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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Abbreviations

amp^R	ampicillin resistance
amu	atomic mass unit
ATP	adenosinetriphosphate
AUDPC	area under the disease progress curve
bp	base pairs
cDNA	complementary DNA
cfu	colony forming unit
cm	centimetre
cpc	cross-pathway control of amino acid biosynthesis
CS	chorismate synthase
DNA	deoxyribonucleic acid
dT	dithymidine
DTT	dithiothreitol
EDTA	ethylene-diamintetraacetate
eIF	elongation initiation factor
Fig.	figure
FMN	flavin mononucleotide
g	gram
h	hour
H_2O	water
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
hygr^R	hygromycn resistance
IgG	immunoglobulin G
IPG	immobilized pH gradient
IT	intron
kan^R	kanamycin resistance
kb	kilobase pairs
kDa	kilodalton
l	litre
MCS	multiple cloning site

mg	milligram
min	minute
ml	millilitre
mRNA	messenger Ribonucleic acid
ms	millisecond
MS	mass spectrometry
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
ng	nanogram
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RT	reverse transcription
SC	synthetic complete
SDS	sodium dodecyl sulphate
sec	second
SH	sulphhydryl
V	volts
2-DE	2-dimensional electrophoresis
2D-PAGE	2-dimensional polyacrylamide gel electrophoresis
°C	degree Celsius
µl	microlitre
µm	micrometre

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 Δ*aro2*. 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.

Chorismat-Synthase ist an der Biosynthese von aromatischen Aminosäuren beteiligt. Das Gen für Chorismat-Synthase *Vlaro2-1* wurde mittels einer cDNA-Genbank von *V. longisporum* durch einen Komplementationstest im Hefestamm Δ*aro2* isoliert. Weil *Vlaro2-1* das bifunktionelle *Aro2* in Hefe komplementieren kann, wird angenommen, dass das Enzym ebenfalls bifunktionell ist, d.h. neben der katalytischen Aktivität weist es eine spezifische NADPH:FMN Oxidoreduktase-Aktivität auf. Es konnte gezeigt werden, dass *Vlaro2-1* ein Isogen *Vlaro2-2* mit identischer genomischer Sequenz besitzt, welches erneut zeigt, daß *V. longisporum* merodiploid ist. Die VLARO2-Proteinsequenz von beiden Isogenen ist identisch zu der entsprechenden Proteinsequenz von *V. dahliae*. Über RNA-vermitteltes „Gen-silencing" wurde eine neue „knock-down" Methode für *V. longisporum* erfolgreich etabliert, mit der die Transkription beider Isogene der Chorismat-Synthase durch RNAi gehemmt werden konnten, damit konnte gezeigt werden, dass RNAi in *V. longissporum* möglich ist. Im Vergleich mit dem Wildtypstamm zeigte die Mutante *Vlaro2* normales Wachstum auf Minimalmedium, aber verlangsamtes Wachstum in Gegenwart von 5-Methyl-DL-Tryptophan, einem Tryptophan-Biosynthese- Inhibitor. Über einen Pathogenitätstest in *B. napus* konnte gezeigt werden, dass Wachstum und Virulenz der Mutante *Vlaro2* stark eingeschränkt waren.

Die Wirkung von Xylemsaft des Wirtes *B. napus* auf die Proteinexpression des Pathogens *V. longisporum* wurde durch eine Proteom-Analyse untersucht. Die 2-dimensionale Gelelektrophorese (2-DE) wurde dazu genutzt, um die in Gegenwart von Xylemsaft unterschiedlich exprimierten Proteine von *V. longisporum* zu identifizieren. Hier wurden 14 Proteine gefunden, von denen 6 hochreguliert und 8 runterreguliert waren. Es wurde festgestellt, dass die hochregulierten Proteine an der Stressantwort des Pilzes beteiligt sein könnten. Eines der hochregulierten Proteine Katalase-Peroxidase von *V. longisporum* VlCPEA, wurde weiter charakterisiert. *V. longisporum* hat auch hier zwei Isogene *VlcpeA-1* und *VlcpeA-2*. Katalase-Peroxidase könnte bei durch die Pflanzen verursachtem oxidativem Stress eine Schutzfunktion ausüben. Die Entwicklung einer „knock-down" Methode und die erste Charakterisierung der beiden Genpaare für Prototrophie oder oxidative Stressantwort, sind die Vorraussetzung für weitere Untersuchungen dieser Pilz-Pflanzen Interaktion.

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).

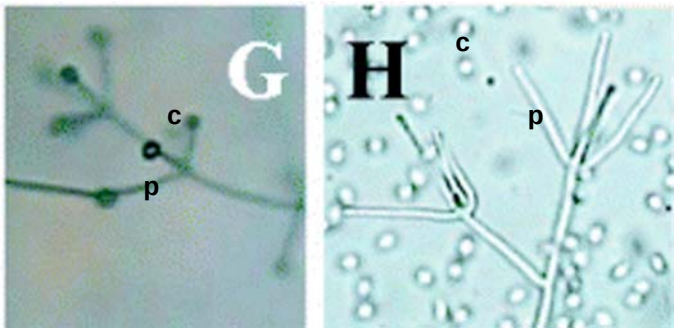


Fig. 1. *V. longisporum* bearing three/four phialides (p) on the conidiophore. The phialide bear elliptical conidia (c) at the apex (modified from Fradin and Thomma, 2006)

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.

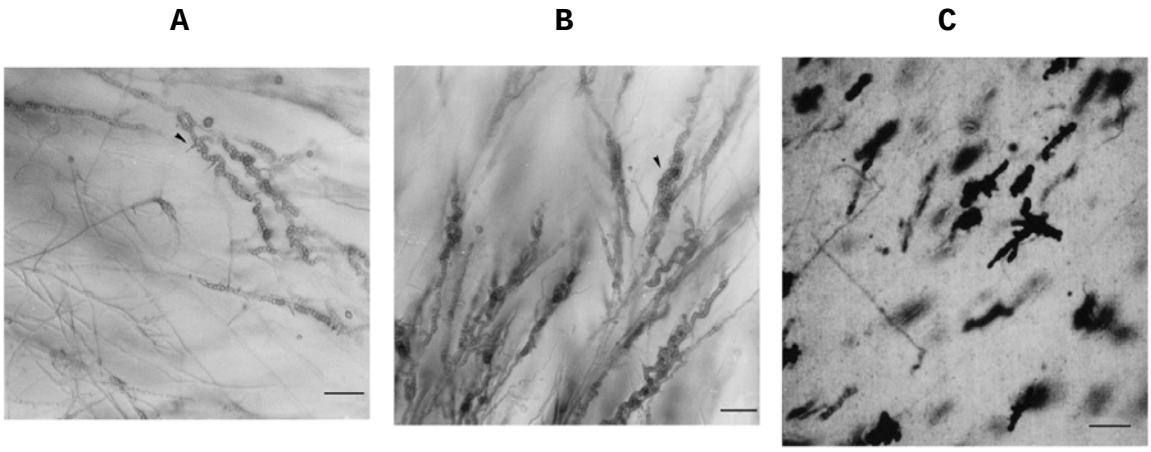


Fig. 2. The three stages of formation of microsclerotia. **A.** Stage I (initiation) of microsclerotia development in *V. longisporum*. **B.** Stage II of microsclerotia formation. Hyphae aggregated (arrow-head) and formed irregular, elongate, strand-like structures. **C.** Stage III of microsclerotia 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*., 1994*a*, Subbarao *et al*., 1995), or melanin deficient mutants (Nagao *et al*., 1994*b*) 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.

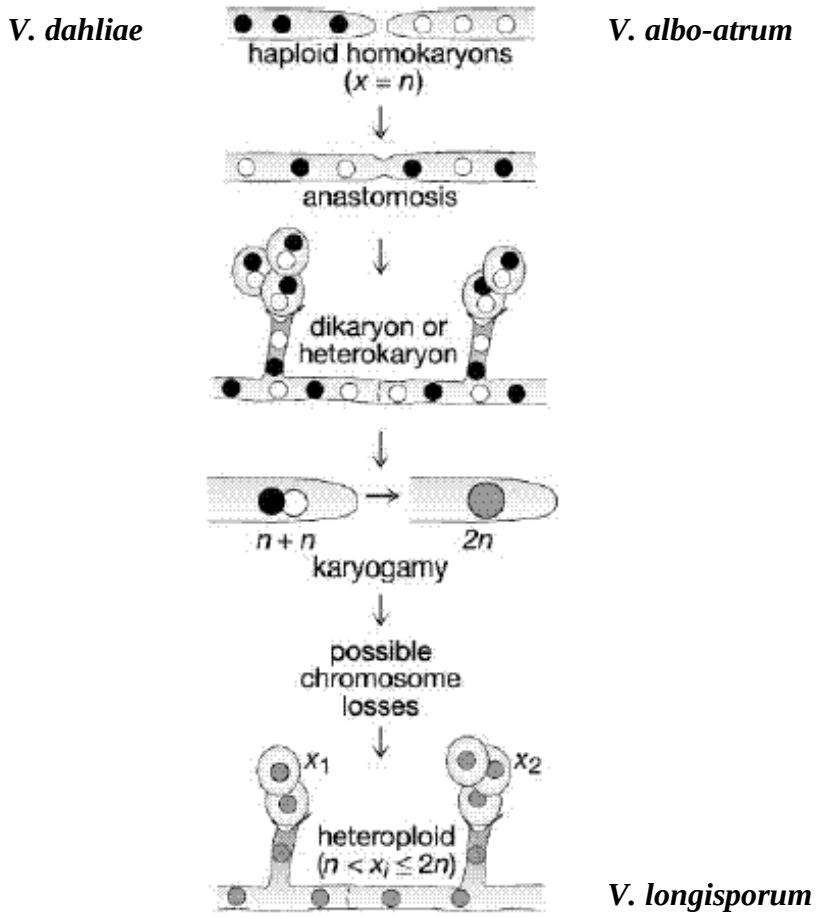


Fig. 3. Karapapa *et al*. (1997) suggested that *V. longisporum* is a hybrid of *V. dahliae* and *V. albo-atrum* evolved via parasexual hybridization. There was a fusion of two different haploid homokaryons to form a diploid heterokaryon. Chromosome rearrangements in the initial heterozygous diploid nucleus could have resulted in a 'near diplod' 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 amphihaploid 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 *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 *et al*., 2002b). Steventon *et al*. (2002) also characterized isolates of *Verticillium* from *Brassica napus* in Sweden and Germany as *V. longisporum.* Although it is also reported that *V. longisporum* can also infect other plant species (Fahleson *et al*., 2003; Johannson *et al*., 2006) and *B. napus* can host other *Verticillium* species when the plant is weak (Collins *et al*., 2003).

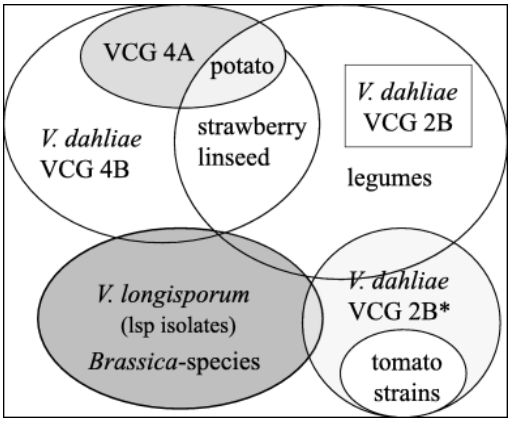


Fig. 4. Host ranges differentiated for VCGs (Vegetative Compatibility Groups) of *V. dahliae* and for *V. longisporum*, based on disease severity (AUDPC) and yield losses induced by representative isolates. *V. longisporum* isolates are host-specific on *Brassica*-species (Zeise *et al*., 2002b).

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 *et al*., 2007; Zhou *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.

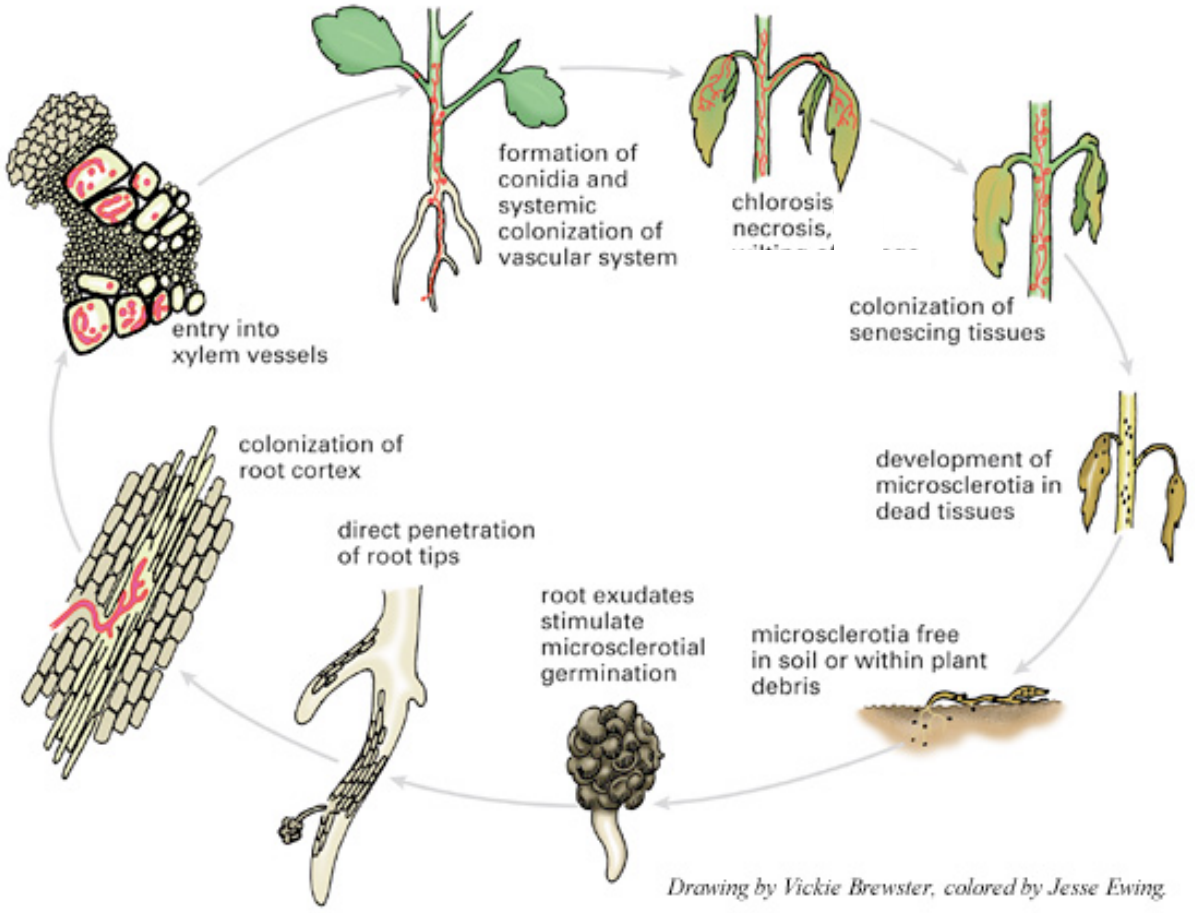


Fig. 5. Infection cycle of *V. longisporum* in *B. napus*. Microsclerotia, the resting spores present in the soil are stimulated to germinate probably by root exudates. The germinating hyphae penetrate directly through the roots, passes through the root cortex and reaches the xylem vessels of the plant. 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.

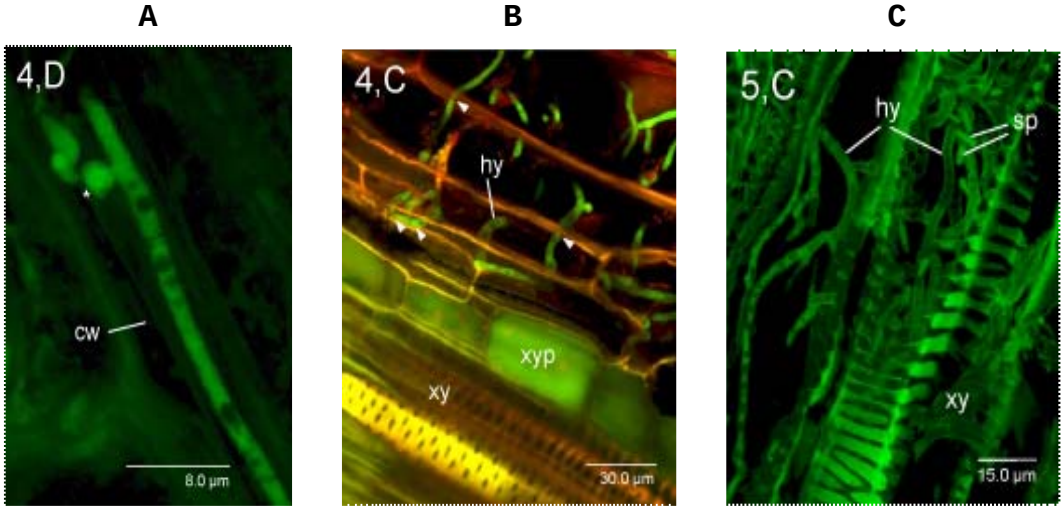


Fig. 6. *V. longisporum* colonization and infection in *B. napus*. Confocal laser scanning microscopy analysis after staining with acid fuchsin and acridine orange. **A**. Magnified view of the penetration of a plant cell wall (cw) by *V. longisporum* in the root cortex. Asterisk marks the swelling of 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).

