Identification of Novel Virulence-Associated Proteins Secreted to Xylem by *Verticillium nonalfalfae* During Colonization of Hop Plants

Marko Flajsman,¹ Stanislav Mandelc,¹ Sebastjan Radisek,² Natasa Stajner,¹ Jernej Jakse,¹ Katarina Kosmelj,¹ and Branka Javornik¹

¹Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia; and

²Slovenian Institute of Hop Research and Brewing, Cesta Zalskega Tabora 2, SI-3310 Zalec, Slovenia

Submitted 20 January 2016. Accepted 10 February 2016.

Plant pathogens employ various secreted proteins to suppress host immunity for their successful host colonization. Identification and characterization of pathogen-secreted proteins can contribute to an understanding of the pathogenicity mechanism and help in disease control. We used proteomics to search for proteins secreted to xylem by the vascular pathogen *Verticillium nonalfalfae* during colonization of hop plants. Three highly abundant fungal proteins were identified: two enzymes, α -N-arabinofuranosidase (VnaAbf4.216) and peroxidase (VnaPRX1.1277), and one small secreted hypothetical protein (VnaSSP4.2). These are the first secreted proteins so far identified in xylem sap following infection with *Verticillium* spp. VnaPRX1.1277, classified as a heme-containing peroxidase from Class II, similar to other *Verticillium* spp. lignin-degrading peroxidases, and VnaSSP4.2, a 14-kDa cysteine-containing protein with unknown function and with a close homolog in related *V. alfalfae* strains, were further examined. The in planta expression of VnaPRX1.1277 and VnaSSP4.2 genes increased with the progression of colonization, implicating their role in fungal virulence. Indeed, *V. nonalfalfae* deletion mutants of both genes exhibited attenuated virulence on hop plants, which returned to the level of the wild-type pathogenicity in the knockout complementation lines, supporting VnaPRX1.1277 and VnaSSP4.2 as virulence factors required to promote *V. nonalfalfae* colonization of hop plants.

Plants interact with numerous potentially pathogenic microbes that can use various routes to penetrate plant surfaces and colonize plant tissue. In the process of infection, plants are able to detect invading pathogens and set up effective defenses, commonly described as pathogen-associated molecular pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Chisholm et al. 2006; Jones and Dangl 2006). To suppress PTI and ETI in order to colonize the plant successfully, pathogens employ secreted molecules, such as effectors, toxins, and cell wall-degrading enzymes (CWDE). CWDE are secreted enzymes

that are important throughout the pathogen life cycle for fungal pathogenicity, decomposition of plant cell tissue, and for obtaining nutrients (Kubicek et al. 2014). Effectors are secreted protein molecules that modulate host cell structures and cell processes to promote pathogen colonization (Hogenhout et al. 2009). In the apoplast, effectors defend the pathogen from plant degrading enzymes or toxic molecules and avoid the activation of defense signaling but, when translocated into the cell, effectors target specific cellular processes to suppress host defense (Doehlemann and Hemetsberger 2013). Effectors can be conserved across species but, generally, they are genus, species, or even isolate specific, contributing to the host range or host specificity of phytopathogens. Functional characterization of fungal effectors has revealed some common plant targets, such as inhibition of plant defense proteases, manipulation of the ubiquitin-proteasome system, interference with plant immune receptors, or suppression of phytohormone synthesis (Lo Presti et al. 2015). Given the importance of effector molecules, which largely determine pathogen lifestyle, attention has been focused on the identification and functional validation of effectors in various pathogens. Better understanding of the mechanisms of pathogenesis and interaction between plant and pathogens on a molecular level might greatly improve applications in plant protection and resistance breeding, leading to better control of pathogens that cause high losses in agriculture.

Verticillium wilt is a widespread vascular disease of many economically important crops, such as tomato, potato, olive, cotton, hop, sunflower, and woody perennials. The causative agents of the disease are soil-borne species of the genus *Verticillium*, mainly *V. dahliae*, *V. albo-atrum* (sensu lato), and *V. longisporum*. *Verticillium* species infect plants through the roots and, then, advance through the vascular (xylem) system, in which the infection spreads, blocks the veins, and causes symptoms such as wilting, chlorotic and necrotic leaves, vascular discoloration, and death of the plant (Cregeen et al. 2014; Fradin and Thomma 2006; Pegg and Brady 2002). In recent years, significant progress has been made in understanding *Verticillium*–host interactions and disease processes. The outcomes of various studies on *Verticillium* spp. pathogenicity and their link to developmental pathways have recently been reviewed by Luo et al. (2014), Daayf (2015), and Klimes et al. (2015).

Although xylem sap is not very rich in nutrients, *Verticillium* spp. find this environment adequate for their biotrophic life-cycle by colonizing the vascular system, employing various secreted molecules to survive in the harsh environment and to combat the plant defense reaction (Yadeta and Thomma 2013). A study of the in vitro secretomes of *V. nonalfalfae* strains

Nucleotide sequence data is available under accession numbers KT981924 for VnaPRX1.1277, KT981925 for VnaSSP4.2, and KT981926 for VnaPRX.

Corresponding author: B. Javornik; E-mail: branka.javornik@bf.uni-lj.si

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

revealed an arsenal of carbohydrate-degrading enzymes with enhanced pectinolytic enzymes and enzymes from carboxylesterase and oxidoreductase groups (Mandelc and Javornik 2015). The importance of CWDE for fungal colonization was shown by the impaired virulence of the *V. dahliae* deletion mutant of the sucrose nonfermenting 1 gene (*VdSNF1*), which regulates expression of some CWDE genes (Tzima et al. 2011). The endoglucanase VdEG-1 (Novo et al. 2006) and specific secreted protein VdSSP1 (Liu et al. 2013) are other potential carbohydrate-degrading enzymes found to be important for *V. dahliae* pathogenicity. In addition to carbohydrate degradation and uptake, a fungus needs to synthesize its own aromatic amino acids during colonization, as shown in experiments in silencing the *V. longisporum* chorismate synthase gene *Vlaro2* (Singh et al. 2010) and cross-pathway control regulator gene *VICPC1* (Timpner et al. 2013), both required for amino acid biosynthesis. On entering the xylem, a fungus encounters a variety of host defense responses, including induction of antimicrobial secondary metabolites such as rutin, which can be degraded by quercetinase (El Hadrami et al. 2015), salicylate-mediated immunity, which can be suppressed by the isochorismatase Vdsc1, which hydrolyzes a precursor of salicylic acid (Liu et al. 2014) or oxidative burst, which can be modulated by H₂O₂ detoxification by secreted catalase-peroxidase under the regulatory control of *Verticillium* transcriptional regulator Vta2 (Tran et al. 2014). Vta2 is an important regulator of *Verticillium* pathogenicity because it regulates expression of a large set of genes, including a number of secreted proteins such as putative adhesins, LysM proteins, or NLP2 (necrosis and ethylene-inducing protein-like proteins). Expansion of NLP gene homologs was found in *Verticillium* genomes (Klosterman et al. 2011), although only VdNLP1 and VdNLP2 displayed cytotoxic activity and involvement in the virulence of *V. dahliae* strains (Santhanam et al. 2013; Zhou et al. 2012). Sge1, a fungal pathogenesis regulator, is another transcriptional factor that regulates at least eight putative effector genes (Santhanam and Thomma 2013), although not the Ave1 effector, a small secreted *V. dahliae* race 1-specific protein that triggers immunity by recognition of the Ve1 tomato resistant receptor. The Ave1 effector is also a virulence factor, since it is required for full *V. dahliae* virulence on susceptible tomatoes (de Jonge et al. 2012). Ave1 is the only small secreted protein (SSP) characterized so far in *Verticillium* spp., although a search of *V. dahliae* and *V. alfalfae* genomes for effectors found around 120 effector candidates, based on the assumption that fungal effectors are SSP with fewer than 400 amino acids and more than four cysteine residues (Klosterman et al. 2011).

Here, we report two novel virulence-associated proteins secreted to xylem sap by *V. nonalfalfae* during colonization of hop plants. Both proteins, lignin-degrading peroxidase and SSP, are required for successful infection and colonization of hop plants by *V. nonalfalfae*, as demonstrated by deletion mutants and their complementation lines.

RESULTS

Identification of fungal proteins in xylem sap.

The vascular pathogen *V. nonalfalfae*, a particularly aggressive lethal strain, causes severe damage in hop fields (Radišek et al. 2006). During infection, the fungus spreads through the xylem vessels, so xylem sap should be rich in plant defense proteins and interacting fungal secreted proteins. Extraction of xylem sap proteins of susceptible and resistant hop cultivars after infection by the lethal *V. nonalfalfae* T2 strain showed a markedly lower sap volume in infected than in control plants, but the protein concentration was the reverse, much higher. Sampling time was at 30 days after infection, with the appearance of the

first symptoms, which was quite late, since the fungus had already well-colonized the particularly susceptible plants, which, in addition to the woody hop bine, hinders the extraction of sap. However, we were able to obtain a sufficient amount of protein to perform proteomic analysis.

