Study of genes of the phytopathogenic fungus

*Verticillium longisporum* involved in the colonization

of xylem vessels of its host *Brassica napus*

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<tbody>
<tr>
<td>amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>ampicillin resistance</td>
</tr>
<tr>
<td>amu</td>
<td>atomic mass unit</td>
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<tr>
<td>ATP</td>
<td>adenosinetriphosphate</td>
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<tr>
<td>AUDPC</td>
<td>area under the disease progress curve</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>cfu</td>
<td>colony forming unit</td>
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<tr>
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<td>centimetre</td>
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<td>cpc</td>
<td>cross-pathway control of amino acid biosynthesis</td>
</tr>
<tr>
<td>CS</td>
<td>chorismate synthase</td>
</tr>
<tr>
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</tr>
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<tr>
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<tr>
<td>eIF</td>
<td>elongation initiation factor</td>
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<td>FMN</td>
<td>flavin mononucleotide</td>
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<td>g</td>
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<td>h</td>
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</tr>
<tr>
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<tr>
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<tr>
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<tr>
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**Summary**

*Verticillium longisporum* is a devastating soil-borne fungal pathogen of the rapeseed (*Brassica napus*). It colonizes the xylem vessels of the host plants but the nutritional status of the fungus and response to the plant defense mechanisms in the xylem vessel is largely unexplored.

The gene for chorismate synthase of *V. longisporum*, *Vlaro2-1* was isolated from the cDNA library of *V. longisporum* by performing a yeast complementation assay in Δaro2. Chorismate synthase is involved in the biosynthesis of the aromatic amino acids. As *Vlaro2-1* complemented the bifunctional *aro2* in yeast, it implied that the fungal enzyme is also bifunctional which means that apart from its catalytic activity it also possess an intrinsic NADPH:FMN oxidoreductase activity. It was determined that *Vlaro2-1* has an isogene, *Vlaro2-2* with identical sequence supporting a ‘near diploid’ genome of *V. longisporum*. The deduced VlARO2 protein sequence of both isogenes is identical to the corresponding protein in *V. dahliae*. A novel knock-down method was established successfully in *V. longisporum* using RNA-mediated gene silencing to silence both isogenes of chorismate synthase which demonstrated that the gene silencing mechanism by RNAi works in the genus *Verticillium*. The *Vlaro2* silenced mutant showed normal growth on minimal medium but marked growth retardation in the presence of 5-methyl-DL-tryptophan, an inhibitor of the tryptophan biosynthesis as compared to the wild type. In a pathogenicity assay in *Brassica napus*, the propagation and virulence of the *Vlaro2* silenced mutant was significantly reduced.

The effect of the xylem sap of the host, *Brassica napus* on the protein expression of the pathogen, *V. longisporum* was further studied using a proteomic approach. The 2-Dimensional Electrophoresis (2-DE) was conducted to identify the differentially expressed proteins of *V. longisporum* in the presence of the xylem sap. Fourteen proteins were observed to be differentially expressed in the 2-DE analysis, of which six were upregulated and eight downregulated. For identification, MS/MS was employed and ten proteins were identified confidently. On functional analysis, it was observed that all the upregulated proteins may be involved in stress response. One of the upregulated proteins, catalase-peroxidase of *V. longisporum*, *VlcpeA*, was further characterized. *V. longisporum* has two isogenes, *VlcpeA-1* and *VlcpeA-2*, which further supports its ‘near diploid’ status. The catalase-peroxidase might play a role in protecting the fungus from the oxidative stress generated by the host plant.

The development of a knock-down method for efficient gene silencing and the first characterization of two gene pairs presumably necessary for prototrophy or oxidative stress response are prerequisites for the further analysis of this fungus-plant interaction.
Zusammenfassung

*Verticillium longisporum* ist ein wirtsspezifischer pathogener Pilz, der Raps (*Brassica napus*) infiziert. *V. longisporum* befällt die Pflanzen über die Wurzeln, dringt in das vaskuläre System der Pflanze ein und kolonisiert die Xylemgefäße der Pflanze. Der Mechanismus der Interaktion zwischen *V. longisporum* und der Wirtspflanze ist noch weitgehend ungeklärt.


1. Introduction

1.1 Characteristics of the plant pathogen *Verticillium longisporum*

The filamentous fungus, *Verticillium longisporum* belongs to the phylum Deuteromycota, a subgroup of the Euascomycota. As it is characteristic of a Deuteromycete, a sexual cycle is not known for *V. longisporum*. It is considered an anamorph of Ascomycetes due to its vegetative structures. The vegetative hypha of *V. longisporum* is hyaline, simple or branched, septate and multinucleate. The conidiophores are septate, hyaline and verticillately branched (in whorls) and due to this feature the genus *Verticillium* received its name. In *V. longisporum*, conidiophores mostly form three, but occasionally four, phialides (elongated conidiogenous cells) per node (Fig.1). The conidia are borne singly at apices of the phialides. The conidia are uninucleate, hyaline, elliptical, rod-shaped and often slightly curved. Most of the cells are monokaryotic but hyphal tips may be multinucleate in other species of the genus *Verticillium* including *V. alboatrum* (MacGarvie and Isaac, 1966) or *V. dahliae* (Tolmsoff, 1973).

It also produces bud spores that bud directly from the vegetative hyphae. It has been described as budding from hyphal tips or other regions, or extruding and growing of a second conidium from the first (Buckley *et al.*, 1969, Garber *et al.*, 1966).

The resting spores or microsclerotia are black and with melanized thick wall. There are three stages of development of microsclerotia (Fig. 2):

I. Initiation: The hyphae start to become swollen and more closely septate.

II. Formation: Swollen hyphae showed a two-dimensional budding and formed a strand-like, elongate and irregular structure of almost spherical cells.

III. Maturation: Structure darkened by deposition of dark pigment within the walls of
individual microsclerotia.

When grown on solid agar culture medium, *V. longisporum* forms white-black coloured colony. Its optimal growth temperature is 25°C.

![Image of microsclerotia formation](image)

**Fig. 2.** The three stages of formation of microsclerotia. **A.** Stage I (initiation) of microsclerotia development in *V. longisporum*. **B.** Stage II of microsclerotia formation. Hyphae aggregated (arrow-head) and formed irregular, elongate, strand-like structures. **C.** Stage III of microsclerotia formation. Mature, irregular, elongate microsclerotia of *V. longisporum* (bar, 50 μm) (Karapapa *et al*., 1997).

### 1.1.1 Distinct Species

Formerly, Verticillium wilt of rapeseed has been described as being caused by *Verticillium dahliae* (Seidel *et al*., 1990; Zeise, 1995). A strain of *V. dahliae* was first reported in 1960s from wilted horseradish with conidia approximately twice as long as that of *V. dahliae* and it was named *V. dahliae* var. *longisporum* (Stark, 1961). Based on pathogenecity tests and other parameters, Karapapa *et al.* (1997) suggested that this fungus, which particularly attacks family Brassicaceae, was *Verticillium longisporum*. Isolates of *V. longisporum* were differentiated from those of *V. dahliae* by three morphological characters i.e. elongate microsclerotia, long conidia (7.1-8.8 μm) and mainly 3 phialides per node on conidiophores whereas those of *V. dahliae* had spherical microsclerotia, short conidia (3.5-5.5 μm) and 4-5 phialides per node. *V. longisporum* isolates were estimated to contain approximately 0.044-0.053 pg of DNA per nucleus as compared with 0.025-0.030 pg per haploid nucleus for *V. dahliae* strains. Isolates of *V. longisporum* lacked extracellular polyphenol oxidase activity whereas it was detectable in isolates of *V. dahliae*. *V. longisporum* isolates showed mean conidial nuclear diameter of 1.85 μm whereas for *V. dahliae* isolates it was 1.16 μm. Isolates of *V. longisporum* were clearly distinguishable from those of *V. dahliae* and *V. albo-atrum* by their RAPD band profile using three oligonucleotide primers. A large intron in the nuclear
SSU-rRNA gene of *V. longisporum*, not present in either *V. albo-atrum* or *V. dahliae*, has also been characterized (Karapapa and Typas, 2001).

### 1.1.2 ‘Near diploid’ status

Isolates of *Verticillium longisporum* showed ‘near diploid’ standardized arbitrary DNA values (Feulgen DNA microdensitometry, *V. dahliae*: 0.57, *V. longisporum*: 1.02) i.e. 1.78 times the amount than those of *V. dahliae* isolates (Karapapa et al., 1997). Earlier also, the analysis of size of conidia and measurement of DNA content by Feulgen DNA microdensitometry showed the values were double for *V. longisporum* compared to that for *V. dahliae*, *V. albo-atrum* or *V. lecanii* (Typas et al., 1977, Typas et al., 1980, Jackson et al., 1985). No auxotrophs could be obtained from this strain by UV irradiation and therefore it was considered as a true diploid (Ingram et al., 1968). Two long-spored isolates from Sweden (one from sugarbeet, and one from rape) which were considered as *V. dahliae*, did not yield microsclerotial colour mutants after UV treatment and thus were assumed to be diploid (Puhalla et al., 1983). Further, other workers failed to obtain nitrate reductase-mutants (Nagao et al., 1994a, Subbarao et al., 1995), or melanin deficient mutants (Nagao et al., 1994b) from such isolates. Attempts to obtain haploid segregants from *V. longisporum* isolates using chloral hydrate or *p*-fluorophenylalanine, were also not successful (Karapapa et al., 1997). RAPD analysis suggested a direct genetic link between *V. longisporum* isolates and isolates of *V. albo-atrum* (Karapapa et al., 1997). Previously, also the group of isolates pathogenic to cruciferous plants were distinguished from other pathogenicity groups that had been identified in Japan by RAPD patterns using decanucleotide primers (Koike et al., 1996). Thus, *V. longisporum* was thought to be a hybrid between *V. dahliae* and *V. albo-atrum* evolved by parasexual hybridization (Karapapa et al., 1997). It was suggested that a hybrid originated in a heterokaryon between two different parental *Verticillium* species, in which there was a fusion of two different haploid nuclei to form a heterozygous diploid nucleus. Subsequently, chromosome rearrangements in the initial heterozygous diploid nucleus, e.g. loss of parts of individual chromosomes, or translocation between relatively non-homologous chromosomes (Geiser et al., 1996) and mitotic recombination, were all possible (Fig. 3.). This could have resulted in two, incomplete, ‘near-haploid’ genomes, each separately deficient for normal growth, but essentially exhibiting balanced lethal effects in the ‘near diploid’ hybrid.
Karapapa et al. (1997) suggested that *V. longisporum* is a hybrid of *V. dahliae* and *V. albo-atrum* evolved via parasexual hybridization. There was a fusion of two different haploid homokaryons to form a diploid heterokaryon. Chromosome rearrangements in the initial heterozygous diploid nucleus could have resulted in a 'near diploid' heteroploid (*V. longisporum*).

Later, a phylogenetic analysis of *Verticillium* species based on nuclear and mitochondrial sequences suggested that *V. albo-atrum*, *V. dahliae* and *V. longisporum* are very similar to each other, with *V. albo-atrum* and *V. longisporum* displaying the closest relationship (Fahleson et al., 2004). But, later *V. longisporum* was described as amphiaploid isolate of *V. dahliae* and based on microsatellite markers it was suggested that not *V. albo-atrum* but a yet unidentified species is the other parent of this interspecific hybrid (Barbara et al., 2005). It has also been argued that *V. longisporum* may not be an appropriate species name as both long and short spored isolates have been cultured from oilseed and have clustered together in phylogenetic groups. It was suggested that molecular analysis is required for accurate identification (Johansson, 2006).
1.2 Verticillium wilt

The genus *Verticillium* includes several pathogenic species affecting trees, herbaceous plants, plantation crops and mushrooms like *V. longisporum*, *V. dahliae* and *V. fungicola*, etc. *Verticillium longisporum* is a devastating vascular pathogen of rapeseed (*Brassica napus*) in Sweden, Germany, France and Poland (Karapapa *et al*., 1997; Zeise *et al*., 2002a, b). Verticillium wilt on oilseed rape is reported in Northern Europe since 1960s (Dixelius *et al*., 2005, Sadowski *et al*., 1995, Svenson *et al*., 1987, Zielinski *et al*., 1995). In Germany, *V. longisporum* infection on rapeseed has increased since 1980s (Daebeler *et al*., 1988, Guenzelmann *et al*., 1990, Zeise *et al*., 1990). The demand for rapeseed oil is rising as it is a healthy vegetable oil and also a renewable resource for the oleochemical industry. The growing area of rapeseed cultivation and the relatively intense crop rotation has rendered this disease an increasing threat to oilseed rape production particularly in Europe (Zielenski *et al*., 1995).

*V. longisporum* in oilseed crops does not induce true wilting, but premature senescence and ripening which can lead to severe yield reductions, up to 50-70% (Dunker *et al*., 2006, Kroeker, 1976). Chemical plant protection by fungicides is ineffective because it is a soil-borne pathogen. Due to this unusual habitat the usual fungicides are hardly applicable. Commercially available breeding material lacks sufficient resistance, therefore, this fungus remains as a major threat to oilseed rape. Gluconasturtin, a product resulting from myrosinase hydrolysis of oilseed rape glucosinolates was found to be highly inhibitory to *V. longisporum* but no difference in resistance, was found between high and low glucosinolate cultivars (Heale *et al*., 1999). Recently, promising *B. oleracea* and *B. rapa*-genotypes with enhanced resistance were identified (Happstadius *et al*., 2003; Dixelius *et al*., 2005, Rygulla *et al*., 2007) but it will take many years of breeding using these gene sources before a resistant oilseed rape variety can reach the market.

1.2.1 Host specificity

*V. longisporum* is host specific on the Brassicaceae family which includes rapeseed (*Brassica napus*), cabbage (*Brassica oleracea*), radish (*Raphanus sativus*), etc. In a pathogenicity assay, all isolates of *V. longisporum* were virulent on rapeseed, whereas *V. dahliae* strains were non pathogenic (Karapapa *et al*., 1997). Previous reports have shown the limited host range and the host-specificity of *V. longisporum* to cruciferous hosts (Baig, 1991, Horiuchi *et al*., 1990, Koike *et al*., 1996). The virulence of ten isolates of *V. dahliae* from eight different hosts on rapid cycling rapeseed genotypes showed that only the three rapeseed
isolates induced severe symptoms on rapeseed, whereas six from other hosts induced only mild symptoms and had no effect on yield (Zeise, 1995). In a test for host specificity on important crops, the pathogenicity of \( V. \) longisporum was restricted to the Brassicaceae family causing losses in plant fresh weight in oilseed rape of 49% and killing about 50% plants within 42 days. \( V. \) longisporum isolates induced the highest AUDPC levels (disease severity) in the four \textit{Brassica} species tested, whereas VCG 4B (Vegetative Compatibility Group 4B) was weakly virulent (Fig. 4). Plant fresh weight was only affected by \( V. \) longisporum isolates, which induced an earlier leaf loss and plant fresh weight losses of 38, 22 and 14% in Pak Choi, cauliflower and broccoli, respectively (Zeise \textit{et al.}, 2002b). Steventon \textit{et al.} (2002) also characterized isolates of \textit{Verticillium} from \textit{Brassica napus} in Sweden and Germany as \( V. \) longisporum. Although it is also reported that \( V. \) longisporum can also infect other plant species (Fahleson \textit{et al.}, 2003; Johannson \textit{et al.}, 2006) and \textit{B. napus} can host other \textit{Verticillium} species when the plant is weak (Collins \textit{et al.}, 2003).

\textbf{Fig. 4.} Host ranges differentiated for VCGs (Vegetative Compatibility Groups) of \textit{V. dahliae} and for \textit{V. longisporum}, based on disease severity (AUDPC) and yield losses induced by representative isolates. \textit{V. longisporum} isolates are host-specific on \textit{Brassica}-species (Zeise \textit{et al.}, 2002b).

\textbf{1.2.2 Infection cycle}

\( V. \) longisporum is a soil-borne hemibiotroph, which infects through the roots, colonizes the plant and then produces microsclerotia in the necrotrophic stage. Infection process and colonization of rapeseed plant has been investigated in previous studies (Eynck \textit{et al.}, 2007; Zhou \textit{et al.}, 2006). \( V. \) longisporum causes monocyclic disease, that is only one cycle of disease and inoculum production occurs during a growing season. \( V. \) longisporum is an opportunistic phytopathogen. The three phases of its life cycle are dormant, parasitic and saprophytic phase. In the dormant phase, microsclerotia, resting dormant spores of \( V. \)
*longisporum* are present in the soil and inhibited to germinate through microbiostasis or mycostasis (Fig. 5.). They are stimulated to germinate probably by root exudates containing excess carbon and nitrogen released in the rhizosphere of plants (Huisman, 1982, Mol *et al.*, 1995). Hyphae that grow out of the germinating microsclerotia can traverse a limited distance, possibly directed by nutrient gradients, to reach potential host plants and then enter the parasitic stage by infecting the host plant.

![Figure 5](http://www.apsnet.org/Education/lessonsPlantPath/Verticillium/discycleFull.htm)

**Fig. 5.** Infection cycle of *V. longisporum* in *B. napus*. Microsclerotia, the resting spores present in the soil are stimulated to germinate probably by root exudates. The germinating hyphae penetrate directly through the roots, passes through the root cortex and reaches the xylem vessels of the plant. They colonizes the xylem vessels, moves up in the plant and causes the disease symptoms of chlorosis, stunting and early senescence. As the diseased plant senesces and begins to die, microsclerotia are produced and released in the soil. (Source: http://www.apsnet.org/Education/lessonsPlantPath/Verticillium/discycleFull.htm)

Previously, it was reported that *V. longisporum* infects through lateral roots and root hairs (Zhou *et al.*, 2006) but in the later study it was observed that the fungus infects through the root surface when the hyphae strictly follow the grooves of the junctions of the epidermal cells and directly penetrate the root epidermal cells in the root hair zone (Eynck *et al.*, 2007).
Slight hyphal swellings were formed before entering epidermal cells but no conspicuous infection structures like appressoria were observed. The plant cell wall was pierced by a thin penetration peg but later the hyphae regained their regular diameter in the lumen of the epidermal cells (Fig. 6A). Thereafter, it grows inter- and intra-cellularly in the cortex towards the central cylinder (Fig. 6B). Whenever growing through cell walls, hyphae showed the typical sequence of swelling, constriction to a narrow infection peg and regaining the regular size after penetration. The colonization of the xylem vessels started three weeks after inoculation. Individual xylem vessels are filled with mycelium and conidia whereas neighbouring vessels remain completely unaffected (Fig. 6C). This might be the reason for no wilt symptoms on rapeseed after *V. longisporum* infection. The adjacent xylem vessels were easily invaded through plasmodesmata. Conidia were formed either by budding or on phialides arranged in a typical verticillate manner. Initiation of flowering in rapeseed was found to be a critical phase when *V. longisporum* is observed even in upper parts of the stem (Zhou *et al.*, 2006). The fungus enters the saprophytic phase during plant senescence. Apart from the vascular tissues, shoots and roots of the plant also now become colonized. As the diseased plant senesces, the fungus produces microsclerotia, which are released into the soil with the decomposition of plant material and persists there for many years in this dormant form.

