Determination of fungal gene expression *in planta* by qRT-PCR and characterization of putative pathogenicity related genes of *Verticillium longisporum*

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Chapter 1: Preface (Literature Review)

1. Gene expression, function and regulation

"Understanding gene function is the key to understanding disease" (Oxford Centre for Gene Function). Analysis of gene expression, function and regulation presently are essential for the phytopathogical research, and is of increasing significance in the development of possible treatments. Gene expression (Figure 1) involves the synthesis of functional gene products like RNA and protein which is responsible for the development and function of all organisms and give rise to its phenotype.

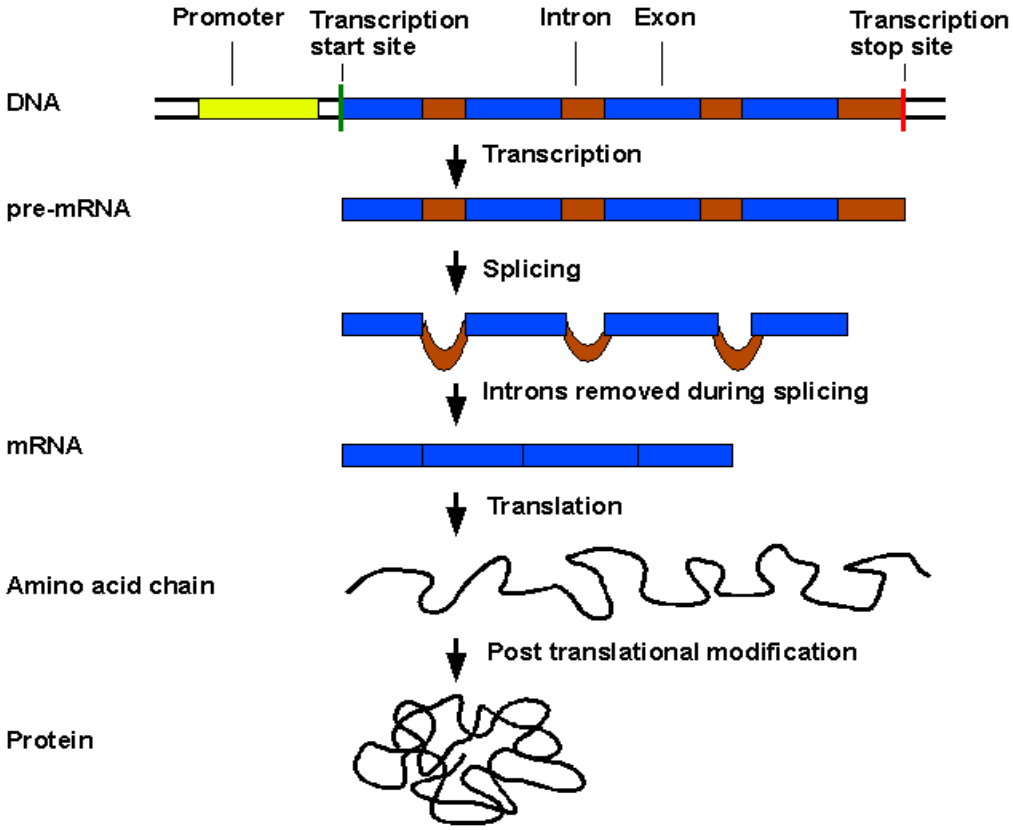

Figure 1: Gene expression Process in higher organisms (http://www.news-medical.net/health/What-is-Gene-Expression.aspx). The gene expression process modulates possible in several steps, including the transcription, RNA splicing, translation, and post-translational modification of a protein.

Gene regulation may control the cellular differentiation, cell growth, timing, location, and amount of gene expression etc. In this work, we demonstrated RNA based antisense and

interference gene knock-down, and GFP (Green Fluorescent Protein) based report gene fusion to determinate the expression, function and regulation of some putative pathogenicity related genes of *Verticillium longisporum.*

2. Determination of gene expression level by qRT-PCR

The polymerase reaction first described by Kleppe and co-workers in 1971 (1) and developed by Kary Mullis into the polymerase chain reaction (PCR) 1983 (2) allows a short DNA sequence to be amplified many times by enzymatic replication therefore the name chain reaction". Today, PCR is thanks to its simplicity widely practised in many organism related areas and for many different applications (3, 4), Which include cloning, sequencing, gene functional analysis and the diseases diagnosis (5). However, the exponential nature of DNA amplification is prone to change the experimental data with significant standard errors because of variable amplification efficiently in the different tubes (6).

The Quantitative Real-Time Reverse Transkriptase PCR (qRT-PCR) was therefore developed to measure the amount of PCR products using fluorescent dye, such as SYBR Green (7), EvaGreen (8) in real time. Then the starting cDNA or RNA of gene expression can be calculated quantitatively in regression. We optimised a procedure in this study for determination *in planta* fungal gene expression and to characterise the gene silenced mutant strains by qRT-PCR.

3. Transcriptome profiling after *Verticillium* infection

3.1. Transcriptome profiling by cDNA-AFLP

The transcriptome screening reflects gene expression variation due to external environmental conditions, it includes all mRNA transcripts which are being actively expressed at any given condition. Recently DNA microarrays have become the standard tool for genome expression analysis with full-sequence information. A cDNA-AFLP based transcript screening method can allow the same analysis without prior sequence knowledge. The metabolites in xylem vessels of host plant rapes were used as elicitor for fungel cultivation *in vitro* for cDNA-AFLP technique based, pathogenicity related transcriptome monitoring (Arne Weiberg 2008). A comparison between treatment with xylem sap from healthy plants and from infected plant gave us a map of a difference of

transcript-derived fragments (TDFs) (Figure 2). An analysis of those TDFs, which were changed especially under induction treatment with xylem sap of *Brassica napus*, was planned as next step.

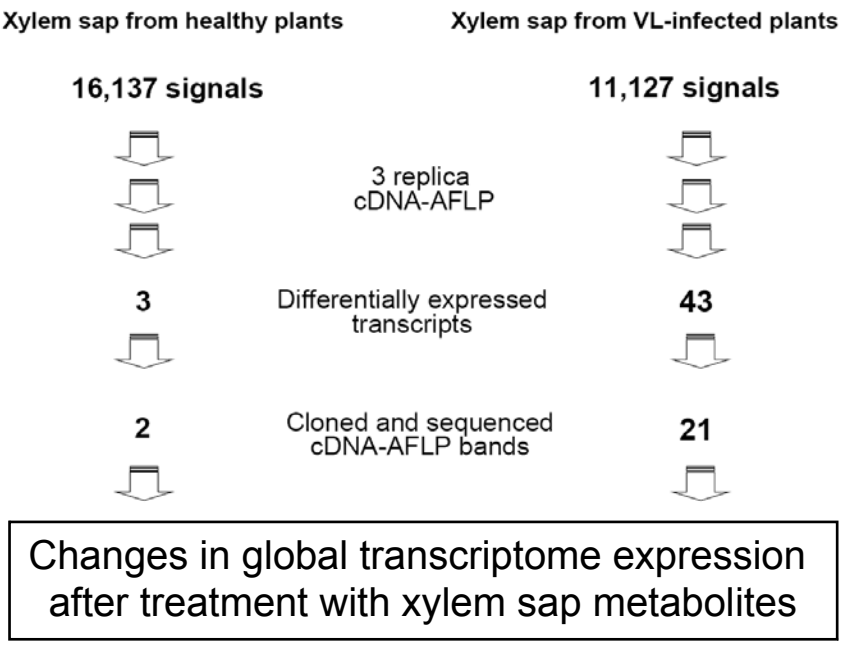

Figure 2: *Verticillium* transcriptome profiling (modified after A. Weiberg 2008). The left line is treated with xylem sap of healthy plants, and the right line is treated with xylem sap of *V.longisporum*-infected plants. The general profiling procedure separated in three steps: 1. cDNA-AFLP (3 replica); 2. Differentially expressed transcripts; 3. Cloned and sequenced cDNA-AFLP bands.

3.2. Sequence screening in databases

Recent electronic databases proliferate the huge amount of cDNA and genomic sequences of the biological organisms. One way to obtain full-length cDNA sequences, which contain the profiled hit TDFs, is to screen the short TDF sequences (with average length 82 bp) using databases correlated with blast search tools. In this case we used *Verticillium* group database (Broad Institute, Massachusetts, United States) and NCBI (National Centre for Biotechnology Information) Genbank databases. As a result, it was only possible in some cases to obtain the several similar cDNA sequences from the related species. For an improvement of the reliability of blast search in databases and a further characterisation of the TDFs related genes, the sequence extension of the short TDFs was necessary.

3.3. TDFs sequence extension and analysis

Rapid amplification of cDNA-ends with Polymerase Chain Reaction (RACE-PCR) is a modification of reverse transcription PCR (RT-PCR) and a practical technique to obtain full-length cDNA sequences. It is used to amplify sequences between a characterized region in the mRNA (cDNA) for example by cDNA-AFLP screening and anchor or adapter sequence that is paired to the 5' or 3' end (9). Inverse Polymerase Chain Reaction (IPCR) (10) is another method for amplifying unknown cDNA or genomic DNA regions (11). This method can amplify DNA sequences of a flanking regions rapidly near the known sequence. These PCR based methods are quite variable from one transcript hit to another and do not always produce successful results. An another method is to generate a genomic library for sequence extension but this is time-consuming and then is used as a second alternative .

There are two types of DNA libraries for an organism, genomic library and cDNA library. A genomic library is a collection of clones constructed of randomly generated overlapping DNA fragments which represent the complete genome of an organism, and the cDNA library contains only complementary DNA molecules synthesized from all mRNA molecules. To generate a cDNA library, the total mRNA is isolated from the organism of interest. For eukaryotes, whose mRNAs bind a poly-A tail, their cDNAs can be synthesized easily with the enzyme reverse transcriptase and changed into double-stranded DNA molecules by DNA polymerase. Then they are completely inserted into standard vectors and cloned. For generation of a genomic DNA library, the genomic DNA is fragmented, for example digested with a “four cutter” restriction enzyme, and then it is inserted into a plasmid or phage vector. The number of clones that are generated to form a genomic library depends on the size of the genome and the size of insert. More than 50 fungi have been sequenced including human and plant pathogens. With the recent genomic information we know a fungal genome is about 1~4 x 10^7 bp long, if a plasmid or a phage vector can carry up to 10 kb fragment, this would require 1~4 x 10^3 recombinant plasmids or phages. However a bacterial phage has been described, which can take a fragment with a relative large size as insert. Generally the work with RNA is more sensitive and expensive than DNA.

Bioinformatic databases and tools (12) are an essential component of molecular biology nowadays. The first bioinformatic/biological databases were constructed during the first

protein sequencing, which was bovine insulin consisting of 51 residues and reported in 1956. A few years later, the first nucleic acid sequence of yeast alanine tRNA with 77 bases was published. Dayhoff collected all the available sequence data and created the first bioinformatic database a year later. The Phage Φ-X174 was sequenced in 1977, since then many genome sequences have been decoded and saved in databases. The Protein Data_Bank followed in 1972 and the Swiss-Prot protein sequence database began in 1987. These development allows scientists to face a growing challenge for sequence analysis, such as genome annotation, gene expression, gene regulation, protein expression, genomics etc. We applied different methods to screen hits of TDFs in databases, to extent DNA sequence of TDFs and used the analysed results as basis for further characterisation.

4. Antisense RNA and RNAi mediated gene silencing

Gene regulation in the cell is controlled at either the transcriptional or post-transcriptional level which achieves respectively via histone modification to inhibit transcriptional machinery such transcription factor, RNA polymerase etc. or via construction of double-stranded RNA to destruct target messenger RNA. Within a cell the single-strand sequence messenger RNA can be translated into a polypeptide, therefore it names “sense-strand” and its complementary strand names “antisense strand”. Gene regulation at post-transcriptional level depends on two important aspects of RNA function, antisense-RNA regulation and RNA interference (RNAi).

The natural antisense-RNAs were found independently by Tomizawas and Nordströms research groups in 1981, they reported that small plasmid-encoded RNA regulators respectively control the copy numbers of the *Escherichia coli* plasmids ColE1 and R1 (13, 14). The phenomenon of clear antisense-RNA regulation was first discovered by scientists in the Netherlands (15). they found some diluted deep color flowers in plant *pegunia* after intergration in *pegunia genome* with a chalcone synthase gene in antisense direction. The chalcone synthase gene plays a key roll for flower pigmentation. This discovery was used for producing the first genetically engineered food tomato “Flavr Savr”, which was intergrated a tomato gene in tomato genome in antisense direction by the company Calgene in Californian, and submitted to the U.S. Food and Drug Administration (FDA) in 1992 (16). The used tomato gene is polygalacturonase which can degrade cell wall component pectin to make tomato more susceptible to be infected by fungal pathogen,

when the antisense gene is expressed in tomato cell, the tomato's susceptible process can be postphoned (17). The antisense-RNA regulation was fund in all three biological kingdoms later (18, 19).

A revolutionary observation of gene silencing in plant attracted many scientists around the world especially in the area of *Caenorhabditis elegans* research (20). In 1998, Craig C. Mello and Andrew Fire reported that double stranded RNA has a catalytic or amplification component in the gene expression interference process. They injected a few molecules of double stranded RNA into *C. elegans* and found a powerful gene silencing effect, neither mRNA nor antisense RNA injections had a similar effect on protein production (21). They were awarded the Nobel Prize in Physiology or Medicine in 2006 for their research (22).

Mechanism of RNAi basis on an immune-like system in an organism. only single-strand RNA molecules exist in the cytoplasm of the cell. They are cut by a Dicer enzyme into small fragments of 21-23 base pairs called small interfering RNAs (siRNAs). One of the RNA pair strands is the antisense strand, which can bind to a complementary sense sequence on mRNA and silence the function of the related gene. The complex of siRNA and protein is called the “RNA-induced silencing complex” (RISC) (23). There is a growing indication that siRNAs can also inhibit the transcription of genes (24).

RNAi is a potential tool in molecular biology which can be used in many areas. In plants, Nature reported that RNAi was used as a tool for producing decaffeinated coffee plants (25). Nature Biotechnology carried a report that a transgenic corn plant expressed a insect “western corn rootworm” inhibiting dsRNA when the plant was eaten by its pest, the dsRNA could inhibit the synthesis of an enzyme from the pest and control it (26). RNAi's target so specifically, it may be possible to use in human therapy. Currently there are trials using siRNA molecules in the clinical treatment to many different diseases (27). We used both methods, antisense-RNA regulation and RNAi, to generate target gene silenced strains for *V. longisporum* gene function analysis.

5. Green fluorescent protein (GFP) as reporter gene

The GFP from the jellyfish *Aequorea victoria*, is a protein composed of 238 amino acid residues (26.9 kDa), which exhibits green fluorescence under excitation by blue light (28, 29). Shimomura researched *Aequorea victoria* successfully in 1962 (30), Chalfie and his co-workers published their results in 1994 about Green fluorescent protein used as a

marker for gene expression in *Escherichia coli* (31). together with Roger Y. Tsien (32) they awarded the 2008 Nobel Prize in chemistry for their discovery and development of the green fluorescent protein. The idea to use GFP as a tracer molecule was got first by Douglas Prasher in 1987, he envisioned that it would be possible to use biomolecular techniques to insert the GFP gene at the end of the hemoglobin gene, right before the stop codon (33) . As a result, the cell would produce a hemoglobin molecule with a GFP attached to it (Figure 3). Today the GFP is often introduced into organisms and integrated in their genome by transformation as a reporter gene in molecular biology (34, 35). Heterologous expression of GFP was first accomplished in *Escherichia coli* and *Caenorhabditis elegans* (36), and then also in many fungi (37), although GFP constructs were not always stable expression, Different forms of GFP controlled by constitutive or inducible fungal promoters or *Neurospora*-GFP fusion proteins were tried but not found to be expressed at levels required for imaging (38). Two new binary vectors each for promoter and gene fusion were constructed in this study.

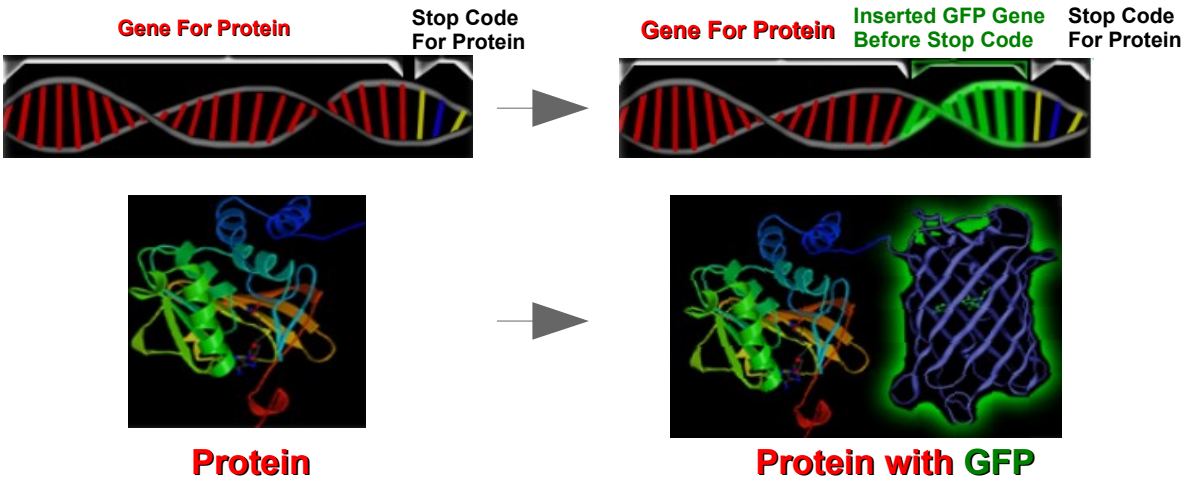

Figure 3: Model for GFP expression after Douglas Prasher's Idea [Pictures modified according to Marc Zimmer (39)]

6. The oilseed rape phytopathogen *V. longisporum*

Verticillium wilt is one of the most important disease causing economic losses in oilseed rape (*Brassica napus* spp. Oleifera). The occurrence is increasing in northern Europe (40, 41, 42, 43). This pathogenic disease is caused by the soil-borne, host-specific, vascular, and “near-diploid” fungal phytopathogen *V. longisporum* comb. Nov (44, 45), which was first reported by Isaac in 1957 and Stark in 1961. The morphology of *V. longisporum* is

very similar to two other important phytopathogen *V. dahliae* and *V. albo-atrum* with a small difference in the length of spores (46). These findings lead to the suggestion of a new subspecies *V. dahliae* f. sp.*longisporum*.

V. longisporum enters through the roots by direct penetration of the epidermal cells or through open wounds (47). The conidia enter the vascular system, move upwards in to the xylem vessels, Typical symptoms on oilseed rape are premature ripening and stunting under greenhouse conditions (Figure 4). Because of its systemically infection, fungicides for direct control of the disease have little effect on host plants infected by the pathogen, and micro-sclerotia can survive 10–15 years in the soil. Control of *Verticillium* wilt is particularly difficult. The knowledge of the pathogenicity related genes involved in the *V.longisporum* may benefit for the development of resistant plant varieties.

Molecular mechanisms through all stages of *Verticillium* differentiation are still unclear. It is not known how the fungus expresses its pathogenicity and which virulence factors are involved specifically during the parasitic phase. Investigation of the parasitic phase of *Verticillium* via functional and comparative genomics, transcriptomics, proteomics and metabolomics can bring an useful output to explain the pathogen-host interaction, and may provide helpful informations for developing resistant plants or new control strategies. Recently *Verticillium* has become a model-system for phytopathology research. In *Verticillium genus* species such as *V. dahliae*, *V. albo-atrum*, *V. longisporum* and *V. tricorpus* etc. are economic very important. Among them a comparative genome project of *V. dahliae* and *V. albo-atrum* was started by the NSF/USDA Microbial Genome Sequencing Program under collaboration with the Broad Institute in 2004 and *V. dahliae'*s complete mitochondrial genome was sequenced in 2006 (48), Genomic DNA and complementary DNA (cDNA) free accessible growing sequence informations allowed the scientists to progress their research in Alignment, Taxonomy, Phylogeny and Motif finding etc. within *Verticillium* and its related species (45, 49, 50, 51, 52, 53). *Verticillium* Gene functional analysis improved to understand the biological development and pathogenesis. Meantime some individual genes of *Verticillium* have been studied, such as a necrosis and ethylene inducing peptide (Vd_NEP) (54), a trypsin protease (50), a mitogen-activated MAP kinase (55), a hydrophobin (56) etc..

7. Goal of this study

In this study, we try to get to know more about function of some candidate genes of *V.longisporum*. With different methods RACE-PCR (rapid amplification of cDNA-ends with polymerase chain reaction), inverse PCR on self circularized genomic DNA, creating a genomic library and direct cloning with primers on the basis of *V. dahliae* to produce full-length sequences of candidate Transcript-derived Fragments (TDFs) after cDNA-AFLP screening.

A genome library of *V. longisporum* was constructed and hierarchical pools of clones was prepared for fast PCR-based screening. These resource allowed us to identify library clones carrying full-length genomic copy of candidate genes (58). In addition to candidate genes identified by cDNA-AFLP further genes of potential interest were selected based on published reports on a related species *V. dahlie* and other pathogenic fungi for transcription analysis *in planta* and other investigations (54). The real-time RT-PCR procedure on the basis of RNA from roots and shoots of *Brassica napus* was established and optimized. Genes found by cDNA-AFLP to be affected by xylem sap *in vitro* were investigated *in planta* using the quantitative real-time RT-PCR. To resolve the pattern of gene expression in space and time, selected candidate genes were fused with reporter genes (59, 60, 61). Some of them were overexpressed and inactivated to learn more about the role of the genes in pathogenicity (62).

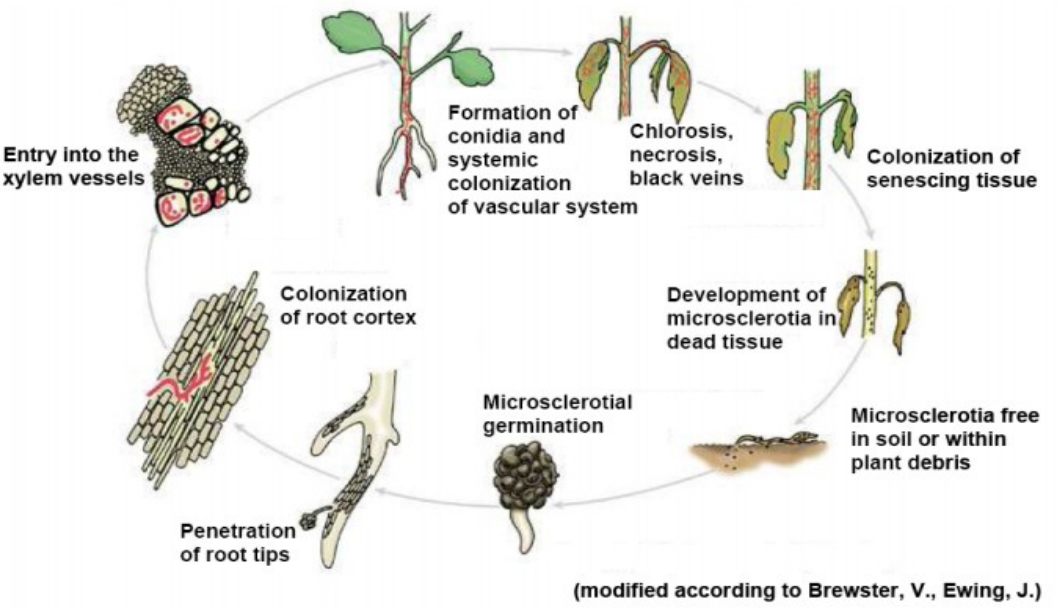

Figure 4: Monocyclic *Verticillium* wilt disease and microsclertia-producing Modified according to Rowe and Powelson (55).

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Chapter 2: Sequence extension

2.1. PCR based methods for sequence extension of TDFs after cDNA-AFLP screening

Introduction

cDNA-AFLP is a PCR-based genome-wide expression analysis technology which does not require any gene sequence information, after identification of differential expression-screening to obtain full sequence of a target gene will be expected (1). Some PCR-based methods, Inverse PCR (2, 3, 4), RACE-PCR (rapid amplification of cDNA-ends with polymerase chain reaction) (5, 6), cloning with amplificons using primers designed with DNA sequences from related species and direct sequencing can be used to extend short TDF (Transcript derived Fragment) DNA sequence. Even they were limited in some cases because of technique limitations.

Methods and materials

1. Inverse PCR

Inverse PCR is a variant of polymerase chain reaction method which uses one known DNA sequence to amplify the flanking unknown DNA fragment and described first by Ochman et al in 1988 (2). The template for IPCR is a with restriction fragment self-formed sequence circle after ligation (Figure 1). The primers for IPCR oriented in the reverse direction of the regular orientation. Fungal gemonic DNA of *V. longisporum* was extracted, digested with endo-restriction enzymes *Eco*RI, *Bam*HI, *Xba*I, *Pst*I, *Hind*III and *Sac*I (Fermentas, St. Leon-Rot, Germany), self-ligated with T4 ligase (Fermentas, St. Leon-Rot, Germany) and provided as template for PCR. Inverse gene specific primers were deduced from cDNA-AFLP fragment sequences. PCR products were separated on a 1% agarose gel, extracted from gel with QIAquick Gel Extraction Kit (Qiagen, Germany), sequenced directly or after cloning in standard vector pBluescript KS(+) (Fermentas, St. Leon-Rot, Germany).

2. 3' RACE-PCR

RACE-PCR (Rapid Amplification of cDNA Ends) sometimes called one-sided PCR, was used to win the full length sequence of an RNA transcript from a small known sequence to

3' end (3' RACE-PCR) or the 5' end (5' RACE-PCR) (6) (Figure 2). The same protocol was used for cDNA synthesis with RNA as template. Total RNA was extracted and purified with mRMA extraction kit. The polyA tail that exists at the 3' end of all eukaryotic mRNAs was applied for designing a reverse Oligo (dT) anchor prime here using $(dT)_{18}$. which was paired with gene specific forward primer from a known sequence within the transcript for amplification. PCR products were separated on a 1.2% agarose gel, extracted from gel with Qiagen-kit and sequenced.

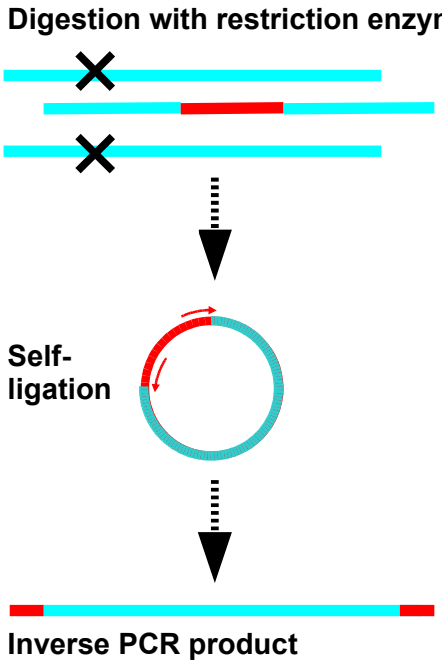

Figure 1: Scheme of IPCR Process. IPCR includes the digestion of genomic DNA, circulation of restricted fragments and using primers designed from the characterized region to amplify unknown flanking sequence by PCR (2).

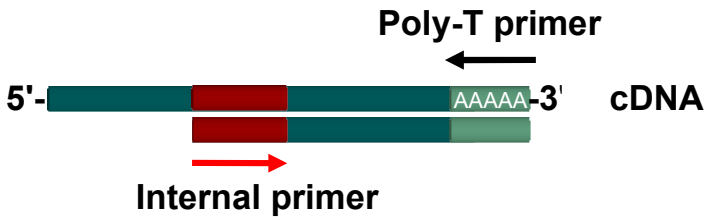

Figure 2: scheme of 3' RACE-PCR Process. All eukaryotic mRNAs exist a polyA tail at the 3' end. For generating cDNA reverse transcription PCR uses a 3' end starting Oligo-dT-anchor primer which is complementary to 3' end natural polyA tail. The 3' end starting Oligo-dT-anchor primer is used then with an characterised 5' internal sense primer for amplification of a unknown DNA region by PCR that is called 3' RACE-PCR (6).

3. PCR-amplicons using primers based on sequences of related species

V. dahliae and *Verticillium albo-altrum* are closely related with *V. longisporum* on genome sequence level (7). Sequences of TDFs were blasted in *Verticillium* group Database (Broad institute), some target genes with high identity to TDFs were investigated and their gene specific primers deduced through the full-length gene sequences. A direct sequencing without cloning for some sequence regions of target genes Vl_6.2, Vl_12.1, Vl_12.2 and Vl_34.2 was applied.

4. Primers for sequence extension

Table 1: Primers for cloning and sequencing of target genes after TDFs.

Nr.	Name of primer	Sequence of primer
1	TDF2.1_SdaI-F	5'-ATCGATCCTGCAGGGACTCCTGTTCATCAAATAA-3'
	TDF2.1_NotI-R	5'-ATCGATGCGGCCGCATTGATCATAACGGTAGCAT-3'
2	TDF11.1-I_SdaI-	5'-ATCGATCCTGCAGGTCGCCATACCTCGTCCTGCC-3'
	TDF11.1-I_NotI-R	5'-ATCGATGCGGCCGCGCAAACTGGTCTGGGTTCTC-3'
3	TDF11.1-II_SdaI-F	5'-ATCGATCCTGCAGGTACCTCTTCAAGTACGAGTG-3'
	TDF11.1-II_NotI-R	5'-ATCGATGCGGCCGCGAATACTGGTCCAGTGAAGT-3'
4	TDF12.1-I_SdaI-F	5'-ATCGATCCTGCAGGAATTTGTTCCGCCCACTTCC-3'
	TDF12.1-I_NotI-R	5'-ATCGATGCGGCCGCATCCGTTAGTGTATGGTCGG-3'
5	TDF12.1-II_SdaI-F	5'-ATCGATCCTGCAGGGGCACCGGTAAGTCAACTCT-3'
	TDF12.1-II_NotI-R	5'-ATCGATGCGGCCGCATCCGTTAGTGTATGGTCGG-3'
6	TDF24.1-I_SdaI-F	5'-ATCGATCCTGCAGGCGATTCGTCCTGGCCCCTCC-3'
	TDF24.1-I_NotI-R	5'-ATCGATGCGGCCGCTGCCTGACCTGGCAGTACTG-3'
7	TDF25.8-I_SdaI-F	5'-ATCGATCCTGCAGGCAACATTGCCTGACCGCTGC-3'
	TDF25.8-I_NotI-R	5'-ATCGATGCGGCCGCCGTTCGAGCAGCTCTTCCAT-3'
8	TDF25.8-II_SdaI-F	5'-ATCGATCCTGCAGGTACAATTTACGAGATGGCAT-3'
	TDF25.8-II_NotI-R	5'-ATCGATGCGGCCGCTCGGCGCTCATCCGTGGTAT-3'
9	TDF33.3_SdaI-F	5'-ATCGATCCTGCAGGTCCTAGTTCCATCGCCAGGC-3'
	TDF33.3_NotI-R	5'-ATCGATGCGGCCGCCTCAAGGCGCAATTCAGGCG-3'
10	TDF34.2_SdaI-F	5'-ATCGATCCTGCAGGAGCAAGCTCAACCTCCATCA-3'
	TDF34.2_NotI-R	5'-ATCGATGCGGCCGCCCAACACAGTGAAGCACAGT-3'
Blue colour: restriction sites.		

5. Vector construction for cloning

The primers (Table 1), the were for sequence extension of target-TDFs using cloning strategy, were designed on bases of sequences of the related species and synthesized by company invitrogen. The target fragments were amplified by PCR and inserted into standard vector pBluescript SK(-) for sequencing by cloning (Figure 3).

6. Sequence analysis

NCBI (National Centre for Biotechnology Information) GenBank and *Verticillium* group database were used for genes hits (TDFs) blast. The DNA sequences isolated from *V. longisporum* was translated using the “Transeq” software in EMBL-EBI. The “ClustalW2” program in EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) was used for construction of the multiple sequences alignment and phylogenetic trees.

A phylogram is a branching diagram exhibiting the inferred evolutionary relationships among various biological species based upon similarities and changes their DNA or protein sequences.

Results and discussion

The sequence extensions brought more sequence information for candidate transcrit derived fragments (genes) such as TDF2.1, TDF6.2, TDF10.2, TDF11.1, TDF12.1, TDF12.2, TDF24.1, TDF25.8 , TDF30.2, TDF33.3, and TDF34.2 etc. (Table 2 and supplementary data). *V. longisporum* may be near-diploidy and hybrid origin, its isolates were estimated to keep double amount of DNA per nucleus as compared with per haploid nucleus in *V. dahliae*. It was suggested that *V. longisporum* may be fused by two different parental *Verticillium* species (8). Under field conditions parasexuality was observed (9, 10). The performance of *V. longisporum* at sequence level may be an additional evidence of the exist of parasexual systems. According to the taxonomic analysis using RAPD etc. methods both *V. dahliae* and *V. albo-atrum* were suggested as *V. longisporum'*s parents. Our phylogenic results confirm this estimation, however the relationship between *V. longisporum* to *V. dahliae* is close than *V. longisporum* to *V. albo-atrum*.

Comparison with the GRIM19 cell death regulator-like gene sequence from these three species, we found that *V. longisporum* presented higher similarity to *V. dahliae* than to *V.*

albo-atrum in alignment score and sequence identities (Table 3) The further investigation demonstrated that *V. longisporum* in 44 nucleotides change positions including 6 gap penalties at 4 positions and 44 mismatches at 40 positions. *V. longisporum* kept absolute similarity to *V. dahliae* than to *V. albo-atrum* with a change ratio in 8.25 times (33 : 4) for mismatch positions. Both *V. longisporum* and *V. dahliae* had 2 same gap penalties, but *V. albo-atrum* monopolized other 4 gap penalties (Figure 4). Table 5 shown phylograms and relationship scales of 8 candidate genes among these three species, 6 of them demonstrated that the evolutionary relationship of *V. longisporum* were close to *V. dahliae* than to *V. albo-atrum*. An another phylogenetic analysis of the candidate genes Vl_2.1, Vl_11.1, Vl_12.1, Vl_33.3 and Vl_34.2 was carried out and compared with sequences of 28 related species classified in appropriate groups at aminoacid sequence levels (Figure 5). The sequences were blasted from NCBI GenBank and *Verticillium* group database (Table 4). The Phylograms showed that *V. longisporum, V. dahliae* and *V. albo-atrum* were closely related than all other species at amino acid levels using five Vl_genes, and Vl_33.3 and Vl_34.2 are absolute identical at amino acid level among three *Verticillium* species. We suggested that these two genes may play the basic roles in fungal biological development during the evolutionary process.

Table 2: Analysis of TDFs and extended sequences using NCBI-Blast.

Name of Candidates	TDFs (bp)	Extended sequences (bp)	Sequence extension used Methods	NCBI-BLAST hits
Vl_2.1	71	724	Cloning	Isochorismate hydrolase
Vl_6.2	78	7873	Genome library and direct sequencing	Hypothetical protein incloding 4 WSC binding domains
Vl_11.1	72	2473	Cloning	Peroxidase/Catalyse
Vl_10.2	68	2437	RACE-PCR, Cloning and direct sequencing	Zinc-finger transcription factor ace1
Vl_12.1	204			
Vl_12.2	172	681 (UTR)	Direct sequencing	hypothetical protein
Vl_24.1	56	1493	Cloning	hypothetical protein
Vl_25.8	135	565	Cloning	IBR (In between Ring) finger domain-containing protein (indirect TF)
Vl_33.3	97	1430	Cloning	DOA4-independent degradation protein (Snf7 super-family)
Vl_34.2	76	925	Inverse PCR and Cloning	NADH:ubiquinone oxidoreductase 14kD subunit) GRIM19 cell death regulator

Table 3: Alignment scores and identities of GRIM19 cell death regulator-like (Vl_34.2) gene sequences among *V. longisporum*, *V. dahliae* VdLs.17 and *V. albo-atrum* VaMs.10.

Nr.	DNA sequence	Length	Nr.	DNA sequence	Length	Score	Identity
1	Vl_TDF_34.2	684	2	Vd_VDAG_09543.1	683	98,0	98.8
2	Vl_TDF_34.2	684	3	Vaa_VDBG_09810.1	682	94.0	94.3
3	Vd_VDAG_09543.1	683	3	Vaa_VDBG_09810.1	682	94.0	94.1
Score = Σ (identities, mismatches) – Σ (gap penalties) Identity = Σ (identities) – Σ (mismatches, gap penalties)							

Table 4: The related species for phylogenetic analysis of the candidate genes Vl_2.1, Vl_11.1, Vl_12.1, Vl_33.3 and Vl_34.2 at aminoacid sequence levels *.

Code	Name of species	Code	Name of species
Ac	*Aspergillus clavatus* NRRL 1	Nf	*Neosartorya fischeri* NRRL 181;
Ad	*Ajellomyces dermatitidis* SLH14081	Nh	*Nectria haematococca* mpVI 77-13-4;
Af	*Aspergillus fumigatus* Af293	Pb	*Paracoccidioides brasiliensis* Pb01
An	*Aspergillus niger*	Pc	*Penicillium chrysogenum* Wisconsin 54-1255
Ao	*Aspergillus oryzae* RIB40	Pd	*Penicillium decumbens*
Bf	*Botryotinia fuckeliana* B05.10	Pg	*Puccinia graminis* f. sp. tritici CRL 75-36-700-3;
Bt	*Burkholderia thailandensis* MSMB43	Pm	*Penicillium marneffei* ATCC 18224
Cg	*Chaetomium globosum* CBS 148.51	Ss	*Sclerotinia sclerotiorum* 1980
Gg	*Glomerella graminicola* M1.001	Tr	*Trichoderma reesei*
Gm	*Gibberella moniliformis*	Ts	*Talaromyces stipitatus* ATCC 10500
Gz	*Gibberella zeae* PH-1	Um	*Ustilago maydis* 521
Lm	*Leptosphaeria maculans*	Vaa	*Verticillium albo-atrum* VaMs.10
Mo	*Magnaporthe oryzae* 70-15	Vd	*Verticillium dahliae* VdLs.17
Nc	*Neurospora crassa* OR74A	Vl	*Verticillium longisporum* VL43
* All related species were selected from NCBI GenBank or *Verticillum* group database and the genes in these species kept identities to target gene fragments from *V. longisporum* more than 60% and e-Value less than 1e-3.			

