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Specific Hypersensitive Response–Associated Recognition of New Apoplastic Effectors from *Cladosporium fulvum* in Wild Tomato

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Tomato leaf mold disease is caused by the biotrophic fungus *Cladosporium fulvum*. During infection, *C. fulvum* produces extracellular small secreted protein (SSP) effectors that function to promote colonization of the leaf apoplast. Resistance to the disease is governed by Cf immune receptor genes that encode receptor-like proteins (RLPs). These RLPs recognize specific SSP effectors to initiate a hypersensitive response (HR) that renders the pathogen avirulent. *C. fulvum* strains capable of overcoming one or more of all cloned Cf genes have now emerged. To combat these strains, new Cf genes are required. An effectoromics approach was employed to identify wild tomato accessions carrying new Cf genes. Proteomics and transcriptome sequencing were first used to identify 70 apoplastic in planta–induced *C. fulvum* SSPs. Based on sequence homology, 61 of these SSPs were novel or lacked known functional domains. Seven, however, had predicted structural homology to antimicrobial proteins, suggesting a possible role in mediating antagonistic microbe-microbe interactions in planta. Wild tomato accessions were then screened for HR-associated recognition of 41 SSPs, using the *Potato virus X*–based transient expression system. Nine SSPs were recognized by one or more accessions, suggesting that these plants carry new Cf genes available for incorporation into cultivated tomato.

Leaf mold disease of tomato (*Solanum lycopersicum*) is caused by the biotrophic Dothideomycete fungal pathogen *Cladosporium fulvum* (syn. *Passalora fulva* and *Fulvia fulva*) (Thomma et al. 2005). The fungus likely originated in South America, the center of origin for tomato (Jenkins 1948), with

the first disease outbreak reported in South Carolina during the late 1800s (Cooke 1883). *C. fulvum* now occurs worldwide but is primarily a problem in greenhouse and high-tunnel environments, in which tomato plants are exposed to both moderate temperatures and high relative humidity. Disease symptoms are typified by pale green to yellow spots on the adaxial leaf surface as well as white to olive-green patches of mold on the abaxial leaf surface that turn brown upon sporulation. In the late stages of disease development, this sporulation is often associated with leaf wilting and partial defoliation, which, in severe infections, can cause death of the plant (Thomma et al. 2005).

During infection (i.e., in a compatible interaction), *C. fulvum* exclusively colonizes the tomato leaf apoplast, in which it grows in close contact with surrounding mesophyll cells (Thomma et al. 2005). This colonization is promoted through a collection of virulence factors, termed effector proteins, which the fungus secretes into the apoplastic environment (Laugé et al. 1997). To date, 13 *C. fulvum* effectors have been identified and the genes encoding these proteins have been cloned (Bolton et al. 2008; Joosten et al. 1994; Laugé et al. 2000; Luderer et al. 2002a; Mesarich et al. 2014; Ökmen et al. 2013; Stergiopoulos et al. 2012; Van den Ackerveken et al. 1993; van Kan et al. 1991; Westerink et al. 2004). The majority (11 of 13) are small secreted proteins (SSPs) of less than 300 amino-acid residues in length with i) an amino (N)-terminal signal peptide for secretion into the tomato leaf apoplast and ii) four or more cysteine (Cys) residues following their signal peptide cleavage site. An intrinsic virulence function has been determined for three of the 11 SSP effectors. The first of these, Avr2, which lacks a known functional domain, targets and inhibits at least four Cys proteases of tomato (Rcr3, Pip1, aleurain, and TDI-65) to prevent the degradation of *C. fulvum* proteins (Krüger et al. 2002; Rooney et al. 2005; Shabab et al. 2008; van Esse et al. 2008). The second, Avr4, possesses a carbohydrate-binding module family 14 domain (CBM_14; PF01607) that binds chitin present in the cell wall of *C. fulvum* to protect against hydrolysis by basic plant chitinases (van den Burg et al. 2004, 2006; van Esse et al. 2007). The third, Ecp6, possesses three lysin motif domains (LysM, PF01476) that function to perturb chitin-triggered immunity (Bolton et al. 2008; de Jonge et al. 2010; Sánchez-Vallet et al. 2013). More specifically, two of the LysM domains cooperate to sequester chitin fragments released from the cell wall of invading hyphae and, in doing so, outcompete host chitin immune receptors for the binding of chitin fragments (Sánchez-Vallet et al. 2013). The third LysM domain has been proposed to perturb chitin-triggered immunity through interference with the host chitin immune receptor complex (Sánchez-Vallet et al. 2013).

Despite their roles in virulence, the same effectors can also be an Achilles heel for *C. fulvum*. In certain accessions of tomato, specific effectors or their modulated targets can be directly or indirectly recognized, respectively, by corresponding Cf immune receptor proteins, to trigger immune responses that render the pathogen avirulent (Cook et al. 2015; de Wit et al. 2009; Wulff et al. 2009b). In such incompatible interactions, the main output of the immune system is the hypersensitive response (HR), a localized form of cell death that arrests growth of the pathogen at the infection site (Heath 2000). So far, 10 of the 11 *C. fulvum* SSP effectors, specifically Avr2, Avr4, Avr4E, Avr5, Avr9, Ecp1, Ecp2-1, Ecp4, Ecp5, and Ecp6, are known to be recognized as avirulence (Avr) determinants by the corresponding Cf immune receptors Cf-2.1/Cf-2.2, Cf-4, Cf-4E, Cf-5, Cf-9, Cf- Ecp1, Cf-Ecp2-1, Cf-Ecp4, Cf-Ecp5, and Cf-Ecp6, respectively (de Wit et al. 2009; Thomma et al. 2011). All Cf immune receptor genes cloned to date encode receptor-like protein (RLP) cell-surface receptors that possess extracytoplasmic leucine-rich repeats (eLRRs), a transmembrane domain, and a short cytoplasmic tail (Dixon et al. 1996, 1998; Jones et al. 1994; Panter et al. 2002; Takken et al. 1999; Thomas et al. 1997).

Several studies suggest that the eLRRs are responsible for the direct or indirect recognition of *C. fulvum* effector proteins in the tomato leaf apoplast (Seear and Dixon 2003; Van der Hoorn et al. 2001a; Wulff et al. 2001, 2009a).

It was determined early on that wild *Solanum* species and landraces are a rich source of resistance against *C. fulvum*. Indeed, all cloned Cf immune receptor genes are derived from wild *Solanum* species or landraces, with *Cf-2.1/Cf-2.2, Cf-9/Cf-9DC*, and *Cf-9B* from *Solanum pimpinellifolium* (Dixon et al. 1996; Jones et al. 1994; Panter et al. 2002; Van der Hoorn et al. 2001b), *Cf-4* and *Cf-4E* from *Solanum habrochaites* (Takken et al. 1999; Thomas et al. 1997), and *Cf-5* from the landrace *Solanum lycopersicum* var. *cerasiforme* (Dixon et al. 1998). Based on this knowledge, Cf immune receptor genes were introgressed from wild *Solanum* species and landraces into cultivated tomato by breeders over several decades (Kerr and Bailey 1964). While largely effective, intensive year-round cultivation of these plants has led to the emergence of natural *C. fulvum* strains capable of overcoming one or more of all cloned Cf immune receptor genes (Hubbeling 1978; Iida et al. 2015; Laterrot 1986; Li et al. 2015). Several types of sequence modification have been shown to occur in *Avr* effector genes that permit the evasion of Cf immune receptor gene–mediated resistance by *C. fulvum*. These are i) gene deletion, ii) the insertion of a transposon-like element (gene disruption), iii) single nucleotide polymorphisms that result in nonsynonymous amino-acid substitutions, and iv) nucleotide insertions or deletions (indels) that result in frameshift mutations (Stergiopoulos et al. 2007). To combat strains capable of overcoming existing resistance specificities, new Cf immune receptor genes need to be identified for incorporation into cultivated tomato.

Laugé et al. (2000) hypothesized that "any stable, extracellular protein produced by a pathogen during colonization is a potential avirulence factor" and, given that all cloned Cf immune receptor genes encode an RLP, we set out to identify wild tomato accessions carrying new Cf immune receptor genes corresponding to apoplastic in planta–induced SSPs of *C. fulvum*, using effectoromics. Effectoromics is a powerful high-throughput functional genomics approach that uses effectors or effector candidates to probe plant germplasm collections for corresponding immune receptors (Domazakis et al. 2017; Du and Vleeshouwers 2014; Vleeshouwers and Oliver 2014). Notably, this approach, which is based on the HR-associated recognition of effectors or effector candidates, has already proven to be successful for the identification of wild accessions and breeding lines of *Solanum*-carrying Cf immune receptor genes corresponding to known effectors of *C. fulvum*. In a pioneering study by Laugé et al. (1998), 21 *S. lycopersicum* lines originating from early *C. fulvum*–resistance breeding programs were screened for their ability to recognize Ecp2-1, using the *Potato virus X* (PVX)-based transient expression system (Hammond-Kosack et al. 1995; Takken et al. 2000), as well as by leaf injection with purified Ecp2-1 protein. Four lines, with the same *S. pimpinellifolium* ancestor, recognized Ecp2-1, indicating for the first time that tomato carries an immune receptor gene corresponding to this effector (*Cf-Ecp2-1*) (Laugé et al. 1998).

In a follow-up study by Laugé et al. (2000), 28 *S. lycopersicum* breeding lines, many of which also have a *S. pimpinellifolium* ancestor, were screened for their ability to recognize purified Ecp1, Ecp2-1, Ecp3 (amino-acid sequence not yet known), Ecp4, or Ecp5 protein. Four lines recognized Ecp2-1, while two different lines recognized Ecp3 and Ecp5, respectively (Laugé et al. 2000). In the same study, a collection of 40 different *S. pimpinellifolium* accessions were also screened for their ability to recognize the same five effectors as well as Avr4 and Avr9, using the PVX-based transient expression system. Three different accessions recognized Ecp1, Ecp2-1, and Ecp3 (purified protein), respectively, while two recognized Ecp4, three recognized Ecp5, and six recognized Avr9

(Laugé et al. 2000). Again, this study indicated for the first time that tomato carries immune receptor genes corresponding to Ecp3 (*Cf-Ecp3*), Ecp4 (*Cf-Ecp4*), and Ecp5 (*Cf-Ecp5*) (Laugé et al. 2000). Three known *C. fulvum* effectors have since been shown to be recognized by wild tomato accessions through infiltration of purified protein, specifically Ecp6 in *S. lycopersicum* (Thomma et al. 2011) as well as Avr4 and Avr9 in *S. pimpinellifolium* (Kruijt et al. 2005; Van der Hoorn et al. 2001b).

As a starting point for our effectoromics approach, we used proteomics and transcriptome sequencing to identify 70 apoplastic in planta–induced SSPs of *C. fulvum*. This set of 70 is made up of all 11 known SSP effectors of this fungus as well as two phialide proteins and 57 new extracellular proteins. We screened 41 of these SSPs for HR-associated recognition by wild tomato accessions, using the PVX-based transient expression system. A total of nine SSPs were recognized by one or more of 14 wild tomato accessions, suggesting that these plants carry new Cf immune receptor genes available for incorporation into cultivated tomato.

RESULTS

Proteomics and transcriptome sequencing identify 70 apoplastic in planta–induced SSPs of *C. fulvum*.

Liquid-chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify fungal peptides corresponding to SSPs present in intercellular washing fluid (IWF) samples of compatible *C. fulvum*–tomato (*S. lycopersicum* cv. Heinz [H]-Cf-0) interactions. Here, SSPs are defined as those proteins of <300 amino-acid residues in length with a predicted N-terminal signal peptide but without a predicted glycophosphatidylinositol (GPI) anchor modification site, one or more transmembrane domains, a carboxyl (C)-terminal endoplasmic reticulum (ER) retention/retention-like signal, or sequence homology to enzymes. Using this approach, 297 unique fungal peptides were mapped to 75 SSPs of *C. fulvum* (Table 1; Supplementary Information S1). Based on pre-existing RNA-Seq transcriptome sequencing data from a compatible *C. fulvum* 0WU–*S. lycopersicum* cv. H-Cf-0 interaction at 4, 8, and 12 days postinoculation (dpi) as well as from *C. fulvum* 0WU grown in vitro in potato-dextrose broth (PDB) or Gamborg B5 liquid media at 4 dpi (Mesarich et al. 2014), 70 of the 75 apoplastic SSPs (approximately 93.3%) were deemed to be encoded by in planta–induced genes (Supplementary Table S1).