Protein separation from the control plants revealed a highly similar two-dimensional pattern of xylem proteomes in both susceptible and resistant cultivars, while striking differences were observed with xylem proteins from infected plants (Fig. 1). Around 200 reproducible protein spots were detected on the gels: 200 and 184 spots, respectively, in control and infected sap of susceptible cultivar Celeia and 213 and 244 spots, respectively, in control and infected sap of resistant cultivar Wye Target. The resistant cultivar showed a weak response to fungal infection, while the xylem proteome of the susceptible cultivar changed markedly. Significant differences in protein abundance between control and infected plants were observed for 39% of the protein spots in Celeia and only 7% of the protein spots in resistant Wye Target. Certain differentially abundant proteins were further analyzed by mass spectrometry and were identified by peptide mapping and homology search (Supplementary Table S1). In addition to plant defense proteins PR-1 and chitinase (Fig. 1, spots 1, 2, and 3), which are not discussed here, three highly abundant fungal proteins were identified only in the xylem sap of infected susceptible plants (Fig. 1, spots 4, 5, 6, 7, 8, 9, 10, and 11). We designated the fungal proteins according to i) species (*Vna* = *V. nonalfalfae*), ii) identified protein, and iii) gene model of the annotated genome of *V. nonalfalfae* T2 (B. Javornik, unpublished).

α -N-arabinofuranosidase (*VnaAbf4.216*), homologous to *V. alfalfae* VaMs.102 (VDBG_04797) and *V. dahliae* (VDAG_04336) α -N-arabinofuranosidases (Klosterman et al. 2011), was identified in spot 4 (Fig. 1). This α -N-arabinofuranosidase is a CWDE with two functional domains (AbfB pfam05270 and ArabFuran-catal pfam09206) that catalyze the hydrolysis of cell-wall polysaccharides such as L-arabinan and arabinoxylan. The full-length *VnaAbf4.216* gene sequence was subsequently extracted from the *V. nonalfalfae* T2 strain-annotated whole-genome sequence. The *VnaAbf4.216* gene is 1,530 bp long, encoding 509 amino acids with a signal peptide sequence containing both domains (AbfB and ArabFuran-catal), and it is one of five arabinofuranosidase genes in *Verticillium* spp. genomes. They all have orthologs in numerous fungi, so are obviously required for successful fungal plant colonization.

Peroxidase (*VnaPRX1.1277*), another fungal enzyme abundant in the xylem sap of the susceptible cultivar after infection with *V. nonalfalfae* (Fig. 1, spots 8, 9, 10, and 11), was identified based on high homology to *V. alfalfae* VaMs.102 versatile peroxidase VPL1 (VDBG_09914) (Klosterman et al. 2011). The retrieved genomic sequence from the *V. nonalfalfae* genome, *VnaPRX1.1277*, translates into a 354 amino acid long protein with N-terminal signal peptide for secretion from the cell. The *VnaPRX1.1277* nucleotide sequence showed homologies with higher identities only with five sequences of *Verticillium* species, while a blastx search found high similarity to VaMs.102 versatile peroxidase VPL1 (98% coverage, 92% identity) and to *V. dahliae* VdLs.17 ligninase H8 (VDAG_06204) (Klosterman et al. 2011) (99% coverage, 62% identity), followed by other fungal hits with lower identity. Around ten different genes code for enzymes with peroxidase activity in *Verticillium* spp. genomes, and phylogenetic analysis revealed that our *VnaPRX1.1277* clustered closely with VaMs.102 VPL1 and VdLs.17 ligninase H8 and formed a clade together with four other peroxidases from VaMs.102 and VdLs.17 strains (Fig. 2). Protein sequence *VnaPRX1.1277* was subjected to the PeroxiScan peroxidase classification tool (available online from PeroxiBase) (Fawal et al. 2013), which placed it in the same nonanimal heme

peroxidases Class II that contains secreted fungal peroxidases, including lignin-modifying peroxidases. These fungal peroxidases carry out a broad spectrum of biosynthetic and degradative functions on various organic and inorganic compounds. Class II-secreted fungal peroxidases have protoporphyrin IX as a heme prosthetic group and three conserved residues (proximal and distal histidines and distal side arginine) for the peroxidative catalytic function, depending on hydrogen peroxide or organic peroxides as the primary oxidant (Hofrichter et al. 2010). The same three conserved residues (H, H, and R) were also predicted for VnaPRX1.1277 and VaMs.102 VPL1, using the ScanProsite tool (available online from ExPASy) (Supplementary Table S2). Fungal peroxidases have already been implicated in pathogen virulence (Mir et al. 2015) and VnaPRX1.1277 was, thus, considered a good candidate to be examined as a virulence factor.

SSP VnaSSP4.2, identified in spots 5, 6, and 7 (Fig. 1), showed high similarity to the *V. alfalfae* VaMs.102 hypothetical protein encoded by the VDBG_05080 gene (Klosterman et al. 2011). This small (14.1 kDa) basic (pH = 8.5) protein was highly abundant in the xylem sap of the infected susceptible hop cultivar but not in the resistant cultivar. The retrieved genomic sequence from the *V. nonalfalfae* T2 genome, VnaSSP4.2, in addition to high similarity to the VaMs.102 protein (99% coverage; 95% identity), showed similarity (96% coverage,

60% identity) to the *V. dahliae* VdLs.17 predicted protein encoded by V DAG_10515 (Klosterman et al. 2011) and *V. dahliae* JR2 sequence V DAG_JR2_chr3g00670 (66% coverage, 68% identity) (de Jonge et al. 2012). All other searches found very low similarities, suggesting that this protein is unique to VaMs.102 and *V. nonalfalfae* T2 isolates, with a related sequence in two other *V. dahliae* sequenced genomes. The VnaSSP4.2 sequence was further examined for some of the characteristics of known fungal effectors (de Jonge 2012; Doehlemann et al. 2012; Saunders et al. 2012). VnaSSP4.2 is a small, 129 amino acids long protein with two cysteine residues, expressed in planta by the determined signal peptide and absence of any other domain. It has an unknown function and had very low hits in databases (Supplementary Table S3). On the basis of these characteristics, the VnaSSP4.2 protein fits many predicted properties of fungal effectors and it thus seemed a good effector candidate.

Sequence analysis of *VnaPRX1.1277* homologs in *Verticillium* spp. isolates.

To examine for the presence and sequence polymorphism of *VnaPRX1.1277* in different isolates and species of the genus, a set of 91 *Verticillium* spp. isolates was analyzed.

VnaPRX1.1277 homologs were amplified in all *V. nonalfalfae* and *V. alfalfae* isolates and in seven of 23 *V. dahliae* isolates but

Fig. 1. Gel images of proteins from xylem sap (40- μ g protein load, silver stain) of *Verticillium nonalfalfae*-infected and control hop plants. **A**, Xylem proteins of control susceptible plants. **B**, Xylem proteins of control resistant plants. **C**, Xylem proteins of infected susceptible plants. **D**, Xylem proteins of infected resistant plants. Spots 1, 2, and 3 represent plant defense proteins (PR-1 in spot 1 and chitinase in spots 2 and 3). In spot 4, α -N-arabinofuranosidase (VnaAbf4.216) was identified. Spots 8, 9, 10, and 11 represent peroxidase VnaPRX1.1277 and spots 5, 6, and 7 represent small secreted protein VnaSSP4.2.

not in the following *Verticillium* spp.: *V. longisporum*, *V. albo-atrum*, *V. tricorpus*, *V. isaacii*, *V. nubilum*, and *Gibellulopsis nigrescens* (formerly *V. nigrescens*) (Supplementary Table S4). Protein sequences of the amplified fragments did not show any variability within the species. VnaPRX1.1277 amplified fragments had 94% similarity on a protein level to VaPRX fragments from *V. alfalfae* isolates and to VaMs.102 VPL1. Similarity to VdPRX fragments from *V. dahliae* isolates was 88%. However, these VdPRX *V. dahliae* sequences display only 63% amino acid identity to the most similar *V. dahliae* peroxidase, VdLs.17 ligninase_H8 (Supplementary Fig. S1). In seven *V. dahliae* isolates, obtained from hop, pepper, and menthe, a new homolog to Class II peroxidase was found, for which the same structural features were predicted by the ScanProsite tool as for VnaPRX1.1277 and VaMs.102. Similarly, PeroxiScan classified the new VdPRX as a nonanimal heme peroxidase Class II type A and it showed closer relatedness to VaMs.102 VPL1 and VnaPRX1.1277 than to *V. dahliae* peroxidase VdLs.17 ligninase_H8 (Fig. 2).

Sequence analysis of VnaSSP4.2 homologs in *Verticillium* spp. isolates.

