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The Plant Host *Brassica napus* Induces in the Pathogen *Verticillium longisporum* the Expression of Functional Catalase Peroxidase Which Is Required for the Late Phase of Disease

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The devastating soilborne fungal pathogen *Verticillium longisporum* is host specific to members of the family Brassicaceae, including oilseed rape (*Brassica napus*) as the economically most important crop. The fungus infects through the roots and causes stunting and early senescence of susceptible host plants and a marked decrease in crop yield. We show here that *V. longisporum* reacts to the presence of *B. napus* xylem sap with the production of six distinct upregulated and eight downregulated proteins visualized by two-dimensional gel electrophoresis. Identification of 10 proteins by mass spectrometry revealed that all upregulated proteins are involved in oxidative stress response. The *V. longisporum* catalase peroxidase (VlCPEA) was the most upregulated protein and is encoded by two isogenes, *VlcpeA-1* and *VlcpeA-2*. Both genes are 98% identical, corroborating the diploid or "amphihaploid" status of the fungus. Knock downs of both *VlcpeA* genes reduced protein expression by 80% and resulted in sensitivity against reactive oxygen species. Whereas saprophytic growth and the initial phase of the plant infection were phenotypically unaffected, the mutants were not able to perform the late phases of disease. We propose that the catalase peroxidase plays a role in protecting the fungus from the oxidative stress generated by the host plant at an advanced phase of the disease.

The genus *Verticillium* includes several pathogenic fungi affecting trees, herbaceous plants, and plantation crops. *Verticillium longisporum, V. dahlia*, and *V. albo-atrum* have become a serious threat to commercially important crops in many agricultural areas (Kroeker 1976; Zielinski and Sadowski 1995). *V. longisporum* is a vascular pathogen of *Brassica napus*. Since the 1960s, Verticillium wilt has become a major threat to oilseed crops in northern Europe, including Sweden, Germany, and Poland, and also France (Karapapa et al. 1997; Zeise and von Tiedemann 2002a and b). In Germany, *V. longisporum* infection on rapeseed has significantly increased since the 1980s (Daebeler et al. 1988; Günzelmann and Paul 1990; Zeise and Seidel 1990). The need for rapeseed oil is increasing due to the fact that it is a healthy vegetable oil and also a renewable resource for the oleochemical industry (Zielinski and Sadowski 1995). With expanding agricultural area for cultivation of rapeseed and the relatively intense crop rotation, this disease has become a menace to oilseed rape production, particularly in Europe (Zielinski and Sadowski 1995). *V. longisporum* does not generate true wilting symptoms but, instead, premature senescence and ripening, which can result in massive yield reductions of up to 50 to 70% (Dunker et al. 2006; Kroeker 1976; Zielinski and Sadowski 1995).

V. longisporum causes only one cycle of disease and inoculum production during a growing season (monocyclic disease). *V. longisporum* is an opportunistic phytopathogen with a life cycle which can be divided into three phases: dormant, parasitic, and saprophytic. In the dormant phase, microsclerotia serve as resting dormant spores of *V. longisporum* and are present in the soil. Their germination is inhibited by microbiostasis or mycostasis. Root exudates containing excess carbon and nitrogen released in the rhizosphere of plants are probably stimulating microsclerotia to germinate (Huisman 1982; Mol et al. 1995). Hyphae that grow out of the germinating microsclerotia can traverse a limited distance, possibly directed by nutrient gradients, to reach potential host plants and then enter the parasitic stage by infecting the host plant.

V. longisporum is remarkable because the fungus is limited to the xylem vessels during the biotrophic phase of its life cycle. In order to propagate in the plant, it must derive nutrients from the xylem sap; however, studies on the nutritional condition for the pathogen in the xylem are limited. The xylem transports mineral-containing water from the soil to the aerial plant parts. In addition, the xylem sap contains amino acids, organic acids, and sugars (Lopez-Millan et al. 2000). It has been reported recently that chorismate synthase silencing in *V. longisporum* reduced the efficiency of plant infection and induced cross-pathway control in the plant xylem (Singh et al. 2010). *V. longisporum* has to react quickly and respond to the host environment by expression of genes that facilitate adaptation to conditions encountered during colonization of the xylem. Such genes might play a role in aiding the fungus to avoid recognition, counteract plant defense responses, use

Nucleotide sequence data is available in the EMBL database under accession numbers FR717672 and FR717673 for *cpeA1* and *cpeA2*, respectively.

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scarce or unique nutrients in the xylem, and influence the plant host, rapeseed, to alter its internal environment to better suit the needs of the invading fungus. In a proteomic analysis of the xylem sap of *B. napus*, 69 proteins belonging to the functional classes of peroxidases, proteases, defense-related proteins, lectins, and cell wall metabolism and remodeling proteins were identified (Kehr et al. 2005). Only some of these proteins were upregulated in response to *V. longisporum* (Floerl et al. 2008). Changes in the composition of the xylem sap due to the infection with *V. longisporum* could be detected (Floerl et al. 2008; Ratzinger et al. 2009).

Proteomics, based on a combination of two-dimensional (2-DE) followed by mass spectrometry (MS) analysis, is a key research tool used to study microbial pathogens in terms of their proteome maps, stage-specific proteomics, and pathogenicity factors (Bhadauria et al. 2007; Jordan et al. 2006). In *V. dahliae*, a fungus very closely related to *V. longisporum*, a comparative analysis of mycelial proteomes has recently been published (El-Bebany et al. 2010). The two species differ in their host preferences, morphology, and geographical distribution. Functional genomics of *V. dahliae* have been investigated in a few studies (Klimes and Dobinson 2006; Klosterman et al. 2011) but a comprehensive proteomics analysis of *V. longisporum* has not been reported. Plants protect themselves from an invading pathogen by various responses which strengthen barriers against the invader or weaken the pathogen (Apostol et al. 1989; Hammond-Kosack and Jones 1996; van Loon et al. 2006). One of the earliest responses to an infection by a pathogen leads to the rapid production of reactive oxygen species (ROS) (e.g., hydrogen peroxide [H_2O_2], superoxide radicals, or hydroxyl ions termed oxidative burst) (Lamb and Dixon 1997; Mayer et al. 2001). The major ROS leading to oxidative burst is H_2O_2 (Wojtaszek 1997). ROS are able to oxidize proteins, lipids, and nucleic acids, which leads to damage in cellular structures (Medentsev et al. 2001). As a response to oxidative burst, plant pathogens can activate various enzymes such as catalases or peroxidases that are capable of removing H_2O_2 or other ROS (Mayer et al. 2001). *Claviceps purpurea* secretes catalases as response to host plant defense mechanisms (Garre et al. 1998). Catalases have been identified as virulence factors in the pathogens *Candida albicans* or *Agrobacterium tumefaciens* (Wysong et al. 1998; Xu and Pan 2000). The catalase CatB of *Magnaporthe grisea* strengthens the fungal cell wall (Skamnioti et al. 2007), whereas Des1 of *M. oryzae* compromises ROS-mediated plant defense (Chi et al. 2009) and MoHyr1 is part of the ROS detoxification mechanism (Huang et al. 2011). The transmembrane protein TmpL is required for intracellular redox homeostasis and virulence in a plant and an animal fungal pathogen (Kim et al. 2009).

Here, we use a proteomic approach to screen for proteins which are expressed when *V. longisporum* senses the xylem sap of the plant. We identified the most prominent statistically relevant reaction as the increase of a bifunctional catalase peroxidase. The two corresponding isogenes of the catalase peroxidase were identified and silenced in *V. longisporum* using RNAi-mediated gene silencing. Plant infection assays in *B. napus* revealed that the catalase peroxidase is required for late phases of the disease.

RESULTS

Xylem sap of *B. napus* inhibits growth of *V. longisporum* and affects fungal development.

We analyzed how the addition of xylem sap of the host plant affects growth or life cycle progression of the pathogen *V. longisporum.* In a first approach, we tested whether xylem sap enhances or inhibits fungal growth. Fungal growth is drastically inhibited during cultivation in xylem sap of infected or uninfected plants (Fig. 1A). Macromolecules larger than 3 kDa were separated from the xylem sap from infected as well as uninfected plants. Both samples were able to inhibit fungal growth when added to a standard complete medium for fungi (Fig. 1B), suggesting that the plant synthesizes proteins which inhibit the fungus.

