from the synergistic complication of the rust and virus infections.

The results suggest that siRNA derived from BPMV expressing the gene fragments for *14349PIG15plike*, *15794unkn*, *05732chit*, and *05026fam26* protected the plants from necrotic death associated

Fig. 2. Relative amounts of *Uromyces appendiculatus* (rust) *14349PIG15plike* RNA with respect to *Phaseolus vulgaris* (plant) elongation factor 1a (EF1a) RNA in plants infected with *Bean pod mottle virus* (BPMV)-1037 (control), BPMV-*14349PIG15plike* (silencing), or BPMV-*15794unkn* (silencing) and challenged with *U. appendiculatus*. Three replicate experiments were performed for each silencing construct. Some plants died due to a synergistic reaction between BPMV-1037 and *U. appendiculatus*. Thus, no RNA could be collected. On some plants infected with silencing viruses, there was not enough rust RNA to yield a detectable signal within 40 polymerase chain reaction cycles (no Ct).

with the virus–rust synergistic reaction. Supporting evidence includes BPMV-15794*unkn* producing siRNA for insert sequence 15794*unkn*. We also provide evidence for reduced fungal expression of 14349*PIG15plike* in bean infected with BPMV-14349*PIG15plike*. This suggests that 14349*PIG15plike* was specifically silenced, although it is not proof because reduced expression is tied to an overall reduction in fungal biomass for an obligate biotroph. Nevertheless, it has already been shown that, when hair-pin loops for a β -glucuronidase (*GUS*) intron were expressed in tobacco and the tobacco was infected with haustoria-forming transgenic *Fusarium verticillioides* expressing *GUS*, the fungus had reduced *GUS* gene expression (Tinoco et al. 2010). This means that a molecular silencing signal can move from a plant to a fungus. Thus, we hypothesize that the bean plant recognized BPMV as part of its natural defense system and produced siRNA to defend itself from the virus (Baulcombe 2004). Consequently, siRNA were generated for 14349*PIG15plike*, 15794*unkn*, 05732*chit*, and 05026*fam26* insert sequences and transferred to *U. appendiculatus* to activate gene silencing and reduce the expression of the cognate genes. It is also possible that longer segments of RNA or viral RNA migrated to the fungus (Baulcombe 2015). Either way, the plant-produced RNA triggered gene silencing in the fungus, which resulted in reduced fungal infection and increased plant resistance. These results also demonstrate that 14349*PIG15plike*, 15794*unkn*, 05732*chit*, and 05026*fam26* are important for bean rust fungal pathogenicity and growth. The biochemical mechanisms by which these proteins function remain to be discovered.

On the basis of our mass spectrometry results and other laboratories' genomics results, there are still many more candidate effectors to be tested (Cooper et al. 2016; De Carvalho et al. 2016; Kunjeti et al. 2016). As we investigate, we may discover that the silencing of some effector genes leads to better protection than others, as demonstrated in the *Puccinia*–BSMV system (Yin et al. 2015). We show here that two of the fungal genes that we interrogated did not provide protection. The aspartic peptidase gene

05556*aspp* was among the sets of effector candidates identified by bioinformatics but differed from the others in that its protein accumulated in bean rust-infected leaves at lower levels and its expression was not limited to infectious stages (Cooper et al. 2016; Link et al. 2014). The lack of protection implies that 05556*aspp* is less important than the others for pathogenicity or that it is expressed in plants in a cell structure other than the haustorium and, therefore, is not accessible to siRNA processed in the haustorium. The latter possibility is made more credible with our test with septin, a noneffector with specific accumulation in uredospores but not haustoria (Cooper et al. 2007). Septin functions in cytokinesis; thus, it is possible that septin exists in the uredospore as a remnant of mitosis or a remnant of separation from the uredial primordia (Chen et al. 2016; Muller et al. 1974). Hence, we imagine that the siRNA for septin gene 02CFH03*sep*, if acquired or produced by the haustorium, do not have an effect on fungal pathogenicity or growth because 02CFH03*sep* is expressed at a much later stage of development. Of course, it is also possible that the constructs we designed for 05556*aspp* and 02CFH03*sep* were, by chance, unsuitable for generating effective gene silencing. At this time, we have a limited understanding of what parts of fungal genes are best suited for inducing silencing through plant-mediated RNAi-silencing mechanisms.

In conclusion, our experiments have shown that four bean rust fungal genes promote pathogenicity, and that the inhibition of these proteins can protect bean plants from infection. Nowara et al. (2010), armed with similar results using BSMV to silence powdery mildew genes, made resistant barley by transforming them to express RNA hairpin loops of the fungal gene targets. It should be possible to do the same in dicotyledonous bean and soybean to protect them against rusts. Alternatively, it may be possible to use RNA sprays to incite siRNA pathways that target fungal genes (Dalakouras et al. 2016; Koch et al. 2016). As we pursue these possibilities, we will likely continue to target effector genes or genes critical for a unique component of rust or fungal biology

Fig. 3. *Phaseolus vulgaris* leaves infected with *Bean pod mottle virus* (BPMV) constructs and subsequently inoculated with *Uromyces appendiculatus*. **A**, Leaves infected with BPMV-05732*chit* showing few pustules; **B**, occasional severe virus symptoms caused by BPMV-05732*chit*; **C**, leaf infected with BPMV-05026*fam26* showing few pustules; **D**, occasional fungal pustules on a leaf infected with BPMV-05026*fam26*; **E**, leaves infected with BPMV-05556*aspp* dying from the synergistic reaction with rust; and **F**, leaves infected with BPMV-02CFH03*sep* dying from the synergistic reaction with rust.

rather than routine genes for basic biochemistry common to plants and pathogens (Koch et al. 2013). It is possible that strong resistance can be achieved when targeting highly expressed effector proteins because of their importance to pathogenicity and because rust effectors have limited similarity to genes from plants or other organisms. This latter feature may help ensure silencing specificity to rust fungal pathogens. Consequently, we support initiatives to sequence a variety of *Uromyces* spp. genomes to find genes to which RNAi-mediated resistance mechanisms can be designed. There may be effector genes common to *U. appendiculatus* races of Andean and Mesoamerican descent that can be targeted to achieve broader protection (Yin et al. 2015).

ACKNOWLEDGMENTS

This research was funded entirely by the United States Department of Agriculture–Agricultural Research Service.

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