The Cpc1 Regulator of the Cross-Pathway Control of Amino Acid Biosynthesis Is Required for Pathogenicity of the Vascular Pathogen *Verticillium longisporum*

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The plant-pathogenic fungus *Verticillium longisporum* is a causal agent of early senescence and ripening in cruciferous crops like *Brassica napus*. *Verticillium* wilts have become serious agricultural threats in recent decades. *Verticillium* species infect host plants through the roots and colonize xylem vessels of the host plant. The xylem fluid provides an environment with limited carbon sources and unbalanced amino acid supply, which requires *V. longisporum* to induce the cross-pathway control of amino acid biosynthesis. RNA-mediated gene silencing reduced the expression of the two *CPC1* isogenes (*VICPC1-1* and *VICPC1-2*) of the allodiploid *V. longisporum* up to 85%. *VICPC1* encodes the conserved transcription factor of the cross-pathway control. The silenced mutants were highly sensitive to amino-acid starvation, and the infected plants showed significantly fewer symptoms such as stunting or early senescence in oilseed rape plant infection assays. Consistently, deletion of single *CPC1* of the haploid *V. dahliae* resulted in strains that are sensitive to amino-acid starvation and cause strongly reduced symptoms in the plant-host tomato (*Solanum lycopersicum*). The allodiploid *V. longisporum* and the haploid *V. dahliae* are the first phytopathogenic fungi that were shown to require *CPC1* for infection and colonization of their respective host plants, oilseed rape and tomato.

With an increasing demand for biofuel, plants like maize, oilseed rape, or oil palm become more important to counteract the limitation of crude oil. In Europe, cultivation of oilseed rape has increased during the last decade. An important problem is the susceptibility toward pathogens such as *Verticillium longisporum*, a soil-borne vascular pathogen of oilseed rape *Brassica napus* (Karapapa et al. 1997; Zeise and von Tiedemann 2002a). Since the 1960s, wilts have become a major threat to oilseed crops in northern Europe, including Sweden, Germany, Poland, and also France (Karapapa et al. 1997; Zeise and von Tiedemann 2001, 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).

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V. longisporum infects its host through the root, colonizes the oilseed rape plant, and produces microsclerotia in the necrotrophic stage (Eynck et al. 2007; Zhou et al. 2006). Microsclerotia rest in the soil and can live for several years in this dormant form (Heale and Karapapa 1999; Schnathorst 1981). *V. longisporum* directly penetrate the epidermal cells in the root-hair zone for infection (Eynck et al. 2007). The fungus grows inter- and intracellularly in the root cortex toward the central cylinder and colonizes the xylem vessels. Whereas several xylem vessels are filled with mycelium and conidia, most of the nearby vessels remain without infection. With progression of ripeness and senescence of the diseased plants the fungus starts to generate microsclerotia. The resting structures are released into the soil with the residues of plant material after harvesting and can again infect healthy plants. For most parts of its life in the host plant, *V. longisporum* is restricted in the vascular system of the oilseed rape *Brassica napus* (Eynck et al. 2007).

The xylem is responsible for the transport of water and soluble mineral nutrients from the roots throughout the plant. Xylem sap, which has been analyzed in several studies (Floerl et al. 2012; Iven et al. 2012; Ratzinger et al. 2009; Singh et al. 2010, 2012), is not rich in nutrients and can contain a number of organic acids (Lopez-Millan et al. 2000). They include low amounts of amino acids (Singh et al. 2010). Glutamic acid, aspartic acid, and serine are the most abundant amino acids in leaf mesophyll cells of *B. napus*, in which the concentration of the aromatic amino acids ranges from 1.2 to 2.1% of the total amino acids under low nitrogen supply conditions (Singh et al. 2010; Tilsner et al. 2005).

Fungi, which are usually prototrophic, prefer to acquire amino acids from their environment by their uptake systems. External amino-acid supply reduces the cellular biosynthesis by multiple enzyme feedback mechanisms. The basal expression of genes for amino-acid biosynthesis is sufficient to provide enough amino acids for cellular translation in a minimal medium, but an imbalanced amino-acid diet requires increased transcription of amino-acid biosynthetic genes. This is provided by a transcriptional network, which is called cross-pathway control for filamentous fungi or general control of amino acids in yeast (Carsiotis and Jones 1974; Carsiotis et al. 1974; Hinnebusch 1986). Numerous genes are activated (Hinnebusch and Natarajan 2002; Kleinschmidt et al. 2005; Natarajan et al. 2001) by a central conserved *c-jun*-like transcriptional activator, which is Gcn4 (general control non-derepressed) in *Saccharomyces cerevisiae* (Hinnebusch 1984; Mösch et al. 1991) and which is conserved to ATF4 in humans (Kilberg et al. 2009; Shan et al. 2009). In filamentous fungi, CpcA (cross-

pathway control) is the counterpart in *Aspergillus* spp. (Eckert et al. 2000; Hoffmann et al. 2001; Krappmann et al. 2004; Wanke et al. 1997) or Cpc1 in *Neurospora crassa* (Paluh et al. 1988).

Protein levels of the yeast Gcn4 activator are low when amino acids are abundant and are increased by enhanced translation and increased protein stability during amino-acid starvation (Albrecht et al. 1998). Filamentous fungi possess an additional transcriptional autoactivation that is not present in yeast. *Aspergillus nidulans* CpcA binds to CPRE (cross-pathway control recognition elements) that locate not only within the promoter region of target genes but also within its own CpcA promoter, resulting in increased transcript levels for the activator (Hoffmann et al. 2001).

A requirement of CpcA for pathogenicity of the human opportunistic pathogen *A. fumigatus* had been demonstrated (Krappmann et al. 2004) but the role of CpcA homologs in plant-pathogenic fungi is unclear. Transcript levels of the CpcA homolog *CPC1* gene of the plant-pathogen *V. longisporum* increased when cultivated in the presence of plant xylem sap and suggested an imbalanced amino-acid supply (Singh et al. 2010). In this study, we examined whether the *CPC1* genes of the *Verticillium* cross-pathway control activator play a role in plant pathogenicity.

