Roles of the Cpc1 regulator of the cross-pathway control in the *Verticillium* plant pathogens

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Christian Timpner

aus Northeim

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Betreuungsausschuss

Prof. Dr. Gerhard Braus, Abteilung f. molekulare Mikrobiologie und Genetik, Institut für Mikrobiologie und Genetik

Mitglieder der Prüfungskommission

Referent: Prof. Dr. Gerhard Braus, Abteilung f. molekulare Mikrobiologie und Genetik, Institut für Mikrobiologie und Genetik

Korreferentin: Prof. Dr. Andrea Polle, Abteilung Forstbotanik u. Baumphysiologie, Büsgeninstitut

Weitere Mitglieder der Prüfungskommission:

Prof. Dr. Stefanie Pöggeler, Abteilung f. Genetik eukaryotischer Mikroorganismen, Institut für Mikrobiologie und Genetik

Jun.-Prof. Dr. Kai Heimel, Abteilung f. Mikrobielle Zellbiologie, Institut f. Mikrobiologie u. Genetik

PD Dr. Stefan Irniger, Abteilung f. molekulare Mikrobiologie und Genetik, Institut f. Mikrobiologie u. Genetik

PD Dr. Thomas Teichmann, Abteilung f. Zellbiologie der Pflanze, Schwann-Schleiden Zentrum

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Abbreviations

AFLP	amplified fragment length polymorphism
AmpR	ampicillin resistance
ATMT	*Agrobacterium tumefaciens*-mediated transformation
Bp	base pair
CDM	Czapek-Dox medium
cDNA	complementary DNA
CPC	Cross-Pathway Control
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetate
h	hour
H_2O_2	hydrogen peroxide
HPH	hygromycin phosphotransferase
IGS	intergenic spacer
ITS	internal transcribed spacer
KanR	kanamycin resistance
kb	kilobase
LB	left border / Luria Bertani medium
LiAc	Lithium acetate
min	minute
ml	milliliter
mM	milimolar
MM	minimal medium
NAT	nourseothricin acetyltransferease
ORF	open reading frame
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose both
RB	right border
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
s	second
S	Svedberg unit
SDS	sodium dodecyl sulphate
SXM	simulated xylem medium
TE	Tris-Cl and EDTA
UV	ultraviolet
Va	*Verticillium albo-atrum*
VCG	vegetative compatibility group
Vd	*Verticillium dahliae*
Vl	*Verticillium longisporum*
WT	wild type
µl	microliter
µm	micrometer

Summary

Verticillium longisporum is a soil-borne fungal pathogen of oilseed rape (*Brassica napus*). Infection is initiated by hyphae from germinating microsclerotia which invade the plant vascular system through penetration of the fine roots. Most of its life cycle, *V. longisporum* is confined to the vascular system of the plant. The xylem fluid provides an environment with limited carbon sources and imbalanced amino acid supply, which requires that *V. longisporum* induces the cross-pathway control of amino acid biosynthesis. *VlCPC1* encodes the conserved transcription factor of the cross-pathway control. RNA-mediated gene silencing reduced the expression of the two *CPC1* isogenes (*VlCPC1-1, VlCPC1-2*) of the allodiploid *V. longisporum* up to 85%. The silenced mutants were highly sensitive to amino acid starvation and the infected plants showed significantly less 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, which 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, which were shown to require *CPC1* for infection and colonization of their respective host plants oilseed rape and tomato. We investigated secondary metabolism as it might be required for pathogenicity. The key regulator of secondary metabolism *LAE1* was further investigated. Silenced mutants reduced the expression of the two isogenes (*VlLAE1-1*, *VlLAE1-2*) up to 80% and showed milder symptoms on plants. The corresponding deletion in *V. dahliae* did not show any contribution to pathogenicity. Furthermore a secreted lipase was silenced as well. The efficiency of silencing reached around 80% and the mutants showed a reduced pathogenicity on oilseed rape in plant infection assays.

Zusammenfassung

Verticillium longisporum ist ein bodenbürtiger pathogener Pilz, welcher Raps (*Brassica napus*) befällt. Eine Infektion geht von Hyphen aus, die aus keimenden Mikrosklerotien entstanden sind und die Pflanze über Penetration der Wurzelhaare infizieren. Der Lebenszyklus von *V. longisporum* ist zu einem Großteil auf das vaskuläre System der Pflanze beschränkt. Xylemsaft stellt einen Lebensraum mit eingeschränkten Kohlenstoffquellen und einer unausgewogenen Aminosäureversorgung dar. Unter diesen Bedingungen aktiviert *V. longisporum* das Gen für den Regulator der „cross pathway control“ für Aminosäurebiosynthesen. Über RNA-vermitteltes Gensilencing konnte die Expression der beiden *CPC1* Isogene (*VlCPC1-1*, *VlCPC1-2*) für diesen Regulator im allodiploiden Pilzes *V. longisporum* um 85% verringert werden. *VlCPC1* codiert den konservierten Transkriptionsfaktor der cross pathway control. Die gesilencenten Mutanten reagierten sehr empfindlich auf Aminosäuremangelbedingungen und die infizierten Pflanzen zeigten schwächer ausgeprägte Symptome der Krankheit wie Stauchung und frühere Reifung in den durchgeführten Pflanzeninfektionsreihen mit Raps. Eine Deletion von *CPC1* im haploiden *V. dahliae* generierte Stämme, welche sensitiv auf Aminosäuremangelbedingungen reagieren mit stark verringerten Symptome auf seine Wirtspflanze Tomate (*Solanum lycopersicum)*. Der allodiploide *V. longisporum* und der haploide *V. dahliae* sind die ersten phytopathogenen Pilze in denen gezeigt werden konnte, dass *CPC1* für Infektion und Kolonisierung ihrer jeweiligen Wirte benötigt wird. Der Sekundärmetabolismus des Pilzes könnte ebenso eine Rolle für die Pathogenität spielen. Der Regulator des Sekundärmetabolismus *LAE1* wurde weitergehend untersucht. Die Expression der beiden Isogene in *V. longisporum* (*VlLAE1-1*, *VlLAE1-2*) wurde um 80% reduziert und zeigten verringerte Symptome auf Raps. Die entsprechende Deletion in *V. dahliae* zeigte keinen Beitrag zur Pathogenität des Pilzes. Weiter wurde eine Lipase identifiziert, die vom Pilz im Xylemsaft sezerniert wird. Diese sekretierte Lipase konnte ebenso gesilenced werden. Die Effizienz lag bei 80% und die Mutanten zeigten eine verringerte Pathogenität in Pflanzeninfektionsreihen auf Raps.

Chapter 1. Introduction

1.1 *Verticillium*: phytopathogenic fungi spreaded worldwide

The *Verticillium* species are soil-borne plant pathogenic fungi. They are widely spread around the world and cause so-called vascular wilting diseases and early senescence in a broad number of economically important crops including alfalfa, cotton, lettuce, hops, olive trees, oilseed rape, cabbages, potato, tomato and strawberries. *Verticillium dahliae*, *V. albo-atrum* and *V. longisporum* are the species which cause the highest losses of crops (Pegg and Brady, 2002; Zeise and von Tiedemann, 2002a; Zeise and von Tiedemann, 2002b; Agrios, 2005). The name *Verticillium* is based on the phialides arrangement in whorls (verticillate shape) around the conidiophores and branching of the conidiophores also occurs in whorls at some levels (Fig. 1). The fungal mycelium is hyaline, simple or branched, septated and multinucleated. Each phialide carries a number of ovoid to elongated conidia (Berlanger and Powelson, 2000; Fradin and Thomma, 2006). The first *Verticillium* strain was detected in 1879 by Reinke and Berthold, who investigated wilt on potato, and isolated the causal agent, which was named *Verticillium albo-atrum* (Reinke and Berthold, 1879; Hastie, 1973; Klosterman *et al.*, 2009).

In 1913 Klebahn described a second species causing wilt on dahlia (*Asteraceae* family) named *V. dahliae* with morphological distinction (Isaac, 1947; Hastie, 1973). In 1961, Stark isolated a *V. dahliae*-like fungus from horseradish in Hamburg, Germany (Stark, 1961). This fungus was named *V. dahliae* var. *longisporum*. It produces microsclerotia like *V. dahliae* but conidia are significantly longer than those of typical *V. dahliae* strains. Therefore it was named *V. dahliae* var. *longisporum*. Besides the fact that isolates from crucifers produced longer conidia, as had already been noted by Stark (1961), they found additional differences with regard to morphological, enzymatic, molecular and virulence characteristics (Fig. 1). On the basis of the morphological differences and other characteristics of many similar strains, Karapapa *et al.* suggested long-spored isolates as a new species, *V. longisporum* (Karapapa *et al.*, 1997). Since scientists have worked with this fungus, there has been a controversy concerning the recognition of *V. longisporum* as a separate host-specific species. Currently, the genomes of *V. dahliae* and *V. albo-atrum* have been sequenced by the Broad Institute, Harvard-Massachusetts (Klosterman *et al.* 2009). Sequencing of *V. longisporum* genome is in progress by the Biofung project at the University of Göttingen.

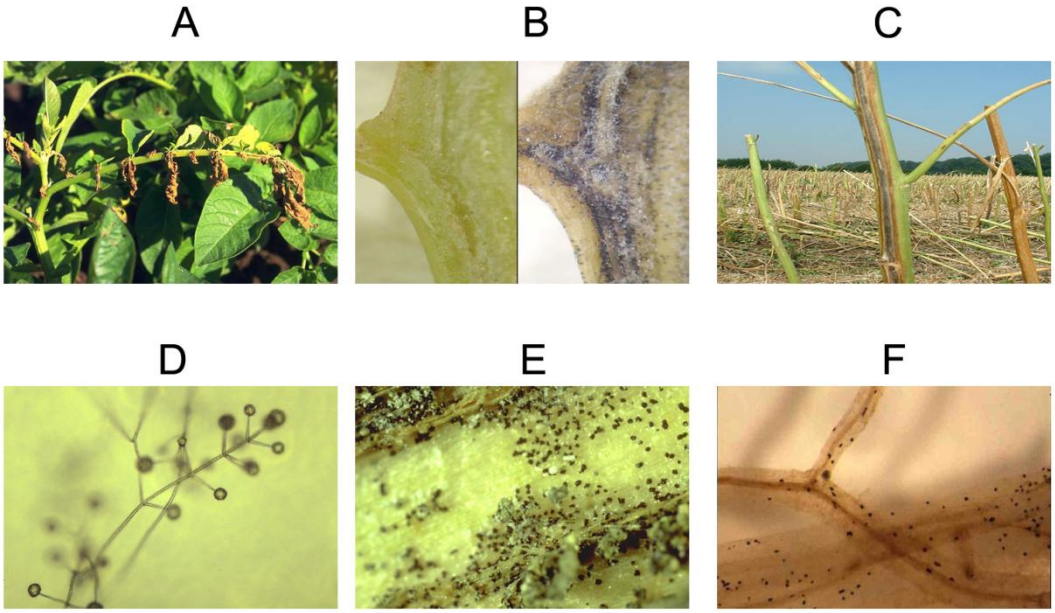

Fig. 1. Wilt disease symptoms and morphological structures of *Verticillium* pathogens. (adapted from Heale and Karapapa, 1999; Berlanger and Powelson, 2000; Andrie *et al.*, 2005; Eynck *et al.*, 2009). **(A)** Leaf necrosis and wilt in potato. **(B)** Longitudinal section through the stem of a healthy spinach plant (left) and a spinach plant infected with *Verticillium dahliae* (right). **(C)** Necrotic symptom of infected rapeseed stems with black microsclerotia. **(D)** *Verticillium* conidia masses on phialides arranged in whorls (verticillate) around conidiophores. **(E)** The survival structure as microsclerotia of *V. dahliae* on infected potato stems. **(F)** Microsclerotia of *V. longisporum* on infected rapeseed.

1.2. Disease symptoms by *Verticillium* species

In comparison with *Fusarium* wilt symptoms induced by *Verticillium* are quite similar and difficult to observe in the field (Babadoost *et al.*, 2004). Symptoms caused by the fungus develop steadier and mainly on the lower and outer parts of plants. Since indicators for disease can vary between different plant hosts, no absolute diagnostic method for *Verticillium* has been established (Rowe and Powelson, 2002). Anyhow infection with *Verticillium* shows some characteristics, like vascular discoloration in stems and roots and premature foliar chlorosis and necrosis. In some hosts, older plants infected with *Verticillium* are usually stunted in various degrees (Kim *et al.*, 2001; Pegg and Brady, 2002; Rowe and Powelson, 2002; Fradin and Thomma, 2006; Gladders *et al.*, 2011).

1.3. *Verticillium* disease cycle

Verticillium wilt is a monocyclic disease, as there is only one cycle of disease per season (Fig. 2). *V. longisporum* life cycle is similar to that of other *Verticillium* species and can be

divided into a dormant, a parasitic and a saprophytic phase. To survive in the soil *V. dahliae* and *V. longisporum* have developed microsclerotia as resting structure, whereas *V. albo-atrum* forms resting mycelium (Karapapa *et al.*, 1997; Rowe and Powelson, 2002; Eynck *et al.*, 2007). Microsclerotia are melanized aggregates of enlarged hyphal cells (Schnathorst, 1981; Heale and Karapapa, 1999). These structures are generated under growth limiting conditions, and remain viable in the soil for several years (Schnathorst, 1981; Heale and Karapapa, 1999); up to 14 years for microsclerotia (Wilhelm, 1955) and around 2-5 years for resting mycelium (Sewell and Wilson, 1964). To compete in the soil ecosystem microsclerotia must withstand microbial degradation, lysis, parasitism and predation, and therefore present an important nutrient state available in the soil (Okubara and Paulitz, 2005). Under favorable conditions microsclerotia can start to germinate again, usually after stimulation with root exudates or contact with the host plant (Zhou *et al.*, 2006; Eynck *et al.*, 2007). These hyphae originating from resting structures grow towards the roots of the plants, and infect through the root tips or follow the root hairs. After breaching the cell wall, fungal hyphae grow through cortical tissues towards developing vascular tissue (Zhou *et al.*, 2006; Eynck *et al.*, 2007).

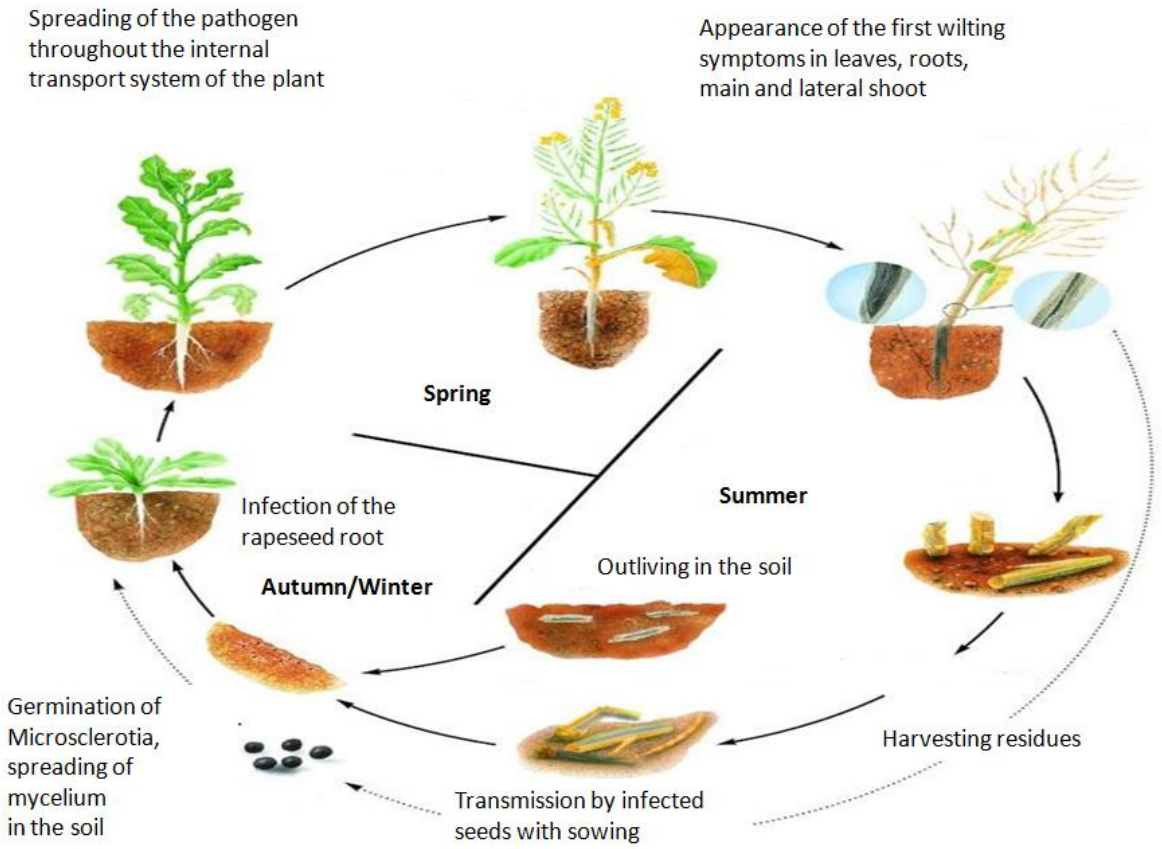

Fig. 2 . The life cycle of *V. longisporum* on oilseed rape (modified from Paul, 2003). Like *V. dahliae*, the infection cycle of *V. longisporum* starts with germination of microsclerotia under the stimulation of root exudates. The fungus enters the plant through the root hairs and grows in the plant vascular system until the first symptoms of stunting and chlorosis can be observed. When the plant becomes old, the fungus produces microsclerotia in plant roots and shoots. These resting structures can be released from dead plant materials into the soil for the next cycle. Microsclerotia can survive in the soil for several years without rapeseed plants.

The fungus colonizes the vascular system and does not leave this environment until senescence and maturity stages of the host plant. The fungal proliferation in this environment is primarily obtained by spore release because colonization by vegetative growth would be too slow due to the poor mycelial growth rate (Presley *et al.*, 1966). Conidia are produced within the xylem vessels and move along with the transpiration stream. Thereafter colonization is pursued via germination of conidia and penetration of germ tubes through full-bordered pits into adjacent vessel elements (Garas *et al.*, 1986), and that way starting another infection cycle. This kind of host colonization may represent on the one hand the observed discontinuous occurrence of mycelium in the plant vascular system (Beckman, 1987; Heinz *et al.*, 1998) and on the other hand the rapidity with which systemic colonization occurs (Zinkernagel, 1982; Gold and Robb, 1995; Heinz *et al.*, 1998; Chen *et al.*, 2004). Consequently, the fungus emerges from the xylem vessels to colonize

neighboring vascular and cortical tissues, resulting in the development of disease symptoms such as wilting, chlorosis and necrosis.

With the progression of disease development, senescence of the host begins, and the pathogen enters a limited saprophytic growth phase in which microsclerotia are formed in the dying stem parenchyma (Schnathorst, 1981; Neumann and Dobinson, 2003). Unlike other *Verticillium* species, *V. longisporum* does not induce wilt symptoms in oilseed rape grown on the field. The colonization of the plant is characterized by an extended latent phase, early symptoms like streaky yellow to brownish discolorations of the stem and half-sided yellowing of the leaves as well as chlorotic patches between veins which themselves turn blackish, are not monitored before the beginning of plant maturity. Past the initiation of maturity the fungus starts to advance out of the xylem vessels and starts to generate large numbers of microsclerotia underneath the stem epidermis, in the stem pith and in the roots. Further symptoms of the disease are premature bloom and maturity as well as stunted growth, the latter occurring particularly under standardized conditions in the greenhouse or climate chamber. Regarding these considerations, a more appropriate term to describe the disease caused by *V. longisporum* on crucifers would rather be *Verticillium* premature senescence (VPS) or *Verticillium* premature ripening (VPR) (Eynck *et al.*, 2009) than *Verticillium* wilt. Resistance to *Verticillium* according to Pegg & Brady (2002) can be defined as ´the total or partial absence of symptoms in comparison with other host species or cultivars similarly exposed to virulent pathotypes and showing severe damage or death`. The systemic nature of *Verticillium* infections makes it difficult to quantify the cellular bases of resistance and susceptibility to wilt disease (Gold and Robb, 1995).

1.4. *V. longisporum* hybrids prefer *Brassicaceae* as host

Due to the limitation of natural resources the demand for bio fuels has been rising within the last ten years, and has increased the cultivation of oil producing plants. In our days oilseed rape is one of the most important oil sources, next to soybean and cottonseed. The opposite site of this development is the progression of pathogens like *V. longisporum* (Heale and Karapapa, 1999) (Heale and Karapapa, 1999; Pua and Douglas, 2004). Infection of *Brassica* crops with this fungus has been reported for many European countries like Germany, France, Poland and Sweden, but it has also been announced for Canada (Svenson and Lerenius, 1987; Zielinski and Sadowski, 1995; Karapapa *et al.*, 1997; Zeise and von Tiedemann, 2002a; Dixelius *et al.*, 2005). *V. longisporum* is a soil-borne fungal pathogen and host-specific on the *Brassicaceae* family such as oilseed rape

(*Brassica napus)*, cabbage (*Brassica oleracea* var. *capitata*), horseradish (*Armoracia rusticana*), cauliflower (*Brassica oleracea* var. *botrytis*). Though infection with *V. longisporum* does not cause true wilting symptoms on oilseed rape, however premature senescence and ripening are accompanied by systemic spread and extensive formation of microsclerotia on shoot tissue. This can cause yield reductions up to 50-70 %. In greenhouse experiments chlorosis and stunting are mainly applied to determine the disease development, but in field studies stunting has not been observed so far (Dunker *et al.*, 2006). Some greenhouse pathogenicity assays with *Brassica* crops showed that *V. longisporum* isolates are most virulent, whereas *V. dahliae* strains are non-pathogenic or weakly virulent (Zeise and von Tiedemann, 2002a). *V. longisporum* infects mainly oilseed rape causing losses in plant fresh weight of 49% and killing about half of the plants at 42 days post inoculation (Zeise and von Tiedemann, 2002a). This pathogen can also infect some other non-host plants (Bhat and Subbarao, 1999; Fahleson *et al.*, 2004; Johannson *et al.*, 2005) and conversely, other *Verticillium* species also weakly infect *B. napus* (Zeise and von Tiedemann, 2002a; Collins *et al.*, 2003).

1.5. *Verticillium* taxonomy

The morphology of *Verticillium* implies characteristic verticilliate arrangement of the three to five asexual spore carrying structures (phialides) forming branches at each node of the conidiophores (Kim *et al.*, 2001). *V. dahliae* and *V. albo-atrum* are two closely related but distinct mature species. *V. dahliae* forms microsclerotia as a resting form which are melanized clumps formed by budding of mycelial cells (Goud *et al.*, 2003), whereas *V. albo-atrum* forms melanized resting mycelium. To distinguish *V. dahliae* and *V. albo-atrum* the most common feature is the formation of these resting structures (Pegg and Brady, 2002). Futhermore *V. albo-atrum* is not able to grow in cultures or to infect its hosts at temperatures of 30°C, while *V. dahliae* still grows and infects plant (Rowe and Powelson, 2002). Via morphological description and rDNA isolates, *V. albo-atrum* can be divided into two groups, Grp1 and Grp2. Most *V. albo-atrum* strains are referred to Grp1 (group1) (Robb *et al.*, 1993; Morton *et al.*, 1995; Barbara and Clewes, 2003; Robinson *et al.*, 2007; Klosterman *et al.*, 2009). Morphological analysis showed that Grp2 (group2) isolates produce resting structures in bundles of melanized hyphae, whereas Grp1 isolates form melanized single hyphae (Mahuku and Platt, 2002). When analysing the sequence of the ITS region it could be demonstrated that the ITS of Grp2 isolates contain 17 bases that

are not found in the Grp1 isolates (Robb *et al.*, 1993; Mahuku and Platt, 2002). The genus *Verticillium* includes four other species: *V. tricorpus, V. nubilum, V. nigrescens* and *V. theobromae* (Barbara and Clewes, 2003). In contrast to *V. dahliae* and *V. albo-atrum*, *V. tricorpus* and *V. nubilum* are soil saprophytes that can grow in the absence of a potential host (Isaac and Milton, 1967). *V. tricorpus* is regarded as a weak pathogen on many hosts and produces survival structures including chlamydospores, microsclerotia and melanized hyphae (Robinson *et al.*, 2007; Qin *et al.*, 2008), whereas *V. nubilum* produces only chlamydospores as the resting structure (Griffiths, 1982; Barbara and Clewes, 2003). The species *V. nigrescens* and *V. theobromae* that are similar to other *Verticillium* species in morphology have been recently classified into the genera *Gibellulopsis* and *Musicillium*, according to the molecular evidences from rDNA (Zare *et al.*, 2007). In addition, *V. lecanii*, an entomopathogen and *V. fungicola*, a pathogen of mushrooms, were assigned to the genus *Lecanicillium* (Zare and Gams, 2008).

V. longisporum was first isolated from horseradish (Stark, 1961), it can be distinguished to the other *Verticillium* species by the number of phialides and the form of microsclerotia. The fungus generates preferentially only three phialides per node and survives by means of black but compared to *V. dahliae* elongated microsclerotia that are arranged in a bead like structure (Fig. 3). Conidia of *V. longisporum* are twice as long (7-9 µm) as those of *V. dahliae* (3.5-5.5 µm) (Subbarao *et al.*, 1995; Karapapa *et al.*, 1997; Zeise and von Tiedemann, 2001; Collins *et al.*, 2003). Isolates of *V. longisporum* have been collected from crucifers throughout the whole world like horseradish from Illinois (Eastburn and Chang, 1994), oilseed rapes from Europe and Canada (Heale and Karapapa, 1999; Zeise and von Tiedemann, 2001; Steventon *et al.*, 2002; Johannson *et al.*, 2005) or cauliflower from California (Koike *et al.*, 1994). Although many isolates have been closely investigated, the taxonomy of *V. longisporum* is still controversially discussed (Klosterman *et al.*, 2009; Inderbitzin *et al.*, 2011). Currently, the sexual stage has not been found in the genus *Verticillium* (Pegg and Brady, 2002; Klosterman *et al.*, 2009).

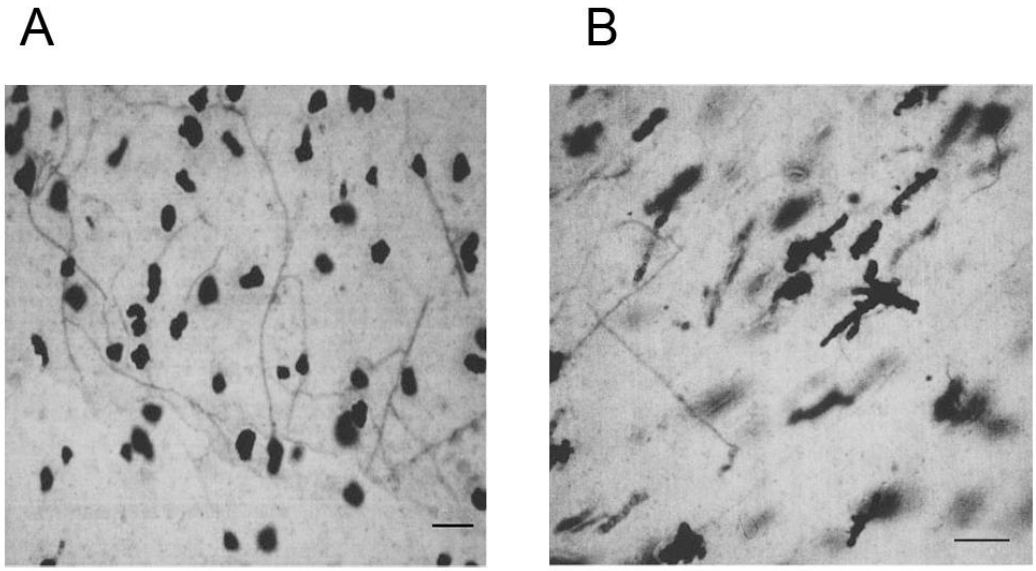

Fig. 3 Microsclerotia of *V. dahliae* and *V. longisporum*. (adapted from Karapapa *et al.* 1997) **A.** Mature, compact, spherical microsclerotia on rape medium of *V. dahliae.* **B.** Mature, irregular, elongate (much less compact) microsclerotia on oilseed rape medium of *V. longisporum.*

Based on sequence data Fahleson *et al.* (2004) suggested that *V. longisporum* should be regarded as a distinct species closely related to *V. albo-atrum*. Regarding the results of other authors, they conceded that the name *V. longisporum* might be somehow misleading because within the new species isolates with relatively short conidia could be grouped with long-spored isolates, at least on the molecular level (Steventon *et al.*, 2002), and a variety of different spore lengths could be observed for the same isolate (Subbarao *et al.*, 1995; Collins *et al.*, 2003). As a matter of fact, in the opinion of the authors, the host specifity of *V. longisporum* is not limited to only *Brassica* host plants as several other *Verticillium* species can use especially very weak plants, as a host, and *V. longisporum* can also infect plant species outside the *Brassicaceae* family (Johannson *et al.*, 2003; Johannson *et al.*, 2005). This latter statement contradicts the results of several investigations according to Zeise & von Tiedemann (2001, 2002a, 2002b) who showed that *V. longisporum* is a strictly host adapted pathogen specific for *Brassica* species. Additional confusion has been caused by some misidentifications of *V. dahliae* and *V. longisporum*. In several publications, *V. dahliae* has been considered to be the causal agent of *Verticillium* wilt in *Brassica* crops (Xiao and Subbarao, 2000; Söchting and Verreet, 2004) or on horseradish (Babadoost *et al.*, 2004), without considering that long-spored isolates may have been involved. On the basis of microsatellite and other marker analyses, Barbara and colleagues (Barbara and Clewes, 2003; Barbara *et al.*, 2005; Clewes and Barbara, 2005) addressed the

parental origin of the interspecific hybrid *Verticillium* isolates. They suggested that long-spored crucifer isolates have emerged through parasexual hybridisation between a species that is probably generally similar to other haploid *V. dahliae* isolates and one that is '*V. albo-atrum*-like' but clearly distinct and of unknown morphology.

V. longisporum was defined as 'near-diploid' or amphihaploid fungus with higher nuclear DNA amounts (about 1.8 times) than those of *V. dahliae* or *V. albo-atrum* isolates (Karapapa *et al.*, 1997; Steventon *et al.*, 2002; Collins *et al.*, 2003). This almost diploid status might be the reason why numerous mutagenesis approaches have failed (Ingram, 1968; Hastie, 1973; Nagao *et al.*, 1994; Subbarao *et al.*, 1995; Karapapa *et al.*, 1997; Zeise and von Tiedemann, 2001; Steventon *et al.*, 2002; Collins *et al.*, 2003). Most filamentous ascomycetes are primarily haploid. Experimental studies with the model fungus *A. nidulans* suggest that during adaptation to a novel environment, haploids deriving from diploids by parasexual recombination reach a higher fitness than the original diploids (Schoustra *et al.*, 2007). *V. longisporum* might therefore represent a nascent species due to changes in ploidy where the subsequent reduction of the genome size has just started. Speciation might not even be accomplished in *V. longisporum*, because shortspored crucifer isolates might be haploid recombinants of long-spored isolates and distinct from non-crucifer isolates of *V. dahliae* (Barbara and Clewes, 2003; Collins *et al.*, 2003; Qin *et al.*, 2006; Clewes and Barbara, 2008; Klosterman *et al.*, 2009). Increase in ploidy is achieved by a hybridization event between two haploid nuclei resulting in a diploid nucleus. Haploidization requires mitotic recombination and a gradual reduction of the genome by chromosome loss due to nondisjunction during mitosis. The fusion of two haploid nuclei can either happen in a homokaryon or in a heterokaryon. Heterokaryon formation is the result of parasexuality between two different compatible *Verticillium* species with different nuclei. Although fusion of nuclei of the same species is not totally excluded (Clewes and Barbara, 2008), it seems likely that the fusion of different haploid nuclei to a heterozygous diploid interspecies hybrid had been the initiation event for *V. longisporum* formation that ultimately resulted in broadening the host range. Amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) support *V. longisporum* as interspecies hybrid between *V. dahliae* and *V. albo-atrum* (Karapapa *et al.*, 1997; Steventon *et al.*, 2002; Collins *et al.*, 2003). The internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA as well as mitochondrial genes suggest a closer relationship of *V. longisporum* to *V. albo-atrum* than to *V. dahliae* (Fahleson *et al.*, 2004). In contrast sequence comparison of the beta-tubulin,

histone 3 and 5S rRNA genes favours an interspecific hybrid between *V. dahliae* and one or even two yet unidentified species excluding *V. albo-atrum* as parents of *V.* longisporum(Clewes and Barbara, 2008; Collado-Romero *et al.*, 2010; Inderbitzin *et al.*, 2011; Tran *et al.*, 2013). Recent studies analysed eight genetic loci including the ribosomal internal transcribed spacer (ITS) regions and genes for two structural proteins (actin, tubulin), two enzymes (glyceraldehyde-3-P-dehydrogenase, tryptophan synthase), the genes for the mating types (MAT1-1), one transporter (mitochondrial oxalacetate transport protein) and one translation factor (EF1-α) (Inderbitzin *et al.*, 2011). These studies proposed that *V. longiporum* hybridized at least three times resulting in three different lineages, A1xD1, A1xD2, A1xD3 (Fig. 4). Species A1xD2 can only be found in the USA, while A1xD1, and A1xD3 have as well been isolated in Europe and Asia. All three lineages share the same A1 parental genome (Inderbitzin *et al.*, 2011; Tran *et al.*, 2013). The A1 and D1 had been described as yet unknown *Verticillium* species, whereas D2 and D3 represent *V. dahliae* lineages (Inderbitzin *et al.*, 2011). *V. longisporum* A1xD1 and A1xD3 hybrids were isolated from oilseed rape in Europe which are virulent or avirulent, respectively, but differ in the second parental genome by several single nucleotide polymorphisms (SNPs) and ribosomal DNA type (Zeise and von Tiedemann, 2001; Tran *et al.*, 2013).

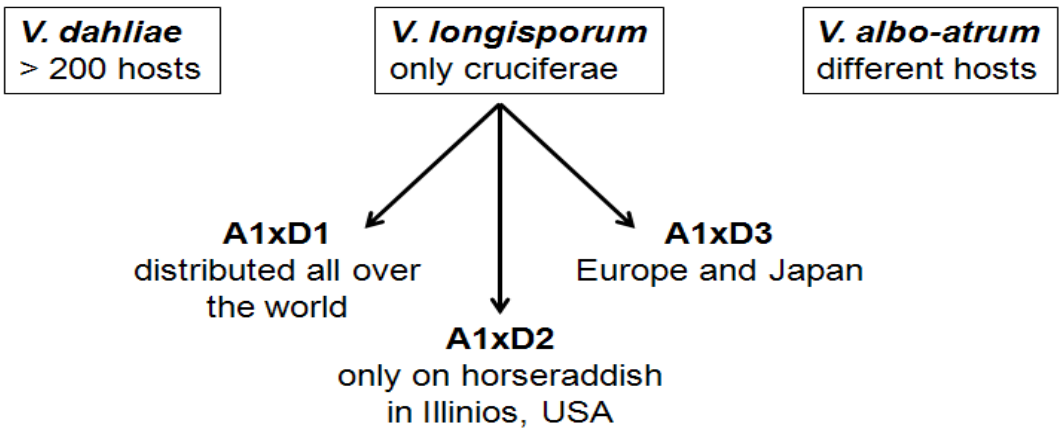

Fig. 4 Schematic distribution of Verticillium plant pathogenic species The haploid species *V. dahliae* and *V. albo-atrum* infect a broad variety of plant host while *V. longisporum* has a narrow host range only infecting cruciferous plants. *V. longisporum* has three different lineages including A1xD1, A1xD2 and A1xD3 with the same A1 parent genome. (adapted from Pegg and Brady, 2002; Inderbitzin *et al.*, 2011; Tran *et al.*, 2013).

