Identification of genes induced in the vascular pathogen *Verticillium longisporum* by xylem sap metabolites of *Brassica napus* using an improved genome-wide quantitative cDNA-AFLP.

Identifizierung von Xylemsaft-induzierten Genen im vaskulären Pathogen *Verticillium longisporum* mittels einer verbesserten cDNA-AFLP Methode für transkriptomeweite Expressionsstudien

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Chapter 1 - General background

Transcriptome analysis is vital to all fields of biology. Total sets of messenger RNA molecules of a living organism, tissue or cell under given physiological or environmental circumstances can be profiled using transcriptome analysis. The regulation of gene expression occurs initially at the transcription level. Thus, the analysis of the transcriptome (syn. transcript profiling, transcriptomics) in different physiological or developmental stages and the comparison of gene expression profiles, for example among states of saprophytic and pathogenic forms, facilitating the assignment of biological functions to genes and gene sets. In this work, we chose cDNA-AFLP (cDNA-amplified fragment length polymorphism) differential display to investigate the genes involved in the pathogenicity of *Verticillium longisporum* infecting oilseed rape (*Brassica napus*).

The plant pathogen *Verticillium*

The fungal genus *Verticillium* (phylum: Ascomycota) includes, among others, hemibiotrophic plant pathogenic species (1, 2) occurring in temperate and subtropical regions around the world. Due to the yield losses caused in several crops by *V. dahliae* and *V. albo-atrum,* they are economically relevant. More than 200 mainly dicotyledonous plant species can be infected by *Verticillium*; among these are tomato, potato, cotton, sunflower, lucerne and different crucifers including oil seed rape. *Verticillium* infection causes diverse visible symptoms, namely wilting, defoliation, stunting, early senescence, chlorosis or even necrosis, depending on the severity of infection and type of host plant (3). Due to these manifold symptoms, which can also be caused by other soilborne pathogens like *Fusarium oxysporum* or by xylem-parasitic bacteria, diagnosis based on molecular assays are a great help in proofing *Verticillium* infection reliably (4, 5).

Verticillium isolated from *Brassica spp.* (Brussels sprout, horse radish) with dissimilar morphology to *V. dahliae* and *V. albo-atrum* in terms of long-spored conidia was first reported by Isaac (6) and Stark (7). These findings resulted in the creation of the new sub-species *V. dahliae* f. sp. *longisporum*. In the following this, a more defined characterization and differentiation of *Brassica*-specific *Verticillium* isolates were established through the analysis of vegetative compatibility groups, the analysis of host specificity, genomic differentiation by the use of DNA fingerprint tools as well as biochemical phenotyping (e.g. extracellular polyphenol oxidase activity assays) (8, 9, 10, 11). Based on these observations the proposal

was made to create the new *Verticillium* species *V. longisporum* (8), but this is still under discussion and not generally accepted by the scientific community. Thus, *Brassica*-specific isolates are often described as amphihaploid, interspecific hybrids of parental haploid *V. dalhiae* and *V. albo-atrum*-like strains (1, 12). Despite the unresolved taxonomic nomenclature (12, 13), the *Verticillium* isolate used in this work will be referred to as *V. longisporum*.

From the practical point of view, the prevention of a *Verticillium* infection of crop plants is difficult. As in other soilborne systemic parasites the application of fungicides is not sufficiently effective to control *Verticillium*. Biological agents against *Verticillium* described in literature (14, 15, 16) lack the proof of sustainability under field conditions. Therefore resistant cultivars of crop plants are required, but efficient sources for *Verticillium* resistance are hard to obtain by means of classical breeding strategies. In terms of *Brassica napus* the production of new resynthesized oil seed rape lines seem to be a promising resistance strategy (17, 18, 19).

Life cycle of plant pathogenic *Verticillium* species

The monocyclic life style of plant pathogenic *Verticillium* spp. (Fig. 1.1) can be divided into three distinct phases, namely a dormant, a parasitic and a saprophytic stage. In the soil the pathogen forms resting structures such as long-living mycelium (*V. albo-atrum*) or microsclerotia (thick-walled, melazined hyphae structures typical for *V. dahliae* and *V. longisporum*). Those can survive for decades (20, 21). Areas contaminated with microsclerotia are consequently endangered by a latent inoculum potential. The germination of microsclerotia, presenting the shift to the parasitic life style of *Verticillium*, is triggered by inductive root exudate released by host plants into the rhizosphere (22). Germ-tubes and initial hyphal structures grow directly to the roots following the exudate and nutrient gradients (C-, N-sources). When contacting the host, *Verticillium* penetrates the plant tissue via the terminal and/or lateral root tips (23). For *B. napus* as host plant it has been shown that *V. longisporum* and *V. dahliae* represent different situations of a host- and non-host interaction (24). Once the pathogen has entered the plant roots, it grows through the root cortex inter- as well as intracellular and penetrates the vessel system (25). *Verticillium* remains restricted to the xylem system during most of its parasitic life (26-32). The fungus sporulates or grows in a yeast-like budding form and spreads from the root part of the plant into the shoot by the transportation of spores with the transpiration stream (23). At eventual

barriers like vessel end walls or pit cavities spores start to germinate and hyphae break through the obstacles to continue fungal propagation (33). Interestingly, cycles of mycelial propagation and fluctuations in biomass were observed during xylem invasion of *Verticillium* (34). Because the pathogen stays restricted to the xylem vessels and contact to living, metabolic active plant cells remains a rare event. Thus, *Verticillium* is well adapted to the nutritional environment of the xylem with low concentrations of sugars and amino acids.