Fig. 6. *V. longisporum* colonization and infection in *B. napus*. Confocal laser scanning microscopy analysis after staining with acid fuchsin and acridine orange. **A.** Magnified view of the penetration of a plant cell wall (cw) by *V. longisporum* in the root cortex. Asterisk marks the swelling of the hypha before penetrating with a thin penetration peg. **B.** Directed growth of *V. longisporum* hypha (hy) in the root cortex towards the xylem (xy). Arrow heads assign points of penetration. xyp, xylem parenchyma. **C.** Hyphal (hy) growth and production of conidia (sp) in a xylem element (xy). (Eynck *et al.*, 2007)
In the infection study, performed by Karapapa et al. (1997), all the virulent *V. longisporum* isolates produced marked stunting in rapeseed 15 days after inoculation. Infection reduced the length of petioles as well as the overall plant height. Chlorosis of the cotyledons of inoculated plants was observed 11-15 days after inoculation, while those of controls remained healthy and green. Mature leaves of infected plants first showed chlorosis with some necrosis 15-18 days after inoculation. The symptoms developed first in the lower leaves and gradually moved upwards. Infected stems and petioles exhibited vascular browning, and defoliation and death of the plants occurred within a month of inoculation with *V. longisporum* isolates. In another study, chlorosis and dark-coloured veins on older leaves were the first disease symptoms observed on *V. longisporum* infected plants 14 days after inoculation. Stunted growth and moderate to less severe disease symptoms were observed at 21 days after inoculation. At 35 days after inoculation, almost 50% of the inoculated plants showed severe disease symptoms (Eynck et al., 2007).

1.2.3 Xylem vessel, the preferred habitat of *V. longisporum*

*V. longisporum* is a serious agricultural problem as it is a fungal pathogen of economically important oilseed crop, rapeseed. *V. longisporum* infection is a difficult disease to control, largely because *V. longisporum* forms large numbers of microsclerotia that are able to remain viable for many years in the soil. Under favourable environmental conditions, they germinate and enter the host plant through the root surface. The fungus grows in the cortex towards the central cylinder and then colonizes the xylem vessels. This shows that the xylem is a preferred rather than accidental habitat. Individual xylem vessels are filled with mycelium and conidia and the fungus grows upward through the xylem and systemically colonizes the host by producing a large number of conidia (Eynck et al., 2007). It is quite fascinating that the fungus is limited to the xylem vessels during the biotrophic phase of its life cycle. In order to propagate in the plant, it must derive nutrition from the xylem sap but studies on the nutritional condition for the pathogen in the xylem are limited. The xylem transports mineral-containing water from the soil to the aerial plant parts. In addition, the xylem sap contains amino acids, organic acids, and sugars (Lopez-Millan et al., 2000). In a proteomic analysis of the xylem sap of *B. napus*, 69 proteins belonging to the functional classes of peroxidases, proteases, defense-related protein, lectins and cell wall metabolism and remodelling were identified (Kehr et al., 2005). In another study, amino acid contents were measured in *B. napus* grown under different nitrogen supply condition to the plant (Tilsner et al., 2005). Glutamic acid, aspartic acid and serine were the most abundant amino acids. The
concentration of tyrosine was found to be 1.2-1.5%, phenyl alanine 1.8-2.1% and tryptophan 1.1-1.5%, of the total amino acids under low nitrogen supply condition in the apoplast (free diffusional space outside the plasma membrane including the xylem). Amino acids and amides appear to be the major sources of nitrogen and carbon available to the vascular pathogen (Dixon and Pegg, 1972). Infection with *V. albo-atrum* reduced the xylem amino acid levels in tomato (Dixon and Pegg, 1972), and in strawberry with *V. dahliae* infection (Springer, 1967). Specific amino acids have been implicated in growth and resistance, e.g. proline (Dixon and Pegg, 1972) and alanine (Singh *et al.*, 1971). *V. longisporum* must quickly sense and respond to the host environment by expressing genes that facilitate adaptation to conditions encountered during colonization of the xylem. Such genes might allow *V. longisporum* to avoid recognition, combat plant defence responses, use scarce or unique nutrients in the xylem and influence the plant host, rapeseed to alter its internal environment to suit better the needs of the invading fungus.

1.3 **Chorismate synthase, the precursor of the aromatic amino acids**

The fungi have very efficient amino acid biosynthesis and uptake system. For the biosynthesis of aromatic amino acids, shikimate pathway is employed. The shikimate pathway is the general aromatic biosynthetic pathway essential in fungi, bacteria, algae and higher plants but is absent in mammals. Shikimate pathway consists of seven reactions for conversion of the substrates, D-erythrose 4-phosphate (E-4-P) and phosphoenolpyruvate (PEP) to chorismate. Chorismate synthase (CS) catalyses the seventh step in the shikimate pathway for conversion of 5-enolpyruvylshikimate-3-phosphate (EPSP) to chorismate, the precursor of the three aromatic amino acids, tryptophan, phenyl alanine and tyrosine (Fig.7). In *S. cerevisiae*, the enzyme is encoded by the *aro2* gene (Braus, 1991). CS can be converted into prephenate for the synthesis of phenyl alanine and tyrosine or, with the help of an additional nitrogen donor, glutamine into anthranilate for the synthesis of tryptophan (Braus, 1991, Haslam, 1974; Weiss and Edwards, 1980). Aromatic secondary metabolites and other aromatic compounds like p-aminobenzoate, amino-hydroxybenzoate, vitamin K and ubiquinone are also derived from chorismate (Bentley *et al.*, 1982, Jones *et al.*, 1991, Knaggs, 2003). A mult-subunit enzyme complex is involved in ubiquinone (coenzyme Q) biosynthesis in *S. cerevisiae* (Hsu *et al.*, 2000). In a microarray assay, terrequinone A (secondary metabolite) biosynthetic gene cluster was revealed in aspergilli (Bok *et al.*, 2006).

CS has been studied in fungi, *Saccharomyces cerevisiae* (Jones *et al.*, 1991), *Neurospora crassa* (Kitzing *et al.*, 2001); several bacterial species (Charles *et al.*, 1990, Ehammer *et al.*, ...
2007, Fitzpatrick et al., 2001); some plant species (Ehammer et al., 2007, Henstrand et al., 1995b, Mousdale and Coggins, 1986); protist, Euglena gracilis (Schaller et al., 1991) and protozoan, Plasmodium falciparum (Ehammer et al., 2007, Fitzpatrick et al., 2001).

**Fig. 7.** Overview of the aromatic amino acid production in fungi. 1-7: seven steps in the shikimate pathway for the production of chorismate, precursor of the aromatic amino acids, phenyl alanine, tyrosine, and tryptophan and secondary metabolites, p-aminobenzoate, ubiquinone, vitamin K and Terrequinone A. The seventh step (7) is catalyzed by chorismate synthase. E-4-P, D-erythrose 4-phosphate, PEP, phosphoenolpyruvate, EPSP, 5-enolpyruvylshikimate 3-phosphate.

CS exclusively catalyses the 1, 4-anti-elimination of the 3-phosphate group and the 6-(pro-R)-hydrogen from 5-enolpyruvylshikimate 3-phosphate (EPSP) to produce chorismate (Hill and Newkome, 1969, Onderka and Floss, 1969). The mechanism of this reaction is stereochemically ambiguous (Hawkes et al., 1990). This enzyme has an absolute requirement for reduced FMN (Morell et al., 1967, Welch et al., 1974) although there is no net change in the redox state.
Chorismate synthases are categorised as monofunctional or bifunctional depending on whether reduced FMN is readily available to them or they have to catalyse its reduction for fulfilling their requirement for catalyzing production of chorismate. Chorismate synthases are defined as monofunctional if they acquire reduced FMN from their environment e.g. in bacteria and plants. As an exception, bacterial *Mycobacterium tuberculosis* chorismate synthase was determined to be bifunctional recently (Ely *et al*., 2008). CS is defined as bifunctional (Fig. 8.) if they possess an intrinsic ability to utilize NADPH for reducing flavin e.g. in fungi and some protozoa. Because the molecular mass of bifunctional fungal *N. crassa* CS was higher as compared to monofunctional bacterial and plant CS, it was thought that *N. crassa* CS contains an additional NADPH binding site (Henstrand *et al*., 1995a). However, attempts to identify this domain by construction of deletion mutants disproved this hypothesis. The organisms with monofunctional CS are thought to generate the cofactor, reduced FMN by NAD(P)H-dependent FMN oxidoreductases (Macheroux *et al*., 1999) and thus have it freely available in their environment. Ehammer *et al.* (2007) suggested that the fungal and protozoan species have bifunctional CS because they lack free reduced FMN in the cellular environment and therefore possess an intrinsic NADPH:FMN oxidoreductase activity.

**Fig. 8.** The catalytic activities of ‘bifunctional’ CS. This class of enzymes can reduce oxidized FMN to the fully reduced cofactor FMNH2 at the expense of NADPH. With the reduced cofactor bound to the active site multiple turnover of the substrate EPSP can occur (Ehammer *et al*., 2007).
as it was possible to substitute a bifunctional CS with a monofunctional CS in conjunction with a NADPH:FMN oxidoreductase. The structure of monofunctional CS was determined for the first time in *Streptococcus pneumoniae* (Maclean and Ali, 2003). The X-ray diffraction structure (Dias *et al.*, 2007) and three dimensional structural model (Fernandes *et al.*, 2007) of *M. tuberculosis* CS was also studied. The structure of bifunctional CS from *S. cerevisiae* has also been studied (Quevillon-Cheruel *et al.*, 2004).

### 1.4 Aim of this work

*V. longisporum* is one of the most important pathogens of the oilseed crop, *B. napus* (rapeseed) and causes huge yield losses. *B. napus* is economically important and its area under cultivation is increasing now because of the healthy food oil produced from it and its potential as a biofuel. *V. longisporum* is a soil-borne pathogen and its resting spores, microsclerotia can persist in the soil for many years. Thus, the usual fungicides are not effective against it. *V. longisporum* is a vascular pathogen and inhabits the xylem vessels of the host plant during infection. It is still not known that why the fungus is confined to the xylem vessels of the plant and how does it survive in this fluid environment.

The aim of this project was to characterize genes (or isogene pairs) which might be important for the biotrophic phase of the fungal life. Isogene pairs should be characterized to the level of the DNA sequence to address the question of the origin of *V. longisporum* that is it is ‘near diploid’ of *V. dahliae* versus it has two different parents’ hypothesis. Two approaches were taken to identify the suitable gene pairs: (i) a candidate approach and (ii) a proteomic based approach. In addition, a method of gene silencing should be established to address the fact that there might be more than one copy of the analysed gene.

The candidate approach aimed to explore the nutritional status of the fungus in the xylem vessels particularly with respect to the procurement of amino acids from the xylem sap. Therefore, chorismates synthase, a key enzyme catalyzing the production of the precursor of the aromatic amino acids in the shikimate pathway was studied. A targeted gene knock-down of the gene for chorismates synthase in *V. longisporum* was generated and *in planta* assay was performed to observe the propagation and infection of the silenced mutant in the plant.

The proteomic approach started with the analysis of the effect of the xylem sap of the host plant, rapeseed on protein expression of the pathogen, *V. longisporum*. The proteome was examined by 2DE and MS/MS was performed to identify and analyse the differentially expressed proteins of *V. longisporum* in the presence of xylem sap from rapeseed. Further, the differentially expressed proteins were functionally classified to identify putative pathogenesis-
related proteins. An upregulated isogene pair which was identified during this process which encodes a catalase-peroxidase was analysed in more detail.
2. Materials and Methods

2.1 Strains, media and growth conditions

The Saccharomyces cerevisiae yeast strains BY4741 (wild type) and Y04515 (BY4741; Mata; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YGL148w::kan MX4) used in this study were obtained from the Euroscarf strain collection. Strains were grown on yeast extract-peptone dextrose (YPD) medium or on synthetic complete (SC) medium at 30°C. YPD medium contains 1% yeast extract, 2% peptone and 2% glucose. SC medium contains yeast nitrogen base (without amino acids and ammonium sulphate) (1.5g/l), ammonium sulphate (5 g/l), 0.1% (v/v) 200 mM myo-inositol and 2% glucose or 2% galactose, supplemented with the appropriate amino acids. For preparing solid medium, 2% agar was added to the medium.

For maintenance and construction of plasmids, Escherichia coli strain DH5α [F–, F80’d/lacZΔM15, Δ(lacZYA-argF’)] M15, (lacZYA-argF), U169, recA1, endA1, hsdR17 (rK-, mK+), supE44, thi1, gyrA96, relA1] (Woodcock et al., 1989), Escherichia coli SURE (STRATAGENE) cells and XL 10-Gold ultracompetent cells (STRATAGENE) were used. They were grown at 37°C in Luria-Bertani (LB) broth or on LB plates, supplemented with ampicillin (100 μg/ml) or kanamycin (100 μg/ml), as required.

Agrobacterium tumefaciens AGL-1 strain (Lazo et al., 1991) was used for plasmid maintenance and transformation of Verticillium longisporum. It was grown in Luria-Bertani (LB) medium supplemented with carbenicillin (50μg/ml) and rifampicin (25μg/ml) at 28°C.

The Verticillium longisporum strain 43 and Verticillium dahliae strain 73 (Zeise et al., 2002b) were used in this study. Minimal Medium (MM) (Bennett and Lasure, 1991) was modified and contained glucose (10g/l), 1M magnesium sulphate (2ml/l), 50x AspA solution (20ml/l) and 1000x trace elements solution (1ml/l). 50x AspA solution and 1000x trace elements solution were prepared according to Kaefer et al. (1977). The Simulated Xylem Fluid Medium (SXM) (Neumann and Dobinson, 2003) was slightly modified and contained sodium polypectate (2g/l), casein hydrolysate (4g/l), 1M magnesium sulphate (2ml/l), 50x AspA solution (20ml/l) and 1000x trace elements (1ml/l). Czapek Dox Agar (CDA) used for growing the fungus was slightly modified and contained sucrose (30g/l), 1M magnesium sulphate (2ml/l), 50x AspA (20ml/l) and ferrous sulphate (0.01g/l). Potato Dextrose Agar (PDA) (Gams et al., 1998) was obtained from SIGMA-ALDRICH. Supplemented antibiotic was hygromycin B (100 μg/ml). To generate fungal spore suspension stock, Czapek Dox medium was inoculated with V. longisporum spores and incubated at 25°C for ten days in
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dark. Then, the culture was filtered through sterilized miracloth (CALBIOCHEM) to remove the mycelium. The spore solution was centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant was discarded and pellet was resuspended in the saline solution (0.96% NaCl, 0.05% Tween 20). The centrifugation step was repeated and the pellet was resuspended in the saline solution. The spores were counted using a haemocytometer, adjusted to 1 x 10^6 spores/ml and preserved as 30% glycerol stock at -80°C.

2.2 The cDNA library of *V. longisporum*

For generation of the cDNA library (by INVITROGEN GMBH), 1 mg total RNA was pooled from RNA extracted from *V. longisporum* growing in different nutrient media. The pooled RNA contained 10% RNA extracted from *V. longisporum* grown in liquid MM for four days, 25% from the fungus grown in liquid MM for ten days, 25% from the fungus grown in liquid SXM for four days, 25% from the fungus grown in liquid SXM for ten days and 15% from the fungus grown on solid PDA for ten days at 25°C. The fungal mycelium was filtered with sterile miracloth filter (CALBIOCHEM) and washed with sterile distilled water. It was ground with seasand in liquid nitrogen to a fine powder using a mortar and pestle. Total RNA was isolated from powdered fungal mycelium using the TRIzol reagent from INVITROGEN according to the manufacturer’s instructions. The uncut custom cDNA library of *V. longisporum* was generated in three vectors. The details and properties of cDNA library in each vector are summarized in table 1.

<table>
<thead>
<tr>
<th>Vector</th>
<th>No. of primary clones (cfu)</th>
<th>Average insert size (kb)</th>
<th>% containing inserts</th>
</tr>
</thead>
<tbody>
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<td>pCMV.SPORT6.1</td>
<td>7.396 x 10⁷</td>
<td>1.314</td>
<td>&gt;99</td>
</tr>
<tr>
<td>(entry vector)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDONR222</td>
<td>6.874 x 10⁷</td>
<td>1.551</td>
<td>&gt;99</td>
</tr>
<tr>
<td>(entry vector)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pYES-DEST52</td>
<td>5.6 x 10⁷</td>
<td>1.814</td>
<td>100</td>
</tr>
<tr>
<td>(destination vector for expression in yeast)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5,652 clones of *V. longisporum* cDNA library were sequenced which as a result generated 4,573 ESTs (Expressed Sequence Tag). The length of the ESTs ranged from 21 bp to 954 bp with average length of 582 bp. The total length of the sequenced DNA is 2,659,599 bp. The 4,573 ESTs resulted in 2,722 different normalized sequences.
2.3 Construction of the plasmid for silencing Vlaro2 in V. longisporum

Plasmids, strains and primers used and constructed during the course of this study are listed and briefly described in table 2, table 3 and table 4.

The pSilent-1 vector (Nakayashiki et al., 2005) was used for constructing the silencing cassette. A fragment of 418 bp was amplified from coding region of Vlaro2-1 of V. longisporum by PCR using primers CSS-Sna-F and CSS-Hind-R to produce the sense strand. Sense product was purified and then digested with SnaBI and HindIII. It was then ligated into SnaBI–HindIII digested pSilent-1 plasmid. The antisense region of the Vlaro2-1 was amplified using primers, CSAS-Apal-F and CSAS-PaeI-R. Antisense product was purified and then digested with ApaI and SphI. It was then ligated with ApaI-SphI digested pSilent-1 plasmid already containing the sense Vlaro2-1. The whole silencing cassette consisting of TrpC promoter, sense Vlaro2-1 strand, spacer, antisense Vlaro2-1 strand and TrpC terminator was isolated by digestion with XbaI. It was then ligated in XbaI digested binary vector, pPK2 (Covert et al., 2001) to generate pME3571 vector for silencing both the isogenes Vlaro2-1 and Vlaro2-2. The pPK2 vector contains the hygromycin B phosphotransferase gene (hph) as the selection marker.

Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPK2</td>
<td>Binary vector (pgpdA::hph::trpC, kanR)</td>
<td>Covert et al., 2001</td>
</tr>
<tr>
<td>pSilent-1</td>
<td>Silencing vector (trpC::MCS::IT::MCS::trpC, ampR)</td>
<td>Nakayashiki et al., 2005</td>
</tr>
<tr>
<td>pME3571</td>
<td>CS silencing binary vector (trpC::CSS::IT::CSAS::trpC, hygrR, kanR)</td>
<td>this study</td>
</tr>
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</table>

Table 3. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>V. longisporum 43</td>
<td>wildtype</td>
<td>Zeise et al., 2002b</td>
</tr>
<tr>
<td>V. dahliae 73</td>
<td>wildtype</td>
<td>Zeise et al., 2002b</td>
</tr>
<tr>
<td>AGB455</td>
<td>V. longisporum strain stably transformed with Vlaro2 silencing plasmid, hygrR</td>
<td>this study</td>
</tr>
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</table>
### Table 4. Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Size</th>
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<tr>
<td>CSS-Sna-F</td>
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<tr>
<td>CSS-Hind-R</td>
<td>30-mer</td>
<td>5'-TTT AAG CTT AGC TTG TCG AAG CAG GGC TCA-3'</td>
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<tr>
<td>CSAS-ApaI-F</td>
<td>34-mer</td>
<td>5'-AAA GGG CCC TGG ACC TAC CTC ACC AAG TAC GGC A-3'</td>
</tr>
<tr>
<td>CSAS-PaeI-R</td>
<td>30-mer</td>
<td>5'-AAA GCA TGC AGC TTG TCG AAG CAG GGC TCA-3'</td>
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<tr>
<td>SS46</td>
<td>28-mer</td>
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<tr>
<td>SS47</td>
<td>27-mer</td>
<td>5'-AAG CTT AGC TTT CAG GGC TCA-3'</td>
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<td>SS15</td>
<td>21-mer</td>
<td>5'-TGG CAC CAC ACC TTC TAC AAC-3'</td>
</tr>
<tr>
<td>SS33</td>
<td>21-mer</td>
<td>5'-CCA GAG TCA AGC ACG ATA CCA-3'</td>
</tr>
<tr>
<td>OLG 70</td>
<td>20-mer</td>
<td>5'-GTC TAG GGG TGT TAG A-3'</td>
</tr>
<tr>
<td>OLG 71</td>
<td>19-mer</td>
<td>5'-GGC TGG TAG GGG GTT TAG A-3'</td>
</tr>
</tbody>
</table>

### 2.4 Genetic manipulations

#### 2.4.1 Transformation procedures

Calcium-treated chemically competent cells of *E. coli* were transformed according to (Hanahan *et al.*, 1991). *V. longisporum* was transformed by *Agrobacterium tumefaciens*-mediated transformation (ATMT) as described by Mullins *et al.* (2001) with slight modifications. For preparing electrocompetent cells, *A. tumefaciens* AGL-1 was cultured in LB medium supplemented with 30 µg/ml carbenicillin until OD$_{660}$ reached 0.5. It was then incubated on ice for 15 min and later centrifuged at 2,500 rpm for 15 min at 4°C. The bacterial pellet was gently resuspended in sterile water and again centrifuged for 10 min. The resuspension and centrifugation of the bacterial pellet was repeated once again. Subsequently, the bacterial pellet was gently resuspended in 10% glycerol and centrifuged again. Finally the bacterial pellet was gently resuspended in 10% glycerol and stored at -80°C in small aliquots. The respective binary vectors were isolated and transformed in electrocompetent *A. tumefaciens* cells by electroporation (2,500V, 5 ms, 0.2 cm cuvettes) using BIORAD GenePulser and plated on LB solid medium with kanamycin (100 µg/ml). AGL-1 strain containing the binary vector was inoculated in LB medium supplemented with 50µg/ml kanamycin and 100 µg/ml carbenicillin and incubated at 28°C for 2 days until OD$_{660}$ reached 1.5. The culture was diluted by adding Induction medium containing acetosyringone to decrease OD$_{660}$ to 0.15 and incubated at 28°C until OD$_{660}$ reached 0.6-0.8. Then, 100 µl of the *A. tumefaciens* culture and 100 µl of *V. longisporum* spore suspension (1 x 10$^6$ spores/ml) were mixed and spread on filter paper (90 mm, SARTORIUS) placed on solid Induction
medium plates containing acetosyringone. They were then incubated at 25°C for 3 days in dark. The filter paper was then transferred on PDA medium containing hygromycin B (100 μg/ml) for selection and cefotaxime (200 μg/ml) to kill A. tumefaciens cells and incubated at 25°C for 10-12 days until transformants appeared. Individual transformants were transferred on selection medium containing 100 μg/ml hygromycin B. The transformants were then propagated on CDA and further on selection medium containing 100μg/ml hygromycin B to obtain single spore isolates. Such monoconidial cultures were used for further experiments and also preserved as 30% glycerol stock at -80°C. The mitotic stability of transformation was examined by culturing transformants on CDA for five generations successively and then transferring to selection medium with hygromycin B.

2.4.2 Yeast complementation assay

V. longisporum cDNA library in the yeast expression vector, pYES-Dest52 was transformed in the yeast deletion mutant of aro2, Y04515 (Δaro2) by the lithium acetate method (Burke et al., 2000). Transformed cells were plated onto SC minus uracil medium supplemented with 2% glucose at 30°C. The uracil prototrophs were subsequently replica plated on SC plates supplemented with 2% galactose, lacking the three aromatic amino acids, tryptophan, phenyl alanine and tyrosine at 30°C.

2.4.3 Recombinant DNA methods

Recombinant DNA technology protocols were followed according to Sambrook et al. (1989). PCR was performed with Pfu proofreading polymerase (STRATAGENE), Hot start Taq DNA polymerase (QIAGEN) or Expand high fidelity PCR system (ROCHE) according to the manufacturer’s instructions. Essential cloning steps were verified by sequencing on an ABI Prism 310 capillary sequencer (APPLEA DEUTSCHLAND GMBH) at the Göttingen Genomics Laboratory. Sequences were analysed using the Lasergene software (DNASTAR INC.). Restriction endonuclease and T4 ligase enzymes were obtained from FERMENTAS GMBH. Primers were synthesized by OPERON. The Qiaprep spin miniprep or Qiagen Plasmid Midi kit (QIAGEN) was used for the isolation of plasmid DNA according to the manual. For extraction of DNA fragments from agarose gels, the QIAquick Gel Extraction Kit (QIAGEN) was used.

2.5 DNA isolation and Southern hybridization analysis
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V. longisporum 43 strain, V. dahliae 73 strain and V. longisporum transformants (1 x 10^6 spores/ml) were inoculated in liquid CDM at 25°C for 10 days. The fungal mycelium was filtered with sterile miracloth filter (CALBIOCHEM) and washed with sterile distilled water. It was ground with sea sand in liquid nitrogen to a fine powder using a mortar and pestle. Genomic DNA of V. longisporum was isolated from powdered mycelium as described by (Kolar et al., 1988). Southern hybridization analyses were performed essentially as described earlier (Southern, 1975). After electrophoresis, DNA was transferred to Hybond-N membranes (AMERSHAM) by alkaline transfer. Gel-purified DNA fragments (QIAquick Gel Extraction Kit, QIAGEN) consisting of a 400 bp fragment from Vlaro2-1, a 1 kb fragment from hph gene in pPK2 (Covert et al., 2001) or a 500 bp fragment from VlcppeA-1 were used as probes and labelled using Amersham AlkPhos Direct labelling Reagents (GE HEALTHCARE). Hybridization of DNA blots was conducted at 55°C, and membranes were washed at 55°C in primary and secondary wash buffers according to the manufacturer’s instructions. Amersham CDP-Star Detection reagent (GE HEALTHCARE) was used for chemiluminescent signal detection.

2.6 RNA isolation and RT-PCR analysis

Total RNA was isolated from powdered fungal mycelium (See 2.5) using the TRIzol reagent from Invitrogen according to the manufacturer’s instructions. 15 μg RNA from each sample was fractionated in formaldehyde, 1.4% agarose, stained with ethidium bromide and then visualised with UV-light. The presence of clear ribosomal bands was used as a criterion for good quality. Total cDNA was generated from 2 μg RNA by reverse transcription using oligo (dT)18 primer in a 20 μl reaction using RevertAid First Strand cDNA synthesis kit (FERMENTAS) according to the manufacturer’s instructions. PCR was performed using the Hot start Taq DNA polymerase (QIAGEN) with 1 μl of RT reaction as template and 0.5 μM of each primer. To detect Vlaro2 mRNA, the forward primer, SS46 and reverse primer, SS47 were used and to detect actin the forward primer, SS15 and the reverse primer, SS33 were used.

2.7 Protein extraction and Western hybridization analysis

In order to resolve proteins, Yeast Protein Extraction Reagent (Y-PER-S reagent, PIERCE) and protease inhibitor (Complete, protease inhibitor cocktail, ROCHE) were added to the powdered fungal mycelium (See 2.5) and incubated for 10 min at room temperature. It was then vortexed and incubated on ice for 20 min, and then centrifuged at 4,500 rpm for 20 min.
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at 4°C. The supernatant containing the solubilized proteins was collected and stored at -20°C. The protein used for 2D-PAGE experiments was purified by chloroform/methanol extraction (Wessel and Flugge, 1984).

For native protein extraction, cold 50 mM potassium phosphate buffer, pH 7.0 and protease inhibitor were added to the powdered mycelium. It was then centrifuged at 8,000 rpm for 15 min at 4°C. The supernatant containing the native proteins was collected and stored at -20°C (Chary and Natvig, 1989).

Protein concentrations were determined using the BCA™ protein assay kit (PIERCE) according to the supplier’s manual.

Western hybridization analysis of the protein extracts was carried out essentially as described by Laxalt et al. (2002). SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970) and then proteins were transferred to a nitrocellulose membrane (SCHLEICHER & SCHUELL BIOSCIENCE GMBH). The PageRuler™ Prestained Protein Ladder (FERMENTAS GMBH) was used as a marker. Chorismate synthase antibody raised against N. crassa (Ehammer et al., 2007) and monoclonal mouse anti-alpha-tubulin antibody (SIGMA ALDRICH CHEMIE GMBH) were used as primary antibodies. As secondary antibodies, peroxidase-coupled goat antirabbit IgG-HRP (SANTA CRUZ BIOTECHNOLOGY) or goat antimouse IgG antibodies (INVITROGEN GMBH) were employed. Cross-reactions were visualised using the ECL technology (AMERSHAM PHARMACIA BIOTECH). The quantification of the bands was performed using the KODAK MI 4.05 software (EASTMAN KODAK COMPANY, Rochester, NY, USA).

2.8 Phenotypic analysis of Vlaro2-silenced mutants

For phenotypic analysis, 5,000 spores (5 μl) of Vlaro2-silenced mutants (Vlaro2-sms) and wild type were point inoculated on CDA and CDA supplemented with the three aromatic amino acids, tryptophan, phenyl alanine and tyrosine or 5 mM 5-methyl-DL-tryptophan at 25°C. The experiment was performed in triplicate. The plates were observed every day until four days post inoculation by light microscopy using Olympus SZX12 binocular (OLYMPUS) or a ZEISS Axiolab light microscope (ZEISS AG). Images were taken using a KAPPA PS30 digital camera and the KAPPA ImageBase software (KAPPA OPTO-ELECTRONICS GMBH). For radial growth rate determination, the procedure described by (Panepinto et al., 2003) was applied by measuring colony diameters after 3, 6, 9 and 12 days post inoculation.
2.9 Xylem sap production and amino acid measurement

Rapid-cycle rape (ACAacc) (Williams and Hill, 1986) was used for xylem sap production. Rapeseed (Brassica napus) plants were harvested when they were 42 days old. For infection, rapeseed plants were inoculated with V. longisporum when they were 7 days old and xylem sap was extracted 35 days post inoculation (dpi) in correspondence to 42 days old uninfected plants. Xylem sap was also extracted at an additional time point, from 35 days old rapeseed plants and from corresponding infected rapeseed plants 28 dpi. The roots were cleaned with water and dried between tissue paper. Shoots were cut just below the node bearing embryonic leaves and fixed into a pressure chamber (Scholander et al., 1965). A pressure of 4 bars was applied to the roots by nitrogen and xylem sap oozing out of the cut end was collected for 15 min (Kehr et al., 2005). The yield was 50-150 µl/plant. Xylem sap was filter-sterilized and stored at -20°C until further use. The amino acids present in the xylem sap were analyzed by HPLC (Pharmacia/LKB) according to Riens et al., (1991) (See 3.1.1 Table 1).

2.10 Pathogenicity assay

A week old Brassica napus seedlings were inoculated with 1 x 10^6 spores/ml of Vlaro2-sms and wild type, and mock-inoculated with tap water for 30 min by root-dipping inoculation. They were then transferred in pots with a sterile sand:soil (1:1) mixture. The plants were incubated in a climate chamber with 14 h light at 23°C and 10 h dark at 20°C. The height of inoculated plants was measured, and they were scored for disease symptoms according to Zeise (1992) (See 3.1.6 Table 2) at 7, 14, 21, 28 and 35 dpi.

2.11 Quantification of V. longisporum DNA in planta by real time PCR

Hypocotyls and stem (5-6 cm from top of plant) were separated from the infected rapeseed plants harvested at 14, 21, 28 and 35 dpi. Eighteen plants were harvested from each treatment. Plant tissue was ground in mortar and pestle in liquid nitrogen to a fine powder. DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN) as per the manufacturer’s instructions. The fungal DNA was quantified in the plant tissue with real time PCR according to Eynck et al. (2007). Primers OLG 70 and OLG 71 amplify a fragment of ITS (Internal Transcribed Spacer) sequence of ribosomal RNA gene, specific for V. longisporum (Eynck et al., 2007). Real-time PCR was carried out by using Fast Start DNA Master SYBR Green I kit (ROCHE) with 0.3 µM of each primer and 2 µl of template in a final reaction volume of 20 µl. For amplification and melting curve analysis, the Light Cycler 2.0 System (ROCHE) was used, initiated by 10 min incubation at 95°C, followed by 45 cycles of 10s at 95°C, 10s at 58°C and...
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25s at 72°C. Fluorescence data were acquired during the elongation step in every cycle. The amount of DNA of *V. longisporum* was estimated by integration of a calibration curve using increasing amounts of genomic *V. longisporum* DNA from 1 pg to 625 pg, in the analysis.

### 2.12 Treatment of *V. longisporum* with xylem sap from *Brassica napus*

To observe the effect of xylem sap on *V. longisporum*, xylem sap from rapeseed uninfected/infected with *V. longisporum* was added in SXM and CDA in a concentration of 1:7 and 1:70, and *V. longisporum* was point inoculated with 5,000 spores in the centre of the petri plate. SXM and CDA without added xylem sap, inoculated with *V. longisporum* were taken as controls. The plates were observed by light microscopy using Olympus SZX12 binocular (OLYMPUS) or a ZEISS Axiolab light microscope (ZEISS AG) until ten days after inoculation. Images were taken using a KAPPA PS30 digital camera and the KAPPA ImageBase software (KAPPA OPTO-ELECTRONICS GMBH). For conidiospore quantification, after eight days of inoculation, the agar with the fungus colony was excised with the end of a disposable 1 ml pipet tip (diameter 1.0 cm) and vortexed for 30 min in 0.5 ml of physiological solution (Busch *et al.*, 2003; Bussink and Osmani, 1998) Then, the number of spores was counted from this solution using a haemocytometer. Counts are based on three different plates.

For the cultures used for 2-D PAGE and native gel electrophoresis, 1 x 10⁶ conidia of *V. longisporum* were used to inoculate 40 ml of SXM. The incubation was done at 25°C for five days in dark. After five days, sterile-filtered xylem sap from rapeseed plants uninfected/infected with *V. longisporum* was added in a concentration of 1:7 and incubated for eight hours.

### 2.13 Isoelectric focusing and 2D-PAGE

2D-PAGE was performed using an immobile/polyacrylamide system. Purified protein samples were used to rehydrate immobilised pH gradient (IPG) strips (pH 4-7, 18cm; AMERSHAM BIOSCIENCES) by applying 340 μl of each sample (300 μg of protein) per IPG strip. After isoelectric focusing for a total of about 40,000 V/h, IPG strips were equilibrated in equilibration buffers I and II (AMERSHAM BIOSCIENCES) for 30 min each. Equilibration buffer I contained DTT for reduction of disulphide bands and equilibration buffer II contained iodoacetamide for alkylation of SH-groups. Then, the IPG strips were placed on top of a vertical 12.5% polyacrylamide gel. Unstained protein molecular weight marker (FERMENTAS) was used for molecular weight determination. 5 μl of the marker was applied on a small piece
of filter paper and coated with agarose. This was placed on the left corner of the SDS-PAGE gel along with the IPG strip. Gels were run with a constant current of 30 mA for 4 h. Gels were stained with fluorescent Ruthenium II tris-bathophenantroline disulfonate (RuBP) (Lamanda et al., 2004). After scanning, RuBP-stained gels were additionally stained with silver nitrate (Blum et al., 1987) to visualize the protein spots for excision for trypsin digestion.

2.14 Image analysis

Images of fluorescent stained gels were acquired by scanning with the Typhoon 8600 laser scanner (GE HEALTHCARE). Image analysis was performed using the PDQuest software (BIO-RAD). By using the spot detection tool, discrete spots were marked by the software. In addition to the automatic spot detection and spot matching procedures provided by the PDQuest, all gels and all matchsets were carefully manually edited and optimized. The signal intensity of each spot was determined in pixel units (optical density) and normalized to the sum of the intensities of all the spots included in a standard gel that is the total spots intensity in all the gels is equal. Each matching analysis included six 2DE gels from control samples (3 biological replicates and each with 2 technical replicates) and similarly six 2DE gels from samples treated with xylem sap (Fig. 1).

2.15 LC-MS/MS and protein identification

Proteins within the excised polyacrylamide gel pieces of the regulated spots were in-gel digested with trypsin according to (Shevchenko et al., 1996). Tryptic peptides extracted from each gel slice were then injected onto a reversed-phase liquid chromatographic column (Dionex NAN75-15-03-C18 PM) by using the ultimate HPLC system (DIONEX) to further reduce sample complexity prior to mass analyses with an LCQ DecaXP mass spectrometer (THERMO ELECTRON CORP.) equipped with a nanoelectrospray ion source. Cycles of MS spectra with m/z ratios of peptides and four data-dependent MS2 spectra were recorded by mass spectrometry. The “peak list” was created with extract ms provided by the Xcalibur software package (BioworksBrowser 3.1). Identification was achieved through homology searching by the TurboSEQUEST analysis software which correlated experimental spectra to theoretical spectra.
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Fig. 1. Experimental set up of the proteomics study. Three independent cultures of *V. longisporum* were untreated (C1, C2, C3) /treated (X1, X2, X3) with xylem sap from *B. napus*. After protein extraction, 2-D PAGE was performed and gels were stained with RuBP and silver nitrate. The PDQuest matching analysis included six gels each (Two 2-DE gel for each biological replicate) from sample untreated/treated with xylem sap. Selected differentially expressed protein spots were digested with trypsin and identified by nano-LC-MS/MS.