RESULTS

V. longisporum carries two *CPC1* regulatory isogenes.

Sequencing of the cDNA library of *V. longisporum* V143 revealed the presence of two transcripts for the cross-pathway

transcriptional regulator VICpc1. The corresponding isogenes that had originally been named *CPCA* (Singh et al. 2010) were renamed (*VICPC1-1* and *VICPC1-2*) to refer to the nomenclature of related *Verticillium* species. Both *CPC1* chromosomal loci of *V. longisporum* were compared with the corresponding genes of haploid *V. dahliae* or *V. albo-atrum* (Fig. 1). Primer pair *CPC1Seq-L/CPC1Seq-R* (Table 1) was designed according to the *CPC1* locus (VDAG_10113) from the Broad Institute (Cambridge, MA, U.S.A.) *V. dahliae* genome assembly (Klosterman et al. 2011). Sequencing of the resulting PCR products from *V. dahliae* and *V. albo-atrum* revealed unique single sequences of 725 bp for *VdCPC1* or 723 bp for *VaCPC1*. In contrast, cloning and sequencing of the polymerase chain reaction (PCR) products from *V. longisporum* revealed two distinct sequences for *VICPC1*, corresponding to isogenes *VICPC1-1* and *VICPC1-2*, with lengths of 725 or 723 bp similar to *VdCPC1* and *VaCPC1*, respectively (Supplementary Fig. S1). In total, there are more than 60 different nucleotides between all four sequences. The deduced open reading frames (ORF) of all four genes consist of two exons, each, of a length of 13 or 653 base pairs (bp), separated by single introns that correspond to two related groups. The introns of *VdCPC1* and *VICPC1-1* have a length of 59 bp, whereas *VaCPC1* and the second isogene *VICPC1-2* share a length of 57 bp.

In contrast to the yeast *GCN4* gene, which is not significantly autoregulated (Albrecht et al. 1998), all *Verticillium* *CPC1* genes carry one TGACTCA CPRE in their own promoter 985 bp upstream of the ORF (Fig. 1A). Conserved CPRE consist of a palindromic sequence element constituted by a central CG pair that is flanked by TGA halvesites (Oliphant et al. 1989).

Fig. 1. The *CPC1* loci of *Verticillium* species. **A**, The black dot indicates the putative cross-pathway control recognition element (CPRE) with the sequence 5'-TGAGTCA-3'. The conserved leucine zipper (bZIP) region at the C-terminus of *CPC1* is in black. The upstream open reading frame (uORF) in the 5' leader region is represented by a white box. **B**, uORF alignments of the deduced amino-acid sequences in comparison to corresponding uORF of *CPCA/CPC1* genes of *Aspergillus fumigatus*, *A. nidulans*, *A. niger*, or *Neurospora crassa*. White letters in black boxes represent conserved and gray boxes partially conserved amino-acid residues. **C**, Alignments of the C-terminal leucine zipper regions of the same genes as in **B**. The bZIP transcription factors basic domain signature (InterProScan PS00036) is indicated by a black line. **D**, Southern hybridization analysis of the single copy *V. albo-atrum* or *V. dahliae* *CPC1* genes in comparison to two isogenes in *V. longisporum*. Genomic DNA was digested with *Bam*HI with a 456-bp *VICPC1* sequence as probe.

CPRE were also found in various promoters of *CPC1* homologs and suggest binding by the activator and transcriptional autoregulation (Hoffmann et al. 2001; Krappmann et al. 2004; Tian et al. 2007). The earlier finding that *VICPC1* mRNAs are induced during infection of *B. napus* or *Arabidopsis thaliana* (Singh et al. 2010) might be due to an induced cross-pathway control.

A small upstream ORF (uORF), located at –424 to –304 bp upstream of the translational start codon, is almost identical in all *Verticillium CPC1* genes (Fig. 1B). uORF are part of the translational control of fungal *CPC1* genes and the deduced primary sequence of 39 amino acids of the *Verticillium* uORF shows 57% identities to the corresponding uORF of *N. crassa CPC1* (Paluh et al. 1988). This suggests that, in addition to the transcriptional autoregulation, *Verticillium CPC1* transcripts are also regulated at the level of translation.

The C-terminal basic leucine zipper region mediates dimerization and DNA binding (Struhl et al. 1988) and is highly conserved in filamentous fungi. The sequences of *VICPC1-1* and *VdCPC1* are 100% identical, whereas *VICPC1-2* and *VaCPC1* are 97% similar to this sequence and differ in two amino acids. The corresponding genes of *N. crassa* and various *Aspergillus* spp. vary between 74 and 47% identical amino acid residues, respectively (Fig. 1C). An overall comparison of the four *CPC1* genes from *V. longisporum*, *V. dahliae*, and *V. albo-atrum* shows specific small nucleotide variations that are shared between *VdCPC1* and the isogene *VICPC1-1* and are distinct to the gene pair *V. albo-atrum VaCPC1* and the other isogene *VICPC1-2*. Southern hybridization confirmed the presence of two *CPC1* isogenes in *V. longisporum*, in contrast to single genes in *V. dahliae* or *V. albo-atrum* (Fig. 1D). This and a phylogenetic analysis (Fig. 2) further support that the *V. longisporum* genome is the result of a hybridization event between two distinct but related parental *Verticillium* species (Inderbitzin et al. 2011; Tran et al. 2013). All *Verticillium CPC1* genes group together with other pathogenic fungi such as *Colletotrichum* or

Fusarium spp. and separate them from saprophytes such as *Aspergillus* spp. or single-cell yeast *S. cerevisiae* (Fig. 2).