Among the 70 apoplastic in planta–induced SSPs are all *C. fulvum* SSP effectors identified in previous studies (Avr2, Avr4, Avr4E, Avr5, Avr9, Ecp1, Ecp2-1, Ecp4, Ecp5, Ecp6, and Ecp7) (Bolton et al. 2008; Joosten et al. 1994; Laugé et al. 2000; Luderer et al. 2002a; Mesarich et al. 2014; Van den Ackerveken et al. 1993; van Kan et al. 1991; Westerink et al. 2004) as well as 32 of 43 (approximately 74.4%) *C. fulvum* candidate effectors recently discovered using a combined bioinformatic and transcriptome sequencing approach (Mesarich et al. 2014). The latter includes CfPhiA-1, a phialide protein previously identified in the IWF sample of a compatible *C. fulvum* (strain IPO 1979)–tomato (*S. lycopersicum* cv. Moneymaker [MM]-Cf-0) interaction at 14 dpi (Bolton et al. 2008). Strikingly, 62 of the 70 apoplastic in planta–induced SSPs (approximately 88.6%) are both Cys-rich (≥4 Cys residues) and have an even number of Cys residues (Table 1).

Basic local alignment search tool (BLAST) homology searches against publicly available sequence databases at the National Center for Biotechnology Information (NCBI) and the Joint Genome Institute (JGI) revealed that 14 of the 70 apoplastic in planta–induced SSPs are novel (20%), while 47 (approximately 67.1%) have homology to proteins of unknown function (Table 1). The nine remaining in planta–induced SSPs (approximately 12.9%) have known or predicted functional domains or have homology to proteins with characterized biological functions. These are Avr4 (CBM_14 domain, PF01607), Ecp2-1 (Hce2 domain,

PF14856), Ecp6 (three LysM domains, PF01476), CfPhiA-1 and CfPhiA-2 (phialide proteins), Ecp14-1 (class II hydrophobin [Fig. 1]), Ecp44 (GPI-anchored superfamily domain, PF10342), Ecp45 (cerato-platanin protein, PF07249), and Ecp50-1 (hydrophobic surface-binding protein A [HsbA] domain, PF12296) (Table 1). BLAST homology searches and Cys spacing comparisons also revealed that 21 in planta–induced SSPs are related to each other at the amino-acid level. These are Ecp4, Ecp7, and Ecp29 (C-terminal region [CTR] [residues 158 to 266]); Ecp9-1 and Ecp9-9; Ecp10-1 and Ecp10-2; Ecp19-1 and Ecp19-2; Ecp20-1, Ecp20-2, and Ecp20-3; Ecp24-1 and Ecp24-2; Ecp28-1, Ecp28-2, Ecp28-3, and Ecp29 (N-terminal region [NTR] [residues 21 to 113]); Ecp32-1 and Ecp32-2 (IgE-binding proteins); and CfPhiA-1 and CfPhiA-2 (Table 1). Consistent with members of the Ecp2 family (Ecp2-1, Ecp2-2, and Ecp2-3) (Stergiopoulos et al. 2012), related extracellular proteins mentioned here have been grouped into families, with each member sequentially numbered using an extension (i.e., -1, -2, and -3). The exceptions are Ecp4 and Ecp7, which were named in previous studies (Bolton et al. 2008; Laugé et al. 2000), and Ecp29, which belongs to both the Ecp4/7 and Ecp28 families.

As 61 of the 70 apoplastic in planta–induced SSPs (approximately 87.1%) are novel or have homology to proteins of unknown function, 10 three-dimensional protein structure prediction servers were employed to infer possible structural relationships between these and proteins of characterized tertiary structure and/or function present in the Research Collaboratory for Structural Bioinformatics protein databank (RCSB protein databank). Three SSPs (Ecp20-1, Ecp20-2, and Ecp20-3) were consistently predicted to have structural homology to Alt a 1 (RCSB protein databank numbers 3V0R and 4AUD), an allergen protein with a β-barrel fold (Chruszcz et al. 2012) from the broad host-range Dothideomycete fungal plant pathogen and saprophyte *Alternaria alternata* (Supplementary Table S2). Four SSPs (Ecp4, Ecp7, Ecp29 [CTR], and Ecp30) were consistently predicted to have structural homology to proteins with a β/γ-crystallin fold, including the plant antimicrobial protein MiAMP1 from *Macadamia integrifolia* (1C01) (McManus et al. 1999) and the yeast killer toxin WmKT from *Williopsis mrakii* (1WKT) (Antuch et al. 1996). Three other SSPs (Ecp28-1, Ecp28-2, and Ecp28-3) were consistently predicted to have structural homology to one or both the α and β subunits of KP6 (1KP6 and 4GVB), a virus-encoded antifungal killer toxin with an α/β-sandwich fold secreted by the fungal corn smut pathogen *Ustilago maydis* (Allen et al. 2013a; Li et al. 1999). Notably, the NTR of Ecp29 was found to share sequence homology with Ecp28-1, Ecp28-2, and Ecp28-3, suggesting that it, too, adopts a KP6-like fold. The NTR and CTR of Ecp29 are separated by a putative kexin protease cleavage site (Supplementary Fig. S1A).

Hidden Markov model (HMM) alignments generated between Ecp20-1 and Alt a 1, Ecp4 and MiAMP1, as well as Ecp28-3 and KP6β (i.e., as part of the HHPred server output [Söding et al. 2005]) are shown in Supplementary Figure S2. In addition to conserved elements of the secondary structure, all three alignments revealed conserved Cys residues. For Ecp20-1 and Alt a 1, two conserved Cys residues at positions 50 and 65 (mature proteins), which are also present in Ecp20-2 and Ecp20-3, were identified. In Alt a 1, these Cys residues are known to form an intramolecular disulphide bond (Chruszcz et al. 2012). Inspection of the predicted Ecp20-1 tertiary structure, which was modeled using Alt a 1 as a template in HHpred (MODELER) (Söding et al. 2005; Webb and Sali 2002) and RaptorX (Källberg et al. 2012), suggests that the conserved Cys50/Cys65 pair forms an intramolecular disulphide bond (Supplementary Fig. S3A). Furthermore, the predicted structure suggests that the two remaining Cys residues, Cys24 and Cys29, which are absent from Alt a 1, may also form an intramolecular disulphide bond, given that they are located in close proximity to each other. This bond, however, would be in a different

location to the second intramolecular disulphide bond of Alt a 1 (Cys104-Cys116) (Chruszcz et al. 2012).

Five of the six Cys residues present in Ecp4 and MiAMP1 were found to be conserved. In MiAMP1, all six Cys residues are known to form intramolecular disulphide bonds (Cys11-Cys65, Cys21-Cys76, and Cys23-Cys49) (McManus et al. 1999). Inspection of the predicted Ecp4 structure, which was modeled using MiAMP1 as a template, suggests that two of the conserved

Table 1. Apoplastic small secreted proteins (SSPs) of *Cladosporium fulvum* produced during colonization of susceptible tomato (*Solanum lycopersicum* cv. Heinz-Cf-0)[a]

SSP name	GenBank accession no.	Protein length (aa)	No. cysteine residues[b]	Brief description and functional domains
Avr2	CAD16675	78	8	Avr effector recognized by the Cf-2 immune receptor. Cysteine protease inhibitor. Similar to hypothetical proteins
Avr4	CAA55403	135	8	Avr effector recognized by the Cf-4 immune receptor. Protects cell wall chitin. CBM_14 domain (PF01607)
Avr4E	AAT28196	121	6	Avr effector recognized by the Cf-4E immune receptor. Novel
Avr5	AHY02126	103	10	Avr effector recognized by the Cf-5 immune receptor. Novel
Avr9	P22287	63	6	Avr effector recognized by the Cf-9 immune receptor. Cysteine knot fold. Similar to hypothetical proteins
Ecp1	CAA78400	96	8	Avr effector recognized by the Cf-Ecp1 immune receptor. Similar to hypothetical proteins
Ecp2-1	CAA78401	165	4	Avr effector recognized by the Cf-Ecp2-1 immune receptor. Hce2 domain (PF14856)
Ecp4	CAC01609	119	6	Avr effector recognized by the Cf-Ecp4 immune receptor. Predicted β/γ-crystallin-like fold. Similar to hypothetical proteins. Paralog of Ecp7. Homolog of Ecp29
Ecp5	CAC01610	115	6	Avr effector recognized by the Cf-Ecp5 immune receptor. Similar to hypothetical proteins
Ecp6	AQA29283	222	8	Avr effector recognized by the Cf-Ecp6 immune receptor. Suppresses chitin-triggered immunity. Three LysM domains (PF01476)
Ecp7	AQA29284	116	6	Predicted β/γ-crystallin-like fold. Similar to hypothetical proteins. Paralog of Ecp4. Homolog of Ecp29
Ecp8	AQA29209	105	8	Possible Avr effector. Novel
Ecp9-1	AQA29212	88	6	Possible Avr effector. Similar to hypothetical proteins. Paralog of Ecp9-9
Ecp9-9	AQA29252	90	6	Similar to hypothetical proteins. Paralog of Ecp9-1
Ecp10-1	AQA29217	70	6	Possible Avr effector. Similar to hypothetical proteins. Paralog of Ecp10-2
Ecp10-2	AQA29234	67	6	Similar to hypothetical proteins. Paralog of Ecp10-1
Ecp11-1	AQA29221	165	10	Possible Avr effector. Homolog of the AvrLm3 Avr effector from *Leptosphaeria maculans*
Ecp12	AQA29229	133	8	Possible Avr effector. Similar to hypothetical proteins
Ecp13	AQA29236	73	10	Possible Avr effector. Similar to hypothetical proteins
Ecp14-1	AQA29258	206	12	Possible Avr effector. Class II hydrophobin
Ecp15	AQA29262	131	8	Possible Avr effector. Similar to hypothetical proteins
Ecp16	AQA29251	101	8	Possible Avr effector. Novel
Ecp17	AQA29222	62	6	Possible Avr effector. Novel
Ecp18	AQA29206	81	10	Novel
Ecp19-1	AQA29207	162	8	Similar to hypothetical proteins. Paralog of Ecp19-2
Ecp19-2	AQA29219	130	8	Similar to hypothetical proteins. Paralog of Ecp19-1
Ecp20-1	AQA29208	166	4	Predicted Alt a 1 allergen-like fold. Similar to hypothetical proteins. Paralog of Ecp20-2 and Ecp20-3
Ecp20-2	AQA29228	149	4	Predicted Alt a 1 allergen-like fold. Similar to hypothetical proteins. Paralog of Ecp20-1 and Ecp20-3

(Continued on next page)

[a] With the exception of Ecp56–Ecp60-1, all SSPs are encoded by genes that are induced in planta. Ecp = extracellular protein; aa = amino acids; Avr = Avirulence.
[b] Number of cysteine residues in each mature SSP (i.e., following their predicted N-terminal signal peptide cleavage site).

Cys pairs, Cys16/Cys84 and Cys35/Cys67, form intramolecular disulphide bonds. Although not conserved, the sixth Cys residue in Ecp4, Cys57, still appears to be in a favorable position for disulphide bond formation with Cys99. All six Cys residues in Ecp4 are conserved across Ecp7 and Ecp29 (CTR), although the latter has an additional pair of Cys residues.