We then analyzed whether the xylem sap without this protein fraction affects the fungus. Two different solid agar growth media—Czapek Dox agar (CDA) (Nell et al. 2006) or simulated xylem fluid medium (SXM)—were tested. Differences in the fungal phenotype when filtered xylem sap in a volume of one-seventh of the media volume was added were already apparent for both media 3 days after inoculation. Filtered xylem sap stimulates fungal growth, melanization, or the formation of

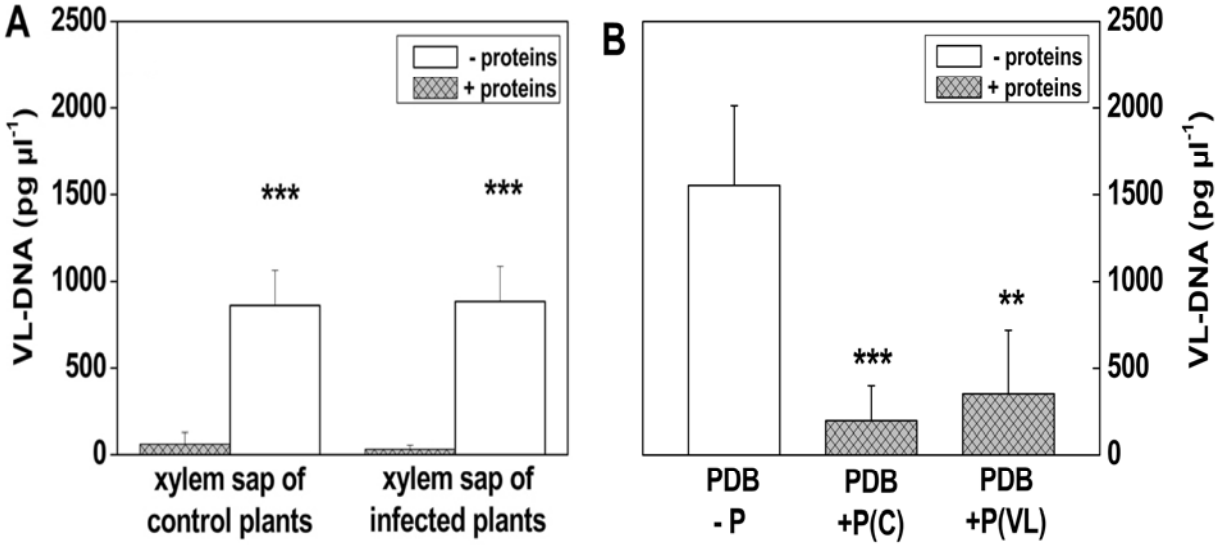

Fig. 1. Inhibition of *Verticillium longisporum* growth by xylem sap proteins of oilseed rape (*Brassica napus*). **A,** *V. longisporum* DNA after 3 days of growth in filtered (white bars) or unfiltered (hatched bars) xylem sap of infected or control plants. **B,** *V. longisporum* DNA after 3 days of growth in potato dextrose broth (PDB) medium with filtered (white bars; without xylem sap proteins: –P) or unfiltered (hatched bars, with xylem sap proteins: +P) of infected (VL) or control (C) plants. Data indicate means ($n = 5$) ± standard deviation. Significant differences between samples with and without proteins are illustrated by asterisks (*** and ** indicate $P \leq 0.001$ and 0.01, respectively). Xylem sap was collected between 25 and 28 days postinfection.

bud spores. The stimulating effect of the filtered xylem sap was quantified after 8 days of cultivation by counting spores. This revealed that filtered xylem sap can increase conidia formation more than twice, independently of the medium (Fig. 2). Further dilution of the xylem sap by a factor of 10 resulted in the same stimulation in fungal growth and melanization and increase in spore formation (data not shown).

V. longisporum showed upregulated as well as downregulated proteins when grown in the presence of xylem sap of *B. napus*.

The stimulating impact of xylem sap of *B. napus* on growth of *V. longisporum* was analyzed in more detail. Xylem sap was extracted from 42-day-old *B. napus* because it corresponded to the plant at 35 days postinoculation, when the disease symptoms are most severe. We examined changes in the intracellular proteome, which may occur after treating *V. longisporum* with the filtered xylem sap of *B. napus* (42 days old) which stimulates fungal growth. To identify the proteins that might be differentially expressed in *V. longisporum* by growth in the presence of xylem sap, we extracted intracellular proteins from 5-day-old liquid fungal cultures incubated with or without xylem sap for 8 h. Comparison of six sets of Ruthenium II Tris-bathophenantroline disulfonate (RuBP) (Lamanda et al. 2004) stained 2-DE gels of these extracts clearly demonstrated that 2-DE analysis of protein samples exhibited reproducible gel images. More than 800 protein spots with a molecular weight between 10 and 120 kDa and a pI ranging from pH 4.0 to 7.0 were resolved for both conditions. A quantitative analysis using PDQuest software revealed six proteins (U1 to U6) upregulated and eight proteins (D1 to D8) downregulated compared with the controls. These are indicated by arrows in Figure 3A. Close-up views of the gels confirmed that the enhanced spots were differentially expressed compared with the control levels (Fig. 3B and C). The proteomic analysis was also performed using xylem sap from 35-day-old *B. napus* plants. Protein spots U1, U4, D2, and D3 showed no significant regulation compared with controls in this analysis. To test whether the xylem sap extracted from *B. napus* that was already infected with *V. longisporum* may influence the *V. longisporum* proteome more, the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis was also performed using the xylem sap extracted from 42-day-old infected *B. napus* plants (35 days postinfection [dpi]) or 35-day-old infected *B. napus* plants (28 dpi). However, there was no significant difference found in protein expression between fungal cultures treated with xylem sap from *B. napus* uninfected versus infected with *V. longisporum*.

To identify the proteins, tryptic digestion of excised, selected differentially expressed protein spots was followed by a tandem MS (MS/MS) run; then, identification was achieved through homology searching with tentative sequences obtained by MS. The resulting identified proteins are shown in Table 1. Of the 14 selected protein spots, 10 protein spots could be successfully identified. Half of the identified proteins were found to be homologous to *V. dahliae* proteins and others to other fungal organisms such as *Aspergillus nidulans* and *Neurospora crassa*. The identification of most of the protein spots was also confirmed through the analysis of the MS/MS data sets with the PEAKS MS program (data not shown). Two upregulated (U5 and U6) and two downregulated (D2 and D7) proteins that were not identified by analysis with TURBOSequest also could not be identified by analysis with PEAKS that used the National Center for Biotechnology Information (NCBI) nr database; therefore, they may be *V. longisporum* proteins that are not so well conserved.

Catalase peroxidase was upregulated in *V. longisporum* as a response to *B. napus* xylem sap.

Spot number U1 was upregulated in the proteomic analysis when *V. longisporum* was treated with xylem sap from its host plant, *B. napus*. It was found to increase more than twofold in the xylem-sap-treated fungal proteome (Fig. 3C). It was confidently identified as the enzyme catalase peroxidase (NCBI accession number CAC59821) encoded by *cpeA* in *A. nidulans* by the TURBOSequest analysis, because two tryptic peptides with X-corr value of 4.75 and 3.03 highly matched with the theoretical spectra (Table 1). The *cpeA* gene in *A. nidulans* encodes an 81-kDa bifunctional enzyme, catalase peroxidase that has a conserved motif for heme coordination (Scherer et al. 2002). In *A. nidulans*, three monofunctional catalases have been described and the *cpeA* gene product was first observed as a fourth catalase activity in native polyacrylamide gel, and named catalase D (Cat D) (Kawasaki and Aguirre, 2001). Cat D activity was induced by glucose starvation, high temperature, and H_2O_2 treatment.

A cDNA library of *V. longisporum* was used to obtain the sequences of candidate genes (Singh et al. 2010). The MS2 spectrum of catalase peroxidase (spot U1) was used to search for matches against the cDNA library sequences, and two cDNA clones from the library matched peptides for confident identification of the protein spot. These two clones were sequenced fully and, subsequently, the full sequence of *V. longisporum* catalase peroxidase was obtained.

The *V. longisporum* catalase peroxidase coding region comprises an open reading frame of 2289 bp (Supplementary Fig.

