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Silencing of *Vlaro2* for chorismate synthase revealed that the phytopathogen *Verticillium longisporum* induces the cross-pathway control in the xylem

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Abstract The first leaky auxotrophic mutant for aromatic amino acids of the near-diploid fungal plant pathogen *Verticillium longisporum* (VL) has been generated. VL enters its host *Brassica napus* through the roots and colonizes the xylem vessels. The xylem contains little nutrients including low concentrations of amino acids. We isolated the gene *Vlaro2* encoding chorismate synthase by complementation of the corresponding yeast mutant strain. Chorismate synthase produces the first branch point intermediate of aromatic amino acid biosynthesis. A novel RNA-mediated gene silencing method reduced gene expression of both isogenes by 80% and resulted in a bradytrophic mutant, which is a leaky auxotroph due to impaired expression of chorismate synthase. In contrast to the wild type, silencing resulted in increased expression of the cross-pathway regulatory gene *VlcpcA* (similar to *cpcA*/*GCN4*) during saprotrophic life. The mutant fungus is still able to infect the host plant *B. napus* and the model *Arabidopsis thaliana* with reduced efficiency. *VlcpcA* expression is increased in planta in the mutant and the wild-type fungus. We assume that xylem colonization requires induction of the cross-pathway control, presumably because the fungus has to overcome imbalanced amino acid supply in the xylem.

Keywords Chorismate synthase · RNA silencing · *Verticillium* wilt · Amino acid metabolism · Cross-pathway control

The EMBL database accession number for the sequence of *Vlaro2* reported in this paper is FM999811.

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Introduction

Natural energy resources are limited, and there is an increasing demand for biofuel to counteract this limitation. This resulted in the past decades in mass plantation of numerous plants including maize, oil palm, sweet sorghum, or rapeseed. The demand for rapeseed oil is rising as it is a healthy vegetable oil and also a renewable resource for the oleochemical industry and the biodiesel production. The dark side of this development is the appearance of unknown and may be even novel specific plant pathogens like *Verticillium longisporum*, a devastating vascular pathogen of rapeseed *Brassica napus* (Karapapa et al. 1997).

V. longisporum is a soil-borne hemibiotroph and opportunistic phytopathogen, which infects through the roots, colonizes the rapeseed plant, and then produces microsclerotia in the necrotrophic stage (Zhou et al. 2006; Eynck et al. 2007). *V. longisporum* can also live saprotrophically and survive in the soil. Microsclerotia of *V. longisporum* are present in the soil and persist there for many years in this dormant form (Schnathorst 1981; Heale and Karapapa 1999). *V. longisporum* infects through the root surface by direct penetration of the epidermal cells in the root hair zone (Eynck et al. 2007). Thereafter, it grows intercellularly and intracellularly in the cortex towards the central cylinder and then colonizes the xylem vessels. Individual xylem vessels are filled with mycelium and conidia whereas neighboring vessels remain completely unaffected. As the diseased plant senesces, the fungus produces microsclerotia, which are released into the soil with the decomposition of plant material and can again infect healthy plants.

V. longisporum is confined for most part of its life in the host plant in the vascular system of rapeseed (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 can also contain a number of organic acids, but it is not rich in nutrients (Lopez-Millan et al. 2000). The amino acid composition of the *B. napus* xylem is unknown. Glutamic acid, aspartic acid, and serine are the most abundant amino acids in leaf mesophyll cells of *B. napus*, where the concentration of the aromatic amino acids ranges from 1.2% to 2.1% of the total amino acids under low nitrogen supply condition (Tilsner et al. 2005).

Fungi are prototroph for amino acids and possess in addition elaborate amino acid uptake systems (Braus et al. 2004). The shikimate pathway is the general aromatic amino acid biosynthetic pathway present in fungi, bacteria, algae, and higher plants but is absent in mammals. The pathway converts D-erythrose 4-phosphate and phosphoenolpyruvate to chorismate and subsequently branches to produce tryptophan, phenylalanine, or tyrosine. In addition, the pathway is required for aromatic compounds like *p*-aminobenzoate, amino-hydroxybenzoate, vitamin K, or ubiquinone (Bentley and Meganathan 1982; Jones et al. 1991; Hsu et al. 2000; Knaggs 2003; Draht et al. 2008) or aromatic secondary metabolites including terrequinone A in *Aspergilli* (Bok et al. 2006). Chorismate synthase (CS) catalyzes the conversion of 5-enolpyruvylshikimate-3-phosphate to chorismate, which is the seventh step of the pathway and is located prior to the branch point (Braus 1991).

A saprotrophic fungus has to adapt according to the surrounding environmental conditions. If nutrients like amino acids are scarce, amino acid biosynthesis has to be activated. However, if amino acids are present in the surrounding environment, they should be procured by amino acid uptake systems and their production should be reduced. Many fungi possess the cross-pathway control system to overcome an imbalanced amino acid diet, which is problematic due to several feedback or cross-feedback controlled multi-amino-acid pathways. The corresponding transcriptional activator is Gcn4 in *Saccharomyces cerevisiae* (Hinnebusch 1984) or CpcA in *Aspergilli* (Wanke et al. 1997; Eckert et al. 1999; Hoffmann et al. 2001; Krappmann et al. 2004). Gcn4 protein is present in low levels when amino acids are abundant in the cell but becomes strongly induced under amino acid starvation condition (Albrecht et al. 1998). In the aromatic amino acid pathway of *S. cerevisiae*, four of the five *TRP* genes (Miozzari et al. 1978), the isogenes *ARO3* and *ARO4* (Teshiba et al. 1986; Paravicini et al. 1989; Künzler et al. 1992), and the *ARO2* gene are derepressed under the general control system. The genes *TRP1* and *TYR1* are not derepressible by this system (Braus 1991; Mannhaupt et al. 1989). The *ARO7* gene, which encodes chorismate mutase (Helmstaedt et al. 2001) producing the precursor to phenylalanine and tyrosine, is not transcriptionally controlled by Gcn4 (Krappmann et al. 2000).

Here, we have identified and characterized *Vlaro2*, which encodes the *V. longisporum* CS required for the production of the three aromatic amino acids. We provide the first RNA-mediated silencing of the two isogenes of *V. longisporum*. The resulting bradytrophic mutant induces during saprotrophic growth and in planta constitutively the transcription of *VlcpcA* encoding the cross-pathway control transcription factor. In wild-type cells, *VlcpcA* transcription is low during saprotrophic growth under nonstarvation condition but is induced in planta. This suggests a low and imbalanced amino acid supply present in the plant xylem.

Methods

Strains, media, and growth conditions The *S. cerevisiae* strains BY4741 (wild type) and Y04515 (BY4741; *Mata*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *YGL148w*::*kanMX4*) were obtained from the Euroscarf (Frankfurt, Germany) collection. Strains were grown on yeast extract-peptone dextrose (YPD) medium or on synthetic complete (SC) medium at 30°C. SC medium contained 2% glucose or 2% galactose, supplemented with the appropriate amino acids.

For maintenance and construction of plasmids, *Escherichia coli* SURE (Stratagene, CA, USA) cells and XL 10-Gold ultracompetent cells (Stratagene, CA, USA) were used. They were grown at 37°C in Luria–Bertani (LB) broth or on LB plates, supplemented with ampicillin (100 μg ml^{-1}) or kanamycin (100 μg ml^{-1}), as required.

A. tumefaciens AGL-1 strain (Lazo et al. 1991) was used for plasmid maintenance and transformation of *V. longisporum*. It was grown in LB medium supplemented with

carbenicillin (50 μg ml^{-1}) and rifampicin (25 μg ml^{-1}) at 28°C.

The *V. longisporum* strain 43 and *V. dahliae* strain 73 (Zeise and von Tiedemann 2002) were used in this study. Czapek Dox Agar (CDA) was inoculated with fungal spores and incubated at 25°C for 4 to 10 days in dark.

Construction of the V. longisporum cDNA library Fungal mycelia growing in different nutrient media were collected with sterile Miracloth filter (Calbiochem, Darmstadt, Germany) and washed with sterile distilled water. They were ground separately in liquid nitrogen to fine powder using a mortar and a pestle. Total RNAs were isolated from the powdered fungal mycelia using the TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions. One milligram of total mixed RNA contained 10% RNA from the fungus growing in liquid minimal medium (MM) for 4 days, 25% RNA from the fungus growing in liquid MM for 10 days, 25% RNA from the fungus growing in liquid simulated xylem medium (SXM) for 4 days, 25% from the fungus growing in liquid SXM for 10 days, and 15% RNA from the fungus growing on potato dextrose agar for 10 days at 25°C. The cDNA library of *V. longisporum* was generated in the yeast expression vector pYES-DEST52 by Invitrogen GmbH (Karlsruhe, Germany), and the DNA sequence of a sample of 5,000 expressed sequence tags was determined.

Yeast complementation assay V. longisporum cDNA library in the yeast expression vector, pYES-DEST52, was transformed in the yeast deletion mutant of CS, Y04515 (Δ*ARO2*; Burke et al. 2000). Transformed cells were plated onto SC minus uracil medium supplemented with 2% glucose at 30°C. The uracil prototrophs were subsequently replica-plated on SC plates supplemented with 2% galactose, lacking the three aromatic amino acids, at 30°C.