For Ecp28-3 and KP6β, six conserved Cys residues, which are also present in Ecp28-1 and Ecp28-2, were identified. In KP6β, these six Cys residues are known to form three intramolecular disulphide bonds (Cys9-Cys74, Cys11-Cys64, and Cys29-Cys46) (Allen et al. 2013a). The predicted Ecp28-3 structure, which was modeled using KP6β as a template, suggests

Table 1. (*Continued from previous page*)

SSP name	GenBank accession no.	Protein length (aa)	No. cysteine residues[b]	Brief description and functional domains
Ecp20-3	AQA29267	153	4	Predicted Alt a 1 allergen-like fold. Similar to hypothetical proteins. Paralog of Ecp20-1 and Ecp20-2
Ecp21-1	AQA29210	184	8	Similar to hypothetical proteins
Ecp22	AQA29211	161	8	Similar to hypothetical proteins
Ecp23	AQA29215	91	4	Novel
Ecp24-1	AQA29216	92	4	Homolog of Ecp24-2. Novel
Ecp24-2	AQA29265	77	1	Homolog of Ecp24-1. Novel
Ecp25	AQA29218	79	8	Similar to hypothetical proteins
Ecp26	AQA29223	65	6	Similar to hypothetical proteins
Ecp27	AQA29225	67	4	Similar to hypothetical proteins
Ecp28-1	AQA29227	101	6	Predicted KP6-like fold. Similar to hypothetical proteins. Homolog of Ecp28-2, Ecp28-3 and Ecp29
Ecp28-2	AQA29259	105	8	Predicted KP6-like fold. Similar to hypothetical proteins. Paralog of Ecp28-3. Homolog of Ecp28-1 and Ecp29
Ecp28-3	AQA29261	105	8	Predicted KP6-like fold. Similar to hypothetical proteins. Paralog of Ecp28-2. Homolog of Ecp28-1 and Ecp29
Ecp29	AQA29274	266	14	Amino (N)-terminal domain has a predicted KP6-like fold. Carboxyl (C)-terminal domain has a predicted β/γ-crystallin-like fold. Similar to hypothetical proteins. Homolog of Ecp4, Ecp7, Ecp28-1, Ecp28-2 and Ecp28-3
Ecp30	AQA29247	141	6	Predicted β/γ-crystallin-like fold. Similar to hypothetical proteins
Ecp31	AQA29230	93	10	Novel
Ecp32-1	AQA29233	197	4	IgE-binding protein. Paralog of Ecp32-2
Ecp32-2	AQA29272	195	2	IgE-binding protein. Paralog of Ecp32-1
Ecp33	AQA29237	210	8	Similar to hypothetical proteins
Ecp34	AQA29238	92	8	Novel
Ecp35	AQA29239	70	8	Novel
Ecp36-1	AQA29240	73	10	Similar to hypothetical proteins
Ecp37	AQA29243	79	6	Novel
Ecp38	AQA29244	84	10	Novel
Ecp39	AQA29245	63	8	Similar to hypothetical proteins
Ecp40	AQA29250	92	8	Similar to hypothetical proteins
Ecp41	AQA29253	133	9	Similar to hypothetical proteins
Ecp42	AQA29254	128	14	Similar to hypothetical proteins
Ecp43-1	AQA29260	94	10	Similar to hypothetical proteins
Ecp44	AQA29263	146	4	Similar to hypothetical proteins. GPI-anchored domain (PF10342)
Ecp45	AQA29264	146	4	Cerato-platanin. Cerato-platanin domain (PF07249)
Ecp46	AQA29266	164	2	Similar to hypothetical proteins
Ecp47	AQA29268	148	10	Similar to hypothetical proteins
Ecp48	AQA29269	78	8	Similar to hypothetical proteins
Ecp49-1	AQA29270	104	7	Similar to hypothetical proteins
Ecp50-1	AQA29271	182	0	Hydrophobic surface-binding protein. HsbA domain (PF12296)
Ecp51	AQA29273	238	8	Similar to hypothetical proteins
Ecp52	AQA29275	170	4	Similar to hypothetical proteins
Ecp53-1	AQA29276	176	2	Similar to hypothetical proteins
Ecp54-1	AQA29278	160	11	Similar to hypothetical proteins
Ecp55	AQA29279	239	20	Similar to hypothetical proteins
Ecp56	AQA29281	60	6	Novel
Ecp57-1	AQA29324	285	5	CAP domain (PF00188)
Ecp58-1	AQA29297	196	4	Similar to hypothetical proteins
Ecp59-1	AQA29364	128	2	Similar to hypothetical proteins
Ecp60-1	AQA29321	249	6	Similar to hypothetical proteins
CfPhiA-1	AQA29214	195	4	Phialide protein. Paralog of CfPhiA-2
CfPhiA-2	AQA29256	218	6	Phialide protein. Paralog of CfPhiA-1

that the three conserved Cys pairs (Cys7/Cys76, Cys9/Cys66, and Cys26/Cys47) form intramolecular disulphide bonds. Both Ecp28-2 and Ecp28-3 possess an additional set of Cys residues (Cys1 and Cys60). Cys1 of Ecp28-3 is located at the extreme N-terminus, which, if flexible, would be expected to make contact with Cys60, located at the base of one of the predicted α-helices.

Most apoplastic in planta–induced SSPs of *C. fulvum* lack an ortholog in *Dothistroma septosporum*.

Of the fungi for which a genome sequence is so far available, *D. septosporum*, which causes red band needle blight disease of pine, is the most closely related to *C. fulvum* (de Wit et al. 2012). Recently, it was shown that homologs of Avr4, Ecp2-1, and Ecp6 are encoded by the genome of *D. septosporum* (de Wit et al. 2012). The same genome was also found to carry pseudogenes of *Ecp4* and *Ecp5* (de Wit et al. 2012). This raises the possibility that, despite differences in host type (angiosperm versus gymnosperm) and mode of host infection (biotroph versus hemibiotroph), substantial overlap exists between the apoplastic SSP effector sets of *C. fulvum* and *D. septosporum*. We set out to investigate this possibility further. To accomplish this, reciprocal BLASTp and tBLASTn searches were used to determine whether the predicted *D. septosporum* protein catalog and genome (de Wit et al. 2012) carry homologs of the 70 *C. fulvum* apoplastic in planta–induced SSPs and their encoding genes, respectively. For 43 of the 70 SSPs, no homologs were identified. Four more showed limited homology to *D. septosporum* SSPs, while five others had homology to pseudogenes. The remaining 18 SSPs had

likely orthologs in *D. septosporum*. However, of these, only 11 were up-regulated during infection of pine (Bradshaw et al. 2016). More specifically, these are the likely orthologs of Ecp2-1, Ecp6, Ecp13, Ecp19-2, Ecp20-1, Ecp20-2, Ecp20-3, Ecp45, Ecp53-1, Ecp55, and CfPhiA-2.

Nine apoplastic in planta–induced SSPs of *C. fulvum* trigger an HR in specific accessions of tomato.

To identify new sources of resistance against *C. fulvum*, wild accessions of tomato were screened for their ability to recognize apoplastic in planta–induced SSPs, using the PVX-based transient expression system (Hammond-Kosack et al. 1995; Takken et al. 2000). In this experiment, recombinant viruses were delivered through agroinfection for local (toothpick wounding) or systemic (cotyledon infiltration) expression of SSPs in tomato, with the pathogenesis-related 1A (PR1A) signal peptide of tobacco (*Nicotiana tabacum*) used to direct secretion of these proteins into the tomato leaf apoplast. Plants that showed a chlorotic or necrotic HR were deemed to have recognized an SSP as an Avr determinant.

As a starting point, 25 predominantly wild accessions of tomato (Supplementary Table S3) were screened for their ability to recognize 41 in planta–induced SSPs using the PVX agroinfection method based on toothpick wounding (Luderer et al. 2002a; Takken et al. 2000). This set of 41 primarily comprises those SSPs with the highest level of expression in planta, as based on pre-existing RNA-Seq data (i.e., those SSPs encoded by a gene with a peak in planta fragments per kilobase of exons per million fragments mapped [FPKM] value of >300). None of the
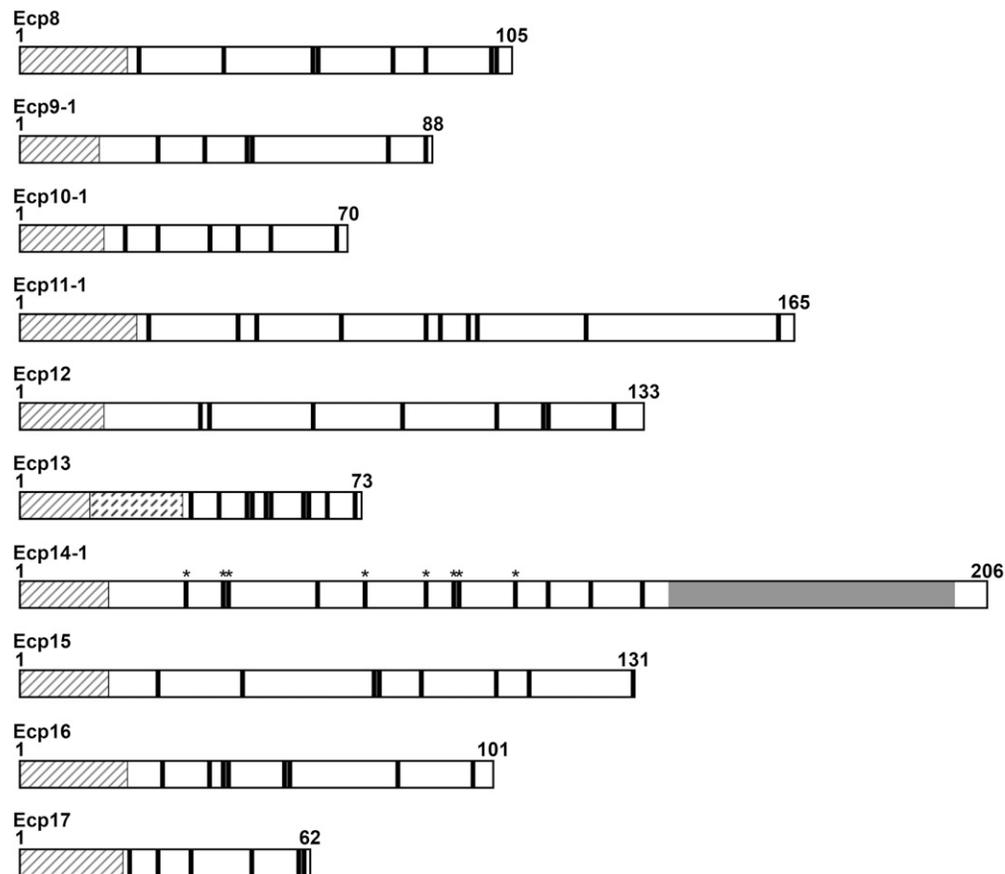
Fig. 1. Schematic representation of 10 apoplastic in planta–induced small secreted proteins (SSPs) from *Cladosporium fulvum* 0WU that trigger a hypersensitive response (HR) in one or more specific accessions of tomato. All 10 SSPs are small, cysteine-rich, and are predicted to possess an amino (N)-terminal signal peptide for extracellular targeting to the tomato leaf apoplast. The predicted signal peptide of each SSP is indicated by black diagonal lines. Cysteine residues are indicated by thick vertical bars. Numbers indicate the first and last amino-acid residue of each protein. The predicted propeptide domain of Ecp13, ending with a predicted kexin protease cleavage site, is indicated by black dashed diagonal lines. A glycine/leucine-rich region present in Ecp14-1 is shaded gray. Cysteine residues of Ecp14-1 that are conserved with fungal hydrophobin proteins are indicated by asterisks.

10 known Avr effectors are among the 41, since these SSPs already have a matching Cf immune receptor gene. Furthermore, a small number of highly expressed SSPs could not be tested, as the genes encoding these proteins could not be cloned from cDNA in time for use in the experiment. A fully expanded leaf from one to three representative plants of each accession was inoculated via toothpick wounding on each side of the main vein, and the presence or absence of an HR was scored at 10 dpi. At the same time, *S. lycopersicum* cv. MM-Cf-0 (no Cf immune receptor genes [Tigchelaar 1984]) was screened to determine whether any of the SSPs trigger a nonspecific HR. Likewise, accessions carrying only the *Cf-1*, *Cf-3*, *Cf-6*, *Cf-9B*, *Cf-11*, or *Cf-Ecp3* immune receptor gene were screened to determine whether any of the SSPs represent one of the yet-unknown Avr effectors Avr1, Avr3, Avr6, Avr9B, Avr11, or Ecp3. As positive controls, *S. lycopersicum* cv. MM-Cf-5, which carries only the *Cf-5* immune receptor (Tigchelaar 1984), as well as the landrace accession CGN 18399 (*S. lycopersicum* var. *cerasiforme*), from which the *Cf-5* gene was originally identified (Kerr et al. 1971), were screened for their ability to recognize the Avr effector Avr5 (Mesarich et al. 2014). Empty vector was used as a negative control to confirm that PVX alone does not trigger a nonspecific HR. For this experiment, recognition of an SSP was deemed to have occurred if an HR was triggered at one or both of the toothpick-wounding sites on a given tomato leaf.