1.6. Secondary metabolism in filamentous fungi

Secondary metabolites are defined as compounds or substances, which are produced by an organism but are not required for primary metabolic processes (Mann, 1986). Fungi have a

huge capability of producing different secondary metabolites, including pharmaceutically important compounds such as antibiotics, as well as mycotoxins that cause poising reactions in animal and plant tissues (Yu and Keller, 2005). Expression of secondary metabolites influences competitive advantage of fungi (Cox, 2007). The metabolites are expressed along with enzymes necessary for extracellular digestion. However, the precise function of many of these compounds in the natural environment is currently unknown. Some of these substances have an influence on the organisms interacting with the fungus (Mattinen et al., 2007; Kulye et al., 2012), and some others like toxins are substances with the potential to kill organisms (Sweeney and Dobson, 1999; Mayer *et al*., 2001; Taborda *et al*., 2008; Kulye *et al*., 2012).

Secondary metabolites are generally produced following active growth, and many have an unusual chemical structure (Vining, 1990). Some metabolites are widely spread throughout different fungal groups, while others are specialized for only one or a few species (Muller and Hausmann, 2011).

Secondary metabolites of fungi are classified into four groups depending on the chemical properties and the manner of synthesis (Keller *et al.*, 2005). The main groups are polyketides, peptides, alkaloids and terpenes. Polyketides represent the biggest group of fungal secondary metabolites including the yellow *A. nidulans* spore pigment intermediate naphthopyrone (WA), the carcinogen aflatoxin (aftatoxin B_1, B_2, G_1 and G_2) and the cholesterol-reducing compound lovastatin. Penicillin G, cyclosporin and gliotoxin are representatives of the group of non-ribosomal peptides (NRP). Terpenes like aristolochenes, caretenoids, gibberellins, trichothecenes are compounds that consist of isoprene units. Indole alkaloids are the derivatives of tryptophan and dimethylallyl pyrophosphate, gibberellin GA3, trichothecene T2 toxin and aristolochene (Keller *et al.*, 2005).

The fungus *Aspergillus nidulans* serves as a model system to investigate secondary metabolism in the genus *Aspergillus*. It can produce the polyketide sterigmatocystin (ST) and aflatoxins (AF) which are related fungal secondary metabolites (Keller and Adams, 1995; Keller and Hohn, 1997). They are among the most toxic, mutagenic and carcinogenic natural products known (Trail *et al.*, 1995; Sweeney and Dobson, 1999). The genes involved in ST biosynthesis are placed in a cluster (Brown *et al.*, 1996). Structural genes involved in fungal secondary metabolism act as one locus (Bok *et al.*, 2006).

Clustered genes are mainly regulated by pathway specific transcription factors (Fernandes *et al.*, 1998; Pedley and Walton, 2001), global regulators (Bok and Keller, 2004) and signal

transduction pathways that for example combine the secondary metabolism with sporulation (Calvo *et al.*, 2002). Transcription of the ST gene cluster is driven by *aflR*, which is the transcription factor embedded within the ST structural gene cluster (Fernandes *et al.*, 1998). Further upstream regulation is conferred by LaeA, which is the master regulator of secondary metabolism in *Aspergillus spp.* It is a methyltransferase possibly acting on histone methylation (Keller *et al.*, 2005). In *A. nidulans*, deletion of *laeA* causes the loss of expression of ST gene cluster including the transcription factor *aflR* (Bok and Keller, 2004), *A. fumigatus laeA*Δ strains have decreased virulence in a murine model (Bok *et al.*, 2005). The regulation of fungal secondary metabolism is controlled by many factors and is depending on a specific stage of fungal development. LaeA is a part of the velvet complex including VelB-VelA-LaeA that controls development and secondary metabolism (Bayram *et al.*, 2008). Velvet proteins regulate development and secondary metabolism in *A. nidulans* and other fungi (Bayram and Braus, 2012).

Secondary metabolism in *Verticillium* is still largely unexplored. Although some common gene clusters for secondary metabolism have been identified, knowledge about their function is limited (Wang *et al.*, 2004; Shi and Li, 2008; Zhou *et al.*, 2012). For *V. dahliae* an elicitor that induces cotton wilting was isolated (Wang *et al.*, 2004). Several of these elicitors have been identified so far and they are related to induction of wilting symptoms in cotton plants (Zhou *et al.*, 2012). Futhermore toxins generated by *V. dahliae* charge a counter reaction of the plant via nitric oxygen (Shi & Li, 2008). Some investigations on interaction between plant and fungus on proteomic or on genetic level revealed putative secondary metabolites (Singh *et al*., 2010; Floerl *et al*., 2012; Iven *et al*., 2012; Singh *et al*., 2012), but so far no deeper insight into secondary metabolism have been described.

1.7. The Fungal Cross-Pathway Control System

Changing environmental conditions are a challenge for microorganisms, as they have to adapt to certain conditions. To increase their chances to survive under rapidly evolving environmental situations, they must have a system that is on the one hand sensitive and that on the other hand enables them to quickly respond on these impacts. Such kinds of regulatory systems were detected in various fungi like the yeast *Saccharomyces cerevisisae* (Hinnebusch, 1984) or in filamentous moulds like *Neurospora crassa* and *Aspergillus nidulans* (Carsiotis *et al.*, 1974; Piotrowska *et al.*, 1980; Sachs, 1996; Davis, 2000) where they were named “General Control of amino acid biosynthesis” (GC) or “Cross-Pathway Control” (CPC), respectively. They are activated under amino acid

starvation conditions and result in a regulation of different sets of genes involved in amino acid biosynthesis, purine biosynthesis, nitrogen and sulfur metabolism and vitamin biosynthesis (Mirande and Waller, 1988; Mösch *et al.*, 1991; Hinnebusch, 1997; Natarajan *et al.*, 2001; Tian *et al.*, 2007). In its core, this signal transduction system consists of a sensor kinase, which is able to phosphorylate a subunit of the eukaryotic translation initiation factor eIF2, and a transcriptional activator that serves as downstream effector to generate a cellular read-out.

1.7.1. Induction and regulation of the CPC/GC

The regulatory gene of the yeast General Control was named *GCN4* (*g*eneral *c*ontrol *n*onderepressible), and its mRNA contains four small coding sequences, which are part of an unusually long leader region preceding the actual coding sequence. The *Aspergillus* gene homologous to *GCN4* is *cpcA*. The *cpcA* gene of *A. fumigatus* has a size of about 2.2 kb, from which the coding sequence spans 810 bp. Two small ORFs exist preceding the *cpcA* coding region, which are similar to the four regulatory uORFS of *GCN4*. Structure and function of the GCN4 gene product as well as the interplay between the two has been studied intensively. The C-terminal part contains a leucine-zipper motif that is important for the dimerisation of the protein. In addition the C-terminal domain comprises a conserved DNA binding region, which is important for transcriptional regulation of Gcn4p target genes (Ellenberger *et al.*, 1992). The N-terminal region is divided in two parts that represent different transcriptional activation domains (Drysdale *et al.*, 1998). Regulation of *cpcA* expression in *A. nidulans* is mediated on the transcriptional as well as the translational level (Hoffmann *et al.*, 2001).

In *S. cerevisiae* expression of *GCN4* is primarily regulated on transcriptional level, whereas in *C. albicans* transcription of *GCN4* is more important than translation (Tournu *et al.*, 2005). In detail, translational regulation of gene expression *via* the upstream open reading frames of *GCN4*/*cpcA* transcripts acts like follows: under sated conditions, expression levels are low at a basal level due to the fact that scanning ribosomes initiate translation on the *GCN4/cpcA* transcript at the first uORF. Important for this are the amounts of so-called tertiary complexes, which consist of the translation initiator eIF2, GTP and a tRNAMet, and these three components are crucial factors for initiation of translation. The tertiary complex enables the ribosomes to bind at the first uORF within the leader sequence of *GCN4/cpcA*. After reaching the stop codon, ribosomes disassemble into the two ribosomal sub-domains. To initiate another round of translation, a new tertiary complex has to be assembled together with the small ribosomal subunit. In case

concentrations of tertiary complexes are high, one of the downstream uORFs will be translated. As the distance between the stop codon of the distal uORF and the start codon of the actual coding sequence is too close translational re-initiation is hampered and almost no gene product is expressed.

Under starvation conditions expression is drastically de-repressed. In case of amino acid starvation, uncharged tRNA molecules accumulate and bind to a sensor kinase called Gcn2p or CpcC, respectively. The activated kinase phosphorylates a subunit of the initiation factor eIF2, resulting in diminished levels of tertiary complexes and therefore lower rates of translation initiation. In consequence, overall cellular translation is down-regulated but translation initiation at the *cpcA/GCN4* coding region increases, as competent ribosomes that have failed to translated the distal uORF are now able to bind at the start codon of the coding sequence to initiate the translation (Hoffmann *et al.*, 2001; Braus *et al.*, 2003; Krappmann *et al.*, 2004).

Regulation of *Aspergillus* CpcA expression on the transcriptional level has been investigated in detail for *A. nidulans* (Hoffmann *et al.*, 2001). There, synthesized CpcA binds to so called CPREs (*C*ross-*P*athway Control *R*ecognition *E*lements) that locate within the promoter region of target genes. It could be shown that two of these CPREs are part of the promoter region of *cpcA* suggesting a functional auto-regulatory loop (Hoffmann *et al.*, 2001). A regulation like this has not been proven for CpcA expression in *A. fumigates*, however, it was shown that two highly conserved CPREs are also present within the *cpcA* promoter region (Krappmann *et al.*, 2004). The proximal one (5'-ATGACTCAC-3', pos. -1062 till -1053) is identical to its *A. nidulans* counterpart, the distal one shows a difference in one site of the sequence in comparison to the *A. nidulans* CPRE (5'-ATGACTCgAC-3', -1273 till -1264). This leads to the suggestion that binding of CpcA to this recognition site might be weaker (Hinnebusch, 1984; Thireos *et al.*, 1984; Arndt and Fink, 1986).

In addition, a variety of other factors that influence activity of the Cross-Pathway Control/General Control activators CpcA/Gcn4p were identified, among them: glucose, nitrogen and purine starvation (Mösch et al., 1991; Grundmann et al., 2001; Hinnebusch and Natarajan, 2002; Braus et al., 2003).

In *Verticillium* species so far the influence of Cross-Pathway Control on development and pathogenicity of the fungus has only been slightly investigated. Singh *et al.* (2010) could show in plant experiments that *CPC1*, the activator of cross pathway control, is upregulated, when the fungus is growing inside the plant (Singh *et al.*, 2010). During

infection and colonization of its host *V. longisporum* activates the cross pathway control to cope with an imbalanced amino acid supply (Singh *et al.*, 2010).

1.8. Aim of this work

The aim of this work was to investigate how the fungus adapts within the plant xylem sap and what regulatory genes might be involved. Xylem sap is the major source of nutrient supply for *Verticillium* when colonizing its host.

Earlier studies of Singh *et al*. (2010) revealed the amount of amino acids avaible for the fungus. Because these amounts are limited the fungus needs to deal with an imbalanced amino acid supply. In a first approach we wanted to see if the regulator of cross pathway control *CPC1* is required for successful infection and colonization of its host. For the survival of the fungus the acquisition of amino acids is necessary to cope with the conditions given by the host plant. Imbalanced amino acid supply requires *CPC1*. To further analyze the influence of *CPC1* in pathogenicity mutants silenced in *CPC1* for *V. longisporum* and mutants with a knockout of *CPC1* for *V. dahliae* were generated. One aim of this work was to generate knockdowns or knockouts of this gene in *V. longisporum* and *V. dahliae*, than to investigate the influence of analogues on these mutants *ex planta*, and later observe the mutant *in planta*. For these mutants assays *in planta* were performed to observe the propagation and infection of mutants in the plant.

For infection and colonization secreted proteins and secondary metabolites play a crucial role for the survival of the fungus in the host plant. They are also known to be important for pathogenicity of the fungus. These proteins or compounds might be useful to adapt to the plants pathogen reaction or either might be necessary to counteract the pathogen response of the host. The key regulator of secondary metabolism is *LAE1*. To analyze the influence of *LAE1* in pathogenicity mutants silenced as well as knockouts of *LAE1* were generated. These mutants were further characterized by assays *in planta* to observe the propagation and infection of mutants in the plant.

Successful infection and colonization of the host requires proteins or enzymes that are capable of acquiring nutrition for the fungal pathogen, or provide opportunities of breaking the plant cell wall. Secreted proteins and secondary metabolites are the key players for these reactions. These proteins or enzymes might be useful to adapt to the plants pathogen reaction or either might be important to counteract the pathogen response of the host plant. Among these enzymes, lipases and cutinases, with their potential roles in the hydrolyzation of the plant cuticle and surface waxes, may contribute to pathogen infection at an early

stage, when the fungus first comes into contact with its host.A secreted lipase has been isolated from a fungal culture treated with xylem sap. As an example for secreted proteins a lipase was characterized as well. This triacylglycerol lipase (*TAGL*) might be required for breaking the plant cell well and enter the host. Silenced mutants of *TAGL* were generated and analysed in plant infection assays.

Chapter 2. Materials and Methods

2.1. Microbial strains and growth conditions

2.1.1. Bacterial strains

Escherichia coli strains DH5α, *ccd*B-resistant strain (Invitrogen, Karlsruhe, Germany) and *Agrobacterium tumefaciens* AGL1 were used for transformation procedures in this work. The *E. coli* strain was cultivated at 37^{o}C, while the *A. tumefaciens* strain was grown at 25-28^{o}C. Both organisms were grown in Luria Bertani (LB) medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl).

2.1.2. *Verticillium* strains

Eight isolates of *Verticillium* species from different hosts were used in this study (Table 1). Most of the *Verticillium* isolates were kindly provided by Prof. Andreas von Tiedemann, Georg-August University Göttingen (according to Zeise and Tiedemann, 2001; 2002a) except the Vl-Bob70 isolate was derived from VdBob70 isolate (Qin *et al.*, 2006), *Vertcillium dahliae* VdJR2 (Fradin *et al.*, 2009), was provided by Bart Thomma from the Laboratory of Phytopathology in Wageningen (The Netherlands). All strains were inoculated in potatoe dextrose broth (PDB) (Sigma-Aldrich Chemie GmbH, Munich, Germany), Minimal medium (MM) (Bennett & Lasure, 1991), Czapek-Dox medium (CDM) (Smith, 1960) or simulated xylem medium (SXM) (Neumann and Dobinson, 2003) and incubated for 7-10 days at 25^{o}C.

2.1.3.1. Spore preparation

The fungal isolates were grown separately in Czapek-Dox liquid medium for 10 days, at 25^{o}C on a shaker, 120 rpm. Fungal conidia were harvested by filtering the culture through miracloth membrane (Calbiochem, Darmstadt, Germany), the filtrate was centrifuged at 5,000 rpm for 20 minutes at 4^{o}C. After a washing step with sterile tap water, the sediment was resuspended in sterile tap water. The number of spores was counted under microscope using a counting chamber and spore density was adjusted to 10^{7} spores/ml. Glycerin was added to the spore suspension at the final concentration of 20% and aliquots of the spore suspension were frozen in liquid nitrogen and stored at -80^{0}C.

Table 1. *Verticillium* isolates used in this study.

Isolate	Species	Host	Geographic origin	Reference
Vd-73	*V. dahliae*	*Linum usitatissimum* (linseed)	Mecklenburg/Germany	Zeise and von Tiedermann, 2001
Vd-JR2	*V. dahliae*	*Solanum lycopersicum*	Canada	Fradin *et al.*, 2009
Vl-40	*V. longisporum*	*Brassica napus* (rapeseed)	Mecklenburg/Germany	Zeise and von Tiedermann, 2002a
Vl-43	*V. longisporum*	*Brassica napus* (rapeseed)	Mecklenburg/Germany	Zeise and von Tiedermann, 2002a
Vl-Bob70	*V. longisporum*	*Brassica oleracea* var. *botrytis* (cauliflower)	California/USA	Qin *et al.* 2009
Va-1	*V. albo-atrum*	*Solanum tuberosum* (potato)	Wiconsin/USA	Zeise and von Tiedermann, 2002b

Table 2. The plasmids used in this study.

Name	Type	Description	Source
pDONR/Zeo	Gateway	Containing a gateway cassette, Zeocin^{R}	Invitrogen
pJET1.2	Cloning	A dephosphorylated cloning vector with blunt ends	Fermentas
pKO2	Cloning	Vector for gene disruption	Tran 2011
pGS1	Cloning	Vector for gene silencing	Tran 2011
pKO*CPC1*	Cloning	Vector for deletion of *CPC1*	Timpner *et al*. 2013
pKO*LAE1*	Cloning	Vector for deletion of *LAE1*	This study
pGS*CPC1*	Cloning	Vector for silencing of *CPC1*	This study
pGS*LAE1*	Cloning	Vector for silencing of *LAE1*	This study
pGS*TAGL1*	Cloning	Vector for silencing of triacyl glycerol lipase	This study

2.2. Bioinformatic methods

2.2.1. Primer designing

The primers used in this study (Table 3) were designed using the Primer3 program (http://frodo.wi.mit.edu/primer3/ (Rozen and Skaletsky, 2000)) and purchased from Invitrogen (Karlsruhe, Germany) or from Eurofins-MWG (Ebersberg, Germany), the amplification efficiency of each primer pair was checked with Mastercycler Gradient (Eppendorf, Hamburg, Germany).

Table 3 Primers used in this study (Underlined part of the sequence represent cutting sites)

Primer	**Size**	**Sequence (5'-3')**	**Reference**
OLG 70 OLG 71	20mer 19mer	CAG CGA AAC GCG ATA TGT AG GGC TTG TAG GGG GTT TAG A	Eynck *et al.* 2007
H2aRTleft H2aRTright	22mer 22mer	CCC GTG ACA ACA AGA AGA CTC G GCA GGA AAG AAA AGC CAA AAC C	Singh *et al.* 2011
Cpc1RTleft Cpc1RTright	22mer 22mer	CAG CCT ACA CAT CCC AAC AAC C TCC GTT TCC AGC AGC AGA TAG T	This study
*Cpc1*gateleft *Cpc1*gateright	50mer 49mer	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GAA CAT CGC GGA TTT CG GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTG CCA GAG TGT TGC GAG C	This study
F1CpcAlc (*Pac*I)	33mer	GGG TTA ATT AAG GCT ATC GAG CAT ACG AAG AAC	This study
F1CpcArc (*Sac*I)	31mer	GGG GAG CTC CCA TCC ATA TAT TCT GGC GAT T	This study
ProbeCpc1left ProbeCpc1right	20mer 20mer	CGG CCC TCA AAA ATT GTT CC GAC TTA AAT TGA GAT ATC CG	This study
*Cpc1*Seqleft *Cpc1*Seqright	19mer 21mer	ATG GCT TCC CTC CAG TTC C CTA GTC GCT GGT TGA CTG ACC	This study
RTCpc1left RTCpc1right	22mer 22mer	CAG CCT ACA CAT CCC AAC AAC C TCC GTT TCC AGC AGC AGA TAG T	This study
VDLaeF2r VDLaeF2l	22mer 22mer	GAA TAC ATC CTG GTA GCC TTC G CTG GAC AGG AAG TAC AAC GAC A	This study

VDLaeF2Lc (PstI) VDLaeF2Rc (BamHI)	29mer 30mer	GGG CTG CAG AGA CCA GAC GCC CCT AAA CT GGG GGA TCC CCT GGA CAG GAA GTA CAA CGA	This study
VDLaeF1l VDLaeF1r	20mer 20mer	GTG CAG CAG GTA CTG GCT TT TGA TAG CTG ACA CGC GAA AC	This study
VDLaeF1Lc (EcoRI) VDLaeF1Rc (EcoRV)	29mer 29mer	GGG GAA TTC GTG CAG CAG GTA CTG GCT TT GGG GAT ATC TGA TAG CTG ACA CGC GAA AC	This study
KOCpcF1Lc (PacI) KOCpcF1Rc (SacI)	31mer 29mer	GGG TTA ATT AAG TAT TCG TGG CCC AAA GAG A GGG GAG CTC CAT ATT CTC CCG AGC TTC CA	This study
RTCpcAleft RTCpcAright	22mer 22mer	CAG CCT ACA CAT CCC AAC AAC C TCC GTT TCC AGC AGC AGA TAG T	This study
LaeART1left LaeART1right	22mer 22mer	AAA CCG CAC TAC AGA CCA GAC G GAA GCG AAG GCT ACC AGG ATG T	This study
LipRT1left LipRT1right	22mer 22mer	ACA TTT TGT CAC GAG GGG GAT T TAC CAC GCT CTT CTT GCT GCT C	This study
ProbeLipleft ProbeLipright	22mer 21mer	TTT CCA GCA AGC TGT ATT CTC A CGA TGG TTA ATT GGC TGT CAT	This study
ProbeLae1left ProbeLae1right	22mer 22mer	CGA GAA CAG CAT TGA TTA CGT C TTT CAA TCT CTT CAG TCG TCC A	This study
VdCPC1KOProbe-L VdCPC1KOProbe-R	20mer 20mer	TCC CAA CAA CCT GCC TAA AC AGA GTC CAG AAC AGC ACG GT	This study
VdLAE1KOProbe-L VdLAE1KOProbe-R	20mer 20mer	TCC CAA CAA CCT GCC TAA AC AGA GTC CAG AAC AGC ACG GT	This study

2.2.2. Tools for DNA sequence analysis

DNA sequences were verified by using the 4Peaks software (www.mekentosj.com) For molecular analyses, DNA sequences were collected from the *Verticillium* group database (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html). The open reading frame (ORF) of each gene was identified and translated into a protein sequence by using the ORF Finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/). The protein sequence was for conserved domains or motifs using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) or Pfam (http://pfam.sanger.ac.uk/) or Prosite

(http://expasy.org/prosite/). The information of DNA sequences was determined by blasting the sequences in Genbank (http://blast.ncbi.nlm.nih.gov/). Restriction sites of the DNA sequences were determined by using NEBcutter V2.0 program from New England Biolabs (http://tools.neb.com/NEBcutter2/).

2.2.3. DNA analysis and comparison

Characterization of *CPC1* and *LAE1* was based on sequences derived from the *Verticillium* group database (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html). The introns and exons were determined by comparing the cDNA sequences with their genomic DNA sequences using the ClustalW program (Thompson *et al.*, 1994). For DNA analysis and comparison in more details, the commercial software Geneious Pro 5.0.4 (Biomatter Ltd, Auckland, New Zealand) was used.

2.2.4. Phylogenetic analysis

The phylogenetic trees were constructed with the MEGA 5.0 software (Tamura *et al.*, 2007) 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.

2.2.5. Drawing of plasmid maps and models

The plasmid maps and models in this study were drawn using the Savvy program (http://www.bioinformatics.org/savvy/) and Adobe Photoshop software.

2.3. Genetic manipulations

2.3.1. *E. coli* transformation

2.3.1.1. Preparation of *E. coli* competent cells

10 fresh colonies of *E. coli* were inoculated into 250 ml of SOB medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) in a 1 liter flask. The flask was incubated on a shaker (100 rpm) at 20°C until the OD_{600} of the culture was about 0.6-0.8. The culture was kept in ice for 10 min and centrifuged at 5,000 rpm for 10 min at 4°C. The sediment was then resuspended in 80 ml TB buffer (10 mM PIPES/HEPES, 15 mM $CaCl_2.2H_2O$, 250 mM KCl; 55 mM $MnCl_2$; pH6.7). This cell suspension was incubated on ice for 10 min followed by centrifugation at 4,000 rpm for 10 min at 4°C. The sediment was resuspended in 20 ml TB followed by the addition of DMSO by gently swirling to a final concentration of 7%. This cell suspension was further

incubated for 10 min on ice and dispensed (400 µl) into aliquots. The aliquots were frozen in liquid nitrogen and stored at -80°C.

2.3.1.2. *E. coli* transformation

An aliquot of competent cells was first thawed on ice. About 5-10 µl of the ligation reaction or 50 ng of a plasmid was added to 100 µl of the competent cells and incubated for 20 min on ice. The cells were then subjected to heat shock at 42°C for 40 sec and then kept on ice for 5 min. About 800 µl of SOC medium (SOB + 20 mM glucose) was added to the mixture followed by a recovery at 37°C with shaking at 180 rpm for 1 h. The cells were collected by centrifugation at 10,000 rpm for 1 min and plated on a LB agar plate supplemented with an appropreate antibiotic for selection (the final concentration of ampicillin or kanamycin is 100 µg/ml and 30 µg/ml for zeocin).

2.3.2. *Agrobaterium tumefaciens* transformation

2.3.2.1.Preparation of competent cells

A. tumefaciens strain AGL1 was used for the mediated transformation of *Verticillium* species. To prepare competent cells, a single colony of this bacterium was grown in 50 ml of LB medium with 50 µg/ml carbenicillin at 28^{o}C, 160 rpm for overnight. The culture (OD_{600} = 0.8) was cooled on ice for 15 min and the cells were collected by centrifugation at 5,000 rpm for 10 min at 4°C. The sediment was resuspended in 20 ml of sterile ice-cold 100 mM $MgCl_2$ solution, and kept on ice for 1 h. The cells were harvested by centrifugation as above, then resuspended in 20 ml of sterile ice-cold 20 mM $CaCl_2$ solution and incubated on ice for 4-5 h to obtain competent cell suspension. Glycerol was added to a final concentration of 20% and aliquots of 200 µl were frozen in liquid nitrogen and stored at -80°C.

2.3.2.2. *Agrobacterium* transformation

The freeze-thaw method (Jyothishwaran *et al.*, 2007) was used for *A. tumefaciens* transformation. A tube of frozen competent cells (200 µl) was kept on ice for 10 min to thaw and 10 µl of a binary vector (100 ng/µl) was mixed to the competent cells. The tube was inverted gently for three times and incubated on ice for 5 min, then frozen in liquid nitrogen for 10 min and thawed at 37°C in a heat block for 5 min. The mixture was added 800 µl of SOC medium without antibiotics and incubated at 28°C for 1 h in a shaker at 160 rpm. The cells collected by centrifugation at 5,000 rpm for 5 min were spread on a LB plate containing 100 µg/ml kanamycin and incubated at 25-28^{o}C or at room temperature

for 48-72 hours to gain colonies. Colony PCR was employed to screen positive colonies using a specific primer pair.

2.3.3. *Agrobacterium*-mediated *Verticillium* transformation

For *A. tumefaciens*-mediated transformation (ATMT) of *Verticillium* species, a plasmid carrying bacterial colony was grown 5 ml of LB medium supplemented with 100 µg/ml kanamycin (also with 50 µg/ml carbenicillin to avoid contamination of other bacteria if necessary) for overnight. About 0.5-1.0 ml of the culture was diluted with induction medium (IM) (5 mM glucose, 0,5% glycerol, 40 mM MES, 1x Salt solution) containing 200 µM acetosyringone (AS) to get an optical density of 0.2 at the wavelength at 600 nm (OD_{600} = 0.2), then the diluted culture was grown at 28^{o}C for 4-5 h at 160 rpm. A mixture of equal volumes (150 µl) of the bacterial culture and the fresh spore suspension (10^6 spores/ml) was spread onto the filter paper of 85-mm diameter (Satorius, Göttingen, Germany) on a agar plate of induction medium containing 200 µM acetosyringone (IMAS) that is identical to liquid IM, except it contains 5 mM of glucose instead of 10mM glucose. Following co-cultivation at 25°C for 48-72h, the filter paper was transferred to a PDA (potato dextrose agar) plate containing hygromycin B (50 µg/ml) or nourseothricin (50 µg/ml) as the selection agent for fungal transformants, and cefotaxime (50 µg/ml) to kill the *A. tumefaciens* cells. The plates were sealed with parafilm, inverted and incubated at 25°C for 8-10 d.

2.3.8. Plasmid isolation from *E. coli*

The plasmids carrying the gateway cassette(s) such as, pDONR/Zeo and pGS1, were maintained and propagated in the *ccd*B-resistant *E. coli* strain (Invitrogen, Karlsruhe, Germany). For other plasmids, the *E. coli* strain DH5α was used instead. A single colony containing a plasmid of interest was inoculated in 5 ml of LB liquid medium with an appropriate antibiotic (100 µg/ml for ampicillin and kanamycin, 30 µg/ml for zeocin) and incubated at 37^{o}C, 180 rpm for overnight. The plasmid was isolated using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.3.9. Isolation of nucleic acids from *Verticillium*

Fungal isolates were grown in potato dextrose broth (PDB) (Sigma-Aldrich Chemie GmbH, Munich, Germany) or in liquid simulated xylem medium (SXM) (Neumann and Dobinson, 2003) for one week, at 25^{o}C with shaking at 100 rpm. The fungal mycelium was harvested with miracloth (Calbiochem, Darmstadt, Germany) and ground to fine powder in

liquid nitrogen using a pestle and mortar. The fungal powder was used directly for nucleic acid extraction or frozen in liquid nitrogen and preserved at -80^{o}C.

Genomic DNA was extracted from the fungal powder according to Kolar *et al.*, 1988 with some modifications. About 800 µg of the fungal powder was transferred to a 2.0 ml tube 800 µl of fresh lysis buffer (50 mM Tris-HCl, pH7.2; 50 mM EDTA; 3% SDS; 1% 2-Mercaptoethanol) was added to the tube. The tube was vortexed at maximum speed for 10 seconds and incubated at 65^{o}C for 1 h. About 800 µl of phenol, chloroform and isoamyl alcohol mixture (25:24:1) was added to the tube and the tube was inverted for several times. The tube was centrifuged at 13,000 rpm for 15 min and 400-500 µl of the supernatant was transferred carefully to a new 2 ml tube. 800 µl of phenol, chloroform, isoamyl alcohol mixture was added to the supernatant and the tube was centrifuged again as discribed above. The supernatant was transferred to a new tube containing 600 µl isopropanol and 25 µl of 3M NaAcOH. The tube was centrifuged at 13000 rpm for 2 min. The sediment was washed twice with 1 ml of 70% ethanol by centrifugation. The sediment was dried at 37^{o}C for 20 min and dissolved in H_2O. The DNA sample was treated with 4 µl of RNase A at 37^{o}C for 30 min to eliminate RNA. DNA concentration was measured using NanoDrop *ND*-1000 spectrophotometer (PEQLAB, Erlangen, Germany). The DNA quality was checked on 1% agarose gel using 5 µl genomic DNA.

Total fungal RNA was extracted by using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) or Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

2.3.10. PCR and DNA purification

Polymerase chain reaction (PCR) amplifications was performed in 25-µl volumes with the PCR cycler (MWG-Biotech Primus, Ebersberg, Germany). For analysis or confirmation purposes, *Taq* polymerase (Fermentas, St. Leon-Rot, Germany) was used. PCR conditions including an initial denaturation at 94°C for 3 min followed by 30-35 cycles of denaturation at 94°C for 1 min, annealing at 55-60°C for 40 s and extension at 72°C for 1 min/kb; a final extension at 72°C for 10 min and storage at 4-8°C until used.

For cloning purposes, the high-fidelity Phusion DNA polymerase (Finnzymes, Espoo, Finland) was used with the constitutions and conditions for PCR based on the manufacturer's instructions. The PCR products were analyzed on a 1% agarose gel. The DNA fragments were excised and purified with QIAquick Gel Extraction Kit (Qiagen,

Hilden, Germany). The purified DNA fragments were used for direct sequencing or for cloning.

2.3.11. Cloning and sequencing

For digestion of DNA fragments or plasmids 250 ng of a PCR product and 500 ng of a plasmid were digested with appropriate restriction enzymes (Fermentas, St. Leon-Rot, Germany) for 2 h. The restricted DNA samples were analyzed on a 1% agarose gel and the expected bands were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the provided manual. For ligation, about 150 ng of the restricted insert DNA was mixed with 50 ng of the restricted plasmid (3:1) along with 1 µl of T4 DNA ligase (Fermentas, St. Leon-Rot, Germany) and 1X T4 DNA ligase buffer in a total volume of 10-20 µl. The ligation reaction was incubated at room temperature for 1 h or at 16^{o}C in a heating block for overnight. The ligation mixture was used to transform *E. coli* competent cells.

For sequencing of the target genes, the PCR product of each gene amplified by Phusion DNA polymerase was purified and cloned directly into the pJET1.2/blunt cloning vector using the CloneJET™ PCR Cloning Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. The whole ligation mixture was transformed into *E. coli* DH5α competent cells. Colony PCR was employed to screen positive colonies from each cloning procedure. At least, 10-15 positive clones were selected and grown in LB (Luria-Bertani) liquid medium added 100 µg/ml of ampicillin, recombinant plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). The sequencing of the recombinant plasmids was performed with specific primers by Göttingen Genomics Laboratory (G2L), Georg-August University of Göttingen, Germany.

2.4. Generation of vector for gene silencing in *V. longisporum*

To investigate the role in the survival of *V. longisporum* when it inhabits the xylem vessel of the host plant, we decided to knock-down the isogenes, *VlCPC1-1* and *VlCPC1-2* by RNA silencing because the conventional knock-out was not practical due to the presence of two isogenes. RNA mediated gene silencing acts in a sequence-specific manner and should silence both the isogenes. It has been successfully implemented in several fungi for targeted gene silencing (Nakayashiki, 2005). Silencing, particularly, by hairpin RNA was found to be most efficient in the filamentous fungus, *M. oryzae* (Kadotani *et al.*, 2003). In previous works (Singh *et al.* 2010, Singh *et al.* 2012) silencing of certain *V. longisporum*

genes was performed. Therefore, a hairpin double-stranded RNA mediated gene silencing approach was applied for RNA silencing of *VlCPC1-1* and *VlCPC1-2* in *V. longisporum*.

To generate silencing plasmid a 544 bp fragment of *VlCPC1* from the coding region was amplified and was cloned into pDONR/Zeo plasmid (Invitrogen GmbH, Karlsruhe, Germany) with BP clonase (Invitrogen GmbH, Karlsruhe, Germany) according to manufacturer's instructions.

This vector generates sense and antisense fragment for silencing (Fig. 5). Using the LR clonase reaction (Invitrogen GmbH, Karlsruhe, Germany) the silencing cassette is transferred to the destination vector pGS1 (Tran, 2011), which contains the hygromycin B phosphotransferase gene (hph) as selection marker.

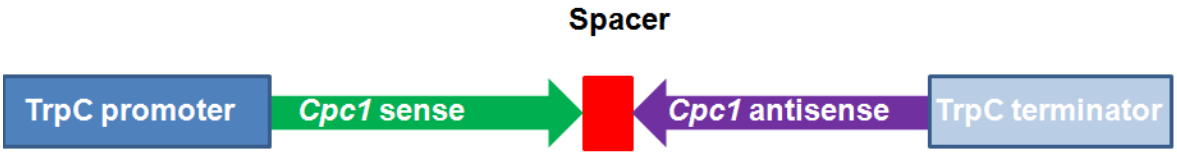

Fig. 5 silencing construct for RNAi of *V. longisporum*. The hairpin construct consists of promotor, sense and antisense part, as well as terminator

This way the vector pGS*CPC1* was constructed and used as gateway silencing vector (Fig. 6).