Generation of the plasmid for silencing Vlaro2 in V. longisporum Recombinant DNA technology protocols were followed according to Sambrook et al. (1989). Polymerase chain reaction (PCR) was performed with *Pfu* proofreading polymerase (Stratagene, CA, USA), Hot start Taq DNA polymerase (Qiagen, Hilden, Germany), or Expand high-fidelity PCR system (Roche, Mannheim, Germany). Primers are listed in Table S1. Calcium-treated chemically competent cells of *E. coli* were transformed according to Hanahan et al. (1991). The pSilent-1 vector (Nakayashiki et al. 2005) was used for constructing the silencing cassette. A fragment of 418 bp was amplified from the coding region of *Vlaro2* of *V. longisporum* by PCR using primers CSS-*Sna*-F and CSS-*Hin*d-R to produce the sense strand. Sense product was purified and then digested with *Sna*BI and *Hin*dIII. It was then ligated into *Sna*BI–*Hin*dIII-digested pSilent-1 plasmid. The antisense region of *Vlaro2* was amplified using CSAS-*Apa*I-F and CSAS-*Pae*I-R. Antisense product was purified and then digested with *Apa*I and *Sph*I. It was then ligated with *Apa*I–*Sph*I-digested pSilent-1 plasmid already containing the sense *Vlaro2*. The whole silencing cassette consisting of *TrpC* promoter, sense *Vlaro2* strand, spacer, antisense *Vlaro2* strand, and *TrpC* terminator was isolated by digestion with *Xba*I. It was then ligated in *Xba*I digested binary vector, pPK2 (Covert et al. 2001) to generate pME3571 vector for silencing both the isogenes *Vlaro2*-1 and *Vlaro2*-2. The pPK2 vector contains the hygromycin B phosphotransferase gene (*hph*) as selection marker.

Agrobacterium tumefaciens-mediated transformation V. longisporum was transformed by *A. tumefaciens*-mediated transformation as described by Mullins et al. (2001) with slight modifications. The pME3571 vector was transformed in *A. tumefaciens* AGL-1 strain by electroporation (2,500 V, 5 ms) and plated on LB medium containing kanamycin (100 μg ml^{-1}). The transformed strain was inoculated in LB medium supplemented with 50 μg ml^{-1} kanamycin and 100 μg ml^{-1} carbenicillin. Filter paper, 90 mm (Sartorius, Göttingen, Germany), was used for cocultivation of the *A. tumefaciens* culture and *V. longisporum* spore suspension. The transformants were obtained on selection medium containing 100 μg ml^{-1} hygromycin B. The mitotic stability of transformation was examined by culturing transformants on CDA for five generations successively and then transferring to selection medium with hygromycin B.

Southern hybridization analysis Genomic DNA of *V. longisporum* was isolated from mycelium grown in liquid culture (Kolar et al. 1988) and used for Southern hybridization (Southern 1975). Gel-purified DNA fragments (QIAquick Gel Extraction Kit from Qiagen, Hilden, Germany) consisting of a 400-bp fragment from *Vlaro2* (Table S1) or a 1-kb fragment from *hph* gene in pPK2 (Covert et al. 2001) were used as probes. Amersham CDP-Star Detection reagent (GE Healthcare, Munich, Germany) was used for chemiluminescent signal detection.

RT-PCR analysis Total RNA was isolated from fungal mycelium using the TRIzol reagent from Invitrogen GmbH, Karlsruhe, Germany according to the manufacturer's instructions. Total cDNA was generated using RevertAid First-Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). PCR was performed using the Hot start Taq DNA polymerase (Qiagen, Hilden, Germany) with 1 μl of reverse transcription (RT) reaction as template and 0.5 μM of each primer. The *VlcpcA* gene was identified in the sequenced sample of the cDNA library of *V. longisporum* by multiple alignments with its fungal counterparts (data

not shown). To detect *VlcpcA* mRNA, the primers SS85 and SS86 were used; for *Vlaro*2 mRNA, the primers SS46 and SS47 were applied, and the transcript for actin was recognized by the usage of the primers SS15 and SS33.

Western hybridization analysis Yeast Protein Extraction Reagent (Y-PER-S reagent, Pierce, Thermo Fisher Scientific, Schwerte, Germany) was used for protein extraction and protease inhibitor (Complete, protease inhibitor cocktail, Roche, Mannheim, Germany) was added during extraction. Protein concentrations were determined using the BCA™ protein assay kit (Pierce, Thermo Fisher Scientific, Schwerte, Germany). Protein extracts were separated by sodium dodecyl sulfate (SDS)–polyacrylamide electrophoresis (Laemmli 1970) for Western hybridization (Laxalt et al. 2002). CS antibody raised against *N. crassa* (Ehammer et al. 2007) and monoclonal mouse anti-α-tubulin antibody (Sigma-Aldrich Chemie, Steinheim am Albuch, Germany) were used as primary antibodies. As secondary antibodies, peroxidase-coupled goat antirabbit immunoglobulin G (IgG)–horse radish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany) or goat antimouse IgG antibodies (Invitrogen GmbH, Karlsruhe, Germany) were employed. Cross-reactions were visualized using the electrochemiluminescence technology (Amersham Pharmacia Biotech, GE Healthcare, Munich, Germany).

Phenotypic analysis of Vlaro2-silenced mutants Five thousand spores of *Vlaro*2-silenced mutants (*Vlaro*2sm) and wild type were inoculated on CDA and CDA supplemented with the three aromatic amino acids tryptophan, phenylalanine, and tyrosine or 5 mM 5-methyl-DL-tryptophan (5-MT) at 25°C. The experiment was performed in triplicate. The plates were observed every day until 4 days postinoculation (dpi) by light microscopy. For radial growth rate determination, the procedure described by Panepinto et al. (2003) was applied.

Xylem sap production and amino acid measurement Rapid-cycle rape (genotype: ACaacc; Williams and Hill 1986) was used for xylem sap production. *B. napus* plants were harvested when they were 42 days old. Xylem sap was collected as described previously (Scholander et al. 1965; Kehr et al. 2005). The yield was 50–150 µl per plant. Xylem sap was filter-sterilized and stored at −20°C until further use. The amino acids present in the xylem sap were analyzed by high-performance liquid chromatography (HPLC; Pharmacia/LKB, Freiburg, Germany) according to Riens et al. (1991).

Pathogenicity assay Week-old *B. napus* seedlings were inoculated with 1×10^6 spores per milliliter of *Vlaro*2-sm and wild type of *V. longisporum* and mock-inoculated with sterile tap water for 30 min by root-dipping inoculation. They were then transferred in pots with sterile sand/soil (1:1) mixture. The plants were incubated in a climate chamber with 14-h light at 23°C and 10-h dark at 20°C. The height of inoculated plants was measured, and they were scored for disease symptoms according to Eynck et al. (2007).

A. thaliana plants were grown in sand/soil mixture (Vitakraft, Nr. 12262, Bremen, Germany) on a layer of seramis (Masterfoods, Viersen, Germany) in a growth chamber (22°C, and ~140 $\mu mol^{-1} m^2 s^{-1}$ PAR) under short-day conditions (8-h-light/16-h-dark photoperiod). Three-week-old plants were inoculated by incubation for 45 min in a conidial suspension (10^6 conidia per milliliter) or mock-inoculated by dipping in sterile tap water. Plants were transplanted into potting mix (steam-sterilized soil) and kept under a transparent cover for 2 days to ensure high humidity. The leaf area of the inoculated plants was measured at 7, 14, 21, and 28 dpi. The fresh weight of the inoculated plants was measured at 28 dpi.

Quantification of gene expression and V. longisporum DNA in planta by real-time PCR Hypocotyls and stems (5–6 cm from top of plant) were separated from the infected rapeseed plants harvested at 14, 21, 28, and 35 dpi. Eighteen plants were harvested from each treatment. Infected *A. thaliana* plants were harvested at 28 dpi. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For induction with 5-MT, fungal mycelium from 4-day-old liquid culture in Czapek Dox (CD) medium was transferred in CD medium supplemented with 5 mM 5-MT and incubated for 8-h. Total cDNA was generated from the fungal mycelium as described before (RT-PCR analysis). The fungal DNA was quantified in the *B. napus* tissue with real-time PCR according to Eynck et al. (2007). Primers OLG 70 and OLG 71 amplify a fragment specific for *V. longisporum* and *V. dahliae* (Eynck et al. 2007). *Vlaro*2 was amplified using primers SS93 and SS94, *VlcpcA* using primers SS85 and SS86, and the gene for ß-tubulin using primers SS57 and SS58 (Table S1).

Real-time PCR was carried out by using Fast Start DNA Master SYBR Green I kit (Roche, Mannheim, Germany) with 0.3 µM of each primer and 2 µl of template in a final reaction volume of 20 µl. Reactions were set up with three replicates per sample. Controls without templates were included for each primer set. For amplification and melting curve analysis, the Light Cycler 2.0 System (Roche, Mannheim, Germany) was used, initiated by 10-min incubation at 95°C, followed by 45 cycles of 10 s at 95°C, 10 s at 58°C, and 25 s at 72°C. 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 ß-tubulin as reference for normalization.