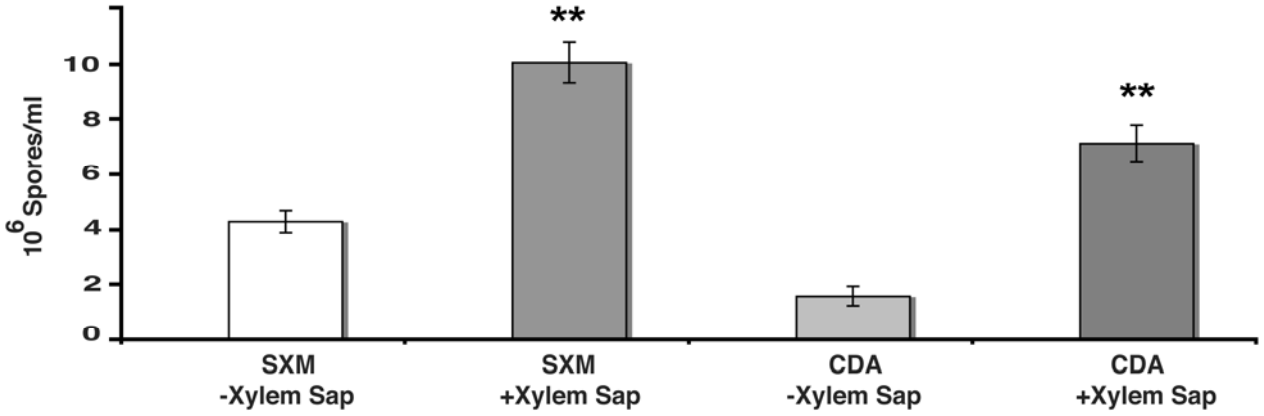

Fig. 2. Influence of filtered xylem sap on *Verticillium longisporum* growth, development, and spore formation. The number of *V. longisporum* spores per milliliter from each growth condition was analyzed 8 days after inoculation. Data indicate means (n = 3) ± standard deviation. Significant differences between samples are illustrated by asterisks (*** and ** indicate $P \leq 0.001$ and 0.01, respectively).

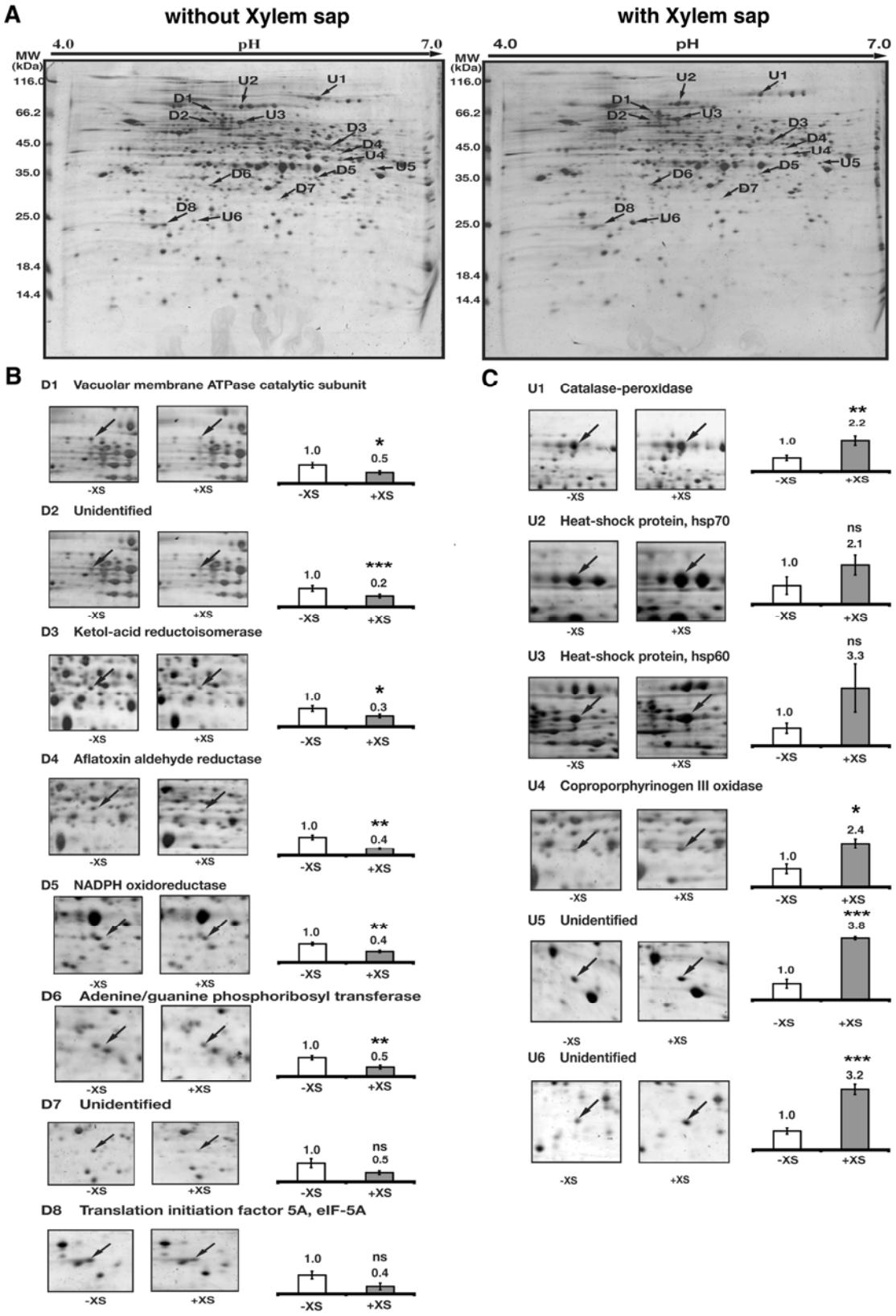

Fig. 3. Differential proteome of *Verticillium longisporum* induced by xylem sap. **A,** Two-dimensional (2-DE) gels of the *V. longisporum* proteome untreated or treated with xylem sap from rapeseed. These are representative figures from three biological and two technical replicates of each condition. Differentially expressed proteins are marked with arrows and labeled: U1–U6 = upregulated protein spots and D1–D8 = downregulated protein spots. Close-up views of the regions of the 2-DE gels that show significant **B,** downregulation or **C,** upregulation in protein expression (untreated [–XS] versus treated [+XS] with xylem sap from rapeseed). Arrowheads indicate the differentially expressed proteins. Expression histograms show the differential protein expression of *V. longisporum* due to treatment with xylem sap from rapeseed (right). The same scale was used on the y-axis for depicting mean protein spot quantity for all the protein spots. Bars represent mean protein spot quantity of the *V. longisporum* proteome untreated (–XS) versus treated (+XS) with xylem sap. Data indicate means ($n = 6$) ± standard deviation. Significant differences between the proteins concentrations are illustrated by asterisks (***, **, and * indicate $P \leq 0.001$, 0.01, and 0.05, respectively; ns indicates $P \geq 0.05$).

S1) which encodes a protein of 762 amino acids with a predicted molecular mass of 83.54 kDa. Two isogenes were cloned and the coding sequences showed 98% identity. *V. longisporum* catalase peroxidase is almost identical (98% identity) to the protein of *V. dahliae,* one of its proposed parental species (Supplementary Fig. S2). It has high sequence similarity with *A. nidulans* CpeA protein (70% identity), to which this protein spot (spot number U1) was found homologous during identification of protein spots in the proteomic analysis. *V. longisporum* protein also shows high sequence similarity with catalase peroxidase proteins from the phytopathogenic fungus *M. grisea* (74% identity) and even from the bacterium *Flavobacterium johnsoniae* (71% identity).

In the in-gel catalase activity assay, equal amounts of the native protein from *V. longisporum* and *V. longisporum* treated with xylem sap were run on a native gel. Two clear bands were observed in each sample against a dark background after staining for catalase activity (Fig. 4A). After quantification of the activity, it was found that the catalase activity has increased significantly in the lower band in the sample treated with xylem sap. Hence, the xylem sap causes an increase in the specific catalase activity in accordance with the result from the 2D-PAGE analysis, where it is upregulated. A second catalase activity was unaffected by the addition of xylem sap (Fig. 4A).