***VICPC1*-silenced mutants grow like wild type on a minimal medium but are strongly reduced in growth under amino acid starvation conditions.**

The presence of two *CPC1* isogenes of *V. longisporum* required RNA silencing (Nakayashiki 2005) to examine the role of the cross-pathway control regulatory gene for the fungal life cycle. A hairpin double-stranded RNA approach combined with the hygromycin B marker selection was applied for RNA silencing of *VICPC1-1* and *VICPC1-2*. The copy number of integrated hairpin constructs was determined by Southern hybridization (Supplementary Fig. S2A). The tested transformants showed one to two copies of the silencing construct in the genome. The extent of silencing was determined for hygromycin B-resistant transformants by reverse transcription (RT)-PCR by the ratio of the amplification of *VICPC1* between the respective transformants and the unsilenced wild type and varied considerably among different transformants. Transformants with a reduced activity of less than 20% were further analyzed (Fig. 3). One day after inoculation on minimal medium (Czapek Dox agar [CDA]), the silenced *V. longisporum* strain could grow like the wild type, and both strains showed increased mycelial growth and first conidia after 2 days of cultivation (data not shown). Radial growth of both strains was similar for 15 days (Fig. 3; Supplementary Fig. S3). Therefore, growth under minimal-nutrition conditions without any amino-acid supply is like wild type when *CPC1* transcription is reduced to less than 20% at the RNA level in the silenced mutants.

In contrast, cultivation on minimal medium supplemented with the amino-acid analog 5-methyl-tryptophan (5-MT), which acts as false feedback inhibitor and causes tryptophan starvation (Schürch et al. 1974), resulted in a significant reduction in the growth rate. After 2 days, wild type showed a mycelial net with few conidia, whereas the *CPC1*-silenced *Verticil-*

Table 1. Primers used in this study

Primer	Sequence (5'–3')	Reference
OLG70	CAGCGAAACGCGATATGTAG (20)	Eynck et al. 2007
OLG71	GGCTTGATGGGGTTTAGA (19)	
H2A-RT1	CCCGTGACAACAAGAAGACTCG (22)	This study
H2A-RT2	GCAGGAAAGAAAAGCCAAAACC (22)	
<i>CPC1</i> RT1	CCCATCGTTGTTGAGAATCCAG (22)	This study
<i>CPC1</i> RT2	GACTGACCCAAAGCCAAAGGTCT (22)	
Probe <i>CPC1</i> -L	CGGCCCTCAAAAATTGTTCC (20)	This study
Probe <i>CPC1</i> -R	GACTTAAATTGAGATATCCG (20)	
<i>CPC1</i> Seq-L	ATGGCTTCCCTCCAGTTCC (19)	This study
<i>CPC1</i> Seq-R	CTAGTCGCTGGTTGACTGACC (21)	
<i>VdCPC1</i> KOF1-L (<i>PacI</i>)	GGGTTAATTGGCTATCGAGCATACGAAGAAC (31)	This study
<i>VdCPC1</i> KOF1-R (<i>SacI</i>)	GGGGAGCTCCCATCCATATATTCTGGCGATT (31)	
<i>VdCPC1</i> KOF2-L (<i>XbaI</i>)	GGGTCTAGAGAGGTTTCTACATTCTGACTTT (32)	This study
<i>VdCPC1</i> KOF2-R (<i>HindIII</i>)	GGGAAGCTTACACTAAGGAATAAGGCCTAAGATT (34)	
<i>VdCPC1</i> PCR-L (P1)	ATGGCTTCCCTCCAGTTCC (19)	This study
<i>VdCPC1</i> PCR-R (P2)	GTATTAACAACTTGTGCCTCG (20)	
<i>VdCPC1</i> KOProbe-L	TCCCAACAACCTGCCTAAAC (20)	This study
<i>VdCPC1</i> KOProbe-R	AGAGTCCAGAACAGCACGGT (20)	
<i>VICPC1</i> gate-L	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAACATCGCGGATTTTCG (50)	This study
<i>VICPC1</i> gate-R	GGGGACCACTTTGTACAAGAAAGCTGGGCTCGCCAGAGTGTGCGAGC (49)	
Arg6RT1L	TTTATTGCTCGGCTTGTGGTTC (22)	This study
Arg6RT1R	CTTCTGCCCTTCGCTTCTCTTC (22)	
Leu4RT1L	ATGCTCAAGGACCCCACTCAAGA (22)	This study
Leu4RT1R	GCCACTTCTCATCGCCACTCAT (22)	
Lys1RT1L	CCGAGATTGTTGAGAGCGACAT (22)	This study
Lys1RT1R	GGTGTAGATGGGGACAGGGTTG (22)	
Trp5RT1L	TGAGTTACGGCGAGGAGAGG (20)	This study
Trp5RT1R	GCGATAAGGGGAACATAGGAGAG (23)	

lium longisporum strain (VICPC1sm) formed short hyphae without conidia (data not shown). Radial growth of the silenced mutant was significantly retarded during 5-MT induced starvation in comparison with the wild type, when the colony diameter was determined after 15 days. Real-time PCR revealed that the wild type shows a significant increase in *CPC1* transcription during starvation, whereas the silenced mutant is unable to achieve even basal wild-type *CPC1* levels of nonstarvation conditions (Fig. 3). Methionine starvation induced by 2 mM methionine sulfoximine, which inhibits glutamine synthetase required for the biosynthesis of glutamine, confirmed these results and also resulted in a specific growth reduction of the silenced strain.

The single *CPC1* gene of *V. dahliae* was deleted as a control to evaluate the impact of the residual activity of less than 20% in the silenced *V. longisporum* mutant under nonstarvation and

starvation condition. The *V. dahliae* deletion mutant (*VdΔCPC1*) was able to grow on minimal medium with a similar colony morphology as wild type (Fig. 3). Growth of the mutant is not affected when the carbon sources were exchanged for cultivation by standard carbon sources like sucrose, glucose, or fructose, but there is a slight growth reduction when plant-derived carbon sources like mannose, lignin, cellulose, or xylan were provided for growth (Fig. 4). In contrast, adding 5-MT to the medium inhibited growth of *VdΔCPC1* mutant completely, and even germinating spores during the initial phase could not be monitored. Real-time PCR showed that *CPC1* transcription is absent in the *VdΔCPC1* deletion strain, whereas the wild type similar to *V. longisporum* increases transcription significantly during starvation (Fig. 3C).