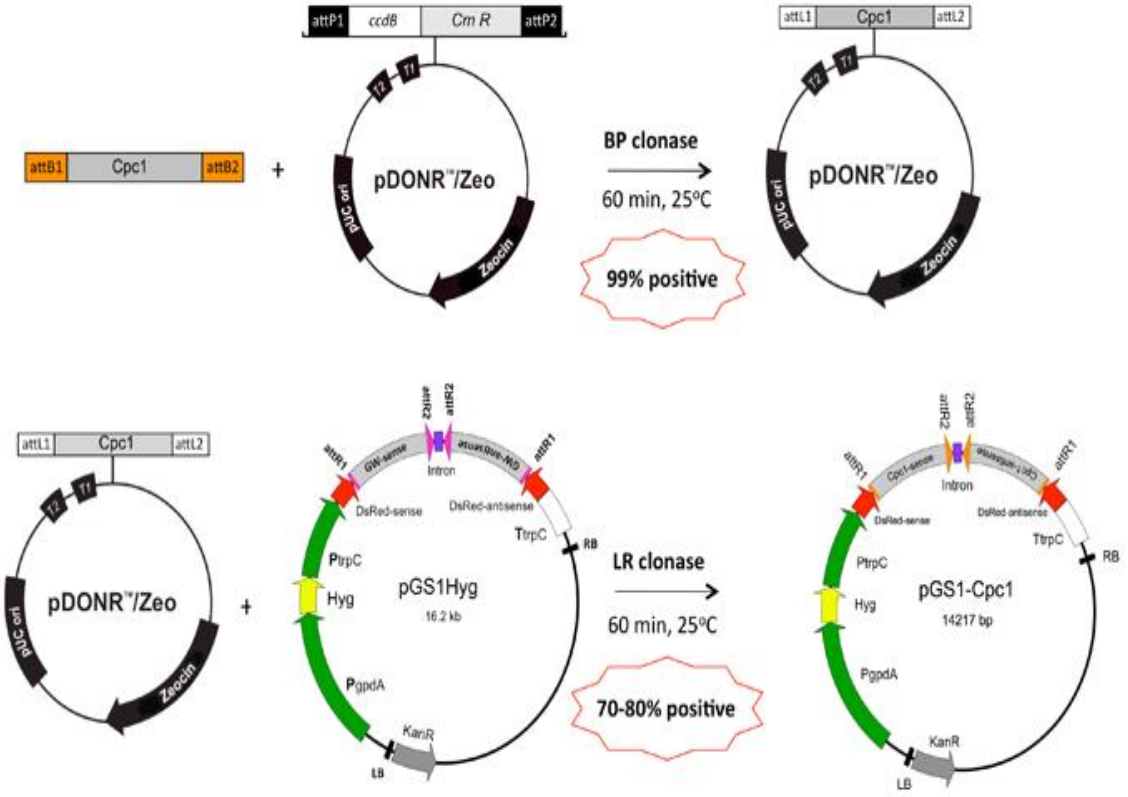

Fig. 6 Two-step procedure to create the hairpin RNA-mediated silencing construct for*Cpc1* gene. A fragment of 550 bp was amplified from the *CPC1* transcripts. The PCR product with *att*B recombination sequences was transferred to the donor vector (pDONR/Zeo) by BP clonase via a reaction between *att*B sequences and *att*P sequences of this vector. The reaction results in pDONR-Cpc1 vector (entry vector) with *att*L recombination sequences. In presence of LR clonase, the entry vector exchanges the *CPC1* fragment with the gateway cassettes in pGS1Hyg via a reaction between *att*L sequences and *att*R sequences. This reaction creates the silencing construct for *CPC1* gene (pGS1-Cpc1i).

The generated plasmid (Fig. 7) was transformed into *V. longisporum* strain 43 by *A. tumefaciens*-mediated transformation (ATMT (Mullins *et al.*, 2001)). *A. tumefaciens* AGL-1 strain (Lazo *et al.*, 1990) was transformed with plasmids using LB medium containing kanamycin (100 µg ml−1). Filter paper, 90 mm (Sartorius), was used for cocultivation of the *A. tumefaciens* culture and the *V. longisporum* spore suspension. The transformants were grown on medium lacking hygromycin B for five generations and then plated on selective medium supplemented with 100 µg ml−1 hygromycin B, where only mitotically stable transformants could grow (Singh *et al.*, 2010).

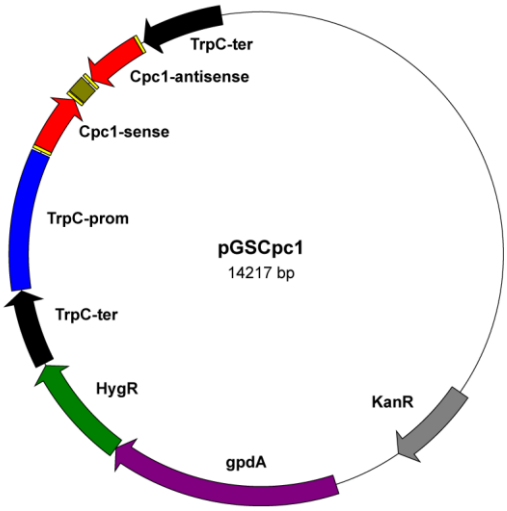

Fig. 7 Silencing construct for *VlCPC1*. Vector is integrated with the gateway cassette in sense and antisense direction for generating hairpin RNA silencing constructs. The same gateway cassettes in both directions contains two special recombination sequences (*att*R1 and *att*R2). They will be replaced automatically with a target gene fragment via two reactions using BP clonase and LR clonase.

A vector for silencing of *LAE1* and *TAGL* was generated in the same way as discribed for *CPC*1.

2.5. Knockout strategy for *V. dahliae*

For fungal species which genomes are not yet sequenced, silencing strategy remains probably the best option to study genes of interest in these organisms. However, silencing suppresses maximally about 80-90% of gene expression in fungi (Nakayashiki, 2005; Janus *et al.*, 2007; Singh *et al.*, 2010). The rest activity of the gene (10-20%) might be still enough for its function in the fungus. Though to analyze the complete functions of genes in *Verticillium* species, the gene must be either disrupted or removed from the genome. Because up to now, the *Agrobacterium tumefaciens*-mediated transformation represents the method with the highest efficiency for gene transfer into *Verticillium* species, strategies of gene disruption in *Verticillium* require a binary vector as a deletion cassette carrier. Gene disruption in *V. dahliae* was performed successfully by inserting a resistant cassette into open reading frames of genes using transposons (Dobinson *et al.*, 2004; Rauyaree *et al.*, 2005; Klimes and Dobinson, 2006) or by multi-step cloning using different plasmids (Tzima *et al.*, 2010). The disadvantage of both strategies is that they are complicated and time-consuming. Therefore, we used a binary vector for gene disruption pKO2 (Tran; 2011) that can be used directly for making deletion constructs as well as for *Verticillium*

transformation via *Agrobacterium*. This binary vector possess nourseothricin resistance gene as selection markers (Fig. 8), so it can be used to perform a single knockout in haploid species *V. dahliae* and *V. albo-atrum.* In *V. longisporum*, silencing of *CPC1* resulted in a phenotype, which was not affected in growth rate, but was sensitive to amino acid starvation conditions when compared with the wild type phenotype. Moreover, pathogenicity inside the host plant was suppressed compared to wild type infection. So in parallel to silencing of *CPC1* in *V. longisporum* we also decided to generate deletion mutants of this gene in *V. dahliae*. Therefore we used this new knockout vector to construct the cassettes for disruption of *CPC1* gene in *V. dahliae*.

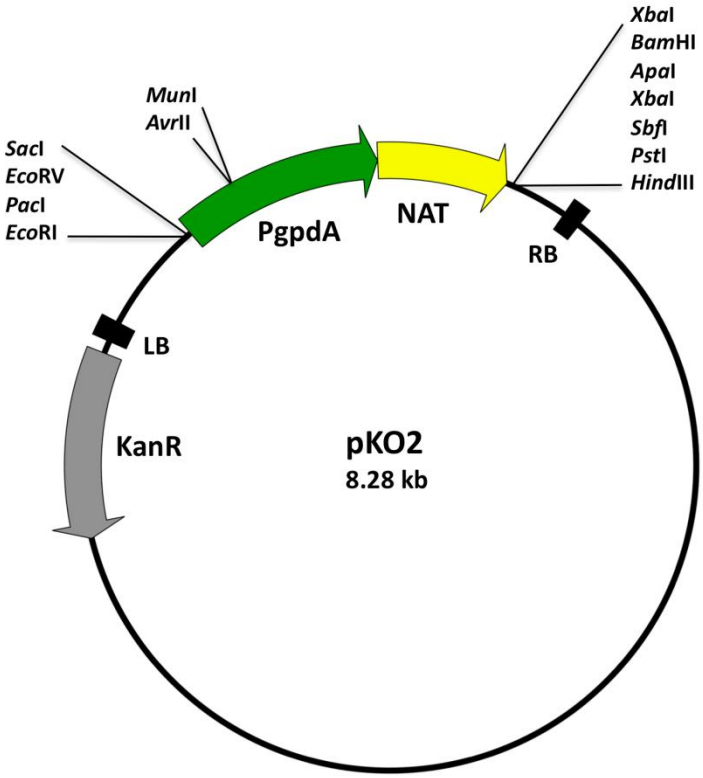

Fig. 8 The structure of plasmid pKO2 for gene disruption (Tran; 2011). pKO2 carries the nourseothricin resistance cassette. The vector possesses multiple cloning sites before and after the resistance cassettes that allow easy generation of constructs to disrupt genes of interest in *Verticillium*.

To generate knockout construct for *V. dahliae* the vector pKO2 was used to generate a *CPC1* mutant allele for gene disruption. The vector was used to disrupt *CPC1* gene in *V. dahliae*. Two fragments of 1.6 kb and 866 bp covering the *CPC1* gene from *V. dahliae* strain VdJR2 with a gap of 252 bp was amplified (Fig. 9) using the pairs *Vdcpc*KOF1l and *Vdcpc*KOF1r and *Vdcpc*KOF2l and *Vdcpc*KOF2r. (Table 3) These primers contained

restriction sites, *Sac*I/*Pac*I and *Xba*I/*Bam*HI respectively at the 5´ and 3´-ends to facilitate cloning of the PCR products. These products were aligned together with the resistance cassette to generate the cpc1 deletion construct. The resulting 3.7-kb *CPC1* fragment was cloned into pKO2 at *Eco*RV and *Xba*I to create p*KO2CPC1* vector (Fig. 9). This *VdCPC1* deletion construct was introduced into *A. tumefaciens* AGL1 for fungal transformation. The resulting transformants were screened by PCR and verified by Southern hybridization.

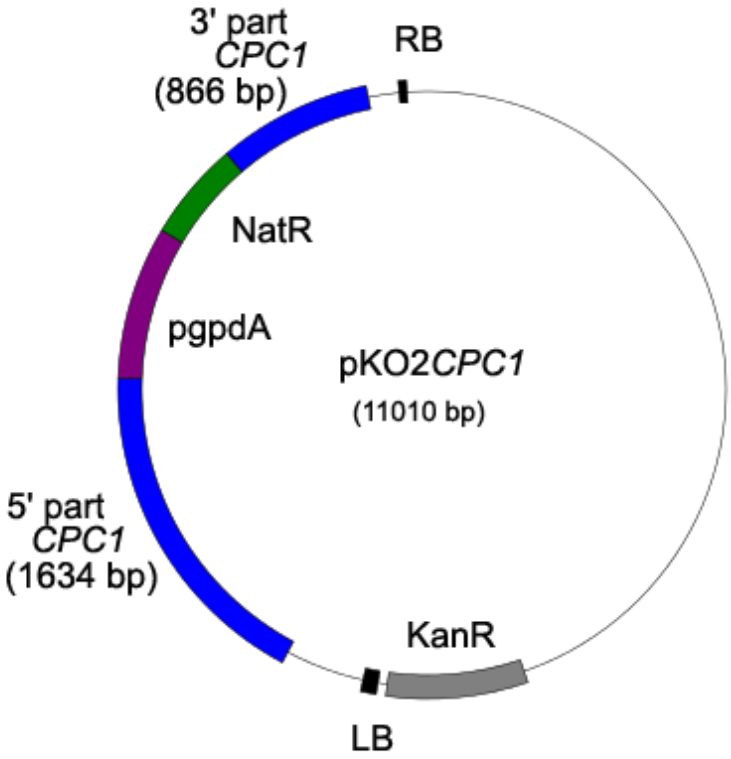

Fig. 9. Knockout construct for *VdCPC1*. The gene for *CPC1* is disrupted by *Nat* resistance cassette. The binary vector transfers the deletion cassette via *A. tumefaciens* mediated transformation into *V. dahliae*.

2.6. Southern hybridization

The Southern hybridization was performed following the recommendations in the provided manuals from GE Healthcare manufacturer. In brief, about 25 µg of genomic DNA was digested for overnight with 3 µl of an appropriate restriction enzyme that uncuts or cuts the target genes at a unique site outside the probe sequence. The digested mixture was analyzed on a 1% agarose gel, DNA was denaturated and transferred to the Amersham Hybond-N membrane (GE Healthcare, Munich, Germany) by blotting. DNA molecules

and the membrane were cross-linked under UV light. A fragment (about 600 bp) of the target gene or the whole gene was amplified and labelled as probe using Amersham AlkPhos Direct labelling Reagent (GE Healthcare, Munich, Germany). The DNA on the membrane was hybridized to the specific probe in hybridization buffer at 60^{o}C for overnight. The membrane was treated carefully with washing buffers to eliminate background. Then, chemiluminescent signals were detected using the Amersham CDP-Star Detection reagent (GE Healthcare, Munich, Germany).

2.7. Quantitative real-time PCR

The total RNA was extracted from 0.1 g of fungal mycelium grown in SXM medium using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Concentration of RNA samples was measured using NanoDrop-1000 spectrophotometer (PEQLAB, Erlangen, Germany). Then, 1 µg of total RNA was transcribed into cDNA in 20 µl using the QuantiTect® Reverse Transcription kit (Qiagen, Hilden, Germany). The expression level of a gene was measured by real-time PCR using a specific primer pair. Reactions contained 2 µl of each 0.5 pM primer (forward and reverse), 1 µl of the cDNA, and reverse transcriptase-grade PCR water to a final volume of 12.5 µl. The 5 PRIME MasterMix (5 PRIME GmbH, Hamburg, Germany) was added to obtain a final running volume of 20 µl per reaction. Each reaction was run in triplicate for both the standard and unknown samples. Reactions were run under the following conditions using the Light Cycler 2.0 System (Roche, Manheim, Germany): 95°C denaturation for 3 min, 42 cycles at 95°C for 10s, 63°C for 15s and 72°C for 25s to calculate cycle threshold values, followed by 95°C for 1 min, 55°C for 1 min and 80 times of 55°C for 10s, increasing temperature by 0.5°C each cycle to obtain melting curves and to enable data analyses. Standard curves were produced with purified DNA products of 10 and 1 pg/µl and starting concentrations of 100, 10, and 1 fg/µl. A baseline subtracted curve fit was used to generate standard curve data. Absolute amounts of transcripts were calculated using a correlation coefficient formula generated from the standard curve in each run.

2.8. Plant experiments

2.8.1. Plant infection assays

Rapeseed *Brassica napus*, *Arabidopsis thaliana* or tomato seedlings of ten days old were inoculated with 10^7 fungal spores/ml (mutants or wild type), and mock with tap water for 40 min by root-dipping. The seedlings were then transferred in pots with a sterile sand:soil (1:2) mixture. The plants were allowed to grow in a climate chamber with 16 hours light at

23°C and 8 hours dark at 20°C. The height of plants or leaf size was measured to calculate disease scores at 7, 14, 21, 28 and 35 days post inoculation (dpi).

Spore injection: 50 µl of 10^7 fungal spore suspension (wild type or mutants) was transferred into each tomato plant of 3 weeks old by injection. For mock, the sterile tap water was used instead. The disease symptoms were observed at 14 days after injection.

2.8.2. Quantification of fungal DNA from infected plants.

Hypocotyls, stem (5–6 cm from top of plant), roots and leaves were separated from the infected rapeseed plants harvested at 28, and 35 dpi. 20 plants were harvested from each treatment and fungal DNA

For extraction of fungal DNA from infected plant the DNeasy Plant Mini Kit (Qiagen Hilden, Germany) was used with modification to manufacturers protocol. Plant material was grinded using a Retsch mill (Retsch, , Germany) at 30000 rpm for 1 min 30 sec. 50-100 mg of grinded material was filled in an E-cup and solved in 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml).The mixture was incubated for 10 min at 65°C. and mixed 2 or 3 times during incubation by inverting tube. To lyse the cells 130 µl Buffer AP2 was pipetted to the lysate, and incubated for 5 min on ice. The samples were centrifuged for 5 min at 10000 rpm.

After centrifugation the supernatant was transfered to a QIAshredder Mini spin column and centrifuged for 2 min at 10000 rpm. The flow-through fraction was pipetted into a new tube without disturbing the pellet. 1.5 volumes of Buffer AP3/E was added to the cleared lysate, and mixed by pipetting. The mixture was transferred into the DNeasy Mini spin column and centrifuged for 1 min at 8000 rpm. The column was placed into a new 2 ml collection tube, and 500 µl Buffer AW was added. The samples were centrifuged for 1 min at 8000 rpm. The flow-through was discarded, and an additional 500 µl Buffer AW was added to the DNeasy Mini spin column. The columns were centrifuged for 2 min at 10000 rpm to dry the membrane. The DNeasy Mini spin column was transferred to a 1.5 ml E-cup and 50 µl of H_2O was pipetted on the column and incubated for 5 min at room temperature. For eluation the samples were centrifuged at 8000 rpm. To increase the amount of eluated DNA an additional 50 µl of H2O was added to the column and centrifugation was repeated. The concentration of the samples was measured using NanoDrop *ND*-1000 spectrophotometer (PEQLAB, Erlangen, Germany). The samples were stored at -20°C.

The expression level of a gene was measured by real-time PCR using a specific primer pair. Reactions contained 2 µl of each 0.5 pM primer (forward and reverse), 1 µl of the DNA sample, and reverse transcriptase-grade PCR water to a final volume of 11 µl. The 5 PRIME MasterMix (5 PRIME GmbH, Hamburg, Germany) was added to obtain a final running volume of 20 µl per reaction. Each reaction was run in triplicate for both the standard and unknown samples. Reactions were run under the following conditions using the Light Cycler 2.0 System (Roche, Manheim, Germany): 95°C denaturation for 3 min, 42 cycles at 95°C for 10s, 63°C for 15s and 72°C for 25s to calculate cycle threshold values, followed by 95°C for 1 min, 55°C for 1 min and 80 times of 55°C for 10s, increasing temperature by 0.5°C each cycle to obtain melting curves and to enable data analyses. The amount of DNA of *V. longisporum* was estimated by integration of a calibration curve using increasing amounts of genomic *V. longisporum* DNA from 100 pg to 100 ng, in the analysis.

2.9. DNA-sequencing

DNA-sequences were analysed using the Sanger-method (Sanger and Coulson, 1975) in Goettingen Genomics Laboratory. The concentration of the DNA was measured using Nano drop (NanoDrop Spectrophotometer ND-1000, PEQLAB BIOTECHNOLOGIE GMBH, Erlangen,D). Then, the necessary amount of DNA was calculated to get a final concentration of 50 ng/µl to 100 ng/µl. 200 ng of DNA together with 5 pmol of primer in a total volume of 5 µl was given for sequencing.

Chapter 3. Results

3.1. Cross pathway control and its role in *V. longisporum*

Microorganisms have to adjust to altering environmental circumstances. To survive under rapidly evolving environmental situations, they need to respond on these impacts. The Cross pathway control (*Cpc*) of amino acid biosynthesis acts in response to the environmental stress conditions amino acid deprivation. *Cpc* has been shown to play a crucial role for fungal life cycle and metabolism. In other organism Cpc is involved in many cellular processes regarding primary and secondary metabolism. The *Verticillium* cross pathway control was upregulated when the fungus is growing inside the plant (Singh *et al.* 2010). However the investigation whether *CPC1* is involved in pathogenicity of the pathogen is still not known. Therefore we had a deeper look into the role of *CPC1* in *V. longisporum* and *V. dahliae*, and to see if mutants lacking *CPC1* by silencing of the gene or by deletion are affected in their pathogenicity. We hypothesized that an active amino acid biosynthetic capability would be required for survival in the host and, therefore, that the *V. longisporum* cross pathway control would play an important role in the pathogenicity of this fungal pathogen.

3.1.1. *V. longisporum* has two copies of *VlCPC1*

V. longisporum has been described as hybrid between two *Verticillium* species (Inderbitzin *et al.* 2011; Tran *et al.* 2013). Therefore it was examined whether *VlCPC1* has two isogenes. To determine the copy number of *VlCPC1* Southern analysis was performed (Fig. 10). So genomic DNA of *V. longisporum*, *V. dahliae*, and *V. albo-atrum* were digested with suitable enzymes, and subjected to Southern hybridization. A fully sequenced 550 bp long fragment of *VlCPC1*, amplified with primers ProbeCpc1left and ProbeCpc1right was used as the probe. In our several independent Southern hybridization analyses, two signals were generated for *V. longisporum* as compared to *V. dahliae*, and *V. albo-atrum* for the genomic DNA digested with *BamH*I. According to these data two isogenes for cross pathway control (*VlCPC1*-1 and *VlCPC1*-2) exist.

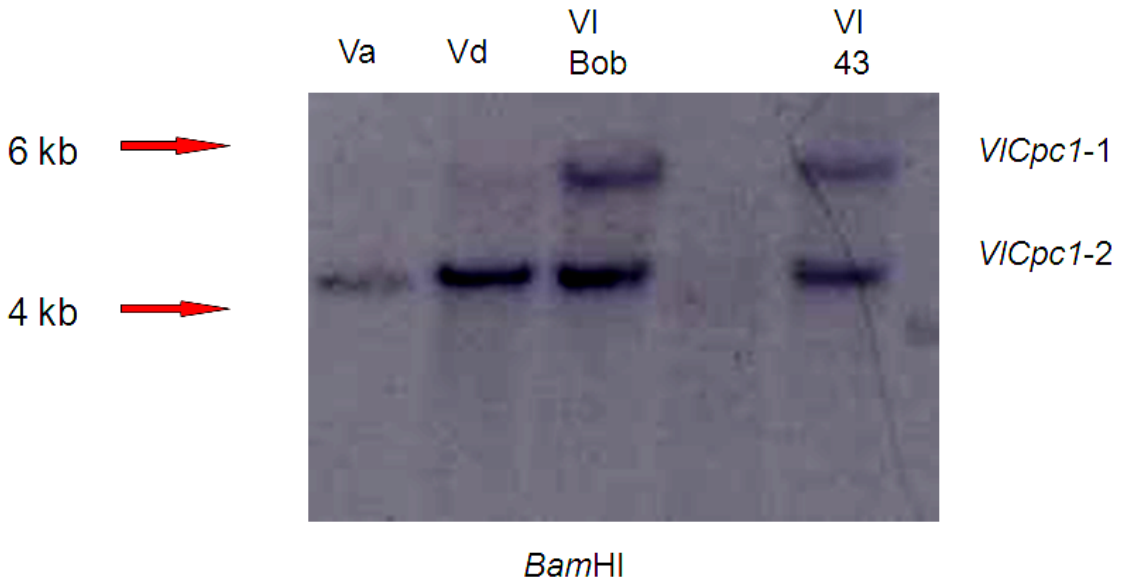

Fig. 10 Determination of the isogene *VlCPC1-2* of *VlCPC1-1*. Southern hybridization analysis of *V. albo-atrum*, *V. dahliae* and *V. longisporum*. The genomic DNA was digested with *BamH*I. A 500 bp sequence of *VlCPC1-1* was used as a probe. For *V. albo-atrum* and *V. dahlia*e one copy of *CPC1 i*s visible, for *V. longisporum* two copies exist.

These results demonstrated that *VlCPC1-1* has an isogene, *VlCPC1-2*. To determine the extent of similarity of these two isogenes in the *V. longisporum* genome, the genomic DNA of *V. longisporum* was digested with *Sal*I and *Xho*I and run on a low percentage agarose gel. DNA was extracted from the four gel fragments corresponding to spots where the signals were generated in the Southern hybridization analysis (Fig. 10). *VlCPC1-1* specific primers were used to amplify the gel extracted DNA and subjected to sequencing. Sequencing of a *V. longisporum* cDNA library (Singh *et al.*, 2010) revealed the presence of both transcripts (*VlCPC1-1* and *VlCPC1-2*) for the cross pathway transcriptional regulator *VlCPC1*. We examined the chromosomal loci of the corresponding *CPC1* genes of the haploids *V. dahliae* and *V. albo-atrum* in comparison to *V. longisporum* (Fig. 10), which has been demonstrated to be a hybrid between two different *Verticillium* species (Inderbitzin *et al.*, 2011). In a first approach, we designed a primer pair *CPC1*Seqleft and *CPC1*Seqright (Table 3) on the basis of the *CPC1* locus (VDAG_10113) from the BROAD *V. dahliae* genome (Klosterman *et al.*, 2011). Sequencing of the PCR products from *V. dahliae* and *V. albo-atrum* resulted in the unique single sequences of 725 bp for *VdCPC1* and 723 bp for *VaCPC1*. In contrast, cloning and sequencing of the PCR product from *V. longisporum* revealed two distinct sequences for *VlCPC1* corresponding to isogenes *VlCPC1-1* and *VlCPC1-2* with the lengths of 725 and 723 bp similar to *VdCPC1* and *VaCPC1*, respectively (Fig. 11 and 12). The DNA sequences were determined and resulted in two, *VlCPC1* and a single *CPC1* sequences for *V. dahliae* or *V. albo-atrum*,

respectively (Fig. 11). In total there are more than 60 SNPS between all four sequences. The deduced open reading frames of all genes consist of two exons with a length of 13 or 653 base pairs (bp) separated by one intron. The introns of *VdCPC1* of *V. dahliae* and *VlCPC1-1* of *V. longisporum* have a length of 59 bp, whereas *V. albo-atrum VaCPC1* and the second isogene *VlCPC1-2* share a length of 57 bp. On sequencing of both loci it was found out that the coding and intronic sequences of both isogenes are identical in *V. longisporum*.

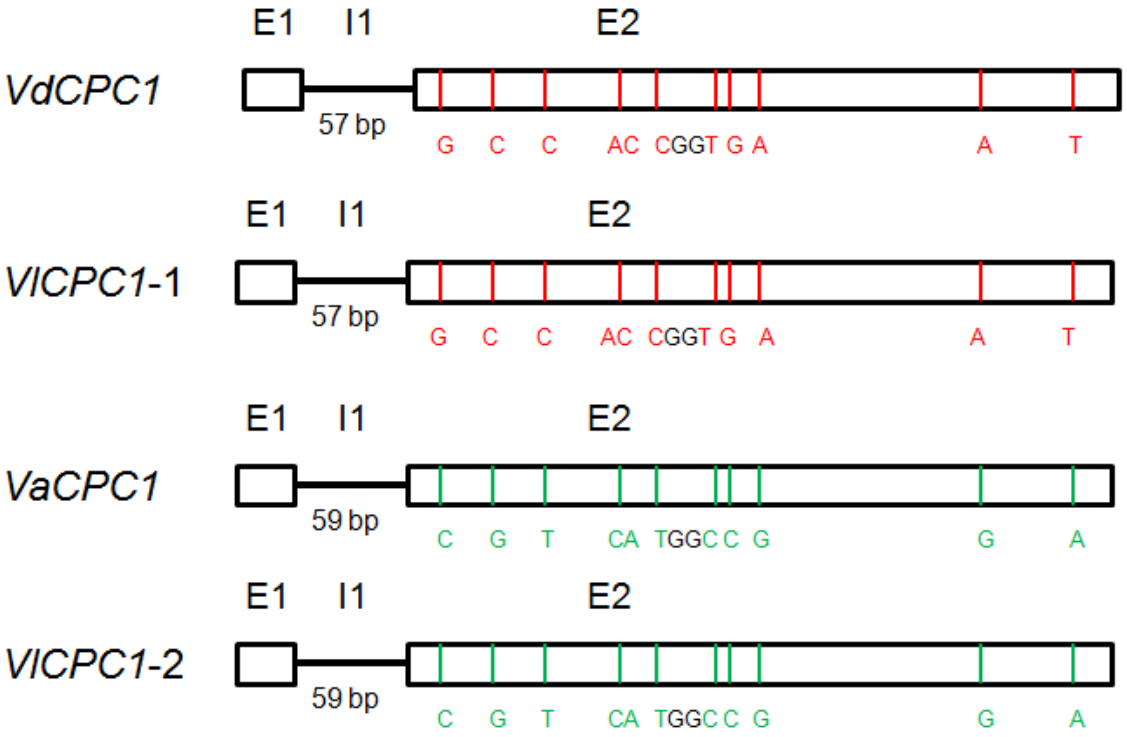

Fig. 11 Schematic of *CPC1* genes of *Verticillium* species. Gene comparison of *V. 1ongisporum* sequences *VlCPC1-1* and *VlCPC1-2*, *V. dahliae VdCPC1* and *V. albo-atrum VaCPC1*. Colors indicate common nucleotides between *VlCPC1-1* and *VdCPC1* (red) or *VlCPC1-2* and *VaCPC1* (green).

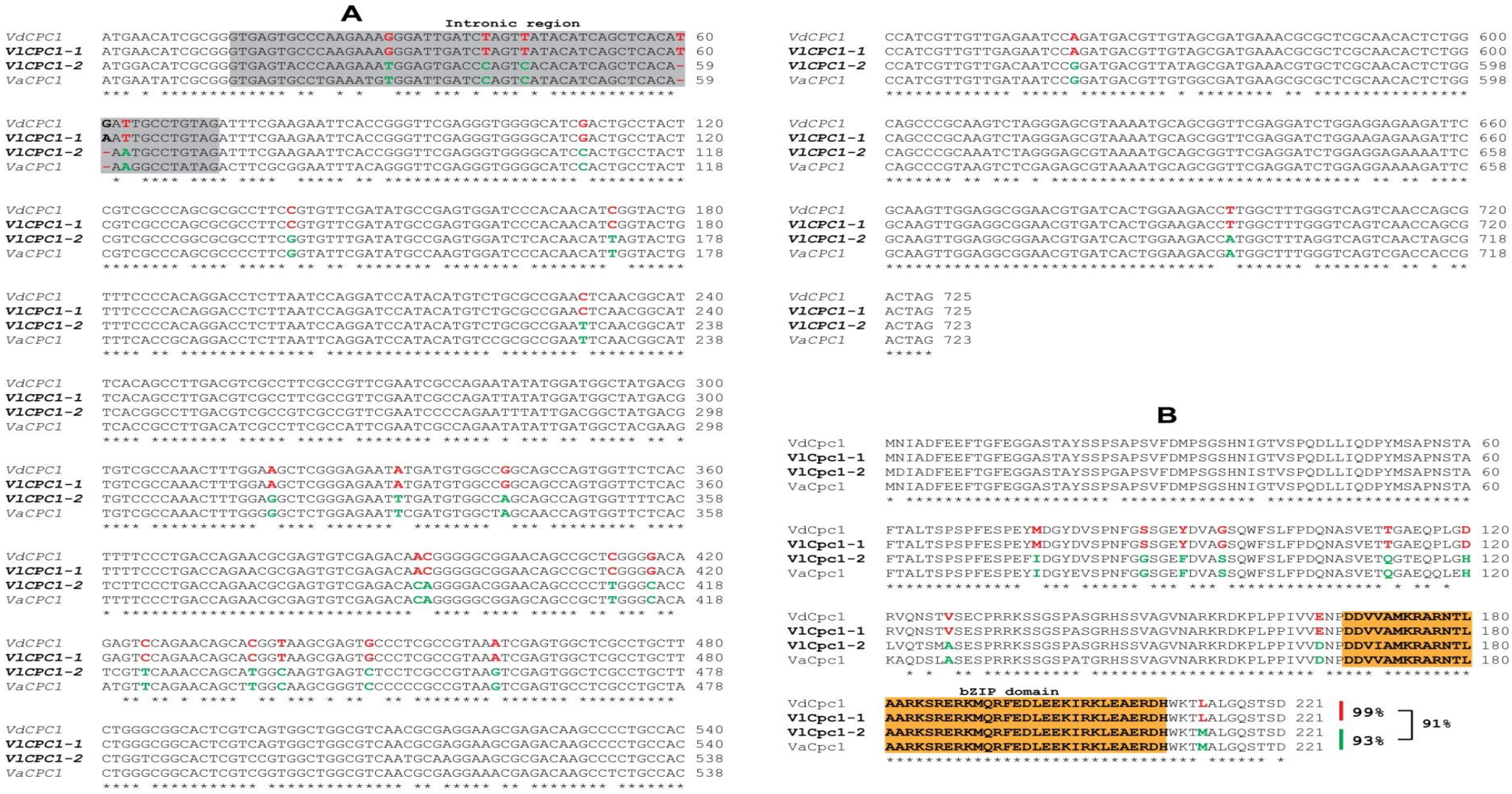

Fig. 12 Sequence alignment for *CPC1* genes of *Verticillium* species. Sequence alignments of *V. 1ongisporum* sequences *VlCPC1-1* and *VlCPC1-2*, *V. dahliae VdCPC1* and *V. albo-atrum VaCPC1*. The grey box indicates intron regions and the bZIP region is orange. Additional colors indicate common nucleotides between *VlCPC1-1* and *VdCPC1* (red) or *VlCPC1-2* and *VaCPC1* (green).

The comparison of the *Verticillium* derived genes revealed that *V. albo-atrum* and *V. dahliae CPC1* are 88% identical and differ in 65 SNPs (Fig 11). The SNPs result in a change of 25 amino acids in the primary protein sequence and therefore the deduced amino acid sequence is 93 % identical (Fig. 12). *V. longisporum VlCPC1-1* differs in 12 and *VlCPC1-2* in 36 nucleotides from the single *V. dahliae VdCPC1* with a remaining identical DNA sequence of 98% and 95%, respectively (Fig. 12). This results in a change of 2 amino acids in the primary protein sequence of *VlCPC1*-1 and therefore the deduced amino acid sequence is 99 % identical for *VlCPC1*-2 there is a change of 12 amino acids in the primary protein sequence and therefore the deduced amino acid sequence is 95 % identical. *VlCPC1-1* has 88% identity with *VdCPC1*, whereas *VlCPC1-2* has at least 92% identity. There are two characteristic sequence patterns which correspond either to *V. dahliae* or to *V. albo-atrum*. *VlCPC1-1* and *V. dahliae VdCPC1* share the CGGCAG signature which is different from the TAGCAA signature which is present in *VlCPC1-2* and *V. albo-atrum VaCPC1* (Figure 12). However this signature does not change the amino acid sequence. These data further corroborate that *V. longisporum* is a hybrid with two different copies for *CPC1*, whereas haploids as *V. dahliae* or *V. albo-atrum* carry a single copy. The closest relatives are the *V. dahliae* copy and *V. longisporum VlCPC1-1*

Transcript analysis suggested that *CPC1* mRNA is induced during infection of *B. napus* and *A. thaliana* (Singh *et al.* 2010). We were interested in analyzing the importance of this plant-specific induction of *CPC1* for fungal pathogenicity on plants. Since we found two *CPC1* genes we analysed whether both of them carry signals which suggest a regulatory control of gene expression. *Verticillium CPC1* genes carry a cross-pathway recognition element in their own promoter (Fig. 13). The cross-pathway recognition element (CPRE) as binding site for target promoters of *CPC1* is conserved and had been originally described for Gcn4p as the *CPC1* orthologue of *S. cerevisiae.* The CPRE consists of a palindromic sequence element constituted by a central C·G pair that is flanked by TGA halfsites (Oliphant *et al.*, 1989). In contrast to the yeast *GCN4* gene which is not significantly autoregulated (Albrecht *et al.*, 1998), the characterized *Aspergillus* and *N. crassa CPC1* homologs carry CPREs within their own promoter region for enhanced expression by autoregulation (Hoffmann *et al.*, 2001; Krappmann *et al.*, 2004; Tian *et al.*, 2007). Inspection of the four chromosomal *CPC1* genes of *Verticillium* revealed a typical CPRE motif TGACTCA which is present 985 bp upstream of the ORFs for the four *CPC1 genes of V. longisporum*, *V. dahliae* and *V. albo-atrum* suggesting that they are also autoregulated by the transcription factor *CPC1* (Fig. 13A).