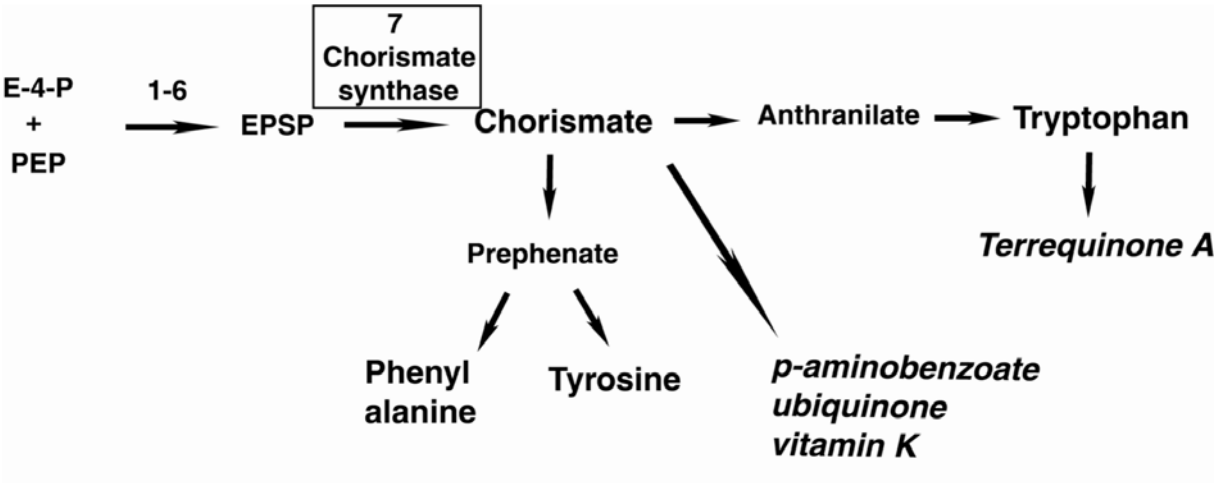


Fig. 7. Overview of the aromatic amino acid production in fungi. 1-7: seven steps in the shikimate pathway for the production of chorismate, precursor of the aromatic amino acids, phenyl alanine, tyrosine, and tryptophan and secondary metabolites, p-aminobenzoate, ubiquinone, vitamin K and Terrequinone 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.

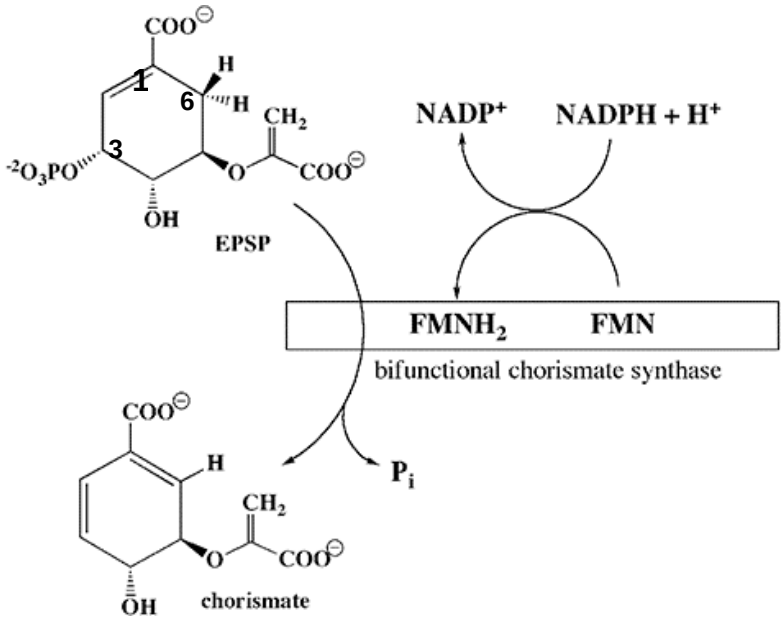


Fig. 8. The catalytic activities of 'bifunctional' CS. This class of enzymes can reduce oxidized FMN to the fully reduced cofactor FMNH2 at the expense of NADPH. With the reduced cofactor bound to the active site multiple turnover of the substrate EPSP can occur (Ehammer *et al*., 2007).

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

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–, F80d/(*lacZ*) *Δ*M15, *Δ*(*lacZYA-argF*), U169, *recA1*, *endA1*, *hsdR17* (r_K-, m_{K+}), *supE44*, λ-, *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

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 1. Details of *V. longisporum* cDNA library

Vector	No. of primary clones (cfu)	Average insert size (kb)	% containing inserts
pCMV.SPORT6.1 (entry vector)	7.396 x 10^7	1.314	>99
pDONR222 (entry vector)	6.874 x 10^7	1.551	>99
pYES-DEST52 (destination vector for expression in yeast)	5.6 x 10^7	1.814	100

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 casette. 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-*Hin*d-R to produce the sense strand. Sense product was purified and then digested with *SnaB*I and *Hin*dIII. It was then ligated into *SnaB*I–*Hin*dIII digested pSilent-1 plasmid. The antisense region of the *Vlaro2-1* was amplified using primers, CSAS-*Apa*I-F and CSAS-*Pae*I-R. Antisense product was purified and then digested with *Apa*I and *Sph*I. It was then ligated with *Apa*I-*Sph*I digested pSilent-1 plasmid already containing the sense *Vlaro2-1*. 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 *Xba*I. It was then ligated in *Xba*I digested binary vector, pPK2 (Covert *et al*., 2001) to generate pME3571 vector for silencing both the isogenes *Vlaro2-1* and *Vlaro2-2*. The pPK2 vector contains the hygromycin B phosphotransferase gene (*hph*) as the selection marker.

Table 2. Plasmids used in this study

Plasmid	Description	Reference
pPK2	Binary vector (^{p}*gpdA::hph:: trpC*^{t}*, kan*^{R})	Covert *et al*., 2001
pSilent-1	Silencing vector (^{p}*trpC::MCS::IT::MCS:: trpC*^{t}*, amp*^{R})	Nakayashiki *et al*., 2005
pME3571	CS silencing binary vector (^{p}*trpC:: CSS::IT::CSAS:: trpC*^{t}*, hygr*^{R}*, kan*^{R})	this study

Table 3. Strains used in this study

Strain	Description	Reference
V. longisporum 43	wildtype	Zeise *et al*., 2002b
V. dahliae 73	wildtype	Zeise *et al*., 2002b
AGB455	*V. longisporum* strain stably transformed with *Vlaro2* silencing plasmid, *hygr*^{R}	this study

Table 4. Primers used in this study

Name	Size	Sequence
CSS-*Sna*-F	34-mer	5'-AAA TAC GTA TGG ACC TAC CTC ACC AAG TAC GGC A-3'
CSS-*Hin*d-R	30-mer	5'-TTT AAG CTT AGC TTG TCG AAG CAG GGC TCA-3'
CSAS-*Apa*I-F	34-mer	5'-AAA GGG CCC TGG ACC TAC CTC ACC AAG TAC GGC A-3'
CSAS-*Pae*I-R	30-mer	5'-AAA GCA TGC AGC TTG TCG AAG CAG GGC TCA-3'
SS46	28-mer	5'-TAC GTA TGG ACC TAC CTC ACC AAG TAC G-3'
SS47	27-mer	5'-AAG CTT AGC TTG TCG AAG CAG GGC TCA-3'
SS15	21-mer	5'-TGG CAC CAC ACC TTC TAC AAC-3'
SS33	21-mer	5'-CCA GAG TCA AGC ACG ATA CCA-3'
OLG 70	20-mer	5'-CAG CGA AAC GCG ATA TGT AG-3'
OLG 71	19-mer	5'-GGC TTG TAG GGG GTT TAG A-3'

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 tranformants 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 (APPLERA 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

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 *VlcpeA-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 gel, 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

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 anti-mouse 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 by10 min incubation at 95°C, followed by 45 cycles of 10s at 95°C, 10s at 58°C and

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 10^6 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 immobiline/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.

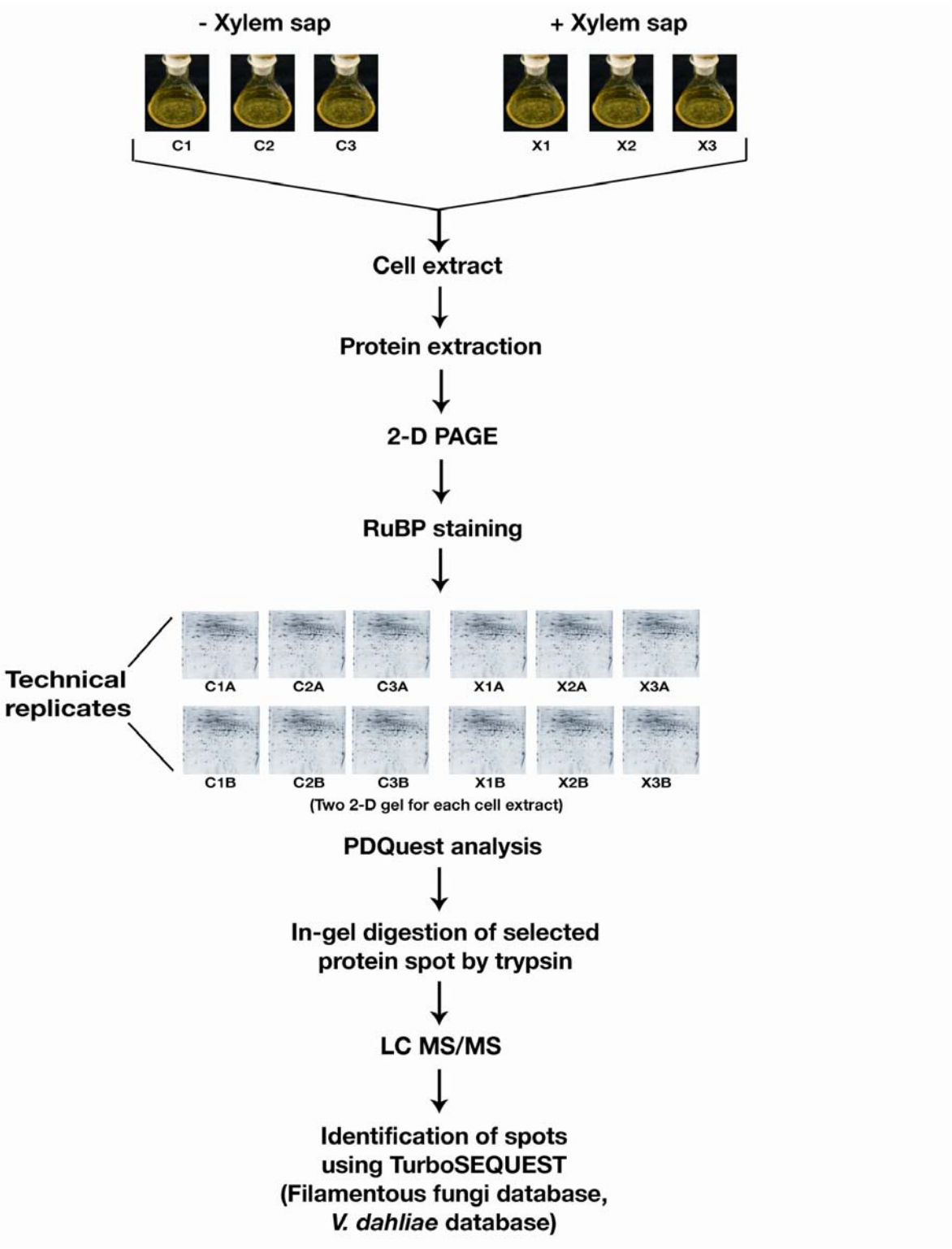


Fig. 1. Experimental set up of the proteomics study. Three independent cultures of *V. longisporum* were untreated (C1, C2, C3) /treated (X1, X2, X3) with xylem sap from *B. napus*. After protein extraction, 2-D PAGE was performed and gels were stained with RuBP and silver nitrate. The PDQuest matching 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 H_2O, suspended in a solution of 0.01 ml 30% H_2O_2 in 100 ml H_2O, and gently

rocked for 10 min. The H_2O_2 solution was then removed and the gel quickly rinsed in H_2O. A freshly prepared mixture of 30 ml each of 2% ferric chloride and 2% potassium ferricyanide, both in H_2O, was poured into a fresh staining pan, and the rinsed gel 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_2O_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 2-177.4µM. 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 arised 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.

S. no.	Amino acid	Concentration (µM)
1.	Asp	52.85
2.	Glu	53.66
3.	Asn	09.72
4.	Ser	37.34
5.	His	04.12
6.	Gln	177.35
7.	Thr	22.82
8.	Gly	17.76
9.	Arg	02.76
10.	Ala	36.32
11.	Gaba (gamma amino butyric acid)	50.58
12.	**Tyr**	07.94
13.	Val	25.75
14.	Met	02.01
15.	**Trp**	02.01
16.	Ile	15.34
17.	**Phe**	06.77
18.	Leu	10.09
19.	Lys	08.83
	Total	544.02

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 Δ*aro2* (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 Δ*aro2*. When the cDNA library of *V. longisporum* was transformed in Δ*aro2*, 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, Δ*aro2* complemented with *Vlaro2-1* (Δ*aro2* + *Vlaro2-1*) could grow as compared to the untransformed Δ*aro2*. 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.

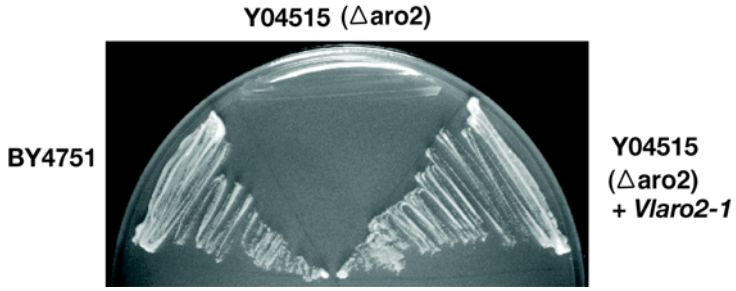


Fig. 1. Δ*aro2* yeast complementation assay on synthetic complete medium lacking the three aromatic amino acids. BY4751, wild type yeast strain; Y04515 (Δ*aro2*), yeast chorismate synthase mutant; Y04515 (Δ*aro2*) + *Vlaro2-1*, yeast chorismate synthase mutant complemented with gene for chorismate synthase in *V. longisporum*.

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.

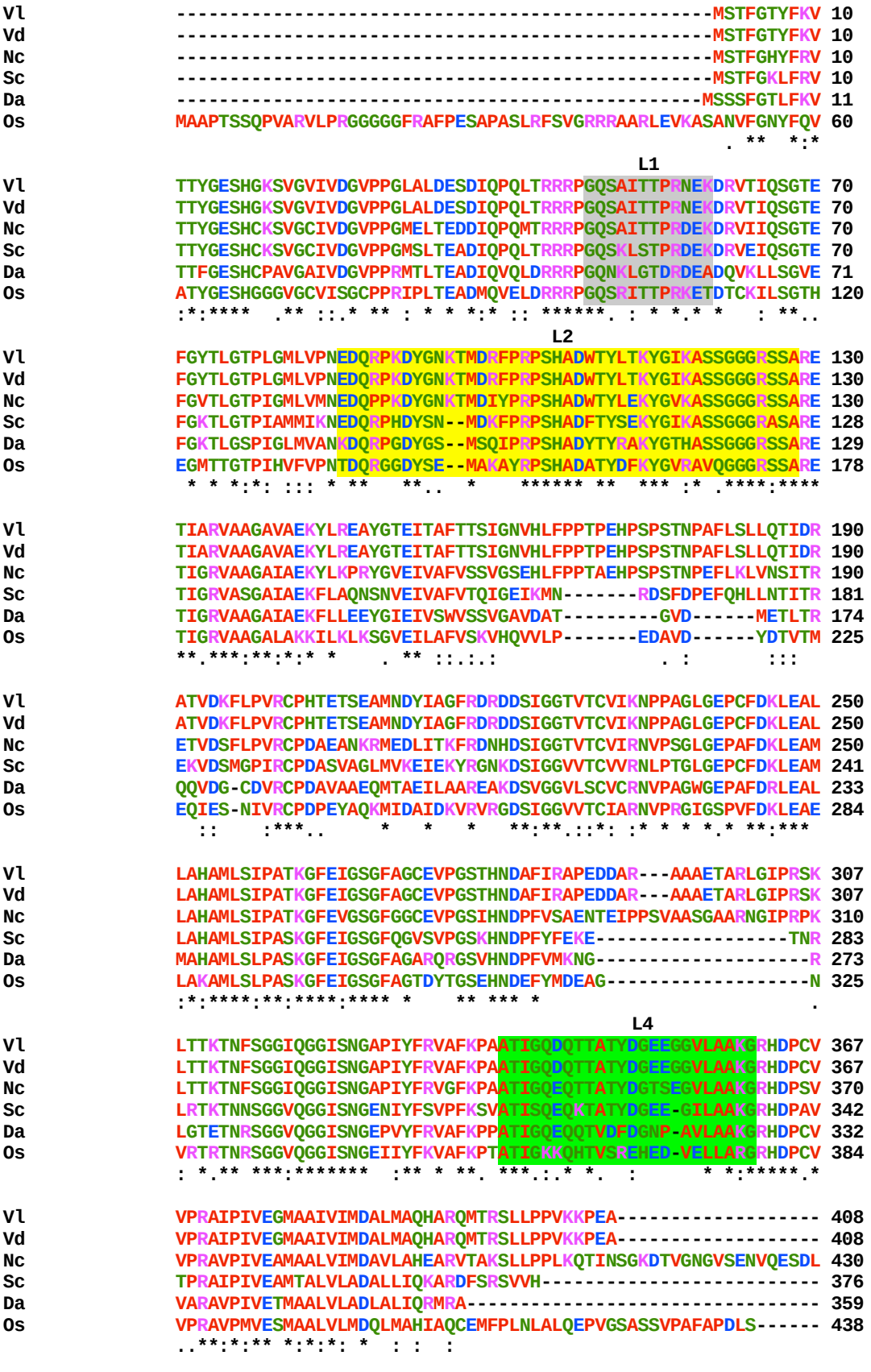
3.1.3 *Vlaro2-1* has an identical isogene *Vlaro2-2*