As expected, the empty vector (negative control) failed to trigger an HR in any tomato accession tested, while Avr5 (positive control) was recognized by only MM-Cf-5 and CGN 18399 (Supplementary Fig. S4), indicating that the PVX agroinfection method is functional, and that no other accessions carry the *Cf-5* immune receptor gene. Ten of the 41 SSPs (Ecp8, Ecp9-1, Ecp10-1, Ecp11-1, Ecp12, Ecp13, Ecp14-1, Ecp15, Ecp16, and Ecp17) were recognized by one to eight predominantly wild accessions of tomato, with HRs ranging from weak chlorosis to strong necrosis. Furthermore, 15 of the 25 accessions recognized between one and four of the 10 SSPs. Importantly, none of the 10 SSPs triggered an HR in MM-Cf-0, suggesting that the observed responses were specific to the accessions tested. None of the accessions carrying the *Cf-1*, *Cf-3*, *Cf-6*, *Cf-9B*, *Cf-11*, or *Cf-Ecp3* immune receptor gene recognized any of the SSPs, indicating that these proteins do not represent the Avr effectors Avr1, Avr3, Avr6, Avr9B, Avr11, or Ecp3. A schematic of the 10 HR-eliciting SSPs is shown in Figure 1.

To further confirm recognition of the 10 SSPs, each was screened for its ability to trigger a systemic HR in the same responding tomato accessions, using the PVX agroinfection method, based on cotyledon infiltration (Mesarich et al. 2014). Here, both cotyledons of five independent plants were infiltrated, and the presence or absence of an HR was scored at 21 dpi. Consistent with the agroinfection assay based on toothpick wounding, the empty vector (negative control) did not trigger an HR in any accession tested (Fig. 2; Supplementary Fig. S5). Similarly, none of the SSPs triggered an HR in MM-Cf-0 (Supplementary Fig. S6). For Ecp8, Ecp12, Ecp13, Ecp14-1, Ecp15, and Ecp16, recognition could be confirmed across all responding accessions identified in the toothpick-wounding agroinfection assay (Fig. 2; Supplementary Fig. S7). Recognition could also be confirmed across most, but not all, previously identified accessions for Ecp9-1, Ecp10-1, and Ecp11-1 (Fig. 2). Indeed, Ecp9-1, Ecp10-1, and Ecp11-1 only failed to trigger an HR in accessions CGN 15392 (*Solanum arcanum*), CGN 14356 (*Solanum peruvianum*), and CGN 14357 (*Solanum corneliomuelleri*), respectively. In all responding accessions, the systemic HR involved weak to strong necrosis and was typically associated with moderate to severe stunting (Fig. 2; Supplementary Fig. S8). The recognition of only one SSP, Ecp17, could not be confirmed (CGN 24034). In some cases, the recognition of an SSP could not be observed across all five plants of a given accession

representing *S. corneliomuelleri* (CGN 14357 and CGN 15793), *S. peruvianum* (CGN 14355, CGN 14356 and CGN 24192), and *S. pimpinellifolium* (CGN 15946) (Fig. 2). This is not surprising, because both *S. corneliomuelleri* and *S. peruvianum* are typically self-incompatible, while *S. pimpinellifolium* is facultatively self-compatible (Peralta and Spooner 2006). In other words, genetic variation is expected to exist between representatives of accessions from these species, with this variation extending to the presence or absence of corresponding functional Cf immune receptor gene alleles. This may explain why Ecp17 gave such a strong HR in accession CGN 24034 using the toothpick assay but no HR in the agroinfiltration assay. This may also be true for Ecp9-1 on CGN 15392 (*S. arcanum*, typically self-incompatible), Ecp10-1 on CGN 14356, and Ecp11-1 on CGN 14357.

Tomato accessions that recognize apoplastic in planta–induced SSPs are resistant to *C. fulvum*.

To determine whether the accessions of tomato that recognize apoplastic in planta–induced SSPs are resistant to *C. fulvum*, each, along with *S. lycopersicum* cv. MM-Cf-0, was inoculated with strain 2.4.5.9.11 IPO of this fungus, and symptoms were inspected on leaves from three independent plants at 14 dpi. Strain 2.4.5.9.11 IPO carries genes corresponding to all nine HR-eliciting SSPs (discussed below) but lacks a functional copy of the previously cloned *Avr2*, *Avr4*, *Avr4E*, *Avr5*, and *Avr9 Avr* effector genes (Mesarich et al. 2014; Stergiopoulos et al. 2007). As expected, *S. lycopersicum* cv. MM-Cf-0 was susceptible to 2.4.5.9.11 IPO (Supplementary Fig. S9). In contrast, all other tomato accessions tested were resistant to this strain. For accessions CGN 14474 (*S. lycopersicum*) and CGN 15820 (*S. lycopersicum* × *cheesmaniae*), this resistance was observed across only two of the three independent plants. While resistant to *C. fulvum*, we cannot exclude the possibility that the set of resistant tomato accessions carries one or more of, for example, the Cf immune receptor genes *Cf-1*, *Cf-3*, *Cf-6*, *Cf-9B*, *Cf-Ecp1*, *Cf-Ecp2-1*, *Cf-Ecp3*, *Cf-Ecp4*, *Cf-Ecp5*, and *Cf-Ecp6*.

Seven HR-eliciting SSPs have one or more homologs in other fungal species, while three HR-eliciting SSPs have one or more paralogs in *C. fulvum*.

To identify homologs of the HR-eliciting SSPs in other fungi, each was screened against the publicly available protein sequence databases at NCBI and JGI using BLASTp. Additionally, in those cases in which no protein homolog could be identified, SSPs were screened against the collection of fungal genome sequences present at JGI using tBLASTn (i.e., to identify homologs without a gene prediction). Except for Ecp8 and Ecp16, homologs of all HR-eliciting SSPs were identified in other fungal species. For Ecp9-1, homologs were identified in the Dothideomycetes *Pseudocercospora fijiensis* (black sigatoka disease of banana), *Septoria musiva* and *Septoria populicola* (leaf spot and canker diseases of poplar), *Teratosphaeria nubilosa* (leaf spot of *Eucalyptus* spp.) and *Zasmidium cellare* (saprobic wine cellar fungus), as well as eight Sordariomycete species (Supplementary Fig. S10). Eight paralogs of Ecp9-1 were found to be encoded by the genome of *C. fulvum* 0WU (Ecp9-2 to Ecp9-9) (Supplementary Fig. S11A), with one pseudogene also identified (*Ecp9-10*; result not shown). A similar expansion was found in the Sordariomycete *Claviceps purpurea* (ergot disease of cereals).

Homologs of Ecp10-1 were identified in the Dothideomycetes *Pseudocercospora eumusae* and *Pseudocercospora musae* (eumusae leaf spot and yellow sigatoka disease of banana, respectively), *A. alternata*, *Septoria musiva*, *Septoria populicola*, *T. nubilosa*, and *Zasmidium cellare* as well as *Zymoseptoria ardabiliae*, *Zymoseptoria pseudotritici*, and *Zymoseptoria tritici* (leaf blotch diseases of grasses), *Venturia inaequalis* and *Venturia pirina* (apple and pear scab disease, respectively), *Clathrospora*

elynae (found growing on curved sedge), *Cochliobolus sativus* and *Cochliobolus victoriae* (cereal pathogens), *Pyrenophora teres* f. *teres* (net blotch disease of barley), *Pyrenophora tritici-repentis* (tan spot disease of wheat), and *Setosphaeria turcica* (northern corn leaf blight disease) (Supplementary Fig. S12; Supplementary Information S2). Homologs of Ecp10-1 were also identified in several Sordariomycete fungi. Interestingly, Ecp10-1 homologs were found to be massively expanded in *V. inaequalis* and *V. pirina*, which is not uncommon for effector candidates from these fungi (Deng et al. 2017). Smaller expansions were also identified in other fungal plant pathogens. Two paralogs of Ecp10-1 (Ecp10-2 and Ecp10-3) were found to be encoded by the genome of *C. fulvum* 0WU.

Homologs of the remaining SSPs were only identified in Dothideomycete fungi. Ecp11-1 was found to have homology to AvrLm3, an Avr effector protein from *Leptosphaeria maculans*

(blackleg disease of *Brassica* species) (Plissonneau et al. 2016) as well as two proteins from *Z. ardabiliae* (Fig. 3). A single pseudogene of *Ecp11-1* (*Ecp11-2*) was also identified in the genome of *C. fulvum* 0WU (result not shown). Ecp12 was found to have multiple homologs in *Septoria musiva* and *Septoria populicola*, with the homologous Cys-rich domain occurring once, or as two or three tandem repeats (Supplementary Fig. S13), as has been found for several other effectors from plant-associated organisms (Mesarich et al. 2015). Homologs of Ecp13 were identified in *D. septosporum*, *P. fijiensis*, *Septoria musiva*, and *Cercospora zeae-maydis* (gray leaf spot disease of maize) (Supplementary Fig. S14), while homologs of Ecp14-1 were found in *Cercospora zeae-maydis*, *D. septosporum*, *P. eumusae P. fijiensis*, *P. musae*, *Septoria musiva*, *Septoria populicola*, *T. nubilosa*, *Trypethelium eluteriae* (lichen-forming fungus), *Z. ardabiliae*, *Zymoseptoria brevis* (leaf blotch disease of barley), *Z. pseudotritici*, *Z. tritici*,
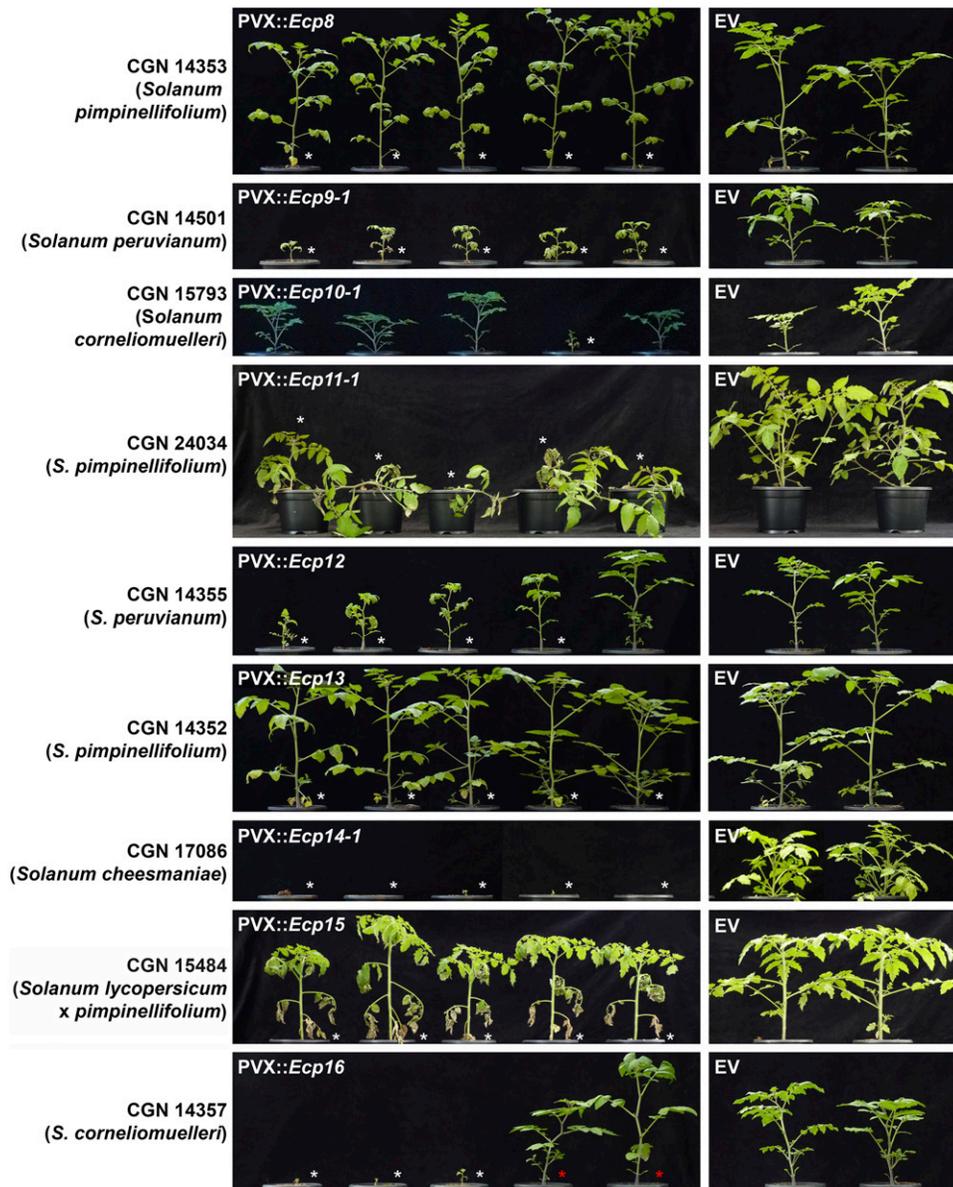
Fig. 2. Nine apoplastic in planta–induced small secreted proteins (SSPs) of *Cladosporium fulvum* 0WU trigger a systemic hypersensitive response (HR) in one or more specific accessions of tomato. Selected examples are shown. SSPs were systemically produced in five representatives of each tomato accession (left) using the *Potato virus X* (PVX) transient expression system. Recombinant viruses were delivered by *Agrobacterium tumefaciens* (agroinfection) through cotyledon infiltration at 10 days post–seed germination. Two representatives of each tomato accession were inoculated with PVX alone (pSfinx empty vector [EV]) (right). White asterisks indicate plants exhibiting a systemic chlorotic or necrotic HR; red asterisks indicate plants without obvious mosaic symptoms (i.e., not infected with PVX). Photographs were taken at 21 days postinfiltration.