V. longisporum DNA in *A. thaliana* was quantified by the iCycler System (Bio-Rad, Munich, Germany). The amplification mix consisted of NH_4 reaction buffer (Bioline, Luckenwalde, Germany); 3 mM $MgCl_2$; 200 µM of each dATP, dTTP, dCTP, and dGTP; 0.3 µM of primer OLG70 and OLG71; 0.25 µM BIOTaq DNA polymerase (Bioline, Luckenwalde, Germany); 10 nM Fluorescein (Bio-Rad, Munich, Germany) 100,000 times diluted SYBR Green I solution (Cambrex, Bio Science Rockland, USA); 20–30 ng of template DNA; and double-distilled water filled to a total volume of 25 µl. PCR program comprised a 2-min denaturation step at 94°C followed by 36 cycles of 20 s at 94°C, 30 s at 59°C, and 40 s at 72°C. The amount of *V. longisporum* DNA was estimated from a calibration curve constructed with purified fungal DNA diluted with plant DNA. To normalize for different DNA preparations, the *Arabidopsis Actin8* gene (At1g49240) was amplified with the primers act8fow and act8rew (Table S1). The amount of *Actin8* DNA in the samples was calculated with a reference plasmid encoding *Actin8* sequences. Copy number of the product was calculated from the threshold cycles of duplicate real-time PCR assays using the standard curve.

Results

Low concentrations of various amino acids are present in the xylem sap of *B. napus*

V. longisporum colonizes the rapeseed xylem, resulting in impairment of plant growth. The xylem primarily carries water and minerals from the root to the aerial parts of the plant. Additionally, the xylem sap also contains some organic acids and amino acids (Schurr and Schulze 1995; Zornoza et al. 1996; Satoh et al. 1998). To address the importance of amino acids as nutrient supply for fungal life, we investigated the amino acid composition in the xylem sap of *B. napus*. For this purpose, xylem sap was extracted from 42-day-old *B. napus* plants. At this time, the *B. napus* plants are mature and flowering, and if infected, they are heavily infected with *V. longisporum*. At this late stage of infection, the fungus spreads from the hypocotyl to the upper parts of the plant. The concentrations of the amino acids present in the xylem sap were determined by HPLC and are listed in Table 1. The amino acids cysteine and proline were not detectable with this HPLC system.

Table 1 Amino acid concentrations in the xylem sap of 42-day-old *B. napus*

Amino acid	Concentration (µM)
Asp	52.85
Glu	53.66
Asn	9.72
Ser	37.34
His	4.12
Gln	177.35
Thr	22.82
Gly	17.76
Arg	2.76
Ala	36.32
GABA[a]	50.58
Tyr[b]	7.94
Val	25.75
Met	2.01
Trp[b]	2.01
Ile	15.34
Phe[b]	6.77
Leu	10.09
Lys	8.83
Total	544.02

All values are an average of two measurements

[a] γ-Aminobutyric acid

[b] Aromatic amino acids

The concentration of the amino acids ranged from 2 to 177 µM. The major amino acids are glutamine, glutamic acid, aspartic acid, and γ-amino butyric acid. γ-Amino butyric acid is produced by decarboxylation of glutamate (Satyanarayan and Nair 1990). Histidine, arginine, methionine, lysine, and aromatic amino acids were below 10 µM. The concentration of all aromatic amino acids was low, as tyrosine was 8 µM, tryptophan 2 µM, and phenylalanine 7 µM, respectively. It is unknown whether aromatic amino acids in these low concentrations are sufficient to support *V. longisporum* colonization of the xylem or whether the fungus primarily depends on its own aromatic amino acid biosynthesis.

V. longisporum Vlaro2 complements the *S. cerevisiae ARO2* deletion mutant of CS

We addressed whether the detected low aromatic amino acid concentration in the rapeseed xylem is sufficient to support fungal growth. We focused on the isogenes for the CS as the last common enzyme, which is required for the synthesis of all the aromatic amino acids of *V. longisporum* as well as other aromatic compounds (Weiss and Edwards 1980; Haslam 1993). The genomic database of the fungus

is not yet available, and therefore we isolated the genes by heterologous complementation. We constructed a *V. longisporum* cDNA library whose expression in yeast is driven by the *GAL1* promoter to complement a yeast Δ*ARO2* mutant strain. *ARO2* encodes the yeast CS, and yeast Δ*ARO2* mutant strain cannot grow if the three aromatic amino acids are not added to the medium (Jones et al. 1991).

Several transformants derived from the cDNA library of *V. longisporum* in the *S. cerevisiae* Δ*ARO2* mutant strain were obtained on SC medium lacking the three aromatic amino acids. For its function, CS has an absolute requirement for reduced FMN. CS from plants and eubacteria depends on external sources for the reduction of FMN. In contrast, CS of *S. cerevisiae* is bifunctional as, along with the catalysis of 5-enolpyruvylshikimate 3-phosphate to chorismate, it also possesses an additional NADPH:FMN oxidoreductase activity (Henstrand et al. 1996). This additional function is required for generating reduced FMN for the catalytic activity of the enzyme. The successful complementation of the yeast Δ*ARO2* mutant strain suggests that *V. longisporum* also encodes a bifunctional CS.

Eight randomly picked complements were analyzed, and the inserts of the plasmids revealed identical cDNA sequences for all plasmids. Comparison of the deduced amino acid sequence of this cDNA with the GenBank National Center for Biotechnology and European Molecular Biology Laboratory database revealed a putative gene for CS. Therefore, the gene was named *Vlaro*2. Retransformation of the isolated plasmid confirmed the complementation resulting in similar growth results as the wild-type yeast strain (BY4751), whereas the untransformed Δ*ARO2* cannot grow on SC medium lacking the three aromatic amino acids (Fig. 1a).

The sequence analysis revealed that the coding region of *Vlaro*2 comprises an open reading frame of 1,227 bp (EMBL database accession number FM999811). The deduced protein Vlaro2 consists of 408 amino acids and has a predicted molecular mass of 43.57 kDa (Fig. 1b). Vlaro2 is identical to the corresponding protein sequence of *V. dahliae* (Locus ID: VDAG_07695.1), but the analysis of the introns showed that the *V. longisporum* gene includes a shorter intron of 74-bp in comparison to the 81-bp intron of *V. dahliae*. In the analysis of the draft genome of *V. dahliae*, only one gene locus on chromosome 5 for CS could be found (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/GeneDetails.html?sp=S7000001884963178, 13 September 2009). There is also high sequence similarity of the deduced primary sequence to CSs from other filamentous fungi including *Neurospora crassa* (76% identity) or *S. cerevisiae* (63% identity; Fig. S1). Sequence similarity was lower with CS proteins from bacteria (*Desulfuromonas acetoxidans* 55% identity) or plants (*Oryza sativa* 52%

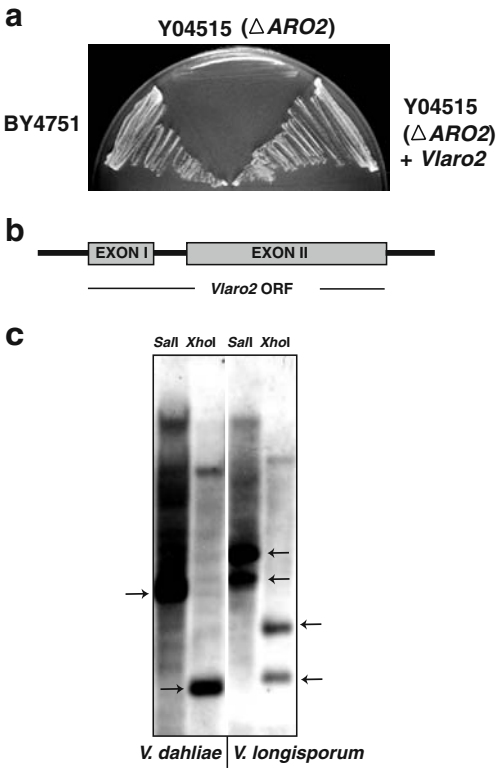


Fig. 1 Isolation of the gene for CS in *V. longisporum*, *Vlaro*2. **a** Δ*ARO2* yeast complementation assay on SC medium lacking the three aromatic amino acids. *BY4751*, wild-type yeast strain; *Y04515* (Δ*ARO2*), yeast CS mutant; *Y04515* (Δ*ARO2*) +*Vlaro*2-1, yeast CS mutant complemented with gene for CS in *V. longisporum*. **b** Illustration of the *Vlaro*2-1 locus consisting of two exons and one intron. **c** Determination of the isogene *Vlaro*2-2 of *Vlaro*2-1 by Southern hybridization analysis of *V. dahliae* and *V. longisporum*. The genomic DNA was digested with *Sal*I and *Xho*I. A 416-bp sequence of *Vlaro*2-1 was used as a probe. *Arrows* indicate the signal generation by probe binding

identity). L1, L2, and L4, the regions that contribute to the substrate binding site in CS of *S. cerevisiae* (Quevillon-Cheruel et al. 2004), could also be identified in Vlaro2 and are shown in Fig. S1. The NADPH binding region is yet unclear. Kitzing et al. (2001) observed that NADPH binds in or near the substrate (5-enolpyruvylshikimate 3-phosphate) binding site, suggesting that NADPH binding to bifunctional CSs is embedded in the general protein structure, and a special NADPH binding domain is not required to generate the intrinsic oxidoreductase activity.