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 hybridizaton. 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 *Sal*I and *Xho*I (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 *Sal*I and *Xho*I and run on a low percentage agarose gel. DNA was extracted from the four gel fragments corresponding to spots where the signals were generated in the Southern hybridization analysis (Fig 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 atgtcgactttcggaacctacttcaaggtcaccacctacggcgag
      M  S  T  F  G  T  Y  F  K  V  T  T  Y  G  E
   46 tcccacggcaaatccgtcggcgtcattgtcgatggtgtgcccccc
      S  H  G  K  S  V  G  V  I  V  D  G  V  P  P
   91 ggcctcgccctcgacgagtccgacattcagccccagcttacacgt
      G  L  A  L  D  E  S  D  I  Q  P  Q  L  T  R
  136 cgccggcccggccagtctgccatcacaacaccccgcaatgagaag
      R  R  P  G  Q  S  A  I  T  T  P  R  N  E  K
  181 gaccgcgtcaccatccagtccggcaccgagtttggctacaccctg
      D  R  V  T  I  Q  S  G  T  E  F  G  Y  T  L
  226 ggcacaccgcttggcatgctcgtgcccaacgaggaccagcgcccc
      G  T  P  L  G  M  L  V  P  N  E  D  Q  R  P
  271 aaggattacggcaacaagacgatggaccgtttcccgcgtccctcg
      K  D  Y  G  N  K  T  M  D  R  F  P  R  P  S
  316 cacgccgactggacctacctcaccaagtacggcatcaaggcttcc
      H  A  D  W  T  Y  L  T  K  Y  G  I  K  A  S
  361 tccggaggcggccgctcctcggcgcgcgagaccattgcccgcgtc
      S  G  G  G  R  S  S  A  R  E  T  I  A  R  V
  406 gccgccggcgccgttgccgaaaagtacctgcgcgaggcctacggc
      A  A  G  A  V  A  E  K  Y  L  R  E  A  Y  G
  451 accgagatcacggccttcacgacgagcatcggcaacgtccacctc
      T  E  I  T  A  F  T  T  S  I  G  N  V  H  L
  496 ttcccgccgacccctgagcacccctcgccctcgacgaaccccgcc
      F  P  P  T  P  E  H  P  S  P  S  T  N  P  A
  541 ttcctctcgctcctgcagaccatcgaccgcgccaccgtcgacaag
      F  L  S  L  L  Q  T  I  D  R  A  T  V  D  K
  586 ttcctgcccgtgcgctgcccacacaccgagacgtccgaggccatg
      F  L  P  V  R  C  P  H  T  E  T  S  E  A  M
  631 aacgactacattgccggcttccgcgaccgtgacgactcgatcggc
      N  D  Y  I  A  G  F  R  D  R  D  D  S  I  G
  676 ggcaccgtcacctgcgtcatcaagaacccgcccgccggcctcggc
      G  T  V  T  C  V  I  K  N  P  P  A  G  L  G
  721 gagccctgcttcgacaagctcgaggccctgctcgcccacgccatg
      E  P  C  F  D  K  L  E  A  L  L  A  H  A  M
  766 ctcagcatcccggccaccaagggcttcgagatcggatcgggcttt
      L  S  I  P  A  T  K  G  F  E  I  G  S  G  F
  811 gccggctgcgaggtccctggctcgacgcacaacgacgcctttatc
      A  G  C  E  V  P  G  S  T  H  N  D  A  F  I
  856 cgcgcccccgaggacgacgcccgcgccgccgccgagacggcccgc
      R  A  P  E  D  D  A  R  A  A  A  E  T  A  R
  901 ctcggcatcccgcgctccaagctcacgaccaagaccaactttagc
      L  G  I  P  R  S  K  L  T  T  K  T  N  F  S
  946 ggcggcatccagggcggcatctccaacggcgcccccatctacttc
      G  G  I  Q  G  G  I  S  N  G  A  P  I  Y  F
  991 cgcgtcgccttcaagcccgccgccaccattggccaggaccagacg
      R  V  A  F  K  P  A  A  T  I  G  Q  D  Q  T
 1036 acggccacctacgatggcgaggagggcggcgtcctcgccgccaag
      T  A  T  Y  D  G  E  E  G  G  V  L  A  A  K
 1081 ggcaggcacgatccctgcgtcgtgccccgcgccatccccattgtc
      G  R  H  D  P  C  V  V  P  R  A  I  P  I  V
 1126 gagggcatggccgccattgtcatcatggacgccctcatggcccag
      E  G  M  A  A  I  V  I  M  D  A  L  M  A  Q
 1171 cacgcccgccagatgacgaggagcctgctgccgcctgtgaagaag
      H  A  R  Q  M  T  R  S  L  L  P  P  V  K  K
 1216 ccggaggcgtag 1227
      P  E  A  *
```

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


L1
L2
L4