The fungal *GCN4/CpcA/CPC1* genes are paradigms for translational control by small open reading frames (uORFs). In the region upstream of the translational start codon, a small ORF was identified, uORFa (-424 to -304 bp upstream), encoding putative polypeptide of 39 amino acids in length. The deduced primary sequence of an uORF shows high similarity to those mapped in the leader regions of *CPC1* from *N. crassa* (Paluh *et al.*, 1988) with a calculated homology of 57% (Fig. 13B).

The c-terminal leucine zipper region is highly conserved in filamentous fungi (Fig. 13C). The sequences of *VlCPC1*-1 and *VdCPC1* are 100% identical, whereas compared to *VlCPC1*-2 and *VaCPC1* are 97% similar to this sequence. They differ in 2 amino acids. Compared to *N.crassa* the sequence is 74% identical and to *A. nidulans, A.fumigatus* and *A. niger* it is 47-55% similar.

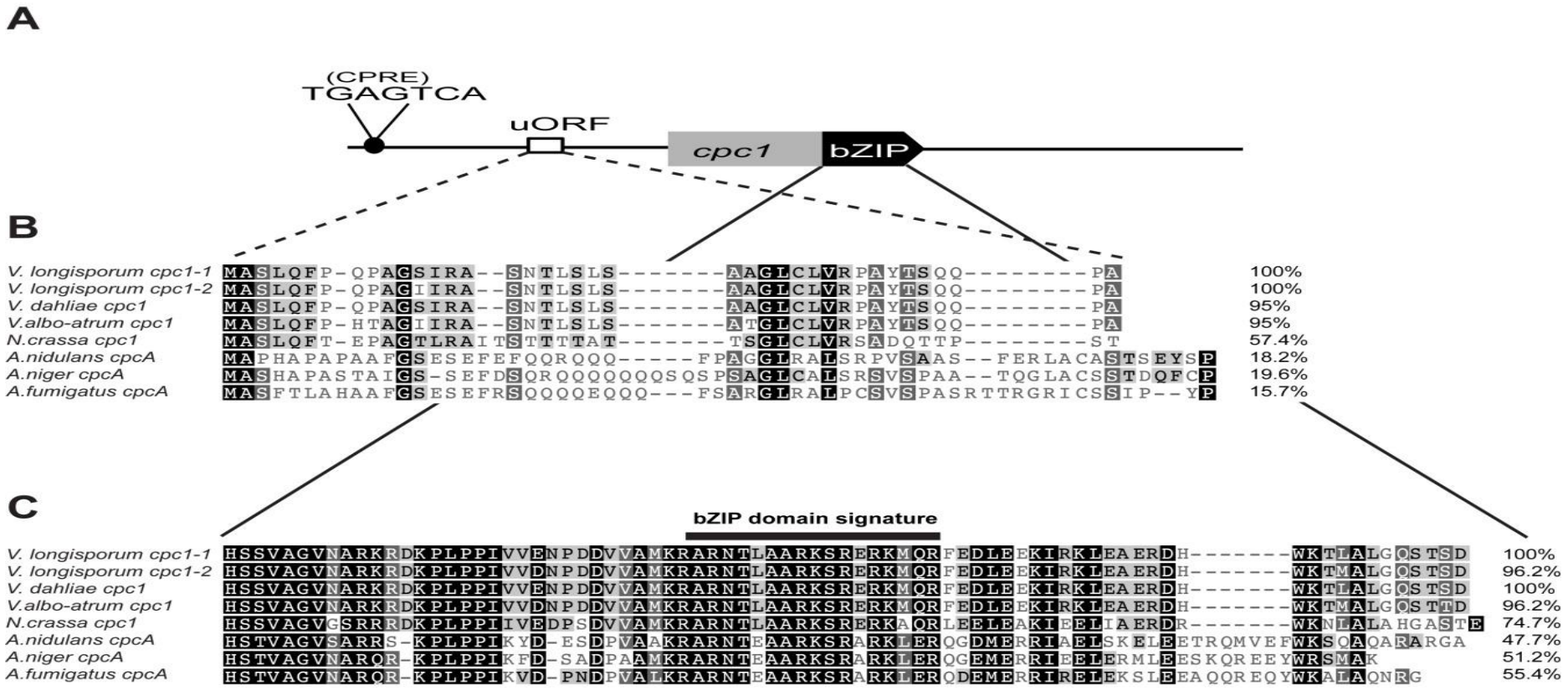

Fig. 13 The *CPC1* loci of *Verticillium* species. **A.** The black dot indicates the putative cross-pathway control recognition element (CPRE) with the sequence 5'TGAGTCA3'. The conserved leucine zipper region at the C-terminus of *CPC1* is in black. The upstream open reading frame uORF in the 5' leader region is represented as white box. **B.** uORF alignments of the deduced amino acid sequences in comparison to corresponding uORFs of *CPCA/CPC1* genes of *A. fumigatus, A. nidulans, A. niger* or *N. crassa*. White letters in black boxes represent conserved, grey 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 as black line. (reprinted from Timpner *et al*. 2013 The Cpc1 regulator of the cross-pathway control of amino acid biosynthesis is required for pathogenicity of the vascular pathogen Verticillium longisporum. Mol Plant Microbe Interact doi.org/10.1094/MPMI-06-13-0181-R)

An overall comparison of the four *CPC1* genes from *V. longisporum*, *V. dahliae*, *V. albo-atrum* with other species group them together with other pathogenic fungi like *Colletotrichium* or *Fusarium* and separate them from saprophytes as *Aspergillus* or single cell yeasts *S. cerevisiae* (Fig. 14).

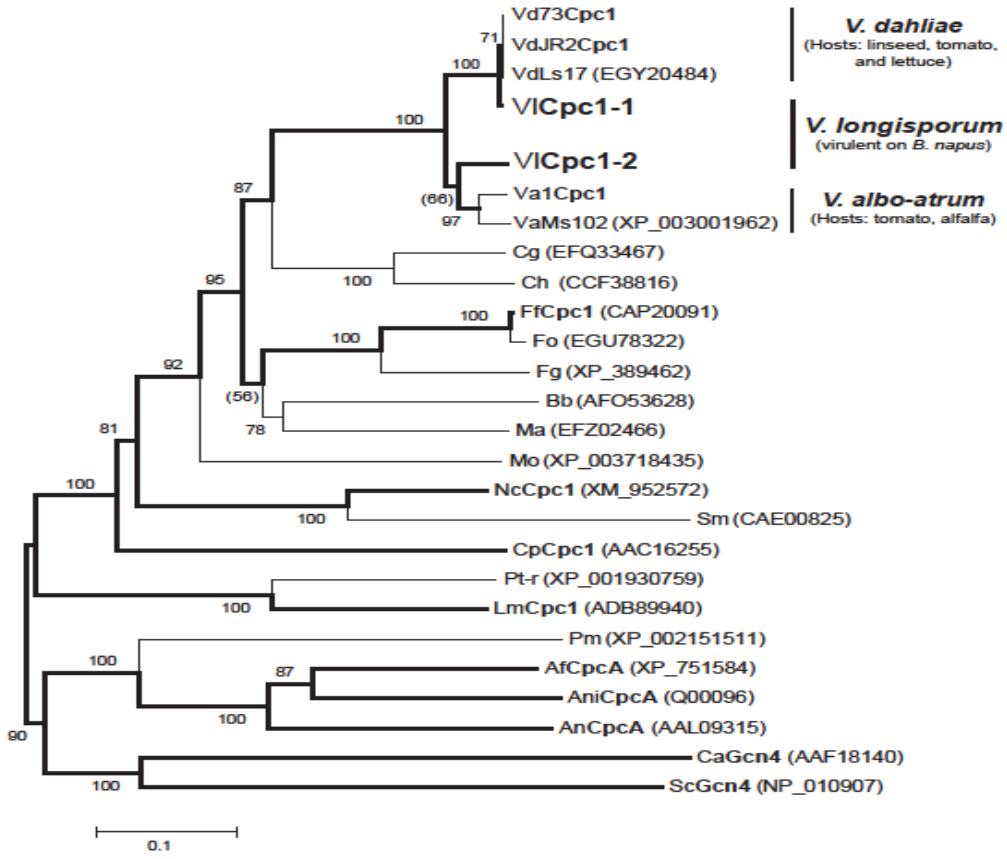

Fig. 14 Phylogenetic analysis for deduced Cpc1 proteins of three *Verticillium* species in comparison to the orthologues of other filamentous fungi. The virulent hybrid pathogen *V. longisporum* possesses two isogenes encoding deduced Cpc1-1 or Cpc1-2 which are similar to *V. dahliae* and *V. albo-atrum*, 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 bracket. *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*, *Sc*: *Saccharomyces cerevisiae*. (reprinted from Timpner *et al*. 2013 The Cpc1 regulator of the cross-pathway control of amino acid biosynthesis is required for pathogenicity of the vascular pathogen Verticillium longisporum. Mol Plant Microbe Interact doi.org/10.1094/MPMI-06-13-0181-R)

Cpc1 from *Verticillium* species is close to other phytopathogenic fungi like *Colletotrichium* and *Fusarium* and further away from *Aspergillus* or *S. cerevisiae*. The plant pathogenic fungi can be divided into one group which is close to each other and farer away from the other fungi.

3.1.2. *VlCPC1* isogenes were silenced up to 85% by RNA-mediated gene silencing

Because two copies of *VlCPC1* exist gene silencing was used as a method. RNA-mediated gene silencing has been established in several fungi for targeted gene silencing instead of a conventional knockout (Nakayashiki, 2005). *A. tumefaciens* mediated transformation of *V. longisporum* was applied and thirty independent hygromycin-B-resistant transformants were selected at random for further analysis. To analyze the efficiency of *VlCPC1* silencing we investigated the mutants by RT-PCR as the silencing of gene expression is the result of posttranscriptional degradation of targeted mRNA. Total RNA was extracted from the *VlCPC1sm* (*VlCPC1* silenced mutant) and wild type. RT-PCR results showed the significant knockdown of *VlCPC1* transcripts (Fig. 15). The extent of silencing varied in the different transformants between high and moderate levels. The degree of silencing of cross pathway control genes was estimated by RT-PCR by the ratio of the amplification of *VlCPC1* between the respective transformants and the unsilenced wild type. For each transformant three biological replicates were analyzed. High-frequency silencing was observed for 54% of the transformants exhibiting reduced gene expression. The extent of silencing varied in the different transformants between high and moderate levels. After 5 generations on SXM the silencing of the *VlCPC1* isogenes was still stable (Fig. 15).

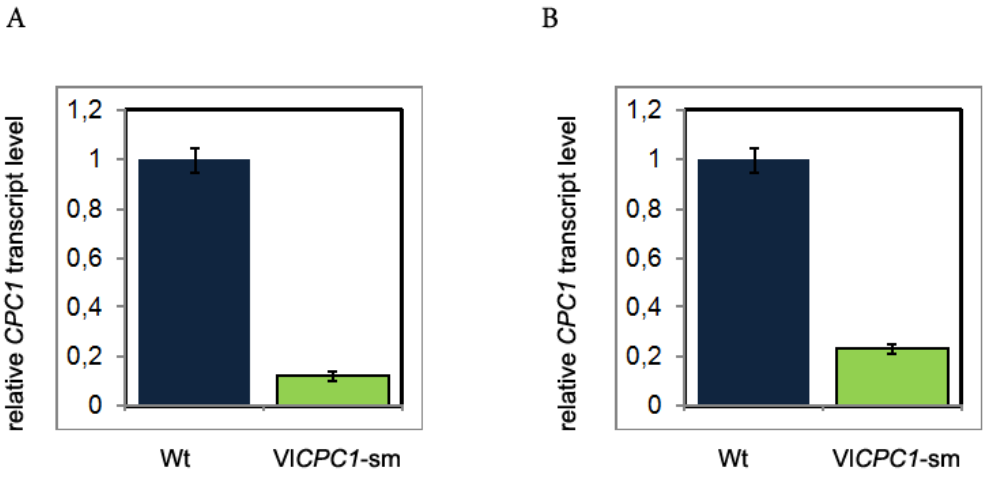

Fig. 15 Expression of cross pathway control encoding genes of *V. longisporum* Relative expression of *VlCPC1* in *Vl* 43 and *VlCPC1*sm measured by quantitative real-time PCR. *VlCPC1* cDNA was normalized to the histone 2a cDNA. *Vl* 43: wild type, *VlCPC1*-sm, *CPC1* silenced mutants. The error bars represent the standard deviation of four different measurements of cDNA. **A.** Transcript levels of *VlCPC1* **B.** Transcript levels of *CPC1* after 5 generations on SXM.

3.1.3. Screening for knockout of *CPC1* in *V. dahliae*.

Silencing strategy is an option for studying genes of interest in fungal species whose genomes are not yet sequenced or for investigating lethal genes. However silencing suppresses maximally about 80-90% of gene expression in fungi (Nakayashiki *et al.*, 2005; Singh *et al.*, 2011; Singh *et al.*, 2012). The rest activity of the gene (10-20%) might be still enough for its function in the fungus. To gain deeper insights into the function of *CPC1* a deletion of the gene is necessary. Therefore we decided to generate a knockout of *CPC1* in *V. dahliae*.To analyse mutants and wildtype strain a part of *CPC1* gene was amplified via PCR with the primer pair P1/P2. As a product a 1.3-kb fragment of *CPC1* gene from the genome of the wild type strain and a 2.5-kb fragment including the resistance cassette from the genome of the mutants was generated by PCR (Fig. 16). For the identification of correct integration of the deletion construct, candidates from the transformants pool were tested by Southern hybridization. To check for the *CPC1* deletion, a probe binding to the 5' region of the gene was used. Genomic DNA isolated from clonal transformants was digested with the enzymes *Bcu*I and *Eco*81I, for comparison wild type DNA was used. From Southern hybridisation, the 550-bp fragment of *CPC1* gene as probe detected only one band for *CPC1* gene in both wild type and mutants (Fig. 16). Two transformants displaying the expected signals at 2.2 kb (*Bcu*I and *Eco*81I) were used for further experiments (Fig. 16). The Southern band from the deletion mutants was 1 kb bigger than the band from the wild type strain explaining the successful exchange between the deletion cassette and locus of *CPC1* gene. For final

validation Southern hybridisation was made with the same probes and enzymes as for the two single knock-outs, and isolates resulting in the correct bands were used for further investigation (Fig. 16).

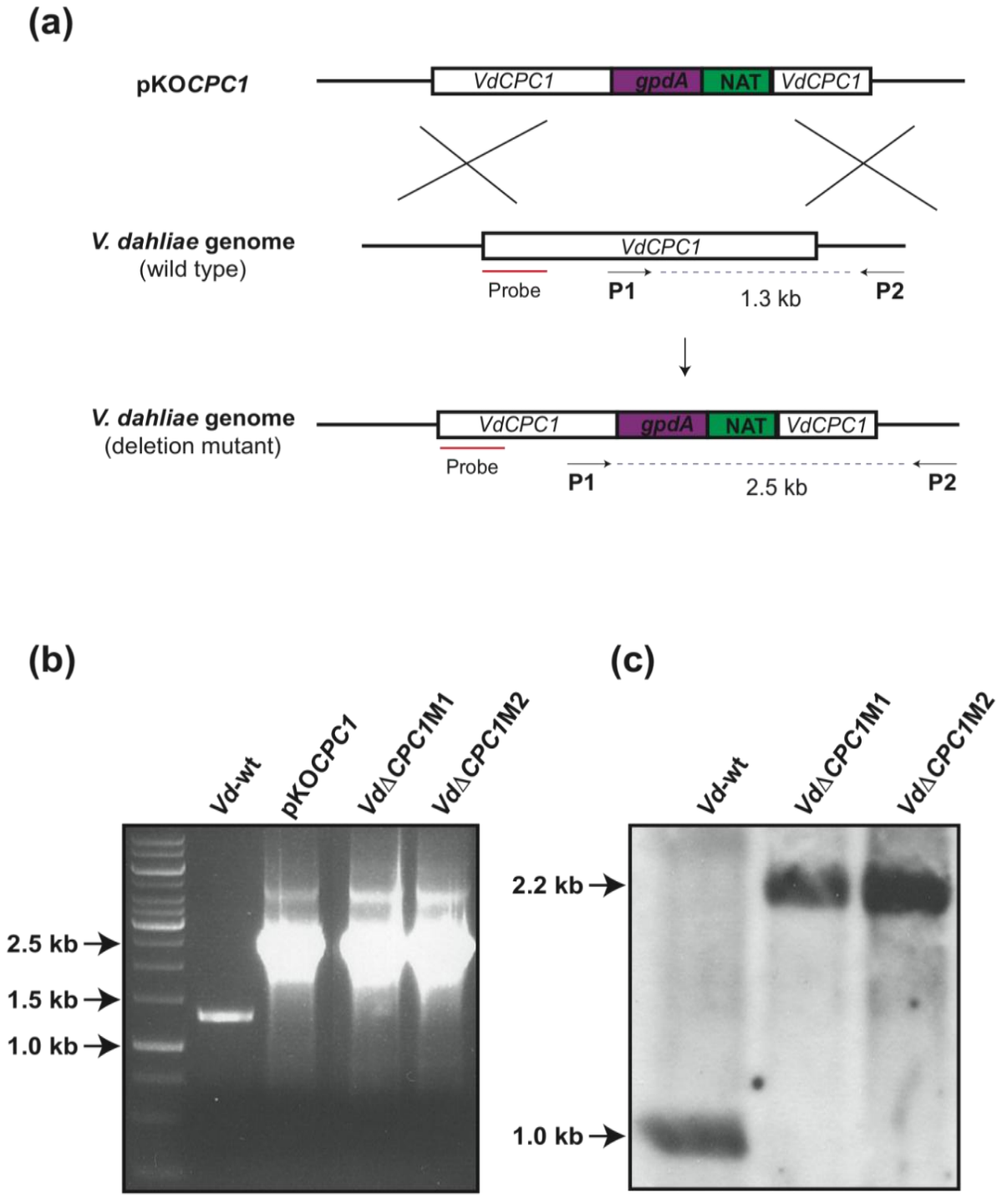

Fig. 16 Strategies for deletion of *CPC1* gene in *V. dahliae* and confirmation of the corresponding deletion mutants. A. Model for exchange between the deletion construct and wild type locus of *CPC1* gene in *V. dahliae*. **B.** Screening *CPC1* deletion mutants using PCR with the primer pair P1/P2. **C.** Confirmation of the *CPC1* deletion mutants by Southern hybridization using *BcuI* and *Eco*81I for genome digestion. The same band (2,2 kb) in the mutants is 1 kb bigger than the one in the wild type VdJR2.

The plasmid was confirmed via digestion with *Sal*I and *XmaJ*I (Fig. 17). We transformed this deletion construct of *CPC1* into *V. dahliae* strain VdJR2 from tomato. Consequently, 100 transformants were selected randomly.

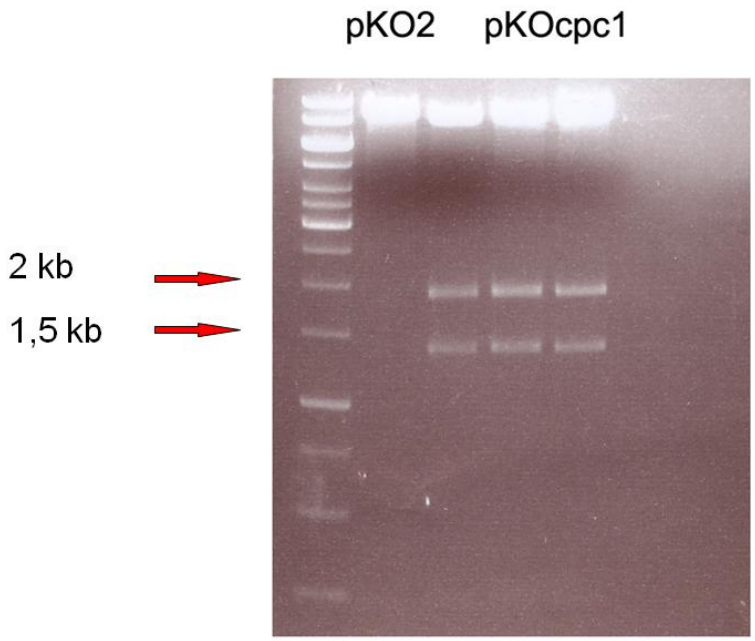

Fig. 17 Control digestion of pKO2 and pKO*CPC1*. Both plasmids were digested with *Sal*I and *XmaJ*I. For pKO*CPC1* this resulted in two fragments with the size of 1,5 kb and 2 kb.

3.1.4. *VlCPC1* silenced mutants are strongly reduced in growth under amino acid starvation conditions

We analyzed the saprotrophic lifestyle of the *VlCPC1*sm and investigated if silencing of *VlCPC1* inhibits the growth of *V. longisporum* in the absence of amino acids, One day after inoculation on CDA, *VlCPC1*sm could grow like the wild type. For both strains short hyphae were visible and the beginning of mycelial growth (Fig. 18). After 2 days of inoculation on CDA, the mycelial growth increased, and the first conidia could be detected. In addition we studied the effect of *VlCPC1* silencing on the radial growth of *V. longisporum*. The radial growth of *VlCPC1*sm measured after 3, 6, 9, 12 or 15 days was similar to wild type (Fig. 18). Therefore, growth under normal conditions does not affect a *VlCPC1* silenced mutant.

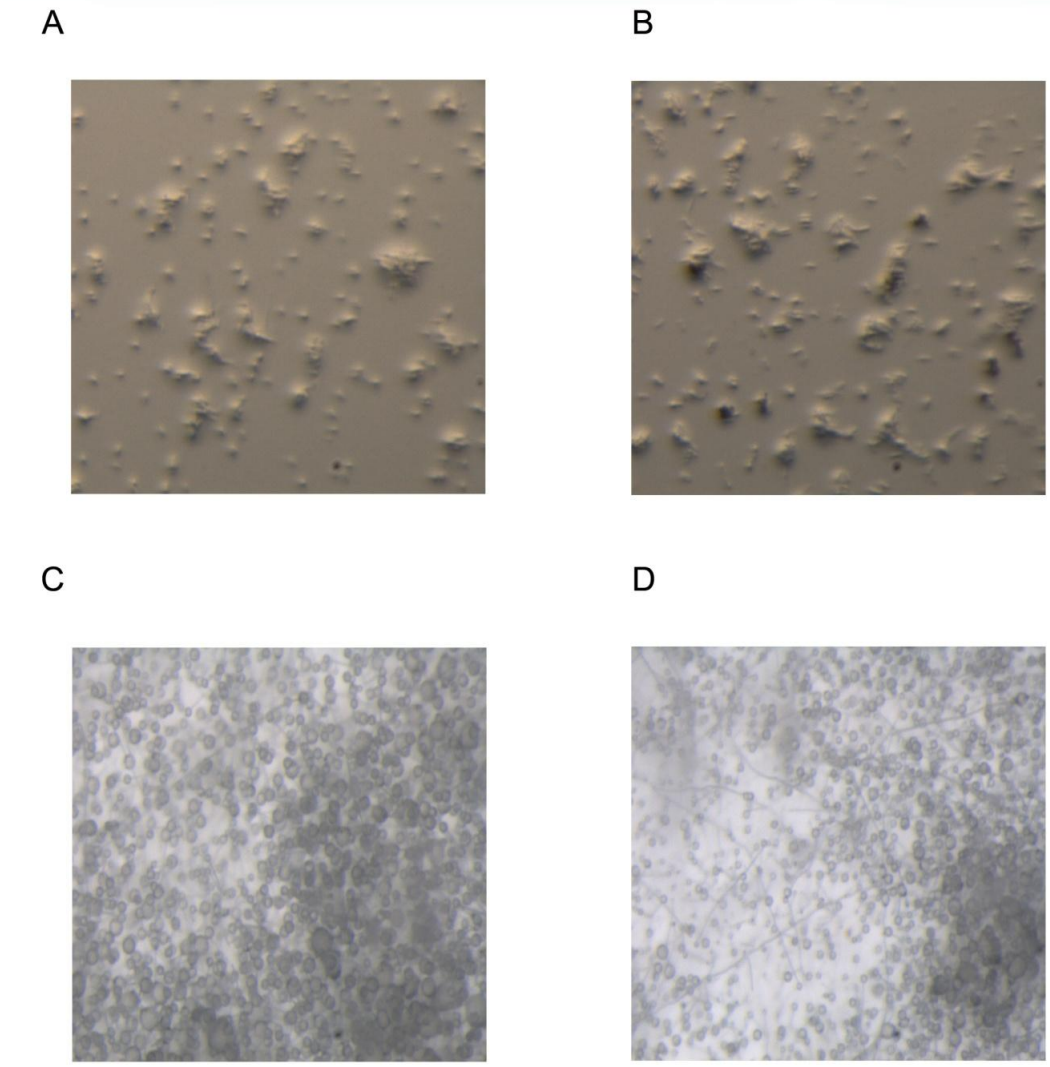

Fig. 18 Light microscopy images of *V. longisporum* wild type (wt) and *VlCPC1* silenced mutant (*VlCPC1*sm). The strains were cultured on CDA as observed at one and 4 days post inoculation. **A. and B.** *V. longisporum* strain (A) and *VlCPC1*sm (B) after one day of growth. **C. and D.** *V. longisporum* strain (C) and *VlCPC1*sm (D) after four days of growth.

To investigate growth limitations of the mutant in comparison to the wild type we analyzed the effect of the tryptophan analog, 5- MT on *VlCPC1*sm. 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(Schürch *et al*., 1974). Spores of *Vl* 43 and *VlCPC1*sm were plated on CDA supplemented with 5 mM 5-MT (Fig. 19A). The strains were grown for 15 days at 25°C. One day after inoculation, the wild type formed short hyphae compared to *VlCPC1*sm in which only initial germination of conidia could be observed. After 2 days, wild type showed a mycelial net with few conidia, whereas *VlCPC1*sm formed short hyphae without conidia. Interestingly, the radial growth of *VlCPC1*sm was significantly lower than in the wild type when the colony diameter was

determined until 15 days (Fig 19B). It started decreasing gradually at 3 and 6 dpi and decreased up to 40% at 9 and 12 dpi. Hence, *VlCPC1*sm showed severe reduction in initial vegetative growth as well as the growth rate observed under saprotrophic conditions on CDA supplemented with 5-MT. After 3 days of growth the diameter of the silenced mutant is limited to roughly 0,5 cm, whereas the wild type strain has reached approximately 1,5 cm. During the following time points the size of the colonies increases to 1,0 cm for the mutant and 2,7 cm for wt. However the mutant is never able to compete with the wild type strain under these limiting conditions. The colony diameter is only 1/3 of that of the wild type, whereas plated on CDA no limitation could be observed (Fig. 19A/B).

Deletion of *CPC1 of V. dahliae* resulted in wild-type like growth on minimal medium. No differences in growth rate or morphology could be observed. *VlCPC1* silencing resulted in a wild type like growth. The mutants show a phenotype after treatment with 5-MT, which reduces the growth of these mutants dramatically. Additionally we could demonstrate that 5-MT induces expression of *CPC1* in mutant and wild type. 5-MT inhibited growth of the *CPC1* knockout in *V. dahliae* completely. Growth under non starvation conditions does not affect a *VlCPC1* silenced mutant. To investigate growth limitations of the mutant in comparison to the wild type we analyzed the effect of the tryptophan analog 5- MT on *VlCPC1*sm. 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 (Schürch *et al.* 1974).

Spores of *Vl* 43 and *VlCPC1*sm were plated on CDA supplemented with 5 mM 5-MT (Fig. 19). The strains were grown for 15 days at 25°C. One day after inoculation, the wild type formed short hyphae compared to *VlCPC1sm* in which only initial germination of conidia could be observed (Fig. 18). Next the influence of 5-MT on expression levels of *VlCPC1* was examined. Therefore quantitative real-time PCR was performed to determine the expression of *VlCPC1* in wild type and *VlCPC1*sm upon induction with 5-MT (Fig. 19C). CDM was inoculated with spores of *Vl* 43 and *VlCPC1*sm. One culture from each strain served as control, the other cultures were treated with 5-MT for 4 h and then harvested to extract total RNA of the fungus. Due to the treatment with 5-MT cross pathway control was significantly upregulated in wild type as well as the mutant. But the silenced strain remains at low rates regarding the expression level, in fact even after induction the expression is lesser than in the untreated wt control (Fig. 19C).

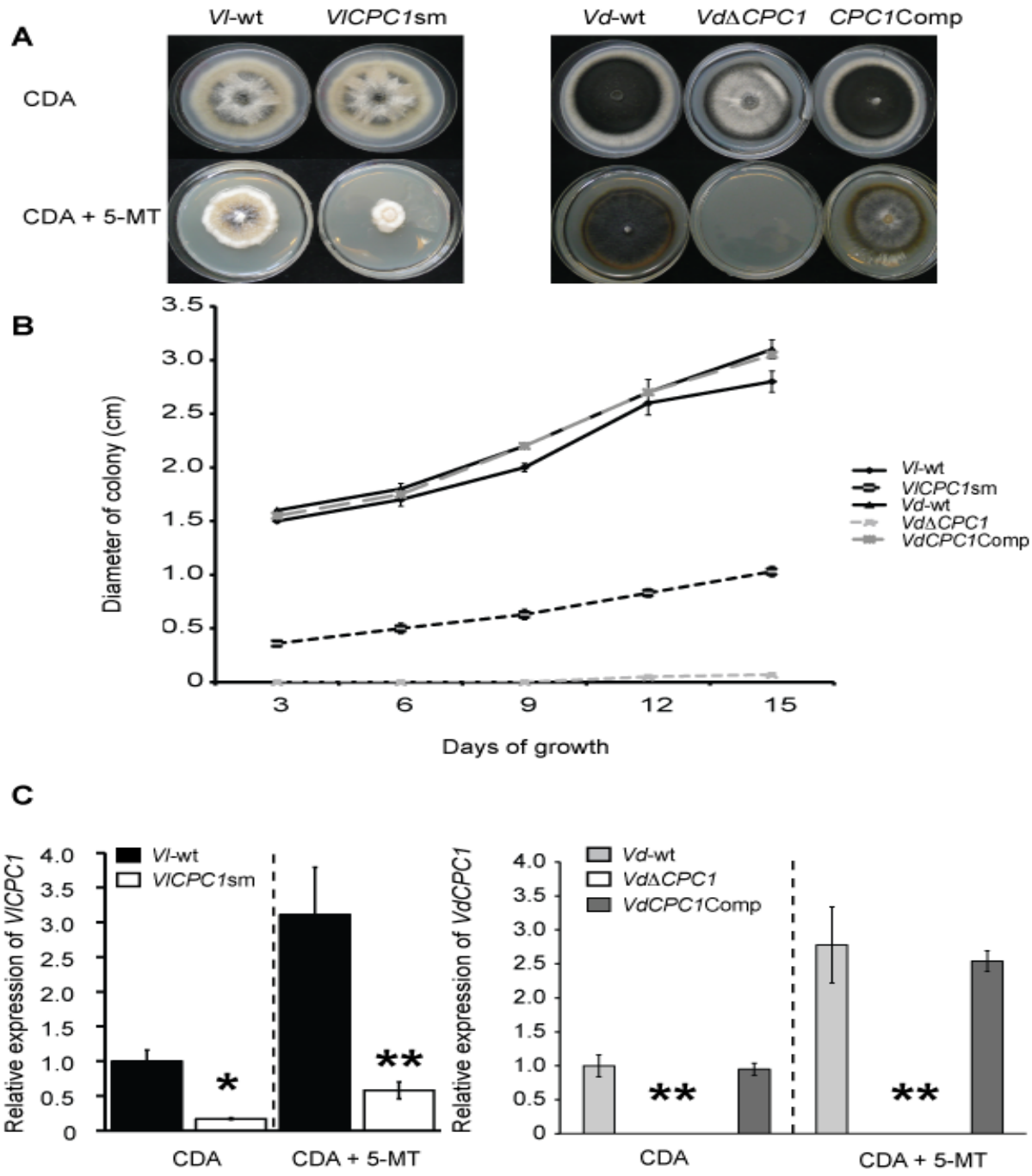

Fig. 19 Influence of 5-methyl-tryptophan (5-MT) on growth of *V. longisporum* and *V. dahliae*. A. Colonies of *V. longisporum*, *VlCPC1* silenced mutants, *V. dahliae* and *V. dahliae* knockout mutant in the presence or absence of amino acid limitation. Colonies were grown on minimal medium (CDA) after 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 (*VlCPC1*sm) as well as *V. dahliae* wild type (*Vd*-wt) and *V. dahliae* knockout mutant (*VdΔCPC1*) 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 *VlCPC1* and *VdCPC1* transcripts in the *V. longisporum* wild type and the silenced mutant *VlCPC1*sm as well as *V. dahliae* wild type and the deletion mutant *VdΔCPC1*, respectively under inducing and non-inducing conditions measured by quantitative real-time PCR. The target cDNAs were normalized to *H2A* histone transcript. Significant differences between the wild type and mutants are illustrated by asterisks (** and * indicate $P \leq 0.01$ and 0.05, respectively). (reprinted from Timpner *et al*. 2013 The Cpc1 regulator of the cross-pathway control of amino acid biosynthesis is required for pathogenicity of the vascular pathogen Verticillium longisporum. Mol Plant Microbe Interact doi.org/10.1094/MPMI-06-13-0181-R)

We analysed the growth of the *Vd*Δ*CPC1* knockout strain in the absence of plants (Fig. 19). One day after inoculation on CDA, *Vd*Δ*CPC1* could grow like the wild type. For both strains short hyphae were visible and the beginning of mycelial growth (data not shown). After 2 days of inoculation on CDA, the mycelial growth increased, and the first conidia could be detected. In addition we studied the effect a deletion might have on the radial growth of *V. dahliae*. The radial growth of *Vd*Δ*CPC1* was measured after 3, 6, 9, 12 or 15 days and was similar to wild type (Fig. 19B). Therefore, growth under normal conditions does not affect a deletion mutant of *CPC1*. Adding 5-MT to the medium inhibited growth of Δ*CPC1* mutant completely (Fig. 19A/B). While mycelial growth could be observed for *Vd* wt the knockout strain was fully retarded in growth. Even the initial phase with germinating spores could not be investigated. Until the the end of the experiment after 15 days no growth was monitored.

Next the influence of 5 methyl-tryptophan (5-MT) on expression levels of *VlCPC1* was examined. Therefore quantitative real-time PCR was performed to determine the expression of *VlCPC1* in wild type and *VlCPC1*sm upon induction with 5-MT (Fig. 19C). The CDB medium was inoculated with spores of Vl43 and *VlCPC1*sm. One culture from each strain served as control, the other cultures were treated with 5-MT for 4 h and then harvested to extract total RNA of the fungus. Due to the treatment with 5-MT cross pathway control was significantly upregulated in wild type as well as the mutant. But the silenced strain remains at low rates regarding the expression level, in fact even after induction the expression is lesser than in the untreated wildtype control (Fig. 19C).

Previous studies analysed the expression of genes involved in amino acid biosynthesis (Schönig *et al*., 2009). We investigated whether *CPC1* of *Verticillium* might also be involved in certain processes (Fig. 20). The regulation of genes from different amino acid synthesis pathways is controlled by *CPC1*. While mutant and complementation show significant differences in their expression levels the *CPC1* deletion strain did not show any expression of the genes.