Similarly as for VnaPRX1.1277, the presence and polymorphism of VnaSSP4.2 was analyzed in 88 different isolates and species of the *Verticillium* genus. The VnaSSP4.2 fragment was amplified in all *V. nonalfalfae* and *V. alfalfae* isolates and in most of the *V. dahliae* (18) isolates but was not detected in the isolates of *V. longisporum*, *V. albo-atrum*, *V. tricorpus*, *V. isaacii*, *V. nubilum*, and *Gibellulopsis nigrescens* (Supplementary Table S5). SSP amplified from *V. nonalfalfae*, *V. alfalfae*, and *V. dahliae* isolates contained the same nucleotide sequence, showing no sequence diversity within species. The SSP fragments amplified in isolates of *V. alfalfae* and *V. dahliae* matched the corresponding genes of VaMs.102 (VDBG_05080) and VdLs.17 (VDAG_10515) in the Broad *Verticillium* database (Klosterman et al. 2011). The highest similarity of the VnaSSP4.2 protein was shown with VaMs.102 and VaSSP (95% identity), while the

VdLs.17 protein shared only 60% identity. The alignment of SSP from *V. nonalfalfae*, *V. alfalfae*, and *V. dahliae* showed a number of conservative sequence motifs, including two cysteine residues in the same position (Supplementary Fig. S2). However, a database search and sequencing of amplified fragments of VnaSSP4.2 in isolates of different *Verticillium* species revealed that VnaSSP4.2 is a specific SSP found only in *V. nonalfalfae*, with a very close homolog in *V. alfalfae*.

Expression patterns of VnaPRX1.1277 and VnaSSP4.2.

In planta expression is probably a prerequisite for molecules to be considered virulence factors (de Jonge 2012). We, therefore, rechecked our two candidates, VnaPRX1.1277 and VnaSSP4.2, for their expression in planta in the stem of the susceptible hop cultivar in a time course experiment by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Fig. 3). VnaPRX1.1277 and VnaSSP4.2 expression was normalized against two stable fungal housekeeping genes to exclude biased interpretation. Both genes of interest resulted in very similar expression levels for all observed treatments. The expression of VnaPRX1.1277 and VnaSSP4.2 steadily increased through time by 13- and 14-fold change on a log₂ scale, showing the highest peak at 18 days postinoculation (dpi) with a slight drop at 30 dpi. The genes were obviously induced and their expression increased with the progression of colonization, which was also shown, via proteomic analysis, by the abundance of proteins of both genes, VnaPRX1.1277 and VnaSSP4.2, in the xylem of susceptible plants at 30 dpi.

Deleted mutants of VnaPRX1.1277 and VnaSSP4.2 showed reduced virulence.

In order to investigate the role of the identified VnaPRX1.1277 and VnaSSP4.2 proteins in the virulence of *V. nonalfalfae*, knockout and complementation mutants were generated by *Agrobacterium tumefaciens*-mediated transformation (ATMT). Independent knockout and complementation mutants were

Fig. 2. Phylogenetic tree of VdLs.17 and VaMs.102 peroxidases and VnaPRX1.1277, VaPRX, and VdPRX from our amplification. VdLs.17 and VaMs.102 are designated with gene models as VDAG_ and VDBG_, respectively. The square highlights the clade of VaMs.102 versatile peroxidase VPL1 and VdLs.17 ligninase H8 peroxidase, together with peroxidases from our amplification (VnaPRX1.1277, VaPRX, and VdPRX). The phylogenetic tree was generated with CLC Genomics Workbench Version 8.5 (Qiagen, Aarhus, Denmark). Statistical reliability was tested using a bootstrap with 500 replications.

randomly chosen (Supplementary Fig. S3) for further analysis. Prior to the pathogenicity assay, the areas of the fungal colonies were measured and conidiation was assessed, in order to verify the effects of the transformation event on the fungus vital functions (Supplementary Table S6). The areas of the colonies of all transformants did not significantly differ from the wild type and conidiation had no impact on disease frequency.

A pathogenicity assay was carried out on the susceptible hop cultivar Celeia and symptoms were evaluated at four time-points, using a disease severity index (DSI) with a 0 to 5 scale (Radišek et al. 2003). After the final evaluation, mycological reisolation was carried out to confirm the presence of the pathogen in inoculated plants. Only infected plants were considered and used for the calculation of relative area under the disease progress curve (rAUDPC) values.

Reduced virulence of knockouts and restored virulence in complement mutants is shown in Figure 4A. All knockouts had DSI values lower than the wild type at each timepoint, except for $\Delta VnaPRX1.1277-1$ at 18 dpi, while DSI values of all complements were very close to those of the wild type (Fig. 5A and B). DSI values of $\Delta VnaPRX1.1277$ knockouts increased more slowly and never reached DSI values of the wild type. Plants infected with wild type were already heavily wilted at 31 dpi (DSI was 4.2) and, at 38 dpi, were practically dead, while, in knockout-infected plants, stunting and wilting of the whole plants did not occur before the last timepoint 45 dpi (DSI was 4.6 and 4.0). The first disease symptoms of the plants infected with complements $\Delta VnaPRX1.1277comp-1$ and $\Delta VnaPRX1.1277comp-2$ occurred at the same time as in plants infected with the wild type, and DSI values increased during timepoints similar to the wild type (Fig. 5A). rAUDPC values (Fig. 6A) demonstrated that disease progression of knockouts was slower than with wild type and complements, because the rAUDPC values of knockouts were lower than those of the wild type and complements. The Dunnett test showed a statistically significant difference ($P < 0.001$) between rAUDPC values of the wild type and $\Delta VnaPRX1.1277-2$. The rAUDPC value of the second mutant $\Delta VnaPRX1.1277-1$ was also lower than those of the wild type, although not statistically

significant, probably due to the small number of assessed plants ($n = 5$). However, there were no significant differences between the two mutants ($P = 0.4608$). The mutant $\Delta VnaPRX1.1277-1$ was tested again in a replicate assay ($n = 12$) and a decreased virulence of the knockout mutant was observed (Fig. 4B). DSI values of the mutant were lower at every timepoint (except the first) (Fig. 5C) and, in this experiment, the rAUDPC of the $\Delta VnaPRX1.1277-1$ mutant significantly ($P < 0.01$) differed from the rAUDPC of the wild type (Fig. 6B). These results support our assumption that the virulence of $VnaPRX1.1277$ mutants was affected by deletion of the gene. Moreover, the rAUDPC values of complement mutants $\Delta VnaPRX1.1277comp-1$ and $\Delta VnaPRX1.1277comp-2$ were not significantly different from the wild type, indicating that ectopic integration of the $VnaPRX1.1277$ gene restored the virulence.

A delay of wilting symptoms was also observed with plants infected with knockouts $\Delta VnaSSP4.2-1$ and $\Delta VnaSSP4.2-2$. Wilting of leaves as the first consequence of fungal infection was first noticed at 24 dpi (DSI was 2.2 and 2.9), whereas, at the same timepoint, complements $\Delta VnaSSP4.2comp-1$ and $\Delta VnaSSP4.2comp-2$ caused severe symptoms (DSI 3.2 and 3.3) similar to those of the wild type (DSI was 3.4). Similar differences in DSI values were also observed at later timepoints. DSI values of wild type and complements reached almost their full potential at 38 dpi, at which the DSI of both knockouts increased steadily. All plants infected with wild type and complements were dead at the last timepoint (45 dpi). This was not the case for knockouts, since some infected plants showed only partially affected green leaves (Fig. 5B). rAUDPC values (Fig. 6A) confirm that disease progression of $VnaSSP4.2$ knockouts was slower than that of the wild type and complements. The rAUDPC values of $\Delta VnaSSP4.2-1$ was statistically ($P < 0.01$) different from the rAUDPC of the wild type, while the rAUDPC of knockout $\Delta VnaSSP4.2-2$ was lower than that of the wild type, though not large enough to be significant. On the other hand, the $\Delta VnaSSP4.2-2$ mutant was similar in virulence to $\Delta VnaSSP4.2-1$, since no statistical differences were found between their rAUDPC values ($P = 0.5494$). $\Delta VnaSSP4.2-2$

Fig. 3. Expression levels of *VnaPRX1.1277* and *VnaSSP4.2* genes in shoots of infected susceptible hop plants measured at 6, 12, 18, and 30 days post-inoculation (dpi), by quantitative polymerase chain reaction. Values of expression levels obtained in wild-type *Verticillium nonalfalfae* grown in Czapek-Dox medium were used for relative quantification and calculation of fold change and difference. Gene expression levels were normalized for each gene according to the level of the constitutively expressed endogenous reference genes topoisomerase and splicing factor. Error bars indicate standard deviation of three biological replicates.

was also tested in a replicate experiment on a larger sample size ($n = 12$), in which reduced virulence (Fig. 4B) and lower DSI values at each timepoint (Fig. 5D) were confirmed by a rAUDPC value statistically ($P < 0.001$) different from the wild type (Fig. 6B). As with *VnaPRX1.1277* results, the rAUDPC of complements $\Delta VnaSSP4.2comp-1$ and $\Delta VnaSSP4.2comp-2$ were also not statistically different from those of the wild type.