```
Vd          --
Nc          AQ 432
Sc          --
Da          --
Os          --
```

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.

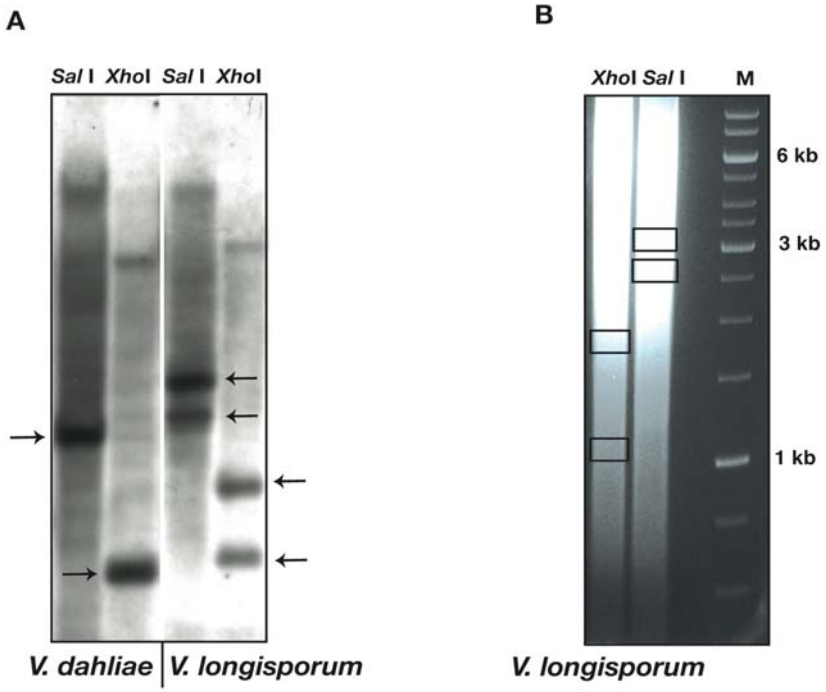


Fig. 4. Determination of the isogene *Vlaro2-2* of *Vlaro2-1* and their corresponding sequence. **A**. Southern hybridization analysis of *V. dahliae* and *V. longisporum*. The genomic DNA was digested with *Sal*I and *Xho*I. A 500 bp sequence of *Vlaro2-1* was used as a probe. Arrows indicate the signal 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        ATGTCGACTTTCGGAACCTACTTCAAGGTCACCAC**ATGAGTCCATTGACAGACTGGACAT** 60
Vlaro2-2        ATGTCGACTTTCGGAACCTACTTCAAGGTCACCAC**ATGAGTCCATTGACAGACTGGACAT** 60
Vd              ATGTCGACTTTCGGAACCTACTTCAAGGTCACCAC**GTGAGTCAATTGACAGACTGGGCAT** 60
                ********************************** ****** ************ ***

Vlaro2-1        **TTTACATGGCCGCCCCACCC------AGACACA-GGCGCTGACAATCATCTCCCAG**CTAC 113
Vlaro2-2        **TTTACATGGCCGCCCCACCC------AGACACA-GGCGCTGACAATCATCTCCCAG**CTAC 113
Vd              **TTTACATGGCCGCCCCCGCCTCCGCCAAACACAAGGAGCTGATAATGGTTTCCCAG**CTAC 120
                ***************  **      * ***** ** ***** ***  * **********
```

```
Vlaro2-1        GGCGAGTCCCACGGCAAATCCGTCGGCGTCATTGTCGATGGTGTGCCCCCTGGCCTCGCC 173
Vlaro2-2        GGCGAGTCCCACGGCAAATCCGTCGGCGTCATTGTCGATGGTGTGCCCCCTGGCCTCGCC 173
Vd              GGCGAGTCCCACGGCAAATCCGTCGGCGTCATTGTCGATGGGGTGCCCCCTGGCCTCGCC 180
                ************************************** ******************

Vlaro2-1        CTCGACGAGTCCGACATTCAGCCCCAGCTTACACGTCGCCGGCCCGGCCAGTCTGCCATC 233
Vlaro2-2        CTCGACGAGTCCGACATTCAGCCCCAGCTTACACGTCGCCGGCCCGGCCAGTCTGCCATC 233
Vd              CTCGACGAGTCCGACATTCAGCCCCAGCTTACACGTCGCCGGCCCGGCCAGTCTGCCATC 240
                ************************************************************

Vlaro2-1        ACAACACCCCGCAATGAGAAGGACCGCGTCACCATCCAGTCCGGCACCGAGTTTGGCTAC 293
Vlaro2-2        ACAACACCCCGCAATGAGAAGGACCGCGTCACCATCCAGTCCGGCACCGAGTTTGGCTAC 293
Vd              ACAACACCCCGCAATGAGAAGGACCGCGTCACCATCCAGTCCGGCACCGAGTTTGGCTAC 300
                ************************************************************

Vlaro2-1        ACCCTGGGCACACCGCTTGGCATGCTCGTGCCCAACGAGGACCAGCGCCCCAAGGATTAC 353
Vlaro2-2        ACCCTGGGCACACCGCTTGGCATGCTCGTGCCCAACGAGGACCAGCGCCCCAAGGATTAC 353
Vd              ACCCTGGGCACACCGCTTGGCATGCTCGTGCCCAACGAGGACCAGCGTCCCAAGGATTAC 360
                ********************************************* ************

Vlaro2-1        GGCAACAAGACGATGGACCGTTTCCCGCGTCCCTCGCACGCCGACTGGACCTACCTCACC 413
Vlaro2-2        GGCAACAAGACGATGGACCGTTTCCCGCGTCCCTCGCACGCCGACTGGACCTACCTCACC 413
Vd              GGCAACAAGACGATGGACCGTTTCCCGCGTCCCTCGCACGCCGACTGGACCTACCTCACC 420
                ************************************************************

Vlaro2-1        AAGTACGGCATCAAGGCTTCCTCCGGAGGCGGCCGCTCCTCGGCGCGCGAGACCATTGCC 473
Vlaro2-2        AAGTACGGCATCAAGGCTTCCTCCGGAGGCGGCCGCTCCTCGGCGCGCGAGACCATTGCC 473
Vd              AAGTACGGCATCAAGGCTTCCTCCGGAGGCGGCCGCTCCTCGGCGCGCGAGACCATTGCC 480
                ************************************************************

Vlaro2-1        CGCGTCGCCGCCGGCGCCGTTGCCGAAAAGTACCTGCGCGAGGCCTACGGCACCGAGATC 533
Vlaro2-2        CGCGTCGCCGCCGGCGCCGTTGCCGAAAAGTACCTGCGCGAGGCCTACGGCACCGAGATC 533
Vd              CGCGTCGCCGCCGGCGCCGTTGCCGAAAAGTACCTGCGCGAGGCCTACGGCACCGAGATC 540
                ************************************************************

Vlaro2-1        ACGGCCTTCACGACGAGCATCGGCAACGTCCACCTCTTCCCGCCGACCCCTGAGCACCCC 593
Vlaro2-2        ACGGCCTTCACGACGAGCATCGGCAACGTCCACCTCTTCCCGCCGACCCCTGAGCACCCC 593
Vd              ACGGCCTTCACGACGAGCATCGGCAACGTCCACCTCTTCCCGCCGACCCCTGAGCACCCC 600
                ************************************************************

Vlaro2-1        TCGCCCTCGACGAACCCCGCCTTCCTCTCGCTCCTGCAGACCATCGACCGCGCCACCGTC 653
Vlaro2-2        TCGCCCTCGACGAACCCCGCCTTCCTCTCGCTCCTGCAGACCATCGACCGCGCCACCGTC 653
Vd              TCGCCCTCGACGAACCCCGCCTTCCTCTCGCTCCTGCAGACCATCGACCGCGCCACCGTC 660
                ************************************************************

Vlaro2-1        GACAAGTTCCTGCCCGTGCGCTGCCCACACACCGAGACGTCCGAGGCCATGAACGACTAC 713
Vlaro2-2        GACAAGTTCCTGCCCGTGCGCTGCCCACACACCGAGACGTCCGAGGCCATGAACGACTAC 713
Vd              GACAAGTTCCTGCCCGTGCGCTGCCCGCACACCGAGACGTCCGAGGCCATGAACGACTAC 720
                ************************** ********************************

Vlaro2-1        ATTGCCGGCTTCCGCGACCGTGACGACTCGATCGGCGGCACCGTCACCTGCGTCATCAAG 773
Vlaro2-2        ATTGCCGGCTTCCGCGACCGTGACGACTCGATCGGCGGCACCGTCACCTGCGTCATCAAG 773
Vd              ATTGCCGGCTTCCGCGACCGTGACGACTCGATCGGCGGCACCGTCACCTGCGTCATCAAG 780
                ************************************************************

Vlaro2-1        AACCCGCCCGCCGGCCTCGGCGAGCCCTGCTTCGACAAGCTCGAGGCCCTGCTCGCCCAC 833
Vlaro2-2        AACCCGCCCGCCGGCCTCGGCGAGCCCTGCTTCGACAAGCTCGAGGCCCTGCTCGCCCAC 833
Vd              AACCCGCCCGCCGGCCTCGGCGAGCCCTGCTTCGACAAGCTCGAGGCCCTGCTCGCCCAC 840
                ************************************************************

Vlaro2-1        GCCATGCTCAGCATCCCGGCCACCAAGGGCTTCGAGATCGGATCGGGCTTTGCCGGCTGC 893
Vlaro2-2        GCCATGCTCAGCATCCCGGCCACCAAGGGCTTCGAGATCGGATCGGGCTTTGCCGGCTGC 893
Vd              GCCATGCTCAGCATCCCGGCCACCAAGGGCTTCGAGATCGGATCGGGCTTTGCCGGCTGC 900
                ************************************************************

Vlaro2-1        GAGGTCCCTGGCTCGACGCACAACGACGCCTTTATCCGCGCCCCCGAGGACGACGCCCGC 953
Vlaro2-2        GAGGTCCCTGGCTCGACGCACAACGACGCCTTTATCCGCGCCCCCGAGGACGACGCCCGC 953
Vd              GAGGTCCCTGGCTCGACGCACAACGACGCCTTTATCCGCGCCCCCGAGGACGACGCCCGC 960
                ************************************************************

Vlaro2-1        GCCGCCGCCGAGACGGCCCGCCTCGGCATCCCGCGCTCCAAGCTCACGACCAAGACCAAC 1013
Vlaro2-2        GCCGCCGCCGAGACGGCCCGCCTCGGCATCCCGCGCTCCAAGCTCACGACCAAGACCAAC 1013
Vd              GCCGCCGCCGAGACGGCCCGCCTCGGCATCCCGCGCTCCAAGCTCACGACCAAGACCAAC 1020
                ************************************************************

Vlaro2-1        TTTAGCGGCGGCATCCAGGGCGGCATCTCCAACGGCGCCCCCATCTACTTCCGCGTCGCC 1073
Vlaro2-2        TTTAGCGGCGGCATCCAGGGCGGCATCTCCAACGGCGCCCCCATCTACTTCCGCGTCGCC 1073
Vd              TTTAGCGGCGGCATCCAGGGCGGCATCTCCAACGGCGCCCCCATCTACTTCCGCGTCGCC 1080
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                ************************************************************

Vlaro2-1        TTCAAGCCCGCCGCCACCATTGGCCAGGACCAGACGACGGCCACCTACGATGGCGAGGAG 1133
Vlaro2-2        TTCAAGCCCGCCGCCACCATTGGCCAGGACCAGACGACGGCCACCTACGATGGCGAGGAG 1133
Vd              TTCAAGCCCGCCGCCACCATTGGCCAGGACCAGACGACGGCCACCTACGATGGCGAGGAG 1140
                ************************************************************

Vlaro2-1        GGCGGCGTCCTCGCCGCCAAGGGCAGGCACGATCCCTGCGTCGTGCCCCGCGCCATCCCC 1193
Vlaro2-2        GGCGGCGTCCTCGCCGCCAAGGGCAGGCACGATCCCTGCGTCGTGCCCCGCGCCATCCCC 1193
Vd              GGCGGCGTCCTCGCCGCCAAGGGCAGGCACGATCCCTGCGTCGTGCCCCGCGCCATCCCC 1200
                ************************************************************

Vlaro2-1        ATTGTCGAGGGCATGGCCGCCATTGTCATCATGGACGCCCTCATGGCCCAGCACGCCCGC 1253
Vlaro2-2        ATTGTCGAGGGCATGGCCGCCATTGTCATCATGGACGCCCTCATGGCCCAGCACGCCCGC 1253
Vd              ATTGTCGAGGGCATGGCCGCCATTGTCATCATGGACGCCCTCATGGCCCAGCACGCCCGC 1260
                ************************************************************

Vlaro2-1        CAGATGACGAGGAGCCTGCTGCCGCCTGTGAAGAAGCCGGAGGCGTAG 1301
Vlaro2-2        CAGATGACGAGGAGCCTGCTGCCGCCTGTGAAGAAGCCGGAGGCGTAG 1301
Vd              CAGATGACGAGGAGCCTGCTGCCGCCTGTGAAGAAGCCGGAGGCGTAG 1308
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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 *Hin*dIII, blotted and probed with hygromycin gene. Analysis of the Southern hybridization showed that all the transformants analysed contained single insertion of T-DNA.

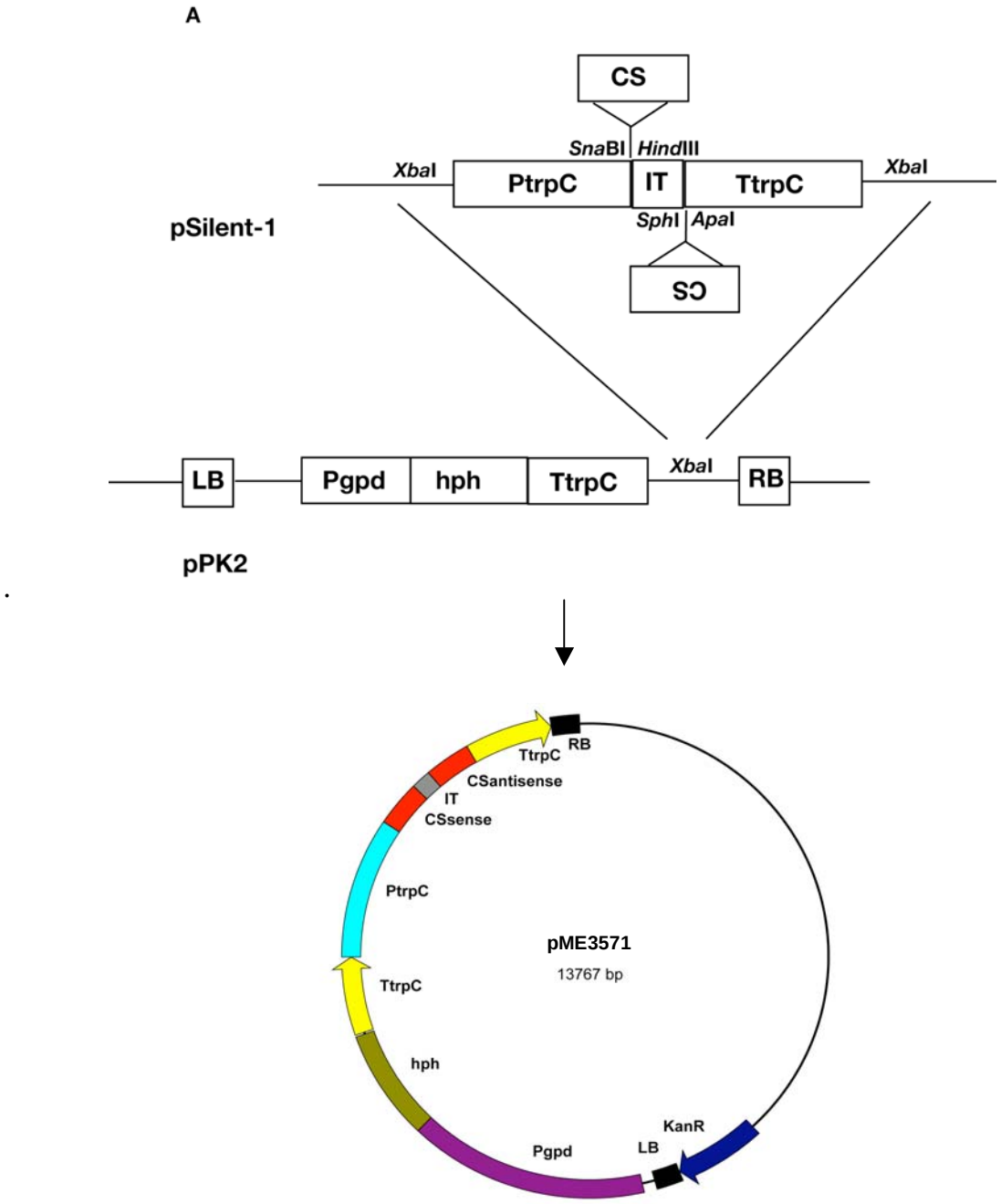


Fig. 6. Schematic representation of the plasmid used for silencing *Vlaro2* in *V. longisporum*. Inverted repeats of *Vlaro2* (CS) were introduced in the pSilent-1 plasmid at the multiple cloning sites (shown in the figure) at 5' and

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. PtrpC, *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.

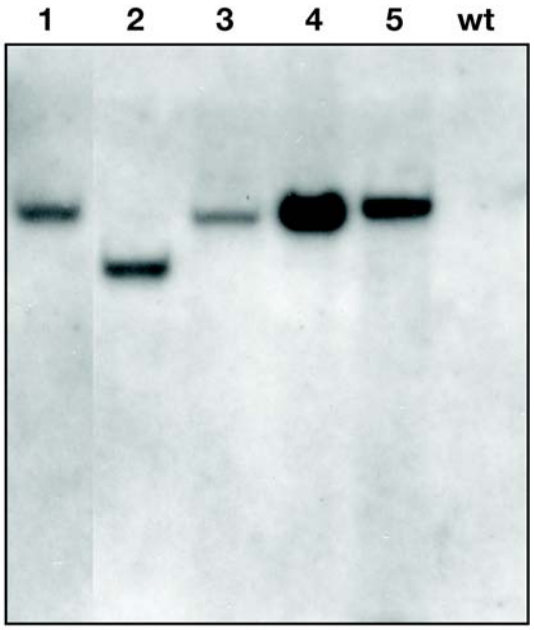


Fig. 7. Southern hybridization analysis of *Vlaro2* silenced mutants (*Vlaro2*sms) to detect integration of T-DNA after ATMT. Five *Vlaro2*sms (1-5) and wild type (wt) gDNA was digested with *Hin*dIII and the hygromycin resistance gene was used as a probe.

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 (*Vlaro2*sms) 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.

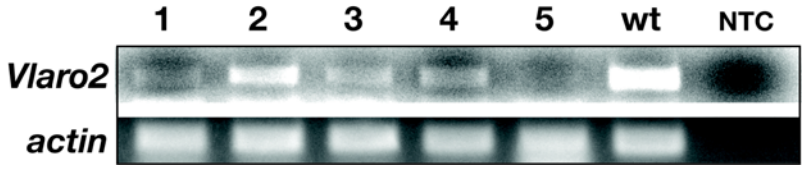


Fig. 8. RT-PCR analysis of *Vlaro2* mRNA expression in the *Vlaro2* silenced mutants. The actin gene was used for control. 1-5, *Vlaro2* silenced mutants; wt, wild type; NTC, no template control; CS, chorismate synthase.

We further examined the knockdown of *Vlaro2* at protein level in the same *Vlaro2*sms by Western hybridization (Fig. 9). Proteins were extracted from the *Vlaro2*sms 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 VlARO2 up to 94% in the *Vlaro2*sms as compared to the wild type. The knockdown of VlARO2 at the protein level corresponds to the RT-PCR analysis of the *Vlaro2*sms. So, the *Vlaro2* silencing via RNAi worked successfully in *V. longisporum* and established gene silencing in *V. longisporum*.

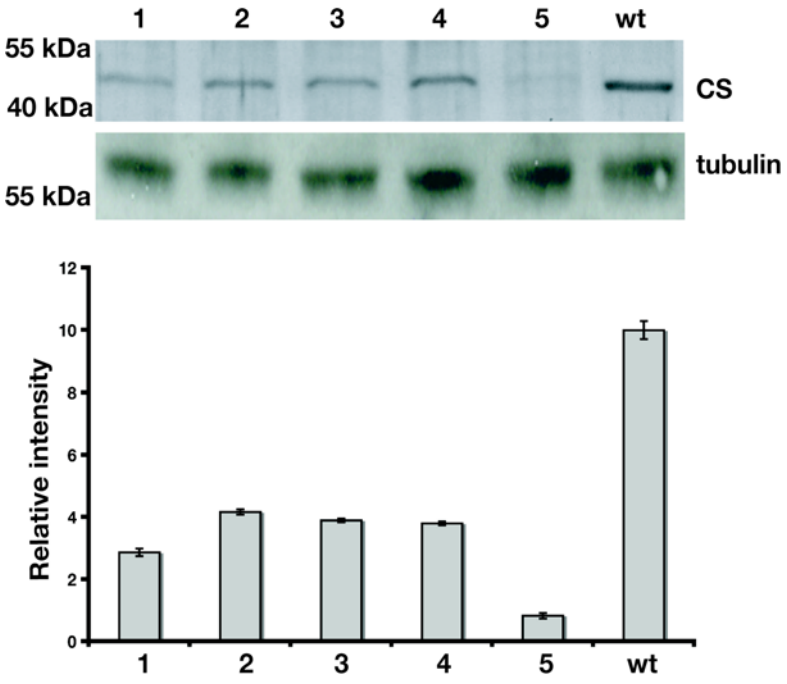


Fig. 9. Western hybridization analysis of CS expression in the *Vlaro2* silenced mutants (*Vlaro2*sms) compared to the wild type. *N. crassa* CS antibody was used for the detection of CS. Rat IgG tubulin was used as a control. 1-5, *Vlaro2*sms; wt, wild type. In the graph, CS expression was quantified and normalized against the tubulin level using Kodak Molecular Imaging software. Data represent average+/- standard deviations of three experimental replicates.

3.1.5 Silencing of *Vlaro2* results in slow growth of *V. longisporum* in presence of 5-MT