Figure 3: Construction maps of vector pBluSK(-) containing target fragments of VI_2.1; VI_11.1; VI_12.1; VI_24.1; VI_25.8; VI_33.3 and VI_34.2. The primers were listed in Table 1.

```
Vl_TDF_34.2           ATGCCCCAGGACATGCCGCCCGTGGGCGGCTATAATGCCGTTCAATACAAGGTGGGTTTA 60
Vd_VDAG_09543.1       ATGCCCCAGGACATGCCGCCCGTGGGCGGCTATAATGCCGTTCAATACAAGGTGGGTTTA 60
Vaa_VDBG_09810.1      ATGCCCCAGGACATGCCGCCCGTGGGCGGCTACAATGCCGTTCAATACAAGGTGGGTTTA 60
                      ****************************** **************************

Vl_TDF_34.2           CCGATCGATCCCGTCGTGGCCGACAGCTAGGCCAATTGGGCTCAATACAGTGCATCAATG 120
Vd_VDAG_09543.1       CCGATCGATCCCATCGTGGCCGACAGCCAGGCCAATTGGGCTCAATACAGTGCATCAATG 120
Vaa_VDBG_09810.1      CCGATCGATCTCGTCGTGGCCGACAGCGATGCCAATCGGGCTCAATATAG-GCGTCAATG 119
                      ********** * ************** * ****** ********** ** ** ******

Vl_TDF_34.2           CCTCTTCAGCACCGATAAACTTCAACGAGACTCCGCATCAGGCCCCAGGCGCCCATACAG 180
Vd_VDAG_09543.1       CCTCTTCAGCACCGATAAACTTCAACGAGACTCCGCATCAGGCCCCAGGCGCCCATACAG 180
Vaa_VDBG_09810.1      CCTCTTGAGCACCAATGAACTTCTACGAGACTCCGCATCAAGCCCCAGGCGCCCATACAG 179
                      ****** ****** ** ****** ********************** ******************

Vl_TDF_34.2           ACTTCTCCCCGAGCAACGCCGGCTAACGCTTCTCCTAGCGTAACCTCCCCGCCCGCGGCT 240
Vd_VDAG_09543.1       ACTTCTCCCCGAGCAACGCCGGCTAACGCTTCTCCTAGCGCAACCTCCCCGCCCGCGGCT 240
Vaa_VDBG_09810.1      ACTTCTCCCCGAGCAACGCCGGCTAACGCTTCCCCTAGCGCAACCTCCCCGCCCGCGGCT 239
                      ******************************** ******* ******************

Vl_TDF_34.2           TCCGCCCTGGAATCCTCCTTCTCGGAATGGGCGCCGTCATGGGCTACGGCTGGTACAAAC 300
Vd_VDAG_09543.1       TCCGCCCTGGAATCCTCCTTCTCGGAATGGGCGCCGTCATGGGCTACGGCTGGTACAAAC 300
Vaa_VDBG_09810.1      TCCGCCCTGGAATCCTCCTCCTCGGAATGGGCGCCGTCATGGGCTACGGCTGGTACAAGC 299
                      ****************** ****************************************

Vl_TDF_34.2           TGATCAAGGGCATCCGCGAGGCCAAGTACGTCCTCGTTCCCCCCTC-CCGCTTTGTCTCG 359
Vd_VDAG_09543.1       TGATCAAGGGCATCCGCGAGGCCAAGTACGTCCTCGTTCCCCCCTC-CCGCTTTGCCTCG 359
Vaa_VDBG_09810.1      TGATCAAGGGCATCCGCGAGGCCAAGTGCGTCCTCGTTTCCCCCTCGCTGCTTTGTCTCG 359
                      ************************** ********** ******* * ****** ****

Vl_TDF_34.2           TCTTGCTTCGGCTGCGTAGCTTGGAGCCGTTGACCAATCCTCCCTCGAACAAAGAACTGA 419
Vd_VDAG_09543.1       TCTTGCTTCGGCTGCGTAGCTTGGAGCCGTTGACCAATCCTCCCTCGAACAAAGAACTGA 419
Vaa_VDBG_09810.1      TCTTGCTTCGGCTGCGTAGCTTGGAGTCGTTGACCAATCCTCCCTCGAACGAAAAGCTAA 419
                      ************************** ********************** ** * ** *

Vl_TDF_34.2           CCACCATGCCAGCGAGCTTGCCCGTGAGAAGATGTGGGCGCGCATCCACCTCATTCCTCT 479
Vd_VDAG_09543.1       CCACCGTGCCAGCGAGCTCGCCCGTGAGAAGATGTGGGCGCGCATCCACCTCATTCCTCT 479
Vaa_VDBG_09810.1      CCACCATGCCAGCGAGCTCGCCCGTGAGAAGATGTGGGCGCGCATTCACCTCATTCCTCT 479
                      ***** ************ ************************** **************

Vl_TDF_34.2           CCTCCAGGCCGAGGAGGACCGCGACCAGATCCGCCGATGGTACGCCGATCAGGCGCGCGA 539
Vd_VDAG_09543.1       CCTCCAGGCCGAGGAGGACCGCGACCAGATCCGCCGATGGTACGCCGACCAGGCGCGCGA 539
Vaa_VDBG_09810.1      CCTCCAGGCCGAGGAGGACCGTGACCAGATCCGCCGATGGTACGCCGACCAGGCGCGCGA 539
                      ******************** ************************* ***********

Vl_TDF_34.2           GAAGGAGCTGCTGGGCGAGAACACGCGTGTGTACCACACGGACAGGTAGGACTGACTGGA 599
Vd_VDAG_09543.1       GAAGGAGCTGCTGGGCGAGAACACGCGTGTGTACCACACGGACAGGTAGGACTGACTGGA 599
Vaa_VDBG_09810.1      GAAGGAGCTGCTGGGCGAGAACACGCGTGTGTACCACACGGACAGGTAGGACTGATAGGG 599
                      ************************************************** **

Vl_TDF_34.2           CGGCTTGCTGTAGAGGGCCGTTTGCTAACGAGGTGCAGGGTTTGTCCGACCCACGTTCGC 659
Vd_VDAG_09543.1       CGGCTTGCTGTAGAGGGCCGTTTGCTAACGAGGCGCAGG-TTTGTCCGACCTACGTTCGC 658
Vaa_VDBG_09810.1      CGGCTGGCTGTAGAGG-TTTTGTGCTAACGAGGCGCAGG-TTTGTCCGACCCACGTTCGC 657
                      ***** **********    * *********** ***** *********** ********

Vl_TDF_34.2           TGTTGCGCCGGAGAAGACGAAATAG 684
Vd_VDAG_09543.1       TGTTGCGCCGGAGAAGACGAAATAG 683
Vaa_VDBG_09810.1      TGTTGCGCCGGAGAAGACGAAATAG 682
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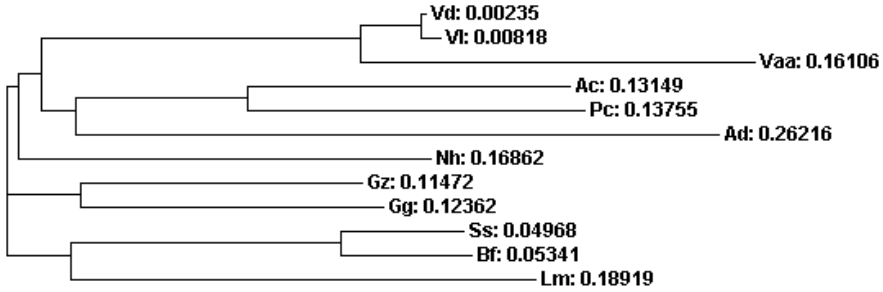
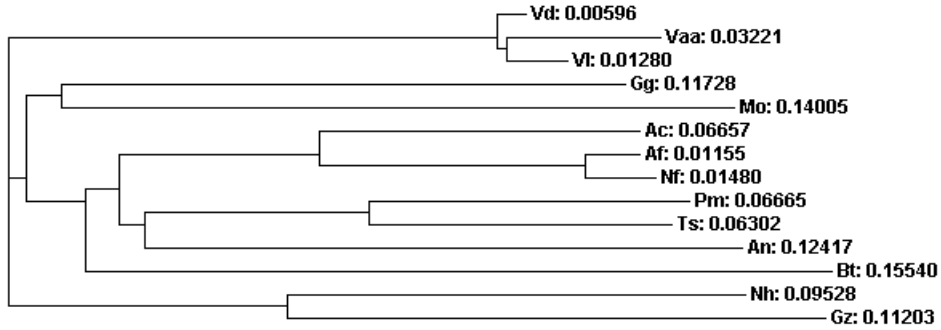
Figure 4: Alignment of the GRIM19 cell death regulator-like (Vl_34.2) gene sequences among *V. longisporum* (Vl_TDF_34.2), *V. dahliae* VdLs.17 (Vd_VDAG_09543.1) and *V. albo-atrum* VaMs.10 (Vaa_VDBG_09810.1). **red colour**: *V. longisporum* = *V. dahliae*; **green colour**: *V. longisporum* = *V. albo-atrum*; **blue colour**: *V. dahliae* = *V. albo-atrum*.

Iolic colour: *V. longisporum* ≠ *V. dahliae* ≠ *V. albo-atrum*.

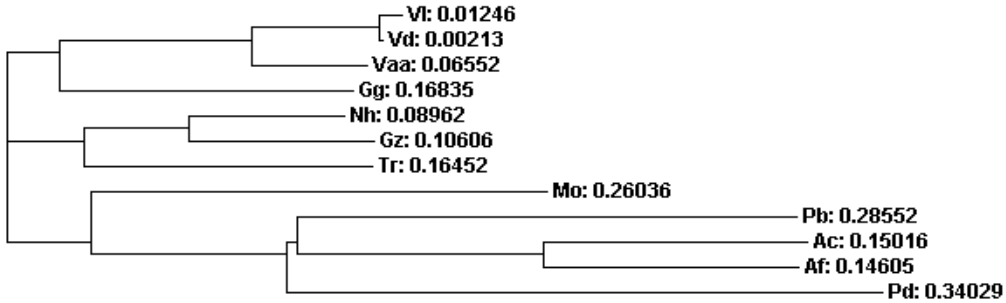
(a) Vl_2.1 and related species



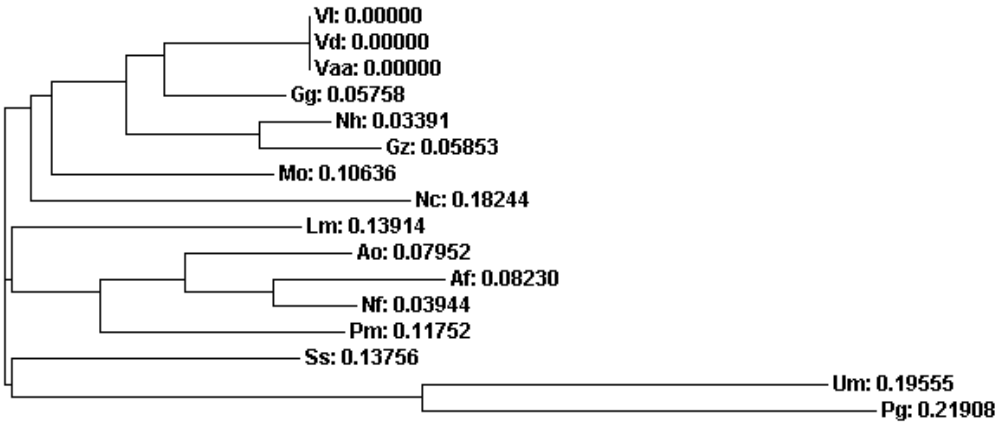
(b) Vl_11.1 and related species



(c) Vl_12.1 and related species



(d) Vl_33.3 and related species



(e) Vl_34.2 and related species

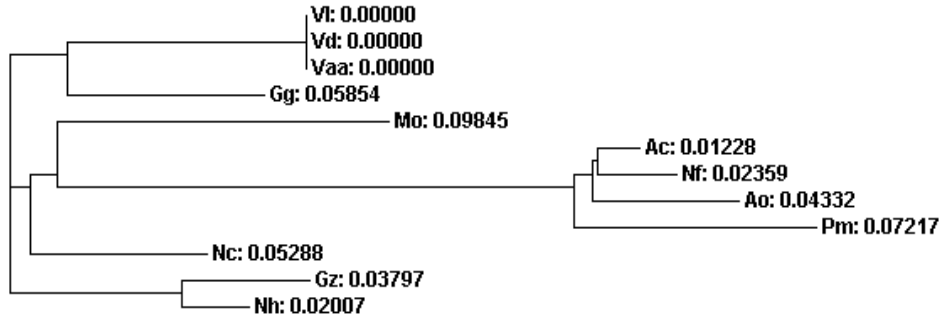
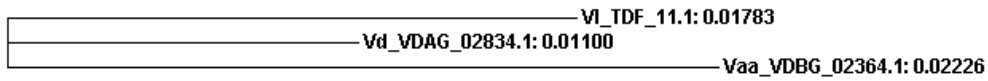
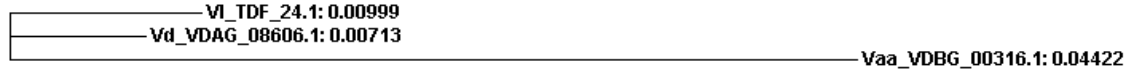
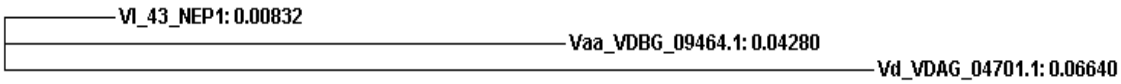
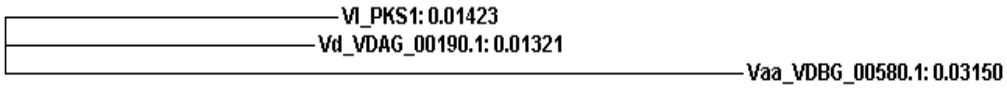


Figure 5: Phylogenetic analysis of candidate genes based on amino acid sequences. The candidate five genes were, (a) Vl_2.1, (b) Vl_11.1, (c) Vl_12.1 (d) Vl_33.3, and (e) Vl_34.2. Phylograms were derived using software ClustalW2. Names of selected species found in Table 4 after the letter-codes in each phylogram. The numbers behind codes indicated the evolutionary relationship scales under a node.

Table 5: Phylograms show genetic distances of 8 candidate genes at DNA level among the species *V. longisporum*, *V. dahliae* VdLs.17 and *V. albo-atrum* VaMs.10.

1	
2	
3	
4	
5	
6	

7	
8	

* Phylograms derived from ClustalW2. Names of species found in Table 4 according to letter-codes in each phylogram. The numbers behind codes indicate the evolutionary relationship scales under a node.

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2.2. Generating a genomic DNA library with microtiter plate monitoring for sequence extension

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Abstract

A genomic library of *Verticillium longisporum* was constructed with a purpose of extension gene sequence of target TDFs after a cDNA-AFLP-based transcript screening in *V. longisporum*. This microtiter plate monitored library contains 9986 clones with an average insert size of 8-12 kb, representing approximately 50% diploid genome equivalents. It was constructed from the wild type isolate VL43 of *V. longisporum*. A selection procedure was improved for identifying a clone with target genes from the library by PCR in three steps. From this library we got Vl_6.2 gene and three Vl_NEP genes with full length sequences of ORF(Open Reading Frame) and UTR (Untranslated Region).

Introduction

Total genomic DNA of filamentous fungi can be fragmented with partially digestion using a restriction endonulease or other methods. The appropriate fragments are fractionated and ligated into a standard cloning vector forming various recombinants. The collection of recombinants can be transferred into *Escherichia coli* or another proper species to build a genomic library which is used to screen the interesting target genes. To generate a genomic library is a conventional method both for genome sequence assembling (1) and extending TDFs (Transcriptome derived Fragments) sequences (2). However, This method is time-consuming (3). Here we present a improved procedure that we have rapidly got the target genes in large size from a genomic library.

The genome of an organism is a complete set of genes. The diploid organisms (like ourselves) contain two genomes. The table 1 presents a list of representative genome sizes from the organisms where the genomes have been sequenced. The table 2 presents a list of vector systems which can be used for generation of a genomic library.

Table 1: Part of representative organisms with sequenced genomes (4)

Name of organisms	Genome size	Genes
E. coli K-12	4,639,221	4,377
Agrobacterium tumefaciens	4,674,062	5,419
Streptomyces coelicolor	6,667,507	7,842
Saccharomyces cerevisiae	12,495,682	5,770
Neurospora crassa	38,639,769	10,082
Arabidopsis thaliana	115,409,949	~28,000
Humans	3.3×10^9	~20,000
Mouse	3.4×10^9	~23,000

Table 2: Capable vector systems (5)

Vector type	Cloned DNA (kb)*
Plasmid	20
lambda phage	25
Cosmid	45
P1 phage	100
BAC (bacterial artificial chromosome)	300
YAC (yeast artificial chromosome	1000
* Approximate maximum length of DNA that can be cloned into vectors.	

The genomic library can be made using each vector according to a requirement after the genome size of an organism (table 1) and the capacity of a vector (table 2). Normally It is efficient to use a vector with a large capacity. For prokaryotes the gene libraries are generated oft using plasmids containing inserts 5~15 kb, because of their smaller genomes. Only a few thousand recombinants are required for a representative library in

this case. The eukaryotic organisms hold larger genomes, their genomic libraries required those vectors which should be inserted the larger DNA fragments (Table 2). The genome of *Verticillium longisporum* is not sequenced. Fungi are eukaryotic microbes with genomes in c. 10~40 Mb. Then a genomic library for *V. longisporum* can use plasmid, lambda phage, cosmid or P1 phage as a vector.

After an equation of Clarke, L. and Carbon, J. (1979) the exact probability of having any given DNA sequence in the library can be calculated as follows:

$$N = \ln (1 - P) / \ln (1 - f)$$

P is the desired probability of gene represented in library; **f** is the fractional proportion of the genome in one insert; **N** is the necessary number of recombinants in a library.

The number of clones required to have a 99% probability (99% chance of getting the desired gene in the library) of finding a desired sequence (an average insert size of 10 kb) represented in a *Verticillium* library with an approximate *V. longisporum* genome size of 2.5 x 10^7.

$$N_{vert} = \ln (1 – 0.99) / \ln[1 – (1 \times 10^4 / 2{,}5 \times 10^7)] = 11510 \text{ clones (recombinants)}$$

Material and methods

1. Fungal, bacterial strain and media

See chapter Reference Gene Selection and Determination of *in Planta* Gene Expression of *V. longisporum* by qRT-PCR. *Escherichia coli* DH5α strain (Hanahan 1983) was used for cloning and grown on LB medium (Sambrook *et al*., 1989). Electro-competent cells for transformation were stored in the freezer at – 80 °C. LB and SOC medium were used for incubation of bacterial celles (Table 3 and 4). PDB and Czapek Dox were applied for incubation of fungal strain *V. longisporum* VL43.

2. Preparation of the electroporation competent cells

1). Preparation of YENB growing media 100 ml. [Mix: 0.75% Yeast Extract 7,5 g l^{-1} and 0.8% Bacto Nutrient Broth (Difco) 8 g l^{-1}. Filled to volume with distilled water and autoclaved]; 2). Cells were grown overnight in 10 ml of YENB media. 3). Overnight growth

of 1 L media with Inoculated 5~10 ml of culture. 4). Grown at 37°C until the OD_{600} is 0.5 to 0.9 [The optimal OD_{600} is 0.6, which takes approximately 3h]. 5). Placed on ice for 30 min and then centrifuged at 4000g for 10 min at 4°C. 6). The pellet was washed twice with 100 ml cold water [centrifuged between washes]. 7). Resuspended in 20 ml of 10% glycerol and centrifuged again. 8). Resuspended to final volume of 2-3 ml in cold 10% glycerol and aliquoted at 50 µl and shot, frozen in liquid nitrogen. [Store at 80°C]. (This protocol was taken from Biotechniques, vol 20 (1): 42-44, Sharme and Schike 1996).

Table 3: Component of LB medium

Name	Amount
Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g
ddH_2O to	1000 ml
Note: adjust pH to 7.0 with 10N NaOH, autoclave to sterilize.	

Table 4: Component of SOC medium

Name	Amount
Bacto-tryptone	20 g
Bacto-yeast extract	5 g
NaCl	0.5 g
1M KCl	2.5 ml
ddH_2O to	1000 ml
Total volume	1000 ml
Note: adjust pH to 7.0 with 10 N NaOH, autoclave to sterilize, add 20 ml of sterile 1 M glucose immediately before use.	

3. Isolation of fungal genomic DNA

Extraction of fungal genomic DNA of *V. longisporum* (see chapter “Reference Gene Selection and Determination of *in Planta* Gene Expression of *Verticillium longisporum* by qRT-PCR “).

4. Partial digestion of gDNA with Sau3AI and isolation of target fragments

A *V. longisporum* genomic library was generated in following steps: First the *V. longisporum* genomic DNA (gDNA) was isolated, 20 µg of gDNA was partially digested for 30 minutes with the restriction enzyme 0.5 U *Sau*3AI (Fermentas, Germany) in a total volume 900 µl. Then the digested DNA fragments were size-fractionated on an 0.5% agarose gel by electrophoresis (20 V / 16 h), and 8-12 kb fragments were cut out and purified with QIAquick Gel Extraction Kit (Figure 1). Then the purified 8-12 kb fragments were inserted into the *Bam*HI site of the pre-cut standard vector pUC57 (GenScript) (Figure 2) by ligation with T4 Ligase (Fermentas, Germany).

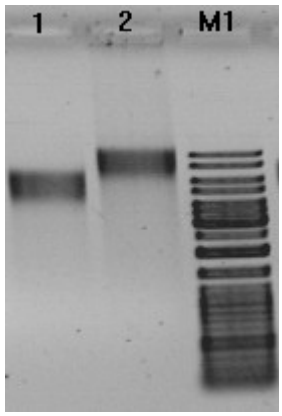

Figure 1: The target 4-8 and 8-12 kb DNA fragments after purification from agarose gel.1: 4-8 kb, 1µl; 2: 8-12 kb, 1 µl; M1: DNA Ladder Mix, 2 µl.

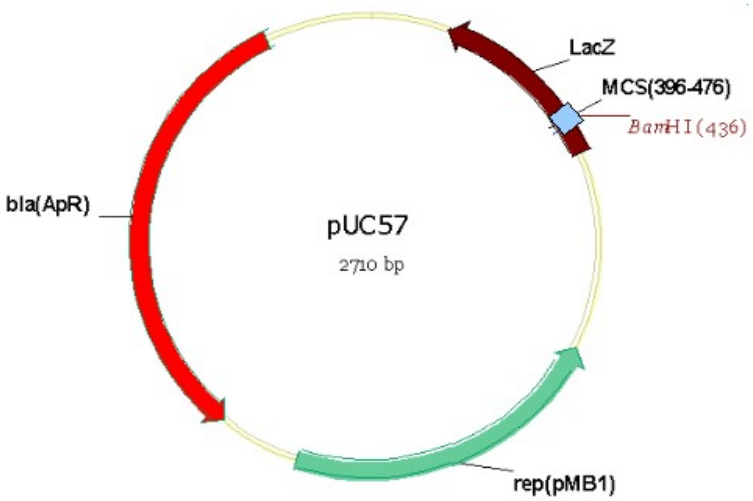

Figure 2: Standard vector pUC57 with *Bam*HI restriction site.

5. Preparation of standard plasmid pUC57 as vector

Plasmid pUC57 was used as a vector for generation of genomic library of *V. longisporum*. pUC57 which is 2710 bp in length, it is a derivative of pUC19. pUC57 MCS contains 6 restriction sites. DNA replication initiates at position 890 (+/-1) and proceeds in the direction indicated. The *bla* gene nucleotides 2510-2442 (compl. strand) code for a signal peptide. The restriction site *Bam*HI was used for insertion of DNA fragments from *V. longisporum* genome (Figure 2).

6. Transformation and monitoring in 96-well microtiter plate

The with inserts recirculated plasmids were transformed in competent *E. coli* DH5α cells by electroporation. To obtain the genomic library, 96-well flat-bottomed microtiter plates (Figure 3) were used to grow the transformants in LB medium with 100 ppm ampicillin. Each transformant was cultured in 150 µl LB per well for two days, and then 100 µl sterile 50% glycerol was added used multipipeter.

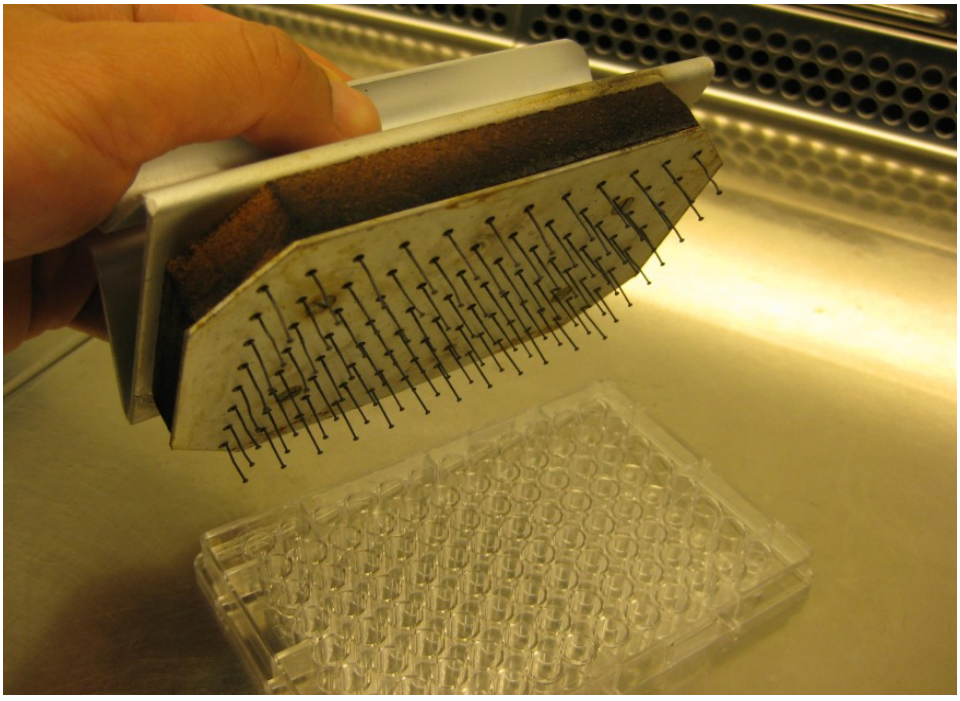

Figure 3: 96-well flat-bottomed microtiter plate for library monitoring and 96-needle inoculating plate for inoculation.

7. Polymerase chain reaction and designed primers for target genes

A standard PCR procedure was used in this experiment. PCR amplification was carried out using a 2 min denaturation at 95°C as first step, followed by 30 reaction cycles consisting

of a 30 s denaturation step at 95°C, an annealing step for 30 s at 58°C or 59°C and a elongation step with a ratio of approximately 1 min for 1 kb at 72°C. An additional final elongation step was performed for 5 min at 72°C. The reaction mixture consisted of NH4-reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01% (v/v) Tween-20, pH 8.8 at 25°C, 3 mM $MgCl_2$, 0.2 mM of each dATP, dTTP, dCTP and dGTP, 0.3 µM of each primer, 0.25 U BioTaq DNA polymerase (Bioline, Luckenwalde, Germany), 1-2 µl of template DNA and ddH_2O up to 25 µl.

The for detection of target genes from a genomic library used primers were designed from TDFs (Weiberg 2008) (Table 5: Nr. 1-3) or related species (*Vercillium* group database) (Table 5: Nr. 4-8).

Table 5: Specific primers of putative pathogenicity related candidate genes of VL.

Nr.	Target gene	Primers
1	Vl_6.2-F	5'-AATTGGCCAACCAGCTCTACG-3'
	Vl_6.2-R	5'-ACGTATTCGCCAGCAGCG-3'
2	Vl_12.1-F	5'-AATTCCAGACTCTGTTCGGCAG-3'
	Vl_12.1-R	5'-ACGTAAAAGAAACAGGCCATCCC-3'
3	Vl_12.2-F	5'-AATTCGTCCTCGGGCCTCAAGGCAGA-3'
	Vl_12.2-R	5'-ACGTAATGTCATCACAGGTCAGAGTGG-3'
4	Vl_NEP1-F	5'-CGGTGAGGTCAAGTAAGACACGC-3'
	Vl_NEP1-R	5'-TGACGACATTCTCCCAGTCG-3'
5	Vl_NEP2-F(Vl_NEPC)	5'-ACGTAGTCGTCTTCGCACGCGGA-3'
	Vl_NEP2-R(Vl_NEPC)	5'-CGGGCTCTTGTAGAACGAGCCTG-3'
6	Vl_NEP3-F(Vl_NEPE)	5'-GCGATTGTCTACCACAAGGACAGCA-3'
	Vl_NEP3-R(Vl_NEPE)	5'-GCCTAGCCTTGCGAATGTTCTCAG-3'
7	Vl_NEP4-F(Vl_NEPF)	5'-TGCCGACGAGCGTAGCCATGCA-3'
	Vl_NEP4-R(Vl_NEPF)	5'-TCCTCATTAGCCCATGCCATGTGA-3'
8	Vl_NEP5-F(Vl_NEPG)	5'-GTACGCTGCTTGGCTGGCACA-3'

	VI_NEP5-R(VI_NEPG)	5'-GTTGTGAAGCCACGTGCCGTCA-3'

8. Procedure of screening and identification

To obtain a clone containing target gene from complete genomic library, a general screening of the target genes from the library was performed by transferring transformants from microtiter plates as plate-pools inoculated with 96-needle inoculating plates in flasks containing 50 ml LB medium, overnight incubation shaking at 150 rpm at 37°C, and plasmids isolation using plasmid extraction kits (Qiagen, Hilden, Germany). 1 µg plasmids DNA from each four plate-pool formed a collective pool. Each 96-well microtiter plate contains 8 rows and 12 lines, they were inoculated with multi-pipette and forms 20 pools (each row or line as one pool). The overnight incubated transformant cultures were used directly, or after plasmid extraction, as template for determination of target genes by PCR. Figure 4 shows a scheme for the screening and identification of a target clone.

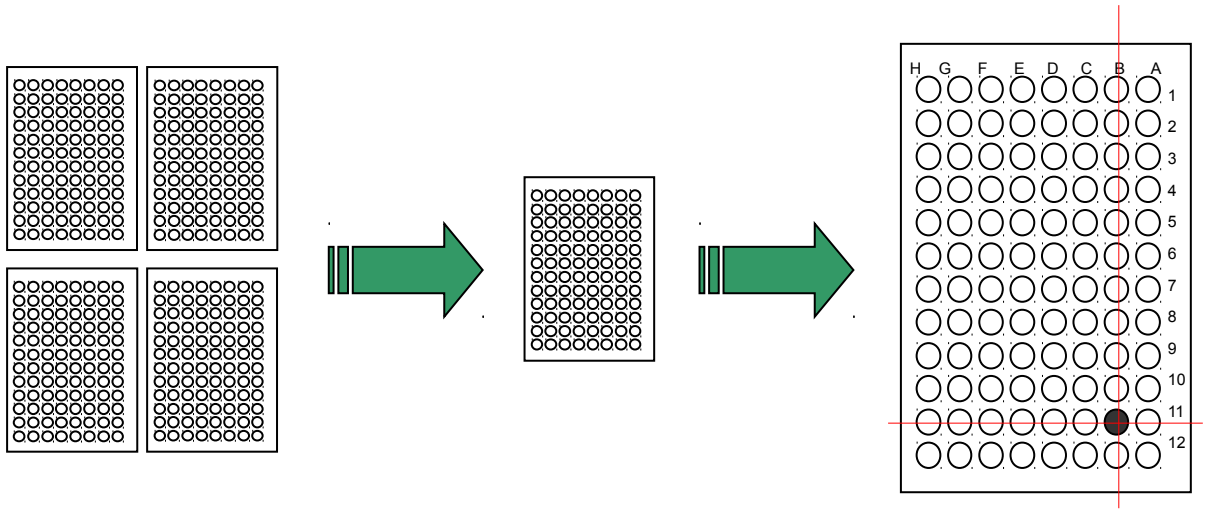

Figure 4: Scheme of the Screening and identification procedure in three steps. First step (left picture): 4 platen form 1 collective pool. Second step (middle picture): 1 plate forms 1 plate-pool. Third step (right picture): Each row or line is 1 pool. If line pool and row pool both were positive by PCR, the target was located on a microtiter plate of genomic library in microtiter plate monitoring..

9. DNA sequencing

DNA sequencing of this work was carried out by Europins MWG Operon (Ebersberg, Germany).

Results

1. Plate monitoring and a rapid procedure for colony identification

A genomic library of *V. longisporum* was generated and contained 9,600 single clones in 100 96-well microtiter flat-bottomed plates. For characterisation of generated genomic library, the transformants of 24 wells each in 3 of 100 microtiter plates were selected randomly, inoculated, plasmids precipitated and inserts checked. About 50% of the clones were checked using agarose gel electrophoresis to see whether the expected insert was present.

A PCR procedure was developed for rapid screening and identification of a clone containing the target gene from the genomic library on 96-well microtiter plates (Sarstedt, Germany). The procedure was divided into three steps. Figure 5 showed one step for screening.

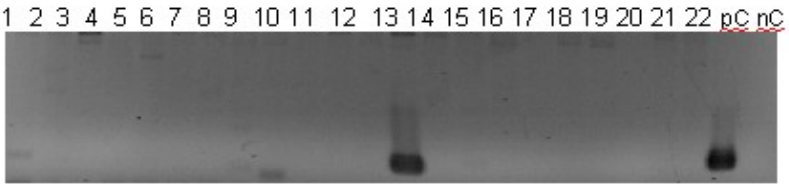

Figure 5: Library screening with plasmids DNA of collective pools as templates by PCR using target gene primers on agarosegel. For pC: positive control genomic DNA of *V. longisporum* was used. nC: negative control used ddH_2O. 1-22: collective pools of genomic library.

2. Detection of target genes from the genomic library

The expected target genes Vl_6.2, Vl_NEP1, Vl_NEP4, Vl_NEP5 and Vl-12.2 etc. were found in library.

3. Analysis and characterisation of Vl_6.2 gene

The from the genomic library isolated Vl_6.2 gene was sequenced completely. Full-length gene sequence of Vl_6.2 with an ORF of 4720 bp nucleotides (supplementary data Vl_6.2 sequence), cDNA translated sequence 1534 aa (Figure 8), 1 signal peptide with 8 aa, 1 fibronectin type III motif with 119 aa and 4 WSC domains contain 88 aa, 93 aa, 72 aa, and 104 aa respectively. A phylogenetic tree (Figure 7) analysis of Vl_6.2-like gene indicates that the relationships and a sense of evolution time among *V. longisporum* VL43, *V.*

dahliae VdLs.17 and *V. albo-atrum* VaMs.102 are very close than with other fungi. The phylogenetic tree was constructed using ClustalW2 software (EMBL-EMI). The protein sequence of *V. longisporum* translated using “Transgen” software (EMBL-EMI). The protein sequences of Vl_6.2-like gene of related species were blasted using the NCBI database. A protein sequence analysis using the software SMART shows Vl_6.2-similar genes in all 11 selected fungal strains containing 3-5 WSC domains. The WSC domain is a putative carbohydrate binding domain which is part of yeast cell wall integrity and a stress response component protein (18). Figure 6 shows a comparison at aminoacid sequence level among four Vl_6.2 WSC domains with a WSC domain as consensus sequence in yeast SLG1 protein (21) using software SMART (Simple Modular Architecture Research Tool) (22). The WSC domain is a putative carbohydrate binding domain. The domain contains up to eight conserved cysteine residues that may be involved in disulphide bridges. The yeast SLG1 protein contains only one WSC domain.

Figure 6: schemetic comparison of aminoacid sequences of four WSC domains with a WSC domain in yeast SLG1 protein (21) as consensus sequence using software SMART. The WSC domain is a putative carbohydrate binding domain. The domain contains up to eight conserved cysteine residues that may be involved in disulphide bridges. The yeast SLG1 protein contains only one WSC domain.

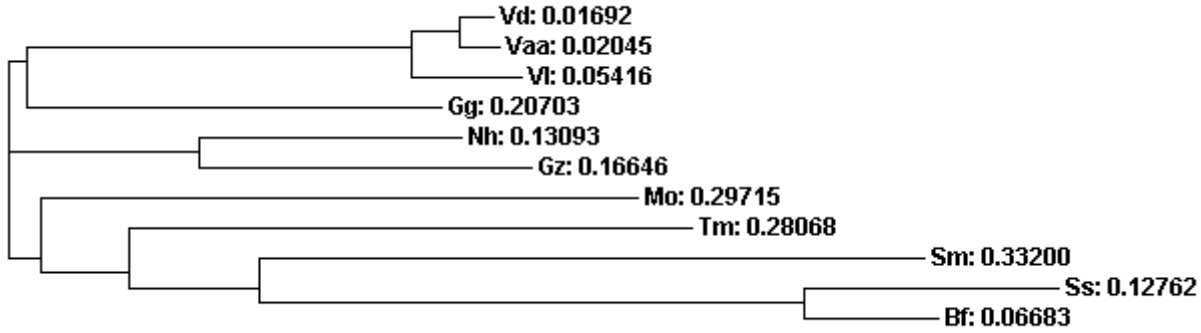

Figure 7: Phylogram of Vl_6.2 gene at aminoacid level with related species containing putative WSC domains. The evolutionary relationship shows with distance. Vd: *Verticillium dahliae* VdLs.17; Vaa: *Verticillium albo-atrum* VaMs.102; Vl: *Verticillium longisporum* VL43; Gg: *Glomerella graminicola* M1.001; Nh: *Nectria haematococca* mpVI 77-13-4; Gz: *Gibberella zeae* PH-1; Mo: *Magnaporthe oryzae* 70-15; Tm: *Tuber melanosporum* Mel28; Sm: *Sordaria macrospora*; Ss: *Sclerotinia sclerotiorum* 1980; Bf: *Botryotinia fuckeliana* B05.10.

MVSFRWLMLAAVAGLANGLADTDTITWGGDNSRAGYQTNHNMDPAVVRSSQFDRIFQTTLPGRYGGRAEQIFSQPLVYTP
DDKQYVYLATTQNNVYKLDAQTGEILASRNLHIPFLSTDLDGCYDIQPHVGVTGTGVIDPDTGTYYLLAKTYENQELVDV
AQGRPAGRYYLHALDVNDLSERPNFPVGLEGTVARNNPDRSFNGGIHLQRPALLHVGQHIYAGLGSHCVKFNFTGWVMGW
DKTTGEQVERFATQGEGVPQNTEGGGLWMAGGGLASDDQGSIFFATGNGYAGQLAEIPVNGRNPPTSLEEAAVHMTIQED
GSLDLVDFFIPWDKRAMDGDDKDLGSSPLQILPSEFSCGSIRRIGVVTGKNKKTYFINLDDMGGYRNGEDRFDNIIQTYE
HENSVYAGAGVYPGEGGYIYINVVQYPTIVFRFSCANGVPSFNKEAETPESNGYTLGVSHGTVTSLNGEPGTAMLWTTDV
QNPPGQLRIYDAVPRDGELALLRKWEIAGVTKFSRAVFGDGIMYLGTTTGLFYGFGAPINRPIECTSPLEFGAVSLEASA
ETRTLTCTALINTVVNDISLREVTDFSISGLPTLPLTLAVGATFTIEAVFAPTDLGLLSTDVNIETENSVAGYRTTTSAR
LTGTGETDNPRLSVSPREIEFDNVITAGAAPPANNVVLSNQGNSVLTVNEIRYSETINSTLQTWDPASGALVIGPFTIRN
IPSTIDANSGATVSVSLSPANGGTFSGHVRFITDGGNTDVTMTAHVGAAPVLLVEFERPDGEGWTTYQEGTAFSFGEVTQ
NNVRNLRMRITNTAPAGGVRLSLTVSKPPHGGSGIIRANNAVDLGEGTNLGPGLSETAVLYCAVPKRQWNMEPYQGEATW
SLNTNDPKVAYQNIQFECTAVSEQSAPLLENGLSQYQYVGCFRENTPGRQLANQLYGNDENTIAMCVEACAAGNYVFCGT
QYHRECWAGPTIPRERVADVNCNFDCDGDLNQICGGNGIATGPGGAYISLFADTLRFDGNETNIPEPEEPVEPTDPIVNP
GVDGYISIGCYTEAPGSRALPFFFATEDQTVAMCVDACSLRDYEYAGVQYGGECWCANAITTGAVPAPDAECNMACNDNA
AEYCGGGSRLNVYQRQSGGSFPSASVSLNGTIPAPTSSLIPASSVPVSSAPIISSIVTSAAPPVPTPGEDHFIGDWSFEG
CYTEGDGVRALNGRFYADDELTLEKCAAFCEGFVYFGAEYGRECWCGDVHGTGSVLASNQGDCNFPCGGDGSQFCGAGNR
LQMYRFGGADAPSGAVSSSLVVPTSAVVSSTVALVSSTAAEVSSAVAEVSSQIAIEASTAPASSSVVEESTSVVETSSTV
AVESSAVQSSSAQPSSAEASSVIEIMSSAVASSSFVPSSGIPSSTTPTSTPSPSVYPGNDLWNYTGCYSEPSPGRLLPSQ
LLNDGDEMDIELCLDVCAGYNYAGVEYGRECWCGDRLNAEGDVPSEGTAVPGELVDDDECAFLCPGNRLNYCGAGVRMSV
YILREREEALAEAS

Figure 8: scheme shows from Vl_6.2 gene cDNA translated amino acids sequence (1534 aa) containing Signal peptide: **green** colour; (18 aa); Fibronectin type III (FN3): **blue** colour (119 aa); Fibronectin is a high-molecular weight (~440 kDa) extracellular matrix glycoprotein that binds to membrane-spanning receptor proteins called integrins (19, 20). Sequence homology analysis revealed the presence of four conserved WSC carbohydrate binding domains (WSC domains) at the C-terminus. WSC domains: **red** colour (WSC1: 88 aa, WSC2: 93 aa, WSC3: 72 aa, WSC4: 104 aa).

4. Characterisation of Vl_NEP1

Vl_NEP1 (Necrosis- and ethylene-inducing protein) was isolated from the genomic library and sequenced completely. Full-length gene sequence of Vl_NEP1 with an ORF 763 bp nucleotides (Figure 9), cDNA translated sequence 233 aa (Figure 10). A phylogenetic tree (Figure 11) analysis of Vl_NEP1(also named NPP1)-like gene indicates the relationships and a time orientation of evolution in the *V. longisporum* VL43*, V. dahliae* VdLs.17 and *V. albo-atrum* VaMs.102 are very close than with other fungi. *V. longisporum* is closer to V. *albo-atrum* than *to V. dahliae.* The phylogenetic tree was created using software "ClustalW" (EMBL-EMI). The protein sequence of *V. longisporum* was translated by software "Transgen" (EMBL-EMI). The protein sequences of Vl_NEP1-like gene of related species were blasted from NCBI database. A protein sequence analysis by software SMART in all 15 selected fungal strains shows they are all NNP1-like necrosis inducing proteins. Infiltration of NPP1 into leaves of *Arabidopsis thaliana* plants result in transcript accumulation of pathogenesis-related (PR) genes, production of HR-like cell death (5).