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Fig. 1. Experimental set up of the proteomics study. Three independent cultures of *V. longisporum* were untreated (C1, C2, C3) /treated (X1, X2, X3) with xylem sap from *B. napus*. After protein extraction, 2-D PAGE was performed and gels were stained with RuBP and silver nitrate. The PDQuest matching analysis included six gels each (Two 2-DE gel for each biological replicate) from sample untreated/treated with xylem sap. Selected differentially expressed protein spots were digested with trypsin and identified by nano-LC-MS/MS.
The MS2 spectra with a total ion current higher than 10,000 were used to search for matches against a filamentous fungi database that consists of 10,082 entries of *Neurospora crassa* (Galagan et al., 2003), 9,541 entries of *Aspergillus nidulans* (Galagan et al., 2005), 9,926 entries of *Aspergillus fumigatus* (Nierman et al., 2005), and 14,063 entries of *Aspergillus oryzae* (Machida et al., 2005), plus 180 entries of the most commonly appearing contaminants, e.g. keratins, proteases, etc., provided with the BioworksBrowser package using the TurboSEQUEST algorithm (Eng et al., 1994). In addition, the *V. dahliae* COGEME EST database that contains 1455 unisequences (Neumann and Dobinson, 2003) was used. The MS2 spectrum of spot U1 was specifically used to search for matches against database of *V. longisporum* cDNA library sequences containing 2,722 unisequences. The search parameters included based on the TurboSEQUEST algorithm were: (i) precursor ion mass tolerance less than 1.4 amu, (ii) fragment ion mass tolerance less than 1.0 amu, (iii) up to three missed tryptic cleavages allowed, and fixed cysteine modification by carboxyamidomethylation (plus 57.05 amu) and variable modification by methionine oxidation (plus 15.99 amu) and phosphorylation of serine, threonine, or tyrosine (plus 79.97 amu). In accordance with the criteria described by (Link et al., 1999) matched peptide sequences of identified proteins had to pass the following criterion: (i) the cross-correlation scores (Xcorr) of matches must be greater than 2.0, 2.5, and 3.0 for peptide ions of charge state 1, 2, and 3, respectively; (ii) Cn values of the best peptide matches must be at least 0.4; and (iii) the primary scores (Sp) must be at least 600. Protein identification required at least two different peptides matching these criteria. The degree of completeness of the b- and y-ion series for each TurboSEQUEST result was manually checked for every protein identified. The MS/MS spectra of all the protein spots was also used to verify the TurboSEQUEST results against the NCBI non-redundant database using PEAKS MS program (BIOINFORMATICS SOLUTIONS INC.) (Ma et al., 2003).

**2.16 In-gel catalase assay**

The native protein extracts were analysed by non-denaturing Tris-glycine polyacrylamide gel electrophoresis, using a modified protocol from Sambrook et al. (1989). Ten μg of the native protein was loaded in each well. Electrophoresis was carried out through a stacking gel (3%, pH 6.8) and a separating gel (7.5%, pH 8.0) in Tris (25 mM) and glycine (192 mM) buffer at pH 8.0. The electrophoresis was performed for about 5 h at 4°C and 100V in a Mini-Protean III cell (BIO-RAD).

To perform the catalase activity assay, the native gel was washed three times for 15 min with distilled H₂O, suspended in a solution of 0.01 ml 30% H₂O₂ in 100 ml H₂O, and gently
rocked for 10 min. The H$_2$O$_2$ solution was then removed and the gel quickly rinsed in H$_2$O. A freshly prepared mixture of 30 ml each of 2% ferric chloride and 2% potassium ferricyanide, both in H$_2$O, was poured into a fresh staining pan, and the rinsed gel was transferred to the ferricyanide mixture (Zou and Schrempf, 2000). The gel tray was gently but steadily rocked by hand over a light box. As soon as a green colour began to appear in the gel itself, the ferricyanide mixture was rapidly removed and replaced with water. The gel was washed twice with water. The gel was scanned using a GS 700 Imaging Densitometer (BIO-RAD). To quantify the catalase activity in the different lanes, the Kodak Molecular Imaging 4.05 software (EASTMAN KODAK COMPANY) was used.

To perform the peroxidase activity assay, the gel was washed thrice in PBS buffer for 10 min and then washed in a freshly prepared solution of 10 μl 30% H$_2$O$_2$ and 50 mg DAB (3,3’-Diaminobenzidine) in 100 ml PBS for 20 to 30 min until brown bands appeared. After that, the gel was washed with water to remove the black DAB particles (Wayne et al., 1986). The gel was scanned using a GS 700 Imaging Densitometer (BIO-RAD).
3. Results

3.1 Silencing of the gene for chorismate synthase in *V. longisporum*

3.1.1 Amino acids in the xylem sap of *B. napus*

The xylem carries water and minerals from the root to the aerial parts of the plant. Additionally, the xylem sap also contains some organic acids and amino acids. We wanted to investigate the amino acid composition in the xylem sap of *B. napus*. For this purpose, xylem sap was extracted from 42 days old *B. napus* plants. The amino acids present in the xylem sap were analyzed by HPLC. The amino acids present in the xylem sap and their respective concentration is listed in table 1. The concentration of the amino acids was found to be in the micromolar range from 2-177.4 μM. The major amino acids are glutamine, glutamic acid, aspartic acid and gamma amino butyric acid. The concentration of the aromatic amino acids was low, as tyrosine was 7.9 μM, tryptophan was 2.01 μM and phenyl alanine was 6.77 μM. The question that arose was if this low concentration of aromatic amino acids could support the growth of *V. longisporum* amino acid biosynthesis mutant in the xylem vessels of the plant.

Table 1. Amino acid concentration in the xylem sap of forty-two days old *Brassica napus* (the values are an average of two measurements). The aromatic amino acids, tyrosine (Tyr), tryptophan (Trp) and phenyl alanine (Phe) are highlighted.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Amino acid</th>
<th>Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Asp</td>
<td>52.85</td>
</tr>
<tr>
<td>2.</td>
<td>Glu</td>
<td>53.66</td>
</tr>
<tr>
<td>3.</td>
<td>Asn</td>
<td>09.72</td>
</tr>
<tr>
<td>4.</td>
<td>Ser</td>
<td>37.34</td>
</tr>
<tr>
<td>5.</td>
<td>His</td>
<td>04.12</td>
</tr>
<tr>
<td>6.</td>
<td>Gln</td>
<td>177.35</td>
</tr>
<tr>
<td>7.</td>
<td>Thr</td>
<td>22.82</td>
</tr>
<tr>
<td>8.</td>
<td>Gly</td>
<td>17.76</td>
</tr>
<tr>
<td>9.</td>
<td>Arg</td>
<td>02.76</td>
</tr>
<tr>
<td>10.</td>
<td>Ala</td>
<td>36.32</td>
</tr>
<tr>
<td>11.</td>
<td>Gaba</td>
<td>50.58</td>
</tr>
<tr>
<td></td>
<td>(gamma amino butyric acid)</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td><strong>Tyr</strong></td>
<td>07.94</td>
</tr>
<tr>
<td>13.</td>
<td>Val</td>
<td>25.75</td>
</tr>
<tr>
<td>14.</td>
<td>Met</td>
<td>02.01</td>
</tr>
<tr>
<td>15.</td>
<td><strong>Trp</strong></td>
<td>02.01</td>
</tr>
<tr>
<td>16.</td>
<td>Ile</td>
<td>15.34</td>
</tr>
<tr>
<td>17.</td>
<td><strong>Phe</strong></td>
<td>06.77</td>
</tr>
<tr>
<td>18.</td>
<td>Leu</td>
<td>10.09</td>
</tr>
<tr>
<td>19.</td>
<td>Lys</td>
<td>08.83</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td>544.02</td>
</tr>
</tbody>
</table>
3.1.2 *V. longisporum* *Vlaro2-1* complements the yeast deletion mutant of chorismate synthase

We wanted to isolate the gene for chorismate synthase from the *V. longisporum* cDNA library to obtain its sequence for its further functional analysis. Chorismate synthase (CS) catalyses the formation of chorismate, the precursor of the three aromatic amino acids, tryptophan, phenyl alanine and tyrosine (Weiss *et al.*, 1980). It is known that Δaro2 (yeast deletion mutant of chorismate synthase) could not grow if the three aromatic amino acids are not added to the medium as it is impaired in the production of these amino acids (Jones *et al.*, 1991). Therefore, the gene for chorismate synthase from the *V. longisporum* cDNA library was targeted to be isolated through the yeast complementation assay in Δaro2. When the cDNA library of *V. longisporum* was transformed in Δaro2, several transformants were obtained on SC medium lacking the three aromatic amino acids. Eight randomly picked complements were isolated and sequenced, and the sequence analysis revealed that all these complements have the same sequence. When this cDNA sequence and deduced amino acid sequence was compared with the Genebank NCBI and EMBL database, it was found out to be a putative gene for chorismate synthase and therefore the gene was named *Vlaro2-1*.

On SC medium lacking the three aromatic amino acids, Δaro2 complemented with *Vlaro2-1* (Δaro2 + *Vlaro2-1*) could grow as compared to the untransformed Δaro2. Here, we used the wild type yeast strain, BY4751 as a control (Fig. 1). For its function, CS enzyme has an absolute requirement for reduced FMN. While CS from plants and eubacteria depend on external sources for reduction of FMN, CS of *S. cerevisiae* is bifunctional as along with the catalysis of 5-enolpyruvylshikimate 3-phosphate (EPSP) to chorismate, it also possess an additional NADPH:FMN oxidoreductase activity. So, these results show that as *Vlaro2-1* could complement the *S. cerevisiae* bifunctional CS mutant, it is also bifunctional.

![Fig. 1. Δaro2 yeast complementation assay on synthetic complete medium lacking the three aromatic amino acids. BY4751, wild type yeast strain; Y04515 (Δaro2), yeast chorismate synthase mutant; Y04515 (Δaro2) + Vlaro2-1, yeast chorismate synthase mutant complemented with gene for chorismate synthase in *V. longisporum*.](image-url)
The sequence analysis revealed that the coding region of Vlaro2-1 comprises an open reading frame of 1,227 bp (Fig. 2) which encodes a protein of 408 amino acids with a predicted molecular mass of 43.57 kDa. The deduced V. longisporum CS protein sequence is identical to the corresponding CS protein sequence of Verticillium dahliae, one of its parental species (Fig. 3). This protein also shows high sequence similarity with other chorismate synthase proteins like from filamentous fungi, Neurospora crassa (76% identity), and from yeast, Saccharomyces cerevisiae (63% identity). Sequence similarity was lower with chorismate synthase proteins from bacteria, Desulfuromonas acetoxidans (55% identity) and plant, Oryza sativa (52% identity). L1, L2 and L4, the regions that contribute to the substrate binding site in CS of S. cerevisiae (Quevillon-Cheruel et al., 2004) are highlighted in fig. 3. However, the NADPH binding region could not be recognised in CS until now.

3.1.3 Vlaro2-1 has an identical isogene Vlaro2-2

V. longisporum has been described as ‘near diploid’ organism because it possess 1.78 times the DNA content than that of V. dahliae, one of its parent species (Karapapa et al., 1997). Therefore, it was determined whether Vlaro2-1 has an isogene. So, V. longisporum and V. dahliae genomic DNA was digested with different restriction enzymes and subjected to Southern hybridization. A unique and fully sequenced Vlaro2-1 fragment from V. longisporum was used as the probe. In our several independent Southern hybridization analyses, two signals were generated for V. longisporum as compared to V. dahliae for the genomic DNA digested with SalI and XhoI (Fig. 4A). We also confirmed this result by using the corresponding gene fragment from V. dahliae as the probe. These results demonstrated that Vlaro2-1 has an isogene, Vlaro2-2. To determine the extent of similarity of these two isogenes in the V. longisporum genome, the genomic DNA of V. longisporum was digested with SalI and XhoI 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 4B). Vlaro2-1 specific primers were used to amplify the gel extracted DNA and subjected to sequencing. On sequencing of both loci it was found out that the coding and intronic sequences of both the isogenes are identical in V. longisporum. The coding sequences of the two isogenes were also found to be 99% identical to that of V. dahliae (Fig. 5). We also studied the extent of similarity of introns of V. dahliae and V. longisporum. The analysis of the introns showed that the isogenes have a shorter intron of 74 bp whereas V. dahliae has an intron of 81 bp. There is 77.8% identity between the two introns. The
nucleotides corresponding to position 46-51 and 59 in the *V. dahliae* intron are missing in the intron of the isogenes, *Vlaro2-1* and *Vlaro2-2* (Fig. 5).

```
1 atg tca gact tt ccg ga acct act ttc aag gto ccc ac ctac gc
M S T F G T K V T T Y G E
46 tccc acgg caa at tcgc tcgc tcatt gtc gat ggt gtt gc cccc
S H G K S V G I V D G V P P
91 ggc tcc gc ccc tc gac ggt tcct gc ac ttc gc cc ccc cgt ctac gc
g L A D E S D I Q P Q L L
136 cg cc gc cc gc gc ag tct gc ctc ctc caca ac ccgc aat gaga g
R P G Q S A I T T P R N E K
181 gac gc gc gtc act ca ccact ccgc gc gc ga gct tgg ctc aac ccg
t D R V I Q S G T F G Y T L
226 ggc aac ccgt tgg cgt gtc gtc cct gc gc ca gc gga ggc cc cc
g P L G M L V P N E D Q R P
271 aag gat ttc gc aaca aag act gat ggc ct tgg cct cc gc gc gc
t K D Y N K T M D R F F R P S
316 cac gc gc gact gc act cc act ccc ca aag ct gc ca ggc tc a cgc
H A D W T Y L T K Y G I K A S
361 tc gc ggc ggc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g S G G R S S A R E T I A R V
g A A G A V A E K Y L R E A Y G
451 ac gc agat ctc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
T E I T A P T T S I G N V H L
496 tt cc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g F P P T F E H P S P S T N P A
541 tt ctc tct gc tc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g F T V T C V I K N P P A G L C
586 tt ctc tct gc tc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g F P V R C P H T E T S E A M
631 aac gc act ac cc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
N D Y I A G F R D R D D S I G
676 gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g G T V T C V I K N P P A G L C
721 gac gc cc tgc tac cc gc gc gc gc gc gc gc gc gc gc gc gc gc
g E P C F D K L E A L L A H A M
766 ttc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g F S I P A T K G F E I G S G F
811 gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
A G C E V P G S T H N D A F I
856 cgc gc cc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g R A P E D D A R A A A E T A R
901 ctc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g L G I R S K L T T K T N F S
946 ggc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g G G I Q G C G I S N G A P I Y F
991 cg cgc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g R V A F K P A A T I G Q D Q T
1036 ac gc gc cc cc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
t A T Y D G E E G G V L A A K
1081 gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g R H D P C V V V P R A I P I V
1126 ga gg gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g E G M A A I V I M D A L M A Q
1171 ca gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g H A R Q M T R S L L P P V K K
1216 cgc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc gc
g P E A *
```

Fig. 2. Open reading frame of *Vlaro2-1* and the deduced amino acid sequence.
Results

Vl    --------------------------------------------------MSTFGTYFKV 10
Vd    --------------------------------------------------MSTFGTYFKV 10
Nc    --------------------------------------------------MSTFGHYFKV 10
Sc    --------------------------------------------------MSTGFHFLFKV 10
Da    -------------------------------------------------MSSSFGTLFKV 11
Os    MAAPTSSQVARLVP/SGGGSFAPFESAPASLAFSVGRRAPAMLVEFASANVFQGNYFQV 60

L1

Vl    TTYGESHIGVAGVFDVGPGVLGDASLDIQMRPQSSAHTPNEQRTIQTSGTE 70
Vd    TTYGESHIGVGFDVGPGVLGDASLDIQMRPQSSAHTPNEQRTIQTSGTE 70
Nc    TTYGESHIGVAGVFDVGPGVLGDASLDIQMRPQSSAHTPNEQRTIQTSGTE 70
Sc    TTYGESHIGVAGVFDVGPGVLGDASLDIQMRPQSSAHTPNEQRTIQTSGTE 70
Da    TTYGESHIFPVGVFDVGPGVLGDASLDIQMRPQSSAHTPNEQRTIQTSGTE 70
Os    ATYGESHIGVAGVFDVGPGVLGDASLDIQMRPQSSAHTPNEQRTIQTSGTE 70

L2

Vl    FGYTLGTLGPTQVFGVGSQGFLPKDQHDFNPRSPSKVNLTFIYKSRV 130
Vd    FGYTLGTLGPTQVFGVGSQGFLPKDQHDFNPRSPSKVNLTFIYKSRV 130
Nc    FGYTLGTLGPTQVFGVGSQGFLPKDQHDFNPRSPSKVNLTFIYKSRV 130
Sc    FGYTLGTLGPTQVFGVGSQGFLPKDQHDFNPRSPSKVNLTFIYKSRV 130
Da    FGYTLGTLGPTQVFGVGSQGFLPKDQHDFNPRSPSKVNLTFIYKSRV 130
Os    FGYTLGTLGPTQVFGVGSQGFLPKDQHDFNPRSPSKVNLTFIYKSRV 130

Vl    LAHAMLSIPATKGFEIGSGFAGCEVPGSTHNDAFIRAPEDDAR---AAAETARLGIPRSK 307
Vd    LAHAMLSIPATKGFEIGSGFAGCEVPGSTHNDAFIRAPEDDAR---AAAETARLGIPRSK 307
Nc    LAHAMLSIPATKGFEIGSGFAGCEVPGSTHNDAFIRAPEDDAR---AAAETARLGIPRSK 307
Sc    LAHAMLSIPATKGFEIGSGFAGCEVPGSTHNDAFIRAPEDDAR---AAAETARLGIPRSK 307
Da    LAHAMLSIPATKGFEIGSGFAGCEVPGSTHNDAFIRAPEDDAR---AAAETARLGIPRSK 307
Os    LAHAMLSIPATKGFEIGSGFAGCEVPGSTHNDAFIRAPEDDAR---AAAETARLGIPRSK 307
**Results**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Vd</td>
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<td>Nc</td>
<td>AQ 432</td>
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<td>--</td>
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<td>Da</td>
<td>--</td>
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<td>Os</td>
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</tbody>
</table>

**Fig. 3.** Alignment of the deduced amino acid sequence of *V. longisporum* (Vl) chorismate synthase (CS) with the sequences of CS of *V. dahliae* (Vd), ascomycetous fungus, *N. crassa* (Nc); yeast, *S. cerevisiae* (Sc); bacteria, *D. acetooxidans* (Da) and plant, *O. sativa* (Os). L1 (grey), L2 (yellow) and L4 (green) are the regions that contribute to substrate binding site in CS of *S. cerevisiae* (Quevillon-Cheruel et al., 2004)."**" identical amino acids, ":" conserved substitutions,"." semi-conserved substitutions.