Taken together, the results of the pathogenicity assay of deletion mutants and complements of *VnaPRX1.1277* and *VnaSSP4.2* genes showed that both genes are virulence-related genes that impair the virulence of *V. nonalfalfae*.

DISCUSSION

Proteomic analysis of xylem sap revealed three abundant fungal proteins in the wilt-susceptible hop cultivar after infection with the vascular pathogen *V. nonalfalfae*. During infection of the vascular system, xylem sap contains an increased amount of plant defense-related proteins as well as pathogen-

secreted proteins, as shown by analysis of tomato xylem sap after infection with the vascular pathogen *Fusarium oxysporum*, in which a number of fungal proteins secreted in xylem (six) were found (Houterman et al. 2007; Rep et al. 2004), and some of them were later characterized as avirulent and virulent factors (Gawehns et al. 2014; Houterman et al. 2008, 2009).

In hop xylem sap, we identified *V. nonalfalfae* α -N-arabinofuranosidases (*VnaAbf4.216*), a lignin-degrading peroxidase (*VnaPRX1.1277*), and a small protein (*VnaSSP4.2*), all secreted proteins in planta and, thus, probably implicated in fungal virulence.

α -N-arabinofuranosidase, a CWDE that catalyzes the hydrolysis of cell-wall polysaccharides such as L-arabinan and arabinoxylan, is common to many fungi. α -N-arabinofuranosidase involvement in virulence was shown by its identification in wheat after infection with the fungal pathogen *Septoria tritici* (Yang et al. 2013) substantiated its participation in plant cell-wall degradation after infection. α -N-arabinofuranosidase is present in five copies in the *V. nonalfalfae* genome, which probably hindered our attempt

Fig. 4. Disease symptoms and mycological reisolation of hop plants. **A**, Disease symptoms of hop plants inoculated with wild-type *Verticillium nonalfalfae*, two *VnaPRX1.1277* deletion strains ($\Delta VnaPRX1.1277-1$ and $\Delta VnaPRX1.1277-2$), two *VnaPRX1.1277* complementation strains ($\Delta VnaPRX1.1277comp-1$ and $\Delta VnaPRX1.1277comp-2$), two *VnaSSP4.2* deletion strains ($\Delta VnaSSP4.2-1$ and $\Delta VnaSSP4.2-2$), two *VnaSSP4.2* complementation strains ($\Delta VnaSSP4.2comp-1$ and $\Delta VnaSSP4.2comp-2$) compared with a mock inoculated asymptomatic plant (top panel). Plants were imaged 31 days after inoculation. Mycological reisolation of hop (bottom panel) plants was carried out 45 days postinoculation and fungal outgrowth is shown 3 days after plating of xylem sections on potato dextrose agar plates. **B**, Replicate pathogenicity assay of $\Delta VnaPRX1.1277-1$ and $\Delta VnaSSP4.2-2$ deletion strains, along with mock and wild-type inoculated hop plants. Plants were imaged 43 days after inoculation.

at the construction of deletion mutants to test its implication in fungal virulence. The high redundancy of CWDE genes probably contributes to relatively few reports on CWDE direct involvement in fungal virulence (Doehlemann and Hemetsberger 2013). However, the *V. dahliae* deletion mutant of the CWDE regulator gene (VdSNF1) showed reduced virulence and, thus, supported a CWDE role in fungus colonization (Tzima et al. 2011).

Peroxidase (VnaPRX1.1277) secreted to hop xylem sap by *V. nonalfalfae* was classified as a heme nonanimal Class II peroxidase, a superfamily of lignin-modifying peroxidases that includes lignin peroxidases, manganese peroxidases, versatile peroxidases, and other Class II peroxidases (PeroxiBase). Lignin peroxidases catalyze oxidative depolymerization of lignin in the presence of H_2O_2 and are best characterized in Basidiomycota white-rot fungi (Wong 2009). However, there are also numerous enzymes classified so far as Class II in Ascomycota species (PeroxiBase). In *Verticillium* genomes (VdLs.17, VaMs.102), three different Class II enzymes and *V. nonalfalfae*

VnaPRX1.1277 peroxidase clustered with lignin-degrading enzymes in a phylogenetic tree (Fig. 2) of 20 different peroxidases annotated in VaMs.102 and VdLs.17 genomes (Klosterman et al. 2011). VnaPRX1.1277 peroxidase showed high (93%) similarity to VaMs.102 versatile peroxidase on a protein level and only 63% similarity to VdLs.17 ligninase H8. A search for VnaPRX1.1277 similar sequences in isolates of *Verticillium* spp. found such sequences only in *V. nonalfalfae* and highly similar ones in *V. alfalfae* isolates. However, it revealed a new *V. dahliae* lignin-degrading peroxidase (VdPRX) with 88% similarity to *V. nonalfalfae* and only 65% to VdLs.17 ligninase H8, which was amplified in seven of 23 *V. dahliae* isolates. The VnaPRX1.1277 peroxidase gene was amplified in all 45 analyzed *V. nonalfalfae* isolates of both pathotypes obtained from different hosts showing no sequence polymorphism. Hop-infecting *V. nonalfalfae* strains are classified as mild and lethal pathotypes; the first causes weak disease symptoms such as wilting, vascular browning, xylem hyperplasia, foliar chlorosis, and necrosis, while severe disease symptoms and plant die-back

Fig. 5. Disease progress curves in disease severity index (DSI) values of hop plants inoculated by root dipping with spores of knockout and complementation mutants and wild type. **A**, DSI values of hop plants inoculated with wild type, two *VnaPRX1.1277* knockouts, and their two complements. **B**, DSI values of hop plants inoculated with wild type, two *VnaSSP4.2* knockouts, and their two complements. **C**, DSI values of hop plants in the replicate experiment inoculated with wild type and a Δ *VnaPRX1.1277-1* knockout. **D**, DSI values of hop plants in the replicate experiment inoculated with wild type and a Δ *VnaSSP4.2-2* knockout.

are the result of infection with the lethal pathotype. The identified peroxidase VnaPRX1.1277 is present in both pathotypes and can be assumed to be required for the pathogenicity of both mild and lethal *V. nonalfalfae* strains. VnaPRX1.1277 peroxidase is highly expressed in planta, as shown by proteomic analysis of xylem sap and RT-qPCR analysis of plant tissue from infected wilt-susceptible hop plants. In planta detection of fungal proteins implicates their involvement in pathogen virulence. Indeed, *V. nonalfalfae* deletion mutants of VnaPRX1.1277 peroxidase gene exhibited attenuated virulence in hop plants, as shown by the pathogenicity test. Moreover, the virulence of the knockout complementation lines returned to the level of the wild-type pathogenicity, supporting VnaPRX1.1277 peroxidase as a virulence factor required to promote *V. nonalfalfae* colonization of hop plants. The mechanism behind this function must be further examined, since our VnaPRX1.1277, as a lignin degrading peroxidase, might act on two levels, first, as a hydrogen peroxide (reactive oxygen species [ROS]) scavenger and second, using scavenged H₂O₂, as a lignolytic enzyme decomposing newly synthesized lignin of the plant defense.

Peroxidases have recently been directly implicated in fungal virulence in relation to ROS detoxification in *Magnaporthe oryzae* (Huang et al. 2011; Tanabe et al. 2011) or indirectly as one of the genes regulated by the transcriptional factors controlling the response to oxidative burst, as shown in *Ustilago maydis* (Molina and Kahmann 2007), *Alternaria alternata* (Lin et al. 2009), *M. oryzae* (Guo et al. 2010, 2011), and *Verticillium* spp. (Tran et al. 2014). During the initial phase of the infection process, the pathogen encounters oxidative burst generated by host-produced ROS as the earliest PTI response and, for successful colonization, the damaging effects and signaling of ROS must be diminished (Doehlemann and Hemetsberger 2013; Heller and Tudzynski 2011). Pathogens have developed various strategies to counter-attack oxidative burst, one of which is employment of peroxidase to detoxify hydrogen peroxide. Systemic investigation of the role of the 27 *M. oryzae* peroxidases in

fungal pathogenesis demonstrated that the ortholog (*MoPRX1*) of thioredoxin peroxidase in *Saccharomyces cerevisiae* is required for host-derived ROS detoxification, although involvement of other peroxidases in response to oxidative burst is not excluded (Mir et al. 2015).

Fewer studies have been devoted to the possibilities of lignin decomposition as a pathogen infection strategy. Soybean pathogen *Fusarium solani* f. sp. *glycines* is actually capable of lignin degradation, which may be an important cause of sudden death syndrome disease of soybean, as demonstrated by tests of lignin degradation and laccase and lignin peroxidase activity (Lozovaya et al. 2006). It is interesting that the activity of *F. solani* F-552 lignolytic enzymes was significantly induced by the introduction of oxidative stress through H₂O₂ in the production line of these enzymes (Obruca et al. 2012), which presumably might be the case in planta, whereby pathogen lignin-degrading peroxidase might detoxify the H₂O₂ produced by the host oxidative burst and use it to degrade lignin and, thus, promote its colonization.