During sequence analysis of Vl_NEP1 we found a conserved hepta-peptide motif GHRHDWE (6, 7) essential for necrosis-inducing activity (8) in 5x Vl_NEP1-homological genes Vl_NEPs (Figure 12), 9x Vd_NEPs (Figure 13) and 7x Vaa_NEPs (Figure 13).

GGATCCTCACAAGCTCGGGGCACAGGATTCAGACAGTGCTTGAAGCTGAGCTGTTCTCAGTTCTCCCTTAGGACACCACA
ACAAATGACCGAACGAACCGGCCGGATGATCAGACCAAAACATGGTGCCGTGTTCATCAGCCCTGAATCGCAACCCCACA
AGCGCACCAAGTAGATCCTGGTACAGATGCGCGCCTACCGTTGAGTCCCGGACATGTTCTGCTCCCATTGTGGCCATAGA
TCCGCACGCCCTATCACCACCGCCTTTCTATCAGCCCCCGCATCACCAGAATGTGCGACAGCCTGGCTTTATTAGTCGTC
GGACATGGCTACGCCTTCTTTCTCCGGTATAGCCTCGGTCCGTGCCGACTCAAATCAGTCTCAGGGGTTCGAATCGGGCT
TACTCTAATGTGTTGGGGGTCCGGCCTTGCTATCCATGACTCGACATCAACCTCTATGGGCGTGCCACCAGTGCACCCGA
ATGGCCCGGGTGGAACAGATGAACCAAAGTATGATAGATGAACCGGCTCCTCTGCCCTTGCCCACCTGCCCAGTCACGAC
TCACTCAGCACCGTCCACGGTCACCGTCCTTGCGATCTCGCATGAATGAGTGCCGAGACATGGGTCACTGCCTACACAGC
ACTTCGCCAAGGCGAGCTCACTGGGGTGGAGATTTATCGAATTATCGAATATCGTATCTGTCGGCCAAGTTCGGGCTGTC
CGGGCCAAGACGACGATTTGATCTTTTGGGATTTGCGCTTAGACGATTGACTGGGCTACTCCAGGGTCTTTGAGCCACGC
CCTTTACTGAAAGAGGCAAGGCATTCTCTTCTAGCAGGTGACAGCTGCTATTCAACAGAGCGCTATTCATGTCCACCTCT
TGGGGGCGGAGCCGCTCCGCAGCAATGTATTTGCCTGGGTAGAGGATACAGCTAAGCCTACCTTAGCGGCTAGCCTCACG
ATGTTCGTGTTCGCATCGTTAACGATGTCCGTCGACCGAGCTCGACATGAGTCGTATGCCTCAAGGACCCCTGGCATCTT
GACACTGCAGCAAACATGACAACCACGACAGAATGAAGTAGGAAGCCCGGTGTGTCAACCAGCCGGTCTGTCTGCAGAAG
GGTGGTCGATGGAAGGCCAAGGCAACTACATCTCCGGACATGATCAAAGTATATAAGAAGGACGATCACCGCTCCTTGAC
TTATTTTGCTTCTCTTCCTCTCATCACCTAGCCTCATTCTCTCTCTCGTCATCTAAACAGCCATCTGCACCTGCTACG**AT**
GCTTCCCTCCACAATCTTCTCGGTCTTTGCCCTCGTCGGCAGCGCCTTGGCTCAGCATCCCCCCAAGGTTAACCACGACA
GTATCAACCCCGTCCGCGATACTCTGGGGCCCAACGGCGACATGATCAGGAAGTTCCAGCCTCTGCTTCACATTGCCCAC
GGTTGCCAGCCTTACTCCGCTGTCAACACCCGCGGTGAGGTCAA**GTAAGACACGCAGCACTCCCTTCAGCTTACACAAAG**
CTCCACGCTAACACATATTCTGCAGCGCCGGTCTCCAAGACAGCGGTACCACCGCAGGCGGCTGCAAGGAAACCAGCAAG
GGCCAGACCTACGCCCGCTCCATGACCCTGAACGGCCAGTTCGGCATCATGTACGCCTGGTACTGGCCCAAGGACCAGCC
CGCCGACGGCAACCTCGCCAGCGGCCACCGCCACGACTGGGAGAACGTCGTCATCTGGTTCAACTCGAACAACGCAAACC
AGGCCGGCATCCTGCGCGGCGCCGCCTCGGGCCACGGCGACTACAAGAAGGTCAACAACCCCCAGCGCAACAACAACAAC
CTCCACGTCGAGTACTTCACCAGCCTCGGCAAGAACCACGAGTTGCAGTTCAAGACGTCGCCCGGCCGCACCTACTGGAT
CTGGGACTGGGACAGGATGGACAGCACCGTCCAGGGCGCCCTCAACCGCGCCGACTTTGGCAGCGCCAACTGCCCCTTCA
ACAACAACAACTTTGAGAGGAACATGCGCGCCGCGTTT**TAA**AGGCTCGACGCGCGGCTCGCCAGTCCTGGATTCCATCTG
GGCACGCATCTTGGCTCTTTCTCCTCCCGCCTTGGCTTGGCTTCTTGAGGCCAGTCGAGTGCTAGCATAGGTCCTGTACA
TAGCTTGTTCTGAATTACATACAACTCTGCCTGTCGAATTTGGTCTTGCATTCTGTGATCGAGTGTGTAAGCTTTTACGC

AGGTCGATAAATGACCAGTGCGTTGATTAGGTTCTTTCGTTGCTCTATTCATGGGTAGGTTCCCAGGAGCCCTGTCGTGA
TCCCGAAAACTCAAATAAACAGCATCATGAACTTTATAAGGCCACCATGCACTGCAGTTGATGTTCCTTTGGAGTGGAAC
ACCCCACTACCTTACCTCAAGCAGTGCAGCGGCAAGCAAGCTTGAGTCTCAACTTAATGTCAAGGAAACTGCTGCGGCAT
TGAGAAATCAGAGCCAATTCCAATGATTGGGGCACTCCGGAACCCAGCACATATGTCAAAAGACCAGACACTCACAACCA
AACACTGCAGATACAGCTCAATTCCAATATGACGATGATCGCATCGACTTGGTATGACATCGGACTGTGATCTCGTTCGA
TAGACGAAATCCAAGTTTGCTGACATACACGTTGGGTGCGACAGCTTGGAGAAATGCTCGGCAACTGGTCCTGTGCCAGG
ACGTACACAATGACGGGACCCGGCGGGGATGGGGCCGGTGGAGAGCTAGATCGACAGGATGACTTCCGCTGAGGAGACTT
CGCGCTCCGGGGAACCGATGCCGCCAGTAATAGTCGAGGTGATCATTAGGGGTGCTAAGCAGTAAAGCAGGAAAGTTTGA
ACTGTCCTACAAGTTCTTTGATGGTTTTATGTTTCGGTAAGCAGTGAGGACATCTCCTTATCATCGTAACGCCGCCCATT
CTTCGTGCCTCGTCCATACGCTGCCCTGAGGATAATTCACCCAAGTATTACACCAAATAGAGAAATCGAGACAGAATGCT
CTACGAAAAAGACGGGCGTCTGGTATGCTCCTATGACGGAGAAAAGCTCTGGATCGAGCCCTGGTCTGCCAATGCTTTCC
GCGTCCGAGCCACCAAGCTCTCCACGATGCCGACAGAGAACTGGGCCCTGCAACAGCCTCTTCAGACCGGATCC

Figure 9: Full length sequence of Vl_NEP1 gene (763 nt) and untranslated region. Coding sequence including one intron in blue colour, start code in green colour and stop code in red colour.

MLPSTIFSVFALVGSALAQHPPKVNHDSINPVRDTLGPNGDMIRKFQPLLHIAHGCQPYSAVNTRG
EVNAGLQDSGTTAGGCKETSKGQTYARSMTLNGQFGIMYAWYWPKDQPADGNLASGHRHDWENVVI
WFNSNNANQAGILRGAASGHGDYKKVNNPQRNNNNLHVEYFTSLGKNHELQFKTSPGRTYWIWDWD
RMDSTVQGALNRADFGSANCPFNNNNFERNMRAAF

Figure 10: Shown from Vl_NEP1 gene cDNA translated amino acids sequence (233 aa).

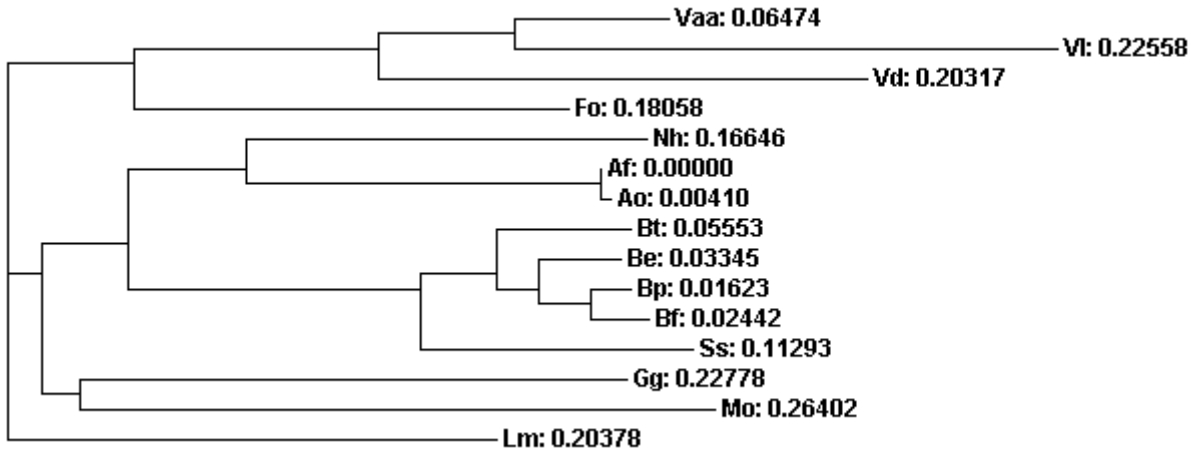

Figure 11: Phylogram of Vl_NEP1 gene at aminoacid level with related species containing NEP1-like gene. The relationship shows with distance. **Vaa**: *Verticillium albo-atrum* VaMs.102; **Vl**: *Verticillium longisporum* VL43; **Vd**: *Verticillium dahliae* VdLs.17*;* **Fo**: *Fusarium oxysporum* f. sp. erythroxyli*;* **Nh**: *Nectria haematococca* mpVI 77-13-4; **Af**: *Aspergillus flavus* NRRL3357; **Ao**: *Aspergillus oryzae* RIB40; **Bt**: *Botrytis tulipae*; **Be**: *Botrytis elliptica*; **Bp**: *Botryotinia pelargonii*; **Bf**: *Botrytis fabae*; **Ss**: *Sclerotinia sclerotiorum* 1980 UF-70; **Gg**: *Glomerella graminicola* M1.001; **Mo**: *Magnaporthe oryzae* 70-15; **Lm**: *Leptosphaeria maculans.*

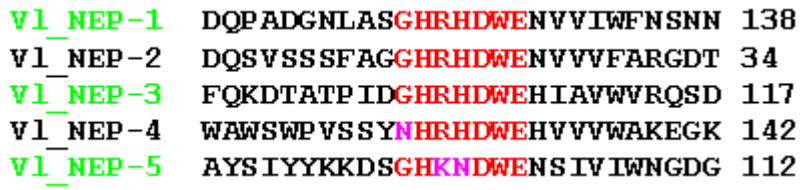

Figure 12*:* Alignment of a aminoacid sequence containing hepta-peptide motif GHRHDWE region from Vl_NEPs in genome sequence of *Verticillium longisporum* VL43 using software ClustalW2 (EMBL-EMI). All candidates contain a conserved hepta-peptide motif GHRHDWE. Vl_NEP-1, 3, 5 were up-regulated in gene relative expression test *in planta* by qRT-PCR (The result is not shown) (9).

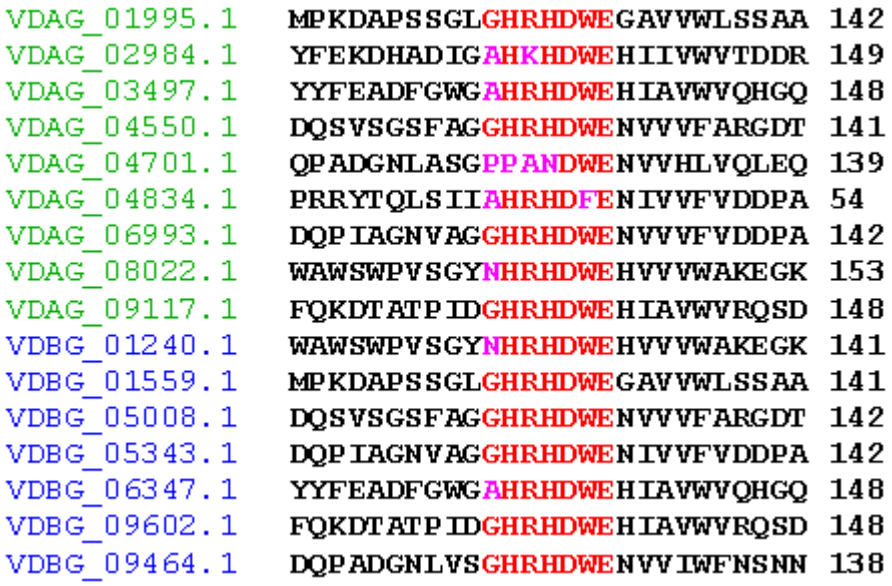

Figure 13: schemetic comparison of aminoacid sequence (in part) in Vd_NEPs (VDAG from genome sequence of *Verticillium dahliae* VdLs.17) and in Vaa_NEPs (VDBG from genome sequence of *Verticillium albo-atrum* VaMs.102) using software ClustalW(EMBL-EMI). Amino acid sequences derived from *Verticillium* Group Database. The results show all selected candidates hold a conserved hepta-peptide motif **GHRHDWE**.

Disscusion

V. longisporum, *V. dahliae* and V. *Verticillium albo-atrum* are closely related species. They are all soilborne fungi causing disease on many economically significant crops (10, 11, 12, 13, 14). Because *Verticillium* isolates collected from horseradish producing long-spores, they were classified as *V. dahliae* var. *longisporum* by Stark in 1961 (15). After detailed

study in morphological, physiological and molecular nature Karapapa et al. classified them as a separate species (16). Even now the taxonomy of *Verticillium* is still discussed (17). However, the host range and specificity, special symptoms after infection and other morphological, physiological and molecular descriptions are evidence for the existence of the separate species *V. longisporum*. This study confirms that *V. longisporum*, *V. dahliae* and *V. albo-atrum* are different, but they are very close related species compared with other selected fungal candidates after an analysis with Vl_6.2 and Vl_NEP1 gene on gene sequence level.

Worldwide access to the genome data gives us great help for sequence analysis which is prerequisite. Especially the successful investment in a genome project for *V. dahliae* and *V. albo-atrum* provides useful sequence information because of the high similarity at sequence level for the *V. longisporum* study. The nucleotide and protein sequence and blast function of *Verticillium* group database enabled us to design primers and to find a conserved necrosis-inducing active hepta-peptide motif **GHRHDWE** also in five NPP1-like Vl_NEPs.

From a genomic library isolated full-length gene sequences with its UTR is very useful for generating target genes containing long sequences. A normal PCR based amplification of target genes is limited to sequence length, and its correctness is dependant on PCR efficiency and polymerase used.

This genomic library is not large enough to represent all gene sequences because it is limited by the total amount of clones. But this can be improved through a further expansion of the library. A monitoring of a genomic library with microtiter plate combined with a rapid colony-PCR based screening procedure is good for a small genomic library generation.

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Chapter 3: Determination of *in planta* gene expression by qRT-PCR

Haiquan Xu, Arne Weiberg and Petr Karlovsky

Abstract

The *in planta* determination of relative gene expression of pathogenicity related genes in *V. longisporum* compared to growth *in vitro* in a xylem-simulating medium (SXM) by quantitative reverse transcription real-time PCR (qRT-PCR) was applied for description of TDFs after cDNA-AFLP screening and sequence extensions. 10 genes have been characterised after their relative expression levels *in planta* by qRT-PCR, 6 of them indicated an up-regulated gene expression, 2 of them were down-regulated and further 2 were time-depended. The isolation, purification and transcription procedures of total RNA and mRNA from *V. longisporum* infected *Brassica* plants were improved and used for the determination of relative gene expression by qRT-PCR. A set of reference genes were tested.

Introduction

V. longisporum (VL) is a pathogen with host specificity on the *Brassicas*. *Verticillium* wilt has become a serious problem in northern Europe. After direct penetration of roots tips the mycelia of pathogen entry into xylem vessels of raps and format conidia and systemic colonise in vascular system. The metabolites in xylem vessels of host plant rapes are used as the elicitors for *Verticillium* cultivation *in vitro* for a pathogenicity related transcriptome screening using cDNA-Amplified fragment length polymorphism (AFLP) technique (1). The transcript derived fragments (TDFs), which are selected by cDNA-AFLP profiling procedure treated with xylem sap from health plant or infected plant. will be investigated by qRT-PCR.

A qRT-PCR is a variant of the standard PCR technique used for quantification of DNA or messenger RNA (mRNA) abundance developed in 1993 (2). The citations of qPCR have increased dramatically (3). Its on sequence specific primers based specificity and on measurement of fluorescence based high sensitivity allowed qRT-PCR to be a most suited

method for evaluation of pathogen gene expression during the infection (4). The real-time PCR machine continuously monitor the fluorescence-based increase, which base upon double-stranded DNA combining with for example SYBR Green I or other similar dyes at each stage during the PCR cycle. A identification of relative copy-numbers between a sequence of target gene relative to a sequence of housekeeping gene performs a relative gene expression level of a target gene. The *in planta* determination of relative gene expression of phytopathogen *V. longisporum* compared to growth *in vitro* in a xylem-simulating medium by qRT-PCR was applied in this study for description of the putative pathogenicity related genes presented through TDFs.

The quantification of relative gene expression is achieved with analysing Ct value. The cycle numbers at the threshold level of log-based fluorescence are defined as Ct value (Figure 1). Several data analysis systems have been developed in the last years for relative quantification such as standard curve method for relative quantification, comparative CT($2^{-\Delta\Delta Ct}$) method and Pfaffl's model etc. (5). All systems include both target and control samples. Many applications of real-time PCR do not require complex models. The efficiency calibrated model (6, 7) and the ΔΔCt model (8) are widely applied (3).

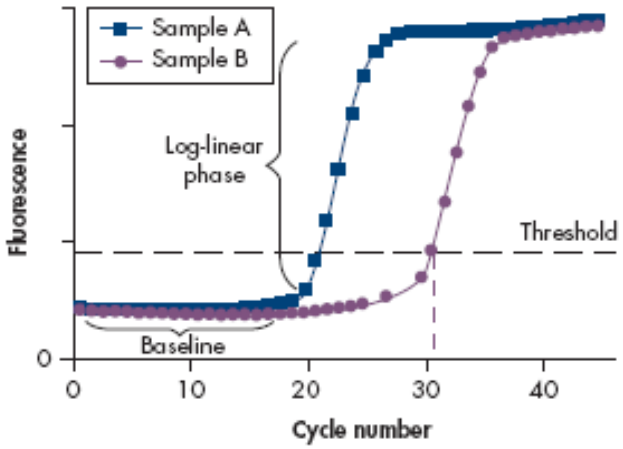

Figure 1: Amplification plots showing increases in fluorescence from 2 samples (A and B). Sample A contains a higher amount of starting template than sample B.

Materials and methods

1. Fungal isolates and preparing inoculum

The strain *V. longisporum* VL43 from *Brassica napus* grown in northern Germany as

described was used in this research (Zeise and von Tiedermann) (10, 11).

For propagation, the spores were plated onto potato dextrose agar (PDA) and incubated at 23°C in the dark. The spore suspensions in a concentration of $1x10^6$ spores ml^{-1} in potato dextrose broth (PDB) with 25% glycerin were used as fungal stocks at –80°C for long time. Shake cultures were started through adding 500 µl spore suspension (1-3 x 10^6 conidia ml^{-1}) to 300ml Potato Dextrose or Czapek Dox broth. The inoculated broth was incubated at 23°C on a rotary shaker at 100 rpm in the dark. After 7-14 days, the culture was filtered with steril gauze. Spore concentration was determined using microscope and diluted to 1 x 10^6 spores ml^{-1}.

2. Plant material and inoculation

Rapid-cycle rape (*Brassica napus* var. *napus*, genome Acaacc[12]) was originally provided by P.H. Williams (Department of Plant Biology, University of Wisconsin-Madison, WI; Crucifer Genetics Cooperative, Stock number 5). Oil-rape seeds were surface sterilised by immersing them in 70% ethanol for 30 s and then rinsed in sterilised tap water for 30 s three times before sowed in sterile silica sand. After 7days the germinated rape seedlings were carefully rinsed from silica substrate, cleaned roots, inoculated in spore suspension (1 x 10^6 spores ml^{-1}) of *V. longisporum* for 30-45 min by dipping, thinned to one plant per pot with an earth/sand mixture (1/1 v/v) and grown in a climatic chamber under constant conditions with a light regime 16/8 h (light/dark) and a temperature 23°C/20°C (day/night). Seedling roots dipped in sterile tap water was as a negative control. Rape plant materials were harvested 14, 21, 28, 35 days post inoculation (dpi) for time-course experiments.

3. Extraction of fungal genome DNA

Fungal genomic DNA was extracted from *V. longisporum* mycelium after CTAB protocol (13). Weight lyophilized mycelium, pulverize 200 mg in the mortar. Transfer ground mycelium to 50 ml tube(s) (chloroform resistant) containing 10ml of TES-Puffer and 4 mg Proteinase K (0.2 ml of stock solution 20mg/ml). Incubate for 45 min at 45°C and then mix by turning the tubes every 10 min. Following add 3.9 ml of 5 M NaCl and mix well. Next add 1.4 ml of 10% CTAB and mix, incubate for 10 min at 65°C and cool tube(s) in ice/water-bath. Add 10 ml Chloroform/Isopentylalcohol, mix throughly and then keep for 30 min in ice/water-bath. Transfer the upper phase and part of the lower phase into another centrifugation tube by pipetting. Spin for 20 min at 4,500 rpm, 5°C (or 5 min at 10,000

rpm). Transfer water phase to new tube(s) containing 10 ml isopropanol (room temperature), mix thoroughly. If DNA precipitates immediately, continue directly with centrifugation (10 min at 4,500 rpm, room temperature). Finally rinse pellet with 70% ethanol, dry and dissolve in TE buffer.

Mix 4 µl DNA solution and 4 µl of a 10-times dilution with 2 µl loading buffer, load on an 0,8% agarose gel with 25, 50, 100 and 200 ng DNA of bacteriphage lambda. Run at 1 V/cm till brompenol blue reaches ¾ of the length of the gel. Stain with ethidium bromide and visualize under UV light.

4. Quantification of fungal DNA *in planta* tissue by qRT-PCR

For quantification of fungal genomic DNA within the total extracted genomic DNA from *Verticillium*-infected plant, quantitative real-time PCR with SybrGreen carried out in iCycler (BioRad, Hercules, CA) was used. A 261 bp fragment of ITS region was amplified with the primer OLG70 (5'-CAGCGAAACGCGATATGTAG-3') and OLG71 (5'-GGCTTGTAGGGGGTTTAGA-3') in PCR and quantified (14). The amount of fungal DNA in the infected plant was derived from a calibration curve formed with a dilution set of fungal DNA.

5. Total RNA extraction, mRNA purification and synthase of cDNA

Total RNA was extracted with a “hot phenol” protocol for quantitative reverse transcription real-time PCR (qRT-PCR). The infected plant material or fungal Mycelium was pulverized under liquid nitrogen in mortars. The powdered material until 500µl volume was transferred into pre-cooled 2ml Eppendorf tube. Addition of 500µl of hot extraction buffer (Tris-HCl, 0.1 M; LiCl, 0.1 M; EDTA, 0.01 M; SDA, 1%) (80 °C) and vortexed. Addition of 500 µl phenol (buffered, pH 7.5) and vortexed. The tubes of samples were centrifuged at 13000 rpm for 5 min. and the supernatant was transferred into a new 2 ml Eppendorf tube. Addition of 500 µl of chloroform / isoaylalcohol mixture (24:1) and vortexed. The tubes of samples were centrifuged at 13000 rpm for 5 min. And the water phase (top) was transferred in a new 2ml Eppendorf tube. Addition of 1 Vol. of LiCl solution (4M) and vortexed. The RNA was precipitated at 4°C (on ice) for at least 3 hrs. After LiCl precipitation RNA samples were keept on ice. The tubes of samples were centrifuged at 13000 rpm for 15min. The supernatant was removed at 4°C. The pellet was dissolved with 15µl DEPC water. Addition of 1.5 µl NaOAc (3 M, pH5.2) and 2.5 Vol. EtOH (96%) and vortexed. The samples were

kept on ice for 25 min. The tubes of samples were centrifuged for 15 min at 4°C and 13000 rpm . The supernatant was removed and the pellet was washed with ice cold EtOH (70%). The RNA pellet was dried and dissolved in 50-200 µl DEPC water. The isolated RNA samples were freezed in -80°C.

RNA was measured by ultraviolet absorbance at 260 nm and 280 nm. Calculation of the RNA concentration is based on the absorbance at 260 nm. Diethyl-pyrocarbonate (DEPC)-treated water is used to dissolve RNA. Add 1 ml of 0.1% DEPC to 1000 l of ddH_2O, Mix and leave at room temperature for at least 1 hour. After Autoclave to be cooled to room temperature prior to use.

For increasing of target gene amount in complete template sample, the pure poly A^+ mRNA was purified from total RNA used with Oligotex mRNA Kits (Qiagen, Hilden, Germany). Oligotex resin consists of polystyrene–latex particles and a perfect spherical shape with dC10T30 oligonucleotides which covalently immobilized on the surface. The useful purification of poly A+ mRNA was provided by efficient hybridization. mRNA recoveries were greater than 90%. The Oligotex requires less time and decreased the risk of degradation by RNases.

6. qRT-PCR assay

Root and hypocotyl of infected plant tissue were harvested on 14, 21, 28, 35 dpi and total RNA was extracted using hot phenol extraction protocol described above. Because of the low quantity of RNA from each plant, each total RNA sample was extracted from a tissue pool of six plants. mRNA was purified from total RNA samples using Oligotex mRNA Purification Kit (Qiagen, Hilden, Germany). About 500 -1000 ng mRNA were used in reverse transcription reactions together with 50pmol oligo-T primer, 1mM dNTPs, 20 U RiboLock RNAse inhibitor and 200 U RevertAid reverse transcriptase (Fermentas, St. Leon-Rot, Germany) in a 20 µl reaction volume. cDNA synthesized from mRNA was purified by PCR Purification Kit (Qiagen, Hilden, Germany) and quantified using a photo-spectrometer at 260 nm (GeneQuant, Cambridge, UK). About 100 ng purified cDNA was used as template in qRT-PCR. SybrGreen system was used in real-time PCR. Housekeeping genes, β-tubulin and ribosomal protein S17 (15, 16). SybrGreen system was used in real-time PCR. Housekeeping genes, ribosomal protein S17, β-tubulin, GADPH, and Histone H3 (15, 16), were used for normalization. ΔΔCt and In efficiency

calibrated ΔΔCt methods were used for quantification of mRNA level between *in vitro* and *in planta* samples (6).

7. Primers used in qRT-PCR

The primers of candidate internal reference genes (Table 1) and putative pathogenicity related genes of *V. longisporum* (Table 2) used in qRT-PR were designed on basis of TDFs from cDNA-AFLP screening and using different methods extended sequences.

Table 1: Primers of candidate internal reference genes

Nr.	Primer Name	Primer Sequences 5'-3'	Tm °C	PCR fragment	
				gDNA	cDNA
1	Vl_GAPDH-f1	CATGCTCAAGTACGACTCCACC	57,5°C	164bp	164bp
	Vl_GAPDH-r1	CAGTGGACTCGACGACGTACT	57,7°C		
2	Vl_GAPDH-f2	TCGCCAAGGTCATCAACGAC	57,3°C	208bp	208bp
	Vl_GAPDH-r2	CAGTGGACTCGACGACGTACT	57,7°C		
3	Vl_GAPDH-f3	GCTCCCATCAAGGTTGGCAT	57,9°C	125bp	125bp
	Vl_GAPDH-r3	GCGTACTTGGTCTCAATGAAGGG	57,8°C		
4	Vl_GAPDH-f3	GCTCCCATCAAGGTTGGCAT	57,9°C	497bp	295bp
	Vl_GAPDH-r1	CAGTGGACTCGACGACGTACT	57,7°C		
5	Vl_GAPDH-f3	GCTCCCATCAAGGTTGGCAT	57,9°C	374bp	172bp
	Vd_GAPDH-R	CACCCTTGAAGACGCCGTG	58,7°C		
6	Histone H3-f	CGCGTGACGAGGAGATGC	58,2°C	163bp	163bp
	Histone H3-r	CGTATCCTTCGTGCCAACTG	55,9°C		
7	Vd_His H3-F	ATGCCACCACGCTCAGGTA	58,3°c	358bp	192bp
	Vd_His H3-R	CATCTCCTCGTCACGCGGT	58,9°C		
8	RP-S17-F	GCATCTGCGATGAGATCGCCA	59,5°C	264bp	196bp
	RP-S17-R	TCGGAGTTCTGGGTAAAGTCGAGA	58,5°C		
9	Vd_RP-S17-F	GCATCTGCGATGAGATCGCC	58,4°C	256bp	188bp
	Vd_RP-S17-R	CTGGGTGAAGTCGAGAGCAGA	58,1°C		
10	Vl_β-tubulin-F	CTACCTGACCTGCTCCGCCATCT	62,1°C	344bp	278bp
	Vl_β-tubulin-R	GCTGGTACTCCGAAACGAGATCG	59,2°C		
11	Vl_β-tubulin-F2	CATGATGGCCGCCTCTGAC	58,4°C	192bp	126bp
	Vl_β-tubulin-R2	CGAAGAGTTCTTGCTCTGGACGT	58,5°C		

12	Vd-β-Tubulin-F	TCCGTAACGGTCGCTACCTG	58,3°C	261bp	193bp
	Vd-β-Tubulin-R	CCGACGAAGGTGGAGGACAT	58,6°C		
13	γ-Tubulin-F1	GGAAACCTTGAAGACTTCGC	54,0°C	288bp	288bp
	γ-Tubulin-R1	ATCGCTACCATCGGCCTCA	57,8°C		
14	γ-Tubulin-F2	TGTCTGCGAGCACCACGAC	59,6°C	281bp	281bp
	γ-Tubulin-R1	GTCGACTTCGCCTTGAATGACG	58,2°C		

Table 2: Designed specific primers of putative pathogenicity related genes of *V. longisporum* after cDNA-AFLP screening

Nr.	cDNA	Primer pair
1	TDF2.1(Vl_2.1)-XF	5'-TCCAACATTGAGGCGTCGC-3'
	TDF2.1(Vl_2.1)-XR	5'-TGGTGTCGGCGAATGAACC-3'
2	TDF6.2(Vl_6.2)-XF	5'-CCTGGAGTCTCAACACCAACG-3'
	TDF6.2(Vl_6.2)-XR	5'-TACGTAGTTGCCAGCAGCGC-3'
3	TDF10.2(Vl_10.2)-F	5'-AATTTGTTCCGCCCACTTCCATCCG-3'
	TDF10.2(Vl_10.2)-R	5'-ACGTGCTGGCTGGCGTAAGAC-3'
4	TDF11.1(Vl_11.1)-F	5'-AATTTGCCCCTACCATGCGAG-3'
	TDF11.1(Vl_11.1)-R	5'-TCGATTTCTTGTCTGTGTCGCC-3'
5	TDF12.1(Vl_12.1)-F	5'-AGCCACAGTATTGCTACGC-3'
	TDF12.1(Vl_12.1)-R	5'-GTATGGGTAGGATGTGTCG-3'
6	TDF12.2(Vl_12.2)-F	5'-AATTCGTCCTCGGGCCTCAAGGCAGA-3'
	TDF12.2(Vl_12.2)-R	5'-ACGTAATGTCATCACAGGTCAGAGTGG-3'
7	TDF24.1(Vl_24.1)-F	5'-AATTTGACAAAGCGACCGAAGCTA-3'
	TDF24.1(Vl_24.1)-R	5'-AGACTGAAGCCGGGCCAGA-3'
8	TDF34.2(Vl_34.2)-F	5'-ATCAGGCGCGCGAGAAGGAGCTG-3'
	TDF34.2(Vl_34.2)-R	5'-ACGTGGGTCGGACAAACCTGTC-3'

9	VINEP-F	5'-CGGTGAGGTCAAGTAAGACACGC-3'
	VINEP-R	5'-TGACGACATTCTCCCAGTCG-3'
10	VIPKS1-F	5'-ACATGTCGCCGCGCGAGCCTAC-3'
	VIPKS1-R	5'-CAGGCTGTATCGATGTTCAGACTAGGTCCAC-3'

8. Data analysis of qRT-PCR

Since qRT-PCR as a powerful tool used for relative quantification of transcriptional level. Several data analysis procedures have been developed. In which ΔΔCt model (Livak and Schmittgen, 2001) and the efficiency calibrated model (Pfaffl, 2001) are widely applied. The experimental systems for both models include treatment and control samples. For each sample, the transcriptional level of target gene and reference gene or housekeeping gene were quantified. Amplification efficiency of reaction was performed from serially diluted aliquots by qRT-PCR. Typically, several biological replicates are used for each diluted concentration.

ΔCt (Sample) = Ct target gene in sample – Ct reference gene in sample

ΔCt (Calibrator) = Ct target gene in calibrator – Ct reference gene in calibrator

$\Delta\Delta$Ct = ΔCt (Sample) - ΔCt (Calibrator)

In efficiency calibrated ΔΔCt model the Ct number was first plotted against cDNA input (or logarithm cDNA input), and the slope of the plot was calculated to determine the amplification efficiency. ΔCt for each gene (target or reference) is then calculated by subtracting the Ct number of target sample from that of control sample.

Statistical considerations are not widely discussed for the analysis of the effect of each experimental factor as well as significance testing. In ΔΔCt model ΔCt for treatment and control was subject to simple t-test, which will yield the estimation of $\Delta\Delta$Ct. In efficiency calibrated ΔΔCt model a substantial statistical analysis of the REST® program (Pfaffl et al., 2002) was applied. The software based on the efficiency-calibrated model and presented randomization tests to obtain the significance level.

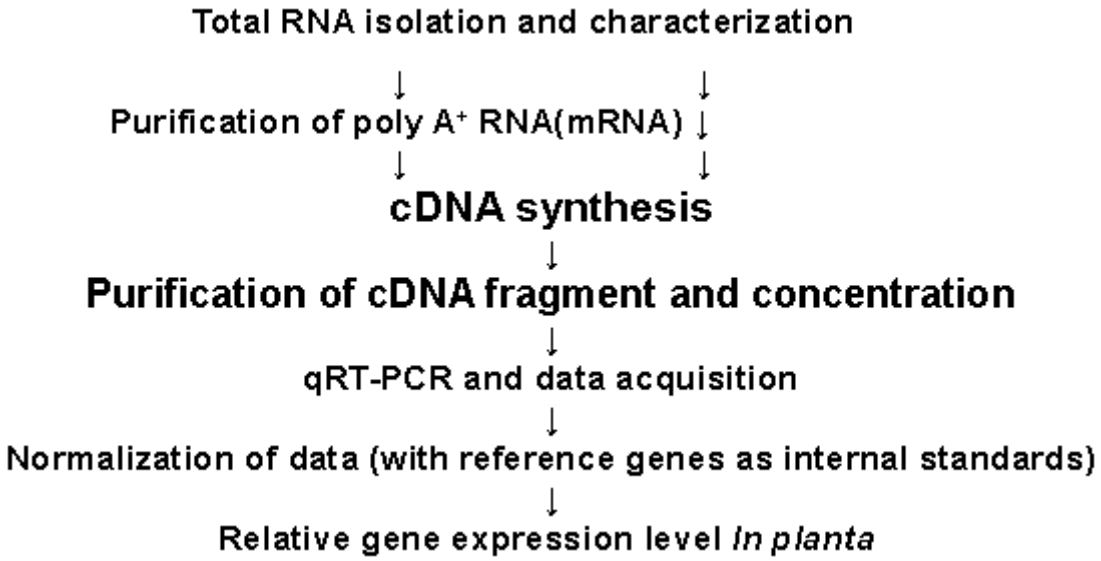

Figure 2: scheme for *in planta* determination of relative gene expression by qRT-PCR.

Results and discussion

1. Relative gene expression of putative pathogenicity related genes *in planta*

For assessment of pathogen *V. longisporum*, oilseed rape plants were inoculated with *V. longisporum* spores via root-dipping. The infection was found in lateral roots, xylem vessels, stems, leaves and pods etc. After 10 dpi the symptoms began to appear in a form of dark-coloured veins, slight chlorosis, a main symptom during the whole infection was clear expanded plant growth stunting (Figure 3).

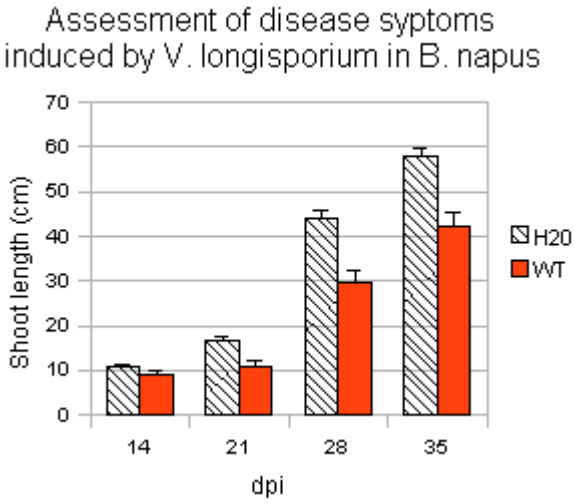

Figure 3: Assessment of disease symptoms on *B. napus* plants inoculated with *V. longisporum* VL43 (concentration 10^6 spores / ml) compared to control (plants treated with water). Error bars indicated the standard deviation of plant growth. significance levels of $p < 0.05$ (student's t-test) between treated with wild type of *V. longisporum* and water.