**Fig. 4.** Determination of the isogene *Vlaro2-2* of *Vlaro2-1* and their corresponding sequence. **A.** Southern hybridization analysis of *V. dahliae* and *V. longisporum*. The genomic DNA was digested with *Sal*I and *Xho*I. A 500 bp sequence of *Vlaro2-1* was used as a probe. Arrows indicate the signal generation by probe binding. **B.** Determination of the sequence of the two isogenes *Vlaro2-1* and *Vlaro2-2* in *V. longisporum*. *V. longisporum* genomic DNA was digested with *Sal*I and *Xho*I and DNA was gel extracted from the spots (shown by open boxes) where chorismate synthase probe bind in the Southern hybridization analysis (A). *Vlaro2-1* and *Vlaro2-2* were amplified from the extracted DNA fragments and sequenced. M is the lane with a DNA size marker with fragment sizes indicated.

---

Vlaro2-1
ATGTCGACCTGTGGAAACCTACTTCAGTCCACTACATGAGTCCATTGACAGACTGACAT

Vlaro2-2
ATGTCGACCTGTGGAAACCTACTTCAGTCCACTACATGAGTCCATTGACAGACTGACAT

Vd
ATGTCGACCTGTGGAAACCTACTTCAGTCCACTACATGAGTCCATTGACAGACTGACAT

---

Vlaro2-1
TTTACATGGCCGCCACCCACCCCCCCACACAGCTAC

Vlaro2-2
TTTACATGGCCGCCACCCACCCCCCCACACAGCTAC

Vd
TTTACATGGCCGCCACCCACCCCCCCACACAGCTAC

---

33
Fig. 5. Alignment of the coding and intron sequence of Vlaro2-1, Vlaro2-2 and the gene for chorismate synthase in V. dahliae (Vd). (Intron sequence is in **bold**)

3.1.4 Establishment of RNA-mediated gene silencing in V. longisporum by knock-down of the expression of Vlaro2

To investigate the role of CS in the survival of *V. longisporum* when it inhabits the xylem vessel of the host plant, we decided to knock-down the isogenes, Vlaro2-1 and Vlaro2-2 by RNA silencing because the conventional knock-out was not feasible 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). Therefore, a hairpin double-stranded RNA mediated gene silencing approach was employed for RNA silencing of Vlaro2-1 and Vlaro2-2 in *V. longisporum*.

The silencing vector was constructed using the pSilent-1 vector. As the sequence of the two isogenes, Vlaro2-1 and Vlaro2-2 is identical it is referred as Vlaro2 in the following text. The binary vector pME3571 (Fig. 6) harbouring the hairpin construct for silencing of Vlaro2 comprising of inverted repeats of Vlaro2 fragment separated by a spacer and hygromycin gene as the selection marker was transformed in *V. longisporum* by ATMT. After transformation, 129 transformants were obtained per 10^6 spores of *V. longisporum* on hygromycin B containing medium. To confirm the mitotic stability of the transformants, they were grown on medium lacking hygromycin B for five generations and then grown on medium supplemented with hygromycin B. The transformants could grow on the latter
medium supporting that they were mitotically stable. Thirty independent hygromycin B resistant transformants were selected at random for further analysis.

The integration of the T-DNA in the genome of the *V. longisporum* was confirmed by Southern hybridization analysis (Fig. 7). Genomic DNA of five *V. longisporum* transformants and wild type was digested with *Hind*III, blotted and probed with hygromycin gene. Analysis of the Southern hybridization showed that all the transformants analysed contained single insertion of T-DNA.

![Diagram of plasmid used for silencing Vlaro2 in V. longisporum](image)

**Fig. 6.** Schematic representation of the plasmid used for silencing *Vlaro2* in *V. longisporum*. Inverted repeats of *Vlaro2* (CS) were introduced in the pSilent-1 plasmid at the multiple cloning sites (shown in the figure) at 5’ and
3’ ends of intron 2 of the cutinase gene from *M. oryzae* (IT). This whole silencing cassette obtained by digesting pSilent-1 plasmid with *Xba*I was introduced in the binary vector, pPK2 at the *Xba*I restriction site. The resulting binary vector pME3571 was used for transformation of *V. longisporum* via ATMT. PptrC, *A. nidulans* trpC promoter; TtrpC: *A. nidulans* trpC terminator; Pgpd, *A. nidulans* gpd promoter; hph, hygromycin resistance gene; LB, left border and RB, right border.

**Fig. 7.** Southern hybridization analysis of Vlaro2 silenced mutants (Vlaro2sms) to detect integration of T-DNA after ATMT. Five Vlaro2sms (1-5) and wild type (wt) gDNA was digested with *Hind*III and the hygromycin resistance gene was used as a probe.

To test the extent of Vlaro2 silencing, RT-PCR was performed as the silencing of gene expression is the result of posttranscriptional degradation of targeted mRNA. Total RNA was extracted from the Vlaro2 silenced mutants (Vlaro2sms) and wild type, and after reverse transcription, cDNA was used for RT-PCR analysis. RT-PCR results showed the significant knockdown of Vlaro2 transcript (Fig. 8), although, the degree of silencing of chorismate synthase varies in the different transformants, from high to moderate levels, as observed by amplification of Vlaro2, in the transformants compared to wild type, which has the highest level of product after amplification. For RNA integrity, actin was used as control. High frequency silencing was observed as 71% of the transformants had reduced gene expression as observed by RT-PCR.
Results

Fig. 8. RT-PCR analysis of Vlaro2 mRNA expression in the Vlaro2 silenced mutants. The actin gene was used for control. 1-5, Vlaro2 silenced mutants; wt, wild type; NTC, no template control; CS, chorismate synthase.

We further examined the knockdown of Vlaro2 at protein level in the same Vlaro2sms by Western hybridization (Fig. 9). Proteins were extracted from the Vlaro2sms and wild type, ran on SDS-polyacrylamide gel, blotted and probed with N. crassa CS specific antibody. To check the integrity of protein, the same blot was stripped and probed again with tubulin antibody. The CS expression was quantified and normalized against the tubulin levels for the different samples as shown in the graph in fig. 9. The data represents average +/- standard deviations of three experimental replicates. Thus, the Western hybridization analysis showed the significant knockdown of VIARO2 up to 94% in the Vlaro2sms as compared to the wild type. The knockdown of VIARO2 at the protein level corresponds to the RT-PCR analysis of the Vlaro2sms. So, the Vlaro2 silencing via RNAi worked successfully in V. longisporum and established gene silencing in V. longisporum.
3.1.5 Silencing of \textit{Vlaro2} results in slow growth of \textit{V. longisporum} in presence of 5-MT

We investigated the question whether \textit{Vlaro2} silencing inhibits the growth of \textit{V. longisporum} in the absence of the aromatic amino acids by using plate-based growth assays. \textit{Vlaro2} silenced mutants (\textit{Vlaro2sms}) and wild type were cultured on CDA and CDA amended with 5-methyl-DL-tryptophan (5-MT) or the three aromatic amino acids, tryptophan, phenyl alanine and tyrosine. The colonies were examined microscopically for spore formation and vegetative growth, and the diameter of the colonies was measured daily.

One day after inoculation on CDA, notably, \textit{Vlaro2sms} grew slowly and only short hyphae were visible in contrast to wild type which showed good mycelial growth (Fig. 10). After two days of inoculation on CDA, wild type mycelial growth increased and many conidia could be observed whereas the \textit{Vlaro2sms} showed less mycelial growth and no conidia were observed. We further analyzed the effect of \textit{Vlaro2} silencing on the radial growth of \textit{V. longisporum}. The radial growth was recorded at 3, 6, 9 and 12 days post inoculation. Three replicates were taken for each measurement. After analysing the results, the radial growth of \textit{Vlaro2sms} was observed to be slightly less than wild type (Fig. 10 graph). Therefore, the \textit{Vlaro2sms}, in particular, showed slower initial vegetative growth but the radial growth was not affected significantly compared to the wild type.

5-MT is an inhibitor of tryptophan biosynthesis as it acts by false feedback inhibition of anthranilate synthase, the first enzyme of the tryptophan biosynthetic pathway (Braus, 1991). To determine the effect of 5-MT on \textit{Vlaro2sms} and wild type, it was added in CDA and three independent experiments were setup to culture the fungus. The growth results showed that, one day post inoculation, the wild type formed short hyphae compared to \textit{Vlaro2sms} in which only initial germination of conidia could be observed (Fig 11). At two days post inoculation, wild type showed a mycelial net with few conidia, but, in contrast, the \textit{Vlaro2sms} formed short hyphae and no conidia were observed. Interestingly, the radial growth of \textit{Vlaro2sms} was noticed to be significantly lower than wild type as observed by measuring the colony diameter 3, 6, 9 and 12 days after inoculation (Fig 11 graph). Three replicates were taken for each
measurement. It started decreasing gradually at 3 and 6 days post inoculation and decreased up to 40% at 9 and 12 days post inoculation. Hence, our study noted that the Vlaro2sms showed severe reduction in initial vegetative growth as well as the growth rate on CDA supplemented with 5-MT. Next, we asked whether addition of 5-methyl-DL-tryptophan and tryptophan simultaneously to CDA medium, affect the growth of Vlaro2sms. For this experiment, Vlaro2sms were inoculated on CDA medium supplemented with 5 mM 5-MT and 196 μM tryptophan. The addition of tryptophan simultaneously to the addition of 5-MT in the medium prevented the severe reduction in growth of the Vlaro2sms as observed in the presence of 5-MT (Fig. 11) alone.

Further, we also studied the effect of the three aromatic amino acids on the Vlaro2sms. To execute this experiment, the three aromatic amino acids were added to CDA, specifically in concentration similar to that present in the xylem sap of B. napus (Table 1), that is, 10 μM phenyl alanine, 10 μM tyrosine and 2 μM tryptophan and Vlaro2sms were inoculated on it. After growth analysis, it was found that the Vlaro2sms again showed slower growth than the wild type. Additionally, we also studied the effect of the three aromatic amino acids in higher concentration that is, 100 μM phenyl alanine, 100 μM tyrosine and 20 μM tryptophan but the Vlaro2sms were again observed to grow slowly. But when 300 μM phenyl alanine, 165 μM tyrosine and 196 μM tryptophan were supplemented in the medium the growth of the Vlaro2sms resembled the wild type strain growth. Therefore, the concentration of the aromatic amino acids in the xylem sap was not found to be high enough to recover the growth of the Vlaro2sms like the wild type strain.
Fig. 10. Light microscopy images of *V. longisporum* wild type (wt) and *Vlaro2* silenced mutant (*Vlaro2sm*) cultured on CDA as observed at one and two days post inoculation. The graph shows the growth rates of wild type (wt) and *Vlaro2* silenced mutant (*Vlaro2sm*) on CDA. Agar plates were inoculated with 5000 spores and colony diameter from three replicates was measured following three, six, nine and twelve days incubation at 25°C. Data represent average±/ standard deviations of three experimental replicates.
Fig. 11. Light microscopy images of wild type (wt) and Vlaro2 silenced mutant (Vlaro2sm) cultured on CDA with 5 mM 5-methyl-DL-tryptophan (5-MT) as observed at one and two days post inoculation. The graph shows the growth rates of wild type (wt) and Vlaro2 silenced mutant (Vlaro2sm) on CDA amended with 5-MT. Agar plates were inoculated with 5,000 spores and colony diameter from three replicates was measured following three, six, nine and twelve days incubation at 25°C. Data represent average+/- standard deviations of three experimental replicates.
3.1.6 *Vlaro2* silenced mutants are less virulent on *B. napus* than wild type

In order to find out if the knock-down of *Vlaro2* in *V. longisporum* had any effect on its propagation and infection, the pathogenicity of *Vlaro2* silenced mutants (*Vlaro2sms*) was tested on *B. napus*. 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 (Fig. 12).

![Fig. 12. Rapeseed infection by root-dipping inoculation. A week old rapeseed seedlings were inoculated by different strains, *Vlaro2* silenced mutant (*Vlaro2sm*), wild type (wt) and water and observed weekly until five weeks after inoculation.](image)

**Table 2.** Key for scoring of disease symptoms due to *V. longisporum* infection on *B. napus* plants (following Zeise 1992, modified).

<table>
<thead>
<tr>
<th>Score</th>
<th>Symptom description</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>no symptoms</td>
</tr>
<tr>
<td>2</td>
<td>slight symptoms on oldest leaves (yellowing)</td>
</tr>
<tr>
<td>3</td>
<td>slight symptoms on next younger leaves</td>
</tr>
<tr>
<td>4</td>
<td>About 50% of leaves show symptoms</td>
</tr>
<tr>
<td>5</td>
<td>&gt;50% of leaves show symptoms</td>
</tr>
<tr>
<td>6</td>
<td>up to 50% of leaves dead</td>
</tr>
<tr>
<td>7</td>
<td>&gt;50% of leaves dead</td>
</tr>
<tr>
<td>8</td>
<td>only apical meristem still alive</td>
</tr>
<tr>
<td>9</td>
<td>plant dead</td>
</tr>
</tbody>
</table>
Verticillium spp. causes the disease symptom of stunting in the host-plants (Zeise et al., 2002b) that is also true for V. longisporum infected rapeseed plants. Therefore, to assess the severity of disease, the height of the plants was measured at 7, 14, 21, 28 and 35 days post inoculation until the plants are highly infected. The plants begin to show disease symptoms at 21 dpi and are heavily infected at 28 and 35 dpi (Fig. 13). The plants infected with Vlaro2sms were smaller than the mock-inoculated plants but were not as stunted as the plants infected with V. longisporum wild type strain (Fig. 14). At 35 days post inoculation, while the wild type infected rapeseed plants were about 60% smaller than the mock-infected plants, the Vlaro2sm infected plants were not as stunted as the wild type infected plants. They were about 35% taller than the wild type infected plants. The infected rapeseed plants were also scored for disease symptoms by assigning disease score from 1-9 corresponding to asymptomatic to dead plant (Zeise, 1992) (Table 2). The plants were observed weekly for disease symptoms until 35 days post inoculation when the plants were highly infected. In the assessment of the symptoms, the yellowing and death of the leaves is considered. Therefore, the mock-infected plants also had disease score more than 1 because of yellowing of leaves.
due to natural senescence. The infected plants do not show any disease symptoms until 14 dpi. The disease score of the Vlaro2sms' infected plants was higher than the mock-inoculated plants but not as high as the plants infected with wild type (Fig. 15). At 35 days post inoculation, while the wild type infected rapeseed plants showed a high disease score of more than 6, the Vlaro2sm infected plants showed a comparatively low disease score of about 4.5 that is only slightly higher than the disease score of mock-infected plants that is 4. Therefore, this investigation showed that the silencing of Vlaro2 in V. longisporum significantly reduced its virulence on rapeseed.

Fig. 14. Assessment of stunting of rapeseed due to V. longisporum infection. The height of 24 replicates each of rapeseed plants infected with wild type (wt) and Vlaro2 silenced mutant (Vlaro2 sm) was measured at 7, 14, 21, 28 and 35 days post inoculation (dpi). For comparison, the height of rapeseed plants mock-inoculated (mock) with tap water was also measured. Data represent average+/− standard deviations of twenty four experimental replicates.
3.1.7 Reduced amount of *V. longisporum* DNA in plant tissue infected with *Vlaro2* silenced mutant

It was observed in the plate-based growth assay that although the *Vlaro2*sm showed reduced initial vegetative growth but could survive without aromatic amino acids (Fig. 10). So, they should survive in the plant also where the concentration of the aromatic amino acids was low. Interestingly, in the *in planta* pathogenicity assay the virulence of the *Vlaro2*sm was noticed to be markedly reduced (Fig. 14 and 15). So, the question was whether the reduced virulence of the *Vlaro2*sm was due to reduced growth of *Vlaro2*sm inside the plant compared to the wild type due to reduced formation of aromatic amino acid based secondary metabolite or other yet unknown factor. Therefore, the total biomass of *V. longisporum* in *B. napus* infected with *Vlaro2* silenced mutant (*Vlaro2*sm) versus wild type was analysed by quantification of the *V. longisporum* DNA in the plant tissue by real-time PCR. Hence, the hypocotyls and top stems were harvested from the rapeseed plants infected with wild type, *Vlaro2*sm or water at 21, 28 and 35 days post inoculation. The hypocotyls were also harvested at 14 days post inoculation. Total DNA was extracted from the plant tissue and the fungal DNA was quantified with real time PCR. The experiment had three biological replicates and three
technical replicates for each biological sample. In the hypocotyls, *V. longisporum* DNA concentration was found to be significantly lower (66-95%) in *Vlaro2sm* infected plant compared to the wild type infected plant throughout the infection study from early stage of infection at 14 dpi to late stage of infection at 35 dpi (Fig. 15’B). In the plant stem, at 21 dpi there is negligible amount of *V. longisporum* DNA in the tissue samples of both wild type and *Vlaro2sm*. At 28 and 35 dpi, the amount of *V. longisporum* DNA was observed to be significantly lower (57-89%) in *Vlaro2sm* infected plants in comparison to wild type infected plant (Fig. 15’A). At 35 dpi, the *V. longisporum* DNA amount increased dramatically in the stem of wild type infected plant probably due to the outbreak of infection in the whole plant at this late stage of infection (Eynck *et al.*, 2007).

Therefore, the *V. longisporum* DNA amount was observed to be significantly low in *Vlaro2sm* infected plant both in the hypocotyl and stem compared to the wild type infected plant at all the stages of infection. It indicates that the growth and propagation of *Vlaro2sm* is significantly less compared to the wild type in the rapeseed.
**Results**

**Fig. 15'.**

A. *V. longisporum* DNA concentration in the plant stem. *V. longisporum* DNA was measured with real-time PCR in stem of *B. napus* inoculated with *Vlaro2* silenced mutant (*Vlaro2sm*) and wild type (wt) at 21, 28 and 35 dpi. 