V. longisporum has been described as "near-diploid" organism because it possesses 1.78 times the DNA content of *V. dahliae*, one of its parent species (Karapapa et al. 1997). Since we found only one type of complementing cDNAs for Vlaro2, we analyzed the copy number of the gene within the genome by Southern hybridization. *V. longisporum* and *V. dahliae* genomic DNA were compared and digested with *Sal*I and *Xho*I (Fig. 1c). Restriction sites for both enzymes are not present within the *Vlaro*2 gene. A

unique and fully sequenced 416-bp *Vlaro*2 fragment from *V. longisporum* was used as probe (Table S1). Two signals were generated for *V. longisporum* as compared to *V. dahliae* for the genomic DNA. These data suggest the presence of two similar isogenes (*Vlaro*2-1 and *Vlaro*2-2) for CS within the genome of *V. longisporum.* It is currently unknown whether and to what degree both genes are expressed.

*Vlaro*2 isogenes were silenced up to 80–90% by RNA-mediated gene silencing

To generate mutant strains impaired in aromatic amino acid biosynthesis, we had to take into account that both isogenes of *Vlaro*2 might be expressed. RNA-mediated gene silencing has been successfully implemented in several fungi for targeted gene silencing instead of a conventional knockout (Nakayashiki et al. 2005). The binary vector pME3571 (Fig. S2) used for the silencing strategy in *V. longisporum* was based on pSilent-1 vector (Nakayashiki et al. 2005). It harbors the hairpin construct for silencing of *Vlaro*2 comprising of inverted repeats of *Vlaro*2 fragment separated by a spacer and the hygromycin gene as selection marker. Transformation in *V. longisporum* was mediated by *A. tumefaciens*. The transformants were grown on medium lacking hygromycin B for five generations and then grown on medium supplemented with hygromycin B, where only mitotically stable transformants could grow. Thirty independent hygromycin-B-resistant transformants were selected at random for further analysis. The integration of the T-DNA in the genome of the *V. longisporum* was confirmed by Southern hybridization analysis with a probe for the hygromycin resistance gene (Fig. 2a).

The extent of *Vlaro*2 silencing was monitored by RT-PCR as the silencing of gene expression is the result of posttranscriptional degradation of targeted mRNA. Total RNA was extracted from the *Vlaro*2sm and wild type. RT-PCR results showed the significant knockdown of *Vlaro*2 transcript (Fig. 2b). The degree of silencing of CS genes was estimated by RT-PCR by the ratio of the amplification of *Vlaro*2 between the respective transformants and the unsilenced wild type. 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.

We verified these results by monitoring the protein levels of *Vlaro*2 for all *Vlaro*2sm in comparison to wild type (Fig. 2c). Western hybridization using a CS-specific antibody and the tubulin levels as control showed the significant knockdown of *Vlaro*2 up to 94% in the *Vlaro*2sm as compared to the wild type. The knockdown of *Vlaro*2 at the protein level corresponded to the RT-PCR analysis of the *Vlaro*2sm and established gene silencing in

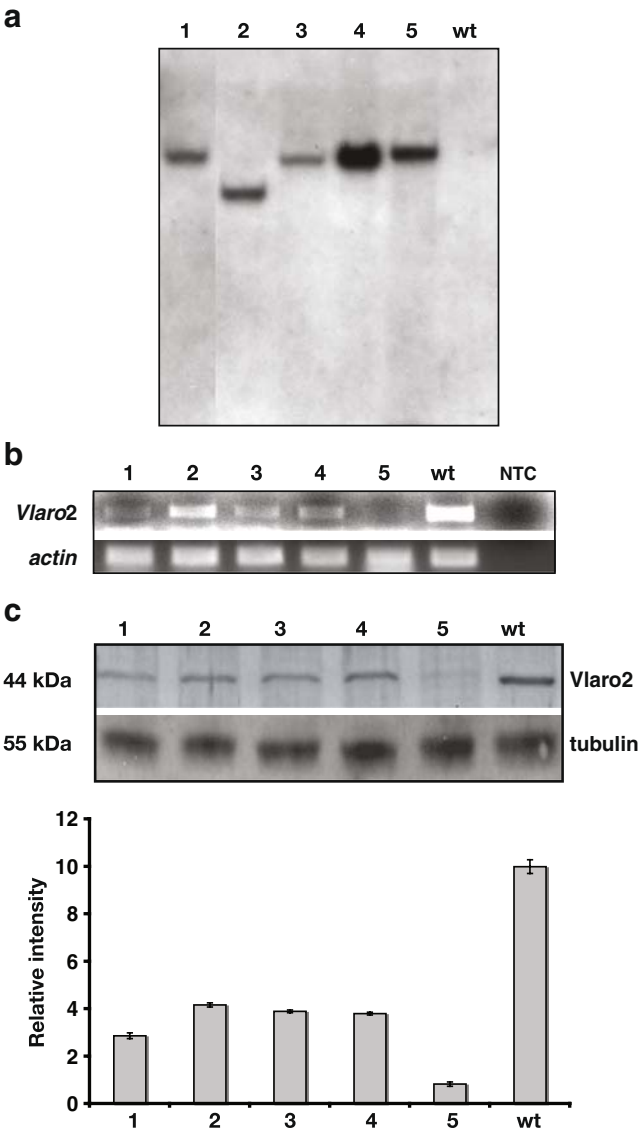


Fig. 2 Characterization of the *Vlaro*2 silenced mutants. **a** Southern hybridization analysis of *Vlaro*2 silenced mutants to detect integration of T-DNA after *A. tumefaciens*-mediated transformation. Five *Vlaro*2 silenced mutants (*1–5*) and wild-type (*wt*) gDNA was digested with *Hin*dIII and the hygromycin resistance gene was used as a probe. All mutants showed single integration of the gene. **b** RT-PCR analysis of *Vlaro*2 mRNA expression in the *Vlaro*2 silenced mutants. For RNA integrity, the *actin* gene was used as a control. *1–5*, *Vlaro*2 silenced mutants; *wt*, wild type; *NTC*, no template control. **c** Western hybridization analysis of *Vlaro*2 expression in the *Vlaro*2 silenced mutants compared to the wild type. Proteins were extracted from the *Vlaro*2 silenced mutants and wild type, ran on SDS-polyacrylamide gel, blotted and probed with *N. crassa* CS antibody. The same blot was stripped and probed again with Rat IgG tubulin antibody as a control. *1–5*, *Vlaro*2 silenced mutants; *wt*, wild type. In the *graph*, CS expression was quantified and normalized against the tubulin level for the different samples using Kodak Molecular Imaging 4.05 software. Data represent average ± standard deviations of three experimental replicates

V. longisporum. *Vlaro*2sm 1 with 80% knockdown of *Vlaro*2 was chosen for further analysis.

Silencing of *Vlaro*2 results in a bradytrophic *V. longisporum* mutant

We analyzed the saprotrophic lifestyle of the *Vlaro*2sm and investigated whether *Vlaro*2 silencing inhibits the growth of *V. longisporum* in the absence of amino acids, specifically aromatic amino acids. One day after inoculation on CDA, notably, *Vlaro*2sm grew slowly. Only short hyphae were visible in contrast to the mycelial growth of wild type (Fig. 3a). After 2 days of inoculation on CDA, wild-type mycelial growth increased, and many conidia could be observed whereas the *Vlaro*2sm showed less mycelial growth and no conidia were observed. We further analyzed the effect of *Vlaro*2 silencing on the radial growth of *V. longisporum.* The radial growth of *Vlaro*2sm recorded after