V. longisporum has been described as a "near-diploid" organism (Inderbitzin et al. 2011; Karapapa et al. 1997), prompting us to ascertain whether *V. longisporum* catalase peroxidase (Vlcpe)A-1 had an isogene. Therefore, *V. longisporum* and *V. dahliae* genomic DNA was digested with different restriction enzymes and subjected to Southern hybridization. A specific and fully sequenced *VlcpeA-1* fragment from *V. longisporum* was used as the probe. In several independent Southern hybridization analyses, two signals were generated for *V. longisporum* compared with *V. dahliae* for the genomic DNA digested with

Table 1. Homologies of differentially expressed proteins of *Verticillium longisporum* after treatment with xylem sap from *Brassica napus*

Spot[a]	Protein	Organism	NCBI[b]	MWe/ pIe[c]	Matched peptides	X-corr[d]	Coverage (%)[e]
U1	Catalase peroxidase	*Aspergillus nidulans*	CAC59821	90/6.1	FLENPDQFADAFAR SPAGAHQYVAK	4.75	3.38
U2	Heat shock protein 70	*Neurospora crassa*	XP_961753	78/5.4	STNGDTHLGGEDFDIHLVR	5.73	9.30
					MLGNFQLVGIPPAHR	4.4	
					MKETAESFLSKPVK	4.09	
U3	Heat shock protein 60	*N. crassa*	XP_956500	60/5.5	TNEVAGDGTTSATVLAR	6.17	20.73
					VEFEKPLILLSEK	4.46	
					FVDALNATR	4.17	
					TIIENAGLEGSVVVGK	3.75	
					AAVEEGILPGGTALIK	3.49	
					NVAAGCNPMDLR	3.36	
					AIFSETVKNVAAGCNPMDLR	2.95	
					GQLQVAAGCNPMDLR	2.88	
					LSGGVAVIK	2.56	
U4	Copropor phyrinogen III oxidase	*Verticillium dahliae*	BQ111120	43/6.2	GGVGVSVVYGGTLPK	4.16	2.76
					YVEFNLVHDR	3.25	
D1	Vacuolar membrane ATPase catalytic subunit A	*V. dahliae*	BQ110481	66/5.2	LGEMPADQGFPAYLSAK	4.39	22.60
					TTLIANTSNMPVAAR	4.29	
					DQGLDVAMMADSSSR	3.56	
D3	Ketol-acid reductoisome-rase precursor	*N. crassa*	XP_961335	46/6.2	NDTLALIGYGSQGHGQGLNLR	3.89	13.18
					VEVPTDVDVILVAPK	3.02	
					NLFDVDEAISR	2.99	
D4	Aflatoxin aldehyde reductase	*V. dahliae*	BQ110032	45/6.2	IILGLMTFGPSESDGAR	4.02	9.37
					ATPFAETLEALDK	3.15	
D5	NADPH oxidoreductase	*V. dahliae*	BQ110643	38/6.1	SALAGIDAVVSTLGAPAVGEPQR	5.39	27.70
					NLVEAAVEAGVQR	4.47	
					VKEVVVDYNDPASLK	4.21	
					EVVVDYNDPASLK	3.49	
					IKTEELLVEK	3.14	
D6	Hypothetical protein AN9083.2 (conserved domain: Adenine phosphoribosyl transferase)	*A. nidulans*	XP_682352	34/5.2	VLIVDDIIATGGSAK	4.98	8.85
					GFLFGPGLALR	3.44	
					LPGPCVTAEYQK	3.12	
					EYGTDFFQMQEDAIKPGQK	2.58	
D8	Translation initiation factor eIF-5A	*V. dahliae*	BQ110791	25/4.9	KLEDLSPSTHNMDVPNVTR	4.31	12.20
					VHIVATDIFTGK	3.57	
					LEDLSPSTHNMDVPNVTR	3.52	

[a] Spot numbers. U1 to U4: upregulated protein spots and D1 to D8 = downregulated protein spots.
[b] National Center for Biotechnology Information (NCBI) accession number.
[c] MWe/pIe = molecular weight (kDa) and isoelectric point of differentially expressed proteins determined experimentally.
[d] Cross-correlation scores of matched peptides (TURBOSequest).
[e] Sequence coverage (percent amino acids).

*Apa*I and *Hin*dIII (Fig. 4B). We also confirmed this result by using the *VlcpeA-1* fragment from *V. dahliae* as the probe (data not shown). These results demonstrated that *VlcpeA-1* has an isogene, *VlcpeA-2*.

VLCPEA isogenes were silenced up to 80% by RNA-mediated gene silencing.

RNA-mediated gene silencing has been successfully implemented in several fungi for targeted gene silencing instead of a conventional knockout (Nakayashiki 2005). The plasmid pME3928 (for details on plasmid construction, discussed below) includes the hairpin construct for silencing of *VlcpeA* and the hygromycin resistance gene as selective marker. *Agrobacterium tumefaciens*-mediated transformation of *V. longisporum* was applied and 30 independent hygromycin-B-resistant transformants were selected at random for further analysis. The extent of *VlcpeA* silencing was monitored by reverse-transcription polymerase chain reaction (RT-PCR) because the silencing of gene expression is the result of posttranscriptional degradation of targeted mRNA. Total RNA was extracted from the *VlcpeA* silenced mutant (*VlcpeAsm*) and the wild type. RNA was extracted for each strain three times from independent cultures. RT-PCR results showed the significant knockdown of *VlcpeA* transcripts (Fig. 5A). The degree of silencing of catalase peroxidase genes was estimated by RT-PCR by the ratio of the amplification of *VlcpeA* between the respective transformants and the unsilenced wild type. For each transformant, three biological replicates were analyzed. High-frequency silencing was observed for 71% of the transformants exhibiting reduced gene expression. The extent of silencing varied in the different transformants between high and moderate levels. After 42 dpi in planta, the silencing of the *VlcpeA* isogenes was still stable (Fig. 5B).

Silencing of VlcpeA does not affect the saprophytic growth phenotype but results in H_2O_2-sensitive mutants of *V. longisporum*.

We analyzed the growth behavior of the silenced mutants compared with that of *V. longisporum* on different media. The strains were plated on CDA and SXM and incubated at 25°C for several days after 48 h and, thereafter, each day, the diameter of the colony was measured and noted (data not shown). The colonies were investigated (dissection microscope) to detect differences between the mutant and wild type. From these analyses, no differences could be observed. The growth phenotype of the *VlcpeA*-silenced mutant is the same as the wild type.

For comparison, the mutants silenced in *VlcpeA* were grown on CDA plates supplemented with different substances inducing oxidative and redox-membrane stress such as H_2O_2, menadione, ethanol, and sodium dodecyl sulfate (SDS) in different concentrations. H_2O_2 inhibited growth of *V. longisporum* in silenced mutants dramatically (Fig. 5C). At a concentration of

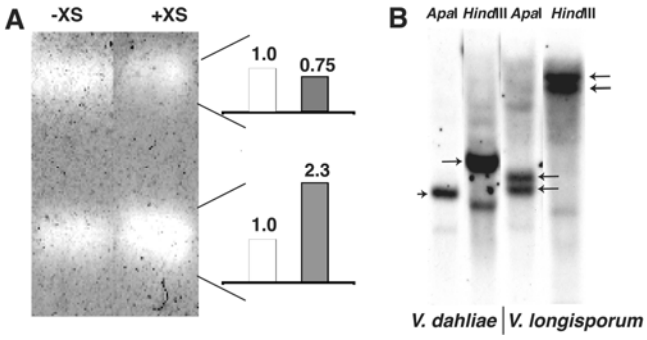

Fig. 4. *Verticillium longisporum* catalase peroxidase isoproteins and encoding isogenes. **A,** In-gel catalase assay. Equal amounts (10 µg) of protein extracts from *V. longisporum* untreated (–XS) or treated (+XS) with xylem sap of *Brassica napus* were separated in native polyacrylamide gel and stained for catalase activity according to Zou and associates (2000). Catalase activities appear as clear bands in the dark-green background. Relative intensity of the catalase activities in each extract was determined using Kodak Molecular Imaging software (right). The experiment was repeated several times and the standard deviation did not exceed 25%. **B,** Southern hybridization. Determination of two copies of *V. longisporum* catalase peroxidase (*VlcpeA*) by Southern hybridization analysis of *V. dahliae* and *V. longisporum*. The genomic DNA was digested with *Apa*I and *Hin*dIII. A 402-bp sequence of *Vlcpe*A-1 was used as a probe. Arrows indicate the signal generation by probe binding.