B. *V. longisporum* DNA concentration in the hypocotyl. *V. longisporum* DNA was measured with real-time PCR in hypocotyl of *B. napus* inoculated with *Vlaro2sm* (CS sm) and wild type (wt) at 14, 21, 28 and 35 dpi. Data represent average ± standard deviations of three experimental replicates. ng VI DNA/g FW: nanogram *V. longisporum* DNA/gram fresh weight of plant tissue. (In the inset a representative rapeseed plant depicting the stem (5-6 cm from the top) and the hypocotyl harvested for the quantification of the *V. longisporum* DNA. Source: [http://usa.loccitane.com/FO/Services/GlossaryDetail.aspx?id=80](http://usa.loccitane.com/FO/Services/GlossaryDetail.aspx?id=80))
3.2 Proteomic analysis of V. longisporum after treatment with xylem sap of B. napus

3.2.1. Xylem sap of B. napus affects the growth of V. longisporum

We wanted to observe whether the addition of xylem sap to the solid agar medium has any effect on growth and/or life cycle progression of V. longisporum cultured on it. The nutrients in the xylem sap could support the growth of the fungus or some plant defense factors present in it could inhibit the fungal growth. For this purpose, sterile xylem sap in a volume of one-seventh of the media volume was added to the growth media, SXM and CDA and inoculated with V. longisporum. The xylem sap was also diluted ten times and added to the two media in a similar manner. SXM (Simulated xylem fluid medium) was used because it reflects the nutritional conditions of the vascular fluid (Neumann and Dobinson, 2003). CDA (Czapek Dox Agar) is one of the most used solid defined minimal medium for the general cultivation of fungi capable of utilizing inorganic nitrogen. Spore formation, melanization and growth of V. longisporum were observed by light microscopy in comparison to the controls without any added xylem sap in a time course of ten days. After two days of growth of V. longisporum on SXM, without addition of xylem sap, small hyphae appeared with few conidia and after three days of growth more mycelial growth with many conidia and small bud spores occurred. The colony became dark hence melanization occurred between the third and the fourth day of growth. On CDA, after two days of V. longisporum growth, hyphae appear with some conidia and growth is faster as compared to growth on SXM. On the third day of growth, there was more mycelial growth and more conidia production but bud spores did not appear before the fourth day of growth in contrast to the SXM. On CDA, the colony became dark and melanization occurred not before the seventh day of growth in contrast to the SXM.

When xylem sap was added to the SXM, the mycelial and conidial growth increased after two days of growth as compared to the control where only small hyphae with very few conidia could be observed (Fig. 16A top left). CDA with added xylem sap, also showed much more mycelial and conidial growth as compared to the control on the second day of growth (Fig. 16A top right). On the third day of growth, V. longisporum is in the later stage of bud spore formation and bigger bud spores could be observed on SXM with added xylem sap compared to the control where bud spores are in the early stage of formation and smaller in size (Fig. 16A middle left). Some bud spores could be observed on CDA with added xylem sap as compared to the control with much mycelial and conidial growth but no bud spores on the third day of growth (Fig. 16A middle right). The number of conidia was counted after eight days of inoculation and it was found out that in SXM with added xylem sap the number
of conidia was increased more than twice and in CDA with added xylem sap, more than four times as compared to the control (Fig. 16B). Another effect of adding xylem sap in CDA was that melanization was hastened that is on the third day of growth only, the fungal colony became dark in colour as compared to the control (Fig. 16A bottom right) but in contrast on SXM melanization occurs in both control and xylem sap added SXM (Fig. 16A bottom left).

The growth behaviour of *V. longisporum* on nutrient media supplemented with or without xylem sap is summarized in table 3. Similar effects were observed when xylem sap extracted from *B. napus* uninfected/infected with *V. longisporum* was used or when xylem sap was diluted ten times and added to the medium.

Therefore, the xylem supported the growth of *V. longisporum* as when xylem sap was added to the medium, the spores and bud spores were produced earlier and the number of spores increased significantly in *V. longisporum*. On CDA with added xylem sap the melanization occurred earlier in the fungus compared to the control.

**Table 3.** Growth characteristics of *V. longisporum* growing on SXM and CDA with/without xylem sap of *B. napus*

<table>
<thead>
<tr>
<th>Nutrient media</th>
<th>Days post inoculation</th>
<th>-Xylem sap</th>
<th>+Xylem sap</th>
</tr>
</thead>
<tbody>
<tr>
<td>SXM</td>
<td>2</td>
<td>Small hyphae, few conidia</td>
<td>longer hyphae, more conidia</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Small bud spores; dark coloured colony</td>
<td>Bigger bud spores; dark coloured colony</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>No. of conidia = 4.31 x 10^6 conidia/ml</td>
<td>No. of conidia = 10.07 x 10^6 conidia/ml</td>
</tr>
<tr>
<td>CDA</td>
<td>2</td>
<td>Long hyphae, some conidia</td>
<td>Good mycelial growth, more conidia</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>No bud spores, good mycelial growth, many conidia; light coloured colony</td>
<td>Bud spores, good mycelial growth, many conidia; dark coloured colony</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>No. of conidia = 1.59 x 10^6 conidia/ml</td>
<td>No. of conidia = 7.13 x 10^6 conidia/ml</td>
</tr>
</tbody>
</table>
Fig. 16 A. Light microscopy images of *V. longisporum* colonies growing on SXM and CDA with/without xylem sap from *B. napus* as observed after two and three days of inoculation. Arrows indicate bud spores. B. The number of *V. longisporum* conidia/ml from each growth condition was analysed eight days after inoculation. Data represent average± standard deviations of three experimental replicates.
3.2.2 *V. longisporum* showed upregulated as well as downregulated proteins when grown in the presence of xylem sap of *B. napus*

We observed that the xylem sap of *B. napus* affects the growth of *V. longisporum* which could be possible due to differential protein expression in *V. longisporum*. Therefore, we set out to analyse changes in the soluble proteome, which may occur after treating *V. longisporum* with the xylem sap of *B. napus* (42 days old). To identify the proteins that might be differentially expressed in *V. longisporum* by growth in the presence of xylem sap, the soluble proteins were extracted from five days’ old liquid fungal cultures incubated with/without xylem sap for eight hours. Equivalent amounts of extracted proteins were run on 2-D gels and identical criteria were employed for each gel. Comparison of six sets of RuBP (Ruthenium II tris-bathophenantroline disulfonate; Lamanda et al., 2004) stained gels clearly demonstrated that 2-DE analysis of protein samples exhibited consistent gel images. More than 800 protein spots with MW between 10 and 120 kDa and a pI ranging from pH 4.0 to pH 7.0 were resolved for both conditions. The proteins separated in this pH range were spread evenly across the pH gradient which allowed detecting up-regulated and down-regulated protein spots. To quantify differences in protein abundance of matched protein spots among the different gels, normalized spot volumes were compared.

![Fig. 17. The 2-DE gels of the *V. longisporum* proteome untreated/treated with xylem sap from rapeseed. These are representative figures from three biological and two technical replicates of each condition. Differentially expressed proteins are marked with arrows and labelled, U1-U6 (Upregulated protein spots); D1-D8 (Downregulated protein spots).](image-url)
Results

U1  Catalase-peroxidase

U2  Heat-shock protein, hsp70

U3  Heat-shock protein, hsp60

U4  Coproporphyrinogen III oxidase

U5  Unidentified
Results

Fig. 18. Close-up views of the regions of the 2-DE gels that show significant upregulation in protein expression (untreated (-XS) versus treated (+XS) with xylem sap from rapeseed). The arrowheads indicate the differentially expressed proteins. The expression histograms show the differential protein expression of *V. longisporum* due to treatment with xylem sap from rapeseed (right). Bars represent mean protein spot quantity of the *V. longisporum* proteome untreated (-XS) versus treated (+XS) with xylem sap. Data represent average +/- standard deviations of six experimental replicates. Fold increase or decrease in the mean protein spot quantity is indicated over the right bar (+XS) in comparison to left bar (-XS).

The proteins from xylem sap treated cultures were compared with untreated controls by quantitative as well as qualitative gel analysis performed with the PD-Quest software to locate the differentially expressed protein spots. In quantitative analysis, protein spots showing at least 40% up-regulation or down-regulation were selected for further analysis. Six proteins (U1-U6) showed upregulation and eight proteins (D1-D8) showed downregulation compared to controls. These are indicated by arrows in fig. 17. Close-up views of the gels confirmed that the enhanced spots were differentially expressed compared to the control levels (Fig. 18 and 19). The proteomic analysis was also performed using xylem sap from 35 days old *B. napus* plants. Protein spots, U1, U4, D2 and D3 showed no significant regulation compared to controls in this analysis. To test whether the xylem sap extracted from *B. napus* that was already infected with *V. longisporum* may influence *V. longisporum* proteome more, the 2D-PAGE analysis was also performed using the xylem sap extracted from 42 days old infected *B. napus* plants (35 dpi) or 35 days old infected *B. napus* plants (28 dpi). But there was no significant difference found in protein expression between fungal cultures treated with xylem sap from *B. napus* uninfected versus infected with *V. longisporum*. 
Results

D1 Vacuolar membrane ATPase catalytic subunit

D2 Unidentified

D3 Ketol-acid reductoisomerase

D4 Aflatoxin aldehyde reductase

D5 NADPH oxidoreductase
Fig. 19. Close-up views of the regions of the 2-DE gels that show significant downregulation in protein expression (untreated (-XS) versus treated (+XS) with xylem sap from rapeseed). The arrowheads indicate the differentially expressed proteins. The expression histograms show the differential protein expression of *V. longisporum* due to treatment with xylem sap from rapeseed (right). Bars represent mean protein spot quantity of the *V. longisporum* proteome untreated (-XS) versus treated (+XS) with xylem sap. Data represent average+/− standard deviations of six experimental replicates. Fold increase or decrease in the mean protein spot quantity is indicated over the right bar (+XS) in comparison to left bar (-XS).

To identify the proteins, tryptic digestion of excised selected differentially expressed protein spots was followed by MS/MS run and then identification was achieved through homology searching with tentative sequences obtained by MS. The resulting identified proteins are shown in Table 4. Of the fourteen selected protein spots, ten protein spots could be successfully identified. Half of the identified proteins were found to be homologous to *V. dahliae* proteins and others to other fungal organisms like *A. nidulans* and *N. crassa*. The identification of most of the protein spots was also confirmed through the analysis of the MS/MS data sets with the PEAKS MS program.
Table 4. Homologies of differentially expressed proteins of *V. longisporum* after treatment with xylem sap from *B. napus*. MW*/pI*: molecular weight (kDa) and isoelectric point of differentially expressed proteins determined experimentally. U1-U4: Upregulated protein spots; D1-D8: Downregulated protein spots. X-corr value: cross-correlation scores of matched peptides (TURBOSequest).

<table>
<thead>
<tr>
<th>Protein (Spot no.)</th>
<th>Organism</th>
<th>NCBI accession no.</th>
<th>MW*/pI*</th>
<th>Matched Peptides</th>
<th>X-corr value</th>
<th>Sequence coverage (%AA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase-peroxidase (U1)</td>
<td><em>Aspergillus nidulans</em></td>
<td>CAC59821</td>
<td>90/6.1</td>
<td>FLENPDQFADAFAR, SPAGAHQYVAK, STNGDTHLGEDFDIHL, VRMLGNQFLVGIPPAHR, MKETAESFLS,KPVK</td>
<td>4.75</td>
<td>3.38</td>
</tr>
<tr>
<td>Heat shock protein 70 (U2)</td>
<td><em>Neurospora crassa</em></td>
<td>XP_961753</td>
<td>78/5.4</td>
<td>TNEVAGDGTTSATVLR, VFKPAFLILLSEK, FVDALNATR, TIENAGLEGSVVVGK, AAVEEGILPGTALK</td>
<td>5.73</td>
<td>9.30</td>
</tr>
<tr>
<td>Heat shock protein 60 (U3)</td>
<td><em>Neurospora crassa</em></td>
<td>XP_956500</td>
<td>60/5.5</td>
<td>TNEVAGDGTTSATVLR, VFKPAFLILLSEK, FVDALNATR, TIENAGLEGSVVVGK, AAVEEGILPGTALK</td>
<td>6.17</td>
<td>20.73</td>
</tr>
<tr>
<td>Copropor-phyrinogen III oxidase (U4)</td>
<td><em>Verticillium dahliae</em></td>
<td>BQ111120</td>
<td>43/6.2</td>
<td>LGEMPADQGFAPYLSAK, TTIANTSNMPVAAR, DQGLDVAMMADSSR</td>
<td>4.16</td>
<td>2.76</td>
</tr>
<tr>
<td>Vacuolar membrane ATPase catalytic subunit A (D1)</td>
<td><em>Verticillium dahliae</em></td>
<td>BQ110481</td>
<td>66/5.2</td>
<td>LGEMPADQGFAPYLSAK, TTIANTSNMPVAAR, DQGLDVAMMADSSR</td>
<td>4.16</td>
<td>2.76</td>
</tr>
<tr>
<td>Ketol-acid reductoisomerase precursor (D3)</td>
<td><em>Neurospora crassa</em></td>
<td>XP_961335</td>
<td>46/6.2</td>
<td>NDTLALIGYSQGHGQG, LNLRLVEPTDVVDVILVAPK, NLFDVDVAEISRL</td>
<td>3.89</td>
<td>13.18</td>
</tr>
<tr>
<td>Aflatoxin aldehyde reductase (D4)</td>
<td><em>Verticillium dahliae</em></td>
<td>BQ110032</td>
<td>45/6.2</td>
<td>NDTLALIGYSQGHGQG, LNLRLVEPTDVVDVILVAPK, NLFDVDVAEISRL</td>
<td>4.02</td>
<td>9.37</td>
</tr>
<tr>
<td>NADPH oxidoreductase (D5)</td>
<td><em>Verticillium dahliae</em></td>
<td>BQ110643</td>
<td>25/6.1</td>
<td>SALAGIDAVVSTLGAPEVGEPQR, NLVEAAAVEAVGVRK, VEVVVYDNPDASLK, EYNTTTEELLVEK</td>
<td>5.39</td>
<td>27.70</td>
</tr>
<tr>
<td>Hypothetical protein AN9083.2 (conserved domain: Adenine phosphoribosyl transferase) (D6)</td>
<td><em>Aspergillus nidulans</em></td>
<td>XP_682352</td>
<td>34/5.2</td>
<td>NDTLALIGYSQGHGQG, LNLRLVEPTDVVDVILVAPK, NLFDVDVAEISRL</td>
<td>4.98</td>
<td>8.85</td>
</tr>
<tr>
<td>Translation initiation factor eIF-5A (D8)</td>
<td><em>Verticillium dahliae</em></td>
<td>BQ110791</td>
<td>25/4.9</td>
<td>KLEDLSPTHNMVDVPNVT, VHIVATDIFTGK, LEDLSPTHNMVDVPNVT, EYNTTTEELLVEK</td>
<td>4.31</td>
<td>12.20</td>
</tr>
</tbody>
</table>
Two upregulated (U5 and U6) and two downregulated (D2 and D7) proteins that were not identified by analysis with TURBOSequest, could also not be identified by analysis with PEAKS that used the NCBI nr database, so, they may be V. longisporum proteins that are not so well conserved. It could also be that the protein amount in the tryptic digest of the protein spots was not enough for their identification by MS.

Therefore, fourteen proteins were observed to be differentially expressed in V. longisporum when it was treated with xylem sap of rapeseed. Six of them were upregulated and eight downregulated and ten protein spots could be identified confidently.

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

Protein spot U1 was upregulated in the proteomic analysis when V. longisporum was treated with xylem sap from its host-plant, B. napus. It was found to increase more than two-fold in the xylem sap treated fungal proteome (Fig. 18). It was confidently identified as the enzyme, catalase-peroxidase (NCBI accession no. CAC59821; EC_number =1.11.1.6) encoded by cpeA in A. nidulans by the TURBOSequest analysis as two tryptic peptides with X-corr value of 4.75 and 3.03 highly matched with the theoretical spectra (Table 4). The catalase-peroxidase was further characterized as it was upregulated probably as a response to oxidative stress produced by the xylem sap and it could have a role in protecting the fungus against oxidative stress produced by the host plant when it colonizes the xylem vessels. This identification was further confirmed by the analysis of the data set with PEAKS. Two matching tryptic peptides, FLENPDQFADAFAR and ADLVFGSHAEAR with score of 99% each were found by this analysis. The first peptide is identical to the result of TURBOSequest and the second is an additional peptide. The sequence coverage was 35.32 % AA. The cpeA gene in A. nidulans encodes an 81-kDa bifunctional enzyme, catalase-peroxidase that has a conserved motif for heme coordination (Scherer et al., 2002). In A. nidulans, three monofunctional catalases have been described and cpeA gene product was first observed as a fourth catalase activity in native polyacrylamide gel, and named as catalase D or Cat D (Kawasaki and Aguirre, 2001). Cat D activity was induced by glucose starvation, high temperature and H₂O₂ treatment.

The cDNA library of V. longisporum was used to obtain the sequence of catalase-peroxidase (spot U1). The MS2 spectrum of catalase-peroxidase (spot U1) was used to search for matches against the database of V. longisporum cDNA library sequences. Two cDNA clones
from the library matched peptides for confident identification of the protein spot (Fig. 20). These two clones were sequenced fully and when the resulting cDNA sequence and deduced amino acid sequence was compared with the Genebank NCBI and EMBL database, it was found out to be the putative gene for catalase-peroxidase and therefore the gene was named *VlcepA*-*I*. 

\[
\begin{align*}
\text{atg} & \text{gccgagtcgagagacagtgcctccaccaacctcaacac} \\
\text{aagcgaggtccgagccacccc} & \text{agctgtgctccaccaacctcaacac} \\
\text{ggagctgtccaccaacctcaacac} & \text{agctgtgctccaccaacctcaacac} \\
\text{ggagctgtccaccaacctcaacac} & \text{agctgtgctccaccaacctcaacac}
\end{align*}
\]
Fig. 20. Open reading frame of VlcpeA-I and the deduced amino acid sequence, VICPEA-1. Underlined: peptides that matched for identification of spot U1 in the proteomic analysis using the database of V. longisporum cDNA library sequences.
**VlcpeA-1** coding region comprises an open reading frame of 2,289 bp (Fig. 20) which encodes a protein (VICPEA-1) of 762 amino acids with a predicted molecular mass of 83.54 kDa. VICPEA-1 is quite similar (98.5% identity) to the catalase-peroxidase protein of *Verticillium dahliae*, one of its parental species (Fig. 21). It has high sequence similarity with *Aspergillus nidulans* CpeA protein (70% identity) to which this protein spot (spot U1) was found homologous during identification of protein spots in the proteomic analysis. VICPEA-1 also shows high sequence similarity with catalase-peroxidase proteins from the phytopathogenic fungus, *Magnaporthe grisea* (74% identity) and even from the bacterium, *Flavobacterium johnsoniae* (71% identity). In the protein alignment it could be seen that the short amino acid triad (R--WH) is conserved in all the catalase-peroxidases. The three regions conserved in the KatGs class of catalase-peroxidase, 'SQXWWPADXGXY', 'AXXMGLIYVN' and 'GXXPXXAXXEXQGLGW' (Zamocky et al., 2001) are also present in *V. longisporum* VICPEA-1 (Fig. 21). Therefore, it belongs to the KatG proteins of Class I of the plant peroxidase superfamily.
Fig. 21. Alignment of VlCPEA-1 (Vl) with the sequences of chorismate synthase of *V. dahliae* (Vd), ascomycetous fungus, *M. grisea* (Mg) and *A. nidulans* (An), and bacteria, *Flavobacterium johnsoniae* (Fj). "*" identical amino acids, ":" conserved substitutions, "." semi-conserved substitutions. The short amino acid triad (R--WH) highlighted in green is conserved in all catalase-peroxidases. The three regions conserved in the KatGs class of catalase-peroxidase: 'SQXWWPADXGXY' is highlighted in yellow, 'AXXMGLIYVN' is highlighted in blue and 'GXXPXXAXXEXQGLGW' is highlighted in grey.
Results

Fig. 22. Determination of the isogene, VlceA-2 of VlceA-1 by Southern hybridization analysis of V. dahliae and V. longisporum. The genomic DNA was digested with Apal and HindIII. A 500 bp sequence of VlceA-1 was used as a probe. Arrows indicate the signal generation by probe binding.