The results of *in planta* determination of relative gene expression in *V. longisporum* during the infection compared to its growth *in vitro* in SXM medium by qRT-PCR were performed using different cDNAs transcripted from total RNA and mRNA respectively (Figure 4 and Figure 5). The expression of pathogen transcripts was measured with infected plant root and hypocotyl tissue in time course 14 dpi, 21 dpi, 28 dpi and 35 dpi. We have not determined the relative gene expression by qRT-PCR using cDNA transcripted from total RNA at 14 dpi. We found all TDFs belong to the putative genes and each of them expressed in certain form and degree. The relative gene expressions for TDF6.2, TDF12.2, TDF34.2, and TDF24.1 were up-regulated by determination using qRT-PCR. The relative gene expression for TDF12.1 was suppressed and for TDF2.1 and TDF11.1 demonstrated time-dependent. TDF11.1 shown the different trends by the determination by qRT-PCR using cDNAs from total RNA and cDNAs from mRNA. TDF10.2 and TDF30.2 were down-regulated both using cDNAs from mRNA and total RNA. The extra selected candidate genes VlNEP1 (encoding necrosis and ethylene inducing peptides) whose hetero-expression induced necrosis *in planta* (unpublished) and VlPKS1 (putative encoding polyketide synthase type I) performed a significant up-regulation in all tested time course. Two reference genes, β-tubulin and ribosomal peptide S17 were used in qRT-PCR using cDNAs transcripted with mRNA. In order to increase the statistic validation, we applied three reference genes glyceraldehyde-3-phosphate dehydrogenase (GADPH),

ribosomal peptide S17 and histone H3 for qRT-PCR using cDNAs transcripted with total RNA.

2. Establishment of *in planta* qRT-PCR and selection of primers

qRT-PCR is a useful tool for determination of relative gene expression. However, *in planta* determination of pathogen *V. longisporum* gene expression during infection in host plant *B. napus* had to establish because of diversity of biological organisms and the special infection and colonisation form of *V. longisporum,* even qRT-PCR is suitable for pathogen biomass in the host in very low level (17). We have improved two procedures for identification of relative gene expression *in planta* (Figure 2) including “hot phenol” total RNA extraction protocol. one procedure used isolated mRNA for cDNA synthesis, the another used directly total RNA, both cDNAs were purified and concentrated in about 100 ng μl^{-1} as template of qRT-PCR.

The specificity, amplification efficiency of all used primers for reference as well as putative pathogenicity related genes were validated respectively. They had no crossing-effect with host plant, the amplification efficiency arriving more than 90% and kept less difference between reference and target fragments. It is clear, not a single universal reference gene has been found, it exists yet at all (18). However, for this study ribosomal peptide S17, β-tubulin, GADPH, and histone H3 were selected as stably expressed both *in vitro* and *in planta* systems, respectively.

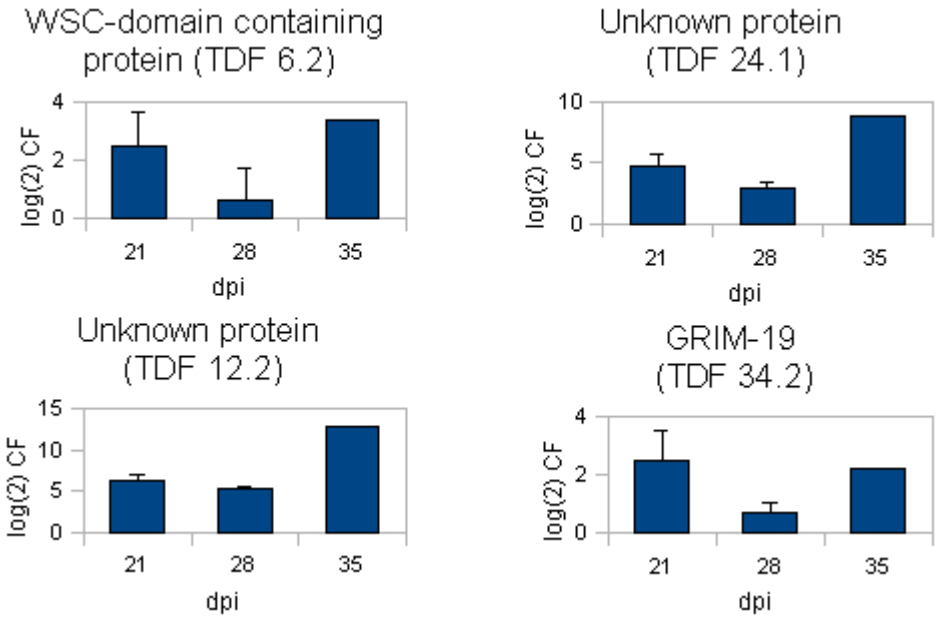

Figure 4a : *In planta* relative gene expression analasis of selected *V. longisporum* target

genes Vl_6.2 (TDF6.2), Vl_24.1 (TDF24.1), Vl_12.2(TDF12.2) and Vl_34.2(TDF34.2) in time course after infection. The with total RNA transcripted cDNAs were used as templates for qRT-PCR.Transcript levels of selected *Verticillium*-genes were measured and normalised using three reference genes, GADPH, ribosomal peptide S17 and histone H3. Ratios of relative expression levels between *Verticillium in vitro* as calibrator and *in planta* as target were calculated as the ΔΔCt values of three biological replicas at each time point. Error bars indicated the standard deviation of calculated ΔΔCt values. Significance level was set to p values < 0.05 and was calculated for three reference genes, respectively. one asterisk indicates significant change in the expression levels regarding to one of the reference genes.

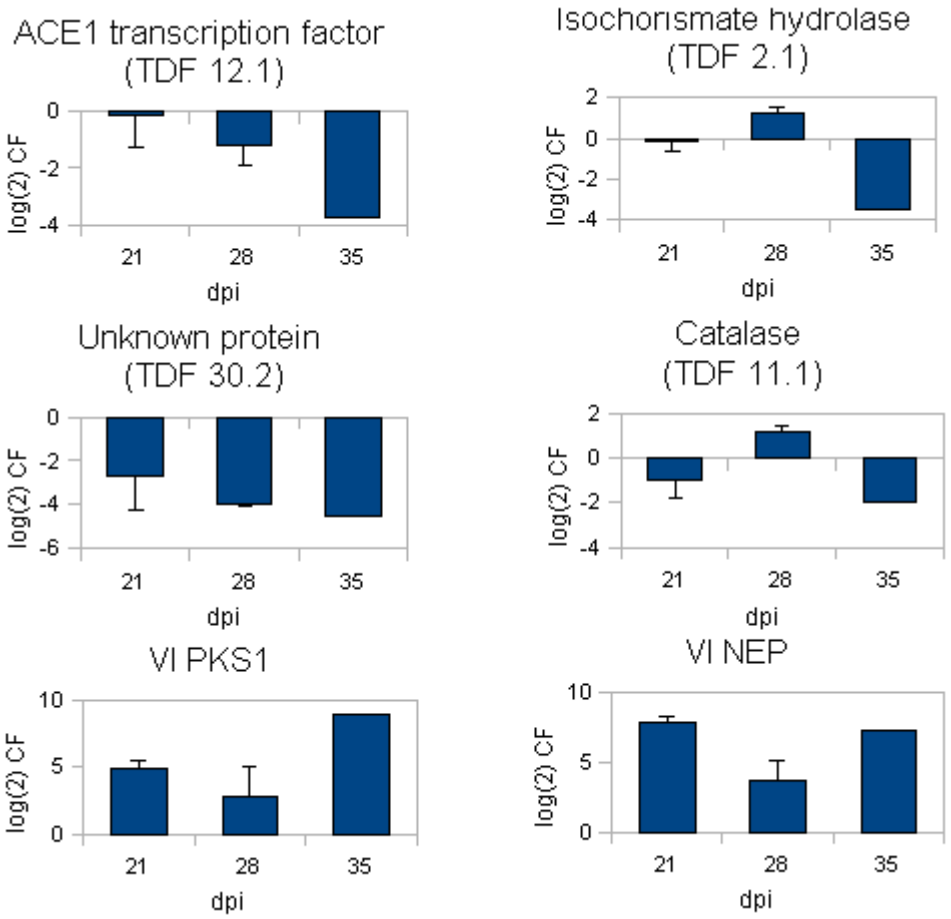

Figure 4b : *In planta* relative gene expression analasis of selected *V. longisporum* target genes Vl_12.1 (TDF12.1), Vl_2.1 (TDF2.1), Vl_30.2(TDF30.2), Vl_11.1(TDF11.1), Vl_PKS1 and Vl_NEP1 in time course after infection. The with total RNA transcripted cDNAs were used as templates for qRT-PCR. Transcript levels of selected *Verticillium*-genes were measured and normalised using three reference genes, GADPH, ribosomal peptide S17 and histone H3. Ratios of relative expression levels between *Verticillium in*

vitro as calibrator and *in planta* as target were calculated as the ΔΔCt values of three biological replicas at each time point. Error bars indicated the standard deviation of calculated ΔΔCt values. Significance level was set to p values < 0.05 and was calculated for three reference genes, respectively.

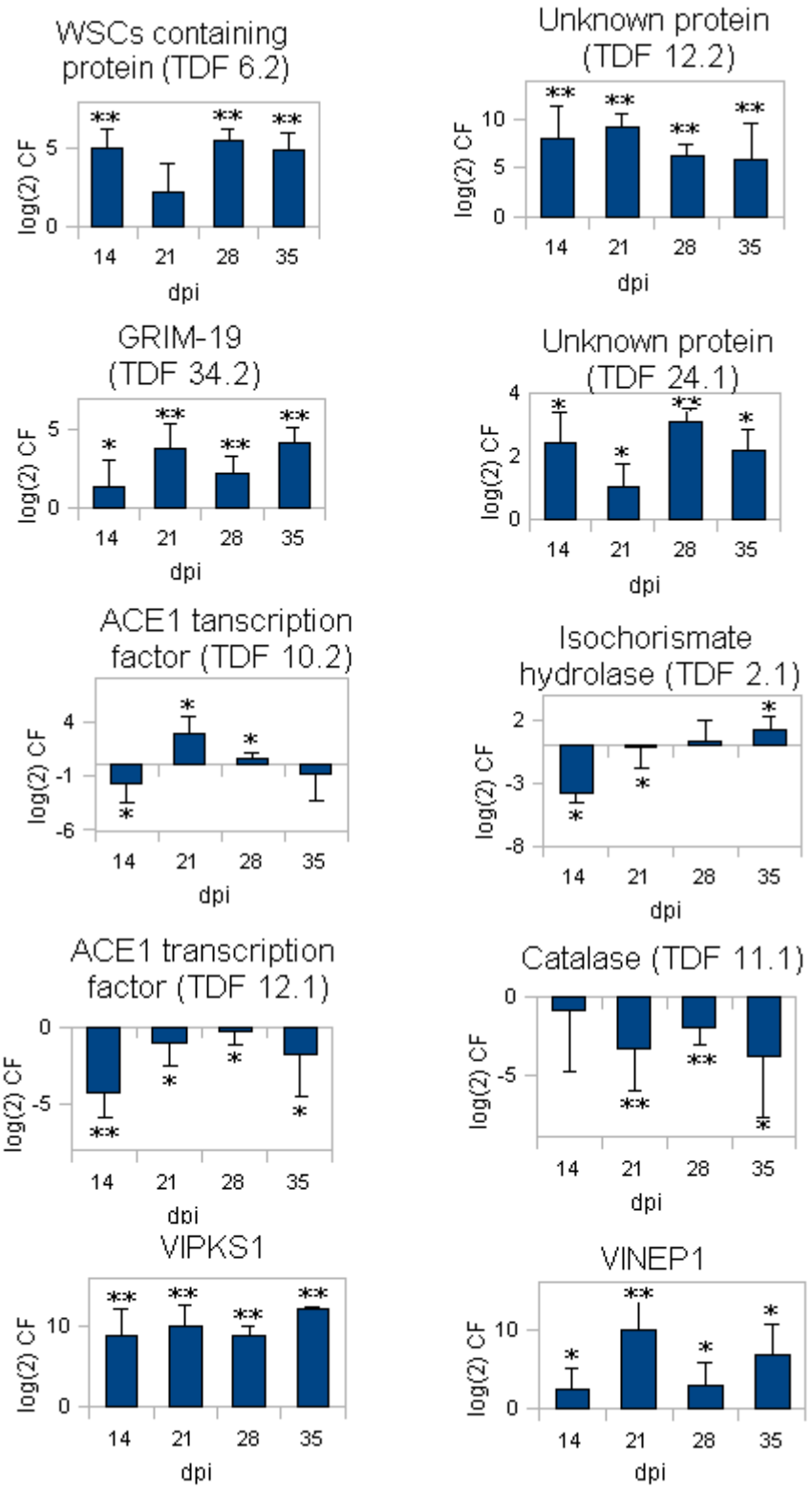

Figure 5: *In planta* relative gene expression analysis of selected *V. longisporum* target

genes Vl_6.2 (TDF6.2), Vl_24.1 (TDF24.1), Vl_12.2 (TDF12.2), Vl_34.2 (TDF34.2), Vl_12.1 (TDF12.1), Vl_2.1 (TDF2.1), Vl_10.2 (TDF10.2), Vl_11.1 (TDF11.1), Vl_PKS1 and Vl_NEP1 in time course after infection. Transcript levels of selected *Verticillium*-genes were measured and normalised using two reference genes, β-tubulin and ribosomal peptide S17. Ratios of relative expression levels between *Verticillium in vitro* as calibrator and *in planta* as target were calculated as the ΔΔCt values of three biological replicas at each time point. Error bars indicated the standard deviation of calculated ΔΔCt values. Significance level was set to p values < 0.05 and was calculated for two reference genes, respectively. one asterisk indicates significant change in the expression levels regarding to one of the reference genes. The with purified mRNA transcripted cDNAs were used as templates for qRT-PCR. (modified from Weiberg, Xu et al. 2008).

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Chapter 4: A potential pathogenicity related gene Vl_6.2 from *Verticillium longisporum* and its role in cell-wall integrity

Haiquan Xu, Arne Weiberg, Petr Karlovsky

Abstract

A transcript we designated as Vl_6.2 was among a set of genes identified by a cDNA-AFLP. Vl_6.2 showed strong regulation when the effects of xylem sap metabolites of the host plant *Brassica napus* on the transcriptome of *V. longisporum* were investigated. This find was confirmed by measuring strongly enhanced transcript accumulation *in planta* during infection of *B. napus* compared to *in vitro* cultivation of *V. longisporum*.

Full gene sequence analysis of Vl_6.2 showed it to comprise of an ORF of 4,722 nucleotides (nt), two introns and translated cDNA is predicted to code for a 1,534 amino acids (aa). Sequence homology analysis (pBLAST, SMART) revealed the presence of four repetitive water soluble carbohydrate (WSC) binding domains at the C-terminus. WSC domains have been reported to influence membrane integrity in different microbes like baker's yeast (*Saccharomyces cerevisiae*).

Through a sequence-specific antisense RNA the gene translation was silenced in *V. longisporum* and transcript accumulation was suppressed to 70% of its activity in transformants to the non transformed wild type determined by quantitative reverse transcription real-time PCR (qRT-PCR).

Mycelial growth of gene-silenced mutants was inhibited in the presence of membrane disrupting detergent SDS (sodium dodecyl sulfate) in PDA plate with a concentration in 0.2 %. A alkaline stress response related melanistic process of mutants was delayed at pH 9. Vl_6.2 gene silenced mutants reduced virulence in *B. napus*.

Introduction

V. longisporum is a soil borne plant pathogen on oilseed rape (*Brassica napus* spp. oleifera) (29, 30, 31) and has been observed to induce rape's vascular wilt in northern Europe. This disease results in increased economic losses in Sweden and Germany since about 50 years (32, 33, 34). Phylogenetic analysis of *V. longisporum* shows a very close relation to *V. dahliae* and *V. albo-atrum* (35). The morphology of *V. longisporum* is very similar to other two important phytopathogen *V. dahliae* and *V. albo-atrum* with a small difference in the length of spores (36, 37, 38).

The fungal cell wall constructs cell form and supports cell integrity to interact with extracellular stress (14, 15). PKC1-MPK1 pathway (Protein kinase C - Mitogen-activated protein kinase) (16, 17, 18, 19, 20, 21, 22) was involved in an activation, which produces a major component 1, 3-beta-D-glucan synthase of cell wall (23, 24, 25), and reacts to disturb the cell wall (26). WSC (Water-soluble carbohydrate binding) domains are members of a protein family in the yeast *Saccharomyces cerevisiae* and are putative upstream activators of the PKC1-MPK1 pathway (15, 17). A deletion of Wsc1, Wsc2 or Wsc3 induced sensitivity of S. cerevisiae cell to extracellular stresses (16, 27, 28). MPK1 pathway affects also microsclerotia formation and pathogenicity in *V. dahliae* (26).

Gene silencing with additional antisense RNA in the cell is a classic method discovered by plant scientists in 1988. Flower pigmentation in petunia and tabacco plants was reduced by a constitutive expression of a chalcone synthase gene in antisense direction (1). A few years later this technique was successfully used for the first genetically engineered food tomato “Flavr Savr”, The tomato gene polygalacturonase, which is responsible for degradation of cell wall component pectin, was suppressed in this way (2). Antisense RNA gene silencing controls have been applied in many cases of fungi (3, 4, 5, 6, 7). This applification is especially useful when the target gene is represented by multiple copies in the fungal genome (8). Antisence RNA-mediated silencing control has also been used in Bacteria (9) and viruses. The short regions of viral RNA sequences, which was uncoded for any viral protein, was carried in plants for increasing of plant resistance against pathogen virus (10, 11). These post-transcriptional gene-silencing phenomena may occur via an increased rate of mRNA degradation (12). Antisense RNA technology exhibits is a useful mild genetic tool (13) in gene regulation and has the following advantages compared to other knock-out methods such as low cost, quick and easy to operate.

Argrobacterium tumefaciens-mediated transformation has been a useful tool for fungal transformation since 1998 (39). An improved protocol has been applied to transform filamentous fungus *V. longisporum* (40).

In this work we characterized a putative WSC domains involved Vl_6.2 gene, which found by cDNA-AFLP profiling induced with xylem sap of oilseed rape (*Brassica napus* spp. oleifera) (41) and silenced with an Antisense RNA method (42).

Materials and Methods

1. Bacterial strains

Escherichia coli strain DH5α and *Agrobacterium tumefaciiens* strain AGL1 (provided by Dr. Susanne Frick, Leibniz Institute of Plant Biochemistry, Halle/Saale,Germany) were used for plasmid construction and fungal transformation (43, 44, 45). The strain was stored in a 9 : 1 (water to glycerol) suspension at -80°C. *Agrobacterium tumefaciens* strain AGL1, containing rifampicin and carbenicillin bacterial selection markers.

2. Fungal isolates and Preparation of fungal inoculum for plant infection

V. longisporum VL43 isolate from *B. Napus* (provided by Prof. A. Von Tiedemann, Gerog-August University Göttingen, Göttingen, Germany), was used in the study. For general cultivation, potato dextrose broth (PDB) or Czapek-Dox broth (Difco) media and with agar were used. Fungal stocks were stored at -80°C in 25% glycerol with about 10^6 conidia ml^{-1}. Shake cultures were begun by adding 500µl spore suspension (1-3 x 10^6 conidia ml^{-1}) to 300 ml Potato Dextrose or Czapek Dox broth. The inoculated broth was incubated at 23°C on a rotary shaker at 100 rpm in the dark. After 7-10 days, the culture was filtered with sterile gauze. Spore concentration was determined using a microscope and diluted to 1 x 10^6 spores ml^{-1}.

For the quantification of transcripts levels of Vl_6.2 silenced mutants *in vitro* by qRT-PCR a liquid simulated xylem fluid medium (SXM) was used (46, 47). *V. longisporum* 6.2 Gene silencing mutants were grown in 100 ml SXM liquid medium for 7 days at 23°C on a rotary shaker (150 rpm), inoculated with 100 µl of 10^6 spores ml^{-1} glycerol spore solution. SXM was designed to reflect the nutritional conditions of the vascular fluid, and contains sodium polypectate (2 g l^{-1}), vitamin-free casamino acids (4 g l^{-1}), 1x trace elements (48), 1x potassium salts, and 0.1 µM biotin.

3. Plant material, inoculation, cultivation and assessment of disease development

Rapid-cycle rape (*Brassica napus* var. *napus*, genome ACaacc[36]) was originally provided by P.H. Williams (Department of Plant Biology, University of Wisconsin-Madison, WI; Crucifer Genetics Cooperative, Stock number 5).

Rapid-cycling rape seeds were surface sterilised by immersing them in 70% ethanol for 30 s and rinsed in sterilised tap water for 30 s three times. Then the Rapid-cycling rape seeds were germinated in sterile silica sand in climate-controlled chambers (30 kLux, 60% humidity, 23/20°C (day/night) temperature and 15/9 h (light/dark) light regime, Philips TL5 HO lamps). Seven-day-old seedling were carefully rinsed from silica sand, the cleaned roots were inoculated in a spore suspension of *V. longisporum* isolate VL43 or its mutants (1 x 106 spores ml-1) by root-dipping for 30-45 min. Seedling roots dipped in sterile tap water were used as negative controls. Subsequently, single seedlings were planted into pots with sterile sand-soil mixture (1 : 1) and grown under the constant conditions described above. Rape plant material was harvested at 14, 21, 28, 35 days post inoculation (dpi) for time-course experiments.

Plants were scored for disease symptoms using an assessment key according to Zeise (Table 1) (49). For plant stunting plant shoot length was measured.

Table 1: Assessment key for scoring disease symptoms induced by *Verticillium* sp. on *B. napus* plants in the greenhouse (following Zeise 1992, Eynck 2007)

Score	Symptom description
1	no symptoms
2	slight symptoms on oldest leaf (yellowing, black veins)
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

4. Genomic DNA extraction and checking the quality and quantity of DNA

Fungal genomic DNA was extracted from *V. longisporum* mycelium following CTAB protocol (50). Lyophilized mycelium (200 mg), was pulverized in mortars. A small amount of sand was used when necessary. (Mortar and Pestle were washed and dried throughly between samples, there was no need to autoclave). The ground mycelium was transferred to 50 ml-tube(s) (chloroform resistant) containing 10 ml of TES-Puffer and 4 mg Proteinase K (0.2 ml of stock solution 20 mg ml^{-1}). Incubated for 45 min. at 45°C, mixed by turning the tubes every 10 min. Addition of 3.9 ml of 5 M NaCl, mixed well. Addition of 1.4 ml of 10% CTAB, mixed, incubated for 10 min at 65°C. Cool tube(s) in ice/water-bath, addition of 10 ml Chloroform/Isoamylalcohol. Mixed throughly, kept for 30 min. in ice/water-bath. Notes: Some perform this incubation at room temperature. The incubation could be prolonged to over night. Processing 8 samples up to this point took 4 h. The upper phase and part of the lower phases were transferred into another centrifugation tube by pipetting (or pouring – avoid sand if reusable tubes are used). Samples were centrifuged for 20 min at 4,500 rpm, 5°C (or 5 min at 10,000 rpm). Water phase was transferred to new tube(s) containing 10 ml isopropanol (room temperature), and mixed thoroughly. If DNA precipitates, continued with centrifugation (10 min at 4,500 rpm, room temperature). If no precipitate was visible, tubes were kept at room temperature for 1 h and then centrifuged as above, Pellet was washed with 70% ethanol, dried and dissolved in TE buffer.

After mixing 4 µl DNA solution and 4 µl of a 10-times dilution of the DNA with 2 µl loading buffer, the DNA was loaded on an 0,8% agarose gel with 25, 50, 100 and 200 ng DNA of bacteriophage lambda. The gel was run at 1 V/cm until brompenol blue reached ¾ of the length of the gel. Then the gel was stained with ethidium bromide and visualized under UV light.

5. Total RNA extraction, measurement and mRNA purification

Total RNA was extracted for quantitative reverse transcription real-time PCR (qRT-PCR) with a “hot phenol” protocol (see chapter 3)

RNA was measured by ultraviolet absorbance at 260 nm and 280 nm. Calculation of the RNA concentration is based on the absorbance at 260 nm. Diethyl-pyrocarbonate(DEPC)-treated water is used to dissolve RNA. Add 1 ml of 0.1% DEPC to 1000 l of ddH_2O, Mixed

and left at room temperature for at least 1 hour. This was cooled to room temperature after autoclaving.

To increase the quantity of target gene in the complete template sample, the pure poly A^+ mRNA was purified from total RNA using Oligotex mRNA Kits (Qiagen, Hilden, Germany).

6. Primers of Vl_6.2 gene for quantitative RT-PCR

Primers of Vl_6.2 gene for qRT-PCR (Table 2 and 3) were designed on the basis of the extended sequence from genomic library .

Table 2: Primers for determination of Vl_6.2 gene expression by qRT-PCR

Name of primer	Sequence of primer
Vl_62-F1	5'-AATTGGCCAACCAGCTCTACG-3'
Vl_62-F(new)	5'-GTGGCTTACCAGAACATTCA-3'
Vl_62-F2	5'-CCTGGAGTCTCAACACCAACG-3'
Vl_62-intF2	5'-CTGTCTCTTCTGCACCGATCATTTCA-3'
Vl_62-R1(new)	5'-GTGGTACTGCGTTCCGCAGA-3'
Vl_62-R(2r) {Vl_62-R(new)}	5'-TACGTAGTTGCCAGCAGCGC-3'
Vl_62-R(plus)	5'-AAGCGTCAACTCGTCGTCGGCGTA-3'

Tabele 3: Expected PCR products

Primer pair conbination	PCR fragment	Primer pair conbination	PCR fragment
Vl_62-F1+Vl_62-R (new)	79 bp	Vl_62-F2+Vl_62-R (new)	232 bp
Vl_62-F1+Vl_62-R1(new)	101 bp	Vl_62-F2+Vl_62-R1(new)	254 bp
Vl_62-F(new)+Vl_62-R (new)	203 bp	Vl_62-intF2+Vl_62-R (plus)	173 bp
Vl_62-F(new)+Vl_62-R1(new)	225 bp		

7. Determination of *in planta* gene expression by qRT-PCR for *V. longisporum*

Root and hypocotyl of infected plant tissue were harvested on 14, 21, 28, 35 dpi and total RNA was extracted using hot phenol extraction protocol described above. Because of the

low quantity of RNA from each plant, each total RNA sample was extracted from a tissue pool of six plants. mRNA was purified from total RNA samples using Oligotex mRNA Purification Kit (Qiagen, Hilden, Germany). About 500 -1000 ng mRNA were used in reverse transcription reactions together with 50pmol oligo-T primer, 1mM dNTPs, 20 U RiboLock RNAse inhibitor and 200 U RevertAid reverse transcriptase (Fermentas, St. Leon-Rot, Germany) in a 20 µl reaction volume. cDNA synthesized from mRNA was purified by PCR Purification Kit (Qiagen, Hilden, Germany) and quantified using a photo-spectrometer at 260 nm (GeneQuant, Cambridge, UK). About 100 ng purified cDNA was used as template in qRT-PCR. SybrGreen system was used in real-time PCR. Housekeeping genes, β-tubulin and ribosomal protein S17 (51, 52), were used for normalization. ΔΔCt and In efficiency calibrated ΔΔCt methods were used for quantification of mRNA level between *in vitro* and *in planta* samples (53).

8. Generation of a genomic library

A genomic library of *V. longisporum* was generated for sequence extension of TDFs (Transcript derived fragments) after cDNA-AFLP screening. The fungal genomic DNA was isolated and partially digested with the restriction enzyme *Sau*3AI (Fermentas, Germany) (54). The DNA fragments were size-fractionated on an agarose gel by electrophoresis, and 8-12 kb fragments were cut out and purified using QIAquick Gel Extraction Kit, and then inserted into the *Bam*HI site of the standard vector pUC57 (GenScript). The ligation mix was transformed into competent cells of *E. coli* DH5α (55) by electroporation .

The clones were monitored in 96-well microtiter plates with LB medium containing ampicillin and incubated for 2 days. Clones with target genes were screened from genomic library by PCR and sequenced by Eurofins MWG Operon (Ebersberg, Germany).

9. Construction of binary Vector with antisense fragment *in vitro*

The expression vector pPK2 with hygromycin resistance cassette was used for gene silencing in *V. longisporum* transformation (56). A physical map of the constructed fungal expression vector is shown in Figure 1. The Primers for Vl_6.2 fragment were synthesized after design (Table 4) by Invitrogen (Germany).

Table 4: Primers for construction of a binary vector pPK2-Antisense-Vl_6.2

Name of primer	Sequence of primer
AS_6.2_(AscI)F	5'-ATCAGTGGCGCGCCATGGACCCAGCTGTCGTCCGTA-3'
AS_6.2_(SbfI)R	5'-ATCAGTCCTGCAGGGTCCAGCGTATCCGTTGCCTG-3'
Blue colour: Restriction sites.	

Antisense PCR fragments of Vl_6.2 were amplified with standard PCR using genomic DNA of *V. longisporum* as a template and under the following conditions: one cycle at 95°C for 2 min., followed by 34 cycles of 95°C for 30 sec., 58°C for 30 sec., 72°C for 1 min. Followed by one cycle of 72°C for 5 min. for final extension.

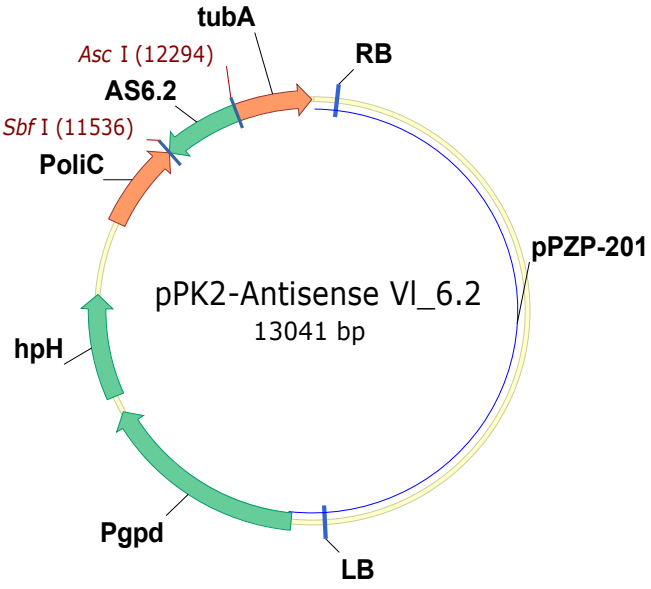

Figure 1: Restriction sites map of the binary vector pPK2-Antisense-Vl_6.2 containing a 303 bp large sequence from Vl_6.2 gene in antisense direction. PoliC (promoter OliC for a gene encodes subunit 9 of the mitochondrial ATP synthase complex) from *Aspergillus nidulans* and tubA (terminator for beta-tubulin gene from *Botrytis cinerea*) were used for control of expression of specific anti-sense RNA-mediated silencing. A *Sbf*I site (CCTGCAGG) was introduced at 3'-direction of Vl_6.2 gene using primer (5'-ATCAGTCCTGCAGGGTCCAGCGTATCCGTTGCCTG-3') in combination with another primer (5'-ATCAGTGGCGCGCCATGGACCCAGCTGTCGTCCGTA-3') containing a *Asc*I site (GGCGCGCC) at 5'-direction of Vl_6.2 gene. Pgpd [promoter for glyceraldehyde-3-phosphate dehydrogenase (gpd) from *Coriolus versicolor*] manages hpH (Hygromycin phosphotransferase resistance gene) and pPZP-201 fragment containing T-DNA region of

Ti plasmid of *Agrobacterium tumefaciens*) is from the host binary vector pPZP201.

10. *Agrobaterium*-mediated fungal transformation of *V. longisporum*

A. tumefaciens-mediated transformation following an optimized protocol (57) was used to generate Vl_6.2 gene silenced mutants. *In vitro* generated binary vector pPK2-Antisense-Vl_6.2 was first transferred into *A. tumefaciens* strain AGL1 (58) provided by Dr. Susanne Frick, Leibniz Institute of Plant Biochemistry (Halle/Saale, Germany) for fungal transformation. The strain was stored in a LB medium with appropriate antibiotics and 15% glycerol at –80°C. The fungal *V. longisporum* 43 strain was inoculated onto PDA or Czapek-Dox agar and incubated for 1-2 weeks at 24°C. Spores were washed from one Petri dish with 2-3 ml sterile tap water. A Drigalski spatula was used to release the spores carefully from the mycelium. The spore suspension was collected and the spore concentration was determined, the spore density was adjusted to 10^7 spores ml^{-1} with sterile tap water. The spore suspension was stored as a recipient for transformation experiments at 4 °C for no more than 50 hours.

A fresh single colony (Φ1-2 mm) of vector pPK2-Antisense-Vl_6.2 held *A. tumefaciens* AGL1 strain was inoculated in 10 ml of LB medium containing antibiotics kanamycin, rifampicin, and carbenicillin at 50, 50, and 25 µg ml^{-1} respectively. The culture was shaken at 28°C and 200 rpm until the OD_{600} reaches 0.5-0.9. 5 ml of the culture was centrifugated at 4000 xg for 5 min at room temperature in sterile 15 ml centrifuge tubes. The supernatant was immediately decanted. 1 ml Induction Medium(IM) was added to the cell pellet (59), this contains Acetonsyringone activating vir genes of *A. tumefaciens,* this was mixed gently until cells were resuspended. The mixture was transferred to a 2 ml microcentrifuge tube and centrifuged at 4000 xg for 5 min at room temperature. The cells were washed briefly by resuspending them. The cell suspension was centrfugated at 4000 xg for 5 min at room temperature and the bacterial pellet was resuspended in 500 µl of IM. After washing and centrifuging the bacterial pellet was resuspended in 150 µl of IM. Dilute OD_{600} of bacterial cells to 0.15 with IM which supplemented with 200 µM acetosyringone. The cells were grown for 8-12 hours in a 100 ml Erlenmeyer flask on a shaker at 200 rpm at 28°C until cells reach OD600 up to 0.3. A cellophane sheet was placed on each IM plate supplemented with 200 µM acetosyringone, 100 µl of this induced culture as donor was mixed with 100 µl of prepared fungal spore suspension (10^7 spores ml^{-1}) as recipient and spread onto the surface of cellophane sheet.