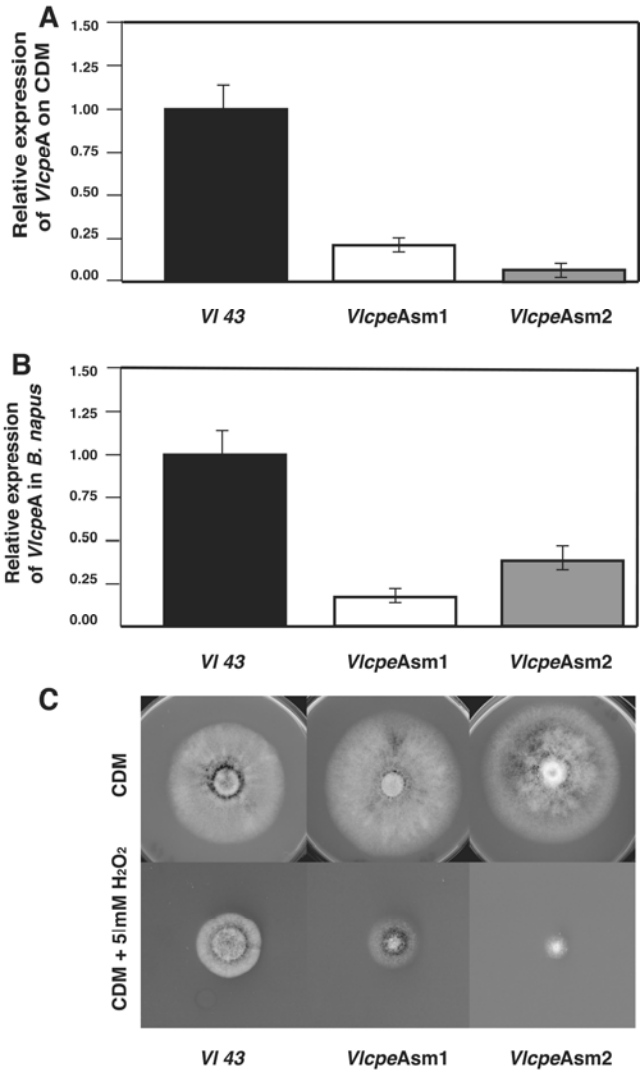

Fig. 5. Expression of catalase peroxidase-encoding genes of *Verticillium longisporum*. **A,** Relative expression of *V. longisporum* catalase peroxidase (*Vlcpe*A) in wild type (*Vl 43*) and silenced mutant (*Vlcpe*Asm) measured by quantitative real-time polymerase chain reaction (PCR). RNA was extracted from three independent cultures. *Vlcpe*A cDNA was normalized to the histone cDNA. *Vlcpe*Asm1 and *Vlcpe*Asm2 = *cpe*A silenced mutants. Error bars represent the standard deviation of four different measurements of cDNA. **B,** Relative expression of *Vlcpe*A in *Brassica napus* measured by quantitative real-time PCR 42 days postinfection. *VlcpeA* cDNA was normalized to the histone cDNA. cDNA from host plants mock inoculated with water served as control. *Vl 43* = cDNA from *B. napus* infected with the wild type; *Vlcpe*Asm1 and *Vlcpe*Asm2 = cDNA from *B. napus* infected with *Vlcpe*A-silenced mutant. Error bars represent the standard deviation of triplicates. **C,** Test for *Vlcpe*Asm sensitivity to H_2O_2. *Vl* 43 and *Vlcpe*A-silenced mutants were cultivated on Czapek Dox agar medium (CDM) and CDM containing 5 mM H_2O_2. Pictures were taken after 4 days of growth.

5 mM H_2O_2, the silenced mutants of *VlcpeA* were strongly reduced in growth. At higher concentrations of H_2O_2 (approximately 10 mM), the growth behavior of the wild type was also affected. Menadione and SDS did not affect growth of the wild type or the mutant fungi, whereas ethanol inhibited all tested fungi equally.

Late-stage plant symptoms decrease significantly during infection in the silenced mutant versus the wild type.

We analyzed whether *VlcpeAsm* mutants were still able to colonize *B. napus* and cause disease symptoms similar to the wild-type strain (Fig. 6). Two different sets of disease symptoms can be observed during the infection of oilseed rape by *V. longisporum*: the host plant becomes stunted and shows signs of early senescence. Both symptoms are normally visible after 21 dpi and are more pronounced at later time points. One-week-old *B. napus* seedlings were infected by root dip inoculation to compare the severity of the disease; height and the signs of early senescence (disease scores) of the plants were measured weekly postinoculation until 35 dpi.

Scoring of the disease symptoms corroborated the requirement of full catalase peroxidase function for plant pathogenicity. The infected rapeseed plants were scored for disease symptoms by assigning disease scores from 1 to 9 corresponding to asymptomatic to dead plants (Eynck et al. 2007). The plants were observed once a week and, in the assessment of the symptoms, the yellowing and death of the leaves were considered. Disease scores of the mock-infected plants of greater than 1 reflect the yellowing of leaves due to natural senescence. Disease symptoms were visible at 21 dpi and grew more pronounced at 35 dpi (Fig. 6B). Both *VlcpeAsm* and the wild type showed similar disease scores only in the initial phase of the disease at 21 and 28 dpi. At 35 dpi, the disease score of the *VlcpeAsm*-infected plants (4.5) was lower than the plants infected with wild-type fungus (5.5) but still significant compared with mock-inoculated control plants (3.7) (Fig. 6B). This reflects a slow down of the disease of the plant when infected by the mutant fungi, corroborating a reduced impact on senescence.

Similar observations for the stunting symptom support this finding. Stunting was barely visible after 21 dpi but became pronounced after 28 dpi for *VlcpeAsm* and the wild type. Consistent with the lowered senescence, the effect on stunting of the plant was significantly less pronounced at 35 dpi (Fig. 6A).

The DNA content was determined in a time window of 35 and 42 dpi where there were no differences within the respective sets of experiments ($P_{(time)} > 0.5$). Very low concentrations of fungal DNA were found in roots (2 to 3.5 ng/g fresh weight [FW]) and did not vary between mock-inoculated and *Vl* 43-, VIcepA1-, and VlcpeA2-infected plants (Fig. 7). However, in the hypocotyls where the highest concentration of fungal DNA (65 to 110 ng/g FW) was present, there were pronounced differences between plants infected with wild-type strain *Vl* 43 or the silenced strain *VlcpeAsm* (Fig. 7). Fungal DNA was significantly lower in stems (14 to 18 ng/g FW) than in the hypocotyl. These data suggest that the mutant strain is able to perform the initial colonization of the plant but has some difficulties in the hypocotyl of the plant (Fig. 7).

The mutant fungi were isolated from the plants and it was verified that they were still silenced. This suggests that the catalase peroxidase which is already induced in the fungus by diluted plant xylem sap represents an alert reaction because significant expression is only essential for later stages of plant infection but not required for the induction of diseases symptoms.

DISCUSSION

The composition of the xylem sap depends on several factors such as age, plant variety, or nitrogen supply (Tilsner et al. 2005; Zornoza et al. 1996) and includes water, inorganic salts, sugars, and some organic compounds which can act as nutrients (Schurr and Schulze 1995). In the xylem, water and inorganic nutrients are translocated from roots to the aerial plant organs. The xylem sap also provides a medium for cross-talk between the pathogen, *V. longisporum*, and its host, *B. napus*. Accordingly, colonization of xylem vessels of the host plant provides the vascular phytopathogen *V. longisporum* with a combination of stimulating substances, including nutrients and, presumably, signal molecules and inhibitory compounds involved in plant defense. The presence of xylem sap even without the plant primarily inhibited fungal growth. The stimulating effect of xylem sap on fungal growth could only be measured when xylem sap was filtered and, therefore, inhibitory compounds were removed.

Xylem sap without macromolecules stimulated mycelial growth of the fungus, earlier bud spore formation, and significantly more conidia. This is presumably due to signal molecules because a 10-fold dilution of the filtered xylem sap resulted in the same stimulation of fungal growth and development. This might be due to molecules that boost the growth of *V. longis-*

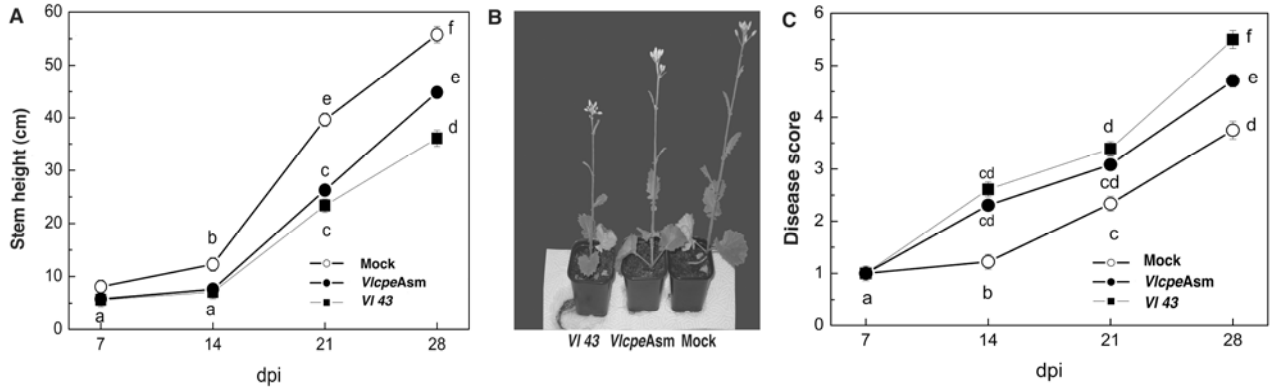

Fig. 6. Assessment of pathogenicity of the *Verticillium longisporum* catalase peroxidase-silenced mutant (*Vlcpe*Asm). **A,** Assessment of stunting of rapeseed due to *V. longisporum* infection. The height of 20 replicates each of rapeseed plants infected with the wild type (*Vl* 43) and *Vlcpe*Asm was measured at 7, 14, 21, and 28 days postinfection (dpi). For comparison, the height of rapeseed plants mock inoculated (mock) with tap water was also measured. Different letters indicate significant differences at $P < 0.05$. The plants are heavily infected at 28 dpi. Data represent average ± standard deviations of 20 experimental replicates. **B,** Stunting and disease development in *Brassica napus* infected with either *Vl* 43, *Vlcpe*Asm, or mock after 28 dpi. **C,** Assessment of disease development by scoring for disease symptoms according to Eynck and associates (2007). Plants were scored for disease symptoms at 7, 14, 21, and 28 dpi. Data represent average ± standard deviations of 20 experimental replicates.