It was determined whether VlceA-1 has an isogene as V. longisporum has been described as ‘near diploid’ organism (Karapapa et al., 1997). Therefore, V. longisporum and V. dahliae genomic DNA was digested with different restriction enzymes and subjected to Southern hybridization. A unique and fully sequenced VlceA-1 fragment from V. longisporum was used as the probe. In several independent Southern hybridization analyses, two signals were generated for V. longisporum as compared to V. dahliae for the genomic DNA digested with Apal and HindIII (Fig. 22). We also confirmed this result by using the VlceA-1 fragment from V. dahliae as the probe. These results demonstrated that VlceA-1 has an isogene, VlceA-2.

As VICPEA was upregulated in the proteomic analysis, the question was whether the catalase-peroxidase enzyme activity is also increased when V. longisporum is treated with xylem sap. Thus, in-gel catalase activity assay was performed where equal amounts of the native protein from V. longisporum and V. longisporum treated with xylem sap were run on a native gel. Two clear bands were observed in each sample against a dark background after staining for catalase activity (Fig. 23). The quantification of the activity showed that the
catalase activity had increased more than twice in the lower band (catalase activity II) in the sample treated with xylem sap. The catalase activity in the upper band (catalase activity I) decreased slightly in the sample treated with xylem sap however it was not significant (25%).

The in-gel peroxidase activity assay was also performed (pers. comm. P. Findeisen) with the native protein from V. longisporum. A single band corresponding to the lower catalase activity II was observed for the peroxidase activity. Hence, it could be a catalase-peroxidase protein of V. longisporum.

In conclusion, the xylem sap caused increase in the catalase activity II corresponding to which peroxidase activity was also observed so it could be a catalase-peroxidase of V. longisporum. It may be VICPEA that was upregulated in the 2DE analysis when V. longisporum was treated with xylem sap.

Fig. 23. In-gel catalase and peroxidase assay. Equal amounts (10 μg) of protein extracts from V. longisporum untreated (-XS) /treated (+XS) with xylem sap of B. napus were separated in native polyacrylamide gel and stained for catalase activity according to Zou et al., 2000. The catalase activity appears as clear bands in the dark green background. Relative intensity of the upper and lower catalase activity (catalase activity I and II) in each extract was determined using Kodak Molecular Imaging software (right). 10 μg of protein extract from V. longisporum was separated in native polyacrylamide gel and stained for peroxidase activity according to Wayne et al., 1986 (left).
4. Discussion

4.1 The knock-down of chorismate synthase in *V. longisporum* affected its propagation and virulence significantly during infection of *B. napus*

The vascular pathogen *V. longisporum* inhabits the xylem of the rapeseed plant during colonization and infection of this oilseed crop. The xylem sap contains water, inorganic compounds, amino acids and organic acids. In the present study, we explored whether *V. longisporum* is exploiting the xylem sap for nutrition or is utilizing its inherent amino acid biosynthetic pathways. Therefore, we studied the *V. longisporum* chorismate synthase (CS) which catalyses the production of chorismate, the last common precursor of the three aromatic amino acids, Phe, Tyr and Trp, in the shikimate pathway.

In this study, the cDNA library of *V. longisporum* was used for the isolation of the gene for CS, *Vlaro2* by the yeast complementation assay in Δaro2 (yeast deletion mutant of CS). The CS of *S. cerevisiae* is defined as bifunctional as apart from its catalytic activity, it also possess an intrinsic FMN:NADPH oxidoreductase activity (Henstrand et al., 1995). This additional function is required for generating reduced FMN for the catalytic activity of the enzyme. The CS from bacteria and plants are defined as monofunctional as they do not possess the additional activity and they obtain the reduced FMN from the cellular environment (Fitzpatrick et al., 2001, Henstrand et al., 1995b) though, bacterial *Mycobacterium tuberculosis* CS was determined to be bifunctional (Ely et al., 2008). Recently, an in vivo screen was conducted using Δaro2 (yeast deletion mutant of CS) to characterize the unclassified CS as bifunctional if they restore prototrophy to the yeast Δaro2 strain and as monofunctional if they failed to do so (Ehammer et al., 2007). It revealed that the protozoan species have bifunctional CS whereas all the tested bacterial and plant species have the monofunctional enzyme. As *V. longisporum* CS, restored the prototrophy in the yeast Δaro2 strain, it is also bifunctional similar to the fungal counterpart in *N. crassa* (Kitzing et al., 2001). On the alignment of the protein sequence, it was found that it is 98% identical to the corresponding fungal protein in *S. cerevisiae* and *N. crassa*. The structure of bifunctional CS from *S. cerevisiae* has also been studied (Quevillon-Cheruel et al., 2004). The crystal structure shows a novel βαβα fold consisting of an alternate tight packing of two α-helical and two β-sheet layers. There were some residues missing from the final structure and are divided in four regions, L1, L2, L3 and L4. Inspection of the location of these disordered regions in the three-dimensional structure of the CS monomer shows that the boundaries of
L1, L2, and L4, the most conserved missing regions, all concentrate around a pocket situated at the center of the helical layer (Fig. 1).

**Fig. 1.** (a) stereo ribbon representation of the CS monomer, with the disordered regions schematically represented as *black interrupted lines*: L1 (12 residues), L2 (41 residues), L3 (8 residues), and L4 (23 residues). L1, L2, and L4 cluster in the same region of the molecule. (b) surface representation of the CS monomer (same orientation as a). The surface is coloured in *shades of red*, according to a score based on the conservation of the surface residue determined after alignment of 50 CS sequences (consurf.tau.ac.il/). The highly conserved region coincides with a cleft on the protein surface and could represent a putative ligand binding pocket surrounded by the flexible regions represented in a (Quevillon-Cheruel *et al*., 2004).

The molecule is arranged as a tight tetramer with four monomers, A, B, C and D and has a brick-like shape. Each monomeric subunit is in contact with the three others, creating an intricate packing arrangement (Fig. 2). The tetrameric association of CS clusters the majority of conserved residues in a pronounced surface pocket, whereas other conserved surface patches are involved in tetramer packing. The boundaries of the L1, L2, and L4 disordered regions are positioned at the rim of this pocket that may harbour part of the active site. The three missing regions (L1, L2, and L4) most likely form a domain and are ideally positioned to fold over a substrate that would bind to the active site. The sequence blocks L1, L2 and L4, described as the disordered regions in *S. cerevisiae* CS that contribute to the active site in this enzyme could also be identified in VIARO2.
Fig. 2. Representation of the CS tetramer with the most prominent elements involved in packing indicated. The four monomers A–D are coloured blue, orange, green, and red, respectively. (a) view showing the packing between the A/D (packing of the S1 sheet) and A/C monomers (a8/a8 interaction). (b) view looking down at the A/B dimer interface illustrating the a6/a6 and β12/β12 interactions. (c) same view as (b) in surface representation, colour-coded according to residue conservation as in fig. 3, showing the putative ligand-binding site at the interface of the A/B/C monomers (Quevillon-Cheruel et al., 2004).

The CS from *S. cerevisiae* should additionally possess a NADPH binding site. The analysis of the crystal structure of CS could not reveal a characteristic dinucleotide binding domain (Baker et al., 1992). It does not possess the classical Rossman fold or other structures specific for NAD-binding proteins. Therefore, the NADPH binding site remained incomprehensible. Kitzing et al. (2001) observed that NADPH binds in or near the substrate (5-enolpyruvylshikimate 3-phosphate) binding site, suggesting that NADPH binding to bifunctional chorismate synthases is embedded in the general protein structure and a special NADPH binding domain is not required to generate the intrinsic oxidoreductase activity.

*V. longisporum* was described as an amphihaploid with one parent as *V. dahliae* and other a yet unidentified *Verticillium* species (Barbara et al., 2005). It was also described as ‘near diploid’ as it showed 1.78 times the amount of DNA than that of *V. dahliae* upon DNA microdensitometric measurement (Karapapa et al., 1997). Therefore, Southern hybridization analysis was performed to determine if there are two isogenes for CS in *V. longisporum* and then to subsequently determine the sequence of the isogenes. It was observed that there are
Discussion

two isogenes Vlaro2-1 and Vlaro2-2 as compared to V. dahliae which has a single gene for CS. The coding and intronic sequences of both the isogenes in V. longisporum were analysed by sequencing and the sequencing analysis revealed that they were identical. The coding sequence of the two isogenes in V. longisporum is 99% identical to the corresponding sequence of the gene for CS in V. dahliae and their respective protein sequences are identical. This sequence analysis of CS in V. longisporum suggests that it is derived from V. dahliae only. It indicates that V. longisporum has arisen from V. dahliae alone contrary to the earlier reports which state that one parent of V. longisporum is V. dahliae and other, a species different from V. dahliae (Barbara et al., 2005, Karapapa et al., 1997). It might be possible that V. longisporum has arisen by diploidization of two haploid V. dahliae nuclei to form a homozygous diploid. Subsequently, chromosome rearrangements in the initial homozygous diploid nucleus, e.g. loss of parts of individual chromosomes and mitotic recombination could have occurred to produce the amphihaploid or ‘near diploid’ V. longisporum (Geiser et al., 1996). The possible evolutionary advantages of diploidy in the organisms include the masking of harmful recessive mutations (Korona et al., 2004, Perrot et al., 1991) and faster adaptation to new environments (Orr et al., 1994). In a study in S. cerevisiae, it was observed that most of the deleterious mutations are recessive, suggesting that mutators produce more deleterious mutations in haploids than they do in diploids. The diploids have an advantage of increased mutation rate over haploids as the beneficial mutations are dominant or semidominant (Thompson et al., 2006). For the plant pathogens, the diploidy could be advantageous in increasing their pathogenicity or host range. In the phytopathogenic fungus, Fusarium oxysporum the somatic diploids showed enhanced pathogenicity compared with their auxotrophic haploid parents and though the diploid attacked the same host varieties as the most virulent haploid parent but some recombinants with a wider host range were also recovered (Buxton, 1956). So, the somatic diploids in plant pathogenic fungi may act as a source of variability. V. longisporum is host-specific on members of family Brassicaceae, which are not infected by V. dahliae but V. dahliae has a wider host range and can infect many plant species (Zeise et al., 2002b).

The objective of this study was to study the loss of function of Vlaro2 in V. longisporum. The frequently used approach of knock-out of gene expression was not feasible here because of the detection of two isogenes Vlaro2-1 and Vlaro2-2 in V. longisporum. Thus, RNA mediated gene silencing was exploited to generate Vlaro2 knock-down strain in V. longisporum. RNA mediated gene silencing acts in a sequence-specific manner (Matityahu et al., 2008) as opposed to the knock-out strategy that functions in a locus-specific manner. It is
suitable for heterokaryons or polyploid organism like *V. longisporum* and should silence both the isogenes (Jong et al., 2006). This technique of suppressing gene expression was first demonstrated successfully in the fungus, *C. neoformans* (Liu et al., 2002) and thereafter has been successfully applied in several other fungi (Nakayashiki et al., 2005, Nguyen et al., 2008). In the filamentous fungus, *M. oryzae*, gene silencing, specifically, by hairpin RNA was found to be most efficient (Kadotani et al., 2003). Gene silencing by hairpin RNA has also been applied in other fungi successfully (Krajaejun et al., 2007, Tanguay et al., 2006). Therefore, a hairpin double-stranded RNA mediated gene silencing approach (Nakayashiki et al., 2005) was employed for RNA silencing of Vlaro2 in *V. longisporum*.

To silence the Vlaro2 in *V. longisporum*, the Vlaro2 silencing vector was transformed in *V. longisporum*, and the integration of the silencing vector was confirmed by Southern hybridization analysis. The extent of Vlaro2 silencing was analyzed by RT-PCR and Western hybridization analysis. The degree of silencing varied in the Vlaro2sms (Vlaro2 silenced mutants) from high to moderate levels at both the transcript and protein levels. The variation in degree of silencing in the silenced mutants has been observed in other fungi also (Fitzgerald et al., 2004, Mouyna et al., 2004). The VIARO2 protein was reduced as high as up to 94% relative to the wild type protein level. A particular disadvantage of silencing is that it is often not absolute or does not completely knock-down the gene expression, thus, some residual activity of the gene always remains. This strategy has the advantage to explore the function of essential genes whose small amounts of gene expression are sufficient to keep the organism viable but whose complete lack of gene expression is lethal. For instance, *FKSI* gene encoding the catalytic subunit of beta (1-3) glucan synthase was silenced in *A. fumigatus* using RNAi (Mouyna et al., 2004). It has been shown to be unique and essential in *A. fumigatus* (Firon et al., 2002). Similarly, it is known that aro2 is an essential gene for viability of the fungus (Jones et al., 1991). *V. longisporum* auxotrophic for CS would have not survived without supplementation of the culture medium by the aromatic amino acids. But it was possible to knock-down Vlaro2 in *V. longisporum* and it established gene silencing in *V. longisporum*. Additionally, it demonstrated that the gene silencing mechanism by RNA interference is effective in *V. longisporum*.

The phenotypic analysis of the Vlaro2sms was performed to observe the effect of Vlaro2 silencing on growth. When 5-methyl-DL-tryptophan (5-MT) was added to the culture medium, the Vlaro2sms showed severe reduction in initial vegetative growth as well as the growth rate compared to the wild type. 5-MT suppressed the growth of the Vlaro2sms as it is an inhibitor of tryptophan biosynthesis and thus completely abolished the production of
tryptophan in the Vlaro2sms. 5-MT is an analog of tryptophan and acts as a feedback inhibitor of anthranilate synthase that acts downstream of CS and is the first enzyme in the aromatic amino acid, tryptophan biosynthesis pathway (Braus, 1991, Miozzari et al., 1978). In addition, the supplementation of tryptophan and 5-MT simultaneously to the culture medium depleted the severe reduction in growth of the Vlaro2sms caused by addition of 5-MT alone because tryptophan is an antagonist of 5-MT (Miozzari et al., 1978, Schurch et al., 1974).

If the Vlaro2 was disrupted by knock-out strategy in V. longisporum, the resulting ΔVlaro2 strain would not have been able to grow without the aromatic amino acids. It would probably not have been able to germinate and then propagate in the rapeseed plant. But, it was possible to employ the Vlaro2sm to study its propagation and infection of the rapeseed plants by in planta assay. The Vlaro2sm infected plants were observed to be significantly less infected than the wild type infected plants as observed by comparatively reduced stunting symptom and low disease score. As the Vlaro2sm, showed slow initial growth compared to the wild type in the culture medium, the propagation of Vlaro2sm and wild type in the rapeseed plants during infection was investigated. It was observed that the V. longisporum DNA amount is always significantly low in the hypocotyl as well as in the top portion of the stem in the Vlaro2sm infected plants compared to the wild type infected plants, at all the observed stages of infection. These results indicate that the Vlaro2sm is not able to propagate in the rapeseed plant like the wild type. The reduced infection in rapeseed plants by the Vlaro2sms could also be due to the less production of secondary metabolite(s) derived from the aromatic amino acids, that are antagonistic to the host plant and which help in propagation of the fungus in the host rapeseed plant. The aromatic amino acids are known to be involved in the production of secondary metabolites like auxins, melanin, terrequinone A etc., in several fungi. The phytopathogenic fungi, Ustilago esculenta, Colletotrichum acutatum can produce the plant hormone indole-3-acetic acid (auxin) efficiently from the aromatic amino acid, tryptophan (Chung et al., 2004, Lahey et al., 2004). A tomato isolate of V. albo-atrum produced indole-3-acetic acid (IAA) in Czapek Dox medium (Pegg et al., 1959). IAA production is reported on a medium containing L-tryptophan by V. dahliae (Bhaskaran, 1972). The enzymes in the shikimate pathway, chorismate mutase and anthranilate synthetase have been described in the synthesis of IAA by V. albo-atrum (Pegg, 1987). Auxins are known to stimulate the production of the gaseous plant hormone, ethylene (Arteca et al., 2008). Ethylene can inhibit growth of the plant stem, and cause chlorosis, necrosis and falling of leaves of the plant (Benavente and Alonso, 2006). V. longisporum infection causes disease symptoms of stunting and early senescence in the rapeseed plants. These could be caused by generation of ethylene
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stimulated by auxin production by the fungus. The Vlaro2sms showed significantly less disease symptoms during infection of rapeseed may be because they are possibly producing less auxin-like metabolite.

4.2 Differentially expressed proteins of V. longisporum after treatment with xylem sap of B. napus

V. longisporum is a vascular phytopathogen which colonizes the xylem vessels of the host plant during infection. 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. Therefore, the effect of the xylem sap of its host, rapeseed on V. longisporum growth was observed in plate-based growth assays. Firstly, V. longisporum growth was observed on the solid agar media, SXM and CDA and it was noticed that bud spore formation and melanization occurred earlier in SXM. The number of conidia was also more in SXM than that in CDA. Bud spores might be formed earlier and the number of conidia is more on SXM as it is a rich medium containing casein hydrolysate as nitrogen source in comparison to CDA, a minimal medium containing inorganic sodium nitrate as nitrogen source. The melanization occurred earlier on SXM than on CDA because it might be that V. longisporum utilized amino acid, tyrosine present in SXM for melanin production as is known for other microorganisms (Claus et al., 2006).

Subsequently, the solid agar media, SXM and CDA were supplemented with xylem sap in a concentration one-seventh of the culture media. In addition, the xylem sap was also diluted ten times and then added to the two media, similarly. When xylem sap was added to both the culture media, it was observed that the mycelial growth increased, and conidia and bud spores were produced earlier in comparison to the control. In addition, the number of conidia also increased more than twice on supplementation of culture media with xylem sap compared to the control. This effect might be because of the nutrients present in the xylem sap as it contains water, inorganic salts, sugars and some organic compounds (Schurr et al., 1995). But the same increase in growth was observed even when xylem sap was diluted ten times and added to the culture media. This indicates that the xylem sap contains a stimulating compound eg. a growth factor that boosts the growth of V. longisporum even when present in low concentrations. In a similar study, bacterial vascular pathogen, Xylella fastidiosa was subjected to xylem fluids with varying chemistries to examine the effects of nutritional components on bacterial growth in vitro. The highly significant differences in planktonic
growth and biofilm formation were correlated to the concentration of citric acid, amino acids and inorganic ions in the xylem fluids (Andersen et al., 2007). It was also observed that the *V. longisporum* colony melanized on CDA supplemented with xylem sap earlier in comparison to the control. It might be because of the production of melanin by utilization of proteins present in the xylem sap of *B. napus* (Kehr et al., 2005). The melanized fungi are less susceptible to the host defence mechanisms (Taborda et al., 2008). So, it might also be that *V. longisporum* melanized as a response to defence-related proteins in the xylem sap (Kehr et al., 2005).