The plates were incubated for 60 hours at 23 °C. Then they were transferred to the celluphane membranes onto the selection plates containing 200 µM kanamycin, cefotaxime and hygromycin B. After 7-10 days, fungal colonies of transformants were visible. The colonies were transferred onto fresh selection plates. The genomic DNA of transformants were extracted and checked with specific primers by PCR.

11. *In vitro* gene expression using qRT-PCR

Expression level of Vl_6.2 gene *in vitro* with cDNA of *V. longisporum* as template and primer paar Vl_62-F2+Vl_62-R(2r) was performed by qRT-PCR using a SYBR Green real-time PCR system. qRT-PCR data analysis was conducted with REST© software and the significance level was set to $p < 0.05$ as indicated by asterisk.

12. *In vitro* test with detergents

Detergents Sodium dodecyl sulfate (SDS) (60, 61) or Polysorbate 20 (commercially also known as Tween 20) (62) were applied to PDA plates at the following concentrations: SDS, 0.2% or Tween-20 (0.2%) (Table 5).

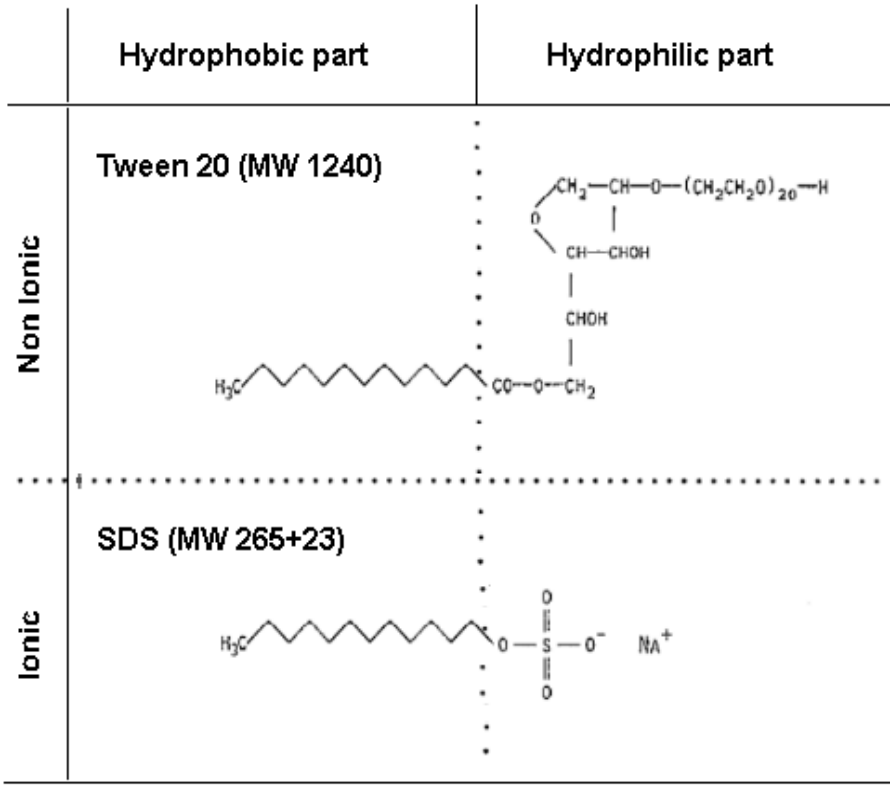

Table 5: Structure formal of Tween 20 and SDS

Results

1. Isolation and characterisation of Vl_6.2 gene containing putative WSC domains.

From the genomic library of *V. longisporum* a full-length gene Vl_6.2 with an ORF of 4720 bp nucleotides (nt) and its UTR was isolated, translated cDNA encoded 1536 amino acids (aa) (see Chapter 3). After comparison of genomic DNA and cDNA sequence the Vl_6.2 gene included two introns and 56 bp each (Figure 2). SMART (Simple Modular Architecture Research Tool) analysis indicated that Vl_6.2 gene contained a signal peptide, a fibronectin type III and four putative WSC (water-soluble carbohydrate binding) domains at the C-terminus (Figure 3).

The transcription analysis in *V. longisporum* by cDNA-AFLP (63), Vl_6.2 gene was determined as an activated gene after a treatment with xylem sap which was extracted from mock treated *B. napus* plants. This phenomenon indicated that Vl_6.2 gene was related in interaction between phytopathogen *V. longisporum* and host plant *B. napus*. *In planta* relative gene expression analysis by qRT-PCR exhibited a strong up-regulation of Vl_6.2 gene normalised with two housekeeping genes (β-tubulin and ribosomal peptide S17) in *V. longisporum* during infection at 14, 21, 28, and 35 dpi (days post inoculation) compared with gene expression of fungal mycelia in SXM (Figure 4).

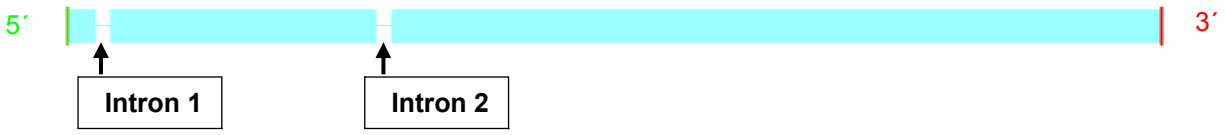

Figure 2: Modular structure shown positions of two introns in Vl_6.2 gene. Full-length of Vl_6.2 gene sequence was isolated from a genomic library of *V. longisporum*. The ORF contains two introns: Intron 1 (at nucleotide positions 114-169 with a 56 bp length sequence GTGAGCGTCCTCGTTCCGTATCCGGCCCCCTTGAAATGGCACTGACGTGTTTC-CAG) and Intron 2 (at nucleotide positions 1324 -1381 with the same length but different sequence GTGCGTTTGGTTCAGGTCTGCGTCTCACCCCCAACATTATTGCTAACTTGCACAA-G). Picture of modular structure modified according to the *Verticillium* group database (64).

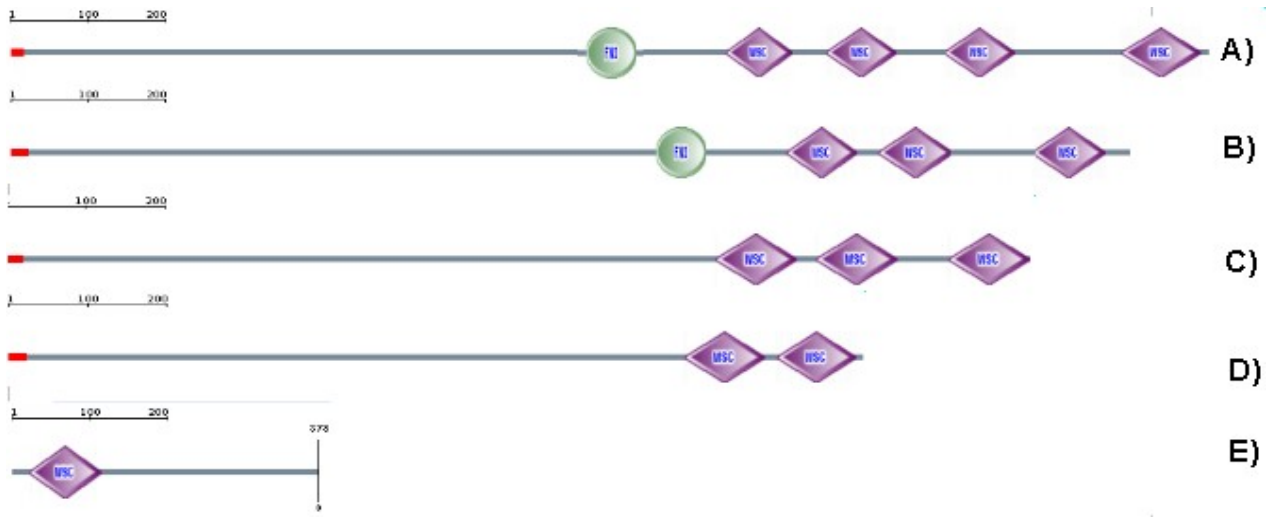

Figure 3: Modular structure shown signal peptide (**red**), Fibronectin type III (**green**) and four putative WSC domains (**lilac**) in amino acids sequences constructed using software SMART (Simple Modular Architecture Research Tool) (65). Sequence homology analysis revealed the presence of four conserved WSC carbohydrate binding domains at the C-terminus for *V. longisporum, V. dahliae* and *V. albu-atrum* in **A)**. Fibronectin is a high-molecular weight (~440 kDa) extracellular matrix glycoprotein that binds to membrane-spanning receptor proteins called integrins (66, 67); **B**) *Magnaporthe oryzae* (Sequence Nr. XP_361287.2) with three WSC domains and one Fibronectin type III; **C)** *Botryotinia fuckeliana* (Sequence Nr. XP_001556864.1) with three WSC domains; **D)** *Trichoderma asperellum* (beta 1, 3 exoglucanase, Sequence Nr. ABY19519.1) with two WSC domains; **E)**. *Saccharomyces cerevisiae* (SLG1 gene, Sequence Nr. YOR008C) with one WSC domain.

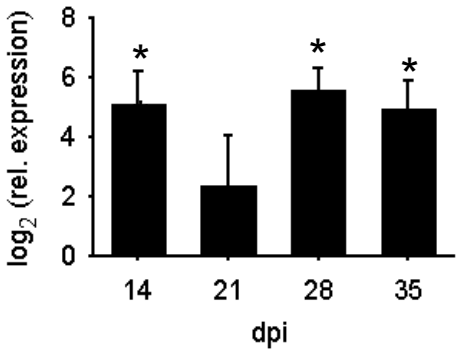

Figure 4: *In planta* gene expression analysis of Vl_6.2 gene of *V. longisporum* represented in time course. Column charts shown ratios of relative expression levels referred to *in vitro* grown mycelium in an artificial medium simulating xylem as control and *in planta* grown

fungal tissue of *V. longisporum* in infected root/hypocotyl material of *B. napus* plant as target, were calculated as ΔΔCt values from three biological replicas at 14, 21, 28, and 35 dpi. Error bars indicated the standard deviation of calculated ΔΔCt values. Significance levels were set to $p < 0.05$ and were given for both reference genes, double asterisks indicate significant change in the expression levels regarding both reference genes.

2. Differential silencing effect of Vl_6.2 gene *in vitro* mediated by antisense RNA

The antisense RNA-mediated post-transcriptional silencing method was used for “knock down” of Vl_6.2 gene,, the specific antisense sequence was inserted in genome of *V. longisporum* by *A. tumefaciens*-mediated transformation with a constructed binary vector pPK2. In order to see whether silencing effects occur, we used qRT-PCR to quantify the expression levels of Vl_6.2 gene transcripts *in vitro.* The results were showed in figure 5. The silencing effect of mutants M5, M6, M7 *in vitro* was suppressed to 70% of its activity in transformants to the non transformed wild type.

3. Pathogenicity test of Vl_6.2 gene silenced mutants *in planta*

The first disease symptoms were visible on *V. longisporum* infected *B. napus* plant after 14 dpi under greenhouse conditions and displayed chlorosis, dark-coloured veins on older leaves and stunting effects on plant growth. At 14 dpi and 21 dpi, most of the plants inoculated with VL43 mutants showed less severe symptoms than VL43 wild type (Table 5). General plant growth especially the growth of stem after inoculation with *V. longisporum* isolate 43 or its anti-sense mutants of Vl_6.2 gene were inhibited (Figure 6). But after comparison with wild type, the effect of inhibition of plant growth infected by Vl_6.2 gene mutants M4, M5, M6, M7 were significant (Figure 6). The picture figure 7 shows *B. napus* plants infected by Vl_6.2 silenced mutants compared to mock treated plants (treated with H_2O) and *V. longisporum* isolate 43 wild type.

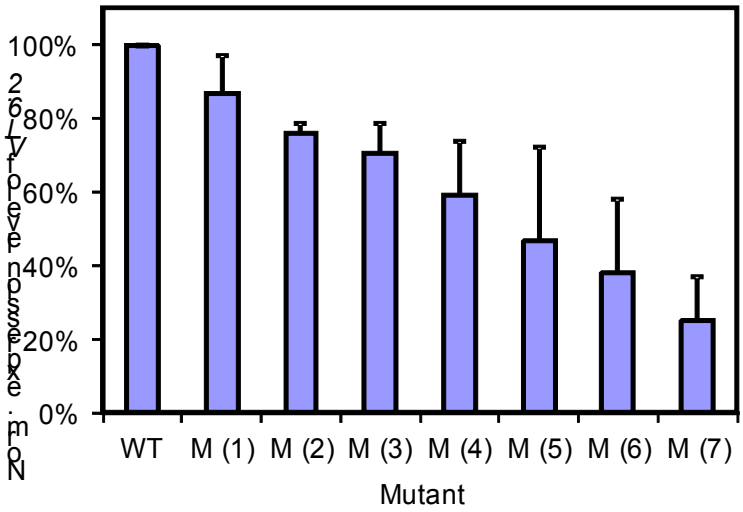

Figure 5: Normalized transcript accumulation of Vl_6.2 was reduced in mutants M (1-7) up to 70% by introducing anti-sense RNA expression in relation to wild type (WT). Analysis was done applying quantitative RT-PCR.

Table 5: Assessment of disease symptoms on host plant *B. napus* with disease score

dpi	Mock treated with H_2O	VL43-WT	VL43-M4	VL43-M5	VL43-M6	VL43-M7
14	1.33± (0.48)	2.42± (1.41)	2.11± (1.37)	2.00± (1.37)	1.68± (0.82)	2.05± (1.47)
21	1.35± (0.40)	2.85± (1.30)	2.74± (1.24)	2.68± (1.49)	1.84± (1.21)	2.53± (1.58)
28	1.38± (0.50)	3.13± (1.45)	-	-	-	-

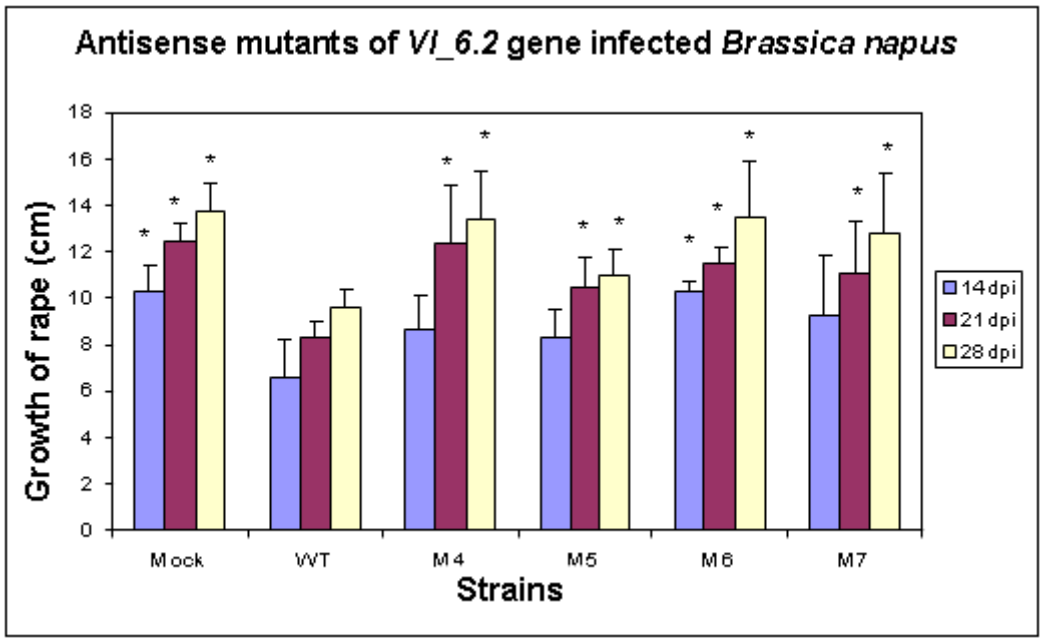

Figure 6: Shoot length of *B. napus* plants was measured in weekly intervals after inoculation. Error bars indicated the standard deviation of calculated average value. Significance level was set to $p < 0.05$ (T-test) and was calculated for wild type as a reference.

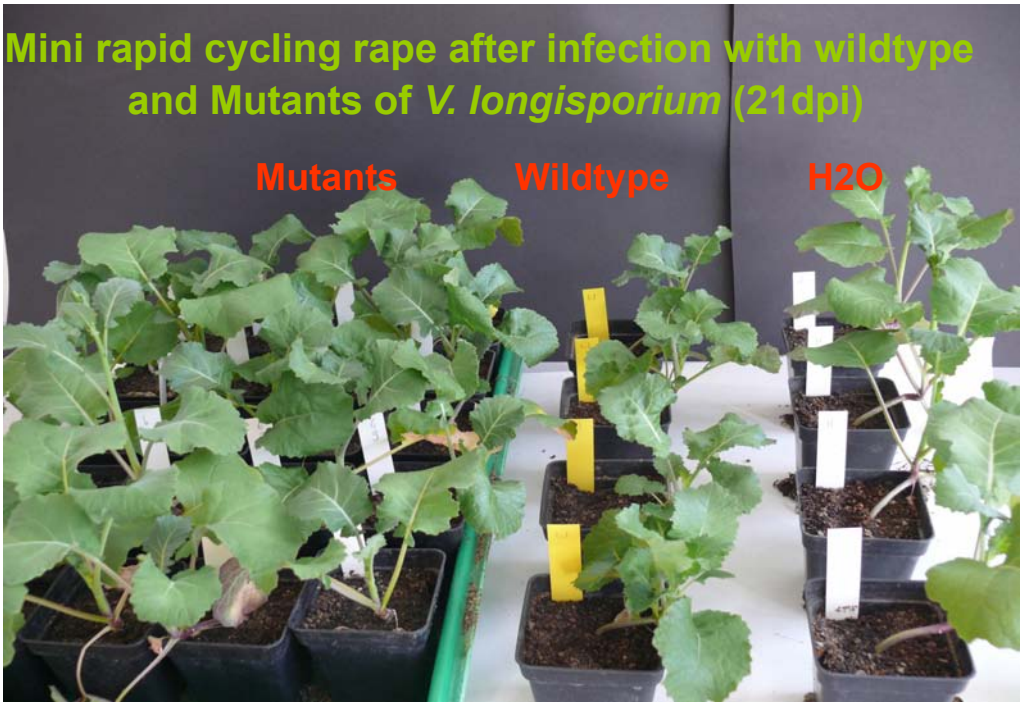

Figure 7: Pathogenicity test of Vl_6.2 silencing mutants *in planta*. Stunting effect as a typical symptom on *B. napus* infected by wild type of *V. longisporum* compared to mock at 21dpi is visible (Middle and right plants of the picture). The Vl_6.2 silencing mutants infected plants showed less stunting effects (left plants of the picture).

4. Growth inhibition assay with Vl_6.2 gene silenced mutants with detergents

The silenced gene Vl_6.2 comprises conserved WSC domains in its peptide sequence putatively involved in stabilizing the fungal cellular membrane integrity (68). We performed a differential growth inhibition assay on PDA agar plates containing either SDS (sodium dodecyl sulfate) or Tween-20 detergent with mutants M (6) and M (7) exhibited the highest silencing efficiency (Figure 8 and Figure 9). the SDS-containing PDA medium (0.2%) inhibited fungal growth of Vl_6.2 silenced mutants with a larger scope.

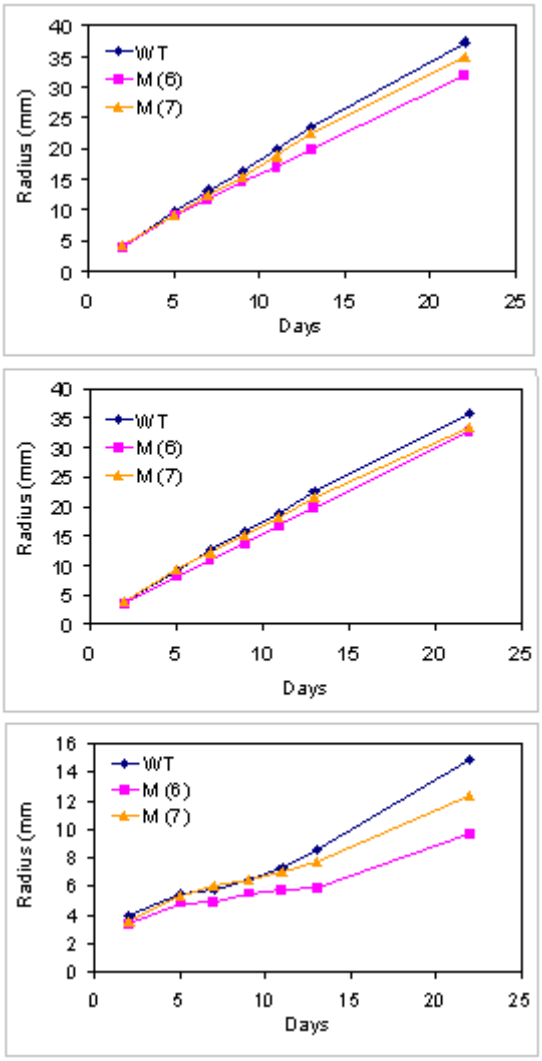

Figure 8: Differential effects of detergents on Vl_6.2 gene silencing mutants. The above picture of figure 8 shows the time course of radial mycelial growth of wild type (WT) and the two Vl_6.2 gene silenced mutants not treated with detergent. While the SDS-containing medium (0.2%) inhibited fungal growth of mutants to a larger extent (below picture in figure 8) than WT. Tween-20 (0.2%) exhibited no detectable effect (middle picture in figure 8).

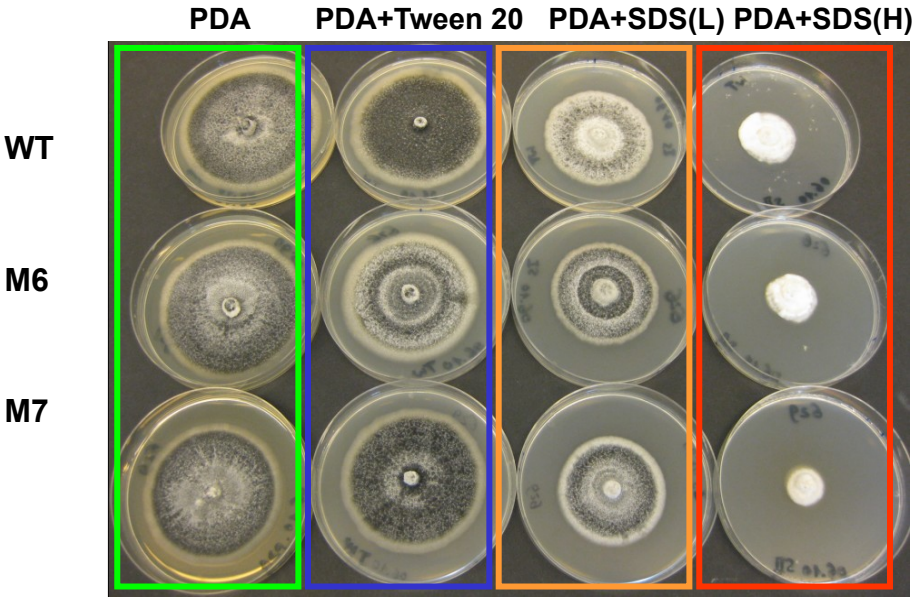

Figure 9: Colony morphology of *V. longisporum* isolate 43 Vl_6.2 gene anti-sense mutants on solid media. Line PDA: PDA without detergent. Line PDA+Tween 20: PDA with detergent Tween 20 (0.2%). Line PDA+SDS(L): PDA with detergent SDS (0.05%). Line PDA+SDS(H): PDA with detergent SDS (0.2%). All PDA plate incubated for 38 days, at 22°C in dark). Colony growth of M6 and M7 mutants were inhibited on PDA plate with SDS detergent with a concentration in 0.2%.

5. Growth inhibition assay with Vl_6.2 gene silenced mutants with different pH-value

The yeast grows better at acidic than at neutral or alkaline pH, Wsc1 participates as an essential cell-surface pH sensor. Lack of Wsc1, removal of specific extracellular and intracellular domains, or substitution of Tyr^{303} in this putative membrane stress sensor rendered cells sensitive to alkali and considerably decreased alkali-induced Slt2 activation (69). Therefore, unexpected alkalinization of the environment provided a stress condition for yeast fungus. Here we presented a alkaline stress result with Vl_6.2 gene silenced mutants whose cell wall integrity may be disrupted. Mutants M5, M6, M7 showed a delayed melanistic process by a alkaline stress.

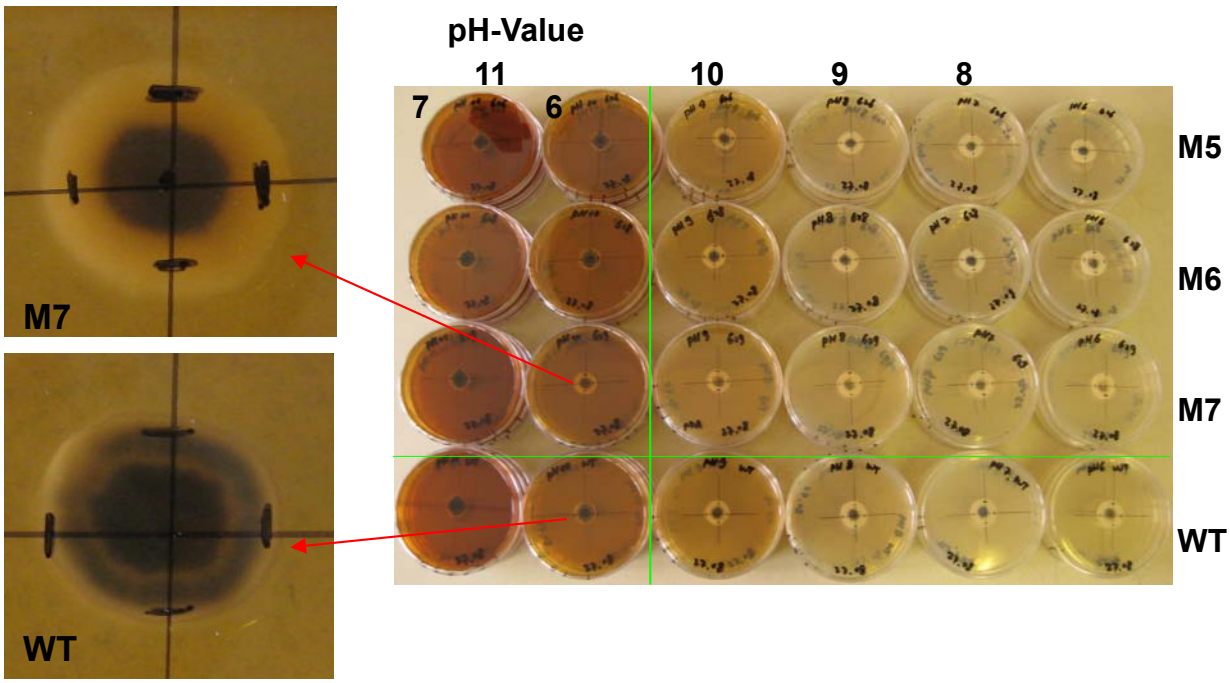

Figure 10: Alkaline stress reflected by delayed melanistic process of Vl_6.2 gene mutants compared with wild type decelerated up pH 10 value after 5 days incubation in dark at 23°C.

6. Growth assay of Vl_6.2 gene silenced mutants with *Trichoderma* species

Mycoparasitic fungi *Trichoderma* species secrete chitinases and glucanases that attack cell wall polymers in other fungi (70, 71, 72). The strain *Trichoderma virens* is a wide spread soil saprophyte that has been applied as a biological control agent to protect plants from fungal pathogens. *T. virens* produces antifungal compounds which assists the killing of its fungal targets (73).

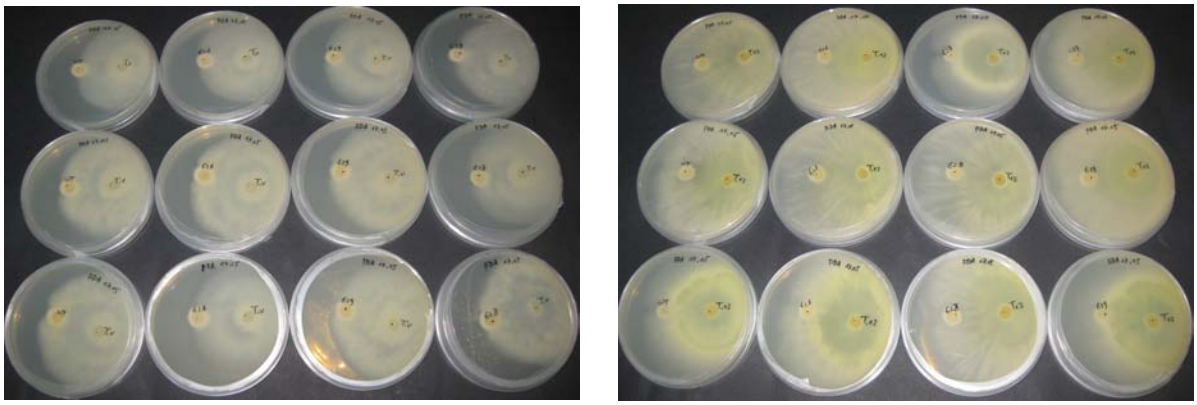

Figure 11: *Terichoderma* strains were used as mycoparasite for test of cell wall integrity of *V. longisporum* and its Vl_6.2 silenced mutants. Because of large differences in growth

rates between of *Terichoderma* and *Verticillium* strains, the mycoparasitic effect was not visible.

Discussion

Vl_6.2 gene was detected from a signal which responded to the *V. longisporum* transcriptome after treatment with extracts of *B. napus* xylem sap by an cDNA-AFLP profiling method. Sequence analysis was performed on Vl_6.2 gene containing four WSC (water-soluble carbohydrate binding) domains at C-terminus. Genetic characterization reports that WSC family are regulators for extracellular stress response and cell wall integrity in the yeast *S. cerevisiae.* A cell wall stress component sensor Wsc1 is a plasma membrane protein that behaves like a linear nano-spring that is capable of resisting high mechanical force and of responding to cell surface stress (74). Our results confirmed anti-sense RNA methods as a mild gene silencing method *in vitro* by qRT-PCR. The with anti-sense RNA methods generated Vl_6.2 silenced mutants demonstrated Vl_6.2 gene as a stress response regulator activating in growth inhibition assays both with ionic detergent SDS with a concentration of 0.2% and with alkaline pH value at 10. A treatment with SDS in low concentration or a non ionic detergent Tween 20 could not effect the growth of *V. longisporum*, This indicates an elastic role of Vl_6.2 gene which behaves in a similar manner to that seen in *S. cerevisiae.* Vl_6.2, Perhaps it also possesses a linear nano-spring-like structure in response to extracellular stress under particular conditions.

In this experiment, Vl_6.2 gene expression *in planta* was up-regulated by determination with qRT-PCR. Vl_6.2 silenced mutants which resulted in a reduced virulence *in planta.* Krishna et al. in 2005 reported that MAP(Mitogen-Activated Protein) kinase-mediated signalling pathways are involved in pathogenicity of *Verticillium dahliae*. Disruption of *Verticillum* MAP Kinase 1 (VMK1) in *V. dahliae* severely decreased virulence in diverse host plants (75). Other researchers presented a deletion of WSC genes in *S. cerevisiae* resulting the WSC binding domains as upstream regulators playing a important role in stress response in PKC1-MAPK1 pathway (15, 16).

VL_6.2 contains a putative fibronectin type III domain (FN3). Fibronectin as a very common constituent of animal proteins was characterized more than 50 years ago and in studies have been carried out on cell adhesion, self-repair, composition, assembly of extracellular matrix and multi-modular protein structure (76). Normally fibronectin is categorised into two classes: cellular and plasma (77). FN3 modules are the major

structural units in fibronectin and are also found in a large number of other proteins. The biologically most important activity of fibronectin is its interaction with cells. The ability of fibronectin to serve as a substrate for cell adhesion, spreading, and on the activities of several modules based migration (78). A present FN3-like domain in gene Vl_6.2 indicates that Vl_6.2 may play a role in cell adhesion and self-repair etc.

In conclusion we postulate that Vl_6.2 gene, a putative plasma membrane protein interacting with carbohydrate and related to the PKC1-MAPK1 pathway, has a considerable impact on the effect of infection *in planta* and external stress response *in vitro.*

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Chapter 5: Identification and characterisation of a ACE1-like gene Vl_12.1 of *Verticillium longisporum*

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Abstract

In a cDNA-AFLP approach investigating the effect of xylem sap metabolites of *Brassica napus* on the transcriptome of *V. longisporum,* we found a Vl_12.1 gene, its gene expression was reduced during infection of *B. napus* in root/hypocotyl tissue in reference to *in vitro* grown mycelium in a xylem simulating artificial medium, indicating a suppression of this gene during the infection process. The full-length gene sequence of Vl_12.1 was isolated from *V. longisporum*. The gene has an ORF of 2,328 nucleotides, one intron and translated cDNA is predicted to code for a 775 amino acids. Sequence analysis of Vl_12.1 showed the high homology to the zinc-finger transcription factor ACE1 (1) containing three ZnF_C2H2 domains. ACE1 in saprophytic fungus *Trichoderma reseii* acts as a regulated transcriptional suppressor of cellulase and xylanase genes. We applied RNAi technology for gene silencing of this ACE1-like Vl_12.1 gene in *V. longisporum* by expressing a gene-specific RNA-hairpin. Gene-silenced mutants did not show any visual difference in triggering typical infection symptoms in *B. napus*. A semi-quantitative testing for alterations in cellulase activities on cellulose-containing agar medium showed less difference between the silenced mutants and *V. longisporum* wild type. Generation of Vl_12.1 gene overexpression mutant strains is in processing.

Introduction

Polysaccharides are polymeric carbohydrates found in plant cell wall and exist in nature. Most filamentous pathogenic or saprophytic fungi can produce polysaccharide-degrading enzymes to degrade different polysaccharides and belong to an important group of microorganism for the global carbon cycle and plant health. They have ability to cause disease of certain plants and bring great economic consequence. *V. tricorpus* secreted endo and exo-enzymes, capable of degrading cellulosic, hemicellulosic and pectinolytic

polysaccharides (8). A large number of enzymes and genes from *Aspergilli* involved in the degradation of plant cell wall polysaccharides. The enzymes covered most of the functions required for the complete degradation of plant cell wall polysaccharides (9). A fungus *Ophiostoma ulmi* (Buism.) Nannf. causing the first epidemic of the Dutch elm disease (DED) shown a significant correlation between the activity of exoglucanase and β-glucosidase *in vitro* and virulence (10). *Verticillium* species are known to infect many host plants include alfafa, potato, strawberry, tomato, mint, sunflower, eggplant, weeds, cotton, oilseed crops and a range of trees like olive and maple trees (2, 3, 4, 5, 6). *V. dahliae* penetrates the host plant through the root epidermis to xylem elements, spreads in the whole vascular system by rapid colonisation (47) and causes different visible symptoms especial necrosis and wilting as a general result. *V. dahliae* is able to secret cellulases degrading crystalline cellulose (Avicel). The cellulases may play a role in penetration (48). *V. longisporum* causes *Verticillium* wilt on *B. napus* and induces, leaf chlorosis, curl and especial growth stunting as a typical symptom. In this work we found *V. longisporum* degraded Carboxymethyl (CM) cellulose *in vitro* on agar plate.

At molecular level the filamentous fungi *Arspergillus niger* and *Trichoderma reesei* (*Hypocrea jecorina*) are two of the best studied organisms for production of cellulase and xynlanase enzymes. XlnR (Xylanase regulator, *A. niger*) gene is a transcriptional activator of xylanase and cellulase expression in *A. niger* (11, 12) and links a range of intracellular carbon metabolisms (13, 14). Xyr1 (Xylanase regulator 1, *T. reesei*) gene is a transcriptional regulator of xylanolystic genes in *T. reesei* that activated by different inducers like cellulose, xylan, mixtures of plant polymers (15, 16, 17). Xyr1 activates generally a hydrolase formation in *T. reesei* (18, 19) and interplays with specific transcriptional regulators ACE1 (Activator of cellulases 1, *T. reesei*) and Ace2 (Activator of cellulases 2, *T. reesei*) (20, 21). Most zinc finger domains through specific binding to nucleic acids or proteins, play important function for transcription, translation and signalling in cell. ACE1 transcriptional factor contains three Cys2His2-type zinc fingers and is a repressor of cellulase and xylanase expression which was performed by *Δace1* mutants. They grew better than the wild type on medium containing cellulose as sole carbon source (22, 23).

Here we report a transcription factor ACE1-like gene of *V. longisporum* related *in vitro* cellulase activity and involved in pathogenic process *in planta* as a repressor as compared to mycelium grown *in vitro* in a xylem-simulating liquid medium (SXM) (33) analysed by

qRT-PCR.

Materials and Methods

1. Plant Material

Rapid-cycle rape (*Brassica napus* var. *napus*, genome ACaacc[26])was originally provided by P.H. Williams (Department of Plant Biology, University of Wisconsin-Madison, WI; Crucifer Genetics Cooperative, Stock number 5).

Rapid-cycling rape seeds were surface sterilised by immersing them in 70% ethanol for 30 s and then rinsed in sterilised tap water for 30 s three times. Then the Rapid-cycling rape seeds were germinated in sterile silica sand in climate-controlled chambers (30kLux, 60% humidity, 23/20°C and 15/9h day/night; Philips TL5 HO lamps). Seven-day-old seedling were carefully rinsed from silica sand, the cleaned roots were inoculated in a spore suspension of *V. longisporum* isolate VL43 or its mutants (1 x 10^6 spores ml^{-1}) by root-dipping for 30-45min. Seedling roots dipped in sterile tap water was as a negative control. After inoculation, subsequently, single seedlings were respectively planted into pots with sterile sand-soil mixture (V:V=1:1) and grown under the constant conditions described above. Rape plant materials were harvested 14, 21, 28, 35 days post inoculation (dpi) for time-course experiments. Plants were scored after time course for disease symptoms using an assessment key according to Zeise (see chapter 4). For plant stunting using plant shoot length by measuring.

2. Fungal strains

V. longisporum isolate VL43 from *Brassica napus* was used in this work (Zeise and von Tiedermann 2001) (27). Conidial suspensions in a concentration of about 10^6 conidia/ml in Czapek Dox medium mixed with 30% sterile glycerol were for long term at -80 °C stored. For propagation, the stored conidia were spread onto Potato Dextrose Agar (PDA) and incubated for two weeks at 23°C in the dark. Spores were gently collected with sterile tap-water. 500 µl spores from the stock solution were inoculated in 250 ml Potato Dextrose Broth (PDB) and incubated for one week at 23 °C by rotary shaking. The resulting suspension was filtered using sterile gauze. Inoculum for root immersing inoculation was concentrated to 1 x 10^6 spores/ml after counting with a haemocytometer.

3. Bacterial strains

Escherichia coli strain DH5α was used for construction of plasmids. *Agrobacterium tumefaciens* strain AGL-1 (28) (provided by Dr. Susanna Frick, Leibniz Institute of Plant Biochemistry, Halle/Saale) was applied for the transformation of fungi. This strain is rifampicin and carbenicilin resistant and contains the hyper-virulent Ti plasmid pEHA105 (pTiBo542ΔTDT-DNA) (29). Cells were stored for long term in the freezer at -80°C.

4. Determination of gene expression *in vitro* and *in planta* by qRT-PCR

For quantification of transcripts level of Vl_12.1 silenced mutants *in planta,* according to time course, root and hypocotyl of infected plant tissue were harvested on 14, 21, 28, 35 dpi and applied for total RNA extraction after a above described “hot phenol” extraction protocol. Because of less amount of RNA from each plant, each total RNA sample was extracted from a tissue pool of six plants. mRNA was purified from total RNA sample by Oligotex mRNA Purification Kit (Qiagen, Hilden, Germany). About 500-1000 ng mRNA was used in reverse transcription reaction together with 50 pmol oligo-T primer, 1 mM dNTPs, 20 U RiboLock RNAse inhibitor and 200 U RevertAid reverse transcriptase (Fermentas, St. Leon-Rot, Germany) in a 20 µl reaction volume. From mRNA synthesized cDNA was purified by PCR Purification Kit (Qiagen, Hilden, Germany) and quantified with photo-spectrometer by 260nm (GeneQuant, Cambridge, UK). About 100ng purified cDNA was used in qRT-PCR as template. SybrGreen system was used in real-time PCR. Housekeeping genes, β-tubulin and ribosomal protein S17 (30, 31), were used for normalization. ΔΔCt method and Pfaffl's method were used for quantification of mRNA level between *in vitro* and *in planta* samples (32).

For quantification of transcripts level of Vl_12.1 silenced mutants *in vitro* by qRT-PCR a SXM medium was used. Vl_12.1 Gene silenced mutants were grown in 100 ml SXM medium for 7 days at 23 °C on a rotary shaker (150 rpm), inoculated with 100 µl of 10^6 spores/ml glycerol spore solution. SXM was designed to reflect the nutritional conditions of the vascular fluid, and contains sodium polypectate (2 g l^{-1}), vitamin-free casamino acids (4 g l^{-1}), 1x trace elements (34), 1x potassium salts, and 0.1 µM biotin.

Table 1: Designed primers for Vl_12.1 gene expression by qRT-PCR

Name of primer	Sequence of primer	PCR fragment
C12-RT-f (Vl_12.1-RT-F)	5'-AGCCACAGTATTGCTACGC-3'	219 bp
C12-RT-r (Vl_12.1-RT-R)	5'-GTATGGGTAGGATGTGTCG-3'	

5. Construction of the binary vector pPK2-RNAi-Hairpin-Vl_12.1

For a knock-down of ACE1-like gene Vl_12.1 we applied a hairpin cassette-based RNAi technique (55). The backbone of pPK2 (35) was used for construction of binary vector pPK2-RNAi-Hairpin-Vl_12.1 (36) (Figure 1). The construction was divided in two steps: At first an antisense sequence of Vl_12.1 and a intron sequence of the putative Hydrophobin from *V. longisporum* as the space were assembled and inserted in vector pPK2. The antsense and the intron sequence were amplified by PCR respectively using genomic DNA extracted from *V. longisporum* mycelium. The amplificons were digested with enzymes NdeI (Fermentas) and ligated with T4-ligase (Fermentas). The result of ligation was used as a template for amplification of antisense-space fragment. As second step, the sense sequence of Vl_12.1 gene was inserted in the vector pPK2 containing antisense-space fragment. All PCRs used bioline polymerase and standard procedure. The for construction of binary vector pPK2-RNAi-Hairpin-Vl_12.1 used primers are listed in Table 2.

Table 2: Primers for construction and detection of target binary vector

Name of primer	Sequence of primer