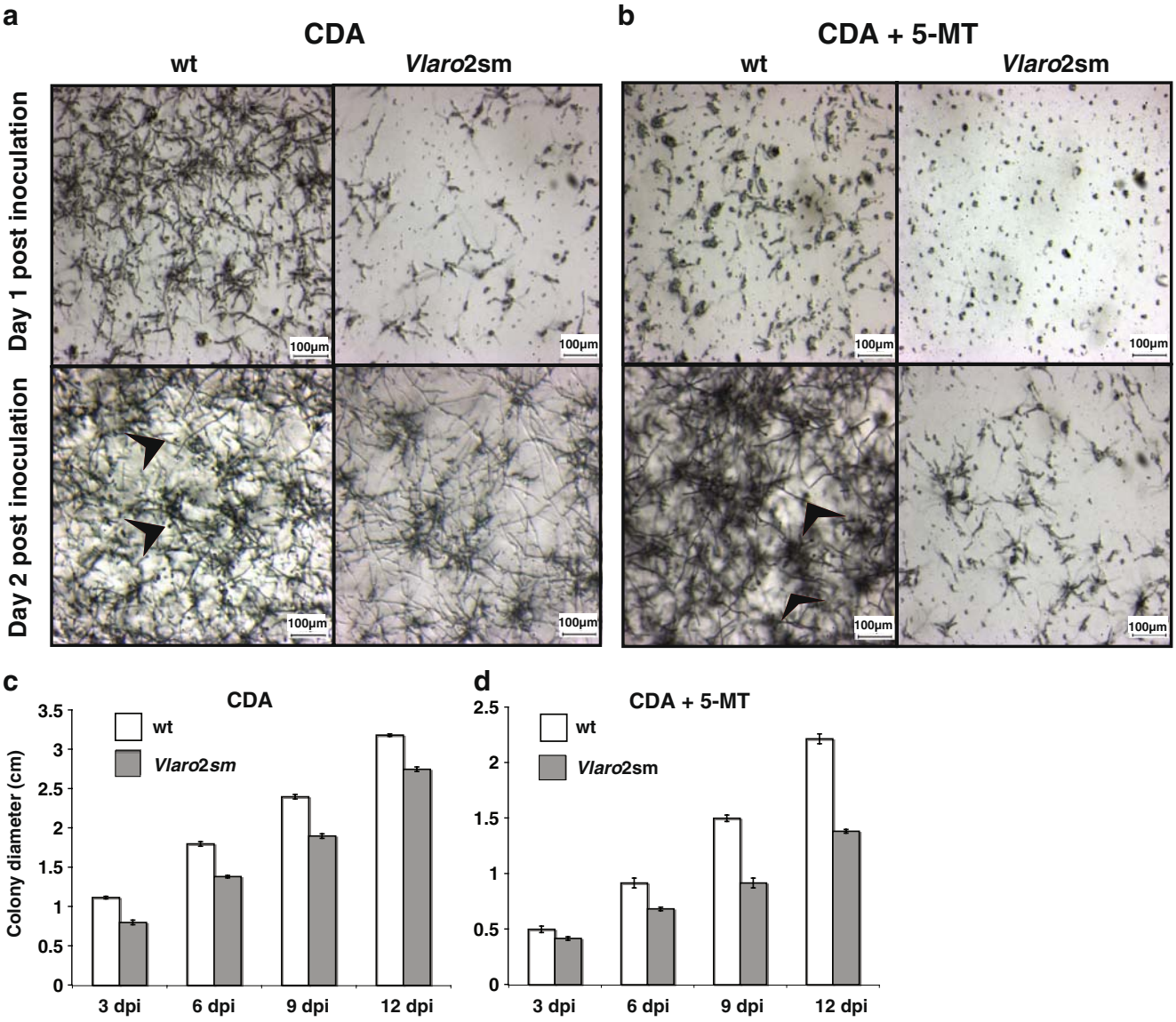


Fig. 3 Phenotypic analysis of the *Vlaro*2 silenced mutants. **a** Light microscopy images of *V. longisporum* wild type (*wt*) and *Vlaro*2 silenced mutant (*Vlaro2sm*) cultured on CDA as observed at 1 and 2 dpi. The colonies were examined microscopically for vegetative growth and spore formation. *Black arrows* indicate conidia. **b** Light microscopy images of *V. longisporum* wild type (wt) and *Vlaro*2sm cultured on CDA supplemented with 5 mM 5-MT as observed at 1 and 2 dpi. The colonies were examined microscopically for vegetative growth and spore formation. *Black arrows* indicate conidia. **c** The *graph* shows the growth rates of wild-type (wt) and *Vlaro*2sm on CDA determined by plate-based growth assays. Agar plates were inoculated with 5,000 spores, and colony diameter from three replicates was measured following 3, 6, 9, and 12 days of incubation at 25°C. Data represent average ± standard deviations of three experimental replicates. **d** The *graph* shows the growth rates of wild type (wt) and *Vlaro*2sm on CDA amended with 5-MT determined as described in **c**

3, 6, 9, or 12 days was similar to wild type (Fig. 3c). Therefore, the *Vlaro*2sm, showed only slower initial vegetative growth, but the radial growth was not affected compared to wild type.

Next, we analyzed the effect of the tryptophan analog, 5-MT, on *Vlaro*2sm. Addition of 5-MT to CDA causes additional tryptophan starvation. 5-MT acts as false feedback inhibitor of anthranilate synthase, the first enzyme of the tryptophan branch of the pathway (Schuerch et al. 1974). One day after inoculation, the wild type formed short hyphae compared to *Vlaro*2sm in which only initial germination of conidia could be observed (Fig. 3b). After 2 days, wild type showed a mycelial net with few conidia, whereas *Vlaro*2sm formed short hyphae without conidia. Interestingly, the radial growth of *Vlaro*2sm was significantly lower than in the wild type when the colony diameter was determined until 12 days (Fig. 3d). It started decreasing gradually at 3 and 6 dpi and decreased up to 40% at 9 and 12 dpi. Hence, *Vlaro*2sm showed severe reduction in initial vegetative growth as well as the growth rate observed under saprotrophic conditions on CDA supplemented with 5-MT. As control, we analyzed whether tryptophan addition can counteract the 5-MT effect on *Vlaro*2sm growth. For this experiment, *Vlaro*2sm was inoculated on CDA medium supplemented with 5 mM 5-MT and 200 µM tryptophan. The addition of tryptophan prevented the severe reduction in growth of the *Vlaro*2sm as observed in the presence of 5-MT alone (data not shown).

We also studied the effect of the three aromatic amino acids on *Vlaro*2sm. The three aromatic amino acids were added to CDA, in concentration similar to the xylem sap of *B. napus* (Table 1), that is, 10 µM phenylalanine, 10 µM tyrosine, and 2 µM tryptophan, respectively. *Vlaro*2sm again showed slower growth than the wild type. Higher concentration of 100 or 300 µM (50 µg ml^{-1}) phenylalanine, 100 or 165 µM (30 µg ml^{-1}) tyrosine, and 20 or 196 µM (40 µg ml^{-1}) tryptophan did not stimulate growth of *Vlaro*2sm. This might be due to the fact that in fungi there are phenylalanine and tyrosine-feedback-inhibited isozymes for DAHP synthase, the initial enzyme of the pathway, but no tryptophan-dependent enzyme (Braus 1991). These data suggest that the concentration of the aromatic amino acids in the xylem sap is not high enough to allow growth of *Vlaro*2sm similar to wild type without support of cellular aromatic amino acid biosynthesis.

*Vlaro*2 silencing induces increased expression of the gene for the putative amino acid cross-pathway control activator, *VlcpcA* during saprotrophic life

The knockdown of *Vlaro*2 resulted in reduced vegetative growth which became more retarded in presence of 5-MT as it produces less chorismate and is not able to cope with tryptophan starvation. Quantitative real-time PCR was performed to determine the expression of *Vlaro*2 in wild type and *Vlaro*2sm under induction with 5-MT. Total RNA was extracted from the liquid cultures, and *Vlaro*2 expression was quantified by real-time PCR using *ß-tubulin* gene for normalization. The expression of *Vlaro*2 in *Vlaro*2sm was drastically reduced by 82% compared to wild type (Fig. 4a). 5-MT only slightly affected *Vlaro*2 expression in wild type or *Vlaro*2sm.

We addressed whether the *Vlaro*2sm counteracts amino acid stress by a cross-pathway control system as described for other fungi. We determined by quantitative real-time PCR whether silencing of *Vlaro*2 induces the expression of the putative amino acid cross-pathway control transcriptional activator, VlcpcA. The samples used for expression study of *VlcpcA* and *Vlaro*2 were identical. *VlcpcA* expression was increased significantly by sixfold in the

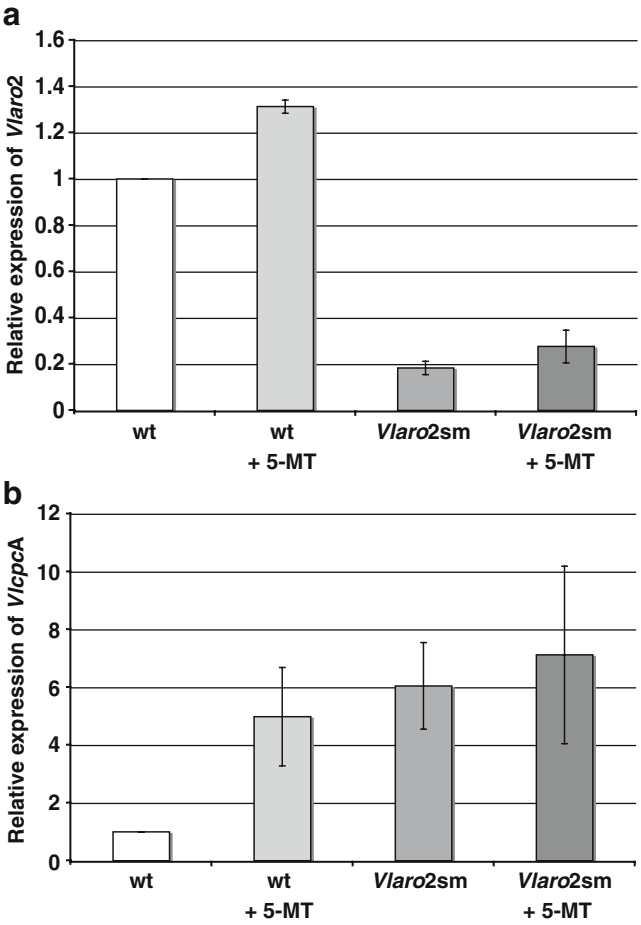


Fig. 4 *Vlaro*2 and *Vlcpc*A expression. **a** Relative expression of *Vlaro*2 measured by quantitative real-time PCR. *Vlaro*2 cDNA was normalized to the *ß-tubulin* cDNA. *wt*, wild type, *Vlaro2sm + 5-MT*, *V. longisporum* strains grown in the presence of 5-MT. The *error bars* represent the standard deviations of three different measurements of the same cDNA. **b** *Vlcpc*A expression measured relative to the ß-tubulin cDNA as described in **a**

bradytrophic *Vlaro*2sm, suggesting that there is here as well amino acid starvation as a counteraction of a cross-pathway control (Fig. 4b). This is similar to the effect observed in *A. nidulans* where *cpcA* mRNA levels were increased when cells were starved for amino acids (Hoffmann et al. 2001). The expression of *VlcpcA* was increased by fivefold and sevenfold in wild type and *Vlaro*2sm cultures induced with 5-MT, respectively. Therefore, our data suggest that there exists a cross-pathway control for amino acid biosynthesis in *V. longisporum*.