The observation that the xylem sap from *B. napus* affected *V. longisporum* growth implied that there was differential protein expression in *V. longisporum* upon treatment with xylem sap. Proteomic analysis has proven to be suitable for the study of alteration of protein expression in an organism under varying conditions. *V. longisporum* has no genome sequence available but MS/MS techniques in proteomic studies had made possible the identification of proteins from *A. fumigatus* which also had little genome sequence available at that time (Medina et al., 2005). Therefore, proteomic analysis was employed to detect the differentially expressed proteins of *V. longisporum* upon treatment with xylem sap. A similar study was conducted on the phytopathogenic bacterium, *Dickeya dadantii* to investigate the modulation of protein expression in the presence of host plant extract (Babujee et al., 2007). Host-extract induced changes in the proteome of phytopathogenic bacterium, *Pectobacterium atrosepticum* was also studied (Mattinen et al., 2007). In the proteomic experiment, *V. longisporum* culture was incubated with/without xylem sap from rapeseed and the protein extracted was subjected to 2-DE. In the 2-DE gel analysis, only fourteen proteins were found to be differentially expressed among more than 800 spots. Among the fourteen protein spots, six proteins (U1-U6) were observed to be upregulated and eight proteins (D1-D8) downregulated, significantly. By applying MS/MS to search for homologous proteins, ten protein spots could be successfully identified from the fourteen differentially expressed protein spots. They were identified as homologous to proteins from *V. dahliae* or other fungi. The remaining four proteins could not be identified possibly due to the low concentration of the proteins in the gel or they may be novel proteins of *V. longisporum*.

In the proteomic analysis performed using xylem sap from 35 days old *B. napus*, the protein spots, U1 (catalase-peroxidase), U4 (coproporphyrinogen III oxidase), D2 (unidentified) and D3 (ketol-acid reductoisomerase precursor) showed no significant regulation compared to the controls. This difference in regulation might be due to the difference in age of the rapeseed used for xylem sap extraction. The xylem is a highly dynamic environment in the plant where
water and inorganic nutrients are translocated from roots to the aerial plant organs. Thus, the composition of the xylem sap varies according to several factors like age, plant variety, nitrogen supply etc. (Tilsner et al., 2005, Zornoza et al., 1996).

The ten identified proteins could be functionally classified in different classes like oxidative reactions, protein folding, biosynthesis, aerobic respiration, metabolism and translation. Fig. 3 shows the schematic representation of the functional classification of the proteins.

Fig. 3. Schematic representation of the functional classification of the proteins identified in the proteomic analysis of *V. longisporum* treated with xylem sap of *B. napus*. All the upregulated proteins are involved in stress response. Upright arrow: upregulation, downright arrow: downregulation.

The upregulated proteins in the proteomic analysis are mainly related to oxidative stress-response (Fig. 3). The upregulated protein, protein spot U1 identified as homologous to catalase-peroxidase from *A. nidulans* was upregulated probably due to the oxidative stress produced by peroxidases in the xylem sap (Kehr et al., 2005). This bifunctional enzyme, catalase-peroxidase is encoded by *cpeA* in *A. nidulans* and it has a conserved motif for heme coordination (Scherer et al., 2002). The protein spot U4 identified as homologous to coproporphyrinogen III oxidase from *V. dahliae* was also upregulated, probably in relation to
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this catalase-peroxidase. Coproporphyrinogen oxidase is an essential enzyme that catalyzes the sixth step of the heme biosynthetic pathway (Lash et al., 1998).

The protein spot U2 and protein spot U3 identified as homologous to heat-shock protein 70 (hsp70) and heat-shock protein 60 (hsp60), respectively, from N. crassa were also upregulated. They are known to be responsible for general stress response (Martin et al., 1992, Petit et al., 1994). The transcripts of hsp60 and hsp70 were upregulated also in another plant pathogen, Phytophthora infestans in the differential gene expression analysis during the early stages of potato infection (Avrova et al., 2003). Hsp60 plays a role in the protection of Saccharomyces cerevisiae against oxidative damage due to hydrogen peroxide and superoxide anions (Cabisco et al., 2002). In a previous study, overexpression of hsp70 rendered tumor cells partially resistant to oxidative stress by hydrogen peroxide (Jaattela, 1993). The chaperone DnaK, a member of the hsp70 family, plays a protective role against protein oxidative damage in Escherichia coli (Echave et al., 2002). Thus, the upregulated protein spots mainly include proteins showing response to stress by treatment with xylem sap of B. napus.

But, protein spot D5 identified as homologous to NADPH oxidoreductase from V. dahliae, which is also known to be involved in redox reactions, was downregulated in the proteome analysis. NADPH oxidoreductases accumulated rapidly in Arabidopsis plants under various oxidative stress conditions and are possibly involved in NAD(P)/NAD(P)H homeostasis (Babiychuk et al., 1995). But it could also be involved in iron uptake in V. longisporum as the ferric reductase gene frp1+ required for ferric iron uptake in the fission yeast, Schizosaccharomyces pombe encodes a protein that is homologous to the human NADPH oxidoreductase (Roman et al., 1993).

Two of the downregulated spots (D3 and D4) may be involved in biosynthesis in V. longisporum. The protein spot D3 identified as homologous to ketol-acid reductoisomerase precursor from N. crassa. Ketolacid reductoisomerase catalyzes a part of the branched chain amino acid biosynthetic pathway, which results in the production of valine, isoleucine and leucine. The ketolacid reductoisomerase expression was also downregulated during infection of potato by Phytophthora infestans, in the early biotrophic stage (12 hpi) of infection which was up-regulated by the end of the biotrophic phase of the interaction (48 hpi). But the expression was again down-regulated by 72 hpi (Grenville-Briggs et al., 2005). This apparent down-regulation of the biosynthesis genes following host penetration could be due to a ready access of amino acids from the plant.
Another downregulated protein, protein spot D4 was identified as homologous to aflatoxin aldehyde reductase from *V. dahliae*. The enzyme, Aflatoxin B1-aldehyde reductase (AFB1-AR) catalyzes the formation of a dialcohol from the cytotoxic dialdehyde form of AFB1-8, 9-dihydrodiol (Judah et al., 1993). Aldo–keto reductases are a family of enzymes known to play important roles in the metabolism of sugars, in the interconversion of biochemical intermediates and in xenobiotic metabolism (Bohren et al., 1989). In a study of three aldo–keto reductases of the yeast, *Saccharomyces cerevisiae*, AAD group of gene products are related to AKR7A1, an aflatoxin-metabolizing aldehyde reductase from rat liver. Recombinant protein of this group has activity towards several aldehyde and ketone substrates (Ford and Ellis, 2001).

Two of the downregulated spots (D1 and D8) are involved in metabolic pathways and energy conversion. Protein spot D1 was identified as homologous to vacuolar membrane ATPase catalytic subunit A from *V. dahliae*. Vacuolar proton-translocating ATPases (V-ATPases) are multisubunit complexes that acidify many organelles of the vacuolar network of eukaryotic cells, including the vacuoles/lysosomes, golgi apparatus and other secretory vesicles (Forgac, 1989). Dschida and Bowman (1995) showed that reducing agents have a stabilizing effect on the V-ATPase from *Neurospora crassa* and oxidizing agents like hydrogen peroxide are potent inhibitors of the V-ATPase in vitro. A study in *S. cerevisiae* provided the *in vivo* evidence that the V-ATPase may be inactivated in a less reducing environment (Oluwatosin and Kane, 1997). So, it might be that this protein is downregulated in the proteomic analysis because the xylem sap has an oxidizing environment.

The downregulated protein, protein spot D8 was identified as homologous to eukaryotic translation initiation factor, eIF-5A from *V. dahliae*. eIF-5A is involved in translation as it promotes the formation of the first peptide bond during the initial stage of protein synthesis (Hershey, 1991). eIF-5A may play a general role in the turnover of mRNAs, perhaps acting downstream of decapping, or cellular RNA export, with an indirect effect on translation (Zuk and Jacobson, 1998). It is important in aspects of protein synthesis including mRNA processing (Kang and Hershey, 1994) and ribosomal synthesis (Valentini et al., 2002).

In addition, spot no. D6 was identified as homologous to a hypothetical protein from *A. nidulans* and it has an adenine phosphoribosyl transferase conserved domain. It has been shown that the phosphoribosyl transferase activities have a positive effect on the rate of external purine uptake by *S. pombe* cells. The transformation of the translocated purines into the mononucleotides accelerates the rate of the uptake by eliminating the product of the uptake reaction (Housset and Nagy, 1977).
In this study, catalase-peroxidase, an upregulated protein in the proteomic analysis, identified as homologous to catalase-peroxidase encoded by *cpeA* in *A. nidulans*, was further investigated. Plants resist pathogen attack or delay pathogen growth by triggering defence responses including the production of reactive oxygen species (ROS) like hydrogen-peroxide, superoxide radical or hydroxyl radical (Lu *et al.*, 2006). A rapid response upon perception of microbial pathogens is termed as oxidative burst (Lamb and Dixon, 1997). Catalase-peroxidase in the fungal pathogen can protect the fungus by decomposing the hydrogen-peroxide generated by the host (Wysong *et al.*, 1998). The catalases and peroxidases of phytopathogenic fungi have attracted attention as potential virulence factors (Garre *et al.*, 1998; Nathues *et al.*, 2004). Thus, the catalase-peroxidase of *V. longisporum*, upregulated probably due to the oxidative stress produced by the xylem sap of *B. napus*, could be a potential virulence factor in the infection of rapeseed.

Catalase-peroxidase of *V. longisporum* was confidently identified as homologous to the catalase-peroxidase encoded by *cpeA* in *A. nidulans*. The complete coding sequence of the gene for catalase-peroxidase, *VlcpeA* was obtained on sequencing two clones from the *V. longisporum* cDNA library. On the alignment of the protein sequence, it was found that it is 98.5% identical to the corresponding protein in *V. dahliae*, one of its parental species. It was also found to be 70-74% identical to the corresponding protein in the fungi, *A. nidulans* and *M. grisea*, and in bacteria, *F. johnsoniae*. VlCPEA belongs to the KatG catalase-peroxidases in the Class I of the plant peroxidase superfamily as it contains the three motifs 'SQXWWPADXGXY', 'AXXMGLIYVN' and 'GXXPXXAXXEXQGLGW' which are conserved in all KatG catalase-peroxidases (Zamocky *et al.*, 2001).

*V. longisporum* has been described as ‘near diploid’ or amphihaploid with atleast one parent as *V. dahliae* (Karapapa *et al.*, 1997, Barbara *et al.*, 2005). The Southern hybridization analysis revealed that there are two isogenes for catalase-peroxidase in *V. longisporum* as compared to *V. dahliae* which has a single gene.

The catalase and peroxidase enzyme activity of *V. longisporum* was observed by in-gel assays using native protein extracted from *V. longisporum* untreated/treated with xylem sap from rapeseed. Two bands were observed for the catalase activity and a single band corresponding to the lower catalase activity II was observed for the peroxidase activity. As the catalase activity II corresponds to the peroxidase activity in the native gel, therefore, it could be a catalase-peroxidase protein of *V. longisporum*. The catalase activity assay was also performed for *V. longisporum* treated with xylem sap from rapeseed and it was observed that the catalase activity had increased more than twice in the lower band (catalase activity II).
after treatment with xylem sap. The catalase activity II increased in the native protein when *V. longisporum* was treated with the xylem sap probably because of the oxidative stress generating proteins present in it as shown in the analysis of the proteins in the xylem sap of *B. napus* (Kehr et al., 2005). The corresponding catalase-peroxidase with increased catalase activity could be VICPEA which was observed to be upregulated in the 2DE analysis when *V. longisporum* was treated with xylem sap. The upregulation of VICPEA might be a reaction of *V. longisporum* to the hostile environment in the xylem sap.
5. Conclusions and Outlook

5.1 The knock-down of chorismate synthase in *V. longisporum* affected its propagation and virulence significantly during infection of *B. napus*

*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. Chorismate synthase (CS) is a key enzyme in the aromatic amino acids biosynthesis and many secondary metabolites produced in fungi are derived from aromatic amino acids. The goal of this study was to find out whether impaired aromatic amino acid biosynthesis and in consequence decreased secondary metabolite production has any effect on the biotrophic growth of *V. longisporum* in the rapeseed. Therefore, we chose to silence Vlaro2-1 and Vlaro2-2, isogenes of the aromatic amino acid biosynthesis pathway.

The gene for chorismate synthase of *V. longisporum*, Vlaro2-1 was isolated by performing the yeast complementation assay in the Δaro2, yeast CS mutant by using the cDNA library of *V. longisporum*. As it complemented the bifunctional aro2 in yeast, it was also determined to be bifunctional. The Vlaro2-1 from *V. longisporum* was characterized and this is the first gene to be studied in this species. It was found that *V. longisporum* possess two isogenes, Vlaro2-1 and Vlaro2-2 for CS. The coding sequences of these two isogenes are identical and they are 99% identical to the corresponding sequence of the gene for CS in *V. dahliae*. The respective protein sequences are identical in *V. longisporum* and *V. dahliae*. *V. longisporum* has a shorter intron of 74 bp whereas *V. dahliae* has an intron of 81 bp with 77.8% identity and 7 nucleotides missing at two positions in *V. longisporum*. This implies that *V. longisporum* could be a homozygous diploid of *V. dahliae*. But study of sequences of more genes in *V. longisporum* and *V. dahliae* is required to ascertain this. Because of the presence of two isogenes Vlaro2-1 and Vlaro2-2 in *V. longisporum*, gene silencing using RNAi was employed to knock-down both the isogenes and for the first time, the gene silencing by RNAi was successfully established in *V. longisporum*. The silencing occurred from high to moderate levels in the silenced mutants as determined by RT-PCR and Western hybridization analysis. After establishing the stable Vlaro2 silenced mutant (Vlaro2sm), the phenotypic analysis was performed. The Vlaro2sm showed slow initial growth compared to the wild type, but it survived in the culture medium lacking the aromatic amino acids. It was also observed that the Vlaro2sm is retarded in growth compared to the wild type when grown in presence of 5-methyl-DL-tryptophan, a feedback inhibitor of the tryptophan biosynthesis. The concentration
of the aromatic amino acids in the xylem sap did not restore the normal growth of Vlaro2sm. The effect of silencing Vlaro2 on the infection of rapeseed was further studied by in planta assays. It was observed that the infection of the fungus in the plant was reduced significantly as the Vlaro2sm infected host plants were less stunted and showed less disease symptoms compared to the wild type infected plants. The propagation of the fungus in the plant was also reduced significantly as the fungal DNA amount in the hypocotyls and stem of the Vlaro2sm infected host plants was less as compared to the control. It could be that the fungus produces lower amounts of secondary metabolites in the Vlaro2sm that are required for fungal self-defence or for altering the plant physiology for the advantage of the fungus. It will be interesting for further studies to determine if V. longisporum is producing secondary metabolites derived from the aromatic amino acids. We would expect that the silencing of Vlaro2 has a direct effect on the production of these secondary metabolites. If Vlaro2sm produces fewer amounts of such secondary metabolites compared to the wild type, it will be possible to study the putative role of these secondary metabolites in the fungus.

On the other hand, under amino acid starvation conditions the regulatory gene for Cross-Pathway Control (CPC) or General Control of amino acid biosynthesis is activated in fungi (Mösch et al., 1991). This results in a regulation of different sets of genes involved in amino acid biosynthesis, purine biosynthesis, vitamin biosynthesis, and nitrogen and sulphur metabolism (Hinnebusch, 1997, Natarajan et al., 2001, Tian et al., 2007). It will be interesting to observe the expression of cpc gene in the Vlaro2sm in comparison to its expression in the wild type and then to determine the impact of its expression on the fungal metabolism and its reduced virulence of the rapeseed.

5.2 Differentially expressed proteins of Verticillium longisporum after treatment with xylem sap of B. napus

The unique feature of V. longisporum is that it is contained in the fluid environment of the xylem vessels only, during most of its life cycle in the host plant, rapeseed. We wanted to study the differential protein expression of V. longisporum on treatment with the xylem sap of rapeseed by applying 2-DE (Two-dimensional electrophoresis) and MS-based proteomics. Two-dimensional electrophoresis is a reliable and reproducible technique used to separate thousands of proteins on a single SDS-PAGE gel, in order to generate a protein array. MS-based proteomics is an effective method to survey the protein arrays generated by 2-DE of the proteins expressed by a filamentous fungus under different growth conditions (Paper et al., 2007).
In this work, the differences in protein expression profiles of *V. longisporum* were displayed in the presence of xylem sap indicating proteins that help the fungus in adapting to the xylem environment in the plant. By using a proteomic approach, the effect of the xylem sap of rapeseed on *V. longisporum* was studied, to identify and analyse the differentially produced fungal proteins. 2-DE gels of protein extracts were produced from *V. longisporum* untreated/treated with the xylem sap of rapeseed. Fourteen proteins were differentially expressed in the gels, of which six were upregulated and eight downregulated. Ten proteins were confidently identified and characterized by using tryptic digestion, mass spectrometry and homology search. The fungal proteins that were affected on interaction with the host plant were analyzed to know their putative roles in the pathogen-host interaction. The upregulated proteins primarily belonged to the class of stress response proteins. The downregulated proteins play a role in the biosynthetic pathways, metabolic pathways, energy conversion and redox reactions.

Catalase-peroxidase of *V. longisporum*, VlCPEA, found to be upregulated in the proteomic analysis was further characterized in relation to its corresponding protein in *V. dahliae* as well as in other fungi. VlCPEA might play a role in protecting the fungus from the oxidative stress generated by the host plant, rapeseed. The complete sequence of VlCPEA-1 was obtained by fully sequencing two cDNA clones from the cDNA library of *V. longisporum*. The protein was found to belong to KatG catalase-peroxidases of Class I of the plant peroxidase superfamily. It showed 98.5% identity to the corresponding protein in *V. dahliae*. On Southern hybridization analysis, it was determined that VlCPEA-1 has an isogene, VlCPEA-2 in contrast to *V. dahliae* that possess a single gene for catalase-peroxidase. It will be interesting to compare the sequences of the two isogenes to ascertain their similarity. The catalase and peroxidase activity of *V. longisporum* was detected by in-gel activity assay. The catalase activity was observed to be increased when *V. longisporum* was treated with the xylem sap of rapeseed. This suggests that the xylem sap produced an oxidative stress on the fungus. In future, generation of knock-down strain of VlCPEA would help in elucidating its function in the fungus and its significance in the fungus-plant interaction.

The detailed study of the other identified proteins in the proteomic analysis could help in understanding their role in adaptation of the fungus to the xylem environment in the host plant.
6. References


References

Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. Nature **438**: 1105-1115.


References


of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature **438**: 1151-1156.


References


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