Previous studies in the plant pathogen *Fusarium fujikuroi* revealed the cross-pathway controlled expression of specific

Fig. 2. Phylogenetic analysis for deduced Cpc1 proteins of three *Verticillium* species in comparison with the orthologs of other filamentous fungi. The virulent hybrid pathogen *Verticillium longisporum* possesses two isogenes encoding deduced Cpc1-1 or Cpc1-2, which are similar to *V. dahliae* and *V. albo-atrum*, respectively. Other Cpc1-like proteins were deduced from the encoding genes with the provided accession numbers (in brackets), which were extracted from GenBank. Thick black lines indicate that the corresponding genes have been verified as cross-pathway regulatory genes. Two bootstrap values smaller than 70 are indicated in brackets. Vl = *V. longisporum*, Vd = *V. dahliae*, Va = *V. albo-atrum*, Cg = *Colletotrichum graminicola*, Ch = *C. higginsianum*, Ff = *Fusarium fujikuroi*, Fo = *F. oxysporum*, Fg = *F. graminearum*, Pt-r = *Pyrenophora tritici-repentis*, Lm = *Leptosphaeria maculans*, Bb = *Beauveria bassiana*, Ma = *Metarhizium anisopliae*, Mo = *Magnaporthe oryzae*, Nc = *Neurospora crassa*, Sm = *Sordaria macrospora*, Cp = *Cryphonectria parasitica*, Pm = *Penicillium marneffei*, Af = *Aspergillus fumigatus*, Ani = *A. niger*, An = *A. nidulans*, Ca = *Candida albicans*, and Sc = *Saccharomyces cerevisiae*.

genes involved in amino-acid biosynthesis (Schönig et al. 2009). We investigated whether *CPC1* of *Verticillium* activates the corresponding genes, including *LYS1* for lysine, *LEU4* for leucine, *ARG6* for arginine, and *TRP5* for tryptophan biosynthesis (Supplementary Fig. S4). Expression of all four genes is regulated by *CPC1*. Whereas wild type or the complementation strain showed significant increases of target-gene expres-

sion during starvation, there was no detectable expression of these genes in the Δ *CPC1* deletion strain. Fungal spore formation was similar for the Δ *CPC1* mutant and the wild-type strain, resulting in comparable amounts of spores and the same capability of the spores to germinate, based on the number of germinated spores 4, 6, and 8 h after inoculation (Supplementary Fig. S5).

Fig. 3. Influence of 5-methyl-tryptophan (5-MT) on *Verticillium longisporum* and *V. dahliae* growth. **A**, Colonies of *V. longisporum*, *Vl* Δ *CPC1*-silenced mutants, *V. dahliae*, and *V. dahliae* knockout mutant in the presence or absence of amino-acid limitation. Colonies were grown on minimal medium (CDA) after 9 days of inoculation. Amino-acid starvation was induced by supplementation with 5 mM 5-MT. **B**, The *V. longisporum* wild type (*Vl*-wt) and the silenced mutant (*Vl* Δ *CPC1*sm), as well as *V. dahliae* wild type (*Vd*-wt), and *V. dahliae* knockout mutant (*Vd* Δ *CPC1*) were plated on CDA containing 5 mM 5-MT. Diameters of colonies were measured at indicated days after inoculation. Error bars represent standard deviation ($n = 5$). **C**, Relative expression of *Vl**CPC1* and *Vd**CPC1* transcripts in the *V. longisporum* wild type and the silenced mutant *Vl* Δ *CPC1*sm as well as *V. dahliae* wild type and the deletion mutant *Vd* Δ *CPC1*, respectively, under inducing and noninducing conditions measured by quantitative real-time polymerase chain reaction. The target cDNAs were normalized to the transcripts of the housekeeping gene *H2A* histone. Significant differences between the wild type and mutants are illustrated by asterisks (** and * indicate $P \leq 0.01$ and 0.05 , respectively).

Fig. 4. Growth of the *Verticillium dahliae* wild type (*Vd-wt*) or the *VdCPC1* deletion mutant (*VdΔCPC1*) on different carbon sources. Czapek Dox agar was used for growth tests with either sucrose or the indicated carbon sources. Diameters of colonies were measured and compared between the wild type and the mutant 10 days after inoculation.

Disease symptoms decrease significantly when plants are infected with the *CPC1* mutant fungi.

We evaluated whether the cross-pathway regulator protein Cpc1 is contributing to plant pathogenicity. The silenced *V.*

longisporum mutant (*VICPC1sm*), which provides a residual transcriptional *CPC1* activity, as well as the *V. dahliae* deletion mutant (*VdΔCPC1*), lacking any Cpc1 protein, were analyzed in their respective hosts for the induction of fungal-caused dis-

Fig. 5. Assessment of pathogenicity of the *VICPC1*-silenced mutant in the plant host *Brassica napus*. **A**, *Verticillium longisporum* induced stunting of *B. napus* plants after infection. Each point represents the average height of 30 oilseed rape plants, either uninfected (mock inoculated with tap water) or infected with the wild-type fungus (*VI-wt*) or the *VICPC1*-silenced mutant (*VICPC1sm*) or a nonsilenced mutant (unsilenced). Plants were measured at indicated days postinfection (dpi) until a significantly marked degree of the disease was visible at 35 dpi. **B**, Examples of uninfected or infected *B. napus* plants at 35 dpi. **C**, Assessment of disease development by scoring for disease symptoms according to Eynck and associates (2007). Plants were scored for disease symptoms at the indicated dpi. The data represent average values $\pm$ standard deviations of 30 experimental replicates. Different letters indicate significant differences at $P < 0.05$.

ease symptoms, such as stunted host plants or signs of early senescence (Figs. 5 and 6).