We also identified one highly abundant fungal small protein (VnaSSP4.2) that was secreted in the xylem sap during *V. nonalfalfae* colonization of hop. VnaSSP4.2 is the first *Verticillium* spp. SSP identified in xylem sap. It resembles Six3 effector from *F. oxysporum* in terms of secretion in xylem, basic pI, low molecular mass and two cysteine residues, although without any sequence similarity (Houterman et al. 2007). This small basic 14-kDa hypothetical protein was also found in a proteomic study of in vitro secretomes of *V. nonalfalfae* hop mild and lethal pathotypes grown on simulated xylem fluid (Mandelic and Javornik 2015). VnaSSP4.2 is present in both pathotypes, as shown by analysis of *V. nonalfalfae* isolates, and is, thus, not pathotype-specific, although it seems to be specific to *V. nonalfalfae*, with a very close homolog in *V. alfalfae*. Pathotype specificity is demonstrated with the specific secreted protein VdSSP1, present only in defoliating and not in non-defoliating strains of cotton-infecting *V. dahliae*, although a homolog of VdSSP1 is found in *V. dahliae* VdLs.17 (VDAG_02988)

Fig. 6. Disease severity expressed as relative area under the disease progress curve (rAUDPC) caused by *VnaPRX1.1277* and *VnaSSP4.2* mutants and wild type. **A**, Mean rAUDPC values of wild-type *Verticillium nonalfalfae*, two *VnaPRX1.1277* deletion strains (Δ VnaPRX1.1277-1 and Δ VnaPRX1.1277-2), two *VnaPRX1.1277* complementation strains (Δ VnaPRX1.1277comp-1 and Δ VnaPRX1.1277comp-2), two *VnaSSP4.2* deletion strains (Δ VnaSSP4.2-1 and Δ VnaSSP4.2-2), two *VnaSSP4.2* complementation strains (Δ VnaSSP4.2comp-1 and Δ VnaSSP4.2comp-2). **B**, Mean rAUDPC values of wild-type *V. nonalfalfae* and Δ VnaPRX1.1277-1 and Δ VnaSSP4.2-2 knockouts in the replicate experiment. In both experiments, rAUDPC values calculated for each treatment were subjected to standard one-way analysis of variance followed by Dunnett's test, which is used to compare each of a number of treatments with a single control. The asterisks show significant differences (***, $P < 0.001$; **, $P < 0.01$) in mean rAUDPC values.

(Liu et al. 2013). Another SSP in *Verticillium* spp., the Ave1 effector, is characteristic of *V. dahliae* race 1 isolates but it has homologs in the plant-pathogenic fungi *F. oxysporum* f. sp. *lycopersici*, *Colletotrichum higginsianum*, and *Cercospora beticola* and in bacterial plant pathogen *Xanthomonas axonopodis* (de Jonge et al. 2012). Of the three *Verticillium* spp. effector proteins, VnaSSP4.2 exhibits the highest species specificity, having only one very closely related homolog in *V. alfalfae*, a species that was recently recognized and taxonomically separated from *V. nonalfalfae* and *V. albo-atrum* (Inderbitzin et al. 2011).

VnaSSP4.2 is the most likely species-specific putative effector that promotes colonization of *V. nonalfalfae* through the hop plant, as shown by the increased expression pattern in a time course in the susceptible hop cultivar. VnaSSP4.2 is required for the full virulence of *V. nonalfalfae* in hop plants, as demonstrated by pathogenicity assay in which reduced virulence was observed in deletion mutants that fully recovered with complementation.

The SSP VnaSSP4.2 has many predicted effector features (de Jonge 2012; Doehlemann et al. 2012; Saunders et al. 2012), such as a small size protein with cysteine residue, expressed in planta with determined signal peptide and the absence of any other domain with unknown function and a very low number of hits in databases (species specific). According to these characteristics, VnaSSP4.2 falls into the category of specialized effectors, which interfere with host-specific processes, unlike common apoplastic virulence factors (ROS detoxification enzymes, Lys M effectors, NLP proteins or protease, and other enzyme inhibitors), which act against central components of innate plant immunity (Doehlemann and Hemetsberger 2013). Apart from the Six effectors of *F. oxysporum* (de Sain and Rep 2015), *V. dahliae*-specific secreted protein VdSSP1 (Liu et al. 2013), and race-specific effector Ave1 (de Jonge et al. 2012), very few secreted proteins of a fungal vascular pathogen with virulent function have, so far, been identified (de Sain and Rep 2015). VnaSSP4.2 SSP is a new *V. nonalfalfae* effector that shares common features with mainly predicted fungal effectors whose mode of action and role in pathogenicity remain to be elucidated.

In summary, two novel virulence-associated proteins were identified in hop xylem sap following infection with the vascular pathogen *V. nonalfalfae*, whose implication in virulence was demonstrated by deletion mutants and their complementation lines. Peroxidase (VnaPRX1.1277) belongs to a family of lignin-degrading enzymes that use hydrogen peroxide to decompose lignin. VnaPRX1.1277 might, thus, be implicated in protection against plant-derived oxidative stress or degradation, or both, of lignin, which poses a physical barrier to the invading fungus. The VnaSSP4.2 SSP is the first *Verticillium* spp. SSP identified in xylem sap and it features genuine effector characteristics whose mechanism and role in virulence still have to be substantiated.

MATERIALS AND METHODS

Fungal and bacterial isolates and media and growth conditions.

Verticillium isolates were obtained from the fungal collection of the Institute of Hop Research and Brewing and the Biotechnical Faculty. All isolates were cultured on ½ Czapek-Dox medium agar plates or on potato dextrose agar (PDA) medium at 24°C in the dark. *Escherichia coli* DH5α was grown in Luria-Bertani medium at 37°C. *A. tumefaciens* LBA4404 was grown in YM medium (per liter, 0.4 g of yeast extract, 10.0 g of mannitol, 0.1 g of NaCl, 0.2 g of MgSO₄·7H₂O, 0.5 g of K₂HPO₄·3 H₂O) at 28°C.

Plant inoculation protocol and sampling.

The protocol for artificial inoculation of hop plants was similar to that previously described by Cregeen et al. (2014)

and Mandelc et al. (2013). The fungal isolates used for artificial inoculation in this experiment were grown in liquid general fungal medium (Kayser 1992) for 5 days at 50 rpm on a rotary shaker at room temperature and in the dark. The conidia inoculum was prepared by filtration of mycelia and the spore concentration was adjusted to 5 × 10⁶ conidia per milliliter. Potted and well-rooted hop plants of susceptible hop cultivar Celeia and resistant cultivar Wye Target were used in this study. Plants were inoculated in phenological stage BBCH 12 by 10-min root dipping in the pathogen inoculum. As a noninfected control, hop plants were similarly mock-inoculated with sterile distilled water. After inoculation, the plants were repotted and were grown in a growth chamber (RK-13300, Kambic) for different periods of time. Growth conditions were a 12-h photoperiod of fluorescent light (L 58 W/77; Fluora, Osram) at a temperature of 22°C and relative humidity of 65% during the light period and 20°C and 70% during the dark period. At the end of all visual disease assessments, mycological reisolation of the pathogen was carried out to confirm the presence of the pathogen in the inoculated plants. Reisolation was done by xylem sections harvested from inoculated plants on PDA plates. Fungal outgrowth was detected after the plates were incubated for 3 to 5 days in the dark at 24°C. Three independent inoculation experiments were performed.

i) For xylem sap extraction, hop plants of susceptible cultivar Celeia and resistant cultivar Wye Target were artificially infected with the lethal pathotype *V. nonalfalfae* T2 and were grown in a growth chamber for 30 days. The sampling time 30 dpi was chosen on the basis of our previous proteomic studies on the hop–*Verticillium* spp. interaction, in which it was shown that the most dramatic changes in proteome abundance occur in infected susceptible plants compared with control plants at this timepoint (Mandelc et al. 2013). Twelve control plants and 48 infected plants for each cultivar were included in the experiment.

ii) To evaluate in planta expression of *VnaPRX1.1277* and *VnaSSP4.2* genes at different timepoints, 48 plants of the cultivar Celeia were artificially inoculated with the lethal pathotype *V. nonalfalfae* T2. Twelve plants were mock-inoculated as a control. Nine Celeia infected plants were sampled at 6, 12, 18, and 30 dpi as biological replications. Three noninfected control plants were also sampled at each timepoint. Samples were immediately frozen in liquid nitrogen and were stored at –80°C until RNA extraction.

iii) For the pathogenicity assay of knockout and complementation mutants of *VnaPRX1.1277* and *VnaSSP4.2* genes, 12 to 15 hop plants of susceptible cultivar Celeia were used for inoculation with each transformant and also for mock-inoculation and inoculation with the wild-type T2 isolate of *V. nonalfalfae*. Symptoms were evaluated 18, 24, 31, 38, and 45 dpi (in the replicate experiment, symptoms were evaluated 15, 25, 32, 39, and 46 dpi), using a DSI with a 0 to 5 scale (Radišek et al. 2003) and the AUDPC was calculated as indicated by Campbell and Madden (1990) and was expressed as rAUDPC (Simko and Piepho 2012). The R programming language was used for the statistical analysis and the R package ggplot2 for the graphic presentation (available online from the R Foundation for Statistical Computing, Vienna, Austria). Standard one-way analysis of variance was followed by Dunnett's test, which is used to compare each of a number of treatments with a single control (Dunnett 1964).