Vl_12.1_RNAiFas_SdaI_F	5'-TAGTGACCTGCAGGTCACAACATCACTCTACTGCT-3'
Vl_12.1_RNAiFas_NdeI_R	5'-GATATGCATATGAATTCCAGACTCTGTTCGGCAG-3'
Vl_12.1_RNAiFs_BglII_F	5'-GATATGAGATCTAATTCCAGACTCTGTTCGGCAG-3'
Vl_12.1_RNAiFs_AscI_R	5'-GAGCTCGGCGCGCCTCACAACATCACTCTACTGCT-3'
Vlh_Intron_NdeI_F	5'-GATATGCATATGTGACTGCTCTACCCGTACGT-3'
Vlh_Intron_BglII_R	5'-GATATGAGATCTCTCCTGGCACTCTTTGCTAT-3'
pPK2-PoliC-F	5'-ATTAAGCGGGAGACGTAT-3'
pPK2-TtubA-R	5'-CCTCGATGAACTCACGCTCA-3'
Blue colour: Restriction sites.	

The restriction sites map of binary vector pPK2-RNAi-Hairpin-Vl_12.1 is following in Figure 1. The constructed vector pPK2-RNAi-Hairpin-Vl_12.1 was transferred into *A. tumefaciens* by electroporation (37) at 2.0 kV, 200 ohms and 25 µF using a cuvette with 1 mm gap between the electrodes.

6. *Agrobacterium*-mediated transformation for knock-down of ACE1-like Vl_12.1

Agrobacterium-mediated transformation was applied for fungal transformation (39). The *A. tumefaciens* strain AGL1 containing the binary vector pPK2-RNAi-Hairpin-Vl_12.1, was incubated in LB medium completed with rifampicin (25 µg ml^{-1}), carbenicillin (25 µg ml^{-1}) and kanamycin (50 µg ml^{-1}) on a rotary shaker with 200 rpm at 28°C for 48 h until OD_{600} of AGL1-cells reaching 0.5-0.9, The bacteria were harvested and washed 2x with IM-solution (induction medium) and resuspended in IM supplemented with 200 µM AS (acetosyringone). Grow cells for 8-12 h under same conditions like before until the cells reach OD_{600} = 0.3. The prepared fresh fungal spores were mixed with pre-induced AGL1 cells and co-cultivated at 23°C in dark for 60 h on a cellophane sheet placed IM agar plate supplemented 200 µM acetosyringone. After co-cultivation, use sterile forceps to transfer the cellophane membranes onto selection plates containing 200 µM cefotaxime and hygromycin B. The plates were incubated at 23°C and after 8-10 days the developed colonies of transformants were visible. To check their stability, all transformants were incubated on PDA plate for at least three generations. To control transformation each colony was checked with specific primers of Vl_12.1 gene (Table 1) combined with pPK2-PoliC primer or pPK2-TtubA primer (Table 2) by PCR.

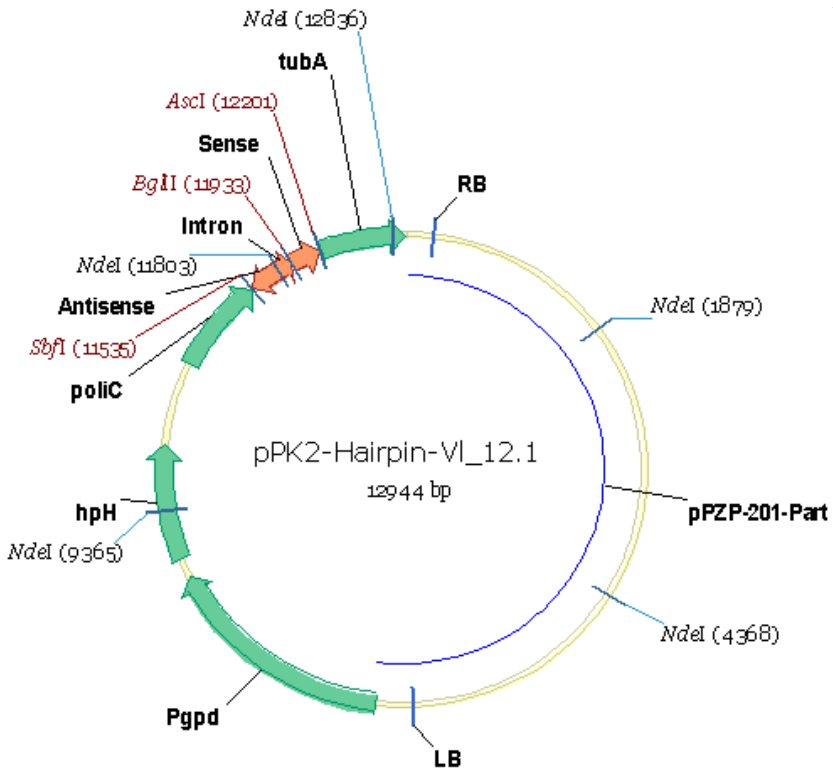

Figure 1: Restriction sites map of pPK2-RNAi-Hairpin-Vl_12.1. For construction of RNAi-Hairpin cassette, a 159 bp large sequence of Vl_12.1 gene was used in both antisense (with primer pair Vl_12.1-RNAiFas_*Sda*I_F and Vl_12.1_RNAiFas_*Nde*I_R) (Table 2) and antisense direction (with primer pair Vl_12.1_RNAiFs_*Bgl*II_F and Vl_12.1-RNAiFs_*Asc*I_R) (Table 2), a native *V. longisporum* putative Hydrophobin intron sequence 129 bp was incorporated as spacer (with primer pair Vlh-Intron_*Nde*I_F and Vlh-Intron_*Bgl*II_R) between the antisense and the sense sequence. The binary vector contained PoliC (promoter OliC for a gene encoding subunit 9 of the mitochondrial ATP synthase complex from *Aspergillus nidulans*) and TtubA (terminator for beta-tubulin gene from *Botrytis cinerea*) for control of RNAi-Hairpin cassette. Pgpd (promoter for glyceraldehyde-3-phosphate dehydrogenase [gpd] from *Coriolus versicolor*) controlled hpH (Hygromycin B phosphotransferase) resistance gene and the pPZP-201 fragment cut out from host binary vector pPZP201 contained T-DNA region including the LB (left-boarder) and RB (right-boarder) from Ti plasmid of *Agrobacterium tumefaciens*) (38).

7. Colorimetric assay of cellulase activity

Water-soluble carboxymethyl-substituted cellulose, labelled covalently with a dye Remazol Brilliant Blue R (RBB) to CM-cellulose-RBB complex was used as substrate for assay of endo-acting cellulase activity (40). Enzyme assay was performed on modified basic

Czepek Dox (CD) agar plate after diffusion effect.

Results

1. Isolation and Characterisation of *V. longisporum* gene Vl_12.1

We found a putative zinc-finger transcription factor ACE1 (activator of cellulase 1 characterized in *T. reesei*) in *V. longisporum* according to *s*equence analysis. The full-length ACE1-like gene Vl_12.1 with an ORF of 2,328 nucleotides (nt) containing one intron (Figure 2) was isolated from *V. longisporum* by the PCR technique based cloning and direct sequencing. Its translated cDNA is predicted to code for a 775 amino acids (aa) (Figure 5). It has three ZnF_C2H2-like motifs (41) respectively containing 25, 29, 26 amino acid residues (Figure 3) (ZnF_C2H2-motif 1: **KKCREPGCNKEFKRPCDLTKHEKTH**; ZnF_C2H2-motif 2: **WKCPVKTCKYHEYGWPTEKGMDRHHNDKH;** ZnF_C2H2-motif 3: **YECLFKPCPYKSKRES-SCKQHMEKAH**) and including paired cysteines and histidines for zinc coordinating and stable fold (Figure 3 and 4). A multiple alignment of putative Vl_12.1 from *V. longisporum* with conserved amino acid sequences of other fungi is partial shown in Figure 4. The nucleotide sequence identity within this gene between *V. longisporum* and *V. dahliae* was determined at a level of 95% (data not shown). Vl_12.1 gene keeps a high similarity like *V. dahliae* on the protein level and contains a close revolutionary relationship with ACE1 in *T. reesei* (Figure 6). In a cDNA-AFLP profiling, the Vl_12.1 gene was investigated as a induced candidate gene in transcriptome from *V. longisporum* after a treatment with xylem sap metabolites of *B. napus.* ACE1-like Vl_12.1 *in planta* relative gene expression was determined as suppressed in time course experiment by qRT-PCR compared with the mycelium grown *in vitro* in a xylem-simulating liquid medium (Figure 5). After sequence extension and transcription analysis an ACE1-like gene Vl_12.1 was identified.

Figure 2: Modular structure shown position of one Intron in Vl_12.1 gene. The 2,328 nucleotides ORF contains one intron at nucleotide positions 745-804 and with a 60 bp length sequence GTAAGTCAACTCTTGTATCAACGCAGCGTTTGGGGAGATACTTATCG-GTCGTTGATGAAG. Modified after modular structure in *Verticillium* group database.

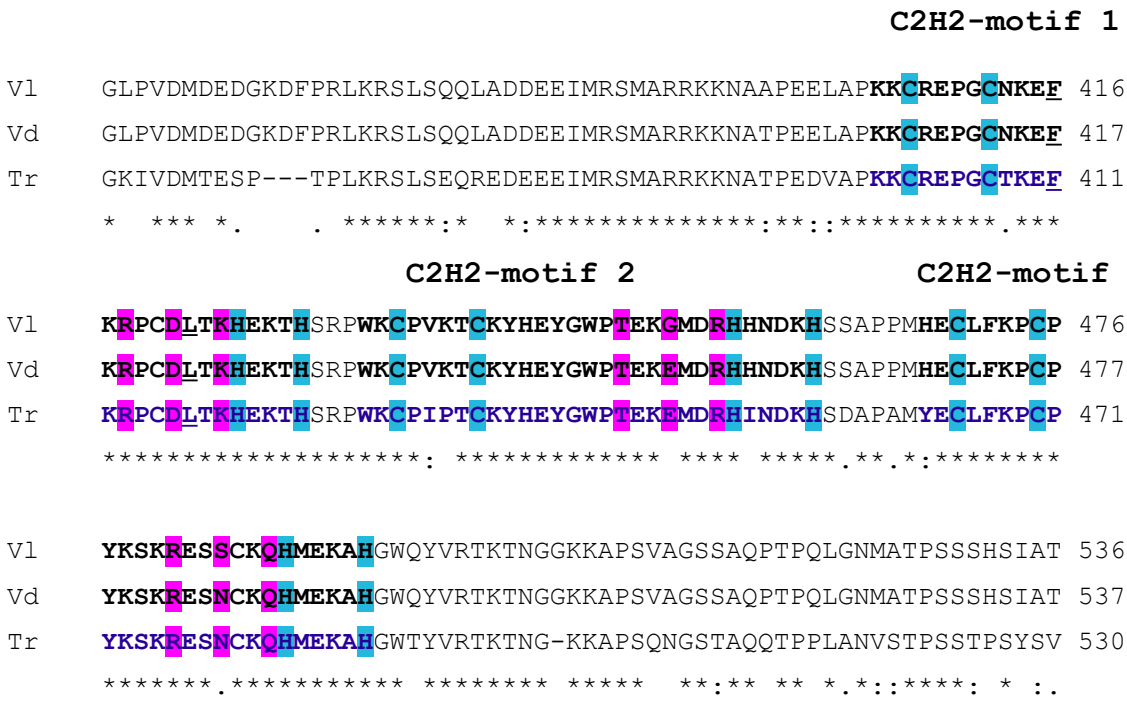

Figure 3: Alignment of three putative ZnF-C_2H_2 Motifs (Nr. 1, Nr. 2 and Nr. 3) in ACE1 from *T. reesei* (**Tr**) and the homologous residues from *V. dahliae* (**Vd**) and *V. longisporum* (**Vl**). ZnF-C_2H_2 Motifs positioned after Saloheimo's work in 2000 (22) and results of analysis with NCBI database (http://www.ncbi.nlm.nih.gov/), simple modular architecture research tool (SMART) (http://smart.embl.de/) and CLUSTAL 2.0.12 multiple sequence alignment. The amino acid sequence of *T. reesei* (**Tr**) appears in NCBI database with the Accession Nr. Q9P8W3. The amino acid sequence of *V. dahliae* is from *Verticillium* group database. ZnF-C_2H_2 Motifs marked with red colour. Blue colour shows the conserved cysteines (C) and histidines (H). Green colour signals the possible primarily responsible binding site for recognition of an extended sequence after Ganss in 2004 (46).

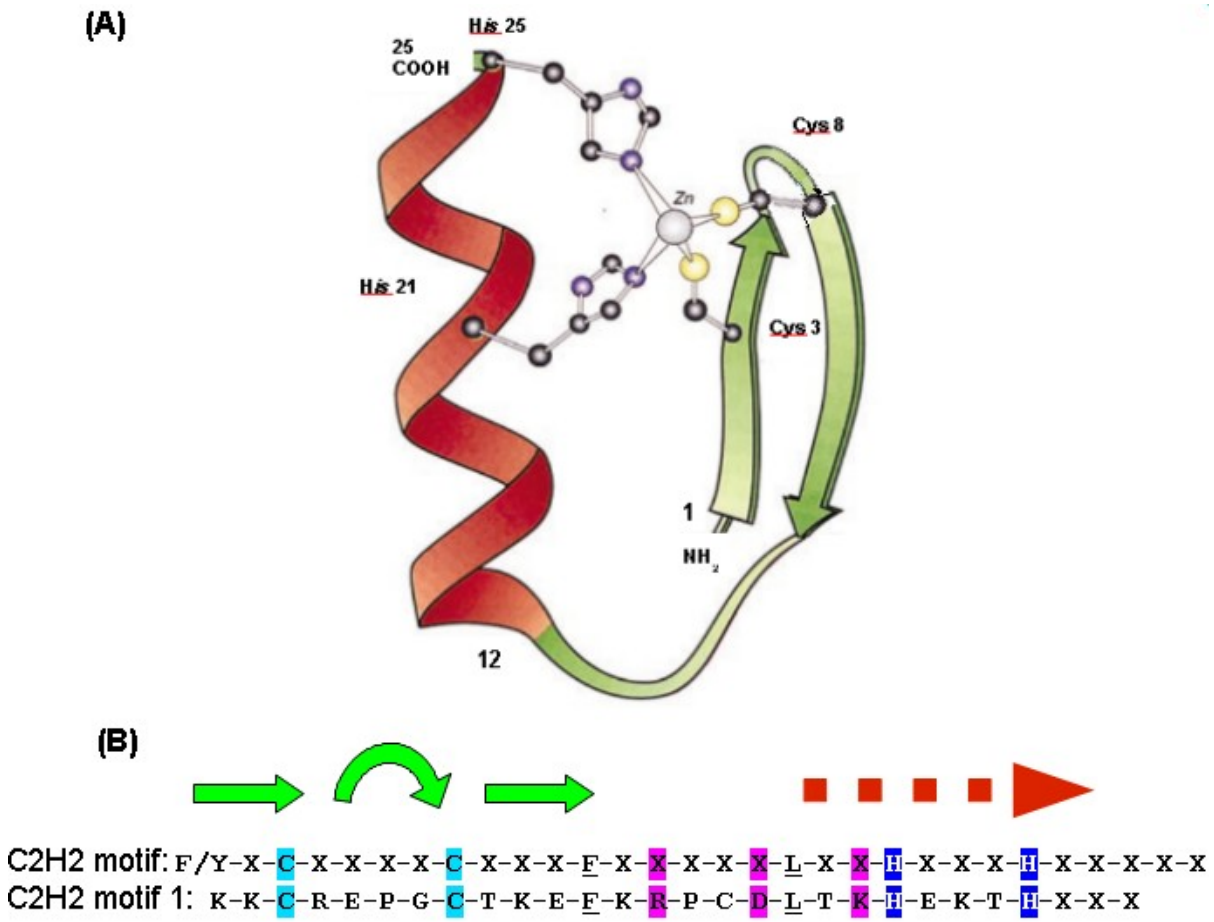

Figure 4: C2H2 zinc finger motif 1. (A) The C2H2 zinc finger motif 1 consists of two cysteins and two histeins residues to form with a Zn ion to a stable fold (modified after Ganss 2004). A short antiparallel β-sheet constructed with two β-strands and a hairpin turn, connected with an α-helix. (B) C2H2 motif 1 is compared with C2H2 motif (consensus): Both have two β-strands (green arrows) containing two cysteins (letters with hell blue background) residues and a hairpin turn (curved green arrow) connected with an α-helix (red dashed arrow) containing two histeins (letters with dark blue background) residues, Conserved amino acids are underlined (46).

MSCQNPRRRSPVTRVGDASSNGLTSLKTNMTLRKGATFHSPTSLDSSSIDAFIPPALGRISDQS*RRVGAHVR
RMEMIVSGIETSLNLNDTPRPASKPSRDECLPRTNGFLGRPTVDPAMAKDTKTSGERRVLRPRHRRSSEQHAS
DSGLGTSLASSVEKQAPSITSKTSKASAITRSAAAPSNTMTKVSGLSSKAVSRVHEHVLRPLRAKPELKDFEP
IVLDIPRRIRDKEIICLRDLEKTLIFMAPVSQLLYQRSVWGDTYRSLMKERAKTAALYLDFCLTSIRCIQATV
EYLSDREQIRPADRPYTNGYFIDLVEQIRQYAGQLATAKEAGVEGREMDVDPTDEVKLFGGISQNGRPAELVR
VRKDGQAISMATGLPVDMDEDGKDFPRLKRSLSQQLADDEEIMRSMARRKKNAAPEELAPKKCREPGCNKEFK
RPCDLTKHEKTHSRPWKCPVKTCKYHEYGWPTEKGMDRHHNDKHSSAPPMHECLFKPCPYKSKRESSCKQHME
KAHGWQYVRTKTNGGKKAPSVAGSSAQPTPQLGNMATPSSSHSIATPPEESTSLFPPFNHDDFPHYVPAEEFA
DTCLGPMGQPPMTLEGIDFNDLGVSPTDYNTPSTDTSYPYTSYQDGPEFVINNDDIYGARVQIPTPAWPEKMM
AGMQNYAPVSACQPQMMPEPLAPHISPIGQGNAMLFTPNSLAEVDEGFDDFGGCGDDFTLFPVNGLDKDAQFQ
TLFGSEMPSSGLGLSQGASQDFFGNGMDWSSMEYHTYSQQPQHQQ

Figure 5: Amino acids sequence of Vl_12.1 gene translated by software Transeq.

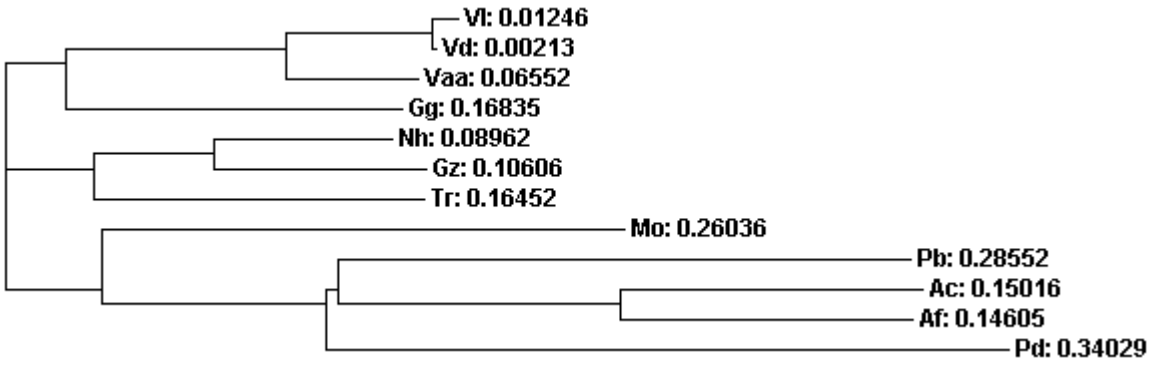

Figure 6: Phylogeny tree showing revolutionary relationship was formed from an alignment of Vl_12.1 protein sequences from *V. longisporum* and related species using alignment program ClustelW2. Vl: *Verticillium longisporum*; Vaa: *Verticillium albo-atrum* VaMs.102; Vd: *Verticillium dahliae* VdLs.17; Gg: *Glomerella graminicola* M1.001; Nh: *Nectria haematococca* mpVI 77-13-4; Gz: *Gibberella zeae* PH-1; Tr: *Trichoderma reesei*; Mo: *Magnaporthe oryzae* 70-15; Pb: *Paracoccidioides brasiliensis* Pb01; Ac: *Aspergillus clavatus* NRRL 1; Af: *Aspergillus fumigatus* Af293; Pd: *Penicillium decumbens*.

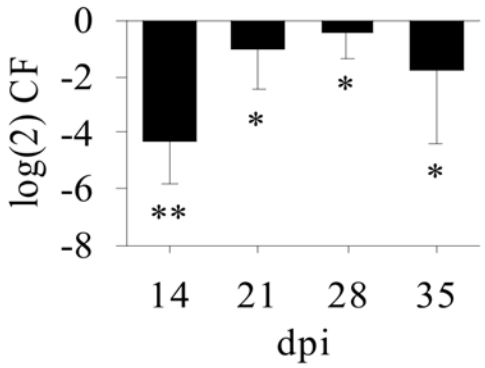

Figure 7: ACE1-like gene Vl_12.1 was identified using root and hypocotyl tissue of VL-infected *Brassica* plants at 14, 21, 28, 35 dpi im comparison to *in vitro* grown mycelium by the treatment with xylem sap metabolites. Gene expression analysis was done by qRT-PCR. All qRT-PCR data were analysed by REST® software (Relative Expression Software Tool) available at http://www.wzw.tum.de/gene quantification. *p-value < 0.05.

2. Generation and characterisation of Vl_12.1 silenced mutants

The *V. longisporum* ACE1-like gene Vl_12.1 was silenced using a RNAi-mediated post-transcriptional gene “knock-down” method with a hairpin construction using intron sequence from the putative Hydrophobin in *V. longisporum* as a space. The expression of

ACE1-like gene Vl_12.1 after gene silencing was suppressed up to 96% in *V. longisporum* quantified by qRT-PCR (Figure 7). Growth rates of transformants were slightly enhanced on PDA agar plates (Figure 8). Gene-silenced mutants did not perform any visual difference in triggering typical infection symptoms in *B. napus.* Figure 7 showed the growth of plants from pathogenicity test.

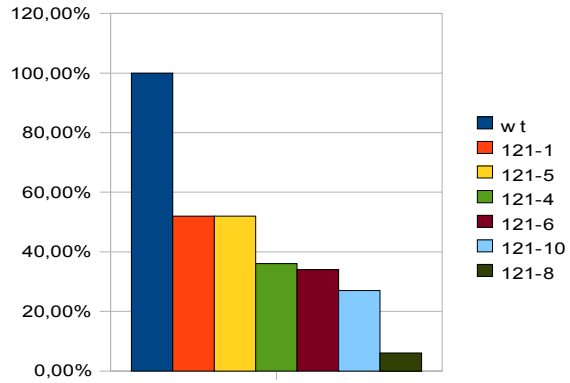

Figure 7: Gene expression levels of ACE1-like gene Vl_12.1 silenced mutants *in vitro* were determined by qRT-PCR and compared with wild type strain. The normalized level of Acel-like gene expression of wild type *in vitro* (calibrator) is always 100%.

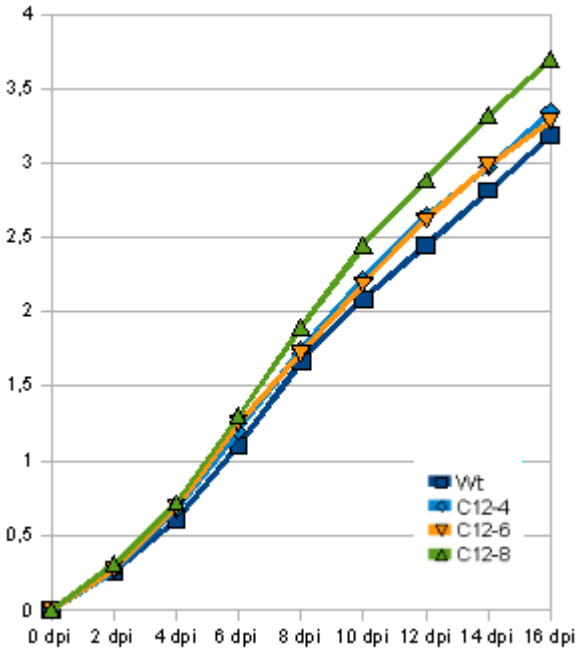

Figure 8: Growth rate of wild type and Acel-like gene mutant strains on PDA plate. The Vl_12.1 mutant strains C12-4, C12-6, C12-8 and wild type VL43 were tested.

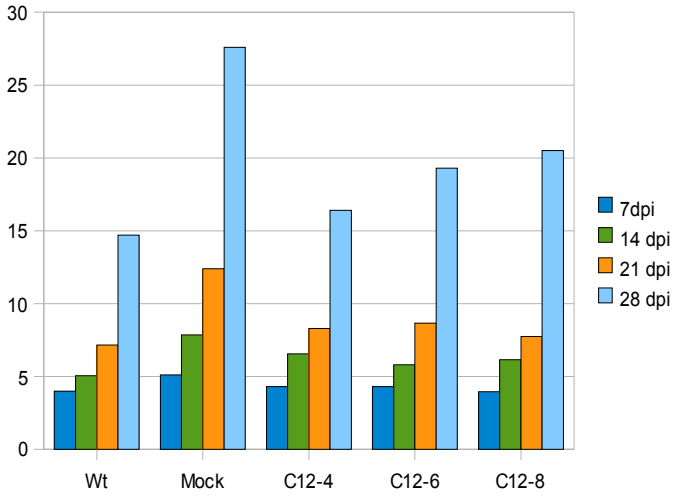

Figure 9: Pathogenicity tests of AceI-like gene silenced mutants *in planta* were determined in time course experiment in green house. The Vl_12.1 mutant strains C12_4, C12_6, C12_8 and wild type VL43 were used. The used *Brassica* plant was “rapid -rolling cycle”.

3. Colorimetric assay of cellulase activity

A colorimetric assay for the determination of cellulase activity was demonstrated on agar plate . A semi-quantitative comparison of cellulase activity on cellulose as a single carbon source showed that AceI-like gene silenced strains grown better than wild type (Figure 10). The mutant strains C12-4, C12-6 and C12-8 showed the bright yard with similar size created by diffusion effect after incubation at 23 °C in dark for 3-5 days. The Samples of *V. longisporum* wild type hold similar diffusion effect with 2-3x more biomass of conidia.

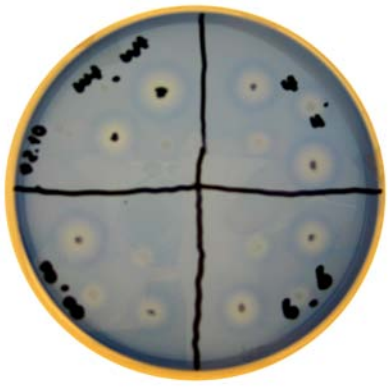

Figure 10: With modified Czapek Dox medium containing Remazol Brilliant Blue and CM-cellulose as a single carbon source. The ACE1-like mutant *V. longisporum* strains C12-4 (above right), C12-6 (below right) and C12-8 (below left) compared with wild type (above

left) were be characterised on agar plate. The bright yard created after incubation at 23°C in dark for 3-5 days by diffusion effect.

Discussion

1. C2H2-Zinc finger as a interaction modular for ACE1 and its homologue in VL

Zinc fingers are small protein domains contained in the most common eukaryotic DNA-binding proteins in which zinc ion plays a important role for the stability of structure. First one of zinc fingers was discovered in the *Xenopus* transcription factor IIIA (TFIIIA) (49, 50). Zinc fingers perform a widely functions in cell, such as transcription, translation, replication, repair, signalling, proliferation, differentiation, metabolism and apoptosis etc. The typically function of zinc fingers is regulation via their interaction modules by binding to variable compounds like small molecules, proteins and nucleic acids. After the functional properties of different zinc fingers they are divided into three groups, C2H2-like finger, gag knuckle and treble clef finger. C2H2 zinc fingers are the most studied (51, 52) and can be divided into two classes after the number of zinc finger motifs in protein sequence (53). The proteins containing fewer than five C2H2 motifs belong to one class which are identified as transcriptional activators or repressors involved in gene regulation. The another class has five or more zinc finger motifs (54). The Vl_12.1 was found during a cDNA_AFLP screening. This gene contained three C2H2-like zinc fingers, each consists of two cysteine, two histeine and the conserved residues in C2H2 type 1 after comparison with ACE1 which contains three C2H2-type motifs (22). The gene expression of Vl_12.1 was suppressed during infection of *B. napus* in root/hypocotyl tissue compared with *in vitro* grown mycelium in a xylem simulating artificial medium. This result confirmed the group of C2H2 zinc finger motifs which functions as regulator in gene expression.

The fungus *T. reesei* produces effective cellulase. It is well-known in addition to inducers such as pophorose, cellubios, lactose and β-cellobiono-1,5-lactone (42, 43) that cellulases in *T. reesei* were regulated also by its carbon source cellulose. The cellulase gene expression regulated by transcriptional factors ACE1 and ACEII (20, 44). Aro and her colleague reported that ACE1 acted on transcriptional level as a repressor and regulated the *cbh1* (cellulase gene in *T. reesei*) promoter by binding to AGGCA element (45) which was found also in the upstream region of a putative cellulase of *V. albo-atrum*. Therefore, we speculate that ACE1-like Vl_12.1 gene possibly via the specific recognition sites of

C2H2 zinc fingers binds to the AGGCA element in cellulase promoter fragment in *V. longisporum* because of high identity between these two species. Thus we recommend that the cellulase regulation mechanism in *T. reesei* could be used as a basic model for *V. longisporum* gene Vl_12.1 study.

After a semi-quantitative colorimetric cellulase activity assay, the visual inspection revealed AceI-like Vl_12.1 mutant strains indicated no significant difference to *V. longisporum* wild type on Czapek Dox agar plate containing CM-cellulose as solo carbon source. Even sometimes the wild type affected weaker than mutants. Hence, generating of overexpression mutant for Vl_12.1 gene and the assay using polysaccharides as inducers under quantitative condition for furthermore investigation of Vl_12.1 are crucial step for successful gene function analysis.

2. ACE1-like gene Vl_12.1 related with pathogenesis

Transcription factor genes encoding regulatory protein exist in all eucaryote. They interplay with promoter in genome and regulate gene expression to control cell cycle and adapt environmental changing. Some transcriptional factor genes were identified in the last years. To delete a gene encoding zinc-finger protein called FOW2 in *Fusarium oxysporum f.sp. Melonis* by mutagenesis, induced to loss pathogenicity of fungus for penetration of host plant roots (24). In another vascular wilt pathogen *F. oxysporum*, a inducible zinc-finger protein XlnR was demonstrated to regulate the expression of xylanase (25).

Pathogenicity test in this work performed two results. One side the Vl_12.1 gene was suppressed during infection of *B. napus* in root/hypocotyl tissue in reference to *in vitro* grown mycelium in a xylem simulating artificial medium; another side the Vl_12.1 gene silenced mutants did not affect the infection on *Brassica* plants. These indicated that Vl_12.1 possibly not direct correlated with pathogenicity, This suggestion confirmed also by similar result with ACE1 expression in fungal pathogen *Magnaporthe grisea.* During infection on rice, the expression of *M. grisea* gene ACE1 connected to the onset of appressorium-mediated penetration without host plant signals (56). ACE1 in *T. reesei* affected as a repressor of cellulase and xylanase expression. *V. dahliae* was able to secrete cellulases, and showed its aggressive isolates degrading crystalline cellulose quickly than the less aggressive isolates (48). Thus we speculated that Vl_12.1 might regulate the production of polysaccharide-degrading enzymes to activate for penetration

through the host cell wall, adaptation to xylem environment and indirectly related with pathogenesis.

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Chapter 6: A putative Vl_PKS1 gene involved in melanistic process

Introduction

Polyketides are large group of secondary metabolites which exist in bacteria, fungi, plants etc.(1, 2, 3, 4). Because of their biosynthetic complexity (5, 6) and special application in pharmaceutical industry were Polyketides intensive researched. These natural resources include numerous toxins [fumonisins (7), aflatoxin (8), zearalenones (9, 10)], antibiotics, and therapeutic compounds (11, 12), fungal melanins (13), and other pigments (14). Microbial polyketides are generally assembled through three types of polyketide synthase (PKS) (15). Fungal PKSs belong to Type I which is large multifunctional polypeptides that consist of a lot of modular units (modulars) determining the length of the carbon backbones of polyketides. Fungal secondary metabolites such as mycotoxins, fungal melanins and pigments are main groups of PKS type I (PKS1). In phytopathogenic fungi they are known often to play a role during the host-pathogen interaction as phytotoxins, pathogenicity or virulence factors.

Results and Discussion

We found a putative PKS1 in *V. longisporum* according to *s*equence analysis compared to sequences in *V. dahliae* and *Bipolaris oryzae*. A fragment 1936 nt (DNA sequence intern from Hanno Wolf et al.) in the gene Vl_PKS1 containing a putative catalytic modules β-ketoacyl synthase (KS) and an acyl transferase (AT) motif (Figure 4) was isolated from *V. longisporum* using the PCR technique based direct sequencing. Its translated cDNA is predicted to code for 656 aa (Figure 4). A multiple alignment of diese fragment in Vl_PKS1 from *V. longisporum* with the amino acid sequences of *V. dahliae* and *Bipolaris oryzae* was shown in Figure 4. Vl_PKS1 gene keeps a high similarity like *V. dahliae* (similarity 100 %) and *Bipolaris oryzae* (similarity 85,8%) on the protein level. Moriwaki et. al demonstrated that PKS1 gene is involved in the melanin biosynthesis pathway of *Bipolaris oryzae* by a disrupting the PKS1 gene (16).

Vl_PKS1 gene was determined *in planta* relative gene expression by qRT-PCR in this study as a up-regulated gene, which was compared with housekeeping genes in *V. longisporum* during infection. This phenomenon indicated that Vl_PKS1 gene may play a role in interaction between pathogen *V. longisporum* and host plant *B. napus*.

The *V. longisporum* PKS1 gene silenced mutant strains were generated using the antisense RNA- and RNAi-mediated methods. The binary vectors containing specific target fragments for Vl_PKS1 gene silencing were constructed using procedures as generating Vl_6.2 gene silenced mutant strains described in chapter 4 and Vl_12.1 gene silencing mutant strains presented in chapter 5, respectively. The for both silencing strategys used target fragments placed at β-ketoacyl synthase motif and Acyl transferase motif in Vl_PKS1 (Figure 3 and Table 1 see primers for construction of binary vectors pPK2-antisense-PKS1-I and pPK2-antisense-PKS1-II). *In vitro* silencing effects of mutant strains were determined by qRT-PCR and resulted *in vitro* until 60% for the specific antisense RNA gene expression regulation and until 90% (This result was reported by Malte Beinhoff in 2009) for the RNAi-mediated gene silencing. The pathogenicity assays *in planta* performed all tested Vl_pKS1 silenced mutant strains which did not affect the growth of the host plant *B. napus,* but a morphological phenomenon of fungal colony with reduced melanistic accumulation was observed on solid medium compared with wild type. They were incubated at 23°C in dark for 4-5 weeks (Figure 1). This Phenomenon was also observed in a competitive assay with *Gliocladium roseum* on solid medium. The *V. longisporum* isolate 43 performed oft a melanistic process in SXM medium or on SXM agar plate during an incubation at 23°C in dark after 3-4 weeks (Figure 2). *In vitro* determination of Vl_PKS1 relative gene expression level by qRT_PCR demonstrated a dramatic enhance of Vl_PKS1 gene expression in comparison with the Vl_PKS1 relative gene expression level at 5 days (Figure 3).

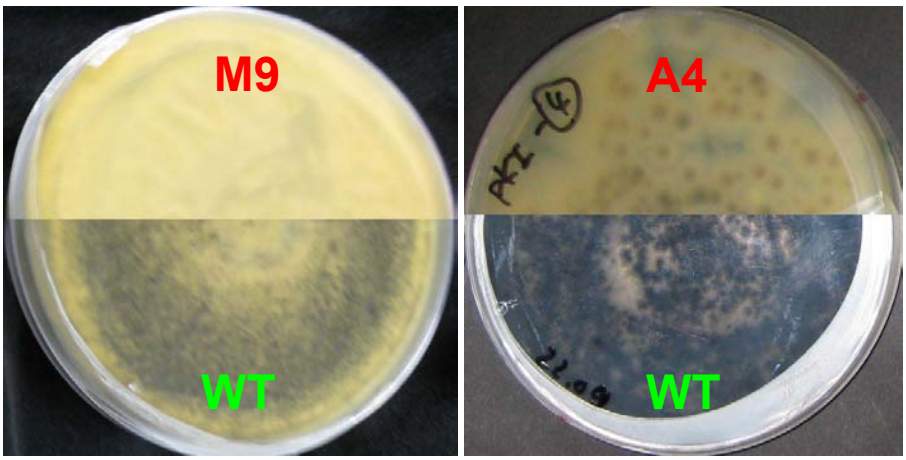

Figure 1: Morphological phenomenon of Vl_PKS1 gene silenced mutant strains. A reduced melanistic accumulation of the colonies was observed after comparison with wild type on PDA and Czapek-Dox plates. Letters in green colour: Wild type strain Vl_43; Letters in red colour: Mutant strains, M9 and A4.

Table 1: Primers for construction of binary vectors pPK2-antisense-PKS1-I and pPK2-antisense-PKS1-II

Name of Primers	Sequence of primers	Length
AS-Vl_PKS1_I_F	5'-ATCAGTGGCGCGCCCATGTCGCCGCGCGAAGCCTA-3'	939 bp
AS-Vl_PKS1_I_R	5'-ATCAGTCCTGCAGGTTTTATGCCGACGTGAGGAGGGA-3'	
AS_Vl_PKS1_II_F	5'-ATCAGTGGCGCGCCCTTCATCTTCACAGGCCAAGG-3'	620 bp
AS_Vl_PKS1_II_R	5'-ATCAGTCCTGCAGGGGAGTGGAAGGCATAGGGAAC-3'	
Blue colour: Restriction sites.		

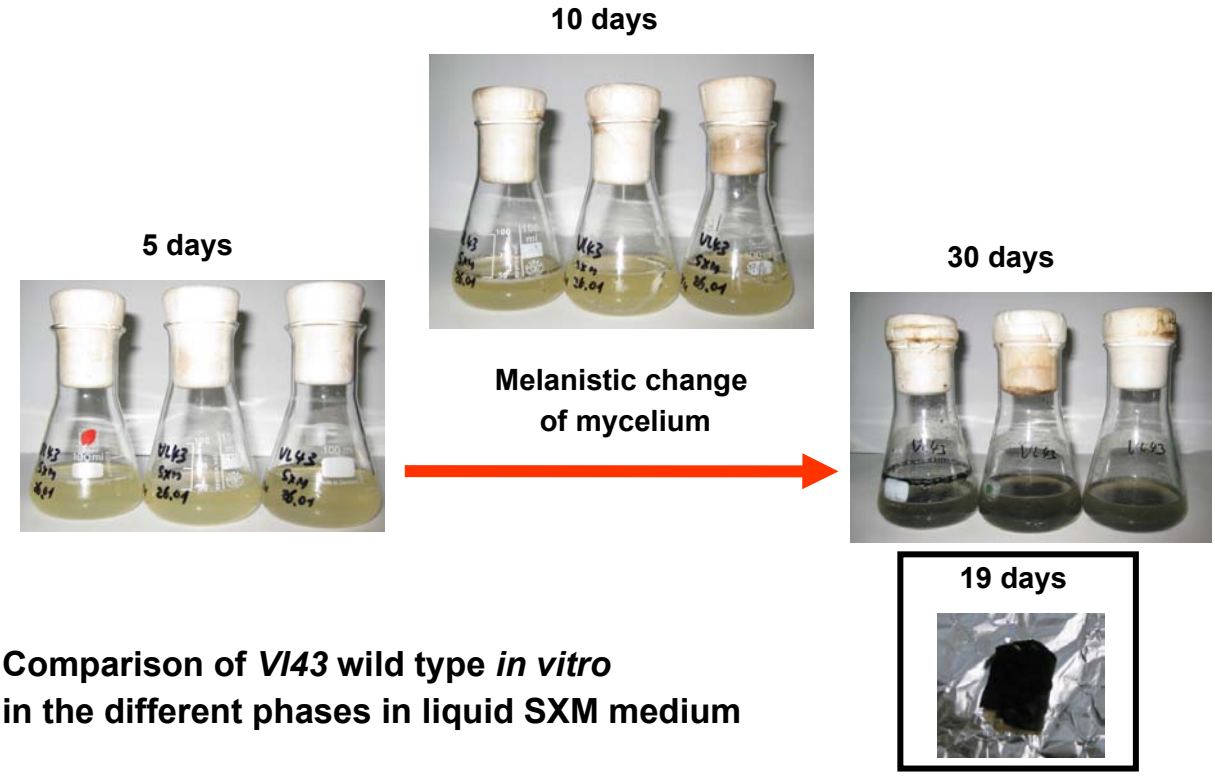

Figure 2: Melanistic process of *V. longisporum* isolate 43 incubated at 23°C in dark.

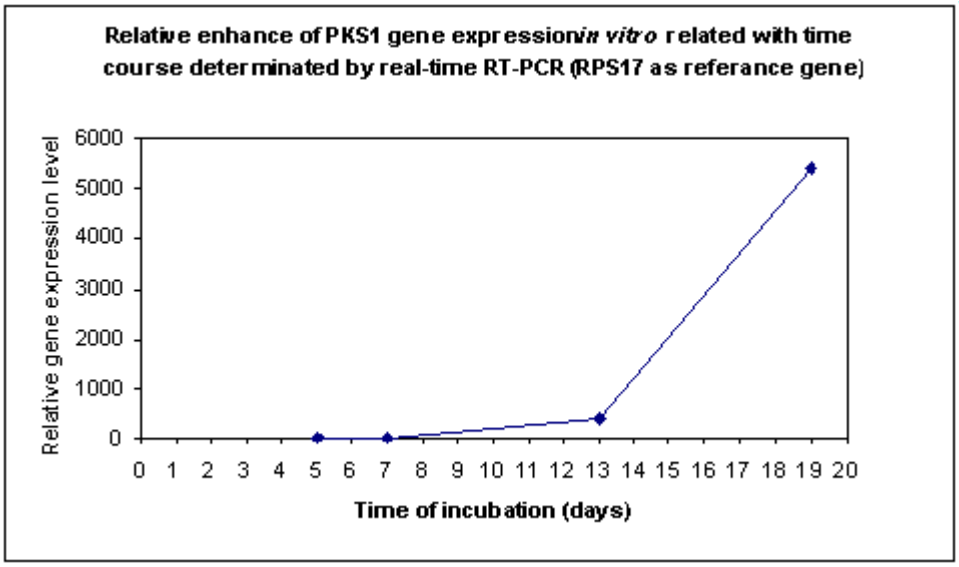

Figure 3: *In vitro* Vl_PKS1 relative gene expressions in time course 5d, 7d, 14d and 19d determined by qRT-PCR. The ribosomal protein S17 (RPS17) was used as a reference gene, Vl_PKS1 relative gene expression level at 5 days was set as a calibrator and a normalisation of qRT-PCR data used ΔΔ CT method (see chapter 3).

Figure 4: Alignments of the amino acid sequences among Vl_PKS1 of *V. longisporum* and its homologous of *V. dahliae* and *Bipolaris oryzae* from *Verticillium* group database and NCBI GenBank. The putative catalytic modules β-ketoacyl synthase (KS) and acyl transferase (AT) motif are found in Vl_PKS1 sequence. KS motif includes an active site cysteine; AT motif includes an active site serine.

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Chapter 7: Labeling *V. longisporum* with GFP and its derivatives

Introduction

The green fluorescent protein (GFP) was extracted from the small, mouse-sized umbrella-shaped glowing Pacific Northwest jellyfish *Aequorea victoria*, and identified as a protein first by Osamu Shimomura in 1962 (1). This jellyfish produces green bioluminescence (2) from small photoorgans located on its umbrella. In order for bioluminescence to occur, *Aequorea* releases calcium ions to bind a photoprotein called aequorin, which released blue light (Figure 1). The blue light *in vivo* (3, 4) or ultraviolet light *in vitro* was absorbed by green fluorescent protein, which in turn emitted the green light (5, 6, 7). This made an advantage for GFP than luciferases which glow under the special conditions such as calcium ions and coelenterazine required for aequrin (8, 9, 10), ATP and magnesium required for luciferase (11, 12, 13) etc. Wild-type GFP had a major excitation peak at 395 nm and a minor peak at 475 nm, gave off bright green light with an emission peak at 509 nm with a shoulder at 540 nm. The GFP research attracted Douglas Prasher, a scientist of biochemistry. He got an revolutionary idea about expression of GFP as tracer molecule in bacteria in 1987 (14). He and co-worker had cloned and sequenced the gene for GFP from jellyfish *Aequorea victoria* in 1992 (6). The result interpreted that GFP was a small protein with 238 amino acids. Its small size allowed it was easy as a to be fused protein. The expression of GFP in *Escherichia coli* and *Caenorhabditis elegans* succeeded in Laboratory of Marty Chalfie in 1994 (15). The crystal structures of wt-GFP (16) and enhanced GFP S65T mutant (17) elucidated both that GFP protein kept a stabil can shape structure constructed with 1 central located α-helix and 1 β-barrel consisted of 11 surrounded tightly packed β-sheets and the chromophore was located in the middle of the β-barrel.