*Vlaro*2sm are able to infect *B. napus* xylem with reduced efficiency

Silencing of *Vlaro*2 resulted in an increase in *VlcpcA* expression that hints to a constitutively active cross-pathway control. We analyzed whether such a *V. longisporum* mutant strain was still able to infect and colonize *B. napus* and the model plant, *A. thaliana*.

To assess the severity of disease, 1-week-old *B. napus* and 3-week-old *A. thaliana* seedlings were infected by root dip inoculation; the height of the plants was measured weekly postinoculation until the plants are highly infected. The plants began to show disease symptoms at 21 dpi and were heavily infected at 35 dpi (Fig. 5a). The plants infected with *Vlaro*2sm were smaller than the mock-inoculated plants, suggesting that the fungus had successfully infected the plant (Fig. 5c). Infection by *Vlaro*2sm was less pronounced than by the wild type resulting in reduced stunting compared to that caused by wild type. At 35 dpi, the wild type and the *Vlaro*2sm-infected rapeseed plants were about 40% and 25% smaller than the mock-inoculated plants, respectively (Fig. 5b). The infected rapeseed plants were also 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 weekly for disease symptoms until 35 dpi when the plants were highly infected. In the assessment of the symptoms, the yellowing and death of the leaves were considered. Therefore, the mock-infected plants also had disease scores more than 1 because of yellowing of leaves due to natural senescence. The infected plants did not show any disease symptoms until 14 dpi. The disease score of the *Vlaro*2sm-infected plants was slightly higher (4.5) than the mock-inoculated plants (4.0) but significantly lower than the plants infected with wild type (6.0; Fig. 5a).

In *A. thaliana*, the leaf area of the infected plants was measured weekly until 28 dpi. The difference in wild-type-inoculated and *Vlaro*2sm-infected plants became evident at 21 dpi and became most pronounced at 28 dpi (Fig. 5d, e). At 28 dpi, when the plants were highly infected, the leaf area of the wild-type-inoculated plants was reduced by 50% than the mock-inoculated plants whereas the leaf area of the *Vlaro*2sm-infected plants was only reduced by 15% (Fig. 5d). The fresh weight of the *A. thaliana* plants was also measured at 28 dpi. The average weight of wild-type-inoculated plants was the least and reduced by 44% compared to the mock-inoculated plants. The fresh weight of *Vlaro*2sm-infected plants was reduced by 22% compared to the mock-inoculated plants. These data suggest that the bradytrophic mutant is able to colonize the plant and to induce disease but with significantly reduced efficiency in comparison to the wild type. It also suggests that *A. thaliana* is suitable for conducting pathogenicity assays with future mutants as it is easier to handle this well-established plant model system. Final conclusions on pathogenicity have always to be verified in the main host plant *B. napus*.

V. longisporum DNA in plant tissue infected with *Vlaro*2sm correlate with reduced virulence

Saprotrophic vegetative growth of *Vlaro*2sm was reduced without aromatic amino acids, and disease symptoms in the plant were also reduced. We analyzed whether there is also reduced growth of the mutant in the plant or whether the plant is able to inhibit fungal growth. Therefore, the total biomass of *V. longisporum* in *B. napus* infected with *Vlaro*2sm versus wild type was analyzed by quantification of the *V. longisporum* DNA in the plant tissue by real-time PCR. The hypocotyls and top stems were harvested from the rapeseed plants infected with wild type or *Vlaro*2sm at various time points. Total DNA was extracted from the plant tissue, and the fungal DNA was quantified with real-time PCR. In the plant stem, at 21 dpi, there was negligible amount of *V. longisporum* DNA in the tissue samples of both wild type and *Vlaro*2sm. At 28 and 35 dpi, the amount of *V. longisporum* DNA was significantly lower (13% and 3%, respectively) in *Vlaro*2sm-infected plants in comparison to wild type (Fig. 6a). At 35 dpi, the *V. longisporum* DNA amount increased dramatically in the stem of wild-type-infected plants probably due to the outbreak of infection in the whole plant at this late stage of infection (Eynck et al. 2007). In the hypocotyls, *V. longisporum* DNA concentration was lower (5–34%) in *Vlaro*2sm-infected plants compared to the wild-type-infected plants throughout the infection study from early stage of infection at 14 dpi to late stage of infection at 35 dpi (Fig. 6b).

Similarly, the DNA of *V. longisporum* in *A. thaliana* infected with *Vlaro*2sm versus wild type was analyzed by real-time PCR. At 28 dpi, *V. longisporum* DNA was present but represented in *Vlaro*2sm-infected plants only 5% of the fungal DNA in wild-type-infected plants (Fig. 6c). Therefore, the reduced amounts of *V. longisporum* DNA in *Vlaro*2sm-infected *B. napus* and *A. thaliana* plants corre-

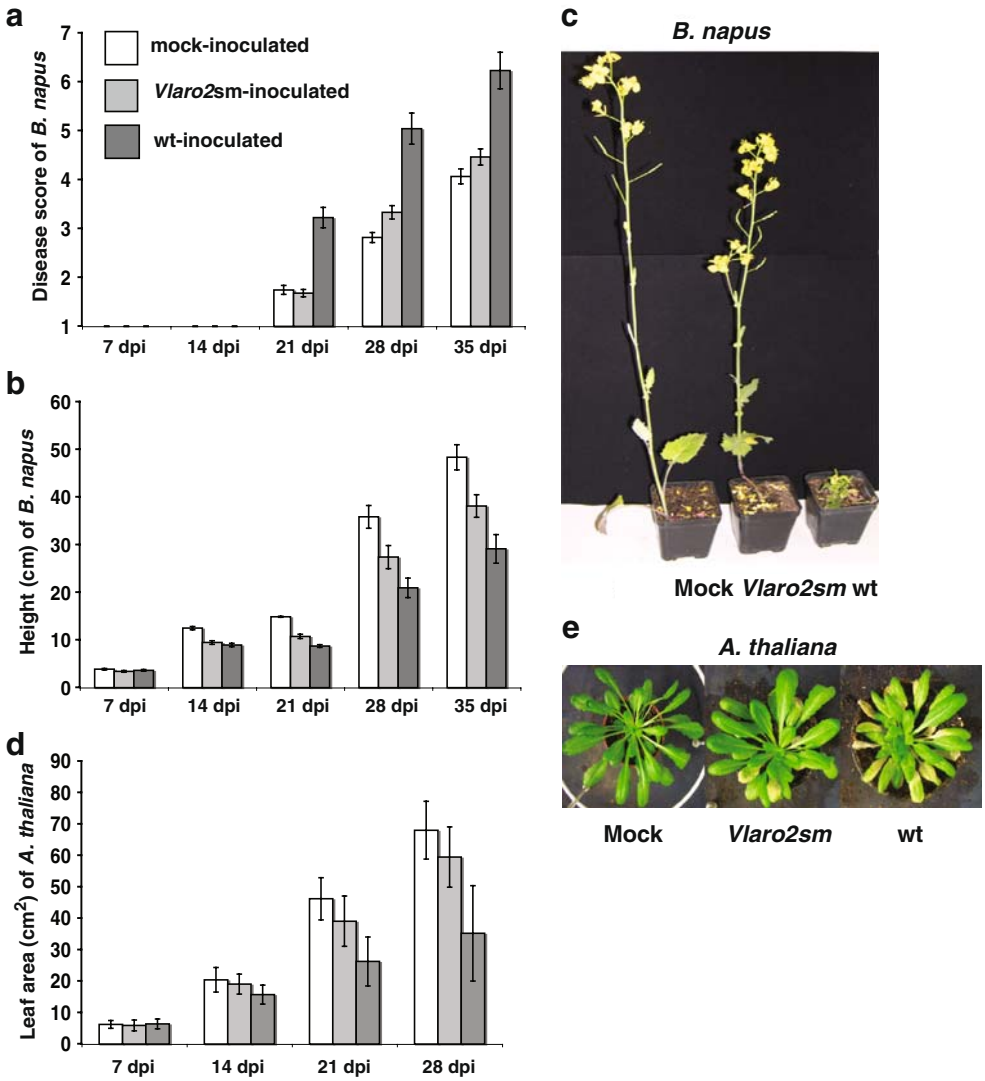
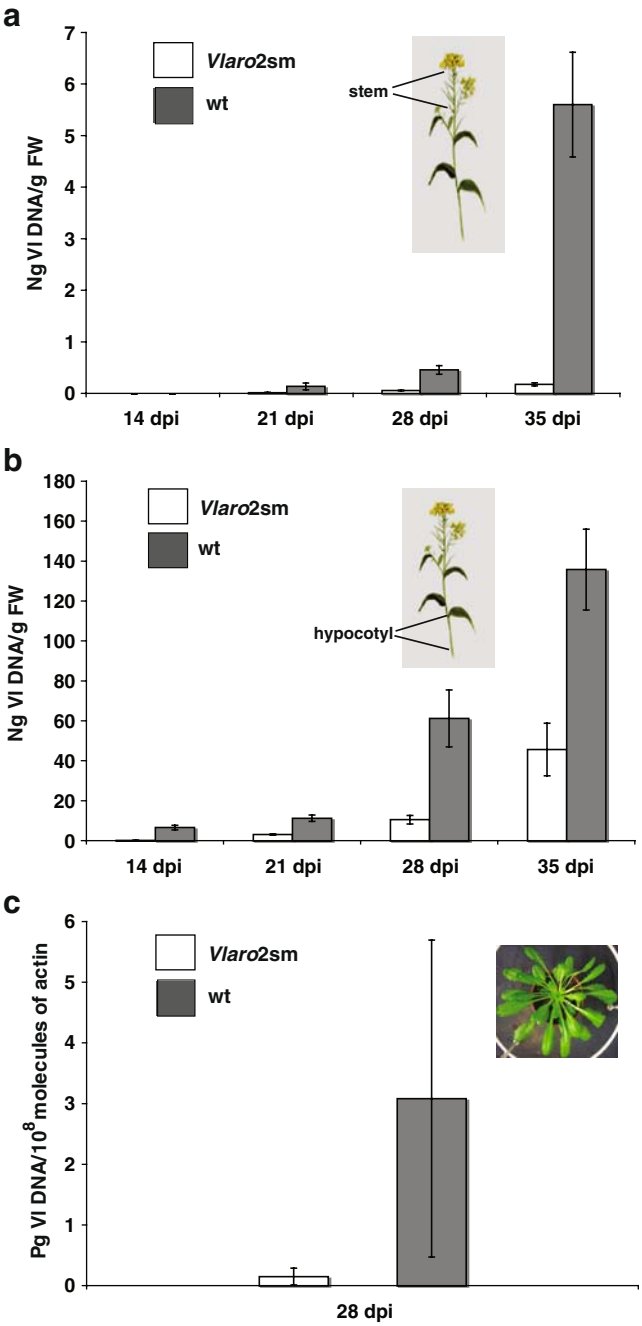