Proteomic analysis.

Extraction of xylem sap. For proteomic analysis, plants of susceptible cultivar Celeia and resistant cultivar Wye Target were artificially infected with the lethal pathotype *V. nonalfalfae* T2. Twelve control plants and 48 infected plants for each cultivar were sampled 30 dpi. Plants were then cut 5 to 10 cm above ground and the cut surface was washed with distilled water. The

root part was placed in a Scholander pressure chamber and a pressure of 0.2 MPa was applied to extract xylem fluid. The first drops of exuding fluid were blotted with filter paper to avoid phloem contamination. The xylem sap was then collected for approximately 120 min in microtubes on ice containing protease inhibitor cocktail. Samples of three to 10 individual plants were pooled into three biological replicates for each treatment, due to the very low protein concentration in xylem. Samples were stored at -80°C until analysis.

Two-dimensional electrophoresis and mass spectrometry identification of proteins.

Proteins were precipitated by adding 100% trichloroacetic acid to a 15% (vol/vol) final concentration and were kept on ice for 30 min. The pellet was washed twice with cold acetone and was resuspended in isoelectric focusing (IEF) sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% immobilized pH gradient buffer pH 3-10 NL, 18 mM dithiothreitol [DTT]). The protein concentration was measured by Bradford assay (Bio-Rad protein assay), according to the manufacturer's instructions, and 40 μg of proteins were applied via anodic cup loading to 13 cm of pH 3-10 NL strips. IEF was performed on an IPGphor3 IEF system (GE Healthcare) for a total of 20 kVh, using the following protocol: step 300 V (300 Vh), step 500 V (500 Vh), gradient to 1,000 V (750 Vh), gradient to 8,000 V (11,250 Vh), step 8,000 V (7,200 Vh). After IEF, strips were equilibrated two times for 15 min in equilibration buffer (6 M urea, 75 mM Tris, pH 8.8, 2% sodium dodecyl sulfate [SDS], 30% glycerol) containing 1% DTT in the first step and 2.5% iodoacetamide in the second step. Strips were then applied to 12.5% polyacrylamide gels for SDS-polyacrylamide gel electrophoresis and were run at 40 mA per gel until the dye front reached the bottom of the gels. After electrophoresis, gels were stained with silver and image analysis was performed using ImageMaster Platinum software (GE Healthcare). Spot detection and spot matching was automatic with manual curation. Comparison was made between control plants of the two cultivars and between control and infected plants within each cultivar. Only spots present in all three biological replicates were considered reliable and spot abundance was quantified using Student's *t* test ($P < 0.05$) of normalized spot volumes, bearing in mind the poor quantitative properties of silver staining. Spots of interest were excised from CBB G-250 stained preparative gels and trypsin digested peptides were subjected to identification with matrix-assisted laser desorption-ionization tandem time of flight mass spectrometry (Ultraflex TOF/TOF; Bruker Daltonics), followed by bioinformatic analysis using Mascot Peptide Mass Fingerprint searches of the NCBI database, using the Matrix Science web server.

Radial growth and conidiation assays.

Seven-day-old *Verticillium* PDA cultures were harvested and diluted to 10^6 conidia per milliliter, and 2- μl droplets of conidial suspension were placed at the center of $\frac{1}{2}$ Czapek-Dox agar plates. Plates were incubated at 22°C in the dark. After 10 days, the colony area was measured by the NIS-Elements BR 3.2 program (Nikon Corporation, Tokyo). The experiment was performed in triplicate for each mutant genotype and also for the wild type.

Prior to the pathogenicity assay, conidiation of mutants and wild type was assessed. The fungal isolates for artificial inoculation were grown in liquid general fungal medium (Kayser 1992) for 5 days at 50 rpm. The inoculum of each fungal isolate was examined under a light microscope by using a Thoma counting chamber and a relative assessment of conidiation was made by comparing conidiation of mutants to that of the wild type.

PCR analysis of *Verticillium* isolates and sequencing PRX1.1.1277 and SSP4.2 genes in *Verticillium* isolates.

DNA from *Verticillium* isolates was extracted as described by Kump et al. (1992). PCR analysis for verification of the presence of *VnaPRX1.1277* and *VnaSSP4.2* genes in *Verticillium* isolates was carried out using gene-specific primers (Supplementary Table S7). PCR conditions consisted of an initial 94°C denaturation step for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The final elongation step was 8 min at 72°C . PCR products of *VnaPRX1.1277* and *VnaSSP4.2* genes were Sanger sequenced on a 3130xl Genetic Analyzer (Applied Biosystems). The sequencing reaction was prepared with each PCR-specific primer with ^{125}I of the recommended BigDye 3.1 reagent. Sequence analysis was performed by CodonCode Aligner V 5.1.5 (CodonCode Co.).

Bioinformatics analysis of *VnaPRX1.1.1277* and *VnaSSP4.2*.

The selected two proteins were further searched using BLAST against the NCBI database collection, the Broad Institute *Verticillium* group database against *V. dahliae* VdLs.17 and *V. alfalfae* VaMs.102 sequences, and Fungal Ensembl database against *V. dahliae* VdLs.17 and JR2. The Peroxisome base was used for classification of *VnaPRX1.1277* sequences. The Peroxiscan peroxidase classification tool was used for classification of *VnaPRX1.1277* sequences and the ScanProsite tool was used for searching for predicted features of peroxidases. ClustalW2 (multiple sequence alignment tool, available online from the European Molecular Biology Laboratory-European Bioinformatics Institute) was used for alignment of the nucleotide and protein sequences of *VnaPRX1.1277* and *VnaSSP4.2*.

Gene expression analysis.

Real-time PCR was used to measure expression levels of *VnaPRX1.1277* and *VnaSSP4.2* genes in infected hop plants. Transcription levels of the target *Verticillium* genes in infected hop plants were quantified relative to the expression of the target genes in the wild type, which was grown in liquid Czapek-Dox medium for 4 days with shaking at 130 rpm, 24°C , in dark conditions. RNA from infected plants and from the wild type of fungus from liquid Czapek-Dox medium was extracted using a Spectrum plant total RNA kit (Sigma-Aldrich). Single-stranded cDNA was synthesized from 1 μg of total RNA, using a High Capacity cDNA reverse transcription kit (Applied Biosystems), following the manufacturer's instructions. FastStart universal SYBR Green master (Rox) (Roche) was used to prepare the PCR reaction mix. Real-time PCR was performed using Fast SYBR Green technology on an ABI PRISM 7500 Fast Sequence detection system (Applied Biosystems). The total volume of the final reaction was 10 μl and it was prepared as follows: 5 μl of Fast SYBR Green master mix, 50 ng of cDNA, and 300 nM of each specific forward and reverse primer. Real-time PCR conditions consisted of an initial 95°C denaturation step for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. All samples were amplified in three technical replicates acquired from the same cDNA sample. The obtained values were normalized using reference genes topoisomerase and splicing factor. The results were analyzed with 7500 Software v2.0.6 (Applied Biosystems), using the $\Delta\Delta\text{Ct}$ method.

Knockout and complementation plasmids.

Knockout plasmids for genes *VnaPRX1.1277* and *VnaSSP4.2* were made by the USER Friendly cloning technique. Approximately 1,000 bp long flanking sequences of each gene were amplified from genomic DNA of *V. nonalfalfae* and were cloned into pRF-HU2 as described previously (Frandsen et al.

2008). To generate complementation plasmid, the hygromycin cassette from the plasmid pRF-HUE (Frandsen et al. 2008) was substituted by the *SpeI/ApaI* fragment from plasmid pBT081 (Houterman et al. 2008), containing the phleomicin-resistant cassette. In detail, pRF-HUE plasmid was digested by *SpeI* and *ApaI* restriction enzyme (New England BioLabs), which enabled cutout of the hygromycin cassette. The backbone of pRF-HUE was purified from the gel using Illustra GFX PCR DNA and a gel band purification kit (GE Healthcare). The phleomicin-resistant cassette was amplified from pBT081 plasmid using primers PHL_*SpeI*_for and PHL_*ApaI*_rev and was then cloned into the backbone of the plasmid pRF-HUE to get pRF-HUEc plasmid. To generate the complementation construct of *VnaPRX1.1277* and *VnaSSP4.2* genes, only the gene sequence of *VnaPRX1.1277* and *VnaSSP4.2* were inserted in the user cloning site of pRF-HUEc plasmid with the phleomicin-resistance cassette, after the PgpDA promotor, which enabled strong expression of the complementation gene.

ATMT of *V. nonalfalfae*.