porum even when present in low concentrations. When the bacterial vascular pathogen *Xylella fastidiosa* was subjected to xylem fluids with varying chemistries, highly significant differences in planktonic growth and biofilm formation were correlated to the concentration of citric acid, amino acids, and inorganic ions in the xylem fluids (Andersen et al. 2007). The enhanced melanization in the presence of xylem sap might be explained by the utilization of tryptophan present in the xylem sap of *B. napus* (Kehr et al. 2005), resulting in melanin production. This might reflect a *V. longisporum* response to the presence of defense-related proteins in xylem sap (Kehr et al. 2005), because melanized fungi are less susceptible to host defense mechanisms (Taborda et al. 2008).

The observation that *B. napus* xylem sap affected *V. logisporum* growth and development implies that the fungus might be perceiving the plant, and that this perception could be manifested by differential protein expression in *V. longisporum*. Host extracts also changed protein expression patterns of the phytopathogenic bacteria *Dickeya dadantii* (Babujee et al. 2007) and *Pectobacterium atrosepticum* (Mattinen et al. 2007). Our proteomic analysis revealed alterations of protein expression for only 14 protein spots among more than 800 fungal spots upon treatment with xylem sap.

The six upregulated proteins are mainly related to oxidative stress response. Upregulation of the analyzed catalase peroxidase was the most prominent statistically relevant effect and might be a consequence of encountering oxidative stress. Germin proteins constitutively present in the xylem sap as well as in leaf apoplast might be possible candidates to evoke this response (Kehr et al. 2005) because some of them can generate H_2O_2. Indeed, increased H_2O_2 levels and diminished *Verticillium* spp. growth were detected in *Arabidopsis thaliana* overexpressing germin-like protein GLP1 from *B. oleracea* (Knecht et al. 2010). The catalase peroxidase detected in our study is a close homologue to a bifunctional catalase peroxidase in *Aspergillus nidulans* that is encoded by cpeA and carries a conserved motif for heme coordination (Scherer et al. 2002). A further upregulated protein homologous to coproporphyrinogen III oxidase from *V. dahliae* might be co-regulated with catalase peroxidase, because it is an essential enzyme to catalyze the sixth step of heme biosynthetic pathway (Lash et al. 1999). The proteins homologous to heat-shock protein 70 (hsp70) and heat-shock protein 60 (hsp60) from *N. crassa* were also upregulated. Transcripts for hsp60 and hsp70 were also upregulated in the plant pathogen *Phytophthora infestans* during early stages of potato infection (Avrova et al. 2003). Hsp60 can protect *Saccharomyces cerevisiae* against oxidative damage due to H_2O_2 and superoxide anions (Cabiscol et al. 2002).

In contrast, a putative NADPH oxidoreductase, which is also known to be involved in redox reactions, was one of the eight downregulated proteins of our proteome analysis. NADPH oxidoreductases accumulate rapidly in *Arabidopsis* plants under various oxidative stress conditions and have been reported to be involved in NAD(P)/NAD(P)H homeostasis (Babiychuk et al. 1995) and even iron uptake (Roman et al. 1993). The downregulated putative subunit of a vacuolar proton-translocating ATPases (V-ATPases) might also reflect that the xylem sap has an oxidizing environment. This protein is part of a complex which acidifies organelles of the vacuolar network, including the vacuoles, Golgi apparatus, or other secretory vesicles (Forgac 1989). Reducing agents have a stabilizing effect on the V-ATPase (Dschida and Bowman 1995), and oxidizing agents like H_2O_2 are potent inhibitors of the V-ATPase in vitro.

Four proteins involved in biochemical processes are downregulated by xylem sap. They include the eukaryotic initiation factor eIF-5A, involved in translation (Hershey 1991), and a putative aflatoxin, B1 aldehyde reductase (Judah et al. 1993). Another downregulated protein carries an adenine phosphoribosyl transferase conserved domain. It has been shown that the phosphoribosyl transferase activities have a positive effect on

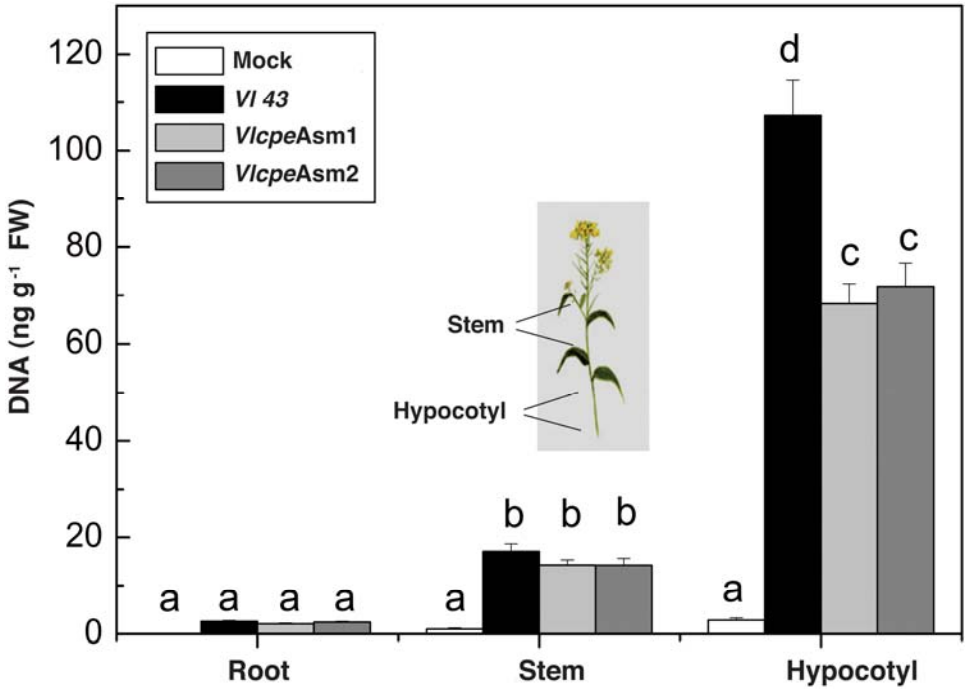

Fig. 7. Determination of the *Verticillium longisporum* DNA concentration in infected plant tissues. Different letters (a to d) indicate significant differences at $P < 0.05$. *V. longisporum* DNA was measured with real-time polymerase chain reaction days postinfection in stems, hypocotyls, and roots of *Brassica napus* inoculated with *V. longisporum* catalase peroxidase-silenced mutants (*Vlcpe*Asm) and the wild type (*Vl 43*) at 42 days postinfection. Data represent average ± standard deviations of five experimental replicates. Mock-inoculated plants as a control did not show presence of any *V. longisporum* DNA; ng Vl DNA/g FW = nanograms of *V. longisporum* DNA per gram fresh weight of plant tissue.

the rate of external purine uptake by *Schizosaccharomyces pombe* cells. The fourth downregulated protein is a precursor of ketol-acid reductoisomerase as part of branched chain amino acid biosynthesis. Ketol-acid reductoisomerase expression is also downregulated following host penetration during infection of potato by *P. infestans* (Grenville-Briggs et al. 2005). This could be due to a ready access of amino acids from the plant.