Fig. 5 Assessment of pathogenicity of the *Vlaro*2 silenced mutant. **a** Assessment of disease development by scoring for disease symptoms according to Eynck et al. (2007). Plants were scored for disease symptoms at 7, 14, 21, 28, and 35 dpi. Data represent average ± standard deviations of 20 experimental replicates. *wt*, wild type; *Vlaro2sm*, *Vlaro*2 silenced mutant; *mock*, mock inoculation with water. **b** Assessment of stunting of rapeseed due to *V. longisporum* infection. The height of 20 replicates each of rapeseed plants infected with wild type (wt) and *Vlaro*2 silenced mutant (*Vlaro*2sm) was measured at 7, 14, 21, 28, and 35 dpi. For comparison, the height of rapeseed plants mock-inoculated (mock) with tap water was also measured. The plants are heavily infected at 28 and 35 dpi. Data represent average ± standard deviations of 20 experimental replicates. **c** Rapeseed infection assay. Representative *B. napus* plants shown at 35 dpi. **d** Assessment of disease development in *A. thaliana* by measuring the leaf area. The area of the leaves of *A. thaliana* plants infected with wild type (wt), *Vlaro*2 silenced mutant (*Vlaro*2sm) or mock-inoculated with water was measured at 7, 14, 21, and 28 dpi. Data represent average ± standard deviations of 30 experimental replicates. **e** *Arabidopsis* infection assay. *A. thaliana* plants are shown at 28 dpi

lated to the slower growth and the reduced virulence of the mutant.

The cross-pathway regulator, VlcpcA is induced during infection of *B. napus* and *A. thaliana*

We compared the expression of *Vlaro*2 in *B. napus* infected with *V. longisporum* wild-type strain and *B. napus* infected with *Vlaro*2sm to analyze whether the expression of *Vlaro*2 is reduced in *Vlaro*2sm after infection of *B. napus.* The hypocotyls were harvested from the rapeseed plants infected with wild type or *Vlaro*2sm at 35 days post inoculation. Total RNA was extracted from the hypocotyls and quantitative real-time PCR was performed for *Vlaro*2 expression in mutant and wild type. *Vlaro*2 was still expressed but significantly reduced to 14% in the hypocotyl from *B. napus* infected with *Vlaro*2sm as compared to the expression in the wild type (Fig. 7a). Similar *Vlaro*2



◀ **Fig. 6** Infection assay and determination of the *V. longisporum* DNA concentration in the infected plant tissue. **a** *V. longisporum* DNA concentration in the plant stem. **b** *V. longisporum* DNA concentration in the hypocotyl. *V. longisporum* DNA was measured with real-time PCR in stem and hypocotyl of *B. napus* inoculated with *Vlaro*2 silenced mutant (*Vlaro2sm*) and wild type (*wt*) at 14, 21, 28, and 35 dpi. Data represent average ± standard deviations of three experimental replicates. The mock-inoculated plants as a control did not show presence of any *V. longisporum* DNA. *Ng VL DNA/g FW* = nanogram *V. longisporum* DNA/gram fresh weight of plant tissue. (In the *inset*, a representative rapeseed plant depicting the stem (5–6 cm from the top) and the hypocotyl harvested for the quantification of the *V. longisporum* DNA is shown). **c** *V. longisporum* DNA concentration in *A. thaliana*. DNA was measured with real-time PCR in *A. thaliana* inoculated with *Vlaro*2 silenced mutant (*Vlaro2sm*) and wild type (*wt*) at 28 dpi. Data represent average ± standard deviations of three experimental replicates. The mock-inoculated plants as a control did not show presence of any *V. longisporum* DNA. *Pg Vl DNA/10^8 molecules of actin*, picogram *V. longisporum* DNA/10^8 molecules of actin of *A. thaliana*

expression was also observed in *Vlaro*2sm infected *A. thaliana* and wild-type-infected *A. thaliana* (Fig. 7b). Therefore, *Vlaro*2 expression was similarly reduced in *Vlaro*2sm in infected plants and in liquid culture (Fig. 4a).

We also compared the expression of the CS encoding gene with the expression of the gene for the cross-pathway regulator CpcA as an indicator for amino acid starvation. The *VlcpcA* expression was increased 3.5-fold and 5.3-fold in the hypocotyl from *B. napus* infected with wild type and *Vlaro*2sm, respectively, compared to the expression in wild-type liquid culture (Fig. 7c). The *VlcpcA* expression in wild-type-infected *A. thaliana* increased 2.6-fold and in *Vlaro*2sm infected *A. thaliana* 5.8-fold compared to the expression in wild-type liquid culture (Fig. 7d). The upregulation of *VlcpcA* in plants infected with wild type suggests that this is due to *V. longisporum* encountering general amino acid starvation during biotrophic growth. We assume that the low levels of amino acids present in the xylem sap create an amino acid imbalance resulting in amino acid starvation and then "turn on" the cross-pathway control.

Discussion

The vascular pathogen *V. longisporum* inhabits the xylem vessels of the *B. napus* plant during colonization and infection of this oilseed crop. The xylem sap contains water, inorganic compounds, amino acids, and organic acids (Satoh et al. 1998; Kehr et al. 2005). In this study, we present the first construction of a *V. longisporum* mutant strain, which is bradytrophic for aromatic amino acids. This strain is suited to explore whether *V. longisporum* is exploiting the xylem sap for nutrition or is utilizing its inherent amino acid biosynthetic pathways during its biotrophic phase. We studied the *V. longisporum* CS which catalyzes the production of chorismate, the last common precursor of the aromatic amino acids in the shikimate pathway. RNA-mediated gene silencing was exploited to generate *Vlaro*2 knockdown strains in *V. longisporum* because it acts in a sequence-specific manner (Matityahu et al. 2008) as opposed to the knockout strategy that functions in a locus-specific manner. Silencing is suitable for heterokaryons or polyploid organism like *V. longisporum* (de Jong et al. 2006) and has been also successfully applied in other fungi (Tanguay et al. 2006;