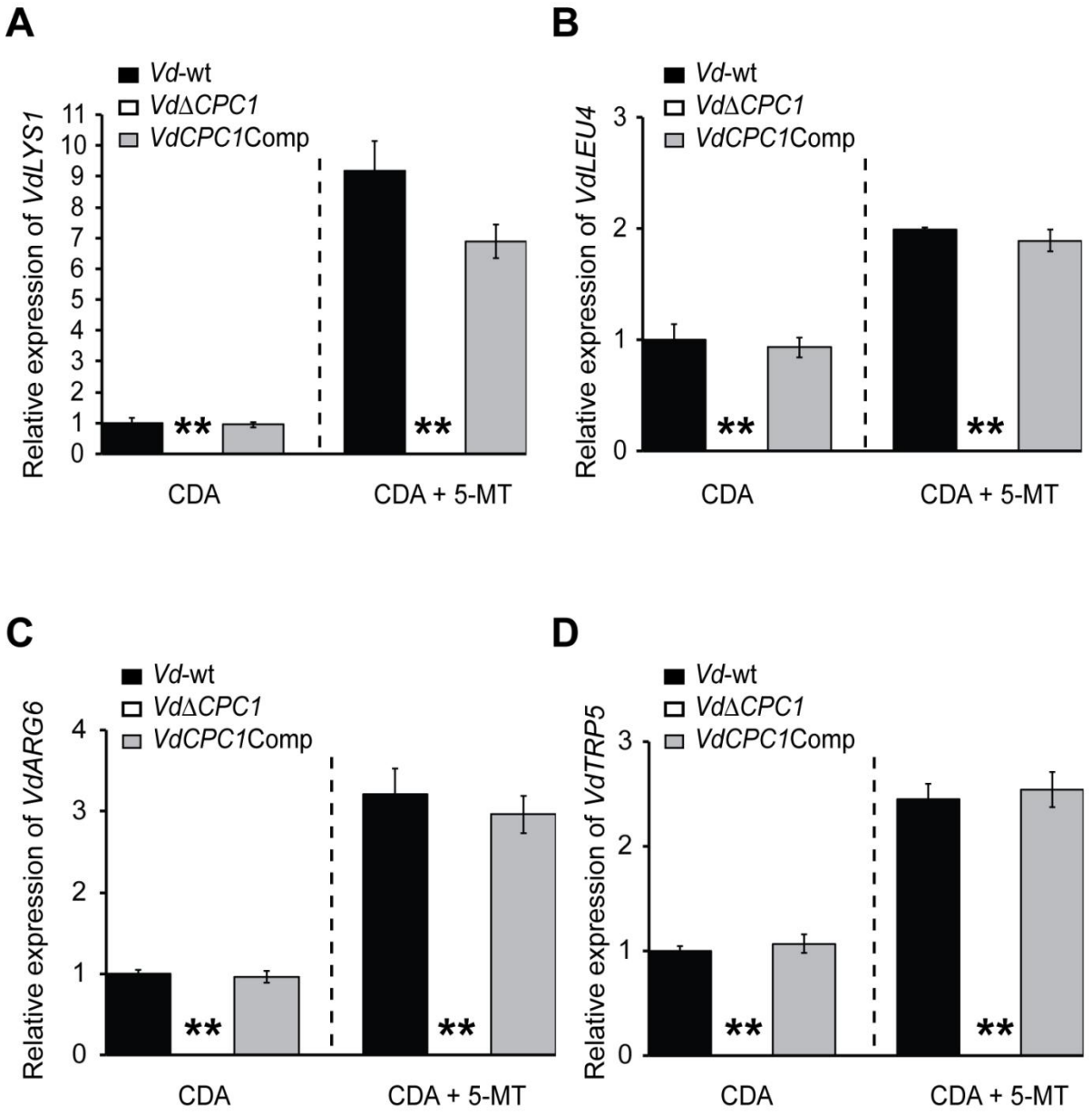

Fig. 20 Comparison of four target genes of amino acid biosynthesis regulated by CPC1 between V. dahliae wild type and the VdΔCPC1 mutant. Relative expression of these targer genes in the fungal wild type, the mutant respectively under inducing and non-inducing conditions measured by quantitative real-time PCR. The target genes of amino acid biosynthesis including VdLYS1 for lysine (A), VdLEU4 for leucine. (B), VdARG6 for arginine (C), and VdTRP5 for tryptophan (D). cDNAs were normalized to H2A histone transcript.. Expression of all four genes is recovered in complementation strain. Significant differences between the wild type and mutants are illustrated by asterisks (** and * indicate $P \leq 0.01$ and 0.05, respectively).

3.1.5. The saprophytic growth of *VdCPC1* knockout mutant is not affected.

In additional experiments the deletion strains of *CPC1* were tested for growth on different kinds of media. As control and for comparison the wild type strain was used. On minimal medium no obvious growth phenotype was evident (Fig. 19/21). Under stress inducing conditions using the deletion strains grew as well as the wild type (Fig. 19). Compared to amino acid starvation conditions which have a strong effect on mutant growth, treatment with

these other substances do not influence the growth of *CPC1* deletion mutant. We analyzed the saprotrophic lifestyle of the *VdCPC1* knockout strain (VDΔ*CPC1*) and investigated if a deletion of *VdCPC1* influences the growth of *V. dahliae.* One day after inoculation on CDA, M1 could grow like the wild type. For both strains short hyphae were visible and the beginning of mycelial growth . After 2 days of inoculation on CDA, the mycelial growth increased, and the first conidia could be detected. In addition we studied the effect a deletion might have on the radial growth of *V. dahliae*. The radial growth of VDΔ*CPC1* was measured after 3, 6, 9, 12 or 15 days and was similar to wild type (Fig. 21). Therefore, growth under normal conditions does not affect a deletion mutant of *CPC1*.

To confirm inhibiting conditions additional testing of amino acid starvation conditions were performed. Therefore CDA was supplemented with 2mM methionine sulfoximine (MSX) (Fig. 21). Under amino acid starvation conditions the silenced mutant is strongly inhibited in growth (Fig. 19/21). Additionally different stress inducing agents were added to the medium (Fig. 21).

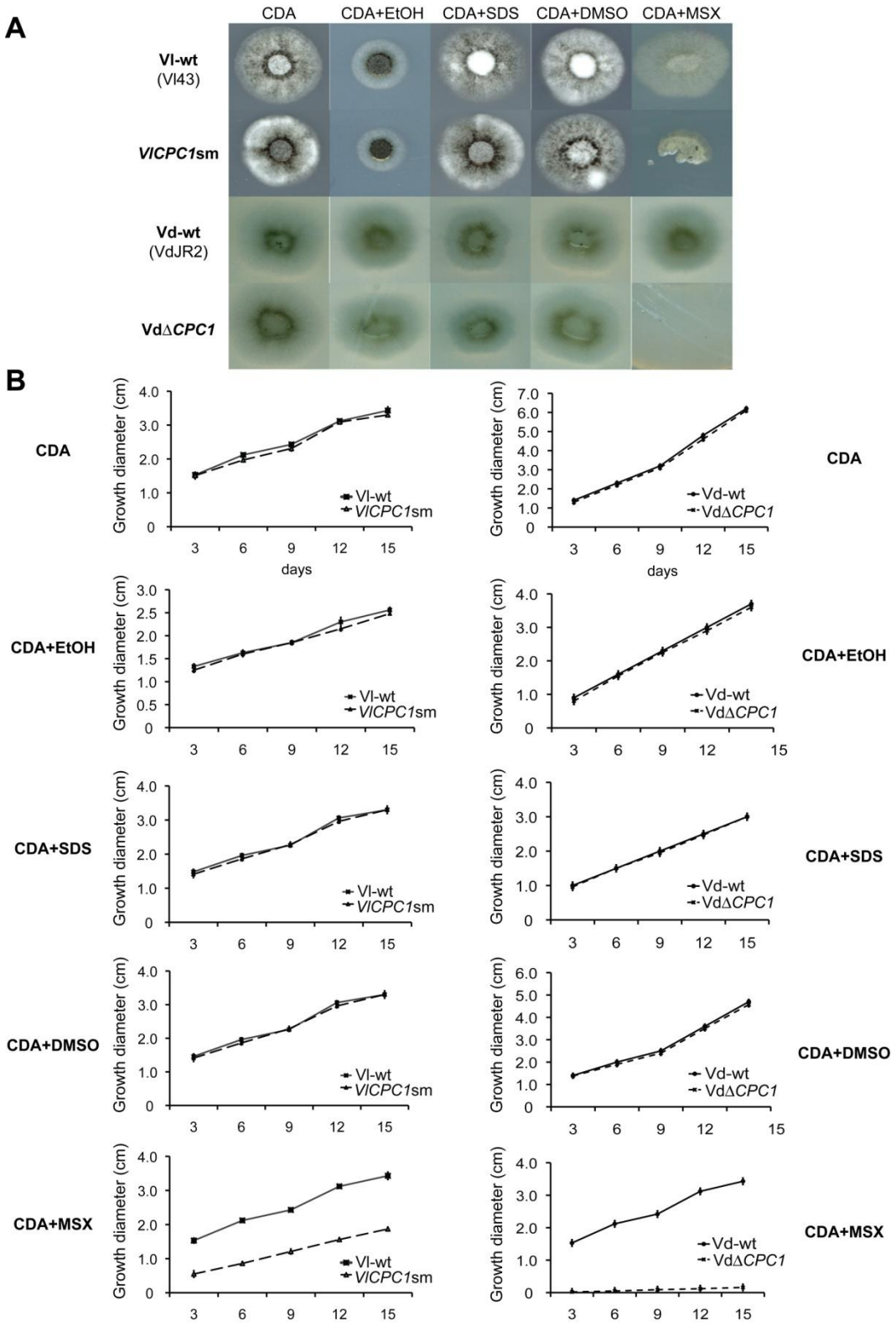

Fig. 21 Influence of different substances on *V. longisporum* growth. A. Images of *V. longisporum* wild type (*Vl*-wt), *VlCPC1* silenced mutant (*VlCPC1*sm), *V. dahliae* wild type (*Vd*-wt) and *VdCPC1* knockout mutant (*VdΔCPC1*). Colonies growing on CDA and on CDA supplemented with EtOH (2%), SDS (0.002%), DMSO (0.5%) or MSX (2 mM) were observed after 9 days of inoculation. **B.** The diameter of the colonies was measured after 3, 6, 9, 12 and 15 days after inoculation. Error bars represent standard deviation (*n*=5).

Furthermore we analyzed whether the number of spores produced by the mutant strain is reduced compared to the wild type (Fig. 22).

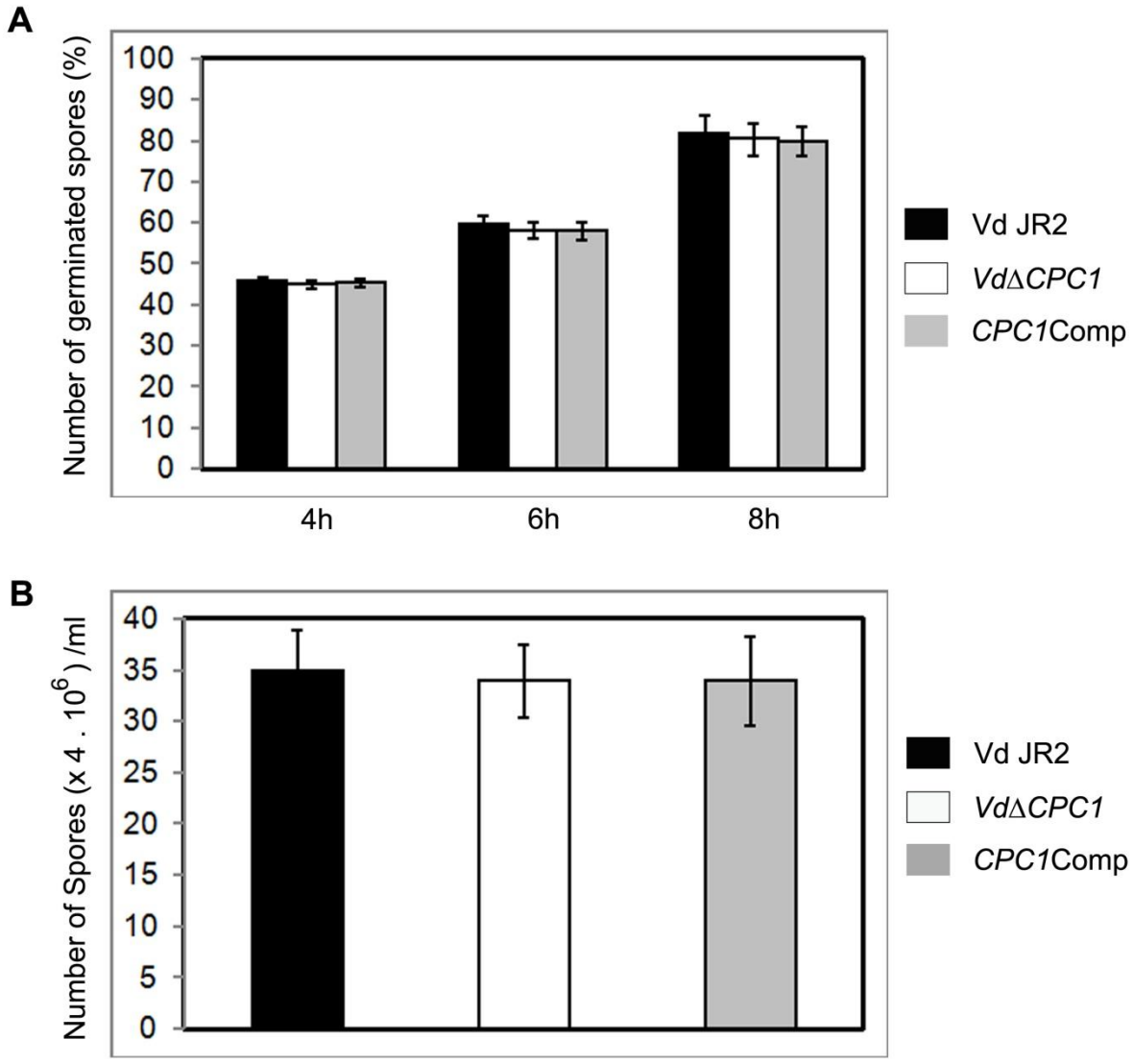

Fig. 22 Comparison of spore levels in WT, *CPC1* deletion mutant and complementation mutant. A. percentage of spores germinating after 4, 6 and 8h. **B.** The mutants show no defect in spore formation on solid as well as in liquid media. This resulted in production of spore level similar to the wild type strain VdJR2. The spore numbers were counted after 8 days.

As we can show a deletion of *CPC1* does not affect the number of spores generated by the fungus. The capability to reproduce is not affected and therefore does not limit the growth of the fungal mutant.

In addition to plant infection we performed a test on different carbon sources for *V. dahliae* wild type and Δ*CPC1* mutant as well as complementation of Δ*CPC1* (Fig. 23). Therefore we use CDA and exchanged the carbon source of this medium by different carbon sources. The

mutant is not affected by growth inhibition regarding standard carbon sources like sucrose, glucose, or fructose, but plant derived carbon sources like mannose, lignin, cellulase or xylan affect the growth of the deletion strain. The diameter of the colony is reduced by 30-40 % compared to the wild type. Especially lignin and xylan reduce Δ*CPC1* mutant's growth rate.

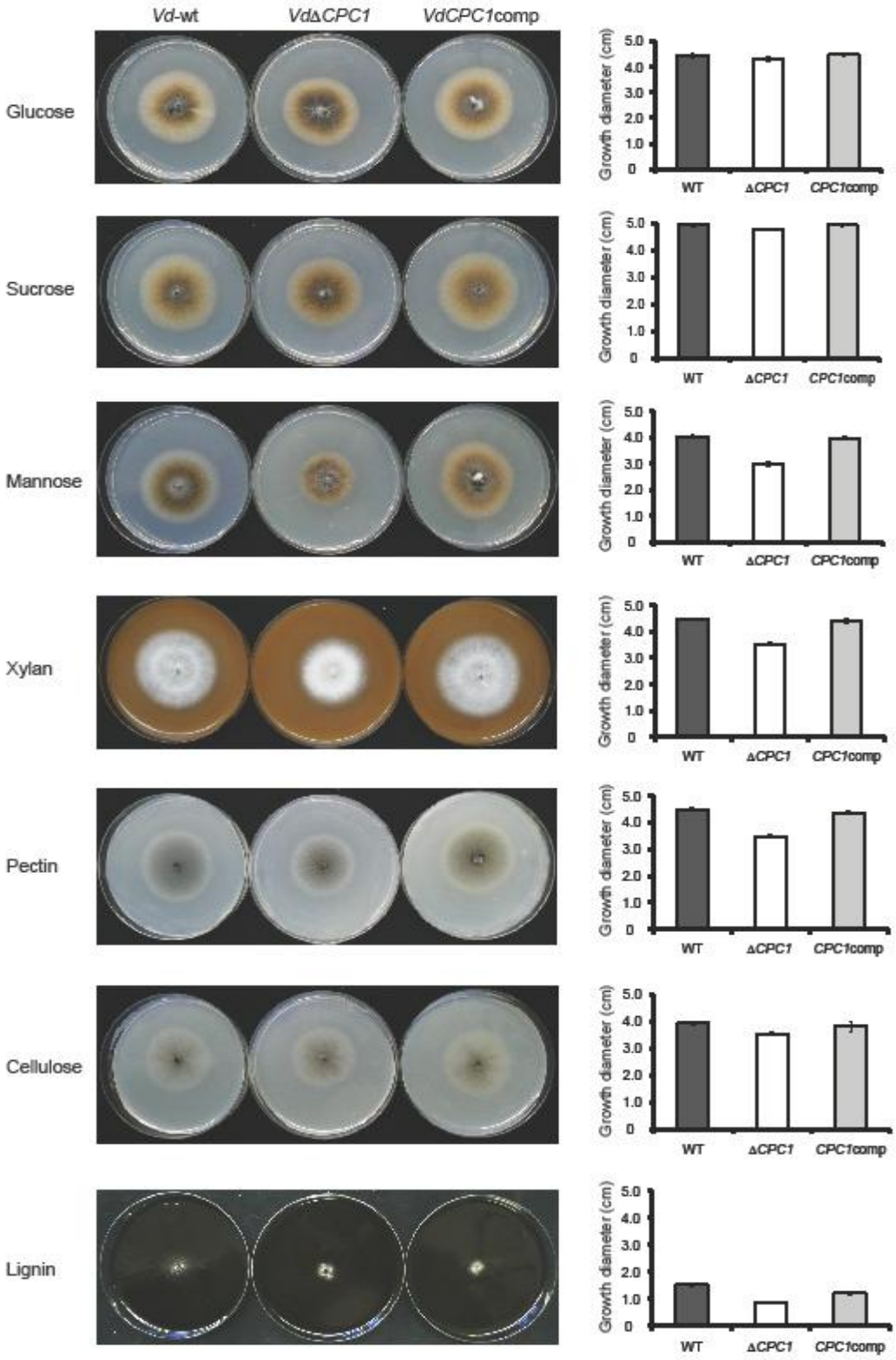

Fig. 23 Growth of the *V. dahliae* wild type (*Vd*-wt) or the *VdCPC1* deletion mutant (*Vd*Δ*CPC1*) on different carbon sources. CDA medium was used for growth tests with either sucrose or the indicated carbon sources. Diameters of colonies were measured and compared between the wild type and the mutant after ten days of inoculation. (reprinted from Timpner *et al*. 2013 The Cpc1 regulator of the cross-pathway control of amino acid biosynthesis is required for pathogenicity of the vascular pathogen Verticillium longisporum. Mol Plant Microbe Interact doi.org/10.1094/MPMI-06-13-0181-R)

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

For further knowledge about pathogenicity and disease development in *planta*, the silenced mutants of *V. longisporum* were investigated when growing inside its host *B. napus*.

To assess the severity of disease, 1-week-old *B. napus* 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. 24).

Two different sets of disease symptoms can be observed during the infection of oilseed rape by *V. longisporum*; the host plant becomes stunted and shows signs of early senescence. Both symptoms are normally visible after 21 days post infection (dpi) and are more distinctive at later time points. One-week-old *B. napus* seedlings were infected by root dip inoculation to compare the severity of the disease; height and the signs of early senescence (disease scores) of the plants were measured weekly post inoculation until 35 dpi. We analyzed whether *VlCPC1*sm mutants were still able to colonize *B. napus* and cause disease, and 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 *VlCPC1*sm versus wild type was analyzed by quantification of the *V. longisporum* DNA in the plant tissue by real-time PCR. The roots hypocotyls, stems and leaves were harvested from the rapeseed plants infected with wild type or *VlCPC1*sm at various time points.

To investigate the influence of cross pathway control on pathogenicity and disease development inside the plant, the plant infection assay was done by root dip-inoculation of a week old *B. napus* seedlings. For comparison, *B. napus* was also infected with *V. longisporum* wild type strain and mock-inoculated with water. The plants were cultivated in a climate chamber and the height of the plants was measured weekly postinoculation until the plants were highly infected. The plants began to show disease symptoms at 21 dpi and were heavily infected at 35 dpi (Fig. 24).

Two different sets of disease symptoms can be observed during the infection of oilseed rape by *V. longisporum*; the host plant becomes stunted and shows signs of early senescence. Both

symptoms are normally visible after 21 days post infection (dpi) and are more distinctive at later time points. One-week-old *B. napus* seedlings were infected by root dip inoculation to compare the severity of the disease; height and the signs of early senescence (disease scores) of the plants were measured weekly post inoculation until 35 dpi.

The infected rapeseed plants were scored for disease symptoms by assigning disease scores from 1 to 9 corresponding to asymptomatic to dead plants (Eynck *et al.*, 2007). The plants were observed once a week and in the assessment of the symptoms the yellowing and death of the leaves were considered. The disease scores of the mock-infected plants of more than 1 reflect the yellowing of leaves due to natural senescence. Disease symptoms were visible at 21 dpi and grew more pronounced at 35 dpi (Fig. 24B). Both *VlCPC1*sm and wildtype showed only similar disease scores in the initial phase of the disease at 21 and 28 dpi. At 35 dpi, the disease score of the *VlCPC1*sm-infected plants was lower than the plants infected with wildtype fungus, but still significant in comparison to mock-inoculated control plants (Fig. 24C). This reflects a slowdown of the disease of the plant when infected by the mutant fungi corroborating a reduced impact on senescence. The infected plants did not show any disease symptoms until 14 dpi. The disease score of the *VlCPC1*sm-infected plants was slightly higher (4.23) than the mock-inoculated plants (3,0) but significantly lower than the plants infected with wild type (6.0; Fig. 25).

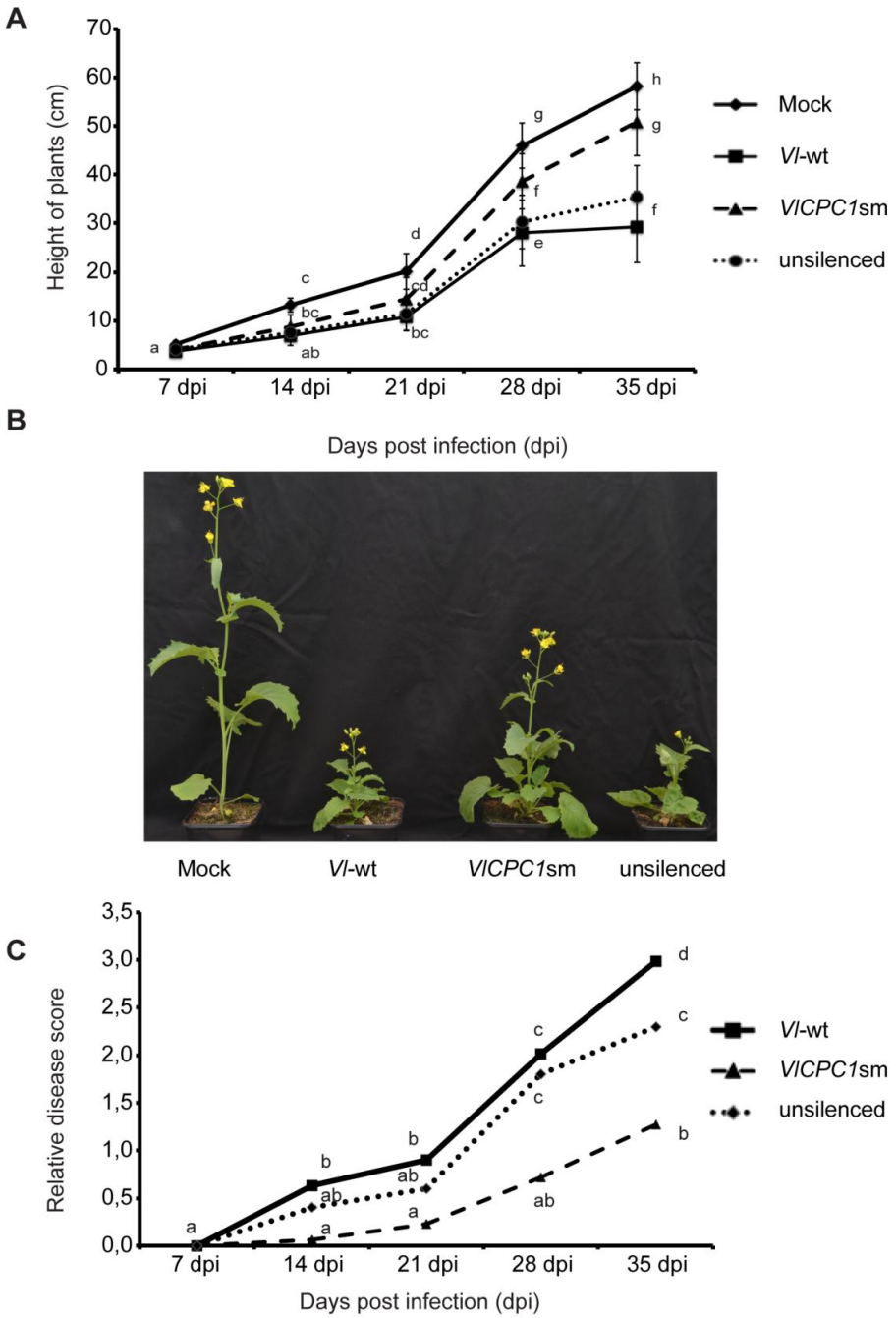

Fig. 24 Assessment of pathogenicity of the *VlCPC1* silenced mutant. A. Assessment of stunting of rapeseed due to *V. longisporum* infection. The height of 30 replicates each of rapeseed plants infected with wild type (wt) and *VlCPC1* silenced mutant (*VlCPC1*sm) was measured at 7, 14, 21, 28, and 35 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 30 experimental replicates. **B.** Rapeseed infection assay. Representative *B. napus* plants shown at 35 dpi**. C.** 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 30 experimental replicates. *Vl* 43, wild type; *VlCPC1*sm, *VlCPC1* silenced mutant. The value for mock was detracted from the values for wild type and mutant**.** Different letters indicate significant differences at $P < 0.05$. (reprinted from Timpner *et al*. 2013 The Cpc1 regulator of the cross-pathway control of amino acid biosynthesis is required for pathogenicity of the vascular pathogen Verticillium longisporum. Mol Plant Microbe Interact doi.org/10.1094/MPMI-06-13-0181-R)

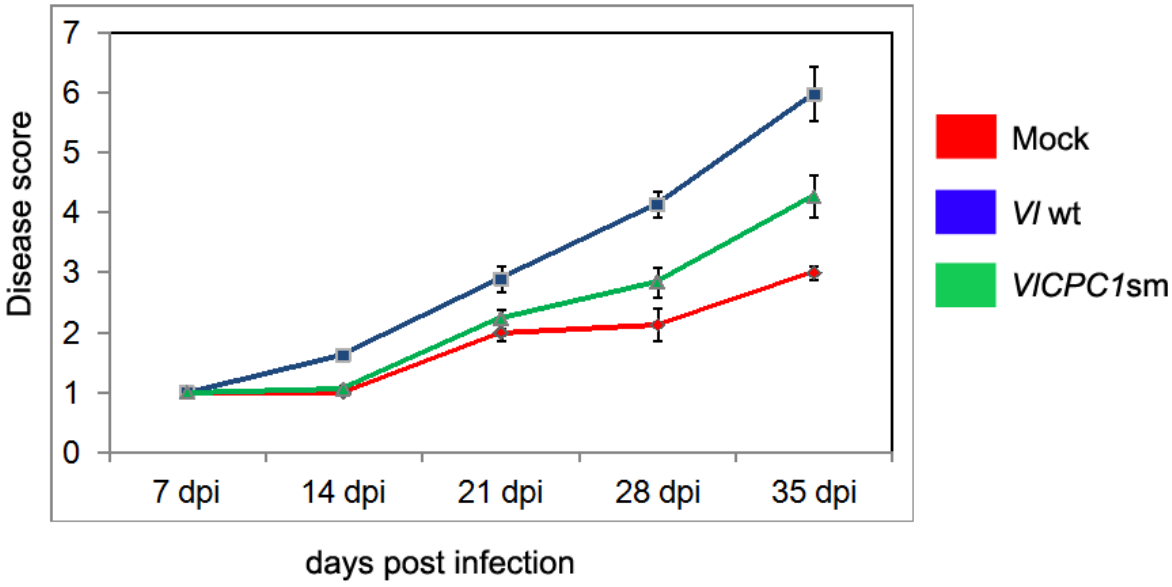

Fig. 25 Assessment of disease development by scoring for disease symptoms according to Eynck *et al.* (2007). Plants were scored for disease symptoms at 7, 14, 21, 28, and 35 dpi. Data represent average ± standard deviations of 30 experimental replicates. *Vl* 43, wild type; *VlCPC1*sm, *VlCPC1* silenced mutant.

To investigate whether a deletion of *CPC1* also affects pathogenicity of *V. dahliae* to its host we performed the infection assays on tomato plants of the *VdCPC1* deletion mutant (VDΔ*CPC1*) and the *V. dahliae* wildtype by root-dipping method. The results showed that the mutant is infecting the plants with reduced efficiency whereas the wild type is successfully colonizing its host (Fig. 26). The wild type *V. dahliae* caused the typical symptoms on tomato including stunting and chlorosis (Fig. 26), whereas the *CPC1* deletion mutant only exhibited a slight symptom of chlorosis.

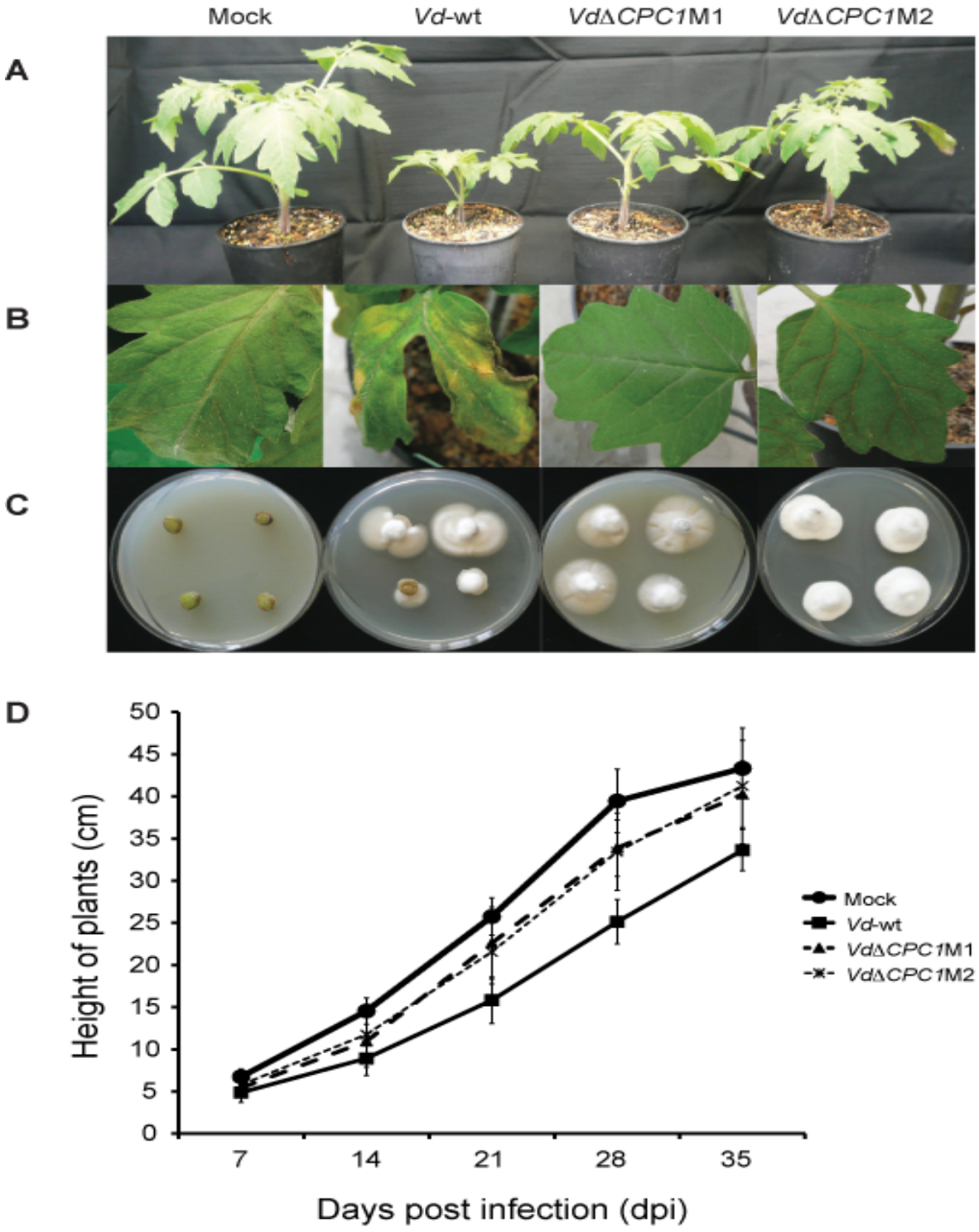

Fig. 26 Assessment of pathogenicity of the *VdCPC1* deletion mutant. A. Tomato infection assay. Representative *S. lycopersicum* plants shown at 21 dpi. **B.** comparison of chlorosis and necrosis symptoms in the leaves of tomato plant. Images of tomato leaves of representative plants from plants infected with *V. dahliae* or *VdCPC1* knockout mutant. As control leaves of Mock plants were taken. **C.** Mutant regrowth test. Image of tomato stem pieces infected with *V. dahliae* or *VdCPC1* knockout mutant (VDΔ*CPC1*) colonies growing PDA plates. As control stem pieces of Mock served as control. **D.** Assessment of stunting of *S. lycopersicum* due to *V. dahliae* infection. The height of 20 replicates each of tomato plants was measured.

We analyzed whether *VlCPC1*sm mutants were still able to colonize *B. napus* and cause disease, and 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 *VlCPC1*sm versus wild type was analyzed by quantification of the *V. longisporum* DNA in the plant tissue by real-time PCR (Fig. 27). The roots hypocotyls, stems and leaves were harvested from the rapeseed plants infected with wild type or *VlCPC1*sm at various time points.