The function of the upregulated catalase peroxidase identified by this proteomic approach was analyzed in more detail to reveal whether this approach is suitable to identify disease-related proteins of the fungus. Plants normally resist pathogen attacks or delay pathogen growth by triggering defense responses, including the production of ROS such as H_2O_2, superoxide radicals, or hydroxyl radicals (Lu et al. 2006). A rapid response upon perception of microbial pathogens termed oxidative burst can be followed by a second ROS burst during plant–pathogen interactions triggering hypersensitive cell death (Lamb and Dixon 1997). Catalase peroxidase can protect a fungal pathogen by decomposing the hydrogen-peroxide generated by the host (Wysong et al. 1998). Catalases and peroxidases of phytopathogenic fungi have attracted attention as potential virulence factors (Garre et al. 1998; Nathues et al. 2004). Silencing of catalase peroxidase reduced growth of the fungus in the hypocotyl, supporting the function as a potential virulence factor in the infection of rapeseed. ROS might play an essential role in the hypocotyl and are possibly preventing the fast spreading of the fungus to other parts of the plant. Alternatively, the catalase peroxidase could also be important to support fungal development in the plant. ROS have been shown to be important developmental signals for fungal differentiation and nutrient scavenging (Egan et al. 2007; Nahlik et al. 2010; Scott and Eaton 2008). The exact role of the fungal catalase peroxidase isogene pair and also whether there are different functions of both isogenes will be an interesting future question.

Cloning and sequencing of both isogenes for catalase peroxidase revealed a 98% identity to the corresponding protein in *V. dahliae*, one of its parental species. It was also found to be 70 to 74% identical to the corresponding proteins in the fungi *A. nidulans* or *M. grisea* and the bacterium *F. johnsoniae*. VlCPEA belongs to the KatG catalase peroxidases in class I of the plant peroxidase superfamily because it contains the three motifs SQXWWPADXGXY, AXXMGLIYVN, and GXXPXX AXXEXQGLGW, which are conserved in all KatG catalase peroxidases (Zamocky et al. 2001). *V. longisporum* has been described as near diploid or amphihaploid, with at least one parent as *V. dahliae* (Barbara et al. 2005; Karapapa et al. 1997). Southern hybridization analysis as well as cloning and sequencing revealed that there are two isogenes for catalase peroxidase present in *V. longisporum*. In contrast, *V. dahliae* carries only a single gene for cpeA.

In summary, our data have identified the upregulation of catalase peroxidase as the strongest reaction of *V. longisporum* to the presence of xylem sap after inhibitory proteins had been removed. This is presumably an early and maybe even primarily prophylactic reaction to the hostile xylem sap environment. Reduced expression of the enzyme is sufficient to allow the performance of the initial phase of the disease. However, the enzyme is essential for completing the disease, resulting in pronounced stunting and early senescence. It will be interesting to examine whether the enzyme might be a putative target for drugs which reduce the impact of the pathogen on the loss of rape seed crops which is encountered in Europe during the last decades. This proteomic approach is promising and could also be applicable for other plant pathogens to identify disease-related proteins.

MATERIAL AND METHODS

Strains, media, and growth conditions.

In all, *V. longisporum* 43 and *V. dahliae* 73 strains were used in this study (Zeise and von Tiedemann 2002a). SXM (Neumann and Dobinson 2003) contained sodium polypectate (2 g/liter), casein hydrolysate (4 g/liter), 1 M magnesium sulfate (2 ml/liter), 50× AspA solution (20 ml/liter), and 1,000× trace elements (1 ml/liter). CDA contained sucrose (30 g/liter), 1 M magnesium sulfate (2 ml/liter), 50× AspA solution (20 ml/liter), and ferrous sulfate (0.01 g/liter). Czapek Dox medium was inoculated with *V. longisporum* spores and incubated at 25°C for 10 days in darkness to generate fungal spore suspension stock. The spores were counted using a hemocytometer, adjusted to 1×10^6 spores/ml, and preserved as 30% glycerol stock at –80°C.

Xylem sap production.

Rapid-cycle rape (ACaacc) (Williams and Hill 1986) was used for xylem sap production as described previously (Singh et al. 2010). Uninfected rapeseed plants were harvested when they were 42 days old. For infection, rapeseed plants were inoculated with *V. longisporum* when they were 7 days old and xylem sap was extracted 35 dpi in correspondence to 42-day-old uninfected plants. Xylem sap was also extracted at an additional time point, from 35-day-old rapeseed plant and from corresponding infected rapeseed plants at 28 dpi. Xylem sap was filter-sterilized (0.2-µm size; Sarstedt AG, Nümbrecht, Germany) and stored at –20°C until further use.

Impact of xylem sap and xylem sap proteins from *B. napus* on fungal growth.

To discriminate between the influence of xylem sap and xylem sap proteins on fungal growth, macromolecules larger than 3 kDa were separated from the xylem sap by filtration during a centrifugation step at 4,000 × *g* for 45 min at 4°C (Vivaspin 6, 3000 MWCO; Sartorius, Goettingen, Germany). Then, 100 µl of a spore solution of 1.8×10^5 spores/ml was added to 900 µl of filtered xylem sap, incubated for 3 days in darkness at 22°C, and used to determine fungal DNA as described previously (Floerl et al. 2008). Xylem sap proteins were diluted to a concentration of 15 µg ml^{-1}. This protein solution (200 µl) and 100 µl of spore solution (1.8×10^5 spores per milliliter) were added to 700 µl of potato dextrose broth (Sigma, Steinheim, Germany) and were grown and used for fungal DNA measurements as described above. Protein concentration was determined using the Coomassie PLUS Assay Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.) following the manufacturer's instructions.

The impact of filtered xylem sap from rapeseed uninfected or infected with *V. longisporum* on fungal growth and development was analyzed by adding to SXM and CDA in a concentration of 1:7 and 1:70 in warm agar. *V. longisporum* was point inoculated with 5,000 spores in the center of the petri plate. SXM and CDA without added xylem sap and inoculated with *V. longisporum* were taken as controls. The plates were observed by light microscopy using an Olympus SZX12 binocular (Olympus, Hamburg, Germany) or a Zeiss Axiolab light microscope (Zeiss AG, Göttingen, Germany) until 10 days after inoculation. Images were taken using a Kappa PS30 digital camera and the Kappa ImageBase software (Kappa Opto-electronics GmbH, Gleichen, Germany). After 8 days of inoculation, for conidiospore quantification, the agar with the fungus colony was excised with the end of a disposable 1-ml pipette tip (1.0 cm in diameter) and vortexed for 30 min in 0.5 ml of physiological solution (Busch et al. 2003; Bussink and Osmani 1998). The number of spores based on three different plates

was counted from this solution using a hemocytometer. For the cultures used for 2D-PAGE and native gel electrophoresis, 1×10^6 conidia of *V. longisporum* were used to inoculate 40 ml of SXM. The fungi were incubated at 25°C for 5 days in darkness. After 5 days, sterile-filtered xylem sap, which contains approximately 40 to 50% less protein than nonfiltered xylem sap, from rapeseed plants uninfected or infected with *V. longisporum* was added in a concentration of 1:7 and incubated for 8 h.

Protein extraction.

Protein Extraction Reagent (Y-PER-S reagent; Pierce, Rockford, IL, U.S.A.) and protease inhibitor (Complete, protease inhibitor cocktail; Roche Diagnostics, Mannheim, Germany) were added to the powdered fungal mycelium and incubated for 10 min at room temperature. It was then vortexed and incubated on ice for 20 min, and then centrifuged at 4,500 rpm for 20 min at 4°C. The supernatant containing the soluble proteins was collected and stored at –20°C. For native protein extraction, cold 50 mM potassium phosphate buffer, pH 7.0 and protease inhibitor were added to the powdered mycelium. It was then centrifuged at 8000 rpm for 15 min at 4°C. The supernatant containing the native proteins was collected and stored at –20°C (Chary and Natvig 1989). Protein concentrations were determined using the BCA protein assay kit (Pierce) according to the supplier's manual. The protein used for 2D-PAGE experiments was purified by chloroform/methanol extraction (Wessel and Flugge 1984).

Isoelectric focusing and 2D-PAGE.

2D-PAGE was performed using an immobiline/polyacrylamide system described in detail by Valerius and coworkers (2007). Gels were stained with fluorescent RuBP (Lamanda et al. 2004). After scanning, RuBP-stained gels were additionally stained with silver nitrate (Blum et al. 1987) to visualize the protein spots for excision for trypsin digestion.

Image analysis.

Images of fluorescent-stained gels were acquired by scanning with the Typhoon 8600 laser scanner (GE Healthcare, Munich). Image analysis was performed using the PDQuest software (Bio-Rad Laboratories GmbH, Munich). By using the spot detection tool, discrete spots were marked by the software. In addition to the automatic spot detection and spot-matching procedures provided by the PDQuest, all gels and all match sets were carefully manually edited and optimized. The signal intensity of each spot was determined in pixel units (optical density) and normalized to the sum of the intensities of all the spots included in a standard gel; that is, the total spots intensity in all the gels is equal. Each matching analysis included six 2DE gels from control samples (three biological replicates, each with two technical replicates) and, similarly, six 2DE gels from samples treated with xylem sap.

Liquid chromatography MS/MS and protein identification.