The silenced *V. longisporum* was analyzed in 1-week-old oilseed rape *Brassica napus* that was infected by root-dip inocu-

lation. Wild-type infection resulted in clearly visible disease symptoms after 21 days postinfection (dpi), when the disease further accelerates and becomes even more distinctive at later time points (Fig. 5A and B). Plant height was measured weekly

Fig. 6. Assessment of pathogenicity of the *VdCPC1* deletion mutant. **A**, Tomato infection assay. Representative *Solanum lycopersicum* plants were shown at 21 dpi. **B**, Fungal regrowth test. Image of fungal colonies growing on PDA plates from tomato stem pieces infected with the *Verticillium dahliae* wild type or the *VdCPC1* knockout mutant. Stem pieces of mock plants were served as control. **C**, Assessment of stunting symptom of *S. lycopersicum* due to *V. dahliae* infection. The height of tomato plants infected with the wild type (*Vd-wt*) and the mutant *VdΔCPC1* was measured at 7, 14, 21, 28 and 35 dpi. For comparison, the height of tomato plants inoculated with tap water (mock) was used as control. The plants show symptoms after 21 dpi. The data represent average values $\pm$ standard deviations of 20 experimental replicates. Different letters indicate significant differences at $P < 0.05$.

after inoculation, and infected oilseed rape plants were scored for senescence disease symptoms by several parameters, including the monitoring of yellowing and death of the leaves. Disease scores from 1 to 9 represent the range between asymptomatic to dead plants (Eynck et al. 2007). Disease symptoms had to be distinguished from natural senescence and, therefore, yellowing of leaves in the uninfected mock plants was monitored for comparison. The silenced mutant (*VICPC1sm*) showed already-reduced disease scores in comparison with the *V. longisporum* wild type in the initial phase of the disease at 21 dpi (Fig. 5C). In contrast to the wild type, the silenced mutant showed no acceleration, but rather, a slowdown of the disease, which is reflected in the lower slope in the relative disease scores between 21 and 35 dpi. In comparison, an unsilenced strain showed an infection pattern similar to *V. longisporum* wild type. Further analysis showed pathogenicity related changes in plant leaves. At 21 days after infection, the *CPC1*-silenced mutant showed, on average, fewer leaves than wild type-infected plants with a larger leaf size. In addition, infected plants showed more leaves per node, resulting in an increased verticillate shape of the plant (Supplementary Fig. S6).

The reduction of pathogenicity of the silenced mutant of *V. longisporum* resulting in a mild version of the disease correlates with the residual reduced *CPC1* transcriptional activity.

Fig. 7. Infection assay and determination of *Verticillium longisporum* and *V. dahliae* DNA concentration in hypocotyls of infected plants after 35 days postinfection (dpi). Fungal DNA was measured with real-time polymerase chain reaction. **A**, Roots, hypocotyls, and leaves of oilseed rape *Brassica napus* infected with the *VICPC1*-silenced mutant (*VICPC1sm*) and *V. longisporum* wild type (*VI-wt*). **B**, Roots, hypocotyls, and leaves of tomato *Solanum lycopersicum* infected with *VdCPC1* knockout mutant (*VdΔCPC1*) and *V. dahliae* wild type (*Vd-wt*) at 35 dpi. The data represent average values $\pm$ standard deviations of five experimental replicates. The tap water-inoculated plants (mock) as a control did not show the presence of any *V. longisporum* or *V. dahliae* DNA per gram of fresh weight of plant tissue. Significant differences between the wild type and mutants are illustrated by asterisks, which indicate $P \leq 0.05$.

For a more comprehensive picture of the role of the *Cpc1* protein, deletion mutants of *V. dahliae* lacking any *CPC1* transcription were analyzed in tomato plants. *Solanum lycopersicum* was root-dipped for infection with *V. dahliae* Δ *CPC1* mutant or wild type. The *V. dahliae* wild type caused the typical symptoms on tomato, including stunting and chlorosis. Similar to the infection of the silenced *V. longisporum* strain, the *CPC1* deletion mutant (*VdΔCPC1*) showed strongly reduced pathogenicity with less stunting symptoms (Fig. 6A). Both data sets support the hypothesis that the gene for the *Cpc1* regulator of the cross-pathway control of amino-acid biosynthesis is required for *Verticillium* species as vascular plant pathogens to cause plant disease.

The plant vascular system might provide amino-acid starvation conditions for the fungi. Therefore, fungal colonization of the wild type and the mutant in planta were monitored and compared. Fungal DNA of mutants and wild types of *V. longisporum* in *B. napus* or *V. dahliae* in tomato were quantified in the plant tissues by real-time PCR. All wild types and mutant fungi were monitored, with low and similar amounts of fungal DNA in roots of the corresponding infected plants, suggesting that this initial phase of colonization does not require an intact cross-pathway control (Fig. 7). Silencing of the two *VICPC1* isogenes was still stable in the oilseed rape after 35 dpi. Highest concentrations of fungal wild-type DNAs were normally present in the hypocotyls of infected plants. *CPC1* knockdowns or knockouts showed significantly less fungal *VICPC1sm* or *VdΔCPC1* DNA when the infected hypocotyls were compared with wild types after 35 dpi. The DNA content of *VdΔCPC1* in tomato hypocotyls was reduced by 40% and the DNA content of *VICPC1sm* in *B. napus* hypocotyls was even reduced by 80% (Fig. 7).

These data demonstrate that the reduced pathogenicity of the *CPC1* mutant strains is not due to a defect in the initial root colonization of the plant but is due to reduced growth in the plant hypocotyls. This suggests that the fungi encounter amino acid-starvation conditions in the hypocotyls, which have to be counteracted by a functional cross-pathway control of amino acids.

DISCUSSION

It was anticipated that, for plant-pathogenic fungi, a knock-down or knockout of *CPC1* might reduce pathogenicity (Shen and Ebbolle 1997), but this hypothesis was not verified so far. Knockdowns in *V. longisporum* and knockouts in *V. dahliae* represent the first examples of phytopathogenic fungi, which require the amino-acid regulatory gene *CPC1* for infection and colonization of their respective host plants. This situation is similar to human fungal pathogens like *A. fumigatus*, in which mutants impaired in the cross-pathway control system had strongly reduced pathogenicity, and the regulatory gene *CpcA* contributes in effectively establishing pulmonary aspergillosis (Abad et al. 2010; Krappmann et al. 2004; Sasse et al. 2008).