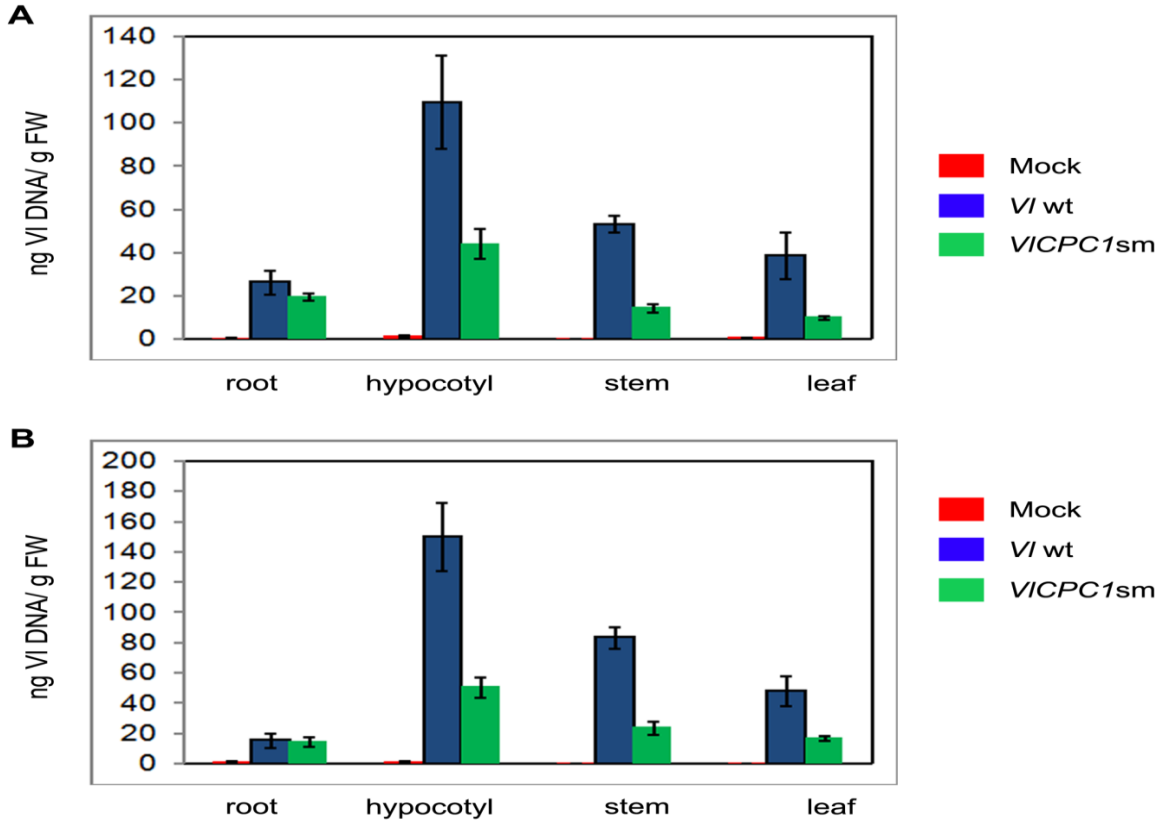

Fig. 27 Infection assay and determination of the *V. longisporum* DNA concentration in infected plant tissues. Different letters indicate significant differences at $P < 0.05$. **A.** *V. longisporum* DNA concentration in root, hypocotyls, stem and leaves after 28 dpi**.** *V. longisporum* DNA was measured with real-time PCR in stem, hypocotyls, root and leaves of *B. napus* inoculated with *VlCPC1* silenced mutant (*VlCPC1*sm) and wild type (wt) at 35 dpi. Data represent average ± standard deviations of five 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. **B.** *V. longisporum* DNA concentration in root, hypocotyls, stem and leaves after 35 dpi**.** *V. longisporum* DNA was measured with real-time PCR in stem, hypocotyls, root and leaves of *B. napus* inoculated with *VlCPC1* silenced mutant (*VlCPC1*sm) and wild type (wt) at 35 dpi. Data represent average ± standard deviations of five 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.

The DNA content was determined in a time window of 35 dpi where there were no differences within the respective sets of experiments ($P_{(time)} > 0.5$). Low concentrations of fungal DNA were found in roots, they did not vary between *Vl* 43, *VIcpc*1sm infected plants, but show a difference towards the mock inoculated plants (Fig. 27). However, in the

hypocotyls where the highest concentration of fungal DNA (100-150 ng/g FW) was present, peculiar differences between plants infected with wild type strain *Vl*43 or the silenced strains *VlCPC1*sm (Fig. 27) could be investigated the mutant's amount did not increase from 28 dpi (Fig. 27A) to 35 dpi like the wild type, in fact it does not change, while the wild type amount increases from 100 to nearly 150 ng/g FW. Fungal DNA lower in stems (40-75 ng/g FW) than in hypocotyl (Fig. 27). These data suggest that the mutant strain is able to perform the initial colonization of the plant but has some difficulties in the hypocotyl of the plant (Fig. 27).

After 35 days post infection (dpi) *in planta*, the silencing of the *VlCPC1* isogenes was still stable (Fig. 28).

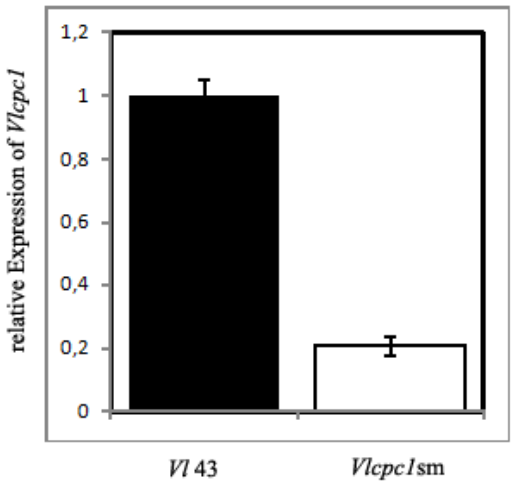

Fig. 28 Expression of cross pathway control encoding genes of *V. longisporum*. Relative expression of *VlCPC1* in *B. napus* measured by quantitative real-time PCR. *VlCPC1* cDNA was normalized to the histone 2a cDNA. Relative expression of *VlCPC1* in *Vl* 43 and *VlCPC1*sm measured by quantitative real-time PCR. *VlCPC1* cDNA was normalized to the histone cDNA. *Vl* 43: wild type, *VlCPC1*-sm, *cpc1* silenced mutants. The error bars represent the standard deviation of four different measurements of the same cDNA. cDNA from 35 days old host plants mock-inoculated with water served as control. *Vl* 43, cDNA from *B. napus* infected with wild type, *VlCPC1*-sm, cDNA from *B. napus* infected with *VlCPC1* silenced mutant. The error bars represent the standard deviation of triplicates.

3.1.7. *VdCPC1* knockout leads to reduced pathogenicity of the fungus.

We performed the infection assays of the *VdCPC1* deletion mutant (*VdΔCPC1*) and the wild type strain VdJR2 on tomato plants by root-dipping method. The results showed that the mutant is infecting the plants with reduced efficiency while the wild type is successfully colonizing its host (Fig. 29). The wild type VdJR2 caused the typical symptoms on tomato

including stunting and chlorosis, whereas the *CPC1* deletion mutant only exhibited a slight symptom of chlorosis. This suggests that *CPC1* gene is involved in virulence of the fungus.

It was also possibly to regrow the strains out of the infected plants. Therefore pieces of the stem were sterilized and plated on PDA plates. After five days the growth of the mutants was obvious.

For Vd∆*CPC1* the amount of fungal DNA in the tomato hypocotyls is also reduced (Fig. 29). Compared to *V. longisporum* in *B. napus* the amount of DNA is lower and the difference between wild type and mutant is less prominent, but the DNA concentration is about 40% lower than in *V. dahliae* wild type.

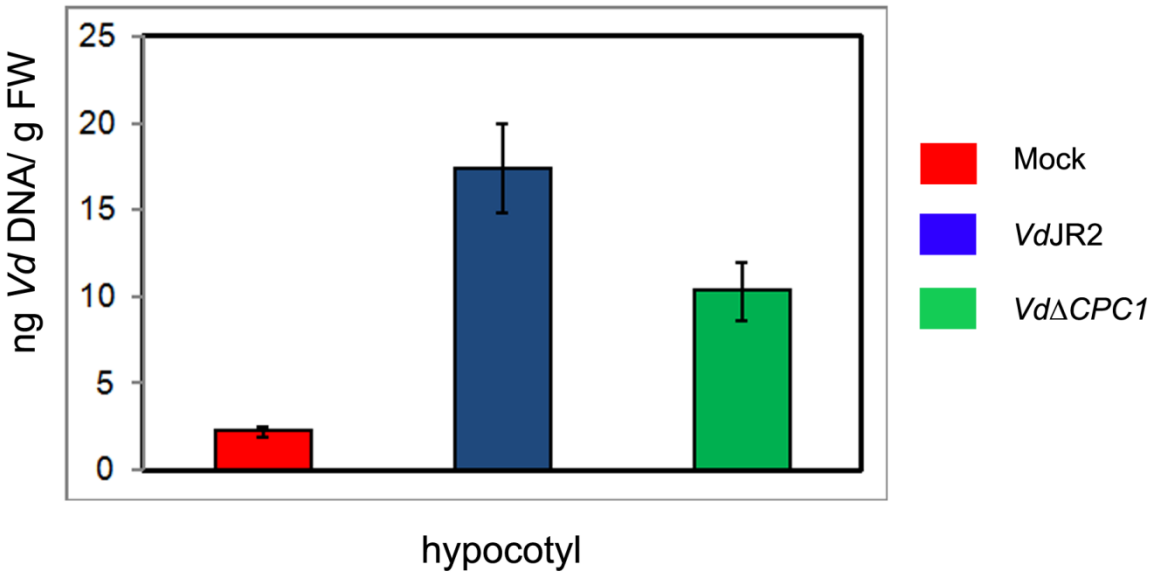

Fig. 29 Infection assay and determination of the *V. dahliae* DNA concentration in infected plant tissues. *V. dahliae* DNA concentration in hypocotyls, after 35 dpi**.** *V. dahliae* DNA was measured with real-time PCR in hypocotyls, of *S. lycopersicum* inoculated with *VdCPC1* knockout srain (*Vd∆CPC1*) and wild type (wt) at 35 dpi. Data represent average ± standard deviations of five experimental replicates. The mock-inoculated plants as a control did not show presence of any *V. dahliae* DNA. ng Vd DNA/g FW = nanogram *V. dahliae* DNA/gram fresh weight of plant tissue.

To confirm whether a deletion of *CPC1* is responsible for reduced pathogenicity of the fungus, a complementation of the Vd∆*CPC1* mutant was tested for plant infection together with Vd∆*CPC1* and Vd-wt (Fig. 30).

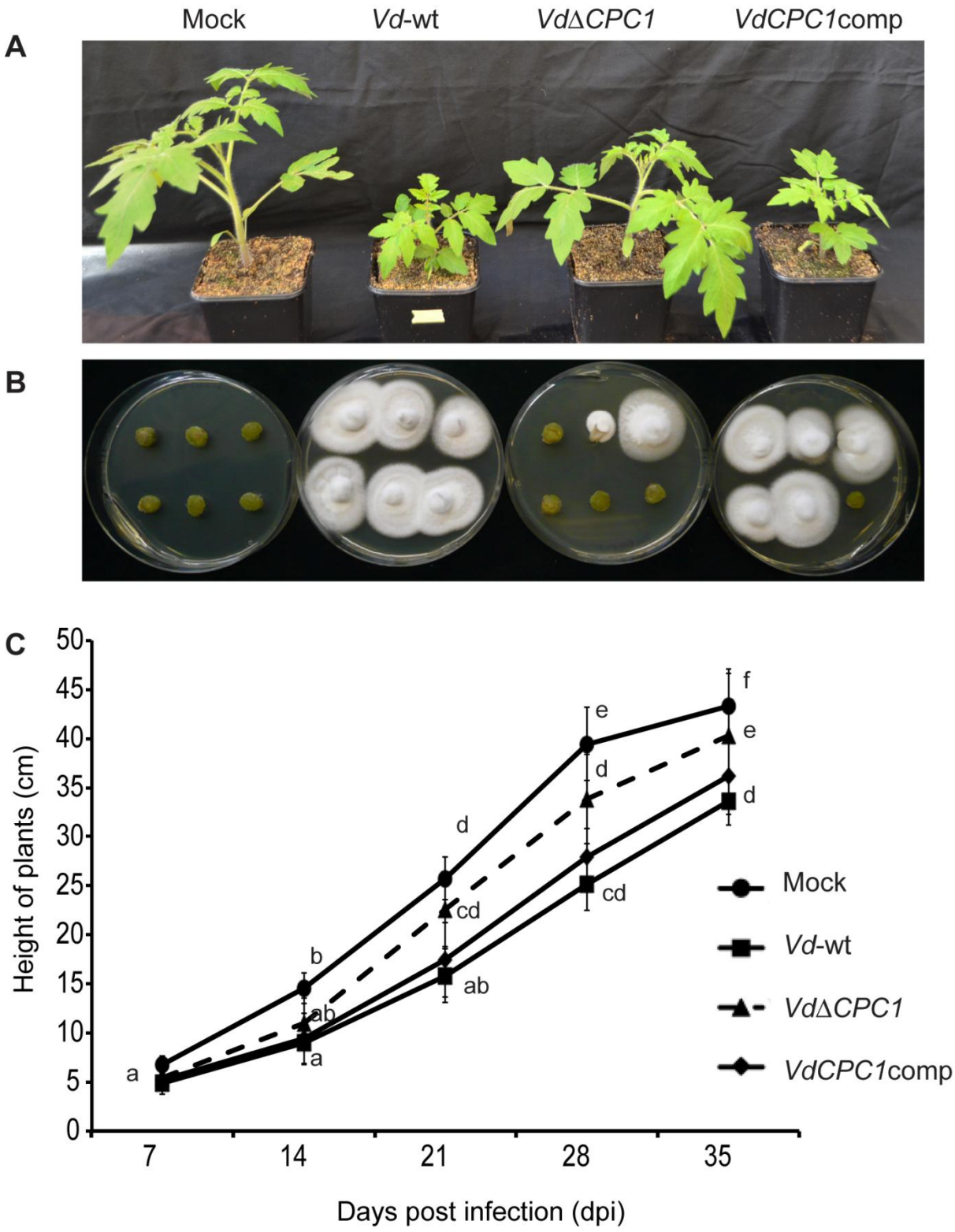

Fig. 30 Assessment of pathogenicity of the *VdCPC1* deletion mutant an complementation. A. Tomato infection assay. Representative *S. lycopersicum* plants shown at 21 dpi. **B.** Mutant regrowth test. Image of tomato stem pieces infected with *V. dahliae*, *VdCPC1* knockout mutant (Vd*ΔCPC1*) or *VdCPC1* complementation mutant (Vd*CPC1*comp) colonies growing PDA plates. As control stem pieces of Mock served as control.. **C.** Assessment of stunting of *S. lycopersicum* due to *V. dahliae* infection. The height of 20 replicates each of tomato plants Different letters indicate significant differences at $P < 0.05$. (reprinted from Timpner *et al.* 2013 The Cpc1 regulator of the cross-pathway control of amino acid biosynthesis is required for pathogenicity of the vascular pathogen Verticillium longisporum. Mol Plant Microbe Interact doi.org/10.1094/MPMI-06-13-0181-R)

The complementation behaves again like a wild type strain. This supports the role *CPC1* plays for pathogenocity of the fungus.

To confirm these data DNA quantification was performed for Vd∆*CPC1* in comparison to the complementation (Fig. 31). The amount of fungal DNA in the tomato roots, hypocotyls and leaves is reduced for the knockout, but the complementation is on the same level as the wild type (Fig. 31). The DNA concentration is about 40% lower than in *V. dahliae* wild type in the hypocotyl and about 50 % for the leaves.

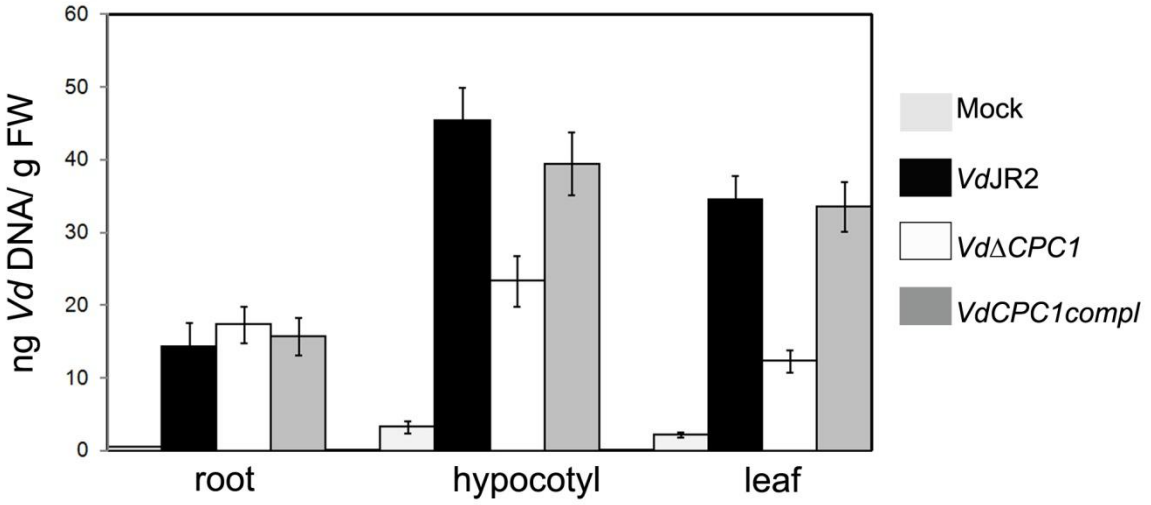

Fig. 31 Infection assay and determination of the *V. dahliae* DNA concentration in infected plant tissues. A. *V. dahliae* DNA concentration in hypocotyls, after 35 dpi**.** *V. dahliae* DNA was measured with real-time PCR in root, hypocotyl and leaves of *S. lycopersicum* inoculated with *VdCPC1* knockout srain (*Vd∆CPC1*), wild type (VdJR2)and complementation of *CPC1* (Vd*CPC1*compl) at 35 dpi. Data represent average ± standard deviations of five experimental replicates. The mock-inoculated plants as a control did not show presence of any *V. dahliae* DNA. ng Vd DNA/g FW = nanogram *V. dahliae* DNA/gram fresh weight of plant tissue.

Silencing of *CPC1* in *V. longisporum* leads to a mutant strain that is significantly reduced in pathogenicity. It still can infect the plant but its ability to successful colonization is strongly restricted. Only low amouts of fungal biomass can be found in the upper parts of the host. A knockout of *CPC1* in *V. dahliae* is also reduced in pathogenicity and shows sensitivity to different plantal carbon sources.

3.2. The key regulator of secondary metabolism Lae1: a first glance in *Verticillium*.

Secondary metabolism has been described to play a key role in the fungal life cycle (Bayram *et al.*, 2008; Amaike and Keller, 2009; Bayram and Braus, 2012). Regarding phytopathogenic fungi effectors and toxins are involved in many processes providing the organism with defence against the host and counteracting the plantal pathogen response (Howlett, 2006; Mukherjee *et al.*, 2012). In the interactions between soil-borne vascular fungi and plants the mechanisms of pathogenicity implicates the exchange of chemical signals within the vascular tissue. In the interaction between *V. longisporum* and *B. napus*, both the pathogen and the host plant are supposed to release metabolites into the xylem which affect the other partner. The main regulator for secondary metabolism Lae1/LaeA represents presumably a methyl transferase and has been described in several fungi (Bok and Keller, 2004; Bok *et al*., 2005; Yu and Keller, 2005; Amaike and Keller, 2009). LaeA has been proposed as an epigenetic regulator which acts on chromatin but exact molecular function is yet elusive. The symptoms caused by the infection indicate that fungal secondary metabolites secreted by the pathogen might play a key role as virulence factors with phytotoxic, elicitor-like or phytohormonal effects, or as suppressors of plant defense. In several studies it has been suggested that *Verticillium* spp. produces a broad spectrum of phytotoxins and elicitor compounds which induce pathogen-specific symptoms in the absence of the fungus (Shi and Li, 2008; Mukherjee *et al.*, 2012; Zhou *et al.*, 2012).

From previous studies (Ratzinger *et al*., 2009; Singh *et al*., 2010; Iven *et al*., 2012) we could conclude that secondary metabolism is involved in pathogenicity of *Verticillium* ssp. Therefore we decided to take a closer look on *LAE1* of *V. longisporum* as well as *V. dahliae*.

3.2.1. There are two copies of *LAE1* in *V. longisporum*

V. longisporum has been described as hybrid between two *Verticillium* species (Tran *et al.*, 2013). Therefore we determined the copy number of *LAE1* in *V. longisporum*. To determine the copy number of *VlLAE1* Southern analysis was performed (Fig. 32). Genomic DNA of *V. longisporum, V. dahliae*, and *V. albo-atrum* were digested with suitable enzymes, and subjected to Southern hybridization. A fully sequenced 520 bp long fragment of *VlLAE1*, amplified with primers ProbeLae1left and ProbeLae1right was used as the probe. Several independent Southern hybridization analyses showed two signals for *V. longisporum* as compared to *V. dahliae*, and *V. albo-atrum* for the genomic DNA digested with *Sal*I.

These results demonstrated that *VlLAE1-1* has an isogene, *VlLAE1-2*. To determine the extent of similarity of these two isogenes in the *V. longisporum* genome, the genomic DNA of

V. longisporum was digested with *Sal*I and run on a low percentage agarose gel. DNA was extracted from the four gel fragments corresponding to spots where the signals were generated in the Southern hybridization analysis (Fig. 32). *VlLAE1-1* specific primers were used to amplify the gel extracted DNA and subjected to sequencing. On sequencing of both loci it was found out that with some exchanges the coding and intronic sequences of both the isogenes are identical in *V. longisporum*. We have analyzed and compared the sequences from *V. longisporum* isolates Polymerase chain reaction (PCR) amplification using primers specific for *VlLAE1* (Table 3) resulted for each primer pair in only one single sequence characteristic for each analysed strain of the three species.

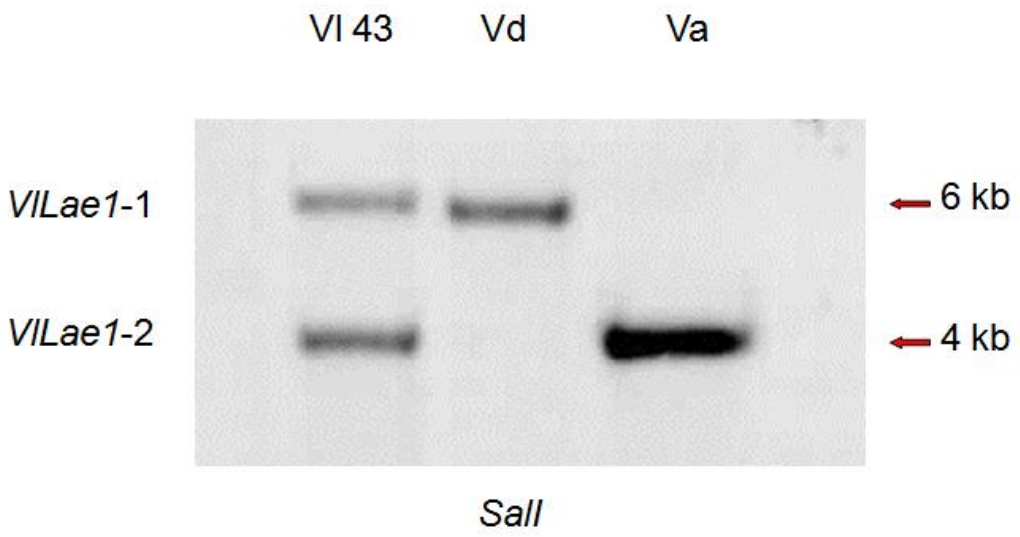

Fig. 32 Determination of the isogene *VlLAE1-2* of *VlLAE1-1* and their corresponding sequence. Southern hybridization analysis of V. albo-atrum, *V. dahliae* and *V. longisporum.* The genomic DNA was digested with *Sal*I. A 500 bp sequence of *VlLAE1-1* was used as a probe.

The sequences for *V. albo-atrum* and *V. dahliae* share 88% of identity. In total there are more than 60 different nucleotides between the two sequences. Comparing the sequences of the two isogenes of *V. longisporum* to *V. dahliae* or *V. albo-atrum* shows that one of those sequences is similar to *V. dahliae* and the other one is related to *V. albo-atrum*.*VlLAE1-1* is 97% identical to *VdLAE1* and they differ in 15 nucleotides. *VlLAE1-2* is 90% identical to *VdLAE1* and they differ in 45 SNPs (Fig. 33). Compared to *VaLAE1 VlLAE1-1* is like *VdLAE1* 82% identical to this sequence, whereas *VlLAE1-2* is at least 89% identical. There

are two characteristic sequence patterns which correspond either to *V. dahliae* or to *V. albo-atrum*. *VlLAE1-1* and *V. dahliae* share three patterns that are identical (TGGCT), (GCCGA) and (GCTTGGT). First *VlLAE1*-2 shares SNPS in the first and the third exon of *V. albo-atrum* (TTACT) and (GATAGGT), but the SNP in the second exon (GCCGA) is similar to *V. dahliae*.

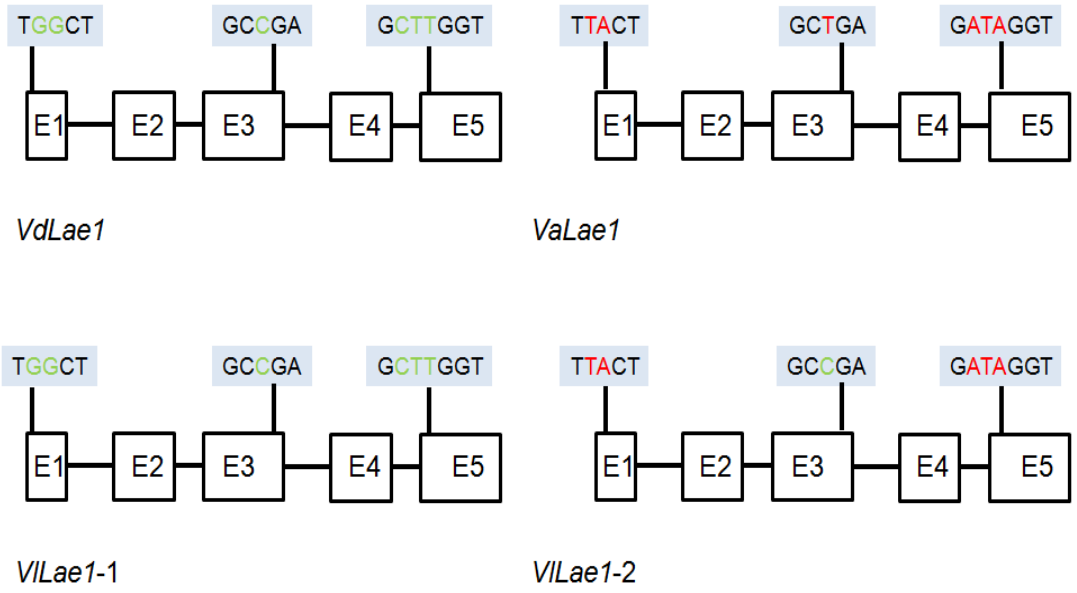

Fig. 33 Sequence comparison of *VlLAE1* sequences. *VlLAE1*-1 and *VlLAE1*-2 with *Vdlae1* and *Valae1*. *VlLAE1-1* and *VlLAE1-2* isogenes of *V. longisporum* are derivatives from *V. dahliae* and *V. albo-atrum*, respectively. Characteristic signatures include two five nucleotide (TGGCT and GCCGA) patterns and a seven nucleotide pattern (GCTTGGT) for *VlLAE1-1* and the *V. dahliae* orthologue. Whereas *VlLAE1-2* corresponds to the *V. albo-atrum* orthologue carries different nucleotide pattern for the first signature (TTACT) the second pattern corresponds to *V. dahliae* (GCCGA) and not to *V. albo-atrum* (GCTGA), while the third one is similar to *V. albo-atrum* again (GATAGGT).

The deduced Lae1 protein sequences of *V. longisporum V. dahliae* and *V. albo-atrum* were compared with corresponding sequences of *A. fumigatus, A. nidulans, P. citrinum* or *N. tetrasperma* (Fig. 34). VlLae1-1 and VdLae1 are 99,7% identical, while VlLae1-2 is 96,9 % identical to both sequences (Fig. 34). VaLae1 is 64,7 % identical to VlLae1-1 and VdLae1, but 79,2% identical to VlLae1-2. We can conclude that the one copy of Lae1 in *V. longisporum* refers to *V. dahliae* while the other one is closer to *V. albo-atrum*, but still closer to the other two copies in *V. longisporum* or *V. dahliae*.

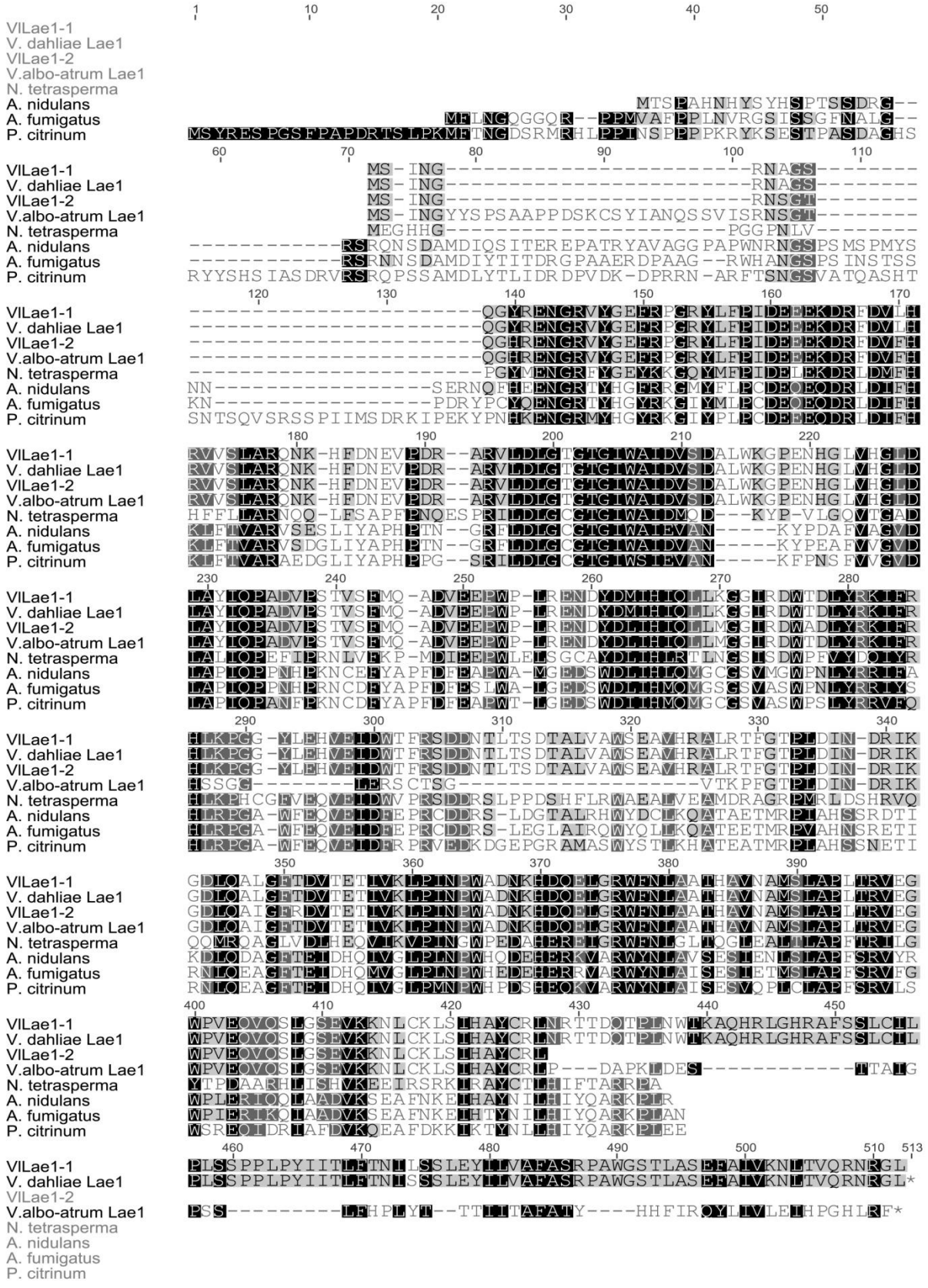

Fig. 34 Sequence alignment of Lae1 sequences. Alignments of the deduced amino acid sequences of Lae1 of *Verticilliae* in comparison to corresponding genes of *A. fumigatus, A. nidulans, P. citrinum* or *N. tetrasperma*. White letters in black boxes represent conserved, grey boxes partially conserved amino acid residues.

Lae1 of *Aspergillus* shares 34,5 % identity with VdLae1 and VlLae1-1, 35,5% for VlLae1-2 and 31,3% with VaLae1. *A. fumigatus* shares 33,1% identity to *Verticillium* sequences, while *N. tetrasperma* is 43,2% identical, *P. citrinum* is 31% identical (Fig. 34).

3.2.2. Generation of mutants silenced in *LAE1*.

Because there are two copies in *V. longisporum* mutants silenced in *VlLAE1* were generated. RNA-mediated gene silencing has been established in several fungi for targeted gene silencing instead of a conventional knockout (Nakayashiki *et al.*, 2005). The plasmid includes a hairpin construct for silencing of *VlLAE1* and the hygromycin resistance gene as selective marker. *A. tumefaciens* mediated transformation of *V. longisporum* was applied and thirty independent hygromycin-B-resistant transformants were selected at random for further analysis. The efficiency of *VlLAE1* silencing was determined by RT-PCR as the silencing of gene expression is the result of posttranscriptional degradation of targeted mRNA. Total RNA was extracted from the *VlLAE1sm* (*VlLAE1* silenced mutant) and wild type. RT-PCR results showed the significant knockdown of *VlLAE1* transcripts (Fig. 35). The extent of silencing varied in the different transformants between high and moderate levels. The degree of silencing of cross pathway control genes was estimated by RT-PCR by the ratio of the amplification of *VlLAE1* between the respective transformants and the unsilenced wild type. For each transformants three biological replicates were analyzed. High-frequency silencing was observed for 45% of the transformants exhibiting reduced gene expression. The extent of silencing varied in the different transformants between high and moderate levels. After 5 generations on SXM Medium, the silencing of the *VlLAE1* isogenes was still stable (Fig. 35).

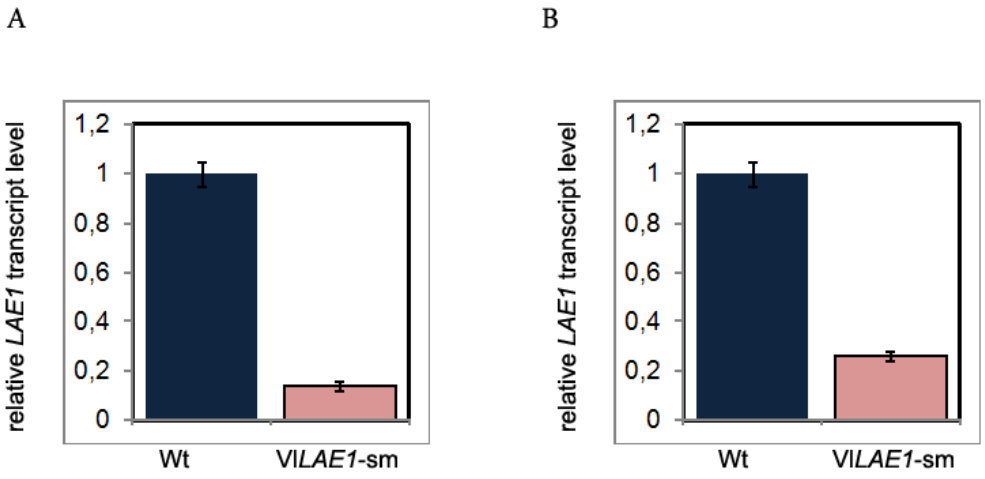

Fig. 35 Expression of *lae1* encoding genes of *V. longisporum*.