and *Zasmidium cellare*, with most, including *C. fulvum*, possessing a paralog (Supplementary Fig. S15). A single pseudogene of *Ecp14-1* (*Ecp14-3*) was identified in the genome of *C. fulvum* 0WU (result not shown). For Ecp15, homologs were found in *P. fijiensis*, *P. musae*, and *Z. ardabiliae* (Supplementary Fig. S16).

Genes encoding HR-eliciting SSPs are induced in planta.

RNA-Seq FPKM values suggested that all genes encoding an HR-eliciting SSP of *C. fulvum*, like those encoding all previously identified Avr effectors of this fungus (Mesarich et al. 2014), are induced during infection of susceptible tomato when compared with expression during growth in vitro in PDB or Gamborg B5 liquid media. To confirm this expression profile, a reverse-transcription quantitative real-time polymerase chain reaction (RT-qrtPCR) experiment was performed. Indeed, all genes encoding an HR-eliciting SSP were found to be induced during infection of susceptible tomato when compared with expression during growth in vitro in PDB or Gamborg B5 liquid media (Fig. 4).

Most genes encoding an HR-eliciting SSP are associated with repetitive elements.

It is common for *C. fulvum* effector genes to be flanked by a mosaic of repetitive elements in the genome of strain 0WU (de Wit et al. 2012; Mesarich et al. 2014). It has been proposed that these elements may assist in the deletion of *Avr* effector genes following Cf immune receptor gene–imposed selection pressure (Mesarich et al. 2014). To determine whether repetitive elements also flank genes encoding the HR-eliciting SSPs, the genome scaffolds harboring each of these genes was screened for repetitive sequence across the *C. fulvum* 0WU genome using BLASTn. Six of the nine *SSP* genes (*Ecp8*, *Ecp9-1*, *Ecp10-1*, *Ecp11-1*, *Ecp12*, and *Ecp15*) were found to be associated with repetitive elements at both their 5 and 3′ flanks (Supplementary Fig. S17). Furthermore, the same six genes were found to reside on small genome scaffolds of less than 35 kb in length (Supplementary Table S4). The latter suggests that the scaffolds harboring these genes are surrounded by even larger flanking repetitive elements, with these elements anticipated to have hampered a larger scaffold assembly (de Wit et al. 2012). The

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Ecp11-1_AQA29221_Clafu          MLSSAKTLWLLLLSMLAYTTKPAYSLDCKAVALKWVHQFRIPGGDNCNFYCSYDSLYQQF 60
AvrLm3_ALS92799_Lepma           MLKPTKVIQILFLLFT-AFFARTCALECHAVAFSSDHQFSLGRNEDCNLYCSKNSMLSIF 59
Scaffold_667:56424-56906_Zymar  MERQNSAMALLLLAFL-GLVLPAFAHDCRSIALSYEARAVAGEFEDCRQYIDVNKMLADM 59
Scaffold_1237:24046-24549_Zymar MGQQFSA-LALLLAFL-GTIAPVFSHDCRAISITYEKRFRIPPYQDCAQYCSLKGMIDEM 58
                                *         *:* :         : :*:::::  :       ::* *    :   :

Ecp11-1_AQA29221_Clafu          NL--------WKKNDACQGADGFSTAIPKIQEAPCS-DCPGSKTCICSVQATAWRVRNG 110
AvrLm3_ALS92799_Lepma           SRV-------LPLDDPCQGDDGFSSLTATIDQISCDTGC----QCRCSVHATAWRVHKS 107
Scaffold_667:56424-56906_Zymar  NRQLVNLPDLDPPRADICMGDDGFGTYNTGCEFPACKQNC--KKNCRCWVRWTAWRVHKE 117
Scaffold_1237:24046-24549_Zymar NKRLDPVMTLGYPRADVCQGDDGFSTFASQIDYPRCSGAC--NTDCRCWVRWTAWRVHKE 116
                                            * * * *** :    :    *   *    * * *: *****::

Ecp11-1_AQA29221_Clafu          --KWFDGQQWFDCDVKPYTERVLGRRWYDESEADKDIYVGYYSRGFISNDNVHCGSQ- 165
AvrLm3_ALS92799_Lepma           GKRYSRRTGWVSCNLEDYVARITGRPFIPVNGALH----EYFSRGFVSKDEVHCDHQ- 160
Scaffold_667:56424-56906_Zymar  N------QGGVSCAIGDFASRLTTKQYKPPNYNGPF--LRTYVLATETNDNVHCGHQR 167
Scaffold_1237:24046-24549_Zymar D------QYGASCQVKDYVARLTGKPFVAPNDPIPGGPPYTFVTAIVSNNNVHCKSQ- 167
                                            * :  :  *:  : :       :         :::::*** *
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Fig. 3. Ecp11-1 of *Cladosporium fulvum* is a homolog of AvrLm3 from *Leptosphaeria maculans*. Conserved (*) and physicochemically similar (:) amino-acid residues shared between all proteins are shown below the alignment. Cysteine residues are highlighted in bold. The predicted amino (N)-terminal signal peptide sequence of each protein is underlined. Clafu = *C. fulvum* 0WU; Lepma = *L. maculans* v23.1.3; and Zymar = *Zymoseptoria ardabiliae* STIR04.
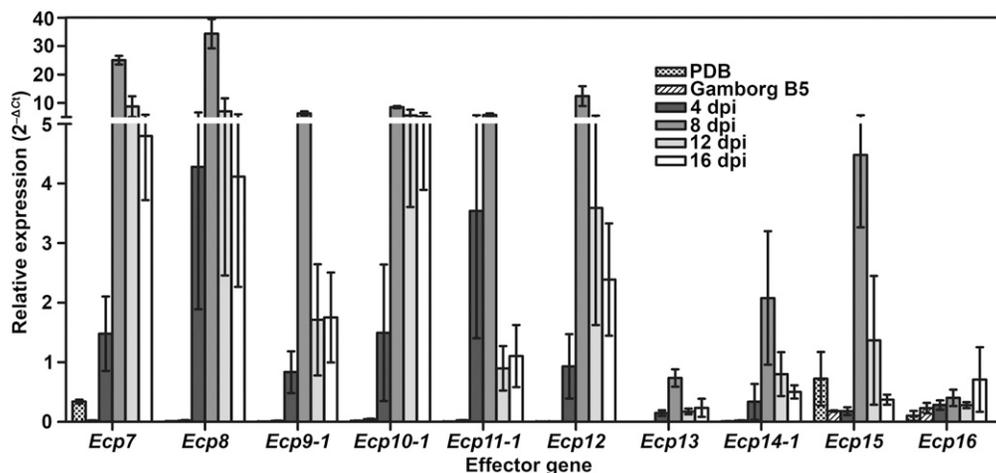
Fig. 4. Genes encoding a hypersensitive response–eliciting small secreted protein (SSP) from *Cladosporium fulvum* 0WU are induced in planta. Expression was monitored by a reverse-transcription-quantitative real-time polymerase chain reaction experiment in planta during a compatible *C. fulvum* 0WU–*Solanum lycopersicum* cv. Heinz Cf-0 interaction at 4, 8, 12, and 16 days postinoculation (dpi) as well as during growth of *C. fulvum* 0WU in vitro in potato-dextrose broth (PDB) and Gamborg B5 liquid media at 4 dpi. The *C. fulvum actin* gene was targeted for normalization of expression, which was calculated using the $2^{-\Delta Ct}$ method. Error bars represent the standard deviation of three biological replicates.

5′ end of *Ecp16* is closely associated with repetitive elements and is present at the 5′ end of an approximately 55-kb scaffold. Likewise, *Ecp13* is located at the 3′ end of an approximately 57-kb scaffold, suggesting the presence of 3′ repeats. In contrast to the *SSP* genes mentioned above, *Ecp14-1* is not surrounded by repetitive elements.

Genes encoding an HR-eliciting SSP exhibit limited allelic variation between strains.

It is common for genes encoding *C. fulvum* Avr effectors to exhibit allelic variation between strains, which is often brought about by selection pressure to avoid recognition by corresponding Cf immune receptor genes (Iida et al. 2015; Joosten et al. 1994; Luderer et al. 2002a; Mesarich et al. 2014; Westerink et al. 2004). To assess the level of allelic variation across genes encoding the HR-eliciting SSPs, each was amplified by PCR from 10 different *C. fulvum* strains (Supplementary Table S5), was sequenced, and was compared with the corresponding sequence from strain 0WU. All nine *SSP* genes could be amplified by PCR from genomic DNA samples representing the 10 *C. fulvum* strains. Of the nine genes, four, namely *Ecp9-1*, *Ecp10-1*, *Ecp13*, and *Ecp15*, exhibited no allelic variation between strains. For *Ecp8* and *Ecp16*, allelic variation was observed; however, this variation did not result in a change of amino-acid sequence. More specifically, in six strains (2.4, 2.4.5, 2.5, 2.9, 4, and 7320), *Ecp8* had a single synonymous CC<u>C</u>→CC<u>T</u> substitution at position 153 (underlined), while, in four strains (2.4, 2.4.5, 2.5, and 4), *Ecp16* had a trinucleotide insertion (CTT) at position 234 in an intron (Fig. 5). For each of the remaining three genes, a single nonsynonymous substitution was identified (underlined): a T<u>T</u>T→<u>G</u>TT (Phe119Val) change at position 355 in *Ecp11-1* of strain 2.9, a <u>G</u>GG→<u>A</u>GG (Gly124Arg) change at position 484 in *Ecp12* of strains 2.9 and 7320, and an A<u>A</u>G→<u>G</u>AG (Lys148Glu) change at position 501 in *Ecp14-1* of strains 2.4, 2.4.5, 2.4.5.9.11 IPO, 2.4.9.11, 2.5, 2.9, and 4 (Fig. 5). A G→T mutation at position 386 of the *Ecp14-1* intron in strains 2.4, 2.4.5, 2.4.5.9.11 IPO, 2.4.9.11, 2.5, and 4, as well as a synonymous GG<u>G</u>→GG<u>A</u> substitution at position 452 in *Ecp14-1* of strains 2.4, 2.4.5, 2.4.5.9.11 IPO, 2.4.9.11, 2.5, 2.9, and 4 were also identified (Fig. 5). It is not yet known whether the nonsynonymous substitutions identified in *Ecp11-1*, *Ecp12*, and *Ecp14-1* allow *C. fulvum* to overcome resistance mediated by the putative *Cf-Ecp11-1*, *Cf-Ecp12*, and *Cf-Ecp14-1* immune receptor genes, respectively.