For generating knockouts, the lethal hop pathotype of *V. nonalfalfae*, T2, was chosen.

ATMT was performed as described previously (Knight et al. 2009), with the following modifications: i) *A. tumefaciens* LBA4404 was used and grown until the optical density reached 0.5 to 0.7; ii) mixtures of bacteria and conidia were incubated on induction media for 72 h; and iii) as selection medium for knockout transformants, 1/2 Czapex-Dox medium with 75 µg of hygromycin B per milliliter (for transformants selection) and 150 µg of timentin per milliliter (to inhibit *A. tumefaciens* growth) was used for knockouts. As selection medium for complementation transformants, 1/2 Czapex-Dox medium with 300 µg of phleomicin per milliliter (for complementation transformants selection) and 150 µg of timentin per milliliter (to inhibit *A. tumefaciens* growth) was used.

Verification of fungal transformants.

Homologous recombination was verified by PCR, using three pairs of primers, the first pair for amplifying *hygromycin B* gene, the second for amplifying the whole-genome region, in which the resistance cassette replaced the targeted gene, and the third pair for amplifying the inner part of the gene of interest. The same primer pairs were used for verification of complementation transformants. PCR conditions of the PCR reaction were the same as described above.

ACKNOWLEDGMENTS

This research was supported by grants from the Slovenian Research Agency, research programs P4-0077 and PhD student grant 1000-11-310096. We thank B. P. H. J. Thomma from the Laboratory of Phytopathology, Department of Plant Sciences of Wageningen University, The Netherlands, for providing pBT081 plasmid and advice on mutational analysis. We are very grateful to A. Vlahou from the Biomedical Research Foundation, Academy of Athens, for mass spectrometry analysis.

LITERATURE CITED

Campbell, C. L., and Madden, L. V. 1990. Introduction to plant disease epidemiology. John Wiley & Sons Inc., New York.

Chisholm, S. T., Coaker, G., Day, B., and Staskiewicz, B. J. 2006. Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell* 124:803-814.

Cregeen, S., Radisek, S., Mandelc, S., Turk, B., Stajner, N., Jakse, J., and Javornik, B. 2014. Different gene expressions of resistant and susceptible hop cultivars in response to infection with a highly aggressive strain of *Verticillium albo-atrum*. *Plant Mol. Biol. Rep.* 33:689-704.

Daayf, F. 2015. *Verticillium* wilts in crop plants: Pathogen invasion and host defence responses. *Can. J. Plant Pathol.* 37:8-20.

de Jonge, R. 2012. *In silico* identification and characterization of effector catalogs. Pages 415-425 in: *Plant fungal Pathogens: Methods and Protocols*. M. D. Bolton, and B. P. H. J. Thomma, eds. Humana Press, New York.

de Jonge, R., van Esse, H. P., Maruthachalam, K., Bolton, M. D., Santhanam, P., Saber, M. K., Zhang, Z., Usami, T., Lievens, B., Subbarao, K. V., and Thomma, B. P. H. J. 2012. Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 109:5110-5115.

de Sain, M., and Rep, M. 2015. The role of pathogen-secreted proteins in fungal vascular wilt diseases. *Int. J. Mol. Sci.* 16:23970-23993.

Doehlemann, G., and Hemetsberger, C. 2013. Apoplastic immunity and its suppression by filamentous plant pathogens. *New Phytol.* 198: 1001-1016.

Doehlemann, G., Schipper, K., and Kahmann, R. 2012. The effectors of smut fungi. Pages 77-99 in: *Effectors in Plant-Microbe Interactions*, 1st Ed., F. Martin, and S. Kamoun, eds. John Wiley & Sons, Oxford.

Dunnett, C. W. 1964. New tables for multiple comparisons with a control. *Biometrics* 20:482-491.

El Hadrami, A., Islam, M. R., Adam, L. R., and Daayf, F. 2015. A cupin domain-containing protein with a quercetinase activity (VdQase) regulates *Verticillium dahliae*'s pathogenicity and contributes to counteracting host defenses. *Front. Plant Sci.* 6:1-18.

Fawal, N., Li, Q., Savelli, B., Brette, M., Passaia, G., Fabre, M., Mathe, C., and Dunand, C. 2013. PeroxiBase: A database for large-scale evolutionary analysis of peroxidases. *Nucleic Acids Res.* 41:D441-D444.

Fradin, E. F., and Thomma, B. P. H. J. 2006. Physiology and molecular aspects of *Verticillium* wilt diseases caused by *V. dahliae* and *V. albo-atrum*. *Mol. Plant Pathol.* 7:71-86.

Frandsen, R. J., Andersson, J. A., Kristensen, M. B., and Giese, H. 2008. Efficient four fragment cloning for the construction of vectors for targeted gene replacement in filamentous fungi. *BMC Mol. Biol.* 9:70.

Gawehns, F., Houterman, P. M., Ichou, A. F., Michiels, C. B., Hijdra, M., Cornelissen, B. J. C., Rep, M., and Takken, F. L. W. 2014. The *Fusarium oxysporum* effector Six6 contributes to virulence and suppresses I-2 mediated cell death. *Mol. Plant-Microbe Interact* 27:336-348.

Guo, M., Guo, W., Chen, Y., Dong, S., Zhang, X., Zhang, H., Song, W., Wang, W., Wang, Q., Lv, R., Zhang, Z., Wang, Y., and Zheng, X. 2010. The basic leucine zipper transcription factor Moatf1 mediates oxidative stress responses and is necessary for full virulence of the rice blast fungus *Magnaporthe oryzae*. *Mol. Plant-Microbe Interact* 23:1053-1068.

Guo, M., Chen, Y., Du, Y., Dong, Y., Guo, W., Zhai, S., Zhang, H., Dong, S., Zhang, Z., Wang, Y., Wang, P., and Zheng, X. 2011. The bZIP transcription factor MoAP1 mediates the oxidative stress response and is critical for pathogenicity of the rice blast fungus *Magnaporthe oryzae*. *PLoS Pathog.* 7:e1001302 Published online. doi:10.1371/journal.ppat.1001302

Heller, J., and Tudzynski, P. 2011. Reactive oxygen species in phytopathogenic fungi: Signaling, development, and disease. *Annu. Rev. Phytopathol.* 49:369-390.

Hofrichter, M., Ullrich, R., Pecyna, M. J., Liers, C., and Lundell, T. 2010. New and classic families of secreted fungal heme peroxidases. *Appl. Microbiol. Biotechnol.* 87:871-897.

Hogenhout, S. A., Van der Hoorn, R. A. L., Terauchi, R., and Kamoun, S. 2009. Emerging concepts in effector biology of plant-associated organisms. *Mol. Plant-Microbe Interact* 22:115-122.

Houterman, P. M., Speijer, D., Dekker, H. L., De Koster, C. G., Cornelissen, B. J. C., and Rep, M. 2007. The mixed xylem sap proteome of *Fusarium oxysporum*-infected tomato plants. *Mol. Plant Pathol.* 8:215-221.

Houterman, P. M., Cornelissen, B. J. C., and Rep, M. 2008. Suppression of plant resistance gene-based immunity by a fungal effector. *PLoS Pathog.* 4:e1000061 Published online. doi:10.1371/journal.ppat.1000061

Houterman, P. M., Ma, L., van Ooijen, G., de Vroomen, M. J., Cornelissen, B. J. C., Takken, F. L. W., and Rep, M. 2009. The effector protein Avr2 of the xylem-colonizing fungus *Fusarium oxysporum* activates the tomato resistance protein I-2 intracellularly. *Plant J.* 58:970-978.

Huang, K., Czysmek, K. J., Caplan, J. L., Sweigard, J. A., and Donofrio, N. M. 2011. HYR1-mediated detoxification of reactive oxygen species is required for full virulence in the rice blast fungus. *PLoS Pathog.* 7:e1001335 Published online. doi:10.1371/journal.ppat.1001335

Inderbitzin, P., Bostock, R. M., Davis, R. M., Usami, T., Platt, H. W., and Subbarao, K. V. 2011. Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. *PLoS One* 6:e28341.

Jones, J. D. G., and Dangl, J. L. 2006. The plant immune system. *Nature* 444:323-329.

Kayser, T. 1992. Protoplasten fusion sowie elektrophoretische Chromosomentrennung und Genkartierung bei filamentösen Pilzen: *Penicillium janthinellum*, *Absidia glauca* und *Cochliobolus heterostrophus*. Dissertation. Humboldt University of Berlin. Berlin.