We investigated the question whether *Vlaro2* silencing inhibits the growth of *V. longisporum* in the absence of the aromatic amino acids by using plate-based growth assays. *Vlaro2* silenced mutants (*Vlaro2*sms) 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, *Vlaro2*sms 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 *Vlaro2*sms showed less mycelial growth and no conidia were observed. We further analyzed the effect of *Vlaro2* silencing on the radial growth of *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 *Vlaro2*sms was observed to be slightly less than wild type (Fig.10 graph). Therefore, the *Vlaro2*sms, 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 *Vlaro2*sms 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 *Vlaro2*sms 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 *Vlaro2*sms formed short hyphae and no conidia were observed. Interestingly, the radial growth of *Vlaro2*sms 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 *Vlaro2*sms 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 *Vlaro2*sms. For this experiment, *Vlaro2*sms 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 *Vlaro2*sms 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 *Vlaro2*sms. 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 *Vlaro2*sms were inoculated on it. After growth analysis, it was found that the *Vlaro2*sms 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 *Vlaro2*sms 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 *Vlaro2*sms 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 *Vlaro2*sms like the wild type strain.

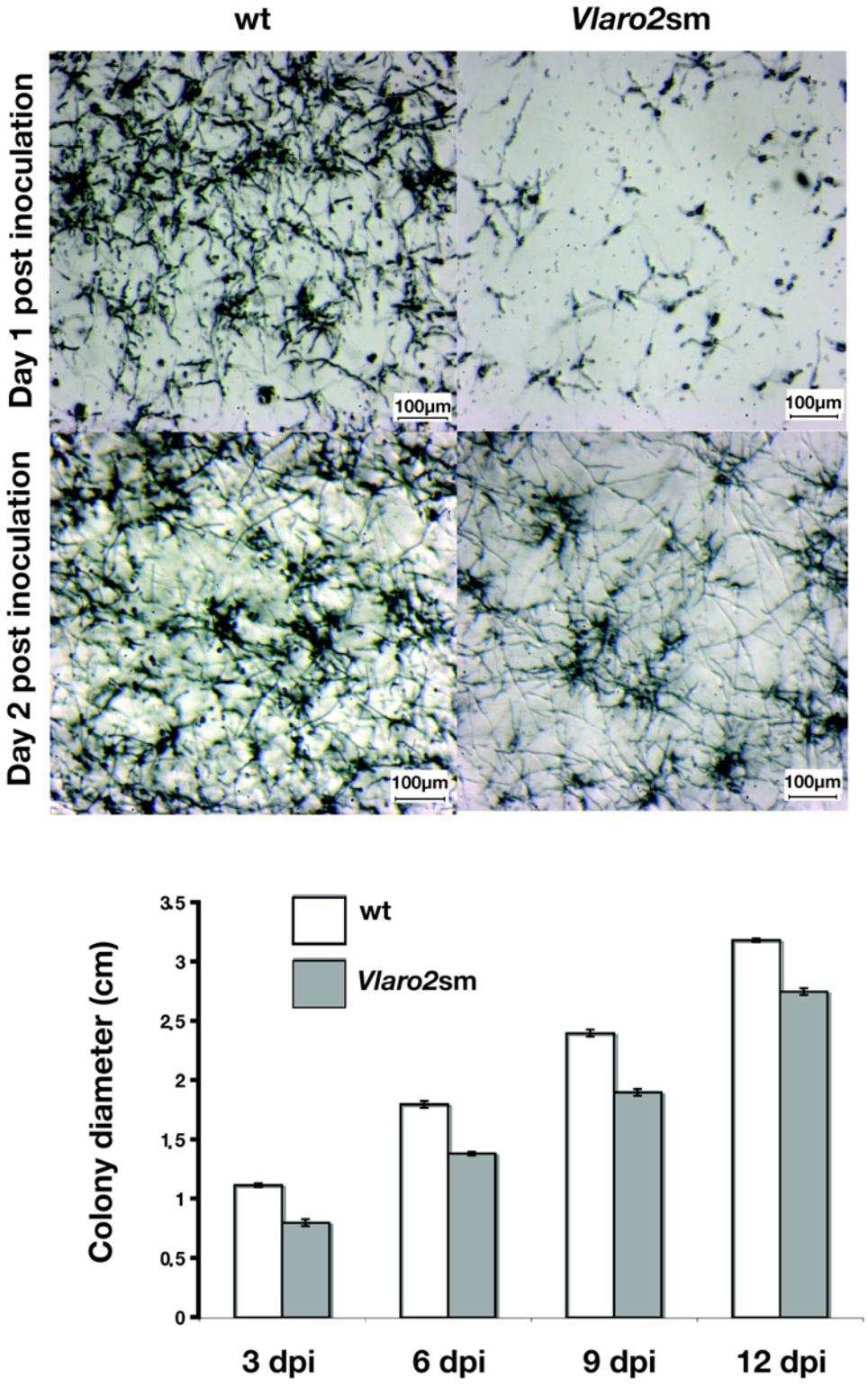


Fig. 10. Light microscopy images of *V. longisporum* wild type (wt) and *Vlaro2* silenced mutant (*Vlaro2*sm) cultured on CDA as observed at one and two days post inoculation. The graph shows the growth rates of wild type (wt) and *Vlaro2* silenced mutant (*Vlaro2*sm) on CDA. Agar plates were inoculated 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.

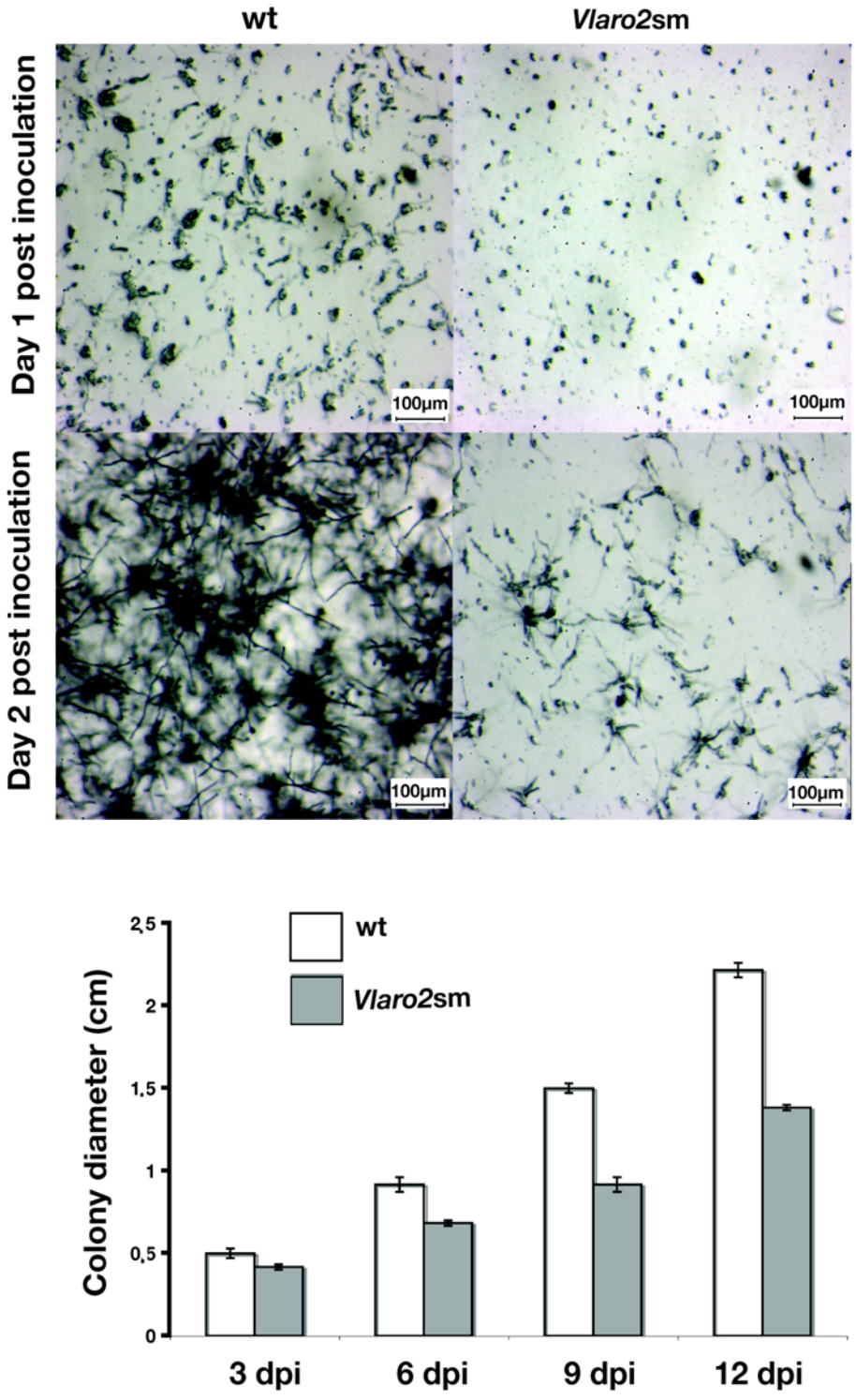


Fig. 11. Light microscopy images of wild type (wt) and *Vlaro2* silenced mutant (*Vlaro2*sm) cultured on CDA with 5 mM 5-methyl-DL-tryptophan (5-MT) as observed at one and two days post inoculation. The graph shows the growth rates of wild type (wt) and *Vlaro2* silenced mutant (*Vlaro2*sm) on CDA amended 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 (*Vlaro2*sms) 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).

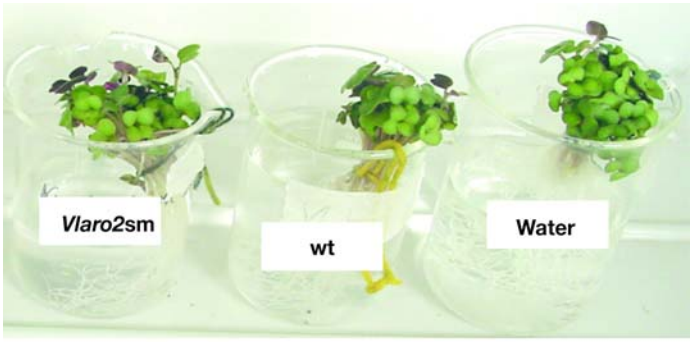


Fig. 12. Rapeseed infection by root-dipping inoculation. A week old rapeseed seedlings were inoculated by different strains, *Vlaro2* silenced mutant (*Vlaro2*sm), wild type (wt) and water and observed weekly until five weeks after inoculation.

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

Score	Symptom description
1	no symptoms
2	slight symptoms on oldest leaves (yellowing)
3	slight symptoms on next younger leaves
4	About 50% of leaves show symptoms
5	>50% of leaves show symptoms
6	up to 50% of leaves dead
7	>50% of leaves dead
8	only apical meristem still alive
9	plant dead

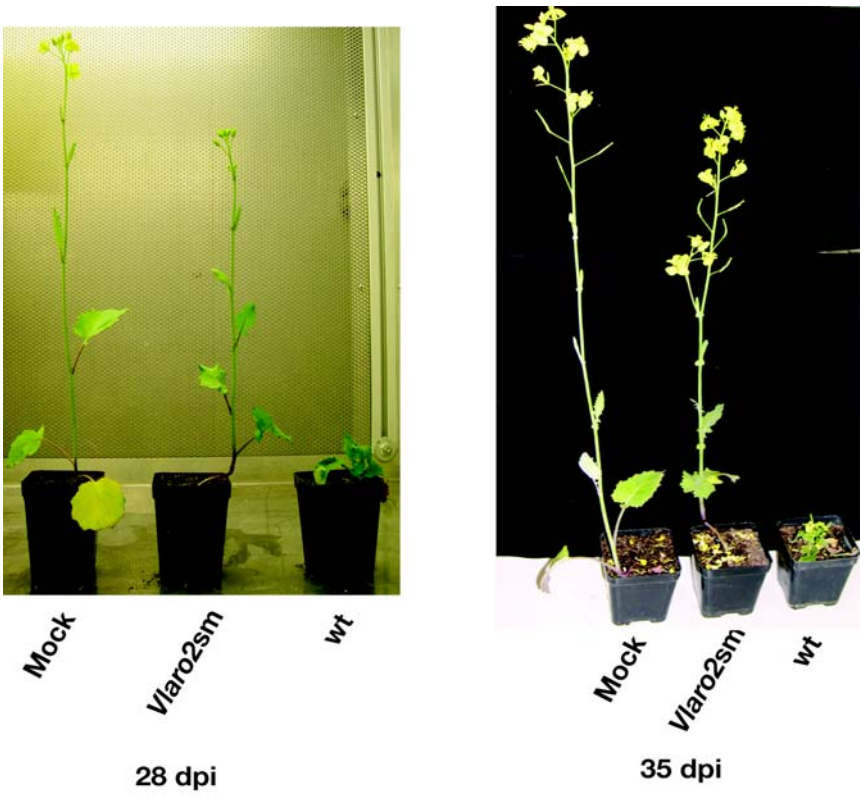


Fig. 13. Rapeseed infection assay. Rapeseed plants inoculated with water (mock), *Vlaro2* silenced mutant (*Vlaro2* sm) and wild type (wt) at 28 and 35 days post inoculation (dpi).

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 *Vlaro2*sms 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 *Vlaro2*sm 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 *Vlaro2*sms' 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 *Vlaro2*sm 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.

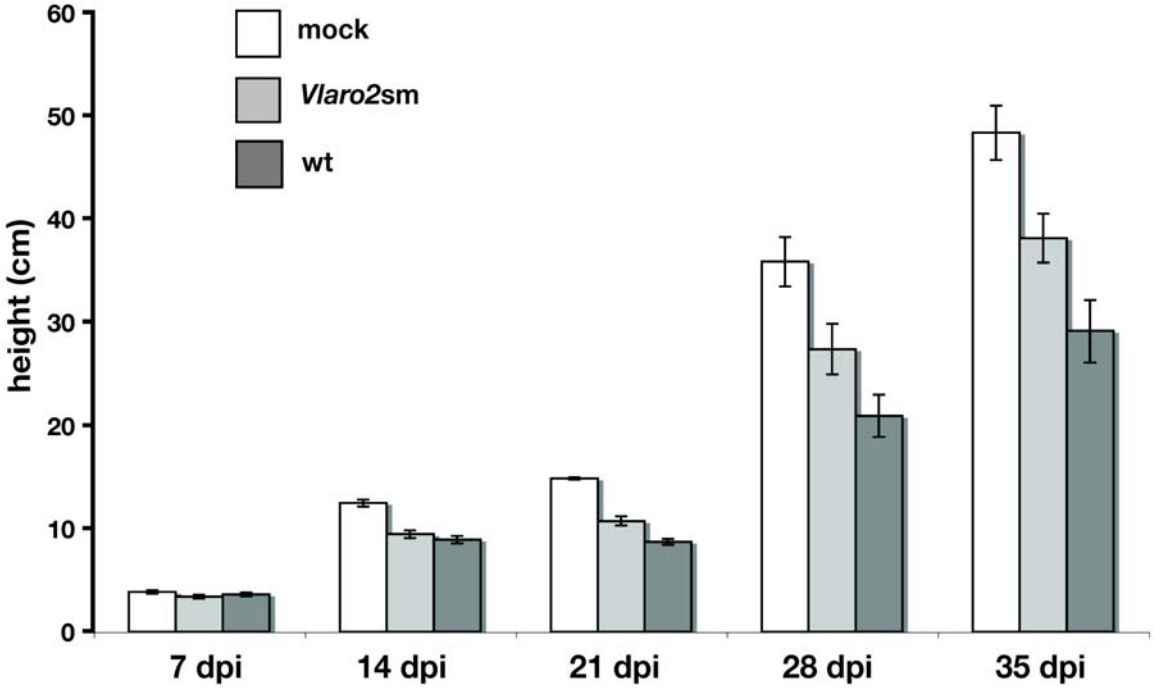


Fig. 14. Assessment of stunting of rapeseed due to *V. longisporum* infection. The height of 24 replicates each of rapeseed plants infected with wild type (wt) and *Vlaro2* silenced mutant (*Vlaro2* sm) was measured at 7, 14, 21, 28 and 35 days post inoculation (dpi). For comparison, the height of rapeseed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

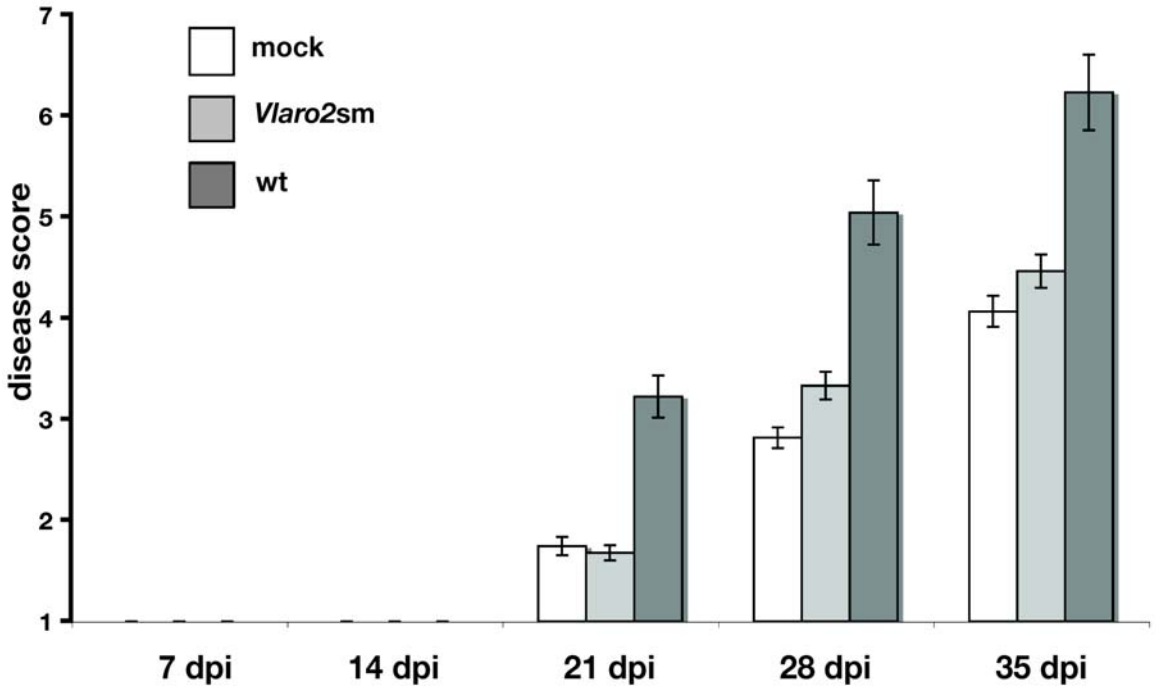


Fig. 15. Assessment of disease development by scoring for disease symptoms according to Zeise, 1992. Plants were scored for disease symptoms at 7, 14, 21, 28 and 35 days post inoculation (dpi). Twenty four replicates for each strain were assessed. Data represent average+/- standard deviations of twenty four experimental replicates. wt: wild type; *Vlaro2* sm: *Vlaro2* silenced mutant ; mock: mock inoculation with water.

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 secondaey 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 *Vlaro2*sm 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 *Vlaro2*sm. At 28 and 35 dpi, the amount of *V. longisporum* DNA was observed to be significantly lower (57-89%) in *Vlaro2*sm 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 *Vlaro2*sm 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 *Vlaro2*sm is significantly less compared to the wild type in the rapeseed.

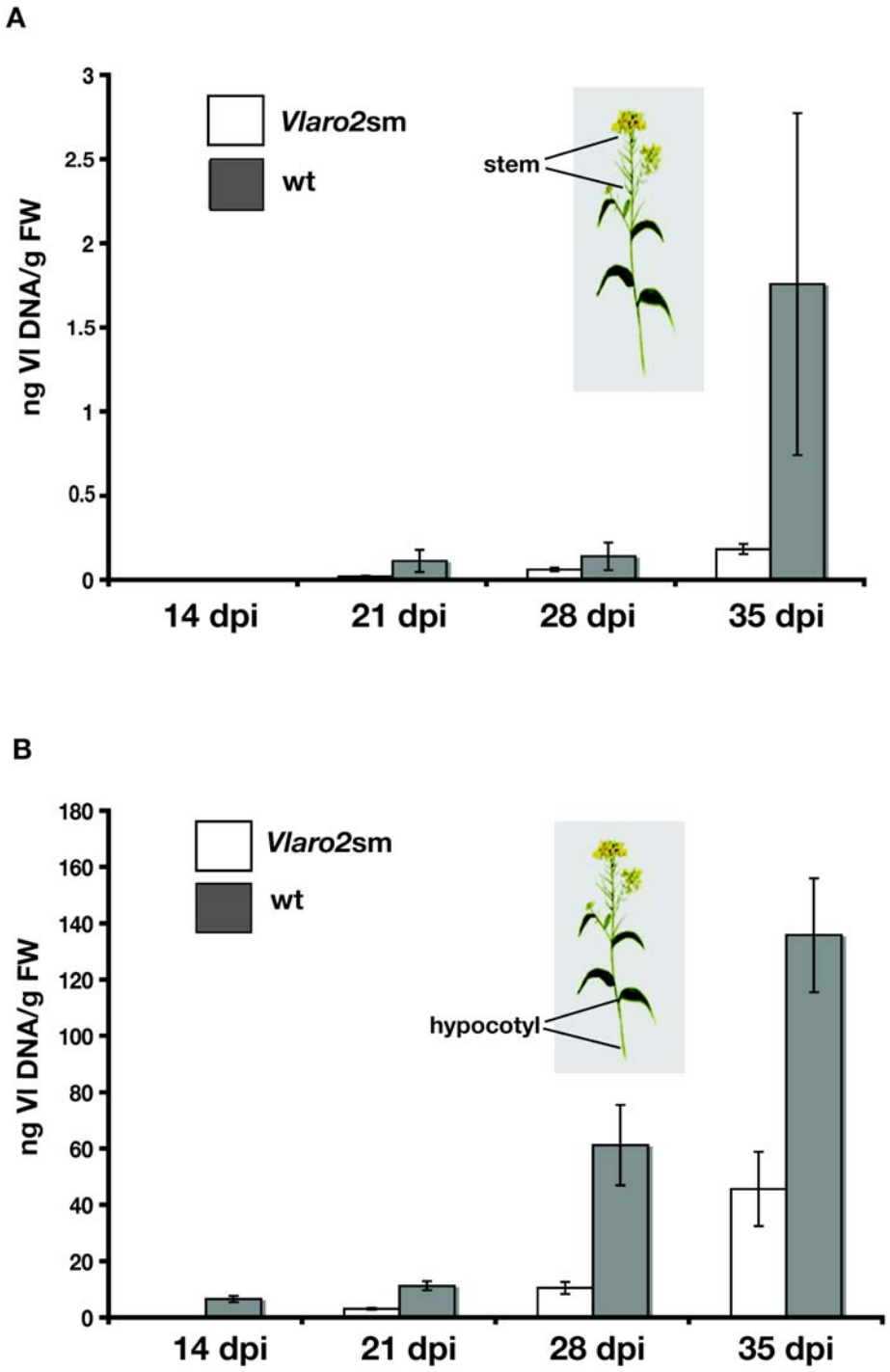


Fig. 15'. A. *V. longisporum* DNA concentration in the plant stem. *V. longisporum* DNA was measured with real-time PCR in stem of *B. napus* inoculated with *Vlaro2* silenced mutant (*Vlaro2*sm) and wild type (wt) at 21, 28 and 35 dpi. **B.** *V. longisporum* DNA concentration in the hypocotyl. *V. longisporum* DNA was measured with real-time PCR in hypocotyl of *B. napus* inoculated with *Vlaro2*sm (CS sm) and wild type (wt) at 14, 21, 28 and 35 dpi. Data represent average+/- standard deviations of three experimental replicates. ng Vl DNA/g FW: nanogram *V. longisporum* DNA / gram fresh weight of plant tissue. (In the inset a representative rapeseed plant depicting the stem (5-6 cm from the top) and the hypocotyl harvested for the quantification of the *V. longisporum* DNA. Source: 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 occured. The colony became dark hence melanization occured 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 appeared before the fourth day of growth in contrast to the SXM. On CDA, the colony became dark and melanization occured 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*

Nutrient media	Days post inoculation	-Xylem sap	+Xylem sap
SXM	2	Small hyphae, few conidia	longer hyphae, more conidia
	3	Small bud spores; dark coloured colony	Bigger bud spores; dark coloured colony
	8	No. of conidia = 4.31×10^6 conidia/ml	No. of conidia = 10.07×10^6 conidia/ml
CDA	2	Long hyphae, some conidia	Good mycelial growth, more conidia
	3	No bud spores, good mycelial growth, many conidia; light coloured colony	Bud spores, good mycelial growth, many conidia; dark coloured colony
	8	No. of conidia = 1.59×10^6 conidia/ml	No. of conidia = 7.13×10^6 conidia/ml

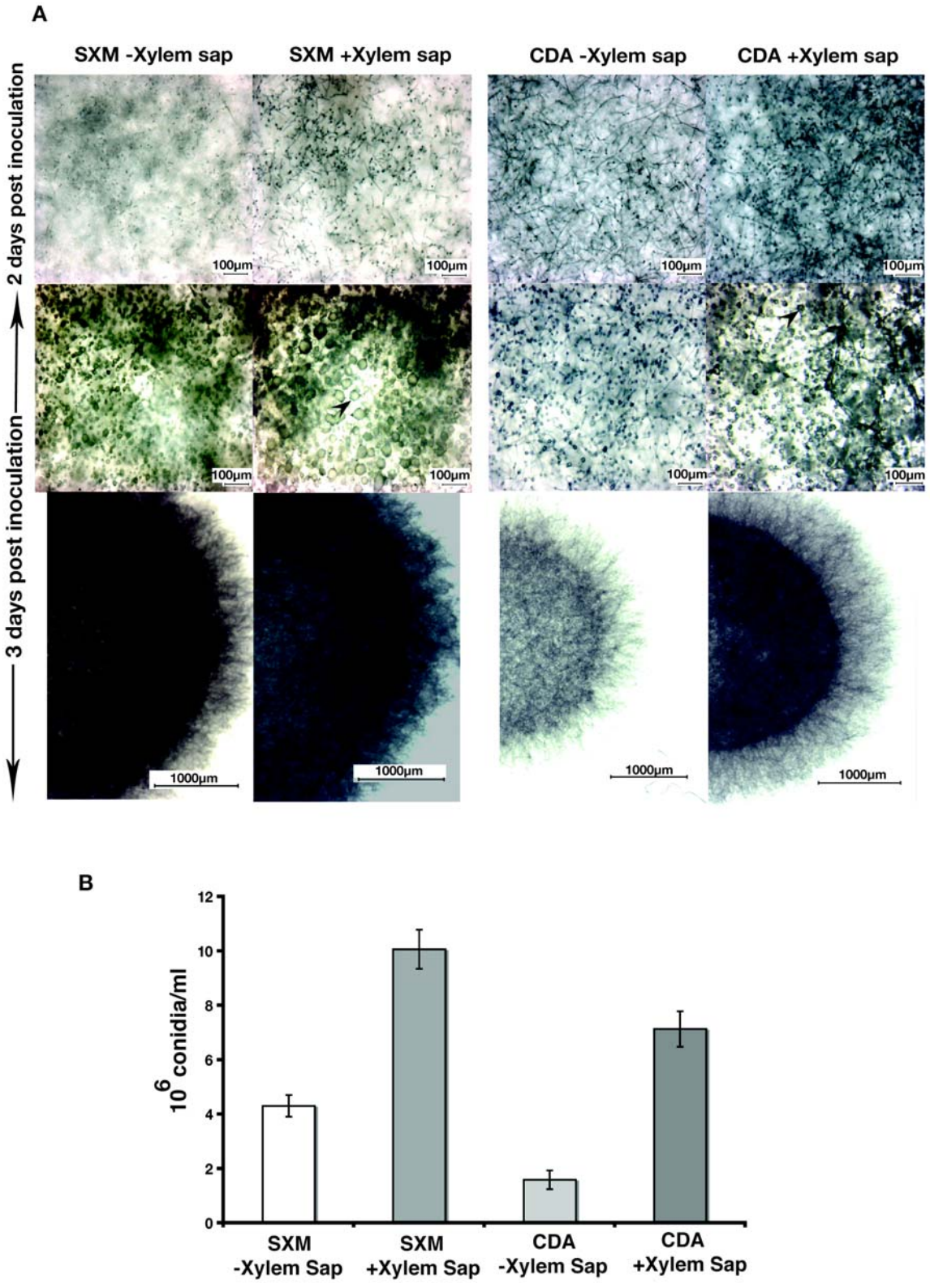


Fig. 16 A. Light microscopy images of *V. longisporum* colonies growing on SXM and CDA with/without xylem sap from *B. napus* as observed after two and three days of inoculation. Arrows indicate bud spores. **B**. The number of *V. longisporum* conidia/ml from each growth condition was analysed eight 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.

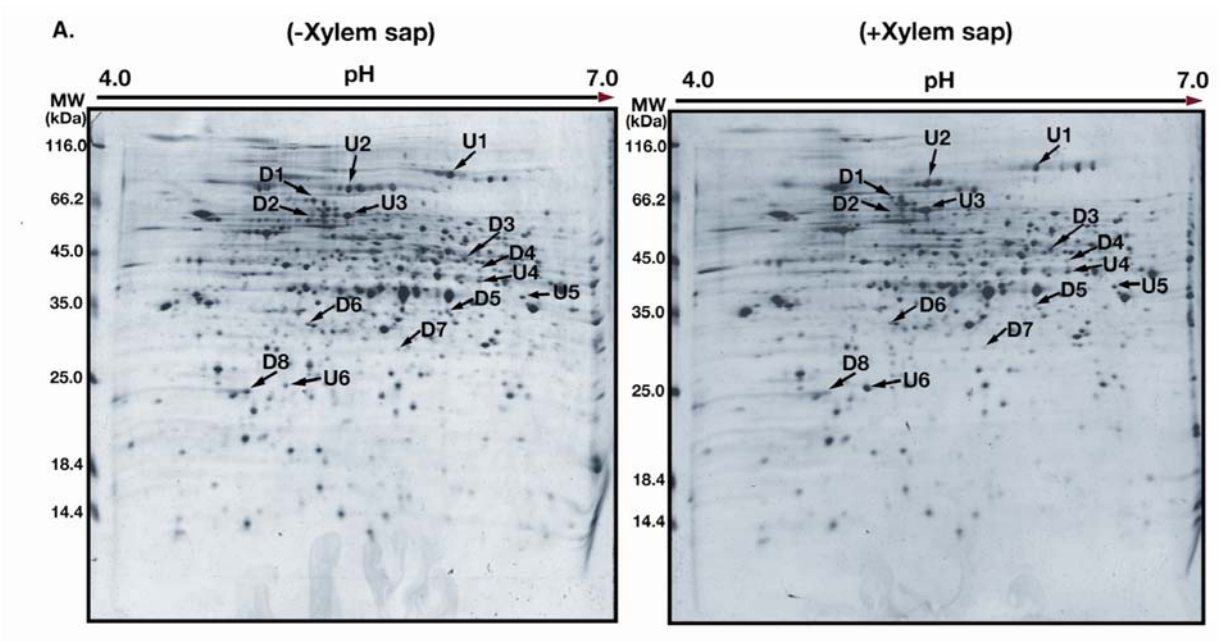
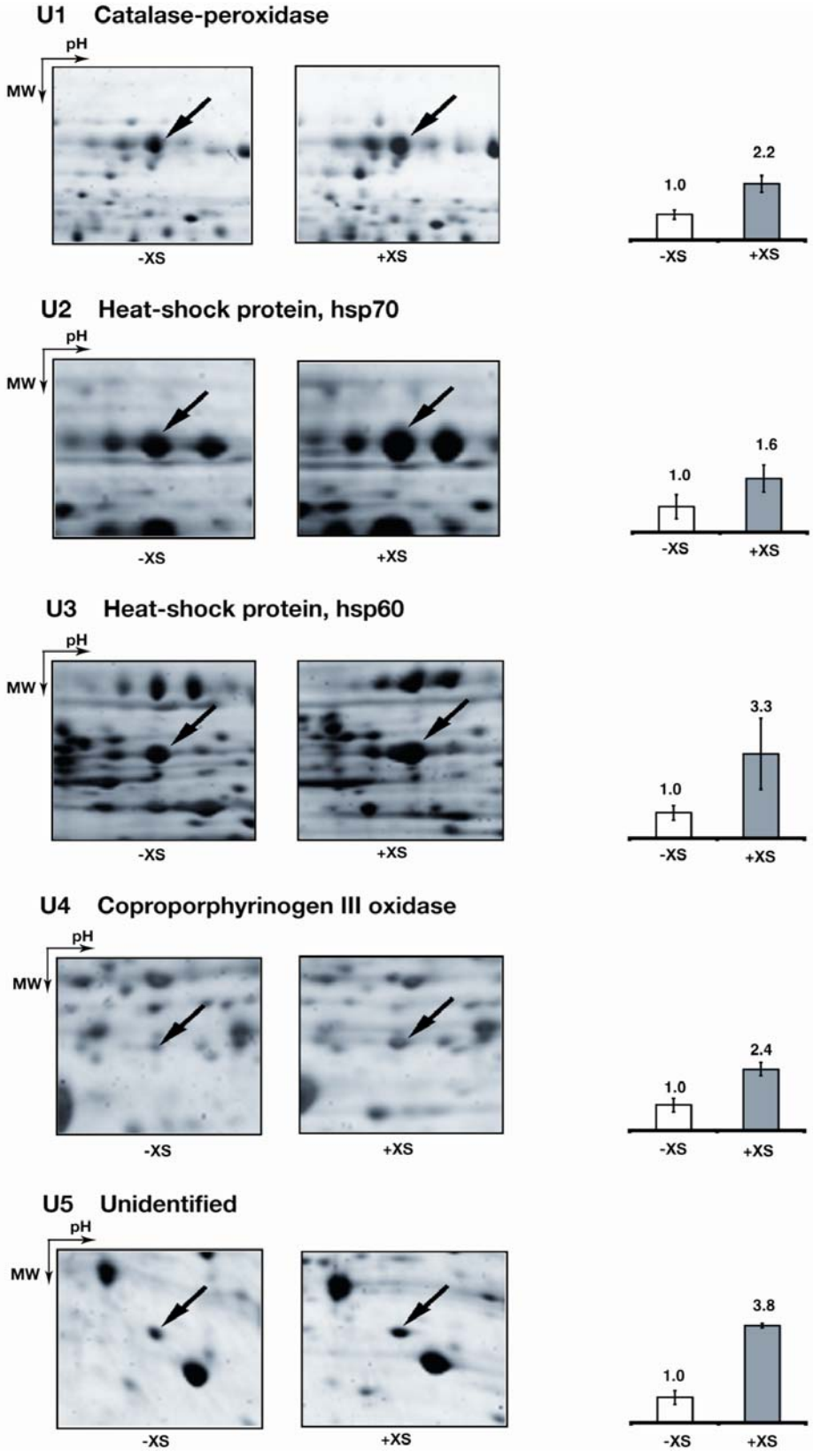


Fig. 17. The 2-DE gels of the *V. longisporum* proteome untreated/treated with xylem sap from rapeseed. These are representative figures from three biological and two technical replicates of each condition. Differentially expressed proteins are marked with arrows and labelled, U1-U6 (Upregulated protein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


U1 Catalase-peroxidase
pH
MW
-XS
+XS
1.0
2.2
-XS
+XS
U2 Heat-shock protein, hsp70
pH
MW
-XS
+XS
1.0
1.6
-XS
+XS
U3 Heat-shock protein, hsp60
pH
MW
-XS
+XS
1.0
3.3
-XS
+XS
U4 Coproporphyrinogen III oxidase
pH
MW
-XS
+XS
1.0
2.4
-XS
+XS
U5 Unidentified
pH
MW
-XS
+XS
1.0
3.8
-XS
+XS

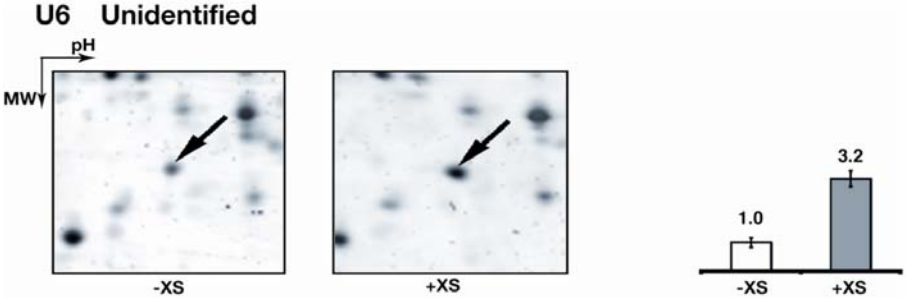
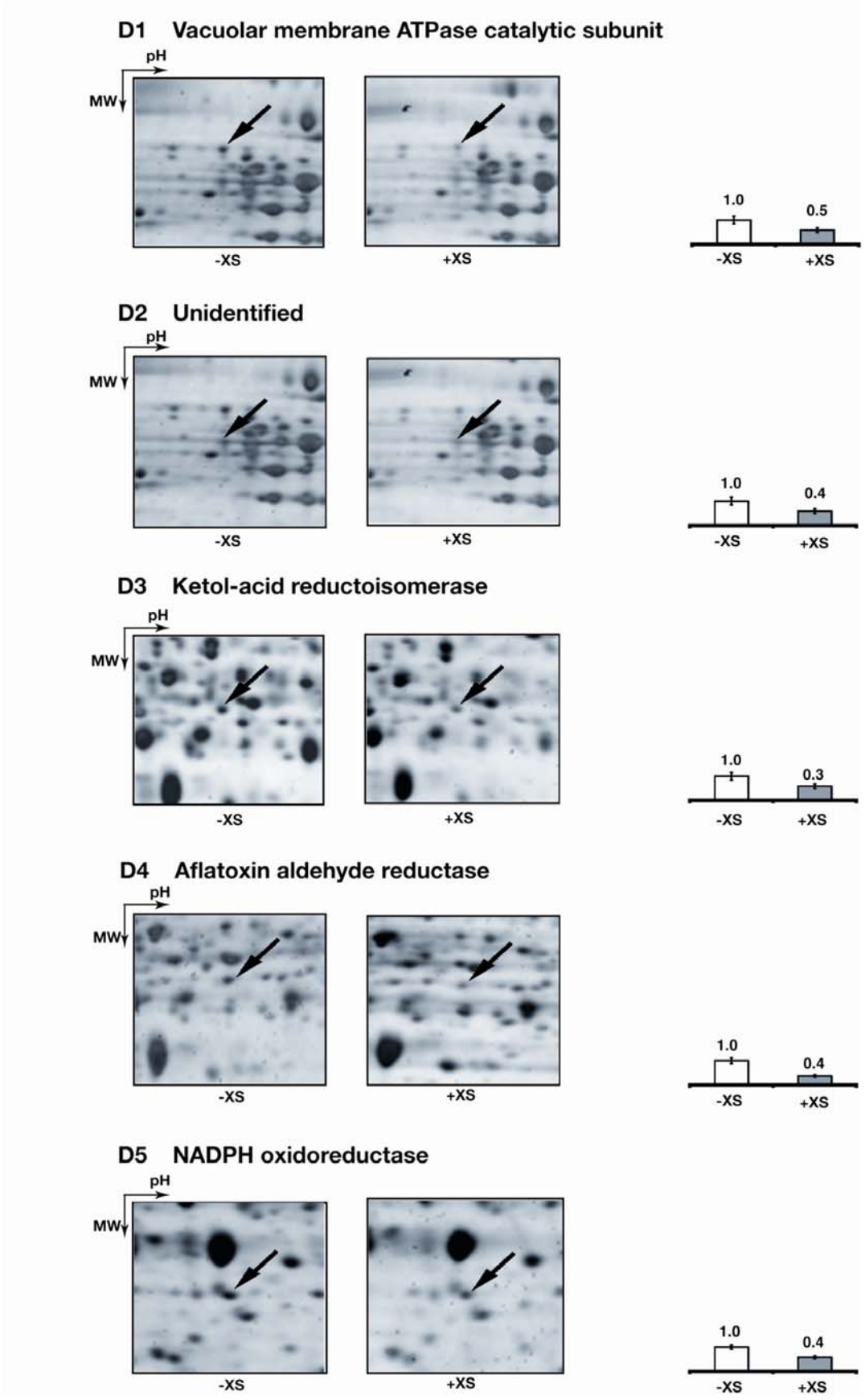


Fig. 18. Close-up views of the regions of the 2-DE gels that show significant upregulation in protein expression (untreated (-XS) versus treated (+XS) with xylem sap from rapeseed). The arrowheads indicate the differentially expressed proteins. The expression histograms show the differential protein 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*.


D1 Vacuolar membrane ATPase catalytic subunit
pH
MW
-XS
+XS
1.0
0.5
-XS
+XS
D2 Unidentified
pH
MW
-XS
+XS
1.0
0.4
-XS
+XS
D3 Ketol-acid reductoisomerase
pH
MW
-XS
+XS
1.0
0.3
-XS
+XS
D4 Aflatoxin aldehyde reductase
pH
MW
-XS
+XS
1.0
0.4
-XS
+XS
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pH
MW
-XS
+XS
1.0
0.4
-XS
+XS

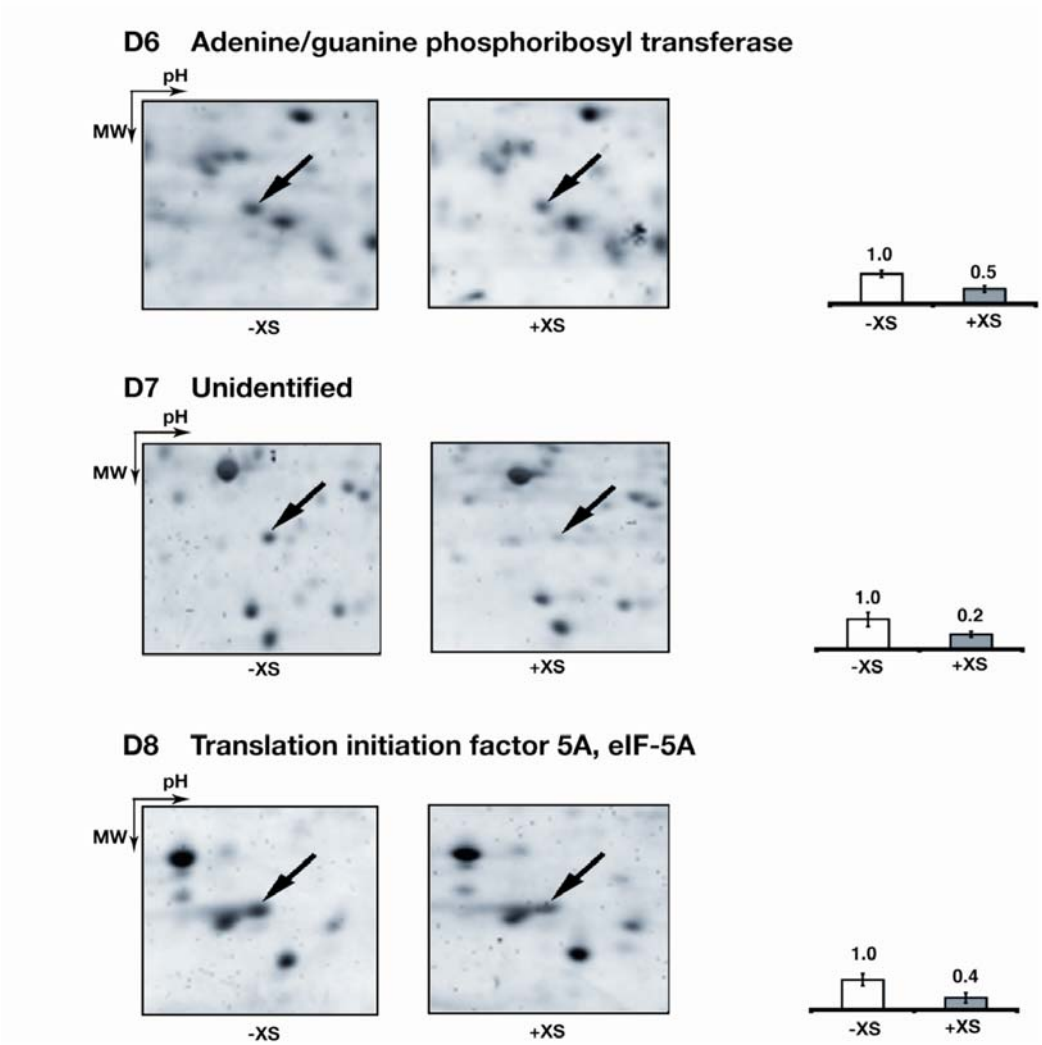

Fig. 19. Close-up views of the regions of the 2-DE gels that show significant downregulation in protein expression (untreated (-XS) versus treated (+XS) with xylem sap from rapeseed). The arrowheads indicate the differentially expressed proteins. The expression histograms show the differential protein 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^e/pI^e: 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).

Protein (Spot no.)	Organism	NCBI accession no.	MW^e/pI^e	Matched Peptides	X-corr value	Sequence coverage (%AA)
Catalase -peroxidase (U1)	*Aspergillus nidulans*	CAC59821	90/ 6.1	FLENPDQFADAFAR SPAGAHQYVAK	4.75 3.03	3.38
Heat shock protein 70 (U2)	*Neurospora crassa*	XP_961753	78/ 5.4	STNGDTHLGGEDFDIHL VR MLGNFQLVGIPPAHR MKETAESFLSKPVK	5.73 4.40 4.09	9.30
Heat shock protein 60 (U3)	*Neurospora crassa*	XP_956500	60/ 5.5	TNEVAGDGTTSATVLAR VEFEKPLILLSEK FVDALNATR TIIENAGLEGSVVVGK AAVEEGILPGGTALIK NVAAGCNPMDLR AIFSETVKNVAAGCNPM DLR GQLQVAAGCNPMDLR LSGGVAVIK	6.17 4.46 4.17 3.75 3.49 3.36 2.95 2.88 2.56	20.73
Copropor -phyrinogen III oxidase (U4)	*Verticillium dahliae*	BQ111120	43/ 6.2	GGVGVSVVYGGTLPK YVEFNLVHDR	4.16 3.25	2.76
Vacuolar membrane ATPase catalytic subunit A (D1)	*Verticillium dahliae*	BQ110481	66/ 5.2	LGEMPADQGFPAYLSAK TTLIANTSNMPVAAR DQGLDVAMMADSSSR	4.39 4.29 3.56	22.60
Ketol-acid reductoisome -rase precursor (D3)	*Neurospora crassa*	XP_961335	46/ 6.2	NDTLALIGYGSQGHGQG LNLR VEVPTDVDVILVAPK NLFDVDEAISR	3.89 3.02 2.99	13.18
Aflatoxin aldehyde reductase (D4)	*Verticillium dahliae*	BQ110032	45/ 6.2	IILGLMTFGPSESDGAR ATPFAETLEALDK	4.02 3.15	9.37
NADPH oxidoreductase (D5)	*Verticillium dahliae*	BQ110643	38/ 6.1	SALAGIDAVVSTLGAPA VGEPQR NLVEAAVEAGVQR VKEVVVDYNDPASLK EVVVDYNDPASLK IKTEELLVEK	5.39 4.47 4.21 3.49 3.14	27.70
Hypothetical protein AN9083.2 (conserved domain: Adenine phosphoribosyl transferase) (D6)	*Aspergillus nidulans*	XP_682352	34/ 5.2	VLIVDDIIATGGSAK GFLFGPGLALR LPGPCVTAEYQK EYGTDFFQMQEDAIKPG QK	4.98 3.44 3.12 2.58	8.85
Translation initiation factor eIF-5A (D8)	*Verticillium dahliae*	BQ110791	25/ 4.9	KLEDLSPSTHNMDVPNV TR VHIVATDIFTGK LEDLSPSTHNMDVPNVT R	4.31 3.57 3.52	12.20

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 ADLVFGSHAELR 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 H_2O_2 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 *VlcpeA-1*.