Proteins within the excised polyacrylamide gel pieces of the regulated spots were in-gel digested with trypsin (Shevchenko et al. 1996). Sample preparation and liquid chromatography MS/MS analysis were performed as described (Valerius et al. 2007). The "peak list" was created with extract ms provided by the Xcalibur software package (BioworksBrowser 3.1; Thermo Electron Corp., San Jose, CA, U.S.A.). Identification was achieved through homology searching by the TurboSEQUEST analysis software (Eng et al. 1994) which correlated experimental spectra to theoretical spectra. The MS2 spectra with a total ion current higher than 10,000 were used to search for matches against a filamentous fungi database that consists of 10,082 entries of *N. crassa* (Galagan et al. 2003), 9,541 entries of *A. nidulans* (Galagan et al. 2005), 9,926 entries of *A. fumigatus* (Nierman et al. 2005), and 14,063 entries of *A. oryzae* (Machida et al. 2005), plus 180 entries of the most commonly appearing contaminants, including keratins or proteases, provided with the BioworksBrowser package using the TurboSEQUEST algorithm (Eng et al. 1994). In addition, the *V. dahliae* COGEME EST database containing 1,455 unisequences (Neumann and Dobinson 2003) was used. The MS2 spectrum of spot U1 was specifically used to search for matches against database of a *V. longisporum* cDNA library sequences containing approximately 2,000 unisequences (Singh et al. 2010). The search parameters included, based on the TurboSEQUEST algorithm, were i) precursor ion mass tolerance less than 1.4 amu, ii) fragment ion mass tolerance less than 1.0 amu, iii) up to three missed tryptic cleavages allowed, and iv) fixed cysteine modification by carboxyamidomethylation (plus 57.05 amu) and variable modification by methionine oxidation (plus 15.99 amu) and phosphorylation of serine, threonine, or tyrosine (plus 79.97 amu). In accordance with the criteria described by Link and associates (1999), matched peptide sequences of identified proteins had to pass the following: i) the cross-correlation scores (Xcorr) of matches must be greater than 2.0, 2.5, and 3.0 for peptide ions of charge state 1, 2, and 3, respectively; ii) Cn (delta cross-correlation score) values of the best peptide matches must be at least 0.4; and iii) the primary scores (Sp) must be at least 600. Protein identification required at least two different peptides matching these criteria. The degree of completeness of the b- and y-ion series for each TurboSEQUEST result was manually checked for every protein identified. The MS/MS spectra of all the protein spots was also used to verify the TurboSEQUEST results against the NCBI nonredundant database using PEAKS MS program (Bioinformatics Solutions Inc.) (Ma et al. 2003).

DNA isolation and Southern hybridization.

Primers are listed in Supplementary Table S1. Genomic DNA of *V. longisporum* was isolated from powdered mycelium as described (Kolar et al. 1988). Southern analyses were performed essentially as described earlier (Southern 1975). Gel-purified DNA fragments (QIAquick gel extraction kit; Qiagen, Hilden, Germany) consisting of a 400-bp fragment from catalase peroxidase was used as a probe and labeled using Amersham AlkPhos direct-labeling reagents (GE Healthcare).

Cloning and sequencing of the catalase peroxidase isogenes.

The two catalase peroxidase-encoding isogenes *cpeA1* and *cpeA2* were cloned from *Apa*I-digested genomic DNA. The DNA fragments which were identified by Southern hybridization were excised, purified (Qiagen QIAquick gel extraction kit), sequenced, and cloned into pJET1.2. Recombinant plasmids were isolated after colony PCR from transformants of DH5α by using the QIAprep spin miniprep kit (Qiagen). The sequences of the two isogenes were deposited with the EMBL European Nucleotide Archive. The *cpeA1* gene has accession number FR717672 and the isogene *cpeA2* has number FR717673.

In-gel catalase activity assay.

The native protein extracts were analyzed by nondenaturing Tris-glycine PAGE, using a modified protocol from Sambrook and associates (1989). The native protein (10 µg) was loaded in each well. Electrophoresis was carried out through a stacking gel (3%, pH 6.8) and a separating gel (7.5%, pH 8.0) in Tris (25 mM) and glycine (192 mM) buffer at pH 8.0. The electrophoresis was performed for about 5 h at 4°C and 100 V in a Mini-Protean III cell (Bio-Rad Laboratories GmbH).

To perform the catalase activity assay, the native gel was washed three times for 15 min with distilled H_2O, suspended in a solution of 0.01 ml of 30% H_2O_2 in 100 ml of H_2O, and gently rocked for 10 min. The H_2O_2 solution was then removed and the gel quickly rinsed in H_2O. A freshly prepared mixture of 30 ml each of 2% ferric chloride and 2% potassium ferricyanide, both in H_2O, was poured into a fresh staining pan, and the rinsed gel transferred to the ferricyanide mixture (Zou and Schrempf 2000). The gel tray was gently but steadily rocked by hand over a light box. As soon as a green color began to appear in the gel itself, the ferricyanide mixture was rapidly removed and replaced with water. The gel was washed twice with water. The gel was scanned using a GS 700 Imaging Densitometer (Bio-Rad Laboratories GmbH). To quantify the catalase activity in the different lanes, the Kodak Molecular Imaging 4.05 software (Eastman Kodak Company) was used.

Silencing *VlcpeA* in *V. longisporum*.

The vector pFANTAi4 (Krajaejun et al. 2007) used for the silencing strategy in *V. longisporum* was based on pSilent-1 vector (Nakayashiki 2005). The 495-bp *VlcpeA* fragment from the coding region was amplified using primers CatPerV-F and CatPerV-R and restricted by *Sna*B1 and *Hin*dIII for the sense strand. Primers CatPerH-F and CatPerH-R were used for the antisense strand and digested by *Apa*I and *Sph*1. Both fragments were inserted into pFANTAi4, resulting in a *VlcpeA* hairpin construct consisting of inverted repeats separated by a spacer. The entire silencing cassette was isolated as an *Xba*I fragment and inserted into the binary vector pPK2 (Covert et al. 2001) which contains the hygromycin B phosphotransferase gene as selection marker. The generated plasmid was pME3928 and was transformed into *V. longisporum* strain 43 by *Agrobacterium tumefaciens*-mediated transformation (Mullins et al. 2001). *A. tumefaciens* AGL-1 strain (Lazo et al. 1991) was transformed with plasmids using Luria-Bertani medium containing kanamycin (100 μg ml^{-1}). Filter paper (90 mm) (Sartorius) was used for co-cultivation of the *A. tumefaciens* culture and the *V. longisporum* spore suspension. The transformants were grown on medium lacking hygromycin B for five generations and then plated on selective medium supplemented with hygromycin B at 100 μg ml^{-1}, where only mitotically stable transformants could grow (Singh et al. 2010).

Stress tests.

V. longisporum wild-type and mutants strains were grown on CDA and on CDA containing different substances inducing oxidative and redox-membrane stress. Experiments were repeated three times. Plates were supplemented with H_2O_2 (2 and 5 mM), EtOH (3 and 4%), SDS (2.5 and 5 mM), or menadione (25 and 50 mM) for 4 days at 25°C.

Pathogenicity assay.

One-week-old *B. napus* seedlings were inoculated with *VlcpeAsm* and the wild type (*Vl* 43) at 1×10^6 spores/ml and mock inoculated with sterile tap water for 30 min by root-dipping inoculation. They were then transferred in pots with a sterile sand/soil (1:1) mixture. The plants were incubated in a climate chamber with 14 h of light at 23°C and 10 h of darkness at 20°C. The height of inoculated plants was measured and disease symptoms were scored (Eynck et al. 2007). Statistical data analyses were performed with a program (Statgraph Centurion XV, St. Louis).

Quantification of gene expression and *V. longisporum* DNA in planta by real-time PCR.

Hypocotyls, stems (5 to 6 cm from top of plant), and roots were separated from the infected rapeseed plants harvested at 14, 21, 28, and 35 dpi. In total, 20 plants were harvested from each treatment and fungal DNA and RNA was quantified using primers OLG 70 and OLG 71 for amplification of a specific *Verticillium* fragment encoding parts of the 5S rRNA (Eynck et al. 2007). *VlcpeA* was amplified using primers Cat2left and Cat2right and the histone gene using primers H2aleft and H2aright. Real-time PCR was carried out as described by Singh and associates (2010). The amount of DNA of *V. longisporum* was estimated by integration of a calibration curve using increasing amounts of genomic *V. longisporum* DNA from 1 to 625 pg in the analysis. The real-time RT-PCR data were analyzed using the $2-\Delta\Delta Ct$ relative quantification method (Livak and Schmittgen 2001), taking the gene for histone as reference for normalization.

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