DISCUSSION

Leaf mold disease of tomato, caused by the fungal pathogen *C. fulvum*, is a re-emerging problem worldwide. This re-emergence is due to the intensive year-round cultivation of resistant tomato cultivars, which have selected for natural strains of this fungus, capable of overcoming, for example, one or more of all cloned Cf immune receptor genes (Hubbeling 1978; Iida et al. 2015; Laterrot 1986; Li et al. 2015). To combat these strains, new Cf immune receptor genes need to be identified. Wild tomato is a rich source of resistance against *C. fulvum* (Kruijt et al. 2005; Laugé et al. 1998, 2000; Van der Hoorn et al. 2001b). In this study, an effectoromics approach (Domazakis et al. 2017; Du and Vleeshouwers 2014) based on apoplastic in planta–induced SSPs of *C. fulvum* was used to identify wild accessions of tomato carrying new Cf immune receptor genes.

As a starting point for this approach, proteomics and transcriptome sequencing were used to identify fungal SSPs most relevant to the *C. fulvum*–tomato interaction. Altogether, 70 apoplastic in planta–induced SSPs, made up of all 11 characterized SSP effectors of this fungus (Bolton et al. 2008; Joosten et al. 1994; Laugé et al. 2000; Luderer et al. 2002a; Mesarich et al. 2014; Van den Ackerveken et al. 1993; van Kan et al. 1991; Westerink et al. 2004), as well as two phialide proteins and 57 new extracellular proteins were identified in IWF samples from compatible *C. fulvum*–*S. lycopersicum* cv. H-Cf-0 interactions. Strikingly, all but eight of these SSPs are Cys-rich and possess an even number of Cys residues. Consistent with that shown for Avr4, Avr9, Ecp1, Ecp2-1, Ecp5, and Ecp6, it is likely that many of these Cys residues form intramolecular disulphide bonds required for stability and function in the protease-rich leaf apoplast of tomato (Joosten et al. 1997; Luderer et al. 2002b; Sánchez-Vallet et al. 2013; van den Burg et al. 2003; van den Hooven et al. 2001).

BLAST homology searches revealed that, in addition to Avr4 (CBM_14 domain, PF01607) (van den Burg et al. 2003), Ecp2-1 (Hce2 domain, PF14856) (Stergiopoulos et al. 2012), Ecp6 (three
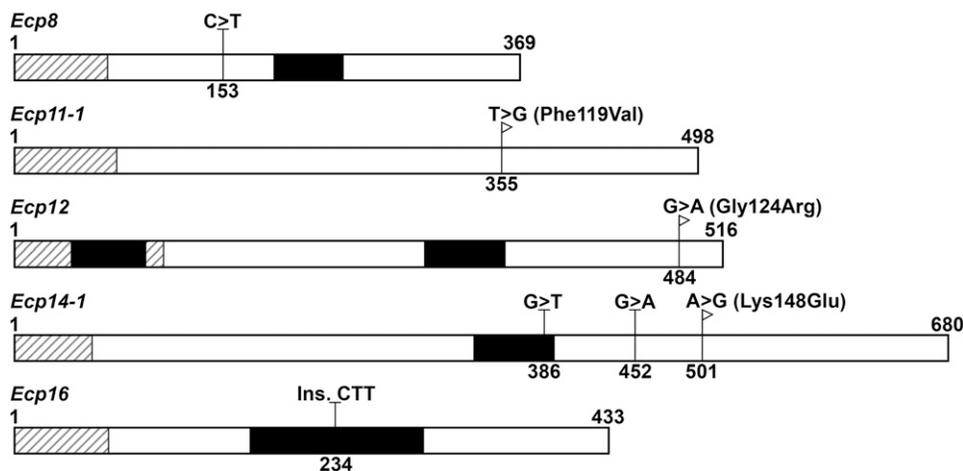
Fig. 5. Genes encoding a hypersensitive response–eliciting small secreted protein (SSP) exhibit limited allelic variation between strains of *Cladosporium fulvum*. Allelic variation was assessed across 10 distinct strains of *C. fulvum* and was compared with strain 0WU. Open reading frames (encoding each mature protein) and introns are shown as white and black boxes, respectively. Regions of each *SSP* gene predicted to encode an amino (N)-terminal signal peptide sequence are indicated by black diagonal lines. DNA modifications leading to nonsynonymous amino-acid substitutions are indicated by white flags. DNA modifications leading to synonymous amino-acid mutations or changes to intronic sequences are indicated by Ts. Numbers above each schematic represent the first and last nucleotide of each gene (i.e., of the ATG to STOP codons, respectively). Numbers on the bottom of each schematic represent the location of each DNA modification.

LysM domains, PF01476) (Bolton et al. 2008), and CfPhiA-1 (phialide protein) (Bolton et al. 2008), five other in planta–induced SSPs, specifically CfPhiA-2, Ecp14-1, Ecp44, Ecp45, and Ecp50-1, possess a known functional domain or have homology to proteins with a characterized biological function. Of these, CfPhiA-2 has homology to CfPhiA-1 and other phialide proteins from Ascomycete fungi. To date, the best-characterized of these homologs is PhiA from *Aspergillus nidulans*, which localizes to the cell wall of phialides and conidia (Melin et al. 2003). PhiA plays an essential role in the development of phialides, which are sporogenous cells that produce and release conidia through a specialized apical budding process (Melin et al. 2003).

Ecp14-1 is a member of the hydrophobins, a class of surface-active proteins specific to fungi (Wessels 1994). Ecp14-1 is the twelfth hydrophobin and sixth class II hydrophobin to be identified from *C. fulvum* (de Wit et al. 2012; Nielsen et al. 2001; Segers et al. 1999; Spanu 1997). It is also the first hydrophobin to be identified from this fungus that is exclusively expressed in planta (Fig. 4). Hydrophobins are initially secreted in a soluble form but, then, spontaneously localize to hydrophilic-hydrophobic interfaces, where they assemble into insoluble, amphipathic layers (Sunde et al. 2017). Hydrophobins are typically found on the outer cell-wall surface of aerial hyphae, fruiting bodies, and spores, in which they reduce wettability or significantly decrease the surface tension of moist environments, allowing these structures to grow in the air (Wösten et al. 1999). Other roles related to surface perception, attachment to hydrophobic surfaces, and plant colonization have also been shown (Kim et al. 2005; Talbot et al. 1993, 1996). So far, only two *C. fulvum* hydrophobins, HCf-1 (class I) and HCf-6 (class II), have been functionally characterized. HCf-1 is required for efficient water-mediated dispersal of conidia (Whiteford and Spanu 2001), while HCf-6 is required for adhesion of hyphae to solid surfaces (Lacroix et al. 2008).

Ecp44 has a GPI-anchored superfamily domain (PF10342) but is not predicted to possess a GPI anchor modification site. Little functional information is available for secreted proteins with this domain. In the Basidiomycete fungus *Lentinula edodes* (shiitake mushroom), the PF10342 domain-containing protein Le.DRMIP, which also possesses a mitochondrial targeting signal peptide and transmembrane domain, interacts with the developmentally regulated mitogen-activated protein kinase Le.MAPK. Both proteins have been proposed to play a role in cell differentiation during fruiting body development (Szeto et al. 2007).

Ecp45 is a member of the cerato-platanins (PF07249), a class of proteins ubiquitous to filamentous fungi that adopts a double Ψβ-barrel fold similar to domain one of expansins (Chen et al. 2013; de Oliveira et al. 2011). Cerato-platanins are predominantly secreted, although several also localize to the cell wall of ascospores, conidia, and hyphae (Boddi et al. 2004; Pazzagli et al. 1999). Cerato-platanins bind chitin but not cellulose (Baccelli et al. 2014; de O. Barsottini et al. 2013; Frischmann et al. 2013), yet several members have expansin-like loosening activity on cellulosic materials in vitro (Baccelli et al. 2014; de O. Barsottini et al. 2013). It has thus been hypothesized that cerato-platanins function as expansins required for fungal cell-wall remodeling and enlargement, possibly by disrupting non-covalent interactions between β-glucan or chitin molecules (de Oliveira et al. 2011). Epl1, a surface-active cerato-platanin from the biocontrol agent *Trichoderma atroviride*, self-assembles at the air-water interface to form protein films that increase the polarity of solutions and surfaces (Frischmann et al. 2013). This suggests an additional role for cerato-platanins in increasing the wettability of hyphae, enabling them to grow in aqueous environments, or in protecting them from desiccation (Frischmann et al. 2013). Other cerato-platanins likely function as effectors

(Jeong et al. 2007). Indeed, preliminary studies have suggested that MpCP5, a cerato-platanin from *Moniliophthora perniciosa* (witches' broom disease of cocoa) may, like Ecp6, perturb chitin-triggered immunity (de O. Barsottini et al. 2013), while cerato-platanins from *Fusarium graminearum* (cereal head blight disease) may, like Avr4, protect the fungal cell wall against hydrolysis by chitinases and β-1,3-glucanases (Quarantin et al. 2016). Some cerato-platanins also trigger a nonspecific HR upon recognition by corresponding host immune receptors (Frías et al. 2011, 2014). This, however, does not appear to be the case for Ecp45, which failed to trigger an HR in tomato.

Ecp50-1 contains an HsbA domain (PF12296), which was originally identified in the HsbA protein from *Aspergillus oryzae* (Ohtaki et al. 2006), a filamentous fungus commonly used in the fermentation industry. In culture, HsbA is secreted in the presence of the hydrophobic polymer polybutylene succinate-*co*-adipate (PBSA). HsbA binds PBSA and, in doing so, recruits CutL1, a polyesterase and cutinase, for its degradation (Ohtaki et al. 2006).

BLAST homology searches revealed that all other *C. fulvum* in planta–induced SSPs are novel or have homology to proteins of unknown function. Remarkably, 10 of these SSPs were consistently predicted to have structural homology to proteins present in the RCSB protein databank. Of these, Ecp20-1, Ecp20-2, and Ecp20-3 were predicted to be structurally homologous to Alt a 1 from *A. alternata*, which adopts a β-barrel fold unique to fungi (Chruszcz et al. 2012; De Vouge et al. 1996). Recent studies have shown that Alt a 1 is an effector protein with multiple roles in promoting host colonization. Initially, Alt a 1 localizes to the cytoplasm and cell wall of *A. alternata* spores (Garrido-Arandia et al. 2016b; Gómez-Casado et al. 2014). In humid settings, these spores then germinate, and, in environments with a pH range of between 5.0 and 6.5, Alt a 1 is released as a tetramer carrying a fungal methoxyflavonol ligand similar to the plant flavonol quercetin (Garrido-Arandia et al. 2016a and b). In the same pH range, which is typical of apoplastic environments, this complex breaks down, releasing Alt a 1 monomers and the flavonol ligand (Garrido-Arandia et al. 2016a and b). The Alt a 1 monomers then function as competitive inhibitors of extracellular plant defense proteins belonging to the pathogenesis-related 5–thaumatin-like protein family (Gómez-Casado et al. 2014), while the flavonol ligand detoxifies reactive oxygen species (Garrido-Arandia et al. 2016b). Interestingly, homologs of Ecp20-1, Ecp20-2, and Ecp20-3 are encoded by the genome of *D. septosporum* (de Wit et al. 2012) and these genes are up-regulated during the infection of pine (Bradshaw et al. 2016). This suggests that Ecp20-1, Ecp20-2, and Ecp20-3, together with Ecp2-1, Ecp6, Ecp14-1, and Ecp45, which are also in planta–induced SSPs of *D. septosporum* (Bradshaw et al. 2016; de Wit et al. 2012), are core effectors that play important roles in the virulence of both pathogens. These *D. septosporum* SSPs have been short-listed for future functional characterization (Hunziker et al. 2016).

Four of the nine in planta–induced SSPs, specifically Ecp4, Ecp7, Ecp29 (CTR), and Ecp30, were predicted to be structurally homologous to proteins with a β/γ-crystallin fold. Of these, Ecp4, Ecp7, and Ecp29 (CTR) were found to share a Cys spacing profile with MiAMP1, a plant antimicrobial protein with a β/γ-crystallin fold from nut kernels of *M. integrifolia* (Marcus et al. 1997; McManus et al. 1999). Purified MiAMP1 exhibits broad-spectrum inhibitory activity against several plant-pathogenic fungi, oomycetes, and gram-positive bacteria in vitro (Marcus et al. 1997). Although a specific mode of action has not yet been determined (Stephens et al. 2005), more functional information is available for Sp-AMP3, a homolog of MiAMP1 from the Scots pine *Pinus sylvestris* (Asiegbu et al. 2003; Sooriyaarachchi et al. 2011). Purified Sp-AMP3 protein has antifungal activity against the

root-rotting Basidiomycete *Heterobasidion annosum* and, as part of this, causes morphological changes in the hyphae and spores of this fungus (Sooriyaarachchi et al. 2011). Carbohydrate-binding assays revealed that Sp-AMP3 binds to both soluble and insoluble β-1,3-glucans but not to insoluble chitin or chitosan (Sooriyaarachchi et al. 2011). It is possible that, through this binding, Sp-AMP3 interferes with glucan assembly. This could then alter cell-wall structure, causing the above-mentioned morphological changes, or could compromise cell-wall integrity to cause cell lysis (Sooriyaarachchi et al. 2011).