The chromophore of GFP was responsible for its fluorescence as the “light in the can”. Osamu Shimomura described a chemical structure of chromophore in *Aequorea* green fluorescent protein first in 1979 (18). Cody and co-workers confirmed the hexapeptide structure of chromophore in 1993. The chromophore formation was a stepwise procedure, which changed a sequence of three amino acids (Ser65, Tyr66 and Gly67) into a single

conjugated system that extended from the side chain of Tyr66 to a heterocycle (19). The surrounding residues forced upon chromophore to influence the fluorescence properties. This structure basis made an artificial engineering of GFP molecule possible (7). Roger Tsien's laboratory developed GFP mutants which started fluorescing faster than wild type, They were brighter and had different colours. First new derivative of GFP with blue colour and oxygen dependence was reported in 1994 (20). A yellow fluorescent T203Y mutant was designed based on crystal structure of S65T GFP mutant in 1996 (17). Cormark and co-workers have successful generated a popular green fluorescent variant EGFP (enhanced GFP) (21). Red fluorescent proteins (DsRed) was discovered in anthozoan corals by Lukyanov' laboratory in 1999 (22) and led to discovery of many new fluorescent proteins and chromoproteins. The biggest difference between green fluorescent protein and its red analogue DsRed, was the chromophore of DsRed, which had an extra double bond extending the chromophores conjugation and causing the red-shift (23). Although the original Y66W mutant of *Aequorea* wild-type GFP was weakly fluorescent, the from it improved variant ECFP was widely used (24).

GFP was used in a range of research areas. In filamentous fungi the GFP was permit to be used in biochemical assays, cell screening and as marker for monitoring of different fungal infection and to follow the development of fungal-plant interaction. GFP and its derivatives as reporter genes were especially used to study the activity of target genes and their transcriptional promoters.

Methods and Results

For *in vitro* construction of transcriptional and translational fusion we applied the binary vector pPK2 as backbone, EGFP and ECFP as reporter gene (Figure 2) and used *Agrobacterium*-mediated transformation to generate respectively EGFP and ECFP marked *V. longisporum* mutants. The high level expression and bright fluorescence were obtained in with EGFP and ECFP marked *V. longisporum* strains (Figure 3 and Figure 4). A Leica TCS SPII Confocal Laser Scanning Microscope (CLSM) was equipped for performance of fluorescent fungal strains. A Melles Griot Argon 488 nm laser was used for excitation for scanning, and 520-560 emission filters to capture the fluorescence of eGFP tagged cultures. A Melles Griot Argon 436 nm laser was used for excitation for scanning, and 440-480 emission filters to capture the fluorescence of eCFP tagged strains. Pictures were viewed using the Leica LAS AF Imaging software.

The binary vector pPK2-eGFP15 was also successfully transferred and expressed in other fungal species *Fusarium graminearum*, *Trichoderma reesei* and *Gliocladium roseum* (Data unpublished).

Figure 1: *Aequorea victoria* (left picture). Photo from the bioluminescence web page of Steve Haddock, Monterey Bay Aquarium Research Institute. Photoreceptors on umbrella of *Aequorea victoria* bioluminescing (right picture). Photo of Osamu Shimomura) [Modified according to GFP web page of Marc Zimmer (http://www.conncoll.edu/ccacad/zimmer/GFP-ww/shimomura.html)]

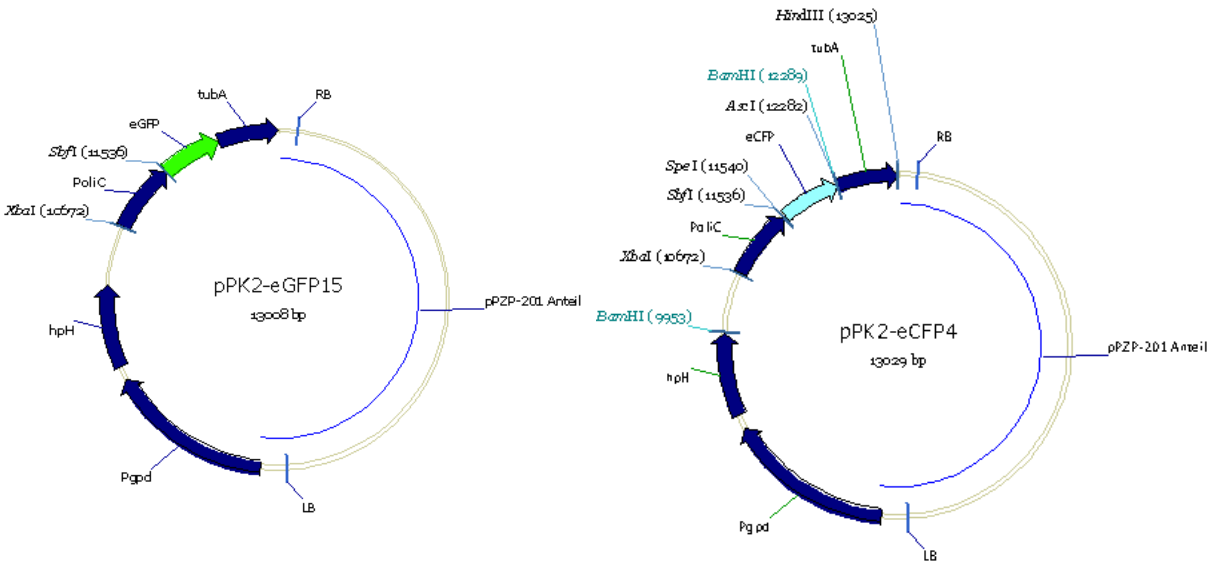

Figure 2: Maps of binary vectors pPK2-eGFP15 and pPK2-eCFP4 for transcriptional and translational reporter in fungi. Promoter OliC for a gene encodes subunit 9 of the mitochondrial ATP synthase complex from *Aspergillus nidulans* and terminator for beta-tubulin gene from *Botrytis cinerea* were used for control of expression of EGFP or ECFP. A SbfI site (CCTGCAGG) was introduced at 3'-direction of Vl_6.2 gene using primer (5'-

ATCAGTCCTGCAGGGTCCAGCGTATCCGTTGCCTG-3') in combination with another primer (5'-ATCAGTGGCGCGCCATGGACCCAGCTGTCGTCCGTA-3') containing a AscI site (GGCGCGCC) at 5'-direction of Vl_6.2 gene. Pgpd [promoter for glyceraldehyde-3-phosphate dehydrogenase (gpd) from Coriolus versicolor] manages hpH (Hygromycin phosphotransferase resistance gene) and pPZP-201 fragment containing T-DNA region of Ti plasmid of *Agrobacterium tumefaciens* is from the host binary vector pPZP201.

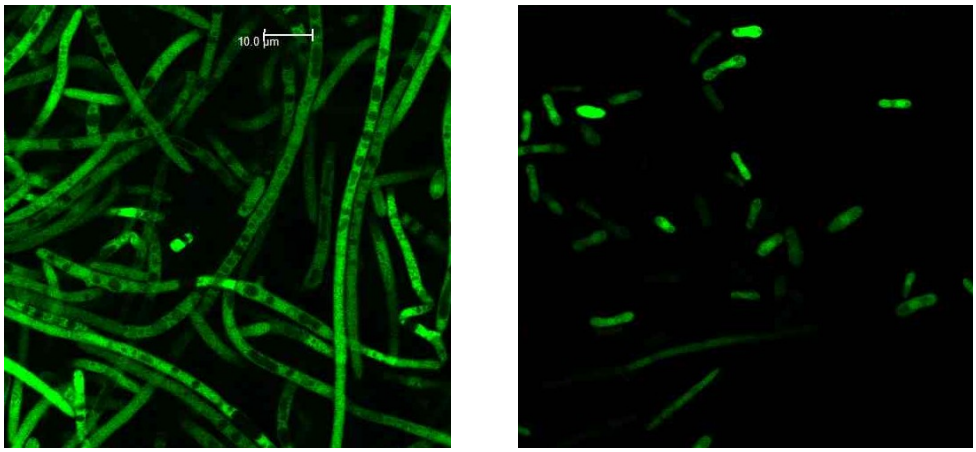

Figure 3: Mycelium and spores of *V. longisporum* isolate 43 expressing eGFP. Fluorescence scanning microscopy of fungal cultures: 488 nm laser was used for excitation for scanning, 520-560 emission filters to capture the fluorescence of eGFP tagged strain.

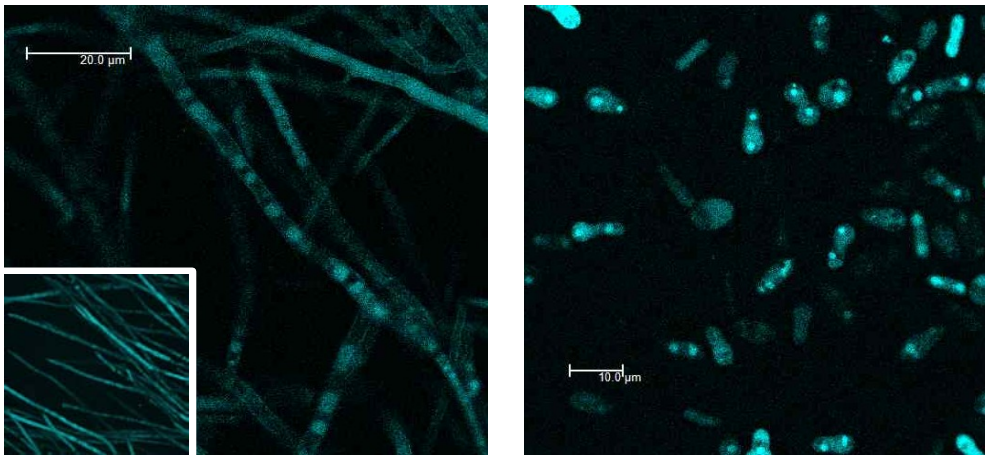

Figure 4: Mycelium and spores of *V. longisporum* isolate 43 expressing eCFP. Fluorescence scanning microscopy of fungal cultures: 436 nm laser was used for excitation for scanning, 440-480 nm emission filters to capture the fluorescence of eCFP tagged strain.

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Chapter 8: General discussion

Research methods

Plant fungal pathogens as plant parasites, cause the most significant plant diseases and hods the largest proportion of organisms. An improved understanding of fungal pathogenesis may lead to improve the plant disease resistance through breeding using genetic engineering. Research using modern tools at morphological, cellular, physiological, biochemical and especially molecular level have developed rapidly in recent years. The advent of electron-, fluorescent-, and confocal-microscopy provided the possibilities to make cytological observation of the ultra structure and interaction between pathogen fungus and host plant. The molecular biological techniques such as PCR, cloning, transformation, sequencing, silencing, over expression, molecular marker and other related disciplines were widely applied to study fungal genetic, metabolic, pathogenic mechanisms and functional analysis. In our work the whole *V. longisporum* transcripts after a treatment with extracts of *B. napus* xylem sap were screened by cDNA-AFLP and compared to water treated controls. The signal results of comparison required genetic identification and primary characterisation. They were completed for TDFs analysis using different functional methods of sequence extension and *in planta* relative gene expression by qRT-PCR to identify the target genes and to see how they related to pathogenicity of *V. longisporum* on *B. napus*. Further gene functional analysis followed with mutant strain generation using other strategies such as gene over expression, post-transcriptional silencing and fusing with green fluorescent protein as a marker etc..

The fluorescence-based qRT-PCR method used in this study provided on the whole an efficient alternative for our experiments with advantages such as sensitivity, simplicity and relative low-costs for the determination of gene expression. The high sensitivity of qRT-PCR limited its usage as an absolute measurement of low expression of target genes because of a frequent occurrence of the variable PCR efficiency. Thus, we used a procedure to determine relative target gene expression using qRT-PCR, which correlated with the reference gene expression levels. The choice of reference genes was based on literature search and a control test of the cross reaction with the host plant and the primer efficiency were tested. Slight changes of quality and quantity of the initial templates, different amplification efficiencies and possibly inhibitors present at low levels can lead to

increased variability of dataanaylsis after qRT-PCR and produce false relative gene expression levels. Total RNA was used for quantification of gene transcripts (1, 2). We conclude that an isolation of mRNA and an additional purification of cDNA of samples are effective for the samples containing low fungal biomass or for candidates tested with low genes expression. A sequence comparison among *V. longisporum, V. dahliae* and *V. albo-atrum* with reference genes at nucleotide and amino acid levels may provide further evidence for their evolutionary relationships.

The gene regulation in fungal cells controlled at post-transcriptional level depends on two important aspects, antisense-RNA regulation and RNA interference. These two aspects have been developed as efficient tools for gene silencing in biotechnology. Although the progress of antisense-RNA regulation at the molecular level is not fully described. The recent trend is towards antisense-RNA regulation in eukaryotes using an RNAi-like mechanism in final phase (3, 4, 5). Both RNAi- and antisense RNA-mediated gene silencing were used for functional analysis of candidate genes. The RNAi method demonstrated a relatively strong silencing effect (up to 90%) compared to the antisense-RNA regulated gene expression (up to 70%) and determined *in vitro* by qRT-PCR. Thus we recommend the use of the RNAi method for a gene silencing applification. Antisense RNA-mediated regulation is more suitable when a mild gene knock-down is required.

Green fluorescent protein (GFP) has been widely used in fungal research areas such as in biochemical assays, cell screening and as marker for monitoring pathogen infection and to follow the pathogen-host interaction etc.. Eynck et al in 2007 (6) have used the GFP marked *Verticillium* strains to demonstrate the fungal infection process on host plant *Brassica napus*. Utermark and Karlovsky in 2006 (7) used GFP to fuse with the zearalenone-sensing promoter-element of the zearalenone lactonase gene zes2 to exploit zearalenone sensing ability of *Gliocladium roseum*. We have successfully constructed two binary vectors fused with enhanced derivatives of GFP for fungal transcriptional and translational fusion. However, GFP expression related to physiological state of fungal mycelium, and the fluorescence reduction in old hyphae (6). A another alternative is to use bioluminescent luciferase, which functions as an enzyme to cut the costly substrates and lead to imaging. Recently these two methods both were used for fusion with target genes or promoters as reporters for functional analysis or for tracing the behaviour of the micro-organisms.

Molecular mechanisms of pathogenicity

Functional analysis of fungal pathogenicity-related genes has attracted more attention in recent years. The xylem sap inducible pathogenicity related genes expressed by *V. longisporum* during the infection process on *B. napus* are involved in a set of molecular mechanisms which related with pathogen activities of aggression and defence. They are host determination, signaltransduction, stress response, biosynthesis of secondary metabolites and activation of the plant cell wall degrading enzymes etc..

Pathogenicity means that an organism is able to cause disease. This ability represents a genetic resources of the pathogen and a established relationship based on the host-pathogen interactions. The pathogen organism cause the damage to the host. However, this disease is not an inevitable result of the host-pathogen interaction. Virulence suggests the degree of pathology caused by the pathogen. A term often used interchangeably with pathogenicity, pathogens can exhibit a wide range of virulence at different levels. Virulence can be extended under a correlation with the ability of the pathogen to multiply within the host and may be affected by other internal factors and external conditions.

Pathogenicity related genes enable the pathogen and host to establish compatible interaction and affect the normal gene function. They can relate with the structure of genes involved in infection. For example, the rice blast fungus disease gene (mpg1) (8, 9) encodies a protein with a small molecule, which is typically hydrophobic, it may function by binding to hydrophobic cuticle on the leaves. VirA and VirG are two virulence genes in *Agrobacterium* (10), they regulate the transformation on plants or other organisms through a two-component regulatory system to sense particular phenolic compounds synthesized by wounded plant tissues. Under the certain conditions, other vir genes are activated, the T-DNA is processed from the Ti-plasmid and transferred into host cells. many plants and non-plant species can not provide enough and correct phenolic compounds, then VirA and VirG can not start regulation (10). Melanins are polymers for dark-pigment and belong to one type of the fungal secondary metabolites. Fungal melanins have been shown on the one side to be important virulence factors in interaction between pathogenic fungal species and host plants. Performing a key role in appressoria-mediated infection (11). Several fungal phytopathogens *Bipolaris oryzae, Pyricularia oryzae, Colletotrichum orbiclare, Verticillium dahliae* etc. were observed which were important for a successful infection on host plant under a melanin accumulation of appressorium (12, 13). The

dihydoxynaphthalene (DHN) plays a very important role (12) for a large accumulation of melanin deposits in the appressorium cell wall of the inner layer, before the invasion of the spores. *Bipolaris oryzae* (Breda de Haan) causes rice brown leaf spot disease, produces 1,8-dihydroxynaphthalene (DHN)-melanin (12), It is similar to *Colletotrichum lagenarium, Magnapothe grisea, and Alternaria alterata* (13, 14, 15). DHN-melanin biosynthesis was speculated to be started by PKS. Moriwaki et. al demonstrated that PKS1 gene is involved in the melanin biosynthesis pathway of *Bipolaris oryzae* by a disrupting the PKS1 gene. On the other side, an accumulation of pigments displayed melanins as an important tolerance factor in stress response of fungal organisms to radical environmental change such as UV radiation (15) and protection against antifungal drugs (16).

In this study, 10 genes from *V. longisporum* were isolated using different methods, they were identified by sequence analysis and characterized. Eight of them were profiled from a cDNA-AFLP screening. After *in planta* relative gene expression analysis by qRT-PCR, they were grouped in three types. up-regulated, suppressed and time-dependent. For further functional analysis, four of them, Vl_6.2, Vl_12.1, Vl_PKS1 and Vl_NEP1 were investigated as the candidates. According to the outcome of these results we proposed their tentative roles in pathogensis by measuring of the putative function of candidate genes. Here we divide them into two types, namely genes function for secondary metabolites and genes function for cell-wall degradation and integrity.

The determination of Vl_PKS1 relative gene expression by qRT-PCR demonstrated that Vl_PKS1 was up-regulated *in planta* and gene silenced mutant strains reduced Vl_PKS1 gene expression *in vitro*. Vl_PKS1 relative gene expression in *V. longisporum* liquid culture presented a huge increase after 3 week incubation and perhaps acted as a factor related stress-response of fungus to nutrient and other conditions. The Vl_PKS1 silenced mutants reduced melanine production compared to wild type and this was displayed very clearly in a competitive assay with *Gliocladium roseum* where a strong decrease of melanine accumulation was observed. Thus we suggest that the Vl_PKS gene expression is involved in aggression and defence mechanisms and its function as a pathogenicity related factor is reduced.

In sequence analysis and *in planta* relative gene expression determination we identified and characterized a small peptide gene encoding necrosis- and ethylene inducing peptide with its homologue with high similarity. We identified a conserved hepta-peptide motif

GHRHDWE which is essential for necrosis-inducing activity (30, 31). This hepta-peptide motif exists in 5 NEP1-homological genes in *V. longisporum*, 7 NEP1-homological genes in *V. albo-atrum* and 9 NEP1-homological genes in *V. dahliae*. Vd_NEP1 was shown to elicit wilt and chlorosis symptoms on leaves of several host plant species (17). A hetero Vl_NEP1 gene expression assay showed Vl_NEP1 gene to be able to induce chlorosis on leaves of oilseed rape and tabaco (intern results). NEP-encoding genes are found in other fungal pathogens *Pythium aphanidermatum* and *Phytophthora parasitica* but with different numbers of copies: 1, 2, and 4. respectively (18, 19). Plant pathogenic species of *V. longisporum*, has two copies of NEP1 gene because of its near-diploid status. A phylogenic analysis showed that the sequence similarity at nucleotide level between *V. longisporum* and *V. dahliae* for NEP1 is decreased, compared with results of other genes. We propose that the number of NEP-like homologues and the change of gene sequences at DNA level as the evidence that for *V. longisporum* uses a another means of aggression for infection than *V. dahliae*.

It is well-known that the cell-wall degrading enzymes cellulases, hemicellulases and pectinases play important roles in the penetration of host plant roots by fungal pathogen. In this study we found that *V. longisporum* degraded Carboxymethyl (CM) cellulose *in vitro*. ACE1 functions as a repressor of cellulase and xylanase expression in *T. reesei*. *V. dahliae,* which were able to secrete cellulases, and showed its aggressive isolates degrading crystalline cellulose quicker than the less aggressive isolates (20). A deletion of FOW2 gene encoding zinc-finger protein in *Fusarium oxysporum* f.sp. Melonis induced to loss pathogenicity to penetrate roots of host plant (21). A XlnR gene encoding inducible zinc-finger protein in vascular wilt *F. oxysporum*, regulated the expression of xylanase (22). During infection on rice, the expression of *M. grisea* gene ACE1 connected to the aggression of appressorium-mediated penetration without host plant signals (23). Pathogenicity tests in this work two results: first the Vl_12.1 gene was suppressed during infection of *B. napus* in root/hypocotyl tissue in reference to *in vitro* grown mycelium in a xylem simulating artificial medium; second the Vl_12.1 gene silenced mutants did not affect the infection on *Brassica* plants. These indicate that Vl_12.1 functions as a regulator for the cell-wall degradation may only be possible with a decrease of its expression products, but this is minimal.

Wsc1 is a a cell wall stress component sensor protein in plasma membrane that behaves like a linear nano-spring that is capable of resisting high mechanical force and responds to

cell surface stress (24). WSC domains play a role in membrane integrity in different microbes like baker's yeast (*Saccharomyces cerevisiae*). The with antisense RNA methods generated Vl_6.2 silenced mutants demonstrated Vl_6.2 gene as a stress response protein activating in growth inhibition assays both with ionic detergent SDS and with alkaline pH value at 10. A treatment with SDS in low concentration or a non ionic detergent Tween 20 could not effect the growth of *V. longisporum*, This indicates an elastic role of Vl_6.2 gene which behaves in a similar manner to that seen in *S. cerevisiae*. It is possible that Vl_6.2 possesses a linear nano-spring-like structure in response to extracellular stress under particular conditions. Disruption of *Verticillum* MAP Kinase 1 in *V. dahliae* severely reduced virulence in host plants (25). Other researchers presented a deletion of WSC genes in *S. cerevisiae* resulting the WSC binding domains as upstream regulators playing a important role in stress response in PKC1-MAPK1 pathway (26, 27). Rauyaree et al. presented that MAP (Mitogen-Activated Protein) kinase-mediated signalling pathways are involved in pathogenicity of *Verticillium dahliae* in 2005 (28). Vl_6.2 was investigated as being up-regulated *in planta* by qRT-PCR compared to *in vitro* cultivation of *V. longisporum* in a xylem-simulating artificial medium. Vl_6.2 silenced mutants which resulted in a reduced virulence *in planta*. thus we propose that Vl_6.2 gene was actively involved in cell-wall integrity related both to the aggression and the defence process of *V. longisporum.*

V. longiporum is a “near-diploid” and host-specific fungal phytopathogen causing wilt disease in oilseed rape and induces, leaf chlorosis, curl and especial growth stunting as typical symptoms. *V. dahliae* is non-pathogenic on *B. napus* (6). The morphology of *V. longisporum* is very similar to two other phytopathogen *V. dahliae* and *V. albo-atrum* with a small difference in the length of the spores (28). The high similarity and identity of the sequences at nucleotide and amino acid levels were confirmed according to the results of comparisons among these three species in this study. We suggest that an infection ability based on the specific pathogenicity related factors of *V. longisporum* on *B. napus* could be a model mechanism for description of a successful balance between heredity and variation of *Verticillium* species along with evolutionary selection under the special environmental dilemma and the internal genetic possibility. The genes variate in the evolution process and cause the changed biological phenotypes. At the same time, the identical kind in order to adapt to the circumstances it is frequently necessary to be able to have a change in phenotype, sometimes even a change of a complete gene. We speculate that some

Verticillium isolates successfully developed this variation. Until today we can find *V. longiporum* only in several regions in northern Europe.

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Chapter 9: Supplementary data

Extended DNA and amino acid sequences of genes and proteins of *V. longisporum*

(Note: **Green colour**: Start code; **Red colour**: Stop code; **Bright blue colour**: Intron)

1). Vl_6.2 gene (Hypothetical protein including 4 WSC binding domains)

Coding sequence (including introns):4722 nt

GTTTGATTTGGGCACGATGAGGGTGTTTATGAATCACAGATATCTCGACATGTTTTGTTTGATTATAGTCGTTGTCGTTA
TCTTTTCCTCGGATGGGCGTCCTGTTGTGAAGGATTTGGGGATCACAGAGTTTAGAGGGCACACAGCGCCGCCATGGATA
TCTTGTTTTCTCTCTTCTCGAGAATCCGTCTGTTCGGAGTTTCAGGACCCTGGGCAACTTTACGCTTCGGAATATATTAT
AGTCCTGGAGTATATTACATGGTACTTTACTTGTGGAACTAATTGACTTGACTTTACCGAAGTTAGATGGTCGTGCTTGG
TGGACATGTCACATCTCATATGTTCCAGCAGCCAGGTTGAGATGCATTGAGTTGGAACGCTGAAAATATGTCTCTGTGGT
ATATGTAAACCTCCGGGAGACCCTCACTTCAGGGCTTAGCGCCTCTACGGCTTCCTTGGGCGAACGGACAGACGCAAACG
CTAACAACGGCTGATCATTACGGCAGAGGGCGGCGGGGCATCGGCCGCAAGCCCTAGCGGCGGGGCGTCTCGGGCTAAAA
GTCAGGTAGCTGTAGGGCAGGTTATTGCCATGGGTCGCAAAAGTCTGGGACAGCATGATTTGGGACCAACGAGGAGCGTA
AATCAGACGCCTTTCGGTCGTTGAGGTGAGCTCTCGCCAGACGCCTTTCGGTCGTTGAGGTGAGCTCTCGCCAAGCACTG
AAAGGTGCTTGTGCATGAGTTCCAGTGCTCTGGGACTGAAAAGCGAGGGACCAACTTGTTCGAACATGCCAATGAATGCC
ACCCACTACTGTAGCGGCATTCAAGGTCGACTCGTGCTAGGGTAGGTGGGAATCCTACCCCTGGCAGTTTGGTGGAGGTG
CATGACGCAAGACGACCGTGAGCTCTGCTGAGTTCGTTTACGCACAGAAGGCAGATGTGCTGGAGGTAAGCTAGGTAAAC
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GGAGAGGATCACTTTATTGGCGACTGGTCGTTCGAAGGCTGCTACACCGAAGGCGATGGCGTGCGGGCCTTGAACGGTCG
CTTCTACGCCGACGACGAGTTGACGCTTGAAAAGTGTGCCGCCTTCTGCGAGGGTTTCGTTTACTTCGGTGCCGAATACG
GGCGCGAGTGTGGTGCGGCGACGTTCATGGCACTGGCAGCGTGTTGGCGTCCAATCAAGGCGACTGCAACTTTCCTTGTG
GCGGCGACGGCTCACAGTTTTGTGGTGCGGGCAACAGGCTTCAGATGTATCGCTTCGGTGGTGCGGACGCGCCTTCGGGG
GCTGTGAGCAGCTCTCTTGTGGTGCCCACGTCTGCGGTCGTCAGTTCTACTGTTGCTCTTGTTAGTTCAACTGCCGCAGA
AGTCAGCTCTGCCGTTGCGGAGGTTAGCTCTCAGATAGCCATCGAGGCTAGCACTGCCCCTGCTTCCAGCTCCGTCGTTG
AAGAATCCAGCACATCAGTCGTCGAGACCAGCTCGACGGTTGCTGTGGAGTCAAGCGCTGTTCAGTCCCAGTTCTGCCCA
ACCCAGCTCCGCAGAGGCCAGCTCGGTCATCGAGATTATGTCTTCCGCCGTTGCATCTTCGAGCTTTGTTCCTAGCTCTG
GGATCCCATCATCCACGACCCCGACCTCGACGCCGTCACCCTCGGTCTATCCAGGCAACGATCTCTGGAACTACACGGGC
TGCTACTCTGAGCCCTCCCCTGGACGCCTTCTCCCCAGTCAGCTCCTGAACGACGGCGATGAGATGGACATTGAGCTCTG
TCTCGACGTCTGCGCTGGGTACAACTATGCAGGTGTCGAGTATGGTCGTGAGTGCTGGTGCGGTGACCGCCTCAACGCCG
AGGGTGACGTCCCCAGCGAGGGCACAGCAGTGCCTGGCGAGCTGGTGGACGACGATGAGTGTGCCTTCCTGTGCCCTGGC
AACAGGCTGAATTACTGCGGTGCAGGTGTCAGAATGAGCGTGTATATCCTGAGAGAGCGCGAGGAGGCGCTGGCTGAGGC
GTCC**TGA**GACAGATAAAGTGGCATGATAGATGGTAAATTGCATGATTGGTTCAGCCCGGCAGGCTCTGCCTTCCAATAGG
CACTTTTTGGTATTTTCTTGTGATAATGAACCTCACGCTGGGGCTTCGTATATAAATTATAAGCAACATATTGCTATTGC
TTGCCGACTCAACTCGCGTGATGGGTAACTTGAAGTCCGCCCACCAGCGGGCTTTTGTAAGGCTCCCATCTCGATACTGA
TAGAACATTCATGGAGTTCAAACTTCCCCTGCTTCAGCTGCTCGTACCTTGAAAACAGGTGTGACGATACCCGTCTCTAC
TCCCCACGTGTTCTTCCGCTTCAGTCGATGATATTTGAGCAGCTCCTCGCGGCCGGGAGACATCTCGATTCCCAGCTGCA
AGAGAATCTCGACGACAGCCGTGTATAAGATCC

Aminoacid sequence: 1534 aa

MVSFRWLMLAAVAGLANGLADTDTITWGGDNSRAGYQTNHNMDPAVVRSSQFDRIFQTTLPGRYGGRAEQIFSQPLVYTP
DDKQYVYLATTQNNVYKLDAQTGEILASRNLHIPFLSTDLDGCYDIQPHVGVTGTGVIDPDTGTYYLLAKTYENQELVDV
AQGRPAGRYYLHALDVNDLSERPNFPVGLEGTVARNNPDRSFNGGIHLQRPALLHVGQHIYAGLGSHCVKFNFTGWVMGW