Relative expression of *VlLAE1* measured by quantitative real-time PCR. *VlLAE1* cDNA was normalized to the histone cDNA. Relative expression of *VlLAE1* in *Vl* 43 and *VlLAE1*-sm measured by quantitative real-time PCR. *VlLAE1* cDNA was normalized to the histone cDNA. *Vl* 43: wild type, *VlLAE1*-sm, *lae1* silenced mutants. The error bars represent the standard deviation of four different measurements of the same cDNA. **A.** Transcript levels of *VlLAE1* **B.** Transcript levels of *LAE1* after 5 generations on SXM.

As mentioned above for *CPC1* silencing strategy suppresses maximally about 80-90% of gene expression in fungi (Nakayashiki *et al.*, 2005; Singh *et al.*, 2010; Singh *et al.*, 2012). The rest activity of the gene (10-20%) might be still enough for its function in the fungus. Therefore we decided to generate a knockout of *LAE1* in *V. dahliae*. To analyse mutants and wildtype strain, a probe binding to the 5' region of the gene was used. Genomic DNA isolated from transformants was digested with the enzymes *Stu*I and *Sal*I, for comparison wild type DNA was used. From Southern hybridisation, the 456-bp fragment of *LAE1* gene as probe detected one band for *LAE1* gene in both wild type and mutant but both bands are visible in the ectopic background (Fig. 36). The transformants displayed the expected signals at 1.5 kb (*Stu*I and *Sal*I) (Fig. 36). The Southern band from the deletion mutants was 1 kb smaller than the band from the wild type strain explaining the successful exchange between the deletion cassette and locus of *LAE1* gene. For final validation Southern hybridisation was made with the same probes and enzymes as for the two single knock-outs, and isolates resulting in the correct bands were used for further investigation (Fig. 36).

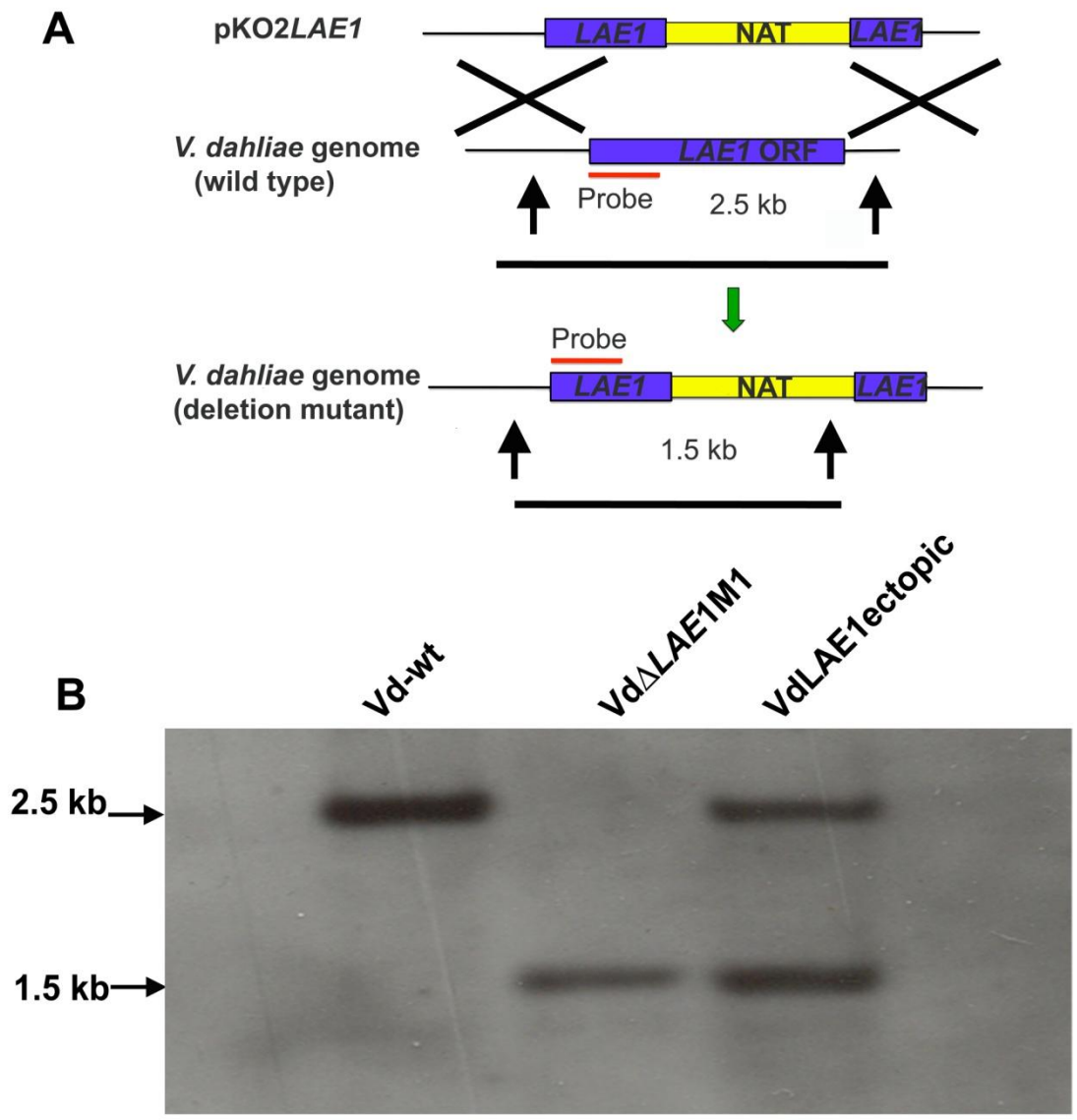

Fig. 36 Strategies for deletion of *LAE1* gene in *V. dahliae* and confirmation of the corresponding deletion mutants. A. Model for exchange between the deletion construct and wild type locus of *LAE1* gene in *V. dahliae*. **B.** Confirmation of the *LAE1* deletion mutants by Southern hybridization using *StuI* and *Sal*I for genome digestion. The same band (1,5 kb) in the mutants is 1 kb smaller than the one in the wild type VdJR2.

3.2.3. Silencing of *LAE1* reduces pathogenicity of *V. longisporum*.

V. longisporum mutants silenced in expression of *LAE1* were tested in a plant infection assay to verify the degree of disease. The same symptoms like for silencing of *CPC1* could be observed for *VlLAE1*sm.

In case of *LAE1* silencing treatment and observation of the plants was carried out like discribed above. In the initial phase of the disease the infected and non infected do not show severe differences, but with development at 21 and 28 dpi distinctions between the wt infected plant and the plants infected with *VlLAE1*sm become obvious. At 35 dpi, the disease

score of the *VlLAE1*sm-infected plants was lower than the plants infected with wildtype fungus, but still significant in comparison to mock-inoculated control plants (Fig. 37). This reflects a slow down of the disease of the plant when infected by the mutant fungi corroborating a reduced impact on senescence. The infected plants did not show any disease symptoms until 14 dpi. The disease score of the *VlLAE1*sm-infected plants was higher (4.2) than the mock-inoculated plants (3,0) but lower than the plants infected with wild type (6.0; Fig. 37). *V. longisporum* DNA in plant tissue infected with *VlLAE1*sm correlate with reduced virulence.

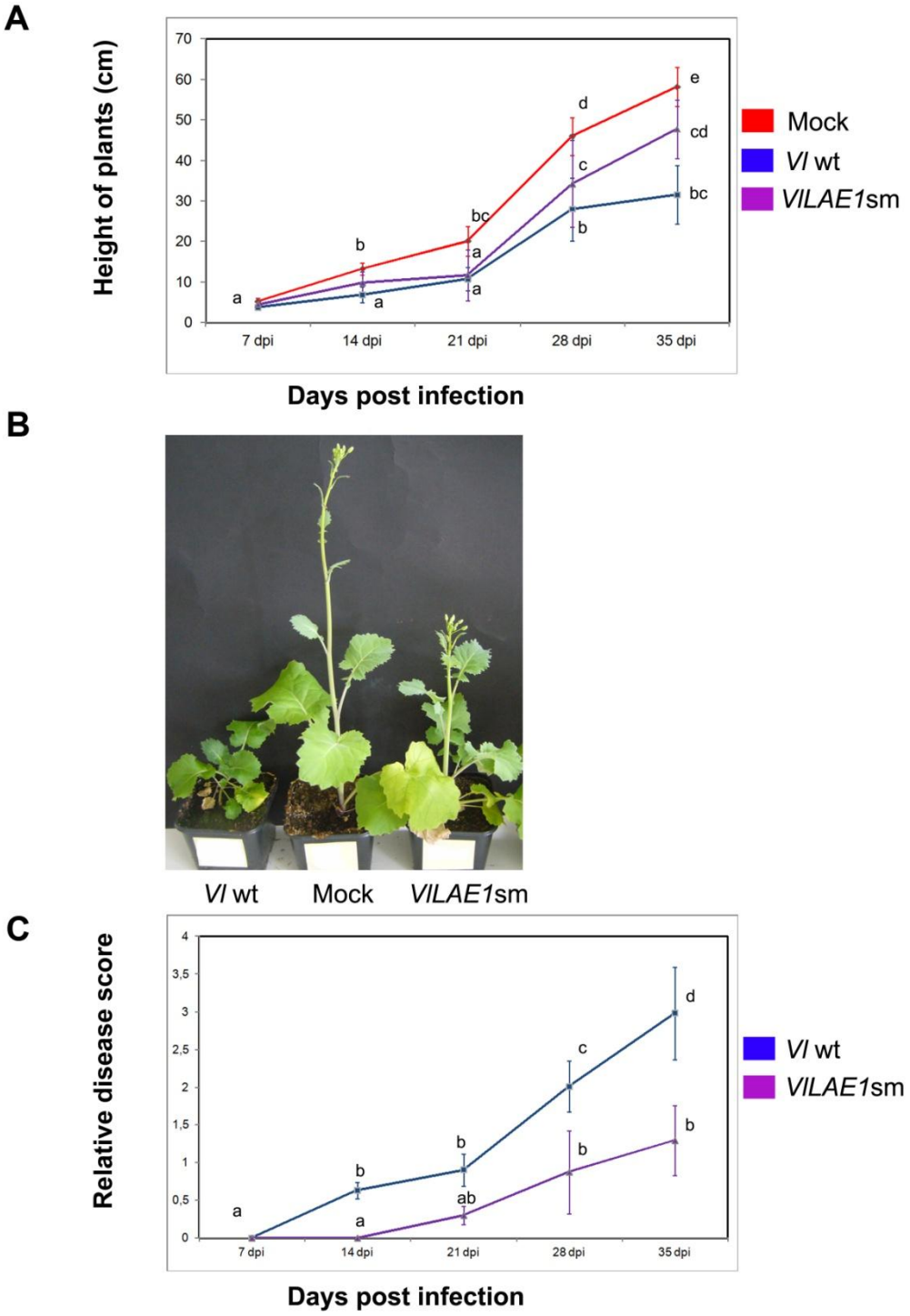

Fig. 37 Assessment of pathogenicity of the *VlLAE11* silenced mutant. **A.** Assessment of stunting of rapeseed due to *V. longisporum* infection. The height of 30 replicates each of rapeseed plants infected with wild type (wt) and *VlLAE1* silenced mutant (*VlLAE1*sm) was measured at 7, 14, 21, 28, 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 30 experimental replicates. **B.** Rapeseed infection assay. Representative *B. napus* plants shown at 35 dpi**. C.** 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 30 experimental replicates. *Vl* 43, wild type; *VlLAE1*sm, *VlLAE1* silenced mutant. Different letters indicate significant differences at $P < 0.05$.

3.2.4. A deletion of *LAE1* doesn't affect the pathogenicity of *V. dahliae*.

To investigate whether a deletion of *LAE1* also affects pathogenicity of *V. dahliae* to its host we performed the infection assays on tomato plants of the *VdLAE1* deletion mutant (Vd*ΔLAE1*) and the *V. dahliae* wildtype by root-dipping method. The results showed that the mutant is infecting the plants with reduced efficiency whereas the wild type is successfully colonizing its host (Fig. 38). The wild type *V. dahliae* caused the typical symptoms on tomato including stunting and chlorosis (Fig. 38), as well as the *LAE1* deletion mutant.

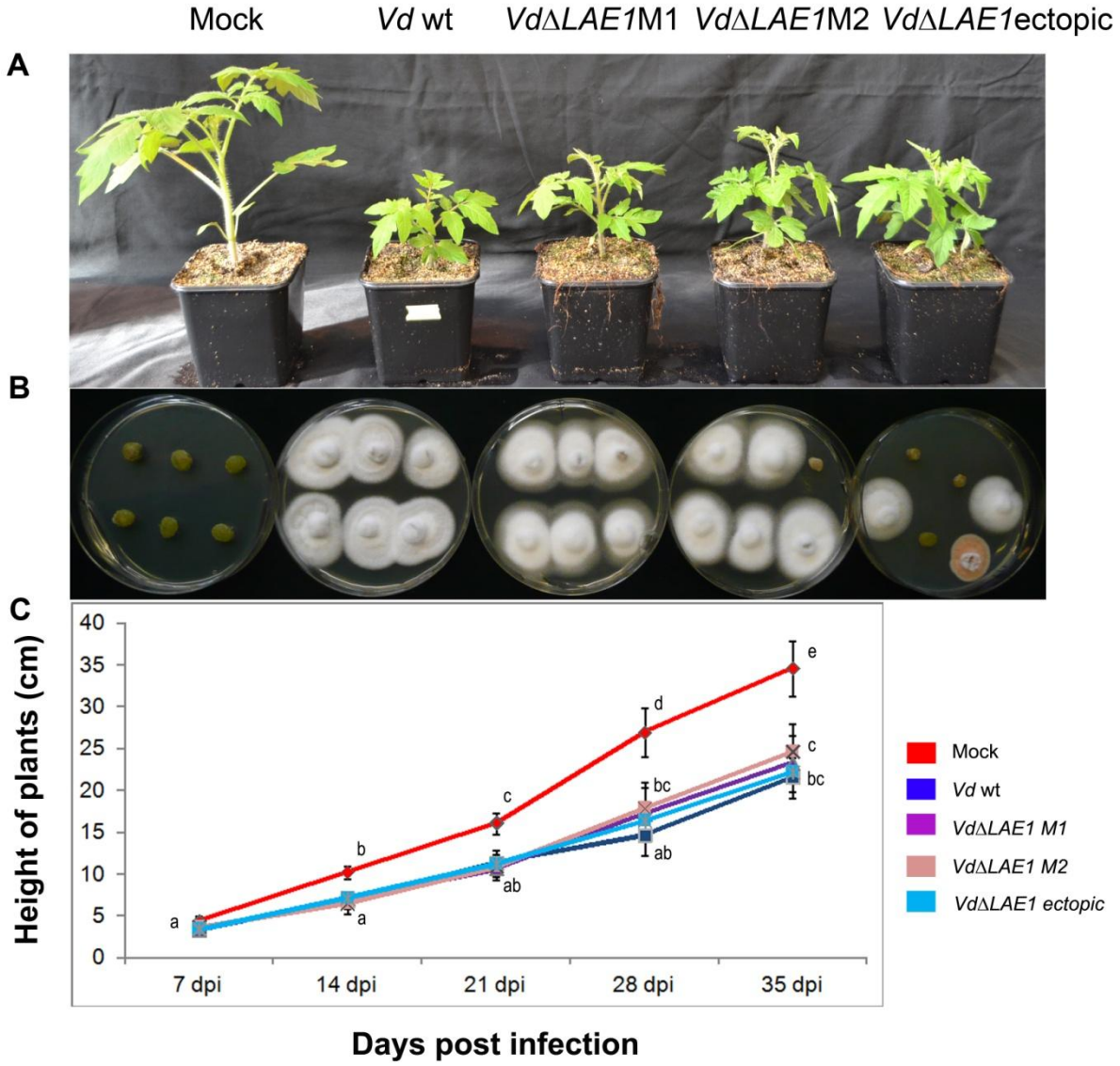

Fig. 38 Assessment of pathogenicity of the *VdLAE1* deletion mutant an complementation. A. Tomato infection assay. Representative *S. lycopersicum* plants shown at 21 dpi. **B.** Mutant regrowth test. Image of

tomato stem pieces infected with *V. dahliae* or *VdLAE1* knockout mutant (Vd*ΔLAE1*) colonies growing PDA plates. As control stem pieces of Mock served as control. **C.** Assessment of stunting of *S. lycopersicum* due to *V. dahliae* infection. The height of 20 replicates each of tomato plants. Different letters indicate significant differences at $P < 0.05$.

Compared to silencing of *LAE1* in *V. longisporum* deletion of the corresponding gene in *V. dahliae* did not show an effect on pathogenicity. The plant height of wild type infected plants was not different from the mutant infected plants. In average they are 22 cm (Vd-wt) until 24 cm (Vd*ΔLAE1*M1). The non infected plants are around 6-8 cm heigher than the infected ones.

We analyzed whether *VlLAE1*sm mutants were still able to colonize *B. napus* and cause disease, and 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 *VlLAE1*sm versus wild type was analyzed by quantification of the *V. longisporum* DNA in the plant tissue by real-time PCR. The roots hypocotyls, stems and leaves were harvested from the rapeseed plants infected with wild type or *VlLAE1*sm at various time points (Fig. 39).

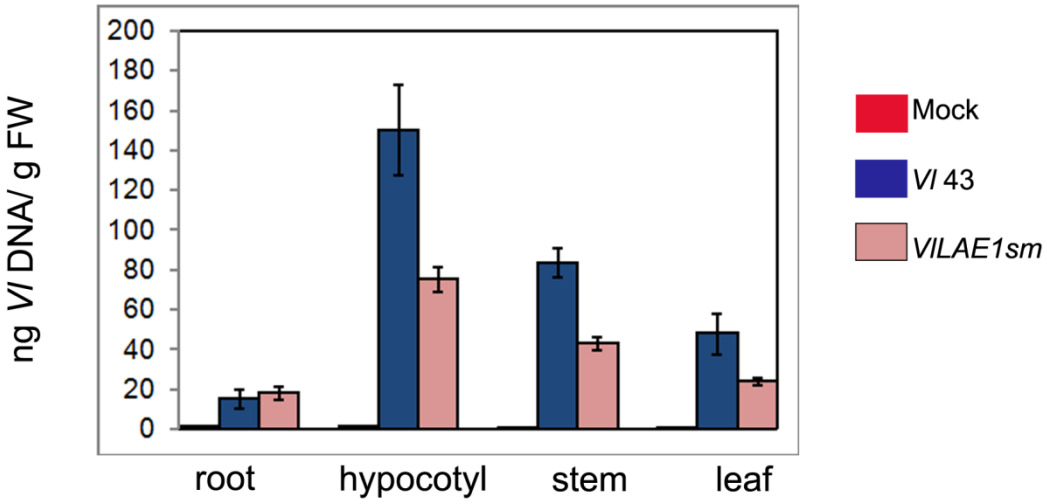

Fig. 39 Infection assay and determination of the *V. longisporum* DNA concentration in infected plant tissues. Different letters indicate significant differences at $P < 0.05$. **A.** *V. longisporum* DNA concentration in root, hypocotyls, stem and leaves after 35 dpi**.** *V. longisporum* DNA was measured with real-time PCR in stem, hypocotyls, root and leaves of *B. napus* inoculated with *VlLAE1* silenced mutant (*VlLAE1*sm) and wild type (wt) at 35 dpi. Data represent average ± standard deviations of five 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.

The DNA content was investigated at 35 dpi where there were no differences within the respective sets of experiments ($P_{(time)} > 0.5$). Low concentrations of fungal DNA were found in roots, they did not vary between *Vl* 43, *VIlAE*1sm infected plants, but show a difference

towards the mock inoculated plants (Fig. 39). In the hypocotyl the concentration of fungal DNA reaches 150 ng/g FW for the wt and around 80 ng/g FW for the *VlLAE1*sm. For the stem amount of DNA reaches 80 ng/g FW for wt infected plant and 50 ng/g FW for the mutant, whereas for the leaves it is 50 ng for wt and 30 for the mutant. The value for mock is only at basal levels. These data suggest that the mutant strain is able to perform the initial colonization of the plant and also reaches the upper parts of the plant but the distribution in these parts seems to be less effective (Fig. 39).

Silencing and deletion of *LAE1* lead to contradictory results. While the silenced mutants of the hybrid *V. longisporum* seems to affect the pathogenicity of the fungus on oilseed rape, deletion of this gene in haploid *V. dahliae* doesn't influence the pathogenicity in tomatoe. Possible Explanations to this problem will be discussed in the Discussion.

3.3. Triacylglycerol lipase (Tagl) a secreted lipase in *V. longisporum*.

Successful infection and colonization of the host requires proteins or enzymes that are capable of acquiring nutrition for the fungal pathogen, or provide opportunities of breaking the plant cell wall. Secreted proteins and secondary metabolites are the key players for these reactions (Mattinen *et al*., 2007; Ratzinger *et al*., 2009; Fu and Wang, 2011; Kulye *et al*., 2012). Furthermore they are directly involved in the pathogenicity of the pathogen. These proteins or enzymes might be useful to adapt to the plants pathogen reaction or either might be important to counteract the pathogen response of the host plant. Among these enzymes, lipases and cutinases, with their potential roles in the hydrolyzation of the plant cuticle and surface waxes, may contribute to pathogen infection at an early stage, when the fungus first comes into contact with its host (Mattinen *et al*., 2007; Floerl *et al*., 2012; Iven *et al*., 2012; Kulye *et al*., 2012; Singh *et al*., 2012).

In previous studies (Timpner, 2008) secreted proteins of *V. longisporum* have been analyzed. Therefore a culture of *V. longisporum* was treated with xylem sap and grown under certain conditions (Fig. 40). The secretome of this culture was harvested and further analyzed. One of the identified proteins was a triacylglycerol lipase (*TAGL*) which was further investigated.

Due to the treatment with xylem sap the lipase was upregulated (Fig. 40). The expression level after treatment increased 2,5 fold compared to non inducing conditions.

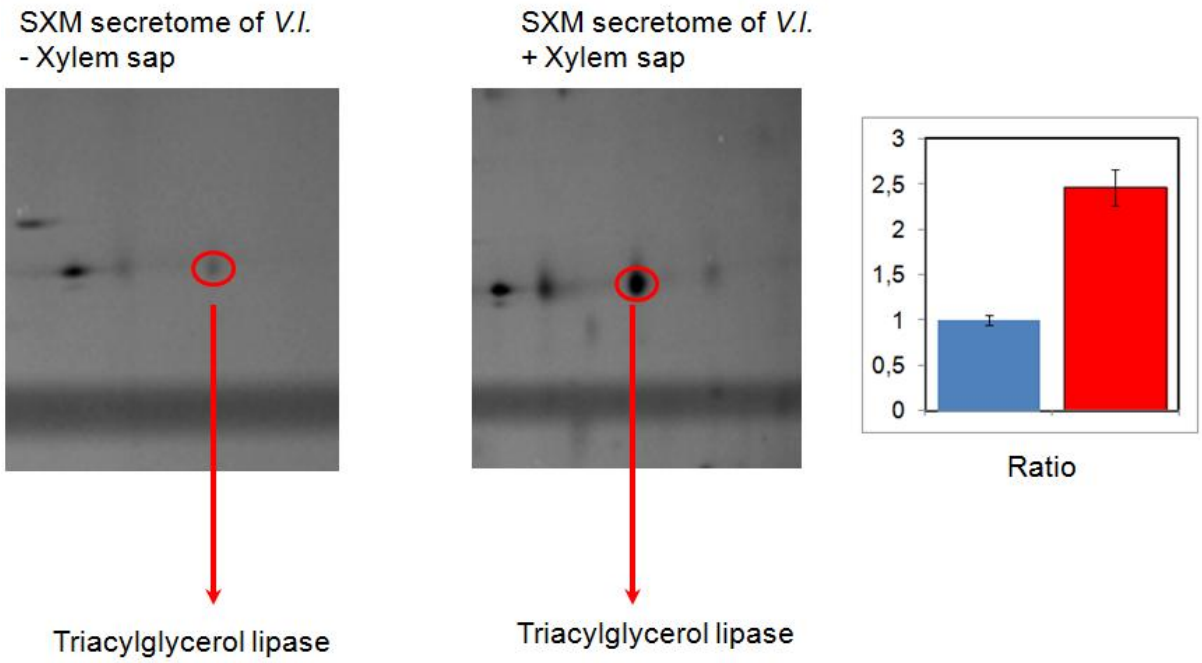

Fig. 40 Expression of triacylglycerol lipase after treatment with xylem sap. Close-up views of the regions of the 2-DE gels that show significant upregulation in protein expression (untreated (-Xylem Sap) versus treated (+Xylem Sap) with xylem sap from rapeseed). The red circles indicate triacylglycerol lipase. The expression histograms show the differential protein expression of *V. longisporum* due to treatment with xylem sap from rapeseed (right).

3.3.1. *V. longisporum* has two copies of triacylglycerol lipase

As mentioned for *CPC1* and *LAE1 V. longisporum* has been described as hybrid between two *Verticillium* species (Inderbitzin *et al.* 2011). Therefore we determined the copy number of *TAGL* in *V. longisporum*. To determine the copy number of *VlTAGL* Southern analysis was performed (Fig. 41). So genomic DNA of *V. longisporum*, *V. dahliae*, and *V. albo-atrum* were digested with suitable enzymes, and subjected to Southern hybridization. A fully sequenced 520 bp long fragment of *VlTAGL*, amplified with primers ProbeLipleft and ProbeLipright was used as the probe. Several independent Southern hybridization analyses showed two signals for *V. longisporum* as compared to *V. dahliae*, and *V. albo-atrum* for the genomic DNA digested with *Bgl*I

These results demonstrated that *VlTAGL-1* has an isogene, *VlTAGL-2*. To determine the extent of similarity of these two isogenes in the *V. longisporum* genome, the genomic DNA of *V. longisporum* was digested with *Sal*I and run on a low percentage agarose gel. DNA was extracted from the four gel fragments corresponding to spots where the signals were generated in the Southern hybridization analysis (Fig. 41). *VlTAGL-1* specific primers were used to amplify the gel extracted DNA and subjected to sequencing. On sequencing of both loci it was found out that with some exchanges the coding and intronic sequences of both the

isogenes are identical in *V. longisporum*. We have analyzed and compared the sequences from *V. longisporum* isolates Polymerase chain reaction (PCR) amplification using primers specific for *VlTAGL* (Table 3) resulted for each primer pair in only one single sequence characteristic for each analysed strain of the three species.

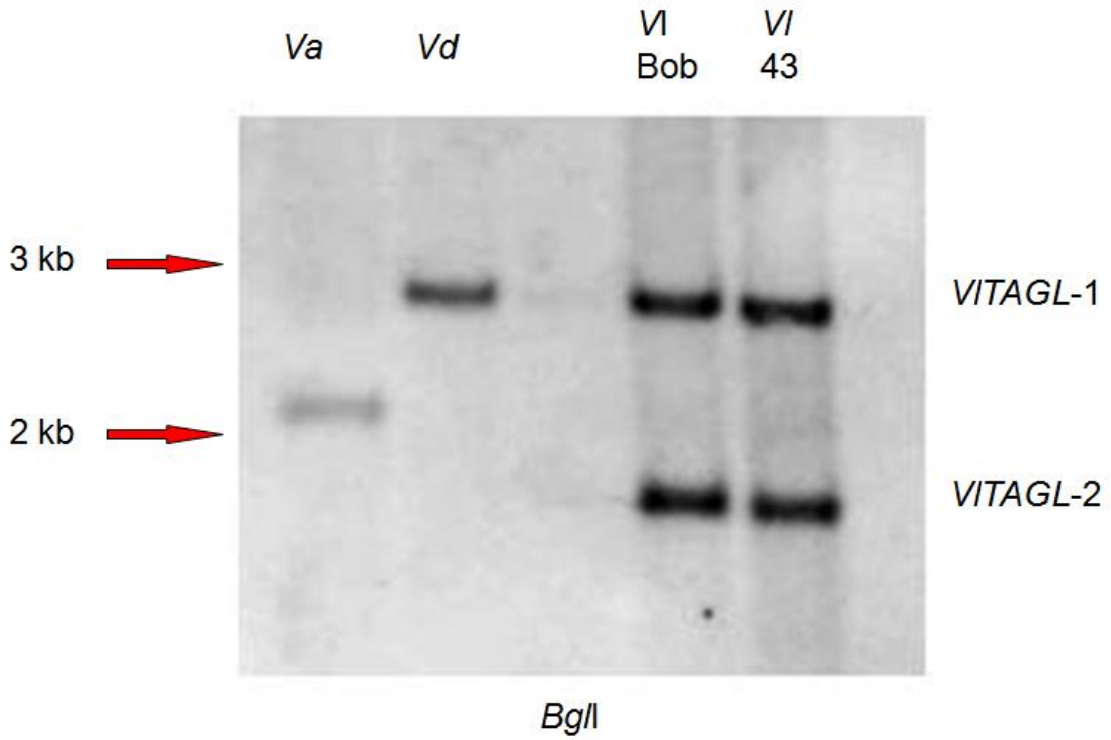

Fig. 41 Determination of the isogene *VlTAGL-1* and *VlTAGL-2* and their corresponding sequence. Southern hybridization analysis of V. albo-atrum, *V. dahliae* and *V. longisporum.* The genomic DNA was digested with *Bgl*I. A 500 bp sequence of *VlTAGL-1* was used as a probe.

The sequences for *V. albo-atrum* and *V. dahliae* share 88% of similarity. In total there are more than 60 different SNPs between the two sequences. Comparing the sequences of the two isogenes of *V. longisporum* to *V. dahliae* or *V. albo-atrum* shows that one of those sequences is close to *V. dahliae* and the other one is close to *V. albo-atrum.VlTAGL-1* is 97% identical to *VdTAGL* and they differ in 15 nucleotides. *VlTAGL-2* is 93% identical to *VdTAGL* and they differ in 36 nucleotides (Fig. 42). Compared to *VaTAGL VlTAGL-1* is like *VdTAGL* 88% identical to this sequence, whereas *VlTAGL-2* is at least 92% identical. There are two characteristic sequence patterns which correspond either to *V. dahliae* or to *V. albo-atrum*. *VlTAGL-1* and *V. dahliae* share two patterns that are identical (GACGGA) and (GCGGGAC). The pattern of *VlTAGL*-2 correspond in both patterns to *V. albo-atrum* (GAC--TAA) and (GCGAGCC).

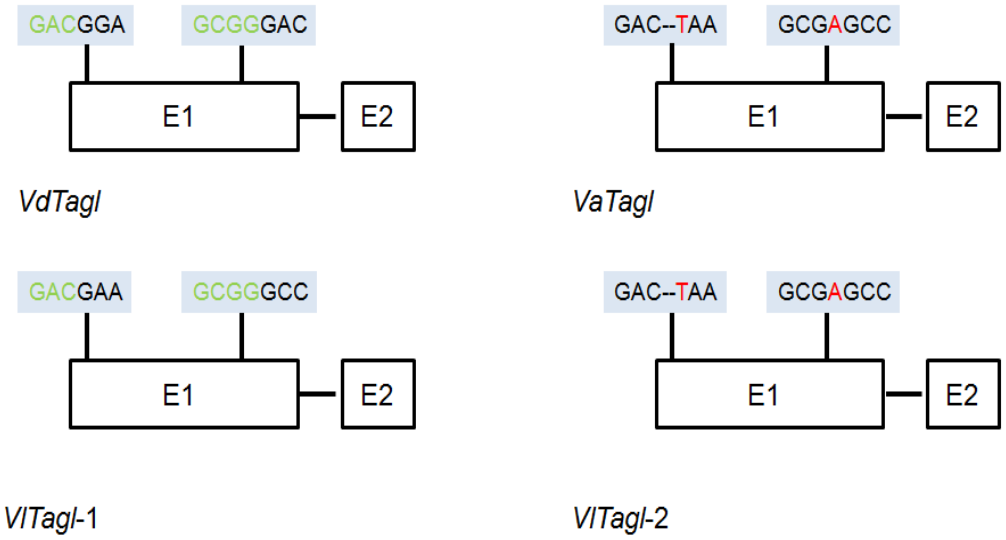

Fig. 42 Sequence comparison of *VlTAGL* sequences. *VlTAGL*-1 and *VlTAGL*-2 with *VdtTAGL*and *VaTAGL*. *VlTAGL-1* and *VlTAGL-2* isogenes of *V. longisporum* are derivatives from *V. dahliae* and *V. albo-atrum*, respectively. Characteristic signatures include a six nucleotide (GACGAA) pattern and a nine nucleotide pattern (GCGGGCC) in the first exon (E1) for *VlTAGL-1* and the *V. dahliae* orthologue. Whereas *VlTAGL-2* corresponds to the *V. albo-atrum* orthologue carries different nucleotide pattern for the first signature (GAC..TAA), as well as the second one (GCGAGCC).

3.3.2. Silencing of triacylglycerol lipase in *V. longisporum*

Like discribed for silencing of *CPC1* mutants silenced in *VlTAGL* were generated might be expressed. RNA-mediated gene silencing has been established in several fungi for targeted gene silencing instead of a conventional knockout (Nakayashiki, 2005). The plasmid (for details on plasmid construction see Materials and Methods) includes a hairpin construct for silencing of *VlTAGL* and the hygromycin resistance gene as selective marker. *A. tumefaciens* mediated transformation of *V. longisporum* was applied and thirty independent hygromycin-B-resistant transformants were selected at random for further analysis. The efficiency of *VlTAGL* silencing was determined by RT-PCR as the silencing of gene expression is the result of posttranscriptional degradation of targeted mRNA. Total RNA was extracted from the *VlTAGLsm* (*VlTAGL* silenced mutant) and wild type. RT-PCR results showed the significant knockdown of *VlTAGL* transcripts (Fig. 43). The extent of silencing varied in the different transformants between high and moderate levels. The degree of silencing of cross pathway control genes was estimated by RT-PCR by the ratio of the amplification of *VlTAGL* between the respective transformants and the unsilenced wild type. For each transformants

three biological replicates were analyzed. High-frequency silencing was observed for 54% of the transformants exhibiting reduced gene expression. The extent of silencing varied in the different transformants between high and moderate levels. After 5 generations on SXM Medium, the silencing of the *VlTAGL* isogenes was still stable (Fig. 43).