- Klimes, A., Dobinson, K. F., Thomma, B. P. H. J., and Klosterman, S. J. 2015. Genomics spurs rapid advances in our understanding of the biology of vascular wilt pathogens in the genus *Verticillium*. *Annu. Rev. Phytopathol.* 53:181-198.
- Klosterman, S. J., Subbarao, K. V., Kang, S., Veronese, P., Gold, S. E., Thomma, B. P. H. J., Chen, Z., Henrissat, B., Lee, Y.-H., Park, J., Garcia-Pedrajas, M. D., Barbara, D. J., Anchieta, A., de Jonge, R., Santhanam, P., Maruthachalam, K., Atallah, Z., Amyotte, S. G., Paz, Z., Inderbitzin, P., Hayes, R. J., Heiman, D. I., Young, S., Zeng, Q., Engels, R., Galagan, J., Cuomo, C. a., Dobinson, K. F., and Ma, L.-J. 2011. Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. *PLoS Pathog.* 7:e1002137. Published online. doi: 10.1371/journal.ppat.1002137
- Knight, C. J., Bailey, A. M., and Foster, G. D. 2009. *Agrobacterium*-mediated transformation of the plant pathogenic fungus *Verticillium albo-atrum*. *J. Plant Pathol.* 91:745-750.
- Kubicek, C. P., Starr, T. L., and Glass, N. L. 2014. Plant cell wall-degrading enzymes and their secretion in plant-pathogenic fungi. *Annu. Rev. Phytopathol.* 52:427-451.
- Kump, B., Svetek, S., and Javornik, B. 1992. Isolation of high molecular weight DNA from plant tissues. *Res. Rep. Biotech. Fac. Univ. Ljubl. Agric.* 59:63-66
- Lin, C.-H., Yang, S. L., and Chung, K.-R. 2009. The YAP1 homolog-mediated oxidative stress tolerance is crucial for pathogenicity of the necrotrophic fungus *Alternaria alternata* in citrus. *Mol. Plant-Microbe Interact* 22:942-952.
- Liu, S.-Y., Chen, J.-Y., Wang, J.-L., Li, L., Xiao, H.-L., Adam, S. M., and Dai, X.-F. 2013. Molecular characterization and functional analysis of a specific secreted protein from highly virulent defoliating *Verticillium dahliae*. *Gene* 529:307-316.
- Liu, T., Song, T., Zhang, X., Yuan, H., Su, L., Li, W., Xu, J., Liu, S., Chen, L., Chen, T., Zhang, M., Gu, L., Zhang, B., and Dou, D. 2014. Unconventionally secreted effectors of two filamentous pathogens target plant salicylate biosynthesis. *Nat. Commun.* 5:4686.
- Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., Zuccaro, A., Reissmann, S., and Kahmann, R. 2015. Fungal effectors and plant susceptibility. *Annu. Rev. Plant Biol.* 66:513-545.
- Lozovaya, V. V., Lygin, A. V., Zernova, O. V., Li, S., Widholm, J. M., and Hartman, G. L. 2006. Lignin degradation by *Fusarium solani* f. sp. *glycines*. *Plant Dis.* 90:77-82.
- Luo, X., Xie, C., Dong, J., Yang, X., and Sui, A. 2014. Interactions between *Verticillium dahliae* and its host: Vegetative growth, pathogenicity, plant immunity. *Appl. Microbiol. Biotechnol.* 98:6921-6932.
- Mandelc, S., and Javornik, B. 2015. The secretome of vascular wilt pathogen *Verticillium albo-atrum* in simulated xylem fluid. *Proteomics* 15:787-797.
- Mandelc, S., Timperman, I., Radisek, S., Devreese, B., Samyn, B., and Javornik, B. 2013. Comparative proteomic profiling in compatible and incompatible interactions between hop roots and *Verticillium albo-atrum*. *Plant Physiol. Biochem.* 68:23-31.
- Mir, A. A., Park, S.-Y., Sadat, M. A., Kim, S., Choi, J., Jeon, J., and Lee, Y.-H. 2015. Systematic characterization of the peroxidase gene family provides new insights into fungal pathogenicity in *Magnaporthe oryzae*. *Sci. Rep.* 5:11831.
- Molina, L., and Kahmann, R. 2007. An *Ustilago maydis* gene involved in H₂O₂ detoxification is required for virulence. *Plant Cell* 19:2293-2309.
- Novo, M., Pomar, F., Gayoso, C., and Merino, F. 2006. Cellulase activity in isolates of *Verticillium dahliae* differing in aggressiveness. *Plant Dis.* 90: 155-160.
- Obruca, S., Marova, I., Matouskova, P., Haronikova, A., and Lichnova, A. 2012. Production of lignocellulose-degrading enzymes employing *Fusarium solani* F-552. *Folia Microbiol. (Praha)* 57:221-227.
- Pegg, G. F., and Brady, B. L., eds. 2002. *Verticillium Wilts*. CABI Publishing, Wallingford, U.K.
- Radisek, S., Jakse, J., Simoncic, A., and Javornik, B. 2003. Characterization of *Verticillium albo-atrum* field isolates using pathogenicity data and AFLP analysis. *Plant Dis.* 87:633-638.
- Radisek, S., Jakse, J., and Javornik, B. 2006. Genetic variability and virulence among *Verticillium albo-atrum* isolates from hop. *Eur. J. Plant Pathol.* 116:301-314.
- Rep, M., van der Does, H. C., Meijer, M., van Wijk, R., Houterman, P. M., Dekker, H. L., de Koster, C. G., and Cornelissen, B. J. C. 2004. A small, cysteine-rich protein secreted by *Fusarium oxysporum* during colonization of xylem vessels is required for I-3-mediated resistance in tomato. *Mol. Microbiol.* 53:1373-1383.
- Santhanam, P., and Thomma, B. P. H. J. 2013. *Verticillium dahliae* Sge1 differentially regulates expression of candidate effector genes. *Mol. Plant-Microbe Interact* 26:249-256.
- Santhanam, P., van Esse, H. P., Albert, I., Faino, L., Nünberger, T., and Thomma, B. P. H. J. 2013. Evidence for functional diversification within a fungal NEP1-Like protein family. *Mol. Plant-Microbe Interact* 26: 278-286.
- Saunders, D. G. O., Win, J., Cano, L. M., Szabo, L. J., Kamoun, S., and Raffaele, S. 2012. Using hierarchical clustering of secreted protein families to classify and rank candidate effectors of rust fungi. *PLoS One* 7:e29847.
- Simko, I., and Piepho, H.-P. 2012. The area under the disease progress stairs: Calculation, advantage, and application. *Phytopathology* 102:381-389.
- Singh, S., Braus-Stromeier, S. A., Timpner, C., Tran, V. T., Lohaus, G., Reusche, M., Knüfer, J., Teichmann, T., von Tiedemann, A., and Braus, G. H. 2010. Silencing of Vlaro2 for chorismate synthase revealed that the phytopathogen *Verticillium longisporum* induces the cross-pathway control in the xylem. *Appl. Microbiol. Biotechnol.* 85:1961-1976.
- Tanabe, S., Ishii-Minami, N., Saitoh, K.-I., Otake, Y., Kaku, H., Shibuya, N., Nishizawa, Y., and Minami, E. 2011. The role of catalase-peroxidase secreted by *Magnaporthe oryzae* during early infection of rice cells. *Mol. Plant-Microbe Interact* 24:163-171.
- Timpner, C., Braus-Stromeier, S. A., Tran, V. T., and Braus, G. H. 2013. The Cpe1 regulator of the cross-pathway control of amino acid biosynthesis is required for pathogenicity of the vascular pathogen *Verticillium longisporum*. *Mol. Plant-Microbe Interact* 26:1312-1324.
- Tran, V. T., Braus-Stromeier, S. A., Kusch, H., Reusche, M., Kaefer, A., Kühn, A., Valerius, O., Landesfeind, M., Abhauer, K., Tech, M., Hoff, K., Pena-Centeno, T., Stanke, M., Lipka, V., and Braus, G. H. 2014. *Verticillium* transcription activator of adhesion Vta2 suppresses micro-sclerotia formation and is required for systemic infection of plant roots. *New Phytol.* 202:565-581.
- Tzima, A. K., Paplomatas, E. J., Rauyaree, P., Ospina-Giraldo, M. D., and Kang, S. 2011. *VdSNF1*, the sucrose nonfermenting protein kinase gene of *Verticillium dahliae*, is required for virulence and expression of genes involved in cell-wall degradation. *Mol. Plant-Microbe Interact* 24: 129-142.
- Wong, D. W. S. 2009. Structure and action mechanism of ligninolytic enzymes. *Appl. Biochem. Biotechnol.* 157:174-209.
- Yadeta, K. A., and Thomma, B. P. H. J. 2013. The xylem as battleground for plant hosts and vascular wilt pathogens. *Front. Plant Sci.* 4:1-12.
- Yang, F., Melo-Braga, M. N., Larsen, M. R., Jorgensen, H. J. L., and Palmisano, G. 2013. Battle through signaling between wheat and the fungal pathogen *Septoria tritici* revealed by proteomics and phospho-proteomics. *Mol. Cell. Proteomics* 12:2497-2508.
- Zhou, B.-J., Jia, P.-S., Gao, F., and Guo, H.-S. 2012. Molecular characterization and functional analysis of a necrosis- and ethylene-inducing, protein-encoding gene family from *Verticillium dahliae*. *Mol. Plant-Microbe Interact* 25:964-975.