The cross-pathway control is a complex global regulatory network that regulates the synthesis of amino acids in numerous fungi. The cross-pathway control has been the subject of examination in several filamentous fungi, including *A. nidulans* (Hoffmann et al. 2001; Piotrowska et al. 1980), *A. niger* (Wanke et al. 1997), *A. fumigatus* (Krappmann et al. 2004; Sasse et al. 2008), *Candida albicans* (Tripathi et al. 2002), *Magnaporthe grisea* (Shen and Ebbolle 1997), *N. crassa* (Carsiotis et al. 1974; Paluh et al. 1988), and *F. fujikuroi* (Schönig et al. 2009). In yeast, the same system was named general control (Hinnebusch 1984; Natarajan et al. 2001). Amino acid-starvation conditions activate this system, resulting in derepression of the cross-pathway control gene *GCN4* in yeast or *CpcA/CpcI* in filamentous fungi. In yeast, the amount of *Gcn4* in the

cell is regulated primarily by a translational control (Albrecht et al. 1998; Braus 1991; Irniger and Braus 2003). There is an additional amino acid starvation-induced transcriptional autoactivation present in filamentous fungi that had been originally described in *A. nidulans* (Hoffmann et al. 2001). The presence of the corresponding Cpc1 upstream DNA-binding element in the promoters of the *Verticillium CPC1* regulatory genes and the finding that a bradytrophic aromatic amino-acid mutant showed increased *VICPC1* transcription (Singh et al. 2010) suggests that this transcriptional autocontrol mechanism is conserved in vascular plant pathogens.

In addition to its role as amino-acid regulator, the cross-pathway control regulator Gcn4/CpcA/Cpc1 has various distinct morphological functions in fungi. Gcn4 controls starvation-induced adhesive and pseudohyphal growth in the yeast *S. cerevisiae* (Braus et al. 2003; Herzog et al. 2011; Valerius et al. 2007). Similarly, histidine starvation controls the homolog CaGcn4 of *C. albicans*, which is involved in morphogenetic responses (Tripathi et al. 2002). In *A. nidulans*, the amino acid starvation-induced cross-pathway control can block fruiting body formation as a complex cellular differentiation process (Hoffmann et al. 2000).

During colonization and infection, *V. longisporum* occupies the xylem vessels of the oilseed crop *B. napus*. The composition of xylem sap consists of water, inorganic compounds, organic acids, and amino acids (Kehr et al. 2005; Sakuta et al. 1998). A reduced activity of the *Vlaro2* isogenes encoding the aromatic amino-acid biosynthetic enzyme chorismate synthase resulted in reduced pathogenicity and was the first hint that the xylem sap does not provide an appropriate composition of aromatic amino acids for fungal growth (Singh et al. 2010). In this study, we present a *V. longisporum* mutant strain that lacks *CPC1* but has an intact basal transcription of amino-acid biosynthetic genes that is sufficient to grow on plates in the absence of any amino acid. The strain is deficient in the transcriptional control of amino-acid biosynthetic genes, which allows an increased transcription during an imbalanced supply of amino acids. This mutant strain is unable to efficiently colonize the plant host hypocotyl, which results in a significantly reduced pathogenicity. *VICPC1* seems to aid in the growth of the fungus in the xylem vessels, in which amino acids are scarce. Acquiring nutrient sources from its host is crucial for a fungal pathogen (Gibson et al. 2011; King et al. 2011). The mutant strain does not reach the upper parts of the plants in later phases of infection. Consistently, different plant tissues, especially hypocotyls, stems, and leaves showed significantly reduced amounts of DNA for the mutant as compared with the *V. longisporum* wild-type strain. The mutant strain is able to infect the plant but is not capable of successfully growing in the upper parts of the host (Fig. 7). Based on our results, this might be due to a limited nutritional supply of the mutant strain with appropriate amounts of amino acids. Accordingly, plants infected with the silenced mutant are considerably stronger and look healthier than plants infected with the wild-type strain.

B. napus xylem sap probably provides not only nutrition to *V. longisporum* but may also contain certain factors, such as phytohormones, for plant defense against *V. longisporum*. Xylem sap is the medium for cross-talk between the pathogen, *V. longisporum*, and its host, *B. napus*. Infection of model plant *Arabi-*

dopsis thaliana by *V. longisporum* induces, in roots, specific tryptophan-derived metabolites, such as indole-3-carboxylic acid and the phytoalexin camalexin. Camalexin is an efficient inhibitor of *Verticillium* growth (Iven et al. 2012; Ratzinger et al. 2009; Singh et al. 2010, 2012). These results support the theory that *CPC1* of *V. longisporum* and *V. dahliae* might also have an influence on secondary metabolism, which might interfere with the communication between fungus and its host. Several researchers describe an impact of the cross-pathway regulator on secondary metabolism (Abad et al. 2010; Elliott et al. 2011). In *A. nidulans*, *CpcA* suppresses the biosynthesis of penicillin and other secondary metabolites under amino-acid starvation. For *Leptosphaeria maculans*, silencing of the *CpcA* homolog gene increases, either directly or indirectly, the production of the toxin sirodesmin. In contrast, Cpc1 of *F. fujikuroi* is not involved in the glutamine synthetase-dependant regulation of secondary metabolism (Schönig et al. 2009). One possible explanation why the *CPC1* genes of *V. longisporum* or *V. dahliae* contribute to plant pathogenicity is that the cross-pathway system allows counteracting an unbalanced amino acid supply. The amino-acid composition of the xylem fluid might correspond to an unequal amino-acid diet for fungal nutrition. Another explanation is a disturbed communication between fungus and host due to a role of *CPC1* in the control of secondary metabolism. Finally, *CPC1* might also be important for both the nutrition in the plant and the correct interplay in exchanging secondary metabolite signals between fungus and host-plant.

MATERIALS AND METHODS

Strains, media, and growth conditions.