DKTTGEQVERFATQGEGVPQNTEGGGLWMAGGGLASDDQGSIFFATGNGYAGQLAEIPVNGRNPPTSLEEAAVHMTIQED
GSLDLVDFFIPWDKRAMDGDDKDLGSSPLQILPSEFSCGSIRRIGVVTGKNKKTYFINLDDMGGYRNGEDRFDNIIQTYE
HENSVYAGAGVYPGEGGYIYINVVQYPTIVFRFSCANGVPSFNKEAETPESNGYTLGVSHGTVTSLNGEPGTAMLWTTDV

QNPPGQLRIYDAVPRDGELALLRKWEIAGVTKFSRAVFGDGIMYLGTTTGLFYGFGAPINRPIECTSPLEFGAVSLEASA
ETRTLTCTALINTVVNDISLREVTDFSISGLPTLPLTLAVGATFTIEAVFAPTDLGLLSTDVNIETENSVAGYRTTTSAR
LTGTGETDNPRLSVSPREIEFDNVITAGAAPPANNVVLSNQGNSVLTVNEIRYSETINSTLQTWDPASGALVIGPFTIRN
IPSTIDANSGATVSVSLSPANGGTFSGHVRFITDGGNTDVTMTAHVGAAPVLLVEFERPDGEGWTTYQEGTAFSFGEVTQ
NNVRNLRMRITNTAPAGGVRLSLTVSKPPHGGSGIIRANNAVDLGEGTNLGPGLSETAVLYCAVPKRQWNMEPYQGEATW
SLNTNDPKVAYQNIQFECTAVSEQSAPLLENGLSQYQYVGCFRENTPGRQLANQLYGNDENTIAMCVEACAAGNYVFCGT
QYHRECWAGPTIPRERVADVNCNFDCDGDLNQICGGNGIATGPGGAYISLFADTLRFDGNETNIPEPEEPVEPTDPIVNP
GVDGYISIGCYTEAPGSRALPFFFATEDQTVAMCVDACSLRDYEYAGVQYGGECWCANAITTGAVPAPDAECNMACNDNA
AEYCGGGSRLNVYQRQSGGSFPSASVSLNGTIPAPTSSLIPASSVPVSSAPIISSIVTSAAPPVPTPGEDHFIGDWSFEG
CYTEGDGVRALNGRFYADDELTLEKCAAFCEGFVYFGAEYGRECWCGDVHGTGSVLASNQGDCNFPCGGDGSQFCGAGNR
LQMYRFGGADAPSGAVSSSLVVPTSAVVSSTVALVSSTAAEVSSAVAEVSSQIAIEASTAPASSSVVEESTSVVETSSTV
AVESSAVQSSSAQPSSAEASSVIEIMSSAVASSSFVPSSGIPSSTTPTSTPSPSVYPGNDLWNYTGCYSEPSPGRLLPSQ
LLNDGDEMDIELCLDVCAGYNYAGVEYGRECWCGDRLNAEGDVPSEGTAVPGELVDDDECAFLCPGNRLNYCGAGVRMSV
YILREREEALAEAS

2). Vl_2.1 gene (Isochorismate hydrolase)

Coding sequence (including introns): 629 nt

GACTCCTGTTCATCAAATAACACACAATCAAGGATTGCCTGTGCCAGATCAGCAACGTTCACC**ATG**TCCTCATTCCGCTC
CATGCTCGGCGTGCCGCCGTCCACGGCCTCCACCAGGACAGTGTCCTCGTCATCATCGACGCCCAGGGCGAATACGCCGA
GGGCAAGCTCAAGATTTCCAACATTGAGGCGTCGCGCCCCAACATCTCTTCCCTGCTGGAGAAGTACCGCGCCGCCAACG
CGCCCATCGTCCACGTCGTCCACGAGACGCCCGCCGGCGCCCCTCTCTTCACGCAGGGCACGAAGCTCGCTGAGATCTTC
GACGAGCTCACGCCCGAGGAAGGCGAGGCTGTCGTGACGAAGCACCACCCCGGTTCATTCGCCGACACCAACCTTCAGGA
GATCCTGGAGAAGTCCGGCAAGAAGAAGATTGTGCTCGTCGGGTACATG**GTGAGTCTTATCAACGAGATGGAAAGCTCGT**
CGTGGAACGTTGCTGACACATTGCAGGCTCACGTCTGCGTCTCGACGACGGCCAGGCAGGGCGCGCAGAGGGGGTGGGAT
GTCATCGTTGCCGAGGACGCTGTCGGTGACAGGGACATTCCCGGCGTGGATGCTGCGCAGCTTGTGAAGGTTGCTCTGGC
TGAGATTGCTGATGTCTTTGGCACTCTTGTCTCGAGCAAGGATATCAAC**TAG**GACGTCACGTGAATGCTACCGTTATGAT
CAAT

Aminoacid sequence: 190 aa

MSSFRSMLGVPPSTASTQDSVLVIIDAQGEYAEGKLKISNIEASRPNISSLLEKYRAANAPIVHVVHETPAGAPLFTQGT
KLAEIFDELTPEEGEAVVTKHHPGSFADTNLQEILEKSGKKKIVLVGYMVHVCVSTTARQGAQRGWDVIVAEDAVGDRDI
PGVDAAQLVKVALAEIADVFGTLVSSKDIN

3). Vl_11.1 gene (Peroxidase/Catalase)

Coding sequence (including introns): 2289 nt

TCGCCATACCTCGTCCTGCCACGCCGACCAGGTATCACCGAGTACACCCCCAATCACACTTTATCAACCGCAGACACAAC
CACACCGACTCACCCAAC**ATG**GCCGAGTCGGAGAGCAAGTGCCCCGTCCACCAACTCAACAACGTCGGCGGCGGCGGCAC
CCGCAACCGTGACTGGTGGCCCAATGCTCTGAAGCTCAACATTCTCCGTCAGCACACAGATGTCACGAACCCCCTCGGCA
ACGACTTTGACTACGCTGCTGCCTTCAACAGCCTCGACTACAATGCCGTCAAGAAGGACCTCAAGGACCTCATGACCGAC
TCTCAGGACTGGTGGCCCGCCGACTTTGGTCACTACGGAGGTCTCTTCGTTCGCATGGCGTGGCACAGCGCCGGTACCTA
CCGCGTCTTCGACGGCCGCGGTGGTGGCGGTCAGGGCCAGCAGCGTTTTGCTCCCCTGAACAGCTGGCCCGACAATGTCT
CGCTCGACAAGGCTCGTCGCCTGTTGTGGCCCATCAAGCAGAAGTACGGCAACAAGATCTCCTGGGCTGATCTGCTGCTC
CTCACCGGCAACGTCGCCCTCGAGTCGATGGACTTCAAGACCTTTGGCTTCGCCGGTGGTCGGGCCGACGTCTGGGAGGC
CGACGAGTCGGTCTACTGGGGTGGCGAGACCACTTGGCTCGGCAACGACGTCCGCTACTCTGGTGGCAACAAGGGCGACA
AGGGTCCGGGCAGCCTCGTCACCGACGAGGGTCATGATAAGAGCACCCACACCCGTGGCTTGCAGAAGCCTCTCGGTGCT
GCCCATATGGGTCTGATCTACGTCAACCCCGAGGGCCCCGATGGCAACCCCGACCCTGTTGCTGCTGCCCACGACATCCG
CACCACCTTTGGCCGCATGGCCATGAACGACGAGGAGACCGTCGCTCTTATTGCTGGTGGCCACTCCTTCGGCAAGACCC
ACGGCGCCGGCCCCAACGACAAGATCGGTGCCGAGCCCGAAGGTGCCCCTCTTGAGGCTCAGGGTTTCGGCTGGCAGAAC
GGATACAAGTCTGGCAAGGGTCCCGACACCATCACCAGCGGTCTCGAGGTCACCTGGACCGCGACCCCGACCAAGTGGAG
TAACAAGTACTTCGAGTACCTCTTCAAGTACGAGTGGGAGCTCACCAAGAGCCCTGCTGGCGCCAACCAGTGGGTGGCCA
AGACCGACGACGAGATCATCCCCGATGCCTACGACTCGTCCAAGAAGCACCGCCCTACAATGCTCACCACCGATCTGTCC
ATGCGCTTCGATCCCGAGTACGAGAAGATCTCTCGCCGTTTCCTCGAGAACCCAGACCAGTTTGCCGATGTTTTCGCTCG
CGCCTGGTTCAAGCTCCTTCACCGTGACCTTGGCCCCAAGGCTCGCTACCTCGGCCCTGAAATCCCTGCCGAAGACCTCC
TGTGGCAGGACCCCATCCCCGCTGTTGACCACCCTCTGATCGACGAGAGCGACATTGCCGCTCTCAAGAAGGAGATCCTC
TCCTCCGGACCTGAGCCCTCGCAGTTCATCTCCGTTGCCTGGGGAGCGTCTTCTTCCTTCCGTGGCAGCGACAAGCGCGG
TGGTGCCAACGGCGCCCGTATCCGCCTCGCCCCCCAGAAGGACTGGGAGGTCAACAACCCCGCCCAGCTGGCCAAGGTTC
TGCAGGCCCTTGAGGGTGTGCAGAAGAGCTTCAACGATGCTCAGCAGGGTGGCAAGAAGGTCTCTCTCGCCGACTTGATC
GTCCTGGCCGGTAACGCCGCTGTTGGGAAGGCCGCGTCGGCCGCCGGCCACAGCGTCACCGTCCCCTTCACTCCTGGCCG

TGGCGACGCCACTCACGAGCAGACCGACATTGAGTCCGTCAGCCACCTCGAGCCTTTTGCCGACGGCTTCCGCAACTACG
GCCACTCGACCGACCGCGTCAAGACGGAGCAGTTCCTTGTTGACCGTGCTCACCTTCTGACGCTGTCGGCGCCTGAGCTG
ACCGTCCTCGTTGGCGGCCTCCGCGTTCTGAACGCCAACTACGACGGATCCCAGCATGGTGTCCTGACCAAGCGCCCCGG
CCAGCTGTCCAACGACTTCTTTGTCAACCTGCTCGACATCAGCACGGCATGGAAGGCTACCGGCAGCGACGAGGAGCTCT
TTGAGGGTAGCGACCGCAAGACTGGCGACAAGAGGTGGACTGCCACGCGTGCGGATCTCGTTTTCGGATCCCACGCCGAG
CTGCGCGCCCTTGCCGAAGTGTATGGCAGCGCCGACGGCGAGAAGAAGTTTGTCAACGACTTTGTCGCGGCGTGGACCAA
GGTCTCGAACCTCGACCGTTTCGATCTCAAGAAGGCATCTGCCGTCAAGACGTCGAGCCGCCTG**TAA**ACGCCTCTAAGAA
GTCAATGACGAAATGTTTGAGTGTTGATAGTAATGAAACCACACTTCATCTCCACTTCACTGGACCAGTATTC

Aminoacid sequence: 762 aa

MAESESKCPVHQLNNVGGGGTRNRDWWPNALKLNILRQHTDVTNPLGNDFDYAAAFNSLDYNAVKKDLKDLMTDSQDWWP
ADFGHYGGLFVRMAWHSAGTYRVFDGRGGGGQGQQRFAPLNSWPDNVSLDKARRLLWPIKQKYGNKISWADLLLLTGNVA
LESMDFKTFGFAGGRADVWEADESVYWGGETTWLGNDVRYSGGNKGDKGPGSLVTDEGHDKSTHTRGLQKPLGAAHMGLI
YVNPEGPDGNPDPVAAAHDIRTTFGRMAMNDEETVALIAGGHSFGKTHGAGPNDKIGAEPEGAPLEAQGFGWQNGYKSGK
GPDTITSGLEVTWTATPTKWSNKYFEYLFKYEWELTKSPAGANQWVAKTDDEIIPDAYDSSKKHRPTMLTTDLSMRFDPE
YEKISRRFLENPDQFADVFARAWFKLLHRDLGPKARYLGPEIPAEDLLWQDPIPAVDHPLIDESDIAALKKEILSSGPEP
SQFISVAWGASSSFRGSDKRGGANGARIRLAPQKDWEVNNPAQLAKVLQALEGVQKSFNDAQQGGKKVSLADLIVLAGNA
AVGKAASAAGHSVTVPFTPGRGDATHEQTDIESVSHLEPFADGFRNYGHSTDRVKTEQFLVDRAHLLTLSAPELTVLVGG
LRVLNANYDGSQHGVLTKRPGQLSNDFFVNLLDISTAWKATGSDEELFEGSDRKTGDKRWTATRADLVFGSHAELRALAE
VYGSADGEKKFVNDFVAAWTKVSNLDRFDLKKASAVKTSSRL

4). Vl_12.1 (Vl_10.2) gene (Zinc-finger transcription factor ace1)

Coding sequence (including introns): 2328 nt

AGAGCTGGCCATCGCCCGCCAGTACCCACTCCCCCTTCCGACACTTTCGTC**ATG**TCGTGCCAGAACCCTCGCCGCAGGTC
CCCTGTGACCCGCGTCGGCGACGCTTCCAGCAATGGCCTCACCAGCCTTAAGACCAACATGACCCTGCGCAAGGGGGCCA
CCTTCCACTCGCCCACCTCTCTCGACTCTTCATCCATCGACGCCTTCATCCCCCCAGCTCTTGGTCGTATCTCAGACCAA
TCTTGAAGACGTGTCGGCGCTCACGTCCGCCGCATGGAGATGATCGTCAGCGGCATCGAGACATCACTCAATCTGAATGA
TACCCCAAGGCCGGCCTCCAAGCCTTCGCGTGACGAGTGCCTGCCTCGCACAAACGGCTTCCTCGGCCGCCCTACTGTCG
ACCCCGCCATGGCAAAAGACACCAAGACCAGCGGGGAGCGCCGCGTTTTGCGCCCAAGACATCGTCGCTCATCGGAGCAG
CACGCTTCGGACAGCGGTCTCGGCACCTCTCTGGCCTCTTCCGTCGAGAAGCAAGCCCCCAGCATCACCTCCAAGACCAG
CAAGGCATCCGCCATTACACGCTCTGCCGCCGCTCCTTCCAACACCATGACCAAGGTCTCTGGCCTGAGCTCCAAGGCGG
TCAGCCGTGTTCACGAACACGTTCTTCGCCCCCTTCGTGCCAAGCCTGAGTTGAAGGACTTCGAGCCCATCGTCCTGGAC
ATTCCCAGGCGAATCCGTGACAAGGAAATCATCTGCCTCAGGGATCTTGAGAAGACTTTGATCTTCATGGCACCG**GTAAG**
TCAACTCTTGTATCAACGCAGCGTTTGGGGAGATACTTATCGGTCGTTGATGAAGGAGAGGGCCAAGACCGCCGCCTTGT
ACCTCGATTTCTGCCTGACGTCCATTCGATGCATTCAAGCCACCGTCGAATACCTCAGCGACCGCGAACAAATTAGACCG
GCCGACCGACCATACACTAACGGATACTTCATTGATCTCGTCGAACAGATTCGCCAATACGCCGGACAACTGGCCACTGC
CAAGGAAGCCGGAGTCGAGGGGCGTGAGATGGACGTCGACCCCACCGACGAGGTTAAGCTGTTCGGTGGCATCTCGCAGA
ACGGCCGCCCCGCCGAGCTTGTCCGCGTCAGAAAGGACGGTCAAGCCATCTCCATGGCCACTGGCCTCCCCGTTGACATG
GATGAGGACGGCAAGGATTTCCCCAGACTGAAGCGCTCCCTGAGCCAGCAGCTGGCAGACGACGAGGAGATCATGCGGTC
CATGGCTCGCAGGAAGAAGAACGCTGCGCCGGAGGAGCTCGCGCCCAAGAAATGCCGCGAGCCTGGCTGCAACAAGGAGT
TCAAGCGTCCCTGTGACCTGACCAAGCACGAGAAGACTCACTCTCGTCCCTGGAAGTGCCCTGTCAAGACGTGCAAGTAC
CACGAGTACGGCTGGCCCACCGAGAAGGGG*ATG*GACCGCCATCACAACGACAAGCACTCCTCAGCGCCCCCCATGCACGA
GTGCCTGTTTAAGCCTTGCCCTTACAAGTCGAAGCGCGAGTCAAGCTGCAAGCAGCACATGGAGAAGGCCCACGGATGGC
AGTACGTCCGCACCAAGACCAACGGCGGCAAGAAGGCGCCCAGCGTTGCTGGAAGCTCGGCACAGCCGACCCCTCAGCTT
GGCAACATGGCAACGCCCTCGAGCAGCCACAGTATTGCTACGCCGCCCGAGGAGAGCACCAGCCTCTTCCCGCCTTTTAA
CCACGATGACTTCCCTCACTACGTCCCGGCCGAGGAGTTTGCTGACACCTGCCTCGGGCCCATGGGACAGCCGCCCATGA
CGCTCGAGGGTATCGACTTTAACGACCTTGGCGTGTCTCCCACTGATTACAACACCCCTTCTACCGACACATCCTACCCA
TACACCTCTTACCAGGATGGACCCGAGTTTGTCATCAACAACGATGACATTTACGGCGCCCGTGTCCAGATCCCGACACC
GGCGTGGCCCGAAAAGATGATGGCTGGCATGCAGAACTACGCCCCAGTGTCTGCATGCCAACCTCAGATGATGCCCGAGC
CGCTCGCCCCACACATCTCCCCGATAGGTCAGGGGAACGCCATGCTCTTCACGCCCAACTCGTTGGCCGAGGTTGACGAA
GGCTTTGATGATTTCGGCGGCTGTGGTGATGATTTCACCTTGTTCCCCGTCAACGGGCTCGACAAGGACGCACAATTCCA
GACTCTGTTCGGCAGCGAGATGCCCAGCAGCGGCCTCGGCTTGTCTCAGGGCGCCTCCCAGGACTTCTTTGGGAACGGCA
TGGACTGGTCCAGCATGGAATACCACACCTACTCCCAGCAGCCCCAGCACCAGCAG**TAG**AGTGATGTTGTGAGACTTCAC
TTACTGGTTGCTTTGGGATGGCCTGTTTCTTTTACGT

Amino acid sequence: 754 aa

MSCQNPRRRSPVTRVGDASSNGLTSLKTNMTLRKGATFHSPTSLDSSSIDAFIPPALGRISDQSRRVGAHVRRMEMIVSG
IETSLNLNDTPRPASKPSRDECLPRTNGFLGRPTVDPAMAKDTKTSGERRVLRPRHRRSSEQHASDSGLGTSLASSVEKQ
APSITSKTSKASAITRSAAAPSNTMTKVSGLSSKAVSRVHEHVLRPLRAKPELKDFEPIVLDIPRRIRDKEIICLRDLEK
TLIFMAPERAKTAALYLDFCLTSIRCIQATVEYLSDREQIRPADRPYTNGYFIDLVEQIRQYAGQLATAKEAGVEGREMD

VDPTDEVKLFGGISQNGRPAELVRVRKDGQAISMATGLPVDMDEDGKDFPRLKRSLSQQLADDEEIMRSMARRKKNAAPE
ELAPKKCREPGCNKEFKRPCDLTKHEKTHSRPWKCPVKTCKYHEYGWPTEKGMDRHHNDKHSSAPPMHECLFKPCPYKSK
RESSCKQHMEKAHGWQYVRTKTNGGKKAPSVAGSSAQPTPQLGNMATPSSSHSIATPPEESTSLFPPFNHDDFPHYVPAE
EFADTCLGPMGQPPMTLEGIDFNDLGVSPTDYNTPSTDTSYPYTSYQDGPEFVINNDDIYGARVQIPTPAWPEKMMAGMQ
NYAPVSACQPQMMPEPLAPHISPIGQGNAMLFTPNSLAEVDEGFDDFGGCGDDFTLFPVNGLDKDAQFQTLFGSEMPSSG
LGLSQGASQDFFGNGMDWSSMEYHTYSQQPQHQQ

5). Vl_24.1 gene (Hypothetical protein)

Coding sequence (including *introns*):1402 nt (incomplete)

CGATTCGTCCTGGCCCCTCCATTTCCATTTCATACCCTTCCGACACGACTGGATAGACCCTTCTTCCAGGAC*ATG*ATCGC
AGCCATCTCCG**ATG**GCTGCCTCACAGCTCGACCTTCCCCTTCCCGGTGCCTCCTTCTTCGATATTCACC*ATGATG*GAACG
ACCACCCGCAGTCCTGTCCCGTTGCCCG*ATG*GGGGCTGCAACTTTGTCGACCTGACGCCCGGTGCCAACGGAGCCAAGTG
TGG*ATG*TCGCCGCTTCTGGAGTCGCACTGTTTCCGGGAGAGGATTCGCAGATACCGTAGGATCGGACCACACGGCCTGGT
GC*ATG*TGCACTCACCACGCTTGTTACCACGACCACACCCGCGACGCAGAAGCCGCCACCCCCGTAATAGGATTCGTTCCC
GGACAGGAGAATACAAAACTCAAGGGACATCGTGGGCCCCTAAGCCCCGTCGTTCAGG*ATG*CTTCGTTCCGCCTGCCTTC
TGGATTCTCTACTTCATTGGATCTT*ATG*AATCTGGACGCCGCG*ATG*CTGCTGCCC*ATG*AGCAAGCCCGAGG*ATG*CCAGGA
ACCCGCGCGCTGGACCCCTCAGCCAGCAGCTCGAATCTACACTACAGGATACGTTAAGTTGGGGCGAGTTTGTCCAGTCT
CAGTCAGCCAACACGACCACACTGCCCCCGATCCCCCCTCA*ATG*CCTT*ATG*CCCTCTCAACCCAGTTCCACCACATCATC
TAGCCAAGCTCGTTACTTGCGCCCTTTTGCCGGGAAAGGATTAAACACGCTCAGTGGTGTGCACCAGCCCGACCCTCGTT
CTCCTCGCCAGGAAAAGCCGCACGACCTGGAGCCC*ATG*GACATTCAGCCCACAAGATCTGGA*ATG*GAA**GTCGTTACCGCG**
GGTGCCGCATCTGCTGTTGGAA*ATG*GTTCAGACACTCCGAAAGCTCGACGATCTCCTGTCCTTACGGTCCAGGCTCCCGC
AGCAATATTTGGGTCACCTTCACGTGATACGTTTCGACACATGTCAGACACAGTCCAAGGACACGAGCAACGGATCGATC
GTTTGGAGAATGTTTCCTTCTCCGCAGGAGGTAATGACGAATGCCATGAAAAGCATGATGCCATGGATCTTCGCGTTACG
GACCTAGAAGGCCGGGTAGAAGAGGTTGAAAAACTCATGAATGATAATACCAGCCACGGAACTGCCCGCCATCTCCGCCA
ACCTGCCATCGATGAGTCGATGAGCAGCGCCGTCTCTGTTTCTACCAGCCCAACTGCTCGTATCTCCGACGCGAGCGAGG
TTTACAACCACATACAGTCCCTTCAGTCTCAACTTCGGCACCTCCAGTCTTTTGTGCCATCTTGTATGCATGCCTGGGAA
GTGGAGGTGGTGTTTCTCCCGTTCGCTCTCCGGCGCATCTGGCAAGAAAGGCACGACTTCAAGCTGGAACCAACATCTAG
CATGGACGAATGGACTCAGCTCCCCAACACCAACAGTACTGCCAGGTCAGGCA

Amino acid sequence: 421 aa (incomplete)

MAASQLDLPLPGASFFDIHHDGTTTRSPVPLPDGGCNFVDLTPGANGAKCGCRRFWSRTVSGRGFADTVGSDHTAWCMCT
HHACYHDHTRDAEAATPVIGFVPGQENTKLKGHRGPLSPVVQDASFRLPSGFSTSLDLMNLDAAMLLPMSKPEDARNPRA
GPLSQQLESTLQDTLSWGEFVQSQSANTTTLPPIPPQCLMPSQPSSTTSSSQARYLRPFAGKGLNTLSGVHQPDPRSPRQ
EKPHDLEPMDIQPTRSGMEVVTAGAASAVGNGSDTPKARRSPVLTVQAPAAIFGSPSRDTFRHMSDTVQGHEQRIDRLEN
VSFSAGGNDECHEKHDAMDLRVTDLEGRVEEVEKLMNDNTSHGTARHLRQPAIDESMSSAVSVSTSPTARISDASEVYNH
IQSLQSQLRHLQSFVPSCMHA

6). Vl_25.8 gene (IBR finger domain- containing protein)

TACAATTTACGAGATGGCATGGCGCGGCTGCACCAGTCCAAGATCAATGTTTTGCGCGACCGGCAGGCCAAGCGCATGGA
AGAGCTGCTCGAACGACAGGCAAACGAGATGGAGAAGCTGGCGGACCGGAAGGAACAGGAACTCGAGGCTCTCGCAAGCG
ACTTTGCTCAAGAAGAAGACGAGCTCGCCCAGGTCTTCAGTGAACGCAAGCGCATGATGCTTCGTGTTTGGTCTCTGCGC
CTCGAGGTTCTGAGAAACGAGCTCGAAGACAAAGACGGCCTTGCCTATGTTGCTGTTGGGTTTCCGCCTTGGAACGGCGA
CTACAGCATTCGGGACGACGCAGCGCACCTAGTGGCAATCGCCGCTTCTTGTTCTTGATCGGCGAACTCTTTCACTCGCC
GAACTGCAAACGGAGTCTTTCTTTGGACACCAATCCTTACA*ATG*CTC*ATG*AAAAAGGATCACTTGGACATAACGTACCTC
GGCTGAGGACCGACGGAGT**TAA**TCATTTCTGGGGTCTGGAGTCCTGGAGGAAGGGGCTGAATCTTATACCACGG*ATG*AGC
GCCGA**GCGGCCGC**CACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATC*ATG*
GTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAG
CCTGGGGTGCCTA*ATG*AGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCG
TGCCAGCTGCATTA*ATG*AATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCAC
TGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGG

Amino acid sequence: 167 aa (incomplete)

YNLRDGMARLHQSKINVLRDRQAKRMEELLERQANEMEKLADRKEQELEALASDFAQEEDELAQVFSERKRMMLRVWSLR
LEVLRNELEDKDGLAYVAVGFPPWNGDYSIRDDAAHLVAIAASCS*RRTLSLAELQTESFFGHQSLQCS*KRITWT*RTS
AEDRRS*

7). Vl_33.3 gene (DOA4-independent degradation protein)

Coding sequence (including introns): 910 nt

TCCTAGTTCCATCGCCAGGCTTCAGGCATACACAACAACCCATCTCGGCCGCGCCTCGTGGATACTCGACCTTTGTATTC
GTGTTGCCAGTCACTTCACCGCCACG**ATG**AATGTAGGACACTCGGCCCGCGTCGCGGACCCTCAAAGGCACAGAGTCGGC
TTGTTGCTAACGTGGGCTGTATAGATCCTGGAATACATGTTCGGGAAGCGTATGACGCCAGCGGAGCGCCTGCGCAAGAA
CCAACGCATGCTCGACAAGGCGATTCGCGAGCTCGACCAAATCCGAGTCAAGCTGGAGAAGCAGGAGAAGACGCTGGTTA
CGCAGATTAAACAGAGTGCGCAAAAGGGACAAATGGGCGCTTGCAAGATCCAGGCCAAGGATCTCGTGCGTACGCGAAGG
TGGGTTCTCTCGGTATCAAGGCCAGACAGGATTGGGCTAACATTATTCAGATATATTGAGAAGTTCTATTCGATGCGCAG
CCAGCTGCAAAAGATCTCTCTCCGCCTACAGGCATGATAACCCCGCCAATCTACCGTGCGCGTGTCAGCTAACCTACGTA
TCAGACCTACCGCACAAATGAGCAGATGATGCAGGCGATGAAGGGAGCTACGACTGCTCTTGGCAGCATGAACCGTTCGA
TGAACTTGCCGGCACTCCAACGAATTGCCATGGAGTTTGAGCGGGAGAACGATGTCATGGAGCAGAGGCAAGAGATGATG
GACGATGCGATCGATGATGCCATGGATGTGGGTGAGGAGGAGGAGGGGGAAGAGGTGGTTGAGCAAGTGTTGGAGGAGAT
TGGCATTGATCTCAACTCCGCGGTACGTCAACTTGATCGAATGGAGACTCACTACGAAACTGACCAGCGTGGCAGCTCGG
CGAGACCCCCACGGGCGTGCAGTCAAGCGCAGTACCGGAACAACGAGTTGCGCAAGCCATTGGCGGCGAAGGCGGCATGG
GCGGAGGCGGGGCGGACGATGATCTACAGGCACGACTTGACAGTCTCAGGCGG**TGA**GGGCTCTCTATGCATTGGTGGAGC
TAGCCAGCAGAGAGTGAACGGTAGTCATGAACTTGCGGGCGGTGTTTTGGAGTACGAGGATTGTGCCAGGTTGCATTTCA
GCTGGCGTTTTGCCTGTTGTGCTTGCGCCGAACGAACTGATATTCAGTTGTCTTTTTGCCTCGCATCGTCGGGAATATTT
AAGATCGCTTCCGGAATAGTGGTTGCGCAGTCTTCAGTGATACCACACCGCGTCTTGAGTCGCCGGGTTTACCGCTGGAC
GGCACCCATGATGTGAACTGTGTCGGCCTTGCCTGGAACATCTTGAGGGGTGTCGCGGGGGGGAGCAGGAGACCACACAG
GGCTGAGATTTTTTCACATATTTCGGCCAGGGACAGACGGCCGAGGATAACGCCTGAATTGCGCCTTGAG

Amino acid sequence: 201 aa

MNILEYMFGKRMTPAERLRKNQRMLDKAIRELDQIRVKLEKQEKTLVTQIKQSAQKGQMGACKIQAKDLTYRTNEQMMQA
MKGATTALGSMNRSMNLPALQRIAMEFERENDVMEQRQEMMDDAIDDAMDVGEEEEGEEVVEQVLEEIGIDLNSALGETP
TGVQSSAVPEQRVAQAIGGEGGMGGGGADDDLQARLDSLRR

8). Vl_34.2 gene (NADH:ubiquinone oxidoreductase 14kD subunit)

Coding sequence (including introns): 684 nt

AGCAAGCTCAACCTCCATCATCGACCACTACTACAATCATATCACGACCTCCCCTCCACGCCAATTGCCTGCTCCGACTG
CGCTGCTTCGAACGCGGCGACCTTGGCCCCCGAAGACCGACCGAATCCCGAGAGACGCGCGACAGCGACATACCGCCATC
ATGCCCCAGGACATGCCGCCCGTGGGCGGCTATAATGCCGTTCAATACAAGGTGGGTTTACCGATCGATCCCGTCGTGGC
CGACAGCTAGGCCAATTGGGCTCAATACAGTGCATCAATGCCTCTTCAGCACCGATAAACTTCAACGAGACTCCGCATCA
GGCCCCAGGCGCCCATACAGACTTCTCCCCGAGCAACGCCGGCTAACGCTTCTCCTAGCGTAACCTCCCCGCCCGCGGCT
TCCGCCCTGGAATCCTCCTTCTCGGAATGGGCGCCGTCATGGGCTACGGCTGGTACAAACTGATCAAGGGCATCCGCGAG
GCCAAGTACGTCCTCGTTCCCCCCTCCCGCTTTGTCTCGTCTTGCTTCGGCTGCGTAGCTTGGAGCCGTTGACCAATCCT
CCCTCGAACAAAGAACTGACCACCATGCCAGCGAGCTTGCCCGTGAGAAGATGTGGGCGCGCATCCACCTCATTCCTCTC
CTCCAGGCCGAGGAGGACCGCGACCAGATCCGCCGATGGTACGCCGATCAGGCGCGCGAGAAGGAGCTGCTGGGCGAGAA
CACGCGTGTGTACCACACGGACAGGTAGGACTGACTGGACGGCTTGCTGTAGAGGGCCGTTTGCTAACGAGGTGCAGGGT
TTGTCCGACCCACGTTCGCTGTTGCGCCGGAGAAGACGAAA**TAG**GCGGGAGGCCGGGTCGGGACAGGAGACTTGGGGGAA
CAGGAAAGAAGAAGAGCCATACCGGGCACGAAGCCGTTGAAGAAG

Amino acid sequence: 118 aa

MPQDMPPVGGYNAVQYKRNLPARGFRPGILLLGMGAVMGYGWYKLIKGIREANELAREKMWARIHLIPLLQAEEDRDQIR
RWYADQAREKELLGENTRVYHTDRFVRPTFAVAPEKTK

9). Vl_NEP1 *(Necrosis- and ethylene-inducing protein)*

Coding sequence (including introns): 763 nt

GGATCCTCACAAGCTCGGGGCACAGGATTCAGACAGTGCTTGAAGCTGAGCTGTTCTCAGTTCTCCCTTAGGACACCACA
ACAAATGACCGAACGAACCGGCCGGATGATCAGACCAAAACATGGTGCCGTGTTCATCAGCCCTGAATCGCAACCCCACA
AGCGCACCAAGTAGATCCTGGTACAGATGCGCGCCTACCGTTGAGTCCCGGACATGTTCTGCTCCCATTGTGGCCATAGA
TCCGCACGCCCTATCACCACCGCCTTTCTATCAGCCCCCGCATCACCAGAATGTGCGACAGCCTGGCTTTATTAGTCGTC
GGACATGGCTACGCCTTCTTTCTCCGGTATAGCCTCGGTCCGTGCCGACTCAAATCAGTCTCAGGGGTTCGAATCGGGCT
TACTCTAATGTGTTGGGGGTCCGGCCTTGCTATCCATGACTCGACATCAACCTCTATGGGCGTGCCACCAGTGCACCCGA
ATGGCCCGGGTGGAACAGATGAACCAAAGTATGATAGATGAACCGGCTCCTCTGCCCTTGCCCACCTGCCCAGTCACGAC
TCACTCAGCACCGTCCACGGTCACCGTCCTTGCGATCTCGCATGAATGAGTGCCGAGACATGGGTCACTGCCTACACAGC
ACTTCGCCAAGGCGAGCTCACTGGGGTGGAGATTTATCGAATTATCGAATATCGTATCTGTCGGCCAAGTTCGGGCTGTC
CGGGCCAAGACGACGATTTGATCTTTTGGGATTTGCGCTTAGACGATTGACTGGGCTACTCCAGGGTCTTTGAGCCACGC

CCTTTACTGAAAGAGGCAAGGCATTCTCTTCTAGCAGGTGACAGCTGCTATTCAACAGAGCGCTATTCATGTCCACCTCT
TGGGGGCGGAGCCGCTCCGCAGCAATGTATTTGCCTGGGTAGAGGATACAGCTAAGCCTACCTTAGCGGCTAGCCTCACG
ATGTTCGTGTTCGCATCGTTAACGATGTCCGTCGACCGAGCTCGACATGAGTCGTATGCCTCAAGGACCCCTGGCATCTT
GACACTGCAGCAAACATGACAACCACGACAGAATGAAGTAGGAAGCCCGGTGTGTCAACCAGCCGGTCTGTCTGCAGAAG
GGTGGTCGATGGAAGGCCAAGGCAACTACATCTCCGGACATGATCAAAGTATATAAGAAGGACGATCACCGCTCCTTGAC
TTATTTTGCTTCTCTTCCTCTCATCACCTAGCCTCATTCTCTCTCTCGTCATCTAAACAGCCATCTGCACCTGCTACGAT
GCTTCCCTCCACAATCTTCTCGGTCTTTGCCCTCGTCGGCAGCGCCTTGGCTCAGCATCCCCCCAAGGTTAACCACGACA
GTATCAACCCCGTCCGCGATACTCTGGGGCCCAACGGCGACATGATCAGGAAGTTCCAGCCTCTGCTTCACATTGCCCAC
GGTTGCCAGCCTTACTCCGCTGTCAACACCCGCGGTGAGGTCAAGTAAGACACGCAGCACTCCCTTCAGCTTACACAAAG
CTCCACGCTAACACATATTCTGCAGCGCCGGTCTCCAAGACAGCGGTACCACCGCAGGCGGCTGCAAGGAAACCAGCAAG
GGCCAGACCTACGCCCGCTCCATGACCCTGAACGGCCAGTTCGGCATCATGTACGCCTGGTACTGGCCCAAGGACCAGCC
CGCCGACGGCAACCTCGCCAGCGGCCACCGCCACGACTGGGAGAACGTCGTCATCTGGTTCAACTCGAACAACGCAAACC
AGGCCGGCATCCTGCGCGGCGCCGCCTCGGGCCACGGCGACTACAAGAAGGTCAACAACCCCCAGCGCAACAACAACAAC
CTCCACGTCGAGTACTTCACCAGCCTCGGCAAGAACCACGAGTTGCAGTTCAAGACGTCGCCCGGCCGCACCTACTGGAT
CTGGGACTGGGACAGGATGGACAGCACCGTCCAGGGCGCCCTCAACCGCGCCGACTTTGGCAGCGCCAACTGCCCCTTCA
ACAACAACAACTTTGAGAGGAACATGCGCGCCGCGTTTTAAAGGCTCGACGCGCGGCTCGCCAGTCCTGGATTCCATCTG
GGCACGCATCTTGGCTCTTTCTCCTCCCGCCTTGGCTTGGCTTCTTGAGGCCAGTCGAGTGCTAGCATAGGTCCTGTACA
TAGCTTGTTCTGAATTACATACAACTCTGCCTGTCGAATTTGGTCTTGCATTCTGTGATCGAGTGTGTAAGCTTTTACGC
AGGTCGATAAATGACCAGTGCGTTGATTAGGTTCTTTCGTTGCTCTATTCATGGGTAGGTTCCCAGGAGCCCTGTCGTGA
TCCCGAAAACTCAAATAAACAGCATCATGAACTTTATAAGGCCACCATGCACTGCAGTTGATGTTCCTTTGGAGTGGAAC
ACCCCACTACCTTACCTCAAGCAGTGCAGCGGCAAGCAAGCTTGAGTCTCAACTTAATGTCAAGGAAACTGCTGCGGCAT
TGAGAAATCAGAGCCAATTCCAATGATTGGGGCACTCCGGAACCCAGCACATATGTCAAAAGACCAGACACTCACAACCA
AACACTGCAGATACAGCTCAATTCCAATATGACGATGATCGCATCGACTTGGTATGACATCGGACTGTGATCTCGTTCGA
TAGACGAAATCCAAGTTTGCTGACATACACGTTGGGTGCGACAGCTTGGAGAAATGCTCGGCAACTGGTCCTGTGCCAGG
ACGTACACAATGACGGGACCCGGCGGGGATGGGGCCGGTGGAGAGCTAGATCGACAGGATGACTTCCGCTGAGGAGACTT
CGCGCTCCGGGGAACCGATGCCGCCAGTAATAGTCGAGGTGATCATTAGGGGTGCTAAGCAGTAAAGCAGGAAAGTTTGA
ACTGTCCTACAAGTTCTTTGATGGTTTTATGTTTCGGTAAGCAGTGAGGACATCTCCTTATCATCGTAACGCCGCCCATT
CTTCGTGCCTCGTCCATACGCTGCCCTGAGGATAATTCACCCAAGTATTACACCAAATAGAGAAATCGAGACAGAATGCT
CTACGAAAAAGACGGGCGTCTGGTATGCTCCTATGACGGAGAAAAGCTCTGGATCGAGCCCTGGTCTGCCAATGCTTTCC
GCGTCCGAGCCACCAAGCTCTCCACGATGCCGACAGAGAACTGGGCCCTGCAACAGCCTCTTCAGACCGGATCC

Amino acid sequence: 118 aa

MLPSTIFSVFALVGSALAQHPPKVNHDSINPVRDTLGPNGDMIRKFQPLLHIAHGCQPYSAVNTRGEVNAGLQDSGTTAG
GCKETSKGQTYARSMTLNGQFGIMYAWYWPKDQPADGNLASGHRHDWENVVIWFNSNNANQAGILRGAASGHGDYKKVNN
PQRNNNNLHVEYFTSLGKNHELQFKTSPGRTYWIWDWDRMDSTVQGALNRADFGSANCPFNNNNFERNMRAAF

10). VI_PKS1 (Polyketide synthase)

Coding sequence (including introns): 1968 nt (incomplete)

GATCCGAGGTTTTTTAACATGTCGCCGCGCGAAGCCTACCAGACCGACCCCATGCAACGCATGGCCCTGACCACGGCTTA
TGAAGCCTTGGAAATGTCTGGGTATGTCCCGAATAGGACAGCCTCTACCAGGCTTGACCGGATTGGTACATTTTACGGTC
AGACCTCCGATGATTGGCGAGAGATCAATGCCGCACAGGAAGTCGACACATACTTCATCACCGGGGGAGTACGCGCCTTT
GGCCCTGGCAGAATCAACTACCACTTCGGTTTCAGTGGACCTAGTCTGAACATCGATACAGCCTGTTCTTCCAGTGCCGC
AGCTCTGCAAATCGCATGCACCTCGCTCTGGGCCAAGGACTGTGACACGGCAGTCGTTGGCGGCTTGTCTTGCATGACCA
ACCCCGATATTTTCTCAGGACTCAGCCGTGGCCAGTTCTTGTCCAAGACTGGTCCCTGTGCTACGTTTGACAATGGAGCT
GATGGTTACTGCCGAGCTGATGGCTGCGCCTCCGTCATTGTGAAGCGTCTGGATGATGCCATTGCCGACAAGGACAATGT
CCTGGCGGTCATCCTGGGCACGGCTACCAACCATTCTGCCGACGCCATATCGATTACCCATCCTCATGGACCTACGCAGT
CGATCCTATCATCAGCCATTCTCGATGAGGCTGGCGTCGATCCTCTCGATGTTGACTACGTCGAGATGCATGGGACAGGC
ACTCAGGCCGGTGATGGCACGGAAATGGTCTCTGTCACAGACGTATTCGCACCTGCCAACCGCAAGCGTCCTGCAAACAG
ACCTCTGTATCTCGGCGCTGTGAAATCTAACATCGGCCACGGTGAGGCTGCTTCAGGCGTGACAGCTCTCTGCAAGGTTC
TCATGATGTTGCAAAAAAACGCCATCCCTCCTCACGTCGGCATAAAAAAGGATATCAACAAGACCTTTCCTAAGGATCTT
GCGGAGCGAAACGTCAACATCGCTTTCCACATGACACCTCTCAAAAGGCATGATGGGAAACCCAGGAGAATCTTCATCAA
CAACTTCAGCGCCGCCGGTGGTAATACCGGTCTCCTCCTTGAGGATGGTCCCAGCCAGACGCCCACACAGGCGGACCCTC
GCAGTGCTCAAGTCATTACTATCACGGCCAAGTCCAAGACGGCCATGATTAAGAATGCTGAACAATTGGTGACGTGGATG
GAGAAGAATCCCGAAACCCCGCTTGCTGATGTTGCTTACACCACAACGGCCCGTCGCATGCAACACTACTGGCGCCTGAA
TGTCGCCGCCTCCACCTTGTCTGAAGCCATGTCAGCCATCAAGGAGAGGCTCACTCAAAACTTTGTGCCCATCTCGACTG
AGCAGCCAAAGGTGGCCTTCATCTTCACAGGCCAAGGCTCTCACTATGCTGGTCTTGGCAAGGACCTCTACGCCCACTAC
GCTGTCTTCAGAGACAGCATCAATGAGTTCAATCATATCGCCGAAGTCCATGGCTTCCCGTCTTTTGTGCCATTGATTGA
TGGCAGCGAACCTGACGTCTCCAAGCTATCACCCGTGGTTGTGCAGCTTGGCCTCTGCTGCTTCGAGATGGCTCTAGCCA
GACTGTGGGCTGCATGGGGTATCAGGCCCGCTGTGGTTCTGGGGCACTCTCTCGGAGAATATGCTGCTCTGAACGCCGCC
GGCGTACTGTCCGCAAGCGACACCATCTATCTCGTCGGCAGTCGCGCTCAGCTTCTCGTCGACAGGTGCACTGCTGGTAC

CCATGCCATGCTTGCTGTGCAGGGCCCGGTTGGGACTGTCAACGAGGCTCTCGGATCCGAATTCGCCTCAGTCAACATCG
CCTGCATCAACGGGCCTCGTGAGACCGTTCTCAGTGGTGAAACTGACCACATGATGAAGATTGCCACGCAATTGGGCGCG
TCTGGATTCAAGTGCACCCAGCTGAATGTTCCCTATGCCTTCCACTCC

Amino acid sequence: 656 aa (incomplete)
DPRFFNMSPREAYQTDPMQRMALTTAYEALEMSGYVPNRTASTRLDRIGTFYGQTSDDWREINAAQEVDTYFITGGVRAF
GPGRINYHFGFSGPSLNIDTACSSSAAALQIACTSLWAKDCDTAVVGGLSCMTNPDIFSGLSRGQFLSKTGPCATFDNGA
DGYCRADGCASVIVKRLDDAIADKDNVLAVILGTATNHSADAISITHPHGPTQSILSSAILDEAGVDPLDVDYVEMHGTG
TQAGDGTEMVSVTDVFAPANRKRPANRPLYLGAVKSNIGHGEAASGVTALCKVLMMLQKNAIPPHVGIKKDINKTFPKDL
AERNVNIAFHMTPLKRHDGKPRRIFINNFSAAGGNTGLLLEDGPSQTPTQADPRSAQVITITAKSKTAMIKNAEQLVTWM
EKNPETPLADVAYTTTARRMQHYWRLNVAASTLSEAMSAIKERLTQNFVPISTEQPKVAFIFTGQGSHYAGLGKDLYAHY
AVFRDSINEFNHIAEVHGFPSFVPLIDGSEPDVSKLSPVVVQLGLCCFEMALARLWAAWGIRPAVVLGHSLGEYAALNAA
GVLSASDTIYLVGSRAQLLVDRCTAGTHAMLAVQGPVGTVNEALGSEFASVNIACINGPRETVLSGETDHMMKIATQLGA
SGFKCTQLNVPYAFHS

11). VI_12.2 gene (Hypothetical protein)

Coding sequence: 172 nt (incomplete)
AATTCGTCCTCGGGCCTCAAGGCAGAGTGTTGGGAGGCACTTGAGATGGACAAGTACATCAACGACTGGATGGCAACCAA
CAGCGCCGCTGCCGGGTGTGACACTCTTGGTTTTGCCCAGTGCTACCTCCAGTTCAACGGCCTGACCACTCTGACCTGTG
ATGACATTACGT

Sequence of the UTR: 681 nt (incomplete)
CTTCGGTATCGGTATAGGGGGGGAGTATCTATTGTTGATGAAAGGAGGAAGAGCAATAATCAACGCGCTTCATTCAGCCT
GATGACGCCCGTGAGTCGGTCATTATCCAGGGTGCTCGTATCCTCCTCGCACCCTCGAGACACCCAGCGTAGCATCCAGT
GGGGGCCAAGGAGAAGGGAACCCCGAGACGATTGTGGGGTGCTGCGACTTGCAGGGTATCACCAAGAAGCCCGCTGCTGG
GAGCCGCACGGGGCTGCCGGCGCATGACGTGCCAAGCACTTTATCGCCCAAGCCATCGTTTCAGCGTTGAGCAGATCGGG
AGCTGTCGTCTTCCATCGTGCAGCTATCATCTCAACTTTGGAATGGTTCGTGGCTCTCAGTGACATTCGTACATACCAAT
GCTCTGTCTGGGCTGAACGAAATCTTGTCGCTCGACCATATGTTAAGCGGGTTGAGGACCTAGCCTACCAGCTACTAACT
ACGTCTTGGTGCTGGTGGCCTTCAACTCAGTCAAATTACCACATTAGAGTGAGCTTGAGCCAAGCGACAGGTTTAGTTGT
CCTTGCTGTCAGGTAGGGCTGTGCAACCATGCTGTGAGCCATAGGGACATGAGTCGCCCAAATGAAGCCAAGGGGCCATG
AATCGCCAAAAGCCTGCCGTGCTGCCCGCTCAATACCCCAT

Presentations at national and international conferences

Xu H.Q., Löfke C., Weiberg A., Karlovsky P. 2007. Identification and Characterization of a gene coding for a putative necrotic peptide Vl-NEP of *Verticillium longisporum*. 8. VAAM Symposium „Molecular Biology of Fungi“ Hamburg, Germany, September 23-26 2007.

Xu H. Q., Weiberg A. & Karlovsky P. (2009) Characterisation of potential pathogenicity related gene Vl_6.2 of *Verticillium longisporum*. 10th International *Verticillium* Symposium Corfu Island, Greece. November 16-20 2009.

Xu H. Q., Beinhoff M., Weiberg A. & Karlovsky P. (2009). Identification and characterisation *Verticillium longisporum* gene encoding a transcription factor similar to repressor of polysaccharide hydrolases AceI of *Trichoderma reesei*. 10th International *Verticillium* Symposium Corfu Island, Greece. November 16-20 2009.

Publication of this Work

Weiberg A., Xu H.Q., Riediger N., von Tiedemann A. & Karlovsky P. (2008) Adaptation of *Verticillium longisporum* to xylem sap enviroment as revealed by cDNA-AFLP analysis. Dissertation online.

Eidesstattliche Erklärung

Hiermit erkläre ich eidesstattlich, dass diese Dissertation selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Göttingen, im Dezember 2010

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Curriculum Vitae

Personal data

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Secondary education

09/1970 until 07/1975	primary school, Jiangsu, P.R.China
08/1975 until 06/1980	grammar school Jiangsu, P.R.China
Graduation:	high school diploma

Tertiary education

1980-1984	Bachlor student at Nanjing Agricultural University
1984	Bachlor in Phytomedicine
10/1997-04/2000	Master student at the Institut Plant Pathology, Georgy-August University Göttingen
04/2000	Master in Phytomedicine
05/2000-/2004	PhD student at the Institut Microbiology, Georgy-August University Göttingen
03/2007-02/2011	PhD student at the Department of Crop Science, Molecular Plant Pathology and Mycotoxin Research Unit, Georgy-August University Göttingen