```
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    V  G  G  G  G  T  R  N  R  D  W  W  P  N  A
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    L  G  N  D  F  D  Y  A  A  A  F  N  S  L  D
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    N  D  V  R  Y  S  G  G  N  K  G  D  K  G  P
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    G  S  L  V  T  D  E  G  H  D  K  S  T  H  T
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    R  G  L  Q  K  P  L  G  A  A  H  M  G  L  I
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    V  T  W  T  A  T  P  T  K  W  S  N  K  Y  F
1036 gagtacctcttcaagtacgagtgggagctcaccaagagccctgct
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```

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   D  A  Y  D  S  S  K  K  H  R  P  T  M  L  T
1171 accgatctgtccatgcgcttcgatcccgagtacgagaagatctct
   T  D  L  S  M  R  F  D  P  E  Y  E  K  I  S
1216 cgccgtttcctcgagaacccagaccagtttgccgatgttttcgct
   R  R  F  L  E  N  P  D  Q  F  A  D  V  F  A
1261 cgcgcctggttcaagctccttcaccgtgaccttggccccaaggct
   R  A  W  F  K  L  L  H  R  D  L  G  P  K  A
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   S  Q  F  I  S  V  A  W  G  A  S  S  T  F  R
1486 ggcagcgacaagcgtggtggtgccaacggcgctcgtatccgcctc
   G  S  D  K  R  G  G  A  N  G  A  R  I  R  L
1531 gcccctcagaaggactgggaggtcaacaaccccgcccagctggcc
   A  P  Q  K  D  W  E  V  N  N  P  A  Q  L  A
1576 aaggttctgcaggccctcgagggtgtgcagaagagcttcaacgac
   K  V  L  Q  A  L  E  G  V  Q  K  S  F  N  D
1621 gctcagcagggtggcaagaaggtgtctctcgccgacttgatcgtt
   A  Q  Q  G  G  K  K  V  S  L  A  D  L  I  V
1666 ctggctggtaacgccgctgttgagaaggctgcctcggccgccggc
   L  A  G  N  A  A  V  E  K  A  A  S  A  A  G
1711 cacagcgtcaccgtccccttcactcctggccgtggcgacgccacg
   H  S  V  T  V  P  F  T  P  G  R  G  D  A  T
1756 caggagcagaccgacattgagtccgtcagccacctcgagcctttc
   Q  E  Q  T  D  I  E  S  V  S  H  L  E  P  F
1801 gccgacggcttccgcaactacggccactcgaccgaccgcgtcaag
   A  D  G  F  R  N  Y  G  H  S  T  D  R  V  K
1846 actgagcagttccttgttgaccgtgctcaccttctgacgctgtcg
   T  E  Q  F  L  V  D  R  A  H  L  L  T  L  S
1891 gcggctgagctgaccgccctcgtcggtggcctccgcgttctgaac
   A  A  E  L  T  A  L  V  G  G  L  R  V  L  N
1936 accaactacgacggatcccagcatggcgtcctgaccaagcgcccc
   T  N  Y  D  G  S  Q  H  G  V  L  T  K  R  P
1981 ggccagctgtccaacgacttctttgtcaacctgctcgacatgagc
   G  Q  L  S  N  D  F  F  V  N  L  L  D  M  S
2026 acggcatggaaggctaccggcagcgacgacgagctcttcgagggt
   T  A  W  K  A  T  G  S  D  D  E  L  F  E  G
2071 agcgaccgcaagactggcgacaagaggtggactgccacgcgtgcg
   S  D  R  K  T  G  D  K  R  W  T  A  T  R  A
2116 gatctcgttttcggatcccacgccgagctgcgcgcccttgccgaa
   D  L  V  F  G  S  H  A  E  L  R  A  L  A  E
2161 gtgtacggcagcgccgatggcgagaagaagtttgtcaacgacttt
   V  Y  G  S  A  D  G  E  K  K  F  V  N  D  F
2206 gtcgcggcgtggaccaaggtctcgaacctcgaccgcttcgatgtc
   V  A  A  W  T  K  V  S  N  L  D  R  F  D  V
2251 aagaaggcgcctgccgtcaagacgtcgagccgcctgtaa 2289
      K  K  A  P  A  V  K  T  S  S  R  L  *
```

Fig. 20. Open reading frame of *VlcpeA-1* and the deduced amino acid sequence, VlCPEA-1. *Underlined* : peptides that matched for identification of spot U1 in the proteomic analyis 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 (VlCPEA-1) of 762 amino acids with a predicted molecular mass of 83.54 kDa. VlCPEA-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. VlCPEA-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* VlCPEA-1 (Fig. 21). Therefore, it belongs to the KatG proteins of Class I of the plant peroxidase superfamily.