All *Verticillium* strains used are listed in Table 2. The simulated xylem fluid medium (SXM) (Neumann and Dobinson 2003) contained sodium polypectate (2 g per liter), casein hydrolysate (4 g per liter), 1 M magnesium sulphate (2 ml per liter), 50× AspA solution (20 ml per liter) and 1,000× trace elements (1 ml per liter). CDA contained sucrose (30 g per liter), 1 M magnesium sulphate (2 ml per liter), 50× AspA solution (20 ml per liter), and ferrous sulphate (0.01 g per liter). Potato dextrose agar was purchased from Sigma Aldrich and was used according to the manufacturer's description. Czapek Dox broth medium was inoculated with *V. longisporum* or *V. dahliae* spores and was incubated at 25°C for 10 days in the dark to generate fungal spore suspension stock. The spores were counted using a Thoma-chamber (Optik Labor, Lancing, U.K.), were adjusted to 1×10^6 spores per milliliter, and were preserved as 30% glycerol stock at -80°C.

Escherichia coli DH5α cells were grown at 37°C for propagation of plasmids in Luria-Bertani (LB) broth or on LB plates supplemented with ampicillin (100 µg per milliliter), phleomycin (50 µg per milliliter), or kanamycin (100 µg per milliliter). The *Agrobacterium tumefaciens* AGL-1 strain (Lazo et al. 1991) was used for plasmid maintenance for transformation of *V. longisporum* in LB medium supplemented with kanamycin (100 µg per milliliter) at 28°C.

Southern hybridization and DNA isolation.

Southern hybridization analyses (Southern 1975) were performed with genomic DNA of *V. longisporum* extracted from

Table 2. *Verticillium* isolates used in this study

Isolate	Species	Host	Geographic origin	Reference
Vl43	<i>Verticillium longisporum</i>	<i>Brassica napus</i> (oilseed rape)	Mecklenburg/Germany	Zeise and von Tiedermann, 2001
Vd73	<i>V. dahliae</i>	<i>Linum usitatissimum</i> (linseed)	Mecklenburg/Germany	Zeise and von Tiedermann, 2001
VdJR2	<i>V. dahliae</i>	<i>Solanum lycopersicum</i> (tomato)	Canada	Fradin et al., 2009
Va1	<i>V. albo-atrum</i>	<i>Solanum tuberosum</i> (potato)	Wisconsin/USA	Zeise and von Tiedermann, 2002b

powdered mycelium (Kolar et al. 1988), using primers Probe-*CPC1*-L and Probe-*CPC1*-R, described in Table 1, for PCR. The 521-bp PCR fragment of the *CPC1* gene was purified with QIAquick gel extraction kit (Qiagen, Hilden, Germany) and was labeled as probe, using Amersham AlkPhos Direct labeling reagents (GE Healthcare, Munich).

Cloning and sequencing of *CPC1*.

CPC1 genes were PCR-amplified from genomic DNA of the haploid *V. dahliae*, *V. albo-atrum*, and the hybrid *V. longisporum*, with an optimized procedure for the hybrid (Tran et al. 2013). The PCR products were purified with QIAprep spin miniprep kit (Qiagen) for direct sequencing or were cloned into pJET1.2 vector (Fermentas, St. Leon-Rot, Germany) for transformation of *E. coli* DH5 α . For plasmid DNA extraction, 20 positive colonies were chosen. DNA sequencing was performed by G2L, Göttingen, Germany.

Silencing of *CPC1* isogenes in *V. longisporum*.

pME3988 was constructed for silencing of the *VICPC1* isogenes. A 544-bp fragment present in both *VICPC1* isogenes was amplified from cDNA by PCR with primers *VICPC1*gate-L and *VICPC1*gate-R and was integrated into the pDONR/Zeo plasmid (Invitrogen, Karlsruhe, Germany) with BP clonase (Invitrogen). The resulting recombinant plasmid was mixed in the presence of LR clonase (Invitrogen) with a modified silencing vector pGS1 based on pSilent1 (Nakayashiki et al. 2005), pPK2 (Covert et al. 2001), and gateway cassettes (Invitrogen). LR clonase promotes the integration of the *VICPC1* fragment in both sense and antisense directions of the silencing vector. The resulting plasmid, pME3988, harbors a T-DNA (transfer DNA) containing the hygromycin resistance gene as selection marker and the *VICPC1* silencing cassette—including the *TrpC* promoter, *VICPC1* sense, spacer, and *VICPC1* antisense-*TrpC* terminator (Supplementary Fig. S7A). pME3988 was transformed into *Agrobacterium tumefaciens* AGL-1 according to Lazo and associates (1991) and was selected on LB medium plates containing kanamycin (100 μ g per milliliter). The T-DNA of the silencing construct was transferred into the genome of *V. longisporum* by *Agrobacterium tumefaciens*-mediated transformation (Mullins et al. 2001). For cocultivation of the *Agrobacterium tumefaciens* carrying the construct and *V. longisporum* spore suspension, 85-mm filter paper (Sartorius, Göttingen, Germany) was used. Stable transformants were grown on medium lacking hygromycin B for five generations and were then plated on selective medium supplemented with 100 μ g per milliliter of hygromycin B, in which only mitotically stable transformants could grow (Singh et al. 2010). The efficiency of *VICPC1* silencing in the transformants was determined by quantitative real-time PCR in comparison to the *V. longisporum* wild type.

Deletion of *CPC1* in the haploid *V. dahliae*.

Plasmid pKOCPC1 was constructed on the basis of the binary vector pPK2 (Covert et al. 2001), with a nourseothricin resistance cassette to delete a single *CPC1* of *V. dahliae* (*VdCPC1*). It includes two PCR fragments of 1.6 kb and 866 bp, covering the *VdCPC1* gene from the *V. dahliae* JR2 genome with a deletion of 252 bp in the open reading frame, which were amplified using primer pairs *VdCPC1*KOF1-L/*VdCPC1*KOF1-R and *VdCPC1*KOF2-L/*VdCPC1*KOF2-R, respectively. The primers contain restriction sites for *PacI*/*SacI* or *XbaI*/*HindIII* at the 5' ends to facilitate cloning of the PCR products. pKOCPC1 was introduced into *Agrobacterium tumefaciens* AGL-1 for fungal transformation of the *V. dahliae* JR2 strain (Table 2), and *VdCPC1* deletion mutants were screened by PCR using genomic DNA, before being confirmed by Southern hybridization (Supplementary Fig. S8).