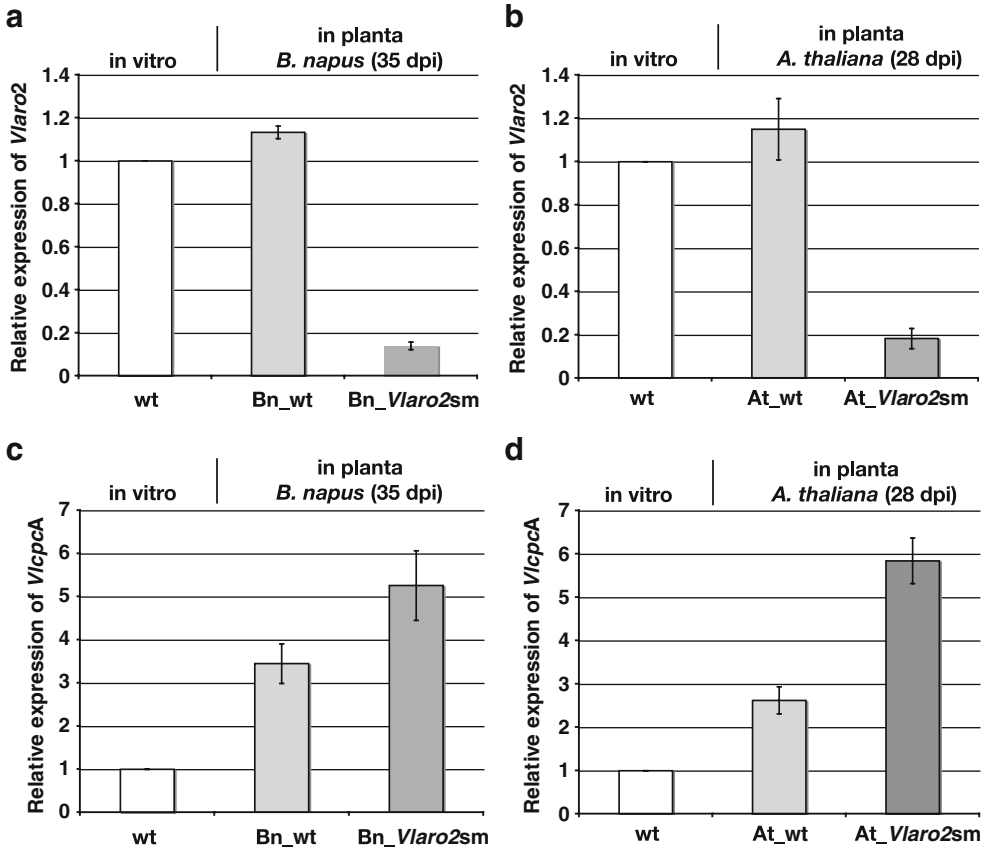


Fig. 7 Quantification of *Vlaro*2 and *Vlcpc* expression in *B. napus* and *A. thaliana.* **a** Relative expression of *Vlaro*2 in *B. napus* measured by quantitative real-time PCR. *Vlaro*2 cDNA was normalized to the ß-tubulin cDNA. cDNA from host plants mock-inoculated with water served as control. *wt*, wild type, *Bn_wt*, cDNA from *B. napus* infected with wild type, *Bn_Vlaro2sm*, cDNA from *B. napus* infected with *Vlaro*2 silenced mutant. The *error bars* represent the standard deviations of triplicates. **b** Expression of *Vlaro*2 relative to the ß-tubulin cDNA measured in *A. thaliana* by quantitative real-time PCR. cDNA from host plants mock-inoculated with water served as control. *wt*, wild type, *At_wt*, cDNA from *A. thaliana* infected with wild type, *At_Vlaro*2sm, cDNA from *A. thaliana* infected with *Vlaro*2 silenced mutant. The *error bars* represent the standard deviations of triplicates. **c** Relative expression of *VlcpcA* in *B. napus* measured by quantitative real-time PCR. *VlcpcA* cDNA was normalized to the ß-tubulin cDNA. cDNA from host plants mock-inoculated with water served as control. *wt*, wild type, Bn_*wt*, cDNA from *B. napus* infected with wild type, *Bn_Vlaro2sm*, cDNA from *B. napus* infected with *Vlaro*2 silenced mutant. The *error bars* represent the standard deviations of triplicates. **d** Relative expression of *VlcpcA* in *A. thaliana* measured by quantitative real-time PCR. *VlcpcA* cDNA was normalized to the ß-tubulin cDNA. cDNA from host plants mock-inoculated with water served as control. *wt*, wild type, *At_wt*, cDNA from *A. thaliana* infected with wild type, *At_Vlaro2sm*, cDNA from *A. thaliana* infected with *Vlaro*2 silenced mutant. The *error bars* represent the standard deviations of triplicates

Krajaejun et al. 2007; Nakayashiki et al. 2005). The degree of silencing varied in the *V. longisporum Vlaro*2sm from high to moderate levels at both the transcript and protein levels. The variation in the degree of silencing in the silenced mutants has also been observed in other fungi (Fitzgerald et al. 2004; Mouyna et al. 2004). The Vlaro2 protein was reduced down to 6% relative to the wild-type protein level. A particular disadvantage of silencing is that it is usually not absolute because residual activity of the gene remains. This strategy has the advantage to explore the function of genes under conditions where they are essential and where small amounts of gene expression are sufficient to keep the organism viable. The yeast *ARO2* mutation results in auxotrophy and therefore has to be supplemented by amino acids for growth (Jones et al. 1991). Auxotrophic mutants for *V. longisporum* CS would not have survived without supplementation of the culture medium by aromatic amino acids. Only the knockdown of *Vlaro*2 with a residual activity allowed the possibility to study the role of amino acids during biotrophic growth in the xylem.

Pathogenicity assays of the *Vlaro*2sm were not only performed in the host, *B. napus* but also in the model plant, *A. thaliana*. All *Vlaro*2sm-infected plants showed reduced stunting symptoms and disease scores. The reduced infection of host plants correlates to highly reduced growth of *V. longisporum* in planta. The successful usage of the *A. thaliana* model system might facilitate further studies, although the impact on the authentic host will be always ultimately necessary.

The reduced rate of chorismate production in the bradytrophic mutant might also reduce the amount of secondary metabolites derived from aromatic amino acids. This could have an additional effect on the biotrophic lifestyle because secondary metabolites might help in propagation of the fungus in the host rapeseed plant. Aromatic amino-acid-derived secondary metabolites include auxins, melanin, or terrequinone A. The phytopathogenic fungi, *Ustilago esculenta* and *Colletotrichum acutatum*, can produce the plant hormone indole-3-acetic acid (IAA; auxin) efficiently from the aromatic amino acid tryptophan (Chung and Tzeng 2004; Lahey et al. 2004). A tomato isolate of *V. albo-atrum* produced IAA in CD medium (Pegg 1959). IAA production is also reported on a medium containing L-tryptophan by *V. dahliae* (Bhaskaran 1972). The enzymes in the shikimate pathway, chorismate mutase, and anthranilate synthetase have been described in the synthesis of IAA by *V. albo-atrum* (Pegg 1987). Auxins are known to stimulate the production of the gaseous plant hormone, ethylene (Arteca and Arteca 2008). Ethylene can inhibit growth of the plant stem and cause chlorosis, necrosis, and falling of leaves of the plant (Benavente and Alonso 2006). *V. longisporum* infection causes disease symptoms of stunting and early senescence in the rapeseed plants. It remains to be elucidated whether this is caused by generation of ethylene stimulated by auxin production by the fungus.

We further find that the expression of the gene for the major regulator of the cross-pathway control system is constitutively increased in *V. longisporum* during its growth in the xylem. The cross-pathway control is a complex global regulatory network, which regulates the synthesis of amino acids in numerous fungi. In the yeast *S. cerevisiae*, the same system had been named general control (Hinnebusch 1984; Natarajan et al. 2001). The cross-pathway control has been studied in several filamentous fungi, including *A. nidulans* (Piotrowska 1980; Hoffmann et al. 2001), *A. niger* (Wanke et al. 1997), *A. fumigatus* (Krappmann et al. 2004), *C. albicans* (Tripathi et al. 2002), and *N. crassa* (Carsiotis et al. 1974; Paluh et al. 1988). Amino acid starvation conditions activate this system resulting in derepression of the cross-pathway control gene *GCN4* in yeast or *cpcA*/*cpc-1* in filamentous fungi, respectively. Whereas in yeast there is primarily a translational control which determines the amount of Gcn4 in the cell (Albrecht et al. 1998), there is an additional amino-acid-starvation-induced autoactivation present in filamentous fungi like *A. nidulans* (Hoffmann et al. 2001). In *V. longisporum*, we found that *Vlaro2* silencing induced the expression of the gene for amino acid cross-pathway control, *VlcpcA* during saprotrophic life. This suggests that there is a similar autoactivation of *VlcpcA* as earlier described for *A. nidulans cpcA*. Consistently, *VlcpcA* was also induced under tryptophan starvation conditions in the wild type, corroborating that the fungus responds by increased *VlcpcA* expression to amino acid starvation. *VlcpcA* was similarly induced in the wild type and in the *Vlaro*2sm during infection of *B. napus* and *A. thaliana.* This suggests that *A. thaliana*, which is much easier to study than *B. napus*, provides at least some similar signals to the fungus. We assume that the signal "amino acid starvation" is induced by the low and imbalanced levels of amino acids in the xylem vessels.

In addition to its role as amino acid regulator, the cross-pathway control regulator Gcn4/CpcA/Cpc-1 has various distinct morphological and pathogenic functions in fungi. Gcn4 controls starvation-induced adhesive growth in the yeast *S. cerevisiae* (Braus et al. 2003), which is required for pseudohyphal development. In *A. nidulans*, cross-pathway control is connected to cellular differentiation as increase in activity of the CpcA resulted in a reversible block of fruit body formation (Hoffmann et al. 2000). Histidine starvation in *C. albicans* induced morphogenetic responses dependent on the global regulator, CaGcn4 (Tripathi et al. 2002). In *A. fumigatus*, mutants impaired in cross-pathway control system had strongly reduced pathogenicity and CpcA seemed to be involved in establishing pulmonary aspergillosis effectively (Krappmann et al. 2004; Sasse et al. 2008). In *V. longisporum*, *VlcpcA* seems to aid in the growth of the fungus in the xylem vessels where amino acids are scarce. It will be interesting to see whether *VlcpcA* might have an additional important role in the establishment of the fungus in the host xylem vessels and subsequently in the pathogenicity of *V. longisporum.* The establishment of an efficient gene silencing system provided by this study is an essential prerequisite for the further analysis of this fungus and for the long-standing goal to control the biotrophic life phase efficiently.

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