```
Vl          MAESE---SKCPVHQ--LNNVGGGGTRNRDWWPNALKLNILRQHTDVTNPLGNDFDYAAA 55
Vd          MAESE---SKCPVHR--LNNVGGGGTRNRDWWPNALKLNILRQHTDVTNPLGSDFDYAAA 55
An          MGS-----NECPYSR-QNANIGGGGQNNRDWWPDDLKLNILRQHNSVSNPLDKGFDYTAA 54
Fj          MENQSNDISKCPFHNGSMDNQAASGTKNNDWWPKQLKVNILRQNSSLSNPLSKDFDYAEA 60
Mg          -------MGECPLRT---ANVAGGGTRNRDWWPNTLKLNILRQHTEATNPYDPNFDYAEA 50
                      .:**       * ...* .*.****. **:*****:.. :** . .***: *
                                 SQXWWPADXGXY     R--WH
Vl          FNSLDYNAVKKDLKDLMTDSQDWWPADFGHYGGLFVRMAWHSAGTYRVFDGRGGGGQGQQ 115
Vd          FNSLDYNAVKKDLRDLMTDSQDWWPADFGHYGGLFVRMAWHSAGTYRVFDGRGGGGQGQQ 115
An          FNSLDYFGLKRDLEALMTDSQDWWPADFGHYGGLFIRMAWHSAGTYRVFDGRGGGGQGQQ 114
Fj          FKTLDLEAVKKDLHVLMTDSQDWWPADFGHYGGLFIRMAWHSAGTYRVHDGRGGAGAGQQ 120
Mg          FKSLDYEGLKKDLRALMTDSQEYWPADFGHYGGLFVRMAWHSAGTYRVMDGRGGGGQGQQ 110
            *::**  .:*:**. ******::***********:*********** *****.* ***

Vl          RFAPLNSWPDNVSLDKARRLLWPIKQKYGNKISWADLLLLTGNVALESMDFKTFGFAGGR 175
Vd          RFAPLNSWPDNVSLDKARRLLWPIKQKYGNKISWADLLLLTGNVALESMDFKTFGFAGGR 175
An          RFAPLNSWPDNVSLDKARRLLWPIKQKYGSKISWADLLILAGNVALESMGFKTFGFAGGR 174
Fj          RFAPLNSWPDNVSLDKARRLLWPIKQKYGQKISWADLMILTGNVALESMGFKTFGFAGGR 180
Mg          RFAPLNSWPDNVSLDKARRLLWPIKQKYGNKISWADLMLLTGNVALEDMGFKTFGFAGGR 170
            *****************************.*******::*:******.*.**********

Vl          ADVWEADESVYWGGETTWLGNDVRYSGGNKG-DKGPGSLVTDEGH--DKSTHTRGLQKPL 232
Vd          ADVWEADESVYWGGETTWLGNDVRYSGGNKG-DKGPGSLVTDEGH--DKGIHTRDLQKPL 232
An          SDTWEADQSVFWGGEKEWLGNDVRYLNG-------------------------ELDNPL 208
Fj          ADVWEPDESVYWGSETTWLGGDERYNNGSDGVPKDHGVVSADDDA--DGKVHSRNLEKPL 238
Mg          PDTWEADESTYWGGETTWLGNEVRYSSGNEG-HKESGVIDGSESKKGHKDIHTRDLEKPV 229
            .*.**.*:*.:**.*. ***.: ** .*                          *::*:
             AXXMGLIYVN
Vl          GAAHMGLIYVNPEGPDGNPDPVAAAHDIRTTFSRMAMNDEETVALIAGGHSFGKTHGAGP 292
Vd          GAAHMGLIYVNPEGPDGNPDPVAAAHDIRTTFGRMAMNDEETVALIAGGHSFGKTHGAGP 292
An          AASHMGLIYVNPEGPNKNPDPVLAAKDIRITFGRMAMNDEETVALIAGGHTFGKTHGAGP 268
Fj          AAVQMGLIYVNPEGPDGNPDPILAAKDIRDTFGRMAMNDEETVALIAGGHTFGKTHGA-A 297
Mg          SAAHMGLIYVNPEGPDGIPDPVAAARDIRTTFSRMAMNDEETVALIAGGHTVGKTHGAAP 289
            .* :***********:  ***: **:*** **.****************:.****** .
```

```
                    GXXPXXAXXEXQGLGW
Vl          NDKIGAEPEGASLEAQGFGWQNGYKSGKGPDTITSGLEVTWTATPTKWSNKYFEYLFKYE 352
Vd          NDKIGAEPEGAPLEAQGFGWQNGYKSGKGPDTITSGLEVTWTATPTKWSNKYFEYLFKYE 352
An          ATHLGKEPHGAGIELQGLGWESGFESGTGRHAITSGLEVIWTKTPTKWSNQFFEYLFKYD 328
Fj          SSHVDKEPEAAGLELQGFGWKNSFGSGKGADAITSGLEVTWTKTPTQWSNNFFENLFAFE 357
Mg          SDNVGPEPEAAPIENQGLGWSNKHGSGKGPDTITSGLEVIWTKEPAKFTMNYLEYLFKYE 349
              ::. **..* :* **:**.. . **.* .:******* **  *:::: :::* ** ::


Vl          WELTKSPAGANQWVAKTDDEIIPDAYDSSKKHRPTMLTTDLSMRFDPEYEKISRRFLENP 412
Vd          WELTKSPAGANQWVAKTDDEIIPDAYDSSKKHRPTMLTTDLSLRFDPEYEKISRRFLENP 412
An          WELTKSPAGAHQYVAKGVEPFIPDPFDPSIKHPPRMLTTDLSLRYDPEYEKISRRFLENP 388
Fj          WELSKSPAGAHQWVAKNAEAIIPDAFDSTKKHLPTMLTTDLSLRLDPEYEKISRRFLENP 417
Mg          WELTKSPAGANQWVAKNAEEFIPDAFDPSKKHKPRMLTTDLSLRFDPEYEKISRRFLENP 409
            ***:******:*:***  : :***.:*.: ** * *******:* ***************


Vl          DQFADVFARAWFKLLHRDLGPKARYLGPEIPAEDLLWQDPIPAVDHPLIDESDIAALKKE 472
Vd          DQFADAFARAWFKLLHRDLGPKARYLGPEIPAEDLLWQDPIPAVDHPLIDESDVAALKKE 472
An          DQFADAFARAWFKLTHRDVGPRVLYQGPEVPSEVLIWQDPVPPLDHPVIDNDDIATLKKA 448
Fj          DQFADAFSRAWFKLTHRDMGPRARYLGPDVPQEVLLWQDPIPEVNHKLIDENDIKQLKEK 477
Mg          EQFKDAFARAWFKLLHRDMGPRSRWLGPEVPKETLLWEDPIPTPDHPIIDGSDVDSLKKA 469
            :** *.*:****** ***:**:  : **::* * *:*:**:*  :* :** .*:  **:


Vl          ILSSGPEPSQFISVAWGASSTFRGSDKRGGANGARIRLAPQKDWEVNNPAQLAKVLQALE 532
Vd          ILSSGPEPSQFISVAWGASSTFRGSDKRGGANGARIRLAPQKDWEVNNPAQLAKVLQALE 532
An          ILNSGISHTDLFSTAWASASTFRGSDKRGGANGARIRLSPQKNWKVNSQPWLSESLAALE 508
Fj          ILNSGLSISQLVAAAWASASTFRGSDKRGGANGARVRLAPQKDWEVNNPAKLAQVLSKLE 537
Mg          ILATGVAPSKLIQTAWASASTFRGGDKRGGANGARIRLEPQNKWEVNNPQQLAEVLKALE 529
            ** :*   :.:. .**.::*****.**********:** **:.*:**.   *:: *  **


Vl          GVQKSFNDAQQGGKKVSLADLIVLAGNAAVEKAASAAGHSVTVPFTPGRGDATQEQTDIE 592
Vd          GVQKSFNDAQQGGKKVSLADLIVLAGNAAVEKAASAAGHSVTVPFTPGRGDATQEQTDIE 592
An          KIQKQFNDAQSTDKRVSLADLIVLAGAASLEKAARDAGHNVSVSFTPGRTDATQEQTDVD 568
Fj          TIQTEFNASQNDGKKVSLADLIVLAGSAGVEKAAKDAGSSVTVSFNPGRMDASAEETDVE 597
Mg          GVKADFEKS---GKKVSIADLIVLAGVAAVEQAAG-----VPVPFTPGRGDATQEQTDVE 581
             :: .*: :   .*:**:******** *.:*:**      *.*.*.*** **: *:**::


Vl          SVSHLEPFADGFRNYGHSTDRVKTEQFLVDRAHLLTLSAAELTALVGGLRVLNTNYDGSQ 652
Vd          SVSHLEPFADGFRNYGHSTDRVKTEQFLVDRAHLLTLSAPELTALVGGLRVLNTNYDGSQ 652
An          SFNNLEPIADGFRNYGRGTPRVLTEDFLIDKAQLLNLSPPELTVLIGGLRVLNNNYDRSN 628
Fj          SFGYLEPKADGFRNYRKTKSAVSTEELLIDKANLLTLTAPELTVLLGGLRVLDINADGSK 657
Mg          SFTHLEPAADAFRNYGKGTSRVTTEQIMVDRAQQLTLTAPELTVLVGGLRVLGANYDGSS 641
            *.  *** **.**** : .  * **::::*:*: *.*:..***.*:******. * * *.