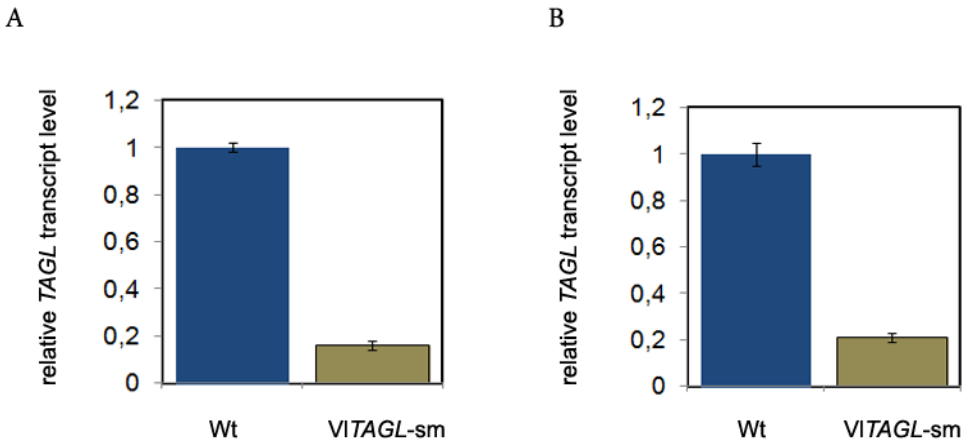

Fig. 43 Expression of *TAGL* encoding genes of *V. longisporum*. Relative expression of *VlTAGL* measured by quantitative real-time PCR. *VlTAGL* cDNA was normalized to the histone cDNA. Relative expression of *VlTAGL* in *Vl* 43 and *VlTAGL*-sm measured by quantitative real-time PCR. *VlTAGL* cDNA was normalized to the histone cDNA. *Vl* 43: wild type, *VlTAGL*-sm, *tagl* silenced mutants. The error bars represent the standard deviation of four different measurements of the same cDNA. **A.** Transcript levels of *VlTAGL* **B.** Transcript levels of *TAGL* after 5 generations on SXM.

3.3.3. Silencing of triacylglycerol lipase in *V. longisporum* shows a reduced pathogenicity of the fungus

Disease symptoms are categorized be two different sets that can be observed during the infection of oilseed rape by *V. longisporum*. Plant of *B. napus* show stunting and signs of early senescence. Both symptoms are normally visible after 21 days post infection (dpi) and are more distinctive at later time points. One-week-old *B. napus* seedlings were infected by root dip inoculation to compare the severity of the disease; height and the signs of early senescence (disease scores) of the plants were measured weekly post inoculation until 35 dpi. *VlTAGL*sm showed the same symptoms like for silencing of *CPC1*, but in comparison the effects are more pronounced than for the other mutants. In case of *TAGL* silencing treatment and observation of the plants was carried out like discribed above. In the initial phase of the disease the infected and non infected do not show severe differences, but with development at 21 and 28 dpi distinctions between the wt infected plant and the plants infected with *Vllae1*sm become obvious. At 35 dpi, the disease score of the *VlTAGL*sm-infected plants was lower than the plants infected with wildtype fungus, but still significant in comparison to mock-inoculated control plants (Fig. 44). This reflects a slow down of the disease of the plant

when infected by the mutant fungi corroborating a reduced impact on senescence. The infected plants did not show any disease symptoms until 14 dpi. The disease score of the *VlTAGL*sm-infected plants was higher (4.5) than the mock-inoculated plants (3,0) but significantly lower than the plants infected with wild type (6.0; Fig. 44). *V. longisporum* DNA in plant tissue infected with *VlTAGL*sm correlate with reduced virulence.

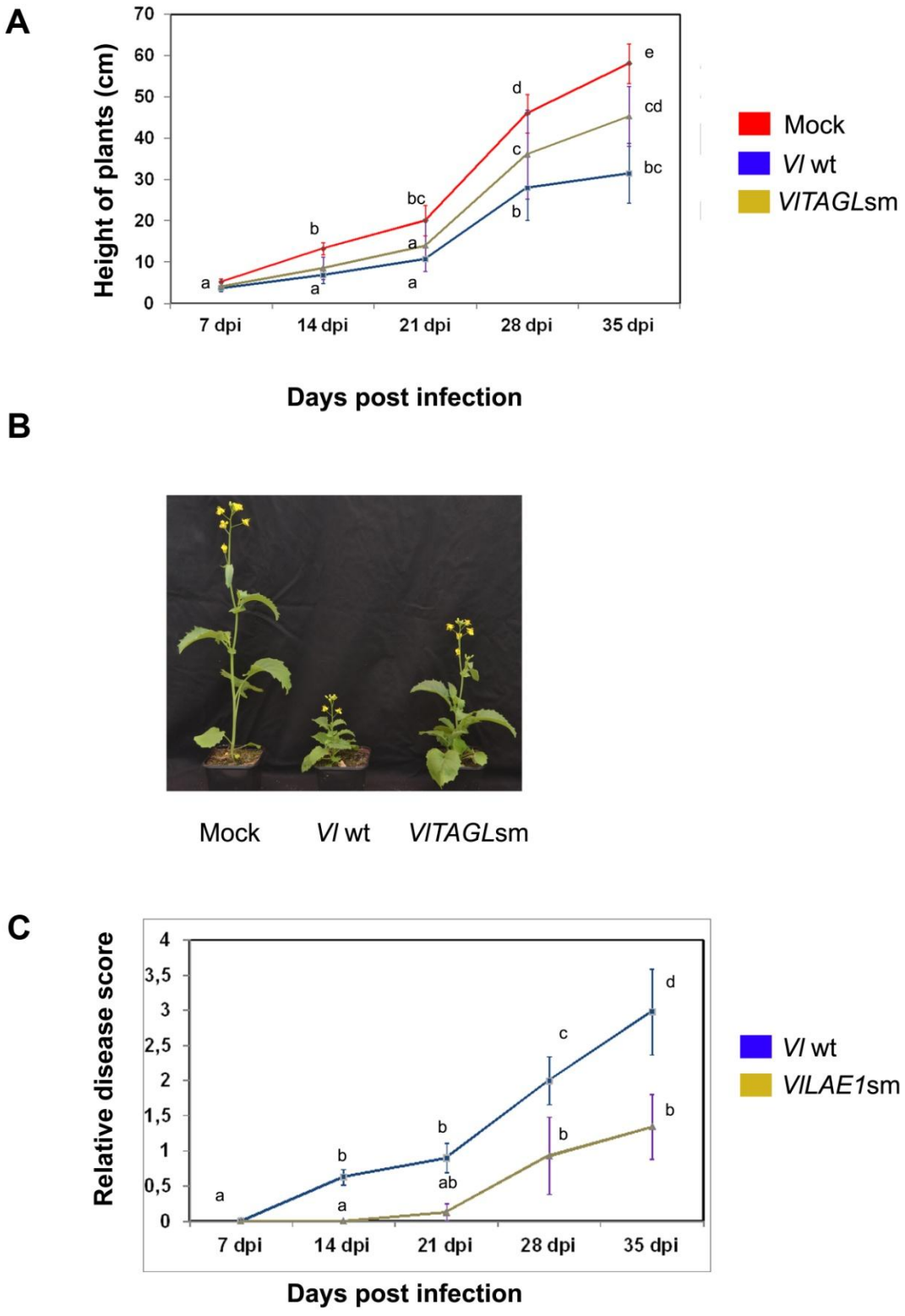

Fig. 44 Assessment of pathogenicity of the *VlTAGL* silenced mutant. **A.** Assessment of stunting of rapeseed due to *V. longisporum* infection. The height of 30 replicates each of rapeseed plants infected with wild type (wt) and *VlTAGL* silenced mutant (*VlTAGL*sm) was measured at 7, 14, 21, 28, and 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 30 experimental replicates. **B.** Rapeseed infection assay. Representative *B. napus* plants shown at 35 dpi**. C.** 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 30 experimental replicates. *Vl* 43, wild type; *VlTAGL*sm, *VlTAGL1* silenced mutant. The value for mock was detracted from the values for wild type and mutant. Different letters indicate significant differences at $P < 0.05$

Chapter 4. Discussion

It was the aim of this work to investigate the role of regulatory genes involved in amino acid metabolism like cross pathway control (Cpc) or in secondary metabolism like *LAE1*. Previous studies (Singh *et al.*, 2010) gave a first hint on the role of *CPC1* in *V. longisporum*. It was demonstrated that the regulatory gene of the cross pathway control *CPC1* was upregulated when the fungus colonizes the xylem vessels of *B. napus*.

In this work it was shown that (i) the genes for the cross pathway control regulator *CPC1* are required for growth in the presence of amino acid analogues and for full virulence of the fungus in *planta*. Furthermore it was shown (ii) that the LaeA/Lae1 global regulator of secondary metabolism has not a major impact on virulence but (iii) that the secreted triacylglycerol lipase Tagl is required for full pathogenicity on plants.

4.1. Amino acids metabolism and its role for pathogenicity.

The cross pathway control has been the subject of examination in several filamentous fungi, including *A. nidulans* (Piotrowska *et al.*, 1980; Hoffmann *et al.*, 2001), *A. niger* (Wanke *et al.*, 1997), *A. fumigatus* (Krappmann *et al.*, 2004; Sasse *et al.*, 2008), *C. albicans* (Tripathi *et al.*, 2002), *M grisea* (Shen and Ebbole, 1997), and *N. crassa* (Carsiotis *et al.*, 1974; Paluh *et al.*, 1988). The cross pathway control system of filamentous fungi is an adaptive system that enables the organism to react on different kinds of environmental stress. The function of this regulatory system has been analysed widely and for several organism, including also pathogenic fungi (Carsiotis *et al.*, 1974; Hinnebusch, 1984; Davis, 2000; Piotrowska, 1980; Sachs, 1996; Hoffmann *et al.*, 2001; Krappmann *et al.*, 2004). For example in *A. fumigatus* this system is required for full virulence (Krappmann *et al.*, 2004). For other plant pathogenic fungi it was anticipated, that a knock down or knock out of *CPC1* might reduce pathogenicity (Shen and Ebbole, 1997), but was so far not proven. For pathogenic fungi like *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; Abad *et al.*, 2010). 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). 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. In yeast the amount of Gcn4 in the cell is regulated primarily

by a translational control (Albrecht *et al.*, 1998), there is an additional amino acid starvation induced auto-activation present in filamentous fungi like *Aspergillus nidulans* (Hoffmann *et al.*, 2001). In *V. longisporum, Vlaro2* silencing induced the expression of the gene for amino acid cross pathway control, *VlCPC1* during saprophytic life (Singh *et al.*, 2010). This suggests that there is a similar auto-activation of *VlCPC1* as earlier described for *A. nidulans cpcA*. However in *V. longisporum* only little is known about the function of cross pathway control and its possible contribution in pathogenicity of the fungus. Throughout its life cycle the pathogen is limited to two basic sources, one is the soil and the other one is the host plant. During colonization and infection *V. longisporum* occupies the xylem vessels of the oilseed crop *B. napus*. The xylem sap probably provides nutrition to *V. longisporum* and it may also contain certain factors such as phytohormones for the plant defence against *V. longisporum*. The xylem sap is the medium for cross-talk between the pathogen, *V. longisporum* and its host, *B. napus*. The composition of xylem sap consists of water, inorganic compounds, amino acids and organic acids (Sakuta *et al.*, 1998; Kehr *et al.*, 2005). Therefore, the effect of the xylem sap of its host, rapeseed on *V. longisporum* growth was observed in plate-based growth assays.

A reduced activity of the *Vlaro2* isogenes encoding the aromatic amino acid biosynthetic enzyme chorismate synthase resulted in reduced pathogenicity and suggested that the xylem sap does not provide sufficient amounts or well-balanced supply of aromatic amino acids (Singh *et al.*, 2010). In this study, we present a *V. longisporum* mutant strain which has an intact basal transcription of amino acid biosynthetic genes. The strain is deficient in the transcriptional control of amino acid biosynthetic genes, which allows an increased transcription during imbalanced supply of amino acids. This mutant strain is significantly reduced in pathogenicity because *VlCPC1* is important for successful colonization of the plant host hypocotyl. *VlCPC1* seems to aid in the growth of the fungus in the xylem vessels where amino acids are scarce. Acquiring nutrient sources from its host is crucial for a fungal pathogen (Gibson *et al.*, 2011; King *et al.*, 2011).

4.2. Cross pathway control is required for pathogenicity of *V. longisporum*

In this work, we present a *V. longisporum* mutant strain, which is significantly reduced in pathogenicity. This strain shows the role cross pathway contol *VlCPC1* plays for pathogenicity of *V. longisporum* and successful colonization of its host. In previous studies *VlCPC1* shown to be induced under tryptophan starvation conditions in the wild type (Singh

et al., 2010), *VlCPC1* was similarly activated in the wild type and in a *vlaro2* silenced mutant during infection of the host plants *B. napus* and *A. thaliana* (Singh *et al.*, 2010). Assuming the fungus responds by increased *VlCPC1* expression to amino acid imbalance or starvation (Singh *et al.*, 2010). Here we can actually prove the role *CPC1* play for the plant pathogen *V. longisporum*. *VlCPC1* seems to aid in the growth of the fungus in the xylem vessels where amino acids are scarce. The mutant strain silenced in *CPC1* is strongly inhibited in pathogenicity, as it is no longer able to infect *B. napus* like the wild type. Furthermore the fungus does not reach the upper parts of the plants in later phases of infection. It is able to infect the plants, but plants infected by the silenced mutant are considerable stronger, and look healthier than plants infected by wild type strain. Measuring the fungal DNA concentration revealed, that the amounts of DNA for mutant and wild type varied strongly for different plant tissues, especially hypocotyls, stems and leaves showed significantly reduced amounts of DNA for the mutant compared to *V. longisporum* wild type strain. From height measurement of the infected plants a clear difference between wild type and *CPC1* silenced mutants could be observed. There is a difference of nearly 10 cm in height, which is very close to the mock infected plants. But still the distance of the other two mutants compared to the wild type infected plants is still significant. This supports the role of *CPC1* for pathogenity in *V. longisporum*.

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; Valerius *et al*., 2007), 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; Abad *et al.*, 2010). It will be interesting to see whether *VlCPC1* might have an additional important role in the secondary metabolism of *V. longisporum.* In several publications (Abad *et al.*, 2010; Elliott *et al.*, 2011) the role of *CPC1* on secondary metabolism has been analysed. For *Leptosphaeria maculans* silencing of *CpcA* gene resulted in a reduced production of the toxin sirodesmin (Elliott *et al.*, 2011). In *A. nidulan*s there is a link between *CpcA* and synthesis of penicillin (Busch *et al.*, 2003). For *V. longisporum* we might consider there is an involvement

in secondary metabolism of this fungus, especially when the fungus is colonizing *B. napus*. Inside its host such substances or metabolites might benefit the survival of the pathogen or might interphere with the defense mechanism of the plant (Fu and Wang, 2011). Other pathogens have developt strategies to counteract plant host defense (Hemetsberger *et al.*, 2012; Kulye *et al.*, 2012; Stergiopoulos *et al.*, 2012; Underwood, 2012).

4.2.1. Knockouts of *CPC1* in *V. dahliae* show similar effects than silencing in *V. longisporum*

Because silencing of *CPC1* in *V. longisporum* did not show a phenotype we decided in parallel to silencing to delete *CPC1* in *V. dahliae*. This resulted in a deletion strain of cross pathway control that behaved similar to *VlCPC1*sm but with stronger effects towards amino acid starvation conditions. In fact the mutant could not grow on CDM plates supplemented with 5 MT, however certain stress conditions like cell wall stress did not affect the growth behavior or the phenotype. The deletion strain was also limited in infection of its host plant *S. lycopersicum*. In Previous studies deletions of *CPC1* or *CpcA* were analyzed in different pathogenic and non pathogenic fungi (Carsiotis *et al.*, 1974; Hinnebusch, 1984; Davis, 2000; Piotrowska, 1980; Sachs, 1996; Hoffmann *et al.*, 2001; Krappmann *et al.*, 2004). In the opportunistic pathogen *A. fumigatus* this system is required for full virulence (Krappmann *et al.*, 2004). In plant pathogenic fungi it was anticipated, that a knock-down or knock-out of *cpc1* might reduce pathogenicity (Shen and Ebbole, 1997), but was so far not proven. In *A. fumigatus*, mutants impaired in cross-pathway control system had strongly reduced pathogenicity and *CpcA* seemed to be required for establishment of pulmonary aspergillosis effectively (Krappmann *et al.*, 2004; Sasse *et al.*, 2008; Abad *et al.*, 2010).

4.3. Efficiency and relieability of the silencing system

Silencing strategy for *V. longisporum* is a reliable system for investigating the pathogenicity of the fungus. The rapeseed pathogen *V. longisporum* is a near diploid fungus with a complex genome. The nuclear DNA content of this pathogen is almost double (Karapapa *et al.*, 1997; Collins *et al.*, 2003). For *V. longisporum* due to the fact that for most genes tow isogenes exist (Singh *et al*., 2010; Inderbitzin *et al*., 2011; Singh *et al*., 2012), strategies for knock-outs are difficult to apply, especially when dealing with an almost diploid genome of this fungus. Therefore, gene silencing seems to be the optimal approach for investigating gene function. In several aproaches it was proven to be functional in *V. longisporum* (Singh *et al*., 2010;

Singh *et al*., 2012) and for our investigation it also was working with high efficiency. However the efficiency of this method is not yet fully investigated for this fungus. In addition, generation of silencing constructs based on cloning techniques is very time-consuming and not feasible for broad investigation of several genes at the same time. 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.

4.4. Secondary metabolism of *V. longisporum*

Interactions of soil-borne vascular fungi and plants include an exchange of signals within the vascular tissue. In the interaction between *V. longisporum* and *B. napus*, both the pathogen and the host plant are supposed to release metabolites into the xylem which affect the other partner.

Infection of the host causes symptoms which indicate the influence of secondary metabolites secreted by the pathogen, acting as elicitor-like and phytohormonal effects, suppressors of plant defense, or as virulence factors with phytotoxic effects, as it has been reported for other *Verticillium* spp. *Verticillium* spp. generate a broad spectrum of phytotoxins and elicitor compounds which induce pathogen-specific symptoms in the absence of the fungus (Nachmias *et al.* 1987; Buchner *et al.* 1989; Meyer *et al.* 1994; Mansoori *et al.* 1995). A glycoprotein elicitor purified from *V. dahliae* culture fluid was found to trigger the synthesis of phytoalexins and oxidative burst (Davis *et al.* 1998). Recently, VdNep (belonging to Nep1-like proteins) was isolated from *V. dahliae* and suggested to play a role as elicitor (Wang *et al.* 2004). *Verticillium* spp. utilizes cell-wall-degrading enzymes for systemic host colonization (Durrands and Cooper, 1988). Particularly pectinolytic enzymes have been shown to be important for fungal virulence on the host (Carder *et al.* 1987) enabling *Verticillium* spp. to overcome pectin-containing pit membranes between xylem elements and at vessel ends (Pegg *et al.* 1976; Bishop and Cooper 1983).

Disease phenotypes like stunting and premature senescence indicate interference of normal phytohormone function in relation to systemic infection and spread of the fungus (Pegg and Brady 2002). The elevation of ethylene production after *Verticillium* infection (Pegg and Cronshaw 1976) is associated with the development of disease symptoms and was found to be involved in disease resistance of *A. thaliana* (Veronese *et al.* 2003; Tjamos *et al.* 2005;

Johansson *et al.* 2006). Elicitation of ethylene was referred to *Verticillium* phytotoxins (Mansoori and Smith 2005). Another group of potential virulence factors represent suppressors of the host defense. In tomato cultivars infected with *V. albo-atrum* or *V. dahliae*, the pathogen can suppress the activity of phenylalanine ammonia-lyase (PAL) (Lee *et al.* 1992; Gold and Robb 1995), an essential enzyme which is involved in the synthesis of suberin and lignin (Hahlbrock and Scheel 1989) but also regulates the synthesis of salicylic acid (SA). Suppression of PAL activity results in less suberin coating in the xylem which is one part of the plant defense response (Lee *et al.* 1992).

The host specificity of many fungi is often determined by whether or not the pathogen has the enzymes to detoxify a particular plant defense product (van Etten *et al.* 1989; Osbourn 1999).

4.5. *LAE1* in *V. longisporum* and *V. dahliae*

It could be demonstrated that amino acid metabolism is required for infection and colonization in *V. longisporum* as well as *V. dahliae*. In a second approach we tried to investigate the role of secondary metabolism in *Verticilliae*. Therefore we decided to observe the role of LAE1, which is the key regulator of secondary metabolism in several fungi (Keller and Hohn, 1997; Keller *et al.*, 2005; Bayram *et al.*, 2008; Bayram and Braus, 2012). A knock out of *LAE1* did not show an effect on pathogenicity of *V. dahliae*, while silencing of this gene in *V. longisporum* revealed stronger effects in pathogenicity. Silencing of genes, especially genes with conserved domains can lead to co-silencing of genes with similar domains (Nakayashiki, 2005). To analyze whether other methyl tranferase containing genes might be co-silenced by the silencing construct for *LAE1*, an alignment of similar potential genes in *V. longisporum* with the sequence of the silencing construct was performed (Fig. 45). The two hosts chosen for plant infection differ in their behavior after infection. Infected plants of *B. napus* shows very strong effects, and differences between infected and non infected plants are clearly visible. Infections with *V. dahliae* on tomato show milder symptoms and the difference between infected and non infected plants is much less pronounced.

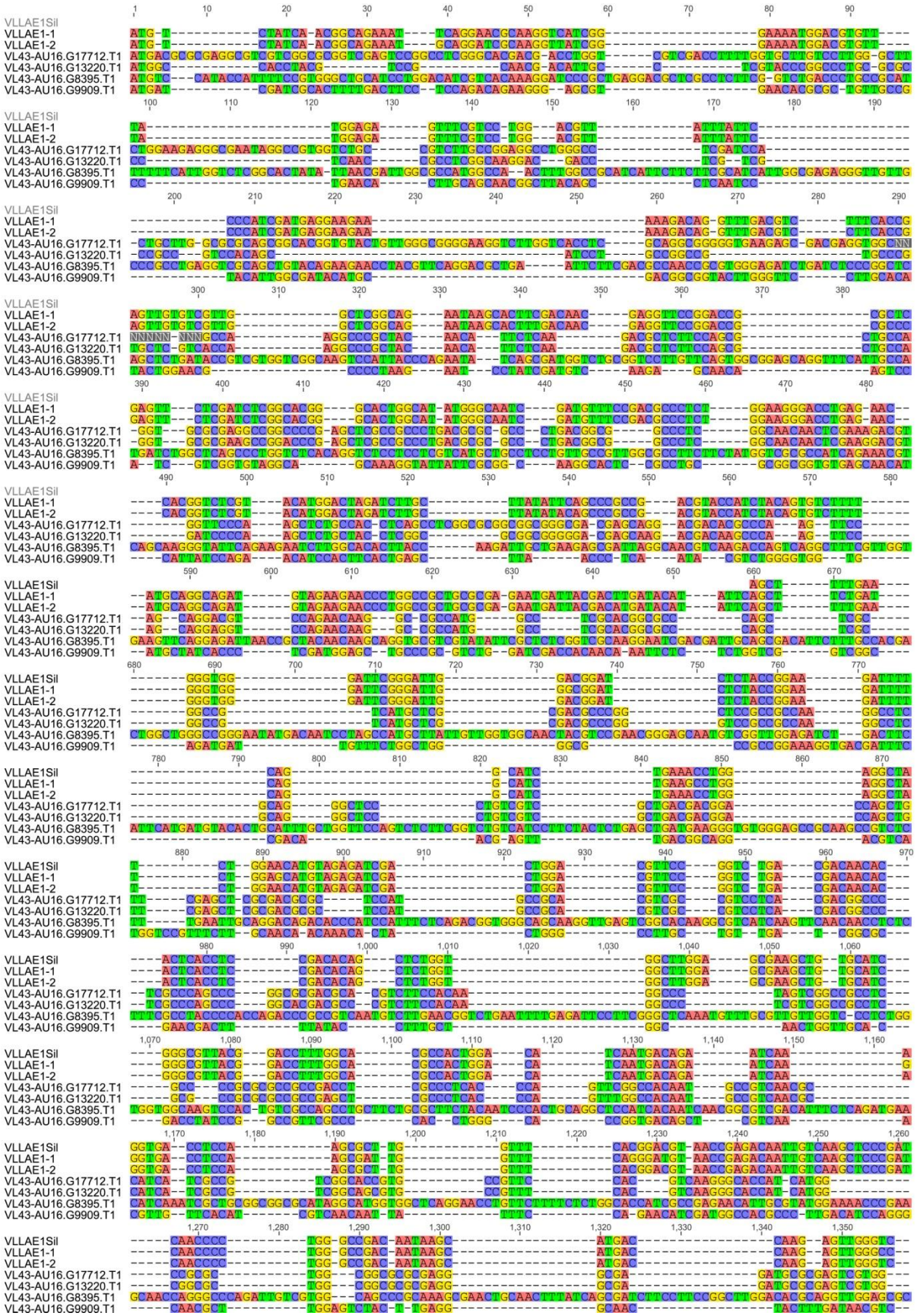

Fig. 45 Sequence alignment of *LAE1* sequences. Alignments of the deduced nucleotide sequences of LAE1 of *V. longisporum* in comparison to methyl transferase domain containing genes. Colors indicate different nucleotides.

The sequence of the silencing construct could align with several other genes containing methyl transferase domains. There is a possibility that these genes might be co-silenced by the *LAE1* silencing construct.

As mentioned for *CPC1* earlier the efficiency of this method is not yet fully investigated for this fungus. In addition, generation of silencing constructs based on cloning techniques is very time-consuming and not feasible for broad investigation of several genes at the same time. A particular disadvantage of silencing is that it is usually not absolute because residual activity of the gene remains. For the silencing of LAE1 there might be an additional problem. As shown in Fig. 44 there are several other sequences beside the target sequence which might interact with the hairpin construct for silencing and be co-silenced by this construct this effect might alter the pathogenicity of the mutant and influence the behaviour inside the plant. All these factors have to be considered when analysing these data.

LAE1 silencing in *V. longisporum* revealed an effect on pathogenicity. In plant infection the silencing had an effect on the pathogenicity of the fungus. The disease score for *VlLAE1*sm is lower than for *V. longisporum* wild type. Compared to *VlCPC1*sm the effect is not as strong but still clear. The mutant strain silenced in *LAE1* is inhibited in pathogenicity. He is capable of infecting its host *B. napus*, but regarding wild type infection a decrease of infection level could be observed. The plants infected with the silenced mutant are considerable stronger, and look healthier than wild type infected plants. The same effect can be investigated on DNA levels. The fungal DNA concentration revealed, that the amounts of DNA for mutant and wild type varied for different plant tissues, especially hypocotyls, stems and leaves showed significantly reduced amounts of DNA for the mutant compared to *V. longisporum* wild type strain. The silenced mutants show a reduction of roughly 1/3 on DNA levels in hypocotyls, stems and leaves. Height measurement of the infected plants also support a clear difference between wild type and *LAE1* silenced mutants. But reduced pathogenicity of the mutant must not correspond with a reduced production of secondary metabolites. We could prove that the gene for *LAE1* is silenced in *V. longisporum*. And for *V. longisporum* we could observe an effect on pathogenicity.

4.5.1. *LAE1* in *V. dahliae*

From the results we received by analysis of *LAE1* silencing we anticipated similar effects for a knockout in *V. dahliae*. A successful deletion of *LAE1* was established in *V. dahliae*. The

mutant did not show any effect on pathogenicity of the fungus. Which might have several reason. Like mentioned above silencing of *LAE1* might have affected some other gene with homology in the sequence which was chosen for silencing, and therefore were co-silenced by the silencing construct. This might give the data we aquired for *LAE1* in *V. longisporum* another meaning. One of the co-silenced genes might as well be involved in the pathogenicity of the fungus. This had an influence on the growth behavior of the fungus in the plant. The silencing construct is randomly integrated into the genome of the fungus. There is a possibility that the construct is integrated into the position of a gene locus and therefore has disrupted the function of a pathogenicity related gene. Another thing is the difference between the hosts chosen for plant infection. Infection of *B. napus* shows very strong effects, the differences between infected and non infected plants are clearly visible. While tomato infections show milder symptoms. The observed effects are still visible but less pronounced than in oilseed rape. The average difference between infected and non infected plants in tomatoe is much smaller than the difference between infected and non infected oilseed rape plants. This might be another reason for the why the effects of these plants are easier to notice than those of the tomatoe plants.

It might also be the reason of a different gene function of *LAE1* in *V. dahliae*. In *Trichoderma reesei* it was demonstrated, that *LAE1* of this fungus has a contrasting role (Karimi-Anghcheh *et al.*, 2013).

Futhermore *LAE1* might not play a crucial role for disease development and pathogenicity in *V. dahliae*. The infection of the plants is mainly limited to the xylem vessels of the plant (Zhou *et al.*, 2006; Eynck *et al.*, 2007), where *LAE1* might not essentially be required for colonization of the plants. Other factors might play a role under these enviroment conditions.

4.6. Triacylglycerol lipase

Successful infection and colonization of the host requires proteins or enzymes that are capable of acquiring nutrition for the fungal pathogen, or provide opportunities of breaking the plant cell wall. Secreted proteins and secondary metabolites are the key players for these reactions. These proteins or enzymes might be useful to adapt to the plants pathogen reaction or either might be important to counteract the pathogen response of the host plant. Among these enzymes, lipases and cutinases, with their potential roles in the hydrolyzation of the plant cuticle and surface waxes, may contribute to pathogen infection at an early stage, when the fungus first comes into contact with its host. Here we could demonstrate with

triacylglycerol lipase (*TAGL*) as an example that their might be a role for breaking the plant cell well and enter the host. The lipase was originally detected in a protein assay were *V. longisporum* cultures were treated with xylem sap. Due to this treatment the lipase was upregulated significantly and was chosen as a candidate for further investigastion.

Silencing of *TAGL* in *V. longisporum* showed reduced pathogenicity. It might be considered to play a role for infection of the plant. The plants are lesser affected by infection with the fungus. Compared to *CPC1* silenced mutants, the effects were less pronounced but still clearly visible in its host *B. napus*. The lipase itself could be required for breaking the plantal cell wall like discribed for other phytopathogens (Voigt *et al.*, 2005). The cutinase activity of this lipase might be involved in breaking the cell wall of the plant.

Regarding the lipase was induced after treatment with xylem sap it gives a strong hint that it might be required inside the plant for several functions. It might provide nutritiens to the fungus. For further investigation a knockout in *V. dahliae* would be interesting. Maybe a knockout would show stronger effects on pathogenicity. The fungus might even not be able to enter its host because the required enzyme is not available. Another test would be for lipase activity which was shown in other studies (Voigt *et al.*, 2005). Clearly a knock out of this gene in *V. dahliae* and plant infection assays to investigate this candidate gene in more detail is neccessary.

4.7. Conclusions and Outlook

Pathogenic organisms like the filamentous fungi *A. fumigatus* require pathways and mechanisms for adaptation within the host. A better understanding of these regulatory systems make easier to find new targets for antifungal drugs and pharmaceutica. One of these systems is the cross pathway control, which is the homologous to the well-studied General Control of amino acid biosynthesis (GC) (Hannig and Hinnebusch, 1988; Hannig *et al.*, 1990; Hinnebusch, 2005). In its core, this global regulatory system contains a sensor kinase (CpcC) and a transcriptional regulator (CpcA) (Hoffmann *et al.*, 2001), for which it was demonstrated that it is required for full virulence of *A. fumigatus* (Krappmann *et al.*, 2004). Therefore, the main focus of this work was set on the regulatory gene *CPC1*. Understanding of this regulatory mechanism enables a more detail insight of a pathogenic filamentous fungus resulting in increasing knowledge that can give hints for new treatments against this plant pathogen.

V. longisporum is pathogenic on oilseed rape and it colonizes the xylem vessels of the plant in its biotrophic phase in the plant. The xylem sap contains water, inorganic minerals, some organic compounds and also amino acids. The fungus is dependent on the xylem sap for its nutrient supply in the plant. Cpc is the global regulator of amino acid biosynthesis and many secondary metabolites produced in fungi are depending on amino acids. The goal of this study was to find out whether *CPC1* has an influence on pathogenicity of *V. longisporum* in the rapeseed. Therefore, we chose to silence the isogenes of *CPC1*. In parallel we also performed a deletion of the gene in *V. dahliae* to whether there is a difference between silencing and deletion of a gene. In *V. longisporum* silcencing of cross pathway control had no influence on growth morphology of the fungus, but lead to a mutant which is sensitive to amino acid starvation conditions. This handicap does not have an influence on normal growth conditions of the pathogen, though when restricted in amino acids the growth rate of the silenced mutant was strongly affected. In plant infection assays with limited supply of amino acids the fungus is significantly reduced in growth and infection rate. Under normal conditions *V. longisporum* spreads throughout the plant after 28 days post infection and becomes detectable via RT-PCR in the upper parts of the plants in significant amounts. For *VlCPC1*sm only 40-50 % of DNA amounts are detectable in the stem and leaves compared to wild type.

LAE1 silencing in *V. longisporum* revealed an effect on pathogenicity. But reduced pathogenicity of the mutant must not correspond with a reduced production of secondary metabolites. We could prove that the gene for *LAE1* is silenced in *V. longisporum*. For further characterization of metabolites a reliable database must be established. Furthermore it requires also some basic experiments were certain gene clusters for secondary metabolism are activated and certain products can be identified. In addition the silencing of the lipase revealed an effect on the pathogenicity which needs to be further analysed. Regarding the lipase was induced after treatment with xylem sap it gives a strong hint that it might be required inside the plant for several functions. It might provide nutritiens to the fungus. Clearly a knock out of this gene in *V. dahliae* and plant infection assays to investigate this candidate gene in more detail is neccessary.

In summary we conclude that the regulation of cross pathway control by Cpc1 plays an important role for the Verticillium pathogens to counteract the imbalanced amino acid supply inside the plant xylem vessels. This is the first time we report Cpc1 is required for plant pathogens to cause disease. Cpc1 appears to be a factor for communication between the fungi and the limited enviroment of amino acids provided by the plant. Furthermore we

investigated roles in pathogenicity of a key regulator LaeA/Lae1 of secondary metabolism and a putative secreted lipase with a conserved cutinase domain in Verticillium. Silencing of the candidate genes in *V. longisporum* resulted in reduced pathogenicity. However deletion of *LAE1* in *V. dahliae* did not lead to similar results like *LAE1* silencing in *V. longisporum*. The role of Lae1 for secondary metabolite production remains characterized. The secreted lipase seem to be an interresting candidate for further investigation. Therefore we suggest that potential function of the lipase gene in pathogenicity needs to be confirmed by gene deletion than only gene silencing.

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Lebenslauf

Persönliche Daten

Name: Christian Timpner
Geburtstag: 10.11.1979
Geburtsort: Northeim, Niedersachsen
Staatsangehörigkeit: deutsch

Wissenschaftlicher Bildungsweg

9/2009 - 5/2013 wissenschaftlicher Mitarbeiter im Institut für Mikrobiologie und Genetik, Göttingen in der Abteilung Molekulare Mikrobiologie und Genetik bei Prof. Dr. Gerhard H. Braus zur Anfertigung der Dissertation mit dem Titel " Roles of the Cpc1 regulator of the cross-pathway control in the *Verticillium* plant pathogens"

5/2008 - 9/2009 wissenschaftlicher Mitarbeiter im Institut für Mikrobiologie und Genetik, Göttingen

2007 - 2008 Institut für Mikrobiologie und Genetik, Göttingen Abteilung: Molekulare Mikrobiologie und Genetik Diplomarbeit zu dem Thema „Identifizierung differentiell exprimierter Biomarker zur Untersuchung der spezifischen Pathogen/Wirt-Interaktion von *Verticillium longisporum* mit *Brassica napus*.
Abschluss: Diplom
2000 – 2007 Georg August-Universität Göttingen
Hauptstudium Diplom Biologie
Hauptfach: Mikrobiologie
Nebenfächer: Botanik
Immunologie

Schulische Ausbildung

Schulabschluss Allgemeine Hochschulreife
1992 - 1999 Gymnasium Corvianum, Northeim
1990 - 1992 Orientierungsstufe Gerhart Hauptmann Schule; Northeim
1986 - 1990 Grundschule Edesheim

Hiermit erkläre ich an Eides Statt, dass die Dissertation mit dem Titel „ Roles of the Cpc1 regulator of the cross-pathway control in the *Verticillium* plant pathogens" selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Göttingen, im September 2013 _____

Christian Timpner