The three remaining in planta–induced SSPs, specifically Ecp28-1, Ecp28-2, and Ecp28-3, were predicted to be structurally homologous to KP6, a killer toxin secreted by specific strains of the fungal corn smut pathogen *U. maydis*. These strains exhibit a "killer" phenotype, which is due to persistent infection by a KP6-producing double-stranded RNA totivirus, P6. Upon secretion, KP6 kills competing, uninfected strains of *U. maydis* (Allen et al. 2013b; Koltin and Day 1975). KP6 is translated as a single polypeptide but is processed into two subunits, KP6α and KP6β, by a kexin protease during passage through the ER-Golgi secretory pathway. Both subunits adopt a core α/β-sandwich fold (Allen et al. 2013a; Li et al. 1999). KP6 functions only as a heterodimer, with both subunits required for cytotoxic activity (Peery et al. 1987). Assays in which sensitive *U. maydis* cells were treated with KP6α or KP6β alone or with one subunit after another, but with a washing step in between, strongly suggest that KP6α is responsible for targeting the cell, while KP6β is cytotoxic (Peery et al. 1987). The specific mode of action for KP6, however, remains unclear, although it is thought that KP6α forms strong interactions with cell-wall carbohydrates or membrane-associated proteins of the target cell, with KP6β subsequently recruited to the plasma membrane or imported to an intracellular target to cause cell lysis (Allen et al. 2013a; Steinlauf et al. 1988). Interestingly, limited amino-acid sequence homology was identified between Ecp29 (NTR) and members of the Ecp28 family, suggesting that Ecp29 (NTR) also adopts a KP6-like fold. A putative kexin protease cleavage site is located between the NTR and CTR (β/γ-crystallin-like domain) of Ecp29, implying that this protein undergoes similar posttranslational processing to KP6 upon passage through the ER-Golgi secretory pathway.

In total, 10% of the *C. fulvum* apoplastic in planta–induced SSPs are predicted to possess a domain typical of antimicrobial proteins. This raises the possibility that *C. fulvum* dedicates a significant proportion of its apoplastic secretome to functions associated with microbial antagonism, perhaps to outcompete other microbial organisms for nutrients and space in the apoplastic environment or to provide a form of self-defense (Rovenich et al. 2014). Of course, it remains possible that the predicted similarities in tertiary structure do not extend to biological function. Instead, these folds may be more common than previously thought, irrespective of whether they have evolved from an ancestral protein or by convergent evolution, providing solutions to typical problems faced at the hostile host-pathogen interface. For example, the above-mentioned folds may provide enhanced stability in protease-rich environments. Alternatively, they may provide a flexible molecular scaffold for either functional diversification, the evasion of recognition by corresponding host immune receptors, or both. Recently, the Avr effectors Avr1-CO39, AVR-Pia, and AvrPiz-t from the rice blast fungus *Magnaporthe oryzae* as well as the ToxB effector from *Pyrenophora tritici-repentis* were found to be structurally related (de Guillen et al. 2015). Structure-informed pattern searches carried out in the same study also revealed that several other effector candidates from Sordariomycete and Dothideomycete plant pathogens likely share this same fold. It has, thus, been hypothesized that "the enormous

number of sequence-unrelated Ascomycete effectors may in fact belong to a restricted set of structurally conserved effector families" (de Guillen et al. 2015). Certainly, the predicted structural relationship between Alt a 1 and members of the Ecp20 family further supports this hypothesis.

Of the 70 apoplastic in planta–induced SSPs from *C. fulvum*, 41 were screened for recognition by wild tomato accessions, using an effectoromics approach based on the PVX transient expression system (Hammond-Kosack et al. 1995; Takken et al. 2000). Such an approach has been successful for the identification of plants carrying immune receptor genes active against other pathogens. For example, of 54 RXLR effectors from the oomycete potato late blight pathogen *Phytophthora infestans*, 31 were found to trigger an HR in one or more of 10 resistant wild *Solanum* accessions, with each accession recognizing between five and 24 effectors (Vleeshouwers et al. 2008). In the current study, nine *C. fulvum* SSPs were found to trigger an HR in one or more of 14 specific wild accessions of tomato. This suggests that nine new Avr effectors of this fungus as well as nine new corresponding Cf immune receptor genes have been uncovered. One of the recognized SSPs, Ecp11-1, is a homolog of AvrLm3, an Avr effector from *Leptosphaeria maculans* (Plissonneau et al. 2016). This suggests that both tomato and *Brassica* spp. carry an immune receptor capable of recognizing this class of effector.

Consistent with *Ecp1*, *Ecp2-1*, *Ecp4*, and *Ecp5* (Stergiopoulos et al. 2007), all genes encoding a new HR-eliciting SSP were found to exhibit limited allelic variation across strains collected from around the world. As has been suggested for *Ecp1*, *Ecp2-1*, *Ecp4*, and *Ecp5* (Stergiopoulos et al. 2007), this limited allelic variation could reflect a lack of selection pressure imposed on the pathogen to overcome Cf immune receptor–mediated resistance, since, so far as we are aware, none of the putative corresponding Cf immune receptor genes have yet been deployed in commercial tomato cultivars. Alternatively, this lack of allelic variation could reflect selective constraints on the SSPs to maintain their protein sequences (i.e., to ensure full virulence of the pathogen). Of note, all genes encoding a new HR-eliciting SSP, except for *Ecp14-1*, are associated with repetitive elements in the genome of *C. fulvum* 0WU. It is, therefore, possible that homologous recombination between flanking repetitive elements could result in the deletion of these genes, as hypothesized for strains lacking the repeat-associated *Avr* effector genes *Avr4E*, *Avr5*, or *Avr9* (Mesarich et al. 2014; van Kan et al. 1991; Westerink et al. 2004). Thus, to increase potential durability, new Cf immune receptor genes should be stacked in resistant tomato cultivars.

Two responding species of wild tomato used in our study, *S. corneliomuelleri* and *S. peruvianum*, are typically self-incompatible (Peralta and Spooner 2006). While Cf immune receptor genes present in self-compatible accessions can be easily introgressed into commercial and breeder's cultivars of *S. lycopersicum* by backcrossing, this is not possible for self-incompatible accessions. In cases of incompatibility, it may be possible to avoid the problems associated with barriers to genetic crossing through a more extensive screen of wild tomato germplasm to identify self-compatible species capable of recognizing the SSPs. This strategy has been successful for the identification of wild potato species that recognize the AVRblb1 Avr effector of *Phythophthora infestans* (Vleeshouwers et al. 2008). Using an effectoromics approach based on the PVX transient expression system, it was initially determined that the wild potato species *Solanum bulbocastanum*, which is not directly sexually compatible with the cultivated potato *Solanum tuberosum*, carries an immune receptor gene, *RB/Rpi-blb1*, corresponding to *AVRblb1* (Vleeshouwers et al. 2008). As direct introgression of *RB/Rpi-blb1* from *S. bulbocastanum* to *S. tuberosum* is not possible, additional screening was carried out to identify wild potato accessions that are

both sexually compatible with cultivated potato and that recognize AVRblb1. HR-associated recognition of AVRblb1 was quickly detected in the sexually compatible species *Solanum stoloniferum*, which was subsequently found to carry *Rpi-sto1*, a functional homolog of *RB/Rpi-blb1* (Vleeshouwers et al. 2008).

The finding that most new HR-eliciting SSPs have homologs in other plant-pathogenic fungi raises the possibility of cross-species resistance. In support of this possibility, the Cf-4 immune receptor has been shown to recognize homologs of Avr4 from *D. septosporum*, *P. fijiensis*, and *Pseudocercospora fuligena* (black leaf mold disease of tomato) (de Wit et al. 2012; Kohler et al. 2016; Stergiopoulos et al. 2010), while the Cf-Ecp2-1 immune receptor has been shown to recognize homologs of Ecp2-1 from *D. septosporum* and *P. fijiensis* (de Wit et al. 2012; Stergiopoulos et al. 2010). It must be pointed out, however, that the Cf-4 immune receptor does not recognize homologs of Avr4 from *Cercospora apii*, *Cercospora beticola*, and *Cercospora nicotianae* (leaf spot disease of celery, beet, and tobacco, respectively) (Mesarich et al. 2016; Stergiopoulos et al. 2010). It is clear that, to provide effective resistance in a recipient plant species, the product of any transferred Cf immune receptor gene must recognize an epitope (direct recognition) or virulence function (indirect recognition) conserved to both the corresponding *C. fulvum* effector and its homolog from the target fungal pathogen.

Conclusions.

In this study, proteomics and transcriptome sequencing were used to identify a set of 70 apoplastic in planta–induced SSPs from *C. fulvum* that is made up of all 11 characterized SSP effectors of this fungus as well as two phialide proteins and 57 new extracellular proteins. These SSPs provide new insights into how *C. fulvum* promotes colonization of the tomato leaf apoplast. Using an effectoromics approach, nine SSPs were found to be recognized by specific wild accessions of tomato. These accessions likely carry new Cf immune receptor genes available for incorporation into cultivated tomato.

MATERIALS AND METHODS

In this study, all kits and reagents were used, unless otherwise specified, in accordance with the manufacturer's instructions.

Isolation of IWF from the leaf apoplast of *C. fulvum*-infected tomato.

Four- to five-week-old H-Cf-0 tomato plants were inoculated with strains 0WU, 4, IPO 1979, or IPO 2559 of *C. fulvum* (compatible interactions). For this purpose, conidia preparation, inoculation, and growth conditions were identical to those described by Mesarich et al. (2014). At 10 to 17 dpi, IWF was harvested from tomato leaves visibly infected with *C. fulvum*, using a previously described protocol (de Wit and Spikman 1982; Joosten 2012). Leaf debris and fungal material were then removed by centrifugation at $12,000 \times g$ and 4°C for 20 min, and the IWF samples stored at −20°C until required.

Preparation of IWF samples for LC-MS/MS analysis.

Frozen IWF samples were thawed on ice, and any precipitant formed during the freeze-thaw process was removed by centrifugation at $12,000 \times g$ and 4°C for 20 min. IWF samples were concentrated 3 to 300× by i) pressure filtration at 4°C, using an Amicon 8400 series Stirred Cell Ultrafiltration Unit (EMD Millipore) fitted with an Ultracel regenerated cellulose PLAC 1-kDa nominal molecular weight limit (NMWL) ultrafiltration membrane disc (EMD Millipore), ii) centrifugation at $4,000 \times g$ and 4°C in a 3-kDa NMWL Amicon Ultra-15 centrifugal filter unit (EMD Millipore) or a Vivaspin 20 3-kDa molecular weight

cut-off polyethersulfone ultrafiltration device (GE Healthcare), or iii) sequential acetone precipitation, as described by May et al. (1996), with final resuspension in 1 ml of distilled H_2O. Following concentration, IWF samples were transferred to 2-ml LoBind microcentrifuge tubes (Eppendorf) and were stored at −20°C until required for further processing. When required, frozen IWF samples were thawed on ice and any precipitant formed during the freeze-thaw process was removed by centrifugation at $12,000 \times g$ and 4°C for 20 min. A filter-aided sample preparation protocol (Lu et al. 2011) or an in-gel digestion protocol (Karimi Jashni et al. 2015), both based on trypsin digestion, were then used to prepare samples for LC-MS/MS analysis.

LC-MS/MS analysis.