Vl          HGVLTKRPGQLSNDFFVNLLDMSTAWKATGSDDELFEGSDRKTGDKRWTATRADLVFGSH 712
Vd          HGVLTKRPGQLSNDFFVNLLDMSTAWKATGSDDELFEGSDRKTGDKRWTATRADLVFGSH 712
An          LGVFTKRPGQLTNDFFVNLLDMGVQWKPADDTNEIFIGSDRKTGQARWKASRADLVFGSH 688
Fj          NGVFTHRPGQLTNDFFVNLLDMNTQWQAVSNDKELYAGNDRSTGQPKWIATRADLVFGSN 717
Mg          HGVWTDKPGKLTNDFFVTLLDPYTSWKSVDG--EVFEGTNSKSGK-KLTGTRADLVFGSH 698
             ** *.:**:*:*****.***  . *:....  *:: *.: .:*. :  .:********:


Vl          AELRALAEVYGSADGEKKFVNDFVAAWTKVSNLDRFDVKKAPAVKTSSRL-- 762
Vd          AELRALAEVYGSADGEKKFVNDFVAAWTKVSNLDRFDVKKAPAVKTSSRL-- 762
An          AELRAISEVYGSSDGEAKFVKDFVAAWEKVSNLDRFDLKQTGLAQRIKPQL- 739
Fj          SELRAVAEVYASTDANEKFVNDFIKAWTKVMNLDRFDLA------------ 756
Mg          SELRALAEVYGSADGQQKFTKDFVAAWDKVMNLDRFDVRRGIYDETRLKSKL 750
            :****::***.*:*.: **.:**: ** ** ******:
```

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.

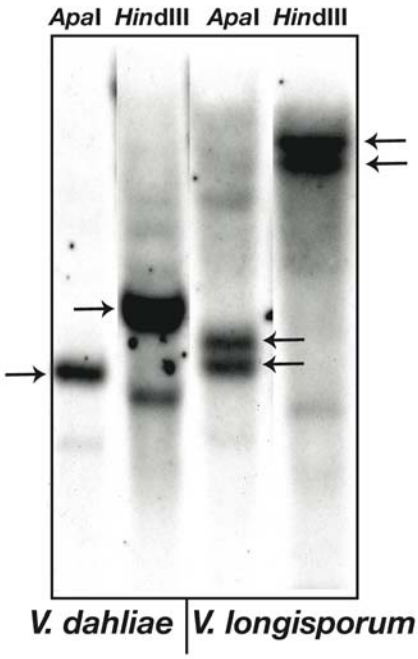

Fig. 22. Determination of the isogene, *VlcpeA-2* of *VlcpeA-1* by Southern hybridization analysis of *V. dahliae* and *V. longisporum*. The genomic DNA was digested with *Apa*I and *Hin*dIII. A 500 bp sequence of *VlcpeA-1* was used as a probe. Arrows indicate the signal generation by probe binding.

It was determined whether *VlcpeA-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 *VlcpeA-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 *Apa*I and *Hin*dIII (Fig. 22). We also confirmed this result by using the *VlcpeA-1* fragment from *V. dahliae* as the probe. These results demonstrated that *VlcpeA-1* has an isogene, *VlcpeA-2*.

As VlCPEA 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 VlCPEA that was upregulated in the 2DE analysis when *V. longisporum* was treated with xylem sap.

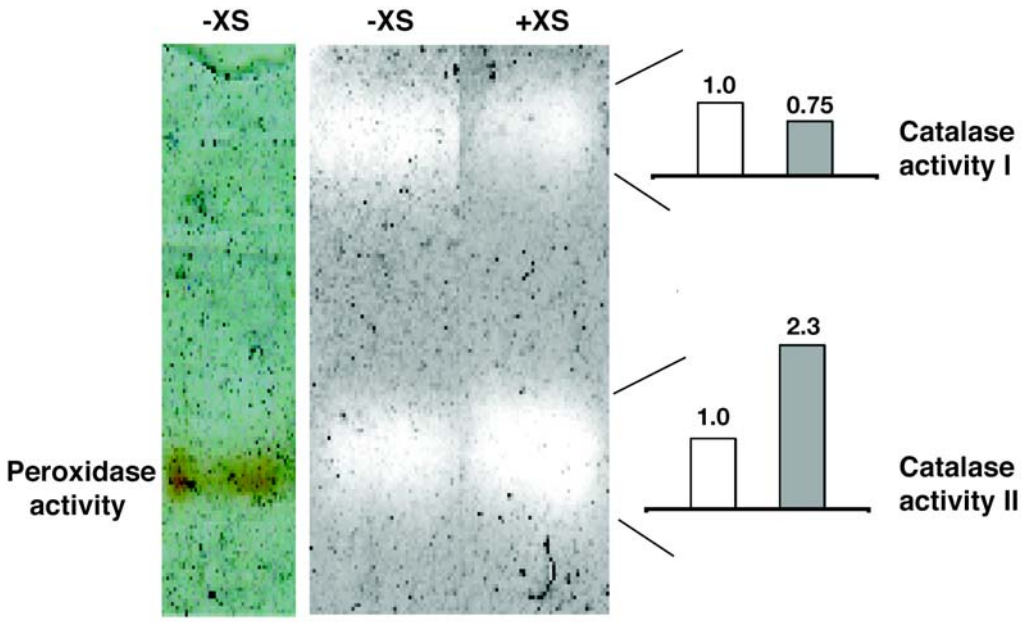

Fig. 23. In-gel catalase and peroxidase assay. Equal amounts (10 µg) of protein extracts from *V. longisporum* untreated (-XS) /treated (+XS) with xylem sap of *B. napus* were separated in native polyacrylamide gel and stained for catalase activity according to Zou *et al*., 2000. The catalase activity 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 (l*eft*).

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 Δ*aro2* (yeast deletion mutant of CS) to characterize the unclassified CS as bifunctional if they restore prototrophy to the yeast Δ*aro2* 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 Δ*aro2* 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).

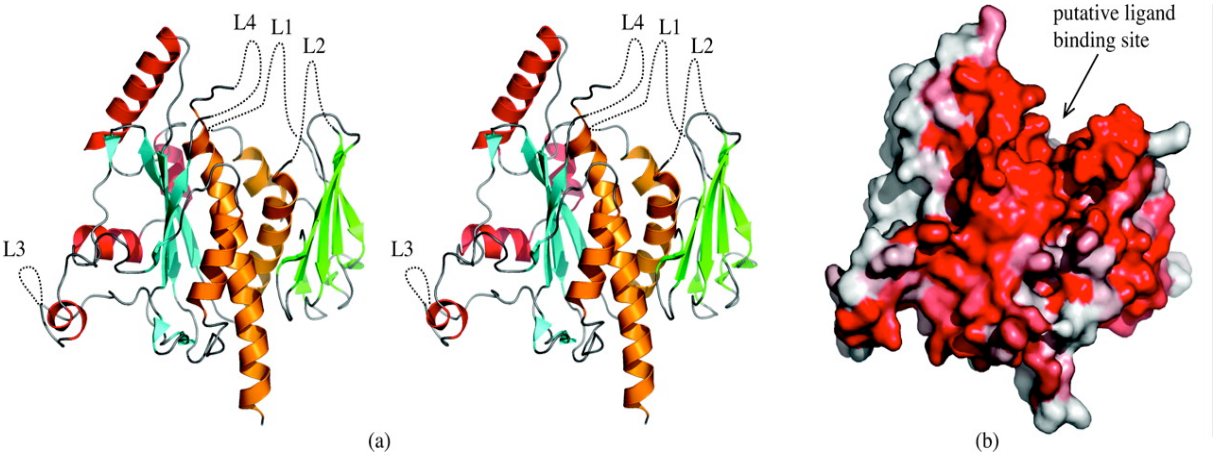

Fig. 1. (a) stereo ribbon representation of the CS monomer, with the disordered regions schematically represented as *black interrupted lines*: *L1* (12 residues), *L2* (41 residues), *L3* (8 residues), and *L4* (23 residues). *L1*, *L2*, and *L4* cluster in the same region of the molecule. **(b)** surface 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 VlARO2.

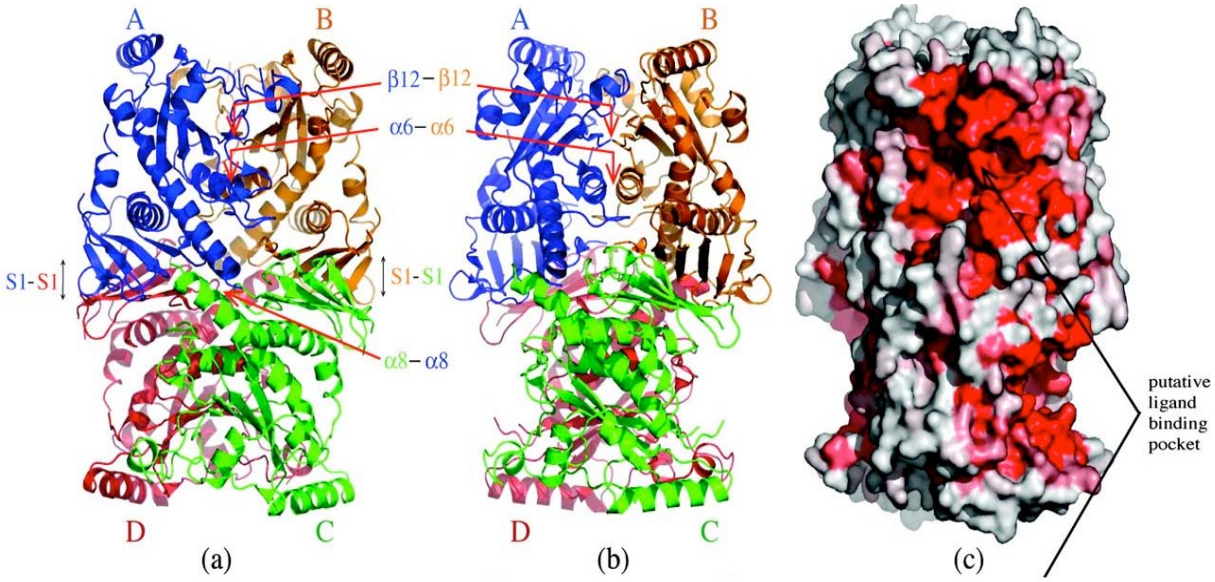

Fig. 2. Representation of the CS tetramer with the most prominent elements involved in packing indicated. The four monomers A–D are coloured *blue*, *orange*, *green*, and *red*, respectively. (*a*) view showing the packing between the A/D (packing of the S1 sheet) and A/C monomers (α8/ α8 interaction). (b) view looking down at the A/B dimer interface illustrating the α6/ α6 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

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 *Vlaro2*sms (*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 VlARO2 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, *FKS1* 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 *Vlaro2*sms 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 *Vlaro2*sms 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 *Vlaro2*sms as it is an inhibitor of tryptophan biosynthesis and thus completely abolished the production of

tryptophan in the *Vlaro2*sms. 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 *Vlaro2*sms 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 Δ*Vlaro2* 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 *Vlaro2*sm to study its propagation and infection of the rapeseed plants by *in planta* assay. The *Vlaro2*sm 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 *Vlaro2*sm, showed slow initial growth compared to the wild type in the culture medium, the propagation of *Vlaro2*sm 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 *Vlaro2*sm infected plants compared to the wild type infected plants, at all the observed stages of infection. These results indicate that the *Vlaro2*sm is not able to propagate in the rapeseed plant like the wild type. The reduced infection in rapeseed plants by the *Vlaro2*sms 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

stimulated by auxin production by the fungus. The *Vlaro2*sms 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 expreseed 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.

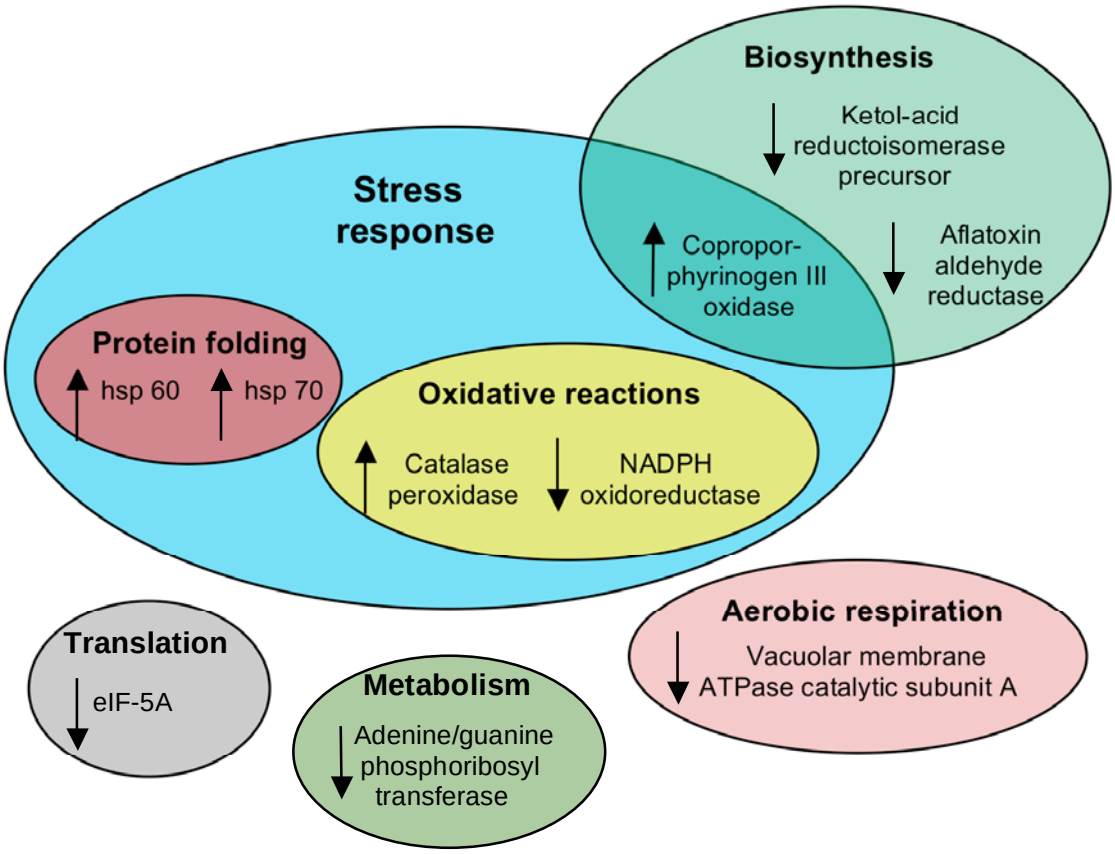

Fig. 3. Schematic representation of the functional classification of the proteins identified in the proteomic analysis of *V. longisporum* treated with xylem sap of *B. napus.* All the upregulated proteins are involved in stress response. *Upright arrow*: upregulation, *downright arrow*: downregulation..

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

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 (Cabiscol *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 VlCPEA which was observed to be upregulated in the 2DE analysis when *V. longisporum* was treated with xylem sap. The upregulation of VlCPEA 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 Δ*aro2*, 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 (*Vlaro2*sm*)*, the phenotypic analysis was performed. The *Vlaro2*sm 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 *Vlaro2*sm 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 *Vlaro2*sm. 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 *Vlaro2*sm 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 *Vlaro2*sm infected host plants was less as compared to the control. It could be that the fungus produces lower amounts of secondary metabolites in the *Vlaro2*sm 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 *Vlaro2*sm 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 *Vlaro2*sm 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.

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