Complementation of Δ *CPC1* strain in *V. dahliae*.

Plasmid pKOCPC1Comp was constructed on the basis of the binary vector pPK2 (Covert et al. 2001). It includes the full-length sequence of the *VdCPC1* gene from the *V. dahliae* JR2 genome, which was amplified using primer pair *VdCPC1*KOF1-L/*VdCPC1*KOF2-R. The primers contain restriction sites for *PacI*/*XbaI* at the 5' ends to facilitate cloning of the PCR products. pKOCPC1Comp was introduced into *Agrobacterium tumefaciens* AGL-1 for fungal transformation of the *V. dahliae* JR2 strain (Table 2).

Phenotypic analysis of *VICPC1* silenced mutants.

Verticillium longisporum wild type and the mutant strains were grown on CDA with or without 5 mM 5-MT for 4 days at 25°C. The analog induces a false-feedback inhibitor, tryptophan starvation. Colony diameters were measured after 3, 6, 9, 12, and 15 dpi. For investigation of *VICPC1* expression, Czapek Dox broth was inoculated with the *V. longisporum* wild type and the silenced mutant *VICPC1*sm, for 7 days at 25°C. After that timepoint, half of the cultures was used as control whereas the other half was induced with 5 mM 5-MT, and the cultures were incubated for an additional 4 h at 25°C.

Plant infection assay.

One-week-old *B. napus* seedlings were inoculated with 1×10^6 spores per milliliter of *VICPC1*sm or wild-type fungus (V143) or with sterile tap water as mock inoculation for 30 min by the root-dipping method. Seedlings were transferred in pots filled with sterile sand and soil (1:1) mixture and were incubated in a climate chamber with 14 h of light at 23°C and 10 h of dark at 20°C. The plants were cultivated until 35 dpi. The inoculated plant heights were measured and disease symptoms were scored (Eynck et al. 2007).

Ten-day-old tomato seedlings were inoculated with 1×10^6 fungal spores per milliliter (mutants or wild type) or mock with tap water for 30 min by root-dipping (Fradin et al. 2009). Seedlings were transferred in pots with a sterile sand and soil (1:1) mixture. Plants were cultivated in a greenhouse with 16 h of light at 23°C and 8 h of dark at 20°C. Plant heights were measured at 7, 14, 21, 28, and 35 dpi. In addition, the leaf size of the third leaf was measured, the number of leaves per plant was counted, and the verticillate shape of the plants was scored starting at 21 dpi. The verticillate shape was scored from 0 = no effect (corresponding to uninfected plants) to 3 = strong effect. For each infection assay, 60 plants per treatment were inoculated. The experiments were repeated two times.

Quantification of *Verticillium* DNA and RNA by real-time PCR.

Hypocotyls were separated from the infected oilseed rape plants harvested at 35 dpi. A sample of 20 single plants was harvested from each treatment. For relative quantification of RNA, *VICPC1* cDNA was amplified with the primer pair *CPC1*-RT1/*CPC1*-RT2; the primers amplify a part of the b-ZIP leucine zipper domain and the histone gene as reference for normalization with the primer pair *H2A*-RT1/*H2A*-RT2 (Table 1). The data were analyzed using the 2 $^{-\Delta\Delta C_t}$ relative quantification method (Livak and Schmittgen 2001). For absolute quantification of DNA, fungal DNA from plants was quantified using the primer pair OLG70/OLG71 for amplification of a specific *Verticillium* fragment of rDNA (parts of the 5.8S rRNA gene and ITS2) (Eynck et al. 2007). For each treatment, 25 ng of DNA was used as starting material.

Total RNA was isolated from fungal mycelium of *V. longisporum*, *V. dahliae*, or the corresponding mutants grown in SXM using the RNeasy plant mini kit (Qiagen). The cultures were inoculated with a spore suspension of 1×10^6 spores per

milliliter and were cultivated for 5 days at 25°C. The concentration of RNA samples was measured with a NanoDrop-ND1000 spectrophotometer (PiqLab, Erlangen, Germany). Total RNA (1 µg) was transcribed into cDNA, using the QuantiTect reverse transcription kit (Qiagen). Gene expression was measured by real-time PCR using a specific primer pair. Reactions contained 2 µl of each 0.5 µM primer (*H2A*-RT1 and *H2A*-RT2 or *CPC1*-RT1 and *CPC1*-RT2 [Table 1]), 1 µl of the cDNA, and reverse transcriptase-grade PCR water to a final volume of 11 µl. To obtain a final volume of 20 µl per reaction, 5 PRIME MasterMix (5 PRIME GmbH, Hamburg, Germany) was added. Each reaction was run in triplicates for all samples. Reactions were run under the following conditions, using the Light Cycler 2.0 System (Roche, Mannheim, Germany): 95°C denaturation for 3 min, 42 cycles at 95°C for 10 s, 63°C for 15 s, and 72°C for 25 s to calculate cycle threshold values, followed by 63°C for 15 s with a continuous increase of temperature by 0.1°C each cycle up to 95°C to obtain a melting curve. Standard curves were produced with purified genomic DNA products of 0.1 to 100 ng per microliter. Absolute amounts of genomic DNA were calculated using a correlation coefficient formula generated from the standard curve.

For relative quantification of RNA, cDNA of amino-acid metabolism genes was amplified with primer pairs Arg6RT1L/Arg6RT1R, Leu4RT1L/Leu4RT1R, Lys1RT1L/Lys1RT1R, and Trp5RT1L/Trp5RT1R. The histone gene was used as reference for normalization with the primer pair *H2A*-RT1/*H2A*-RT2 (Table 1).

Phylogenetic analysis of *CPC1* genes.

The phylogenetic trees were constructed with the MEGA 5.0 software (Tamura et al. 2011) based on the neighbor-joining method (Saitou and Nei 1987). The statistical reliabilities of the internal branches were assessed for all trees by using the bootstraps of 1,000 replicates. The *p*-distance model was used and gaps or missing data were treated with pairwise deletion (Tran et al. 2013).

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