IWF samples were analyzed by nano-scale (n)LC-MS/MS with a Proxeon EASY nLC system connected to a LTQ-Orbitrap XL mass spectrometer (Lu et al. 2011) at the Laboratory of Biochemistry, Wageningen University. LC-MS runs and associated MS/MS spectra were analyzed with the MaxQuant v1.3.0.5 suite (Cox and Mann 2008), with default settings applied to the integrated Andromeda peptide search engine (Cox et al. 2011), with one exception, i.e., extra variable modifications were set for the deamidation of Asn and Gln. MS/MS spectra were searched against one of four sequence databases. These were built from i) a collection of common contaminants including, for example, bovine serum albumin (BSA) (P02769, BSA precursor), trypsin (P00760, bovine), trypsin (P00761, porcine), keratin K22E (P35908, human), keratin K1C9 (P35527, human), keratin K2C1 (P04264, human), and keratin K1CI (P35527, human); ii) a six-frame translation of tomato (*S. lycopersicum* cv. H-Cf-0) genome sequence (Tomato Genome Consortium 2012); iii) the predicted protein catalog of *C. fulvum* 0WU (de Wit et al. 2012; Mesarich et al. 2014) as well as a six-frame translation of the most highly abundant de novo–assembled in vitro and in planta RNA-Seq reads of this fungus (Mesarich et al. 2014); and iv) a six-frame translation of the repeat-masked *C. fulvum* 0WU genome sequence (de Wit et al. 2012). The 'label-free quantification' and 'match between runs' (set to 2 min) options were enabled. Deamidated peptides were 'allowed' to be used for protein quantification. All other quantification settings were kept at default. Filtering and further bioinformatic analysis of the MaxQuant/Andromeda workflow output and the analysis of abundances for the identified proteins were performed with the Perseus v1.3.0.4 module as part of the MaxQuant suite. Peptides and proteins with a false discovery rate of less than 1% as well as proteins with at least one peptide across two or more IWF samples or two or more independent peptides in a single IWF sample were considered as reliable identification. Reversed hits were deleted from the MaxQuant results table, as were tomato and contamination hits.

Identification of apoplastic in planta–induced SSPs from *C. fulvum*.

C. fulvum SSPs directed to the apoplastic environment via the classical, conventional secretory pathway (i.e., SSPs that possess an N-terminal signal peptide but lack a GPI anchor modification site, a transmembrane domain, or a putative C-terminal ER retention or retention-like signal) were targeted for identification in the protein set identified by LC-MS/MS analysis. The SignalP v3.0 (Dyrløv Bendtsen et al. 2004) and v4.1 (Petersen et al. 2011) servers were used for signal peptide prediction, while the big-PI Fungal Predictor (Eisenhaber et al. 2004) and TMHMM v2.0 (Krogh et al. 2001) servers were used for the prediction of GPI anchor modification sites and transmembrane domains, respectively.

Pre-existing RNA-Seq transcriptome sequencing data (Mesarich et al. 2014) from a compatible in planta time course involving *C. fulvum* 0WU and *S. lycopersicum* cv. H-Cf-0 (4, 8, and 12 dpi) as well as from strain 0WU grown in vitro in PDB or Gamborg B5 liquid media (4 dpi) were used to predict which of the SSPs identified by LC-MS/MS analysis are encoded by in planta–induced genes. Although these data lack biological replicates, they have been extensively validated through RT-qrtPCR experiments (Mesarich et al. 2014; this study). Paired-end RNA-Seq reads were remapped to the strain 0WU genome sequence (de Wit et al. 2012) with Bowtie v2-2.1.0 (Langmead and Salzberg 2012) and TopHat v2.0.12 (Kim et al. 2013), using a custom script. Transcript assembly and abundance estimations were then performed using Cufflinks v2.0.2 (Trapnell et al. 2010), with transcript abundance expressed as FPKM values. SSPs were deemed to be in planta–induced if they were encoded by genes that had a maximum in planta FPKM value of ≥50 at 4, 8, or 12 dpi that exceeded their maximum in vitro FPKM value at 4 dpi by a factor of ≥1.5. Gene exon-intron boundaries were confirmed using the same RNA-Seq data.

General homology screening and alignments.

BLASTp screens (Altschul et al. 1997) were used to identify the top homolog of each *C. fulvum* apoplastic SSP in NCBI and JGI databases (Grigoriev et al. 2012). Only those hits with an expect (E)-value of <1E-02 or a conserved Cys-spacing were considered. Iterative BLASTp screens (Stergiopoulos et al. 2012) were used till saturation to identify homologs of each HR-eliciting SSP in the NCBI and JGI databases (i.e., homologs were used to identify additional homologs in subsequent rounds of BLASTp). Only those hits with an E-value of <1E-02 and the same number of Cys residues as the query sequence were considered. For those proteins for which a homolog could not be identified in JGI, a tBLASTn screen was carried out against the genome collection with the same E-value cut-off. Homologous proteins were aligned using the Clustal Omega server (Sievers et al. 2011).

Structural modeling.

Three-dimensional protein structure prediction servers were used to infer possible structural relationships between apoplastic in planta–induced SSPs of *C. fulvum* and proteins with characterized tertiary structures in the RCSB protein databank (Berman et al. 2000). Only those SSPs with no homology to proteins present in NCBI or JGI databases or those with homology to hypothetical proteins of unknown function in these databases were investigated. The prediction servers employed were HHPred (Hildebrand et al. 2009; Söding et al. 2005), SPARKS-X (Yang et al. 2011), MUSTER (Wu and Zhang 2008), FFAS03/FFAS-3D (Jaroszewski et al. 2005; Xu et al. 2013), FUGUE v2.0 (Shi et al. 2001), RaptorX (Källberg et al. 2012), pGenTHREADER (Lobley et al. 2009), Phyre2 (Kelley et al. 2015), and I-TASSER (Zhang 2008). For each server, default settings were used. A consistency of hits across server outputs, rather than significance values, was used to evaluate potential structural homology. Structural modeling was done with MODELER (HHPred) (Webb and Sali 2002) and RaptorX and was visualized using PyMOL (DeLano 2002).

Repeat identification.

BLASTn was used to identify repetitive nucleotide sequences shared between the genome scaffolds harboring an *SSP* gene and the rest of the *C. fulvum* 0WU genome. Only those sequence repeats of ≥100 nucleotides in length and sharing ≥80% identity with an E-value threshold of 1E-05 were considered. The maximum total number of sequence alignments considered per scaffold was set to 5,000.

Homology screening and expression profiling in *Dothistroma septosporum*.

Reciprocal BLASTp and tBLASTn screens were used to identify homologs of the apoplastic SSPs from *C. fulvum* in the *D. septosporum* NZE10 protein catalog and genome (de Wit et al. 2012) in the JGI database, respectively, with only those hits possessing an E-value of <1E-02 considered. RNA-Seq data from *D. septosporum* NZE10 (Bradshaw et al. 2016) were used to determine which of the homologs are most relevant to the *D. septosporum–Pinus radiata* interaction. More specifically, transcript abundance data from one in vitro growth condition (fungal mycelia in *Dothistroma* liquid medium) and three in planta growth conditions (epiphytic/biotrophic [early], initial necrosis [mid], and mature sporulating lesion [late]), expressed as reads per million per kilobase (RPMK) values, were used. Genes deemed relevant to the interaction had to have a maximum in planta RNA-Seq RPMK value of ≥50 at the early, mid, or late time point. Furthermore, this value had to exceed the gene in vitro RPMK value by a factor of at least 1.5.

PVX-mediated transient expression assays.

Tomato accessions were screened for their ability to recognize apoplastic SSPs through the elicitation of an HR, using the PVX-based transient expression system (Hammond-Kosack et al. 1995; Takken et al. 2000). For this purpose, the cDNA sequence encoding a mature SSP was fused downstream of the cDNA sequence encoding the *N. tabacum* PR1A signal peptide (i.e., for secretion into the apoplastic environment) and was cloned into the binary PVX vector pSfinx behind the *Cauliflower mosaic virus* 35S promoter (Takken et al. 2000). These steps were carried out using protocols of Mesarich et al. (2014) (overlap extension PCR and restriction enzyme–mediated cloning) or Mesarich et al. (2016) (overlap extension PCR and Gateway cloning [Invitrogen]) with the primer pairs listed in Supplementary Table S6. Constructs were transformed into *Agrobacterium tumefaciens* GV3101 for agroinfection of tomato by electroporation, using the method of Takken et al. (2000). For localized transient expression assays in tomato, transformants were prepared, using the protocol described by Stergiopoulos et al. (2010) but with resuspension in a final volume of 0.5 ml of MMA (2% wt/vol sucrose, 0.5% wt/vol Murashige and Skoog salts without vitamins, 0.195% wt/vol MES [pH 5.6], 200 μM acetosyringone), and were inoculated into fully expanded leaves by localized wounding on each side of the main vein with a toothpick (Luderer et al. 2002a; Takken et al. 2000). For systemic transient expression assays, transformants were again prepared, using the method of Stergiopoulos et al. (2010) with final resuspension in MMA to an optical density at 600 nm of 1.0, and were infiltrated into both cotyledons of a seedling at 10 days postgermination with a 1-ml needleless syringe (Mesarich et al. 2014). The presence or absence of an HR was visually assessed at 10 days postwounding and 3 weeks postinfiltration for systemic and localized transient expression assays, respectively.

Tomato infection assays.

Tomato accessions were inoculated with *C. fulvum* 2.4.5.9.11 IPO, using the method described by Mesarich et al. (2014), with resistance or susceptibility to this strain visually assessed across three independent plants at 14 dpi.

RT-qrtPCR gene expression analysis.

Leaf samples from compatible *C. fulvum* 0WU–*S. lycopersicum* cv. H-Cf-0 interactions at 4, 8, 12, and 16 dpi as well as fungal samples from *C. fulvum* 0WU PDB and Gamborg B5 liquid media cultures at 4 dpi were collected according to Mesarich et al. (2014) and were stored at −80°C. Total RNA extraction from each sample as well as subsequent cDNA synthesis was carried out according

to the protocol of Griffiths et al. (2017). RT-qrtPCR experiments were performed on cDNA samples using the method described by Ökmen et al. (2013). The *C. fulvum actin* gene was targeted as a reference for normalization of gene expression, and results were analyzed according to the cycle threshold ($2^{-\Delta Ct}$) method (Livak and Schmittgen 2001). Results were the average of three biological replicates.

Allelic variation analysis.

C. fulvum strains were grown in PDB, with conidia preparation, PDB inoculation, and culture conditions identical to those described by Mesarich et al. (2014). Genomic DNA was extracted from each strain according to the method of van Kan et al. (1991). Genes targeted for an analysis of allelic variation were amplified from genomic DNA by PCR using the protocol and reagents described by Mesarich et al. (2014). PCR amplicons were purified using an illustra GFX PCR DNA and gel band purification kit (GE Healthcare) and were directly sequenced at Macrogen Inc. (Seoul, South Korea), using the same gene-specific primers employed for PCR amplification.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Big-PI Fungal Predictor server: http://mendel.imp.ac.at/gpi/fungi_server.html
Clustal Omega server: https://www.ebi.ac.uk/Tools/msa/clustalo
FFAS03/FFAS-3D server:
  http://ffas.sanfordburnham.org/ffas-cgi/cgi/ffas.pl
FUGUE v2.0 server: http://mizuguchilab.org/fugue/prfsearch.html
HHPred server: https://toolkit.tuebingen.mpg.de/tools/hhpred
I-TASSER server: http://zhanglab.ccmb.med.umich.edu/I-TASSER
JGI BLAST server:
  http://genome.jgi.doe.gov/pages/blast-query.jsf?db=fungi
MEME v4.11.2 server: http://meme-suite.org/tools/meme
MUSTER server: http://zhanglab.ccmb.med.umich.edu/MUSTER
NCBI BLAST server: https://blast.ncbi.nlm.nih.gov/Blast.cgi
pGenTHREADER server: http://bioinf.cs.ucl.ac.uk/psipred/
Phyre2 server: http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index
RaptorX server: http://raptorx.uchicago.edu/StructurePrediction/predict
RCSB protein databank: http://www.rcsb.org/pdb/home/home.do
SignalP v3.0 server: http://www.cbs.dtu.dk/services/SignalP-3.0
SignalP v4.1 server: http://www.cbs.dtu.dk/services/SignalP
SPARKS-X server: http://sparks-lab.org/yueyang/server/SPARKS-X
TMHMM v2.0 server: http://www.cbs.dtu.dk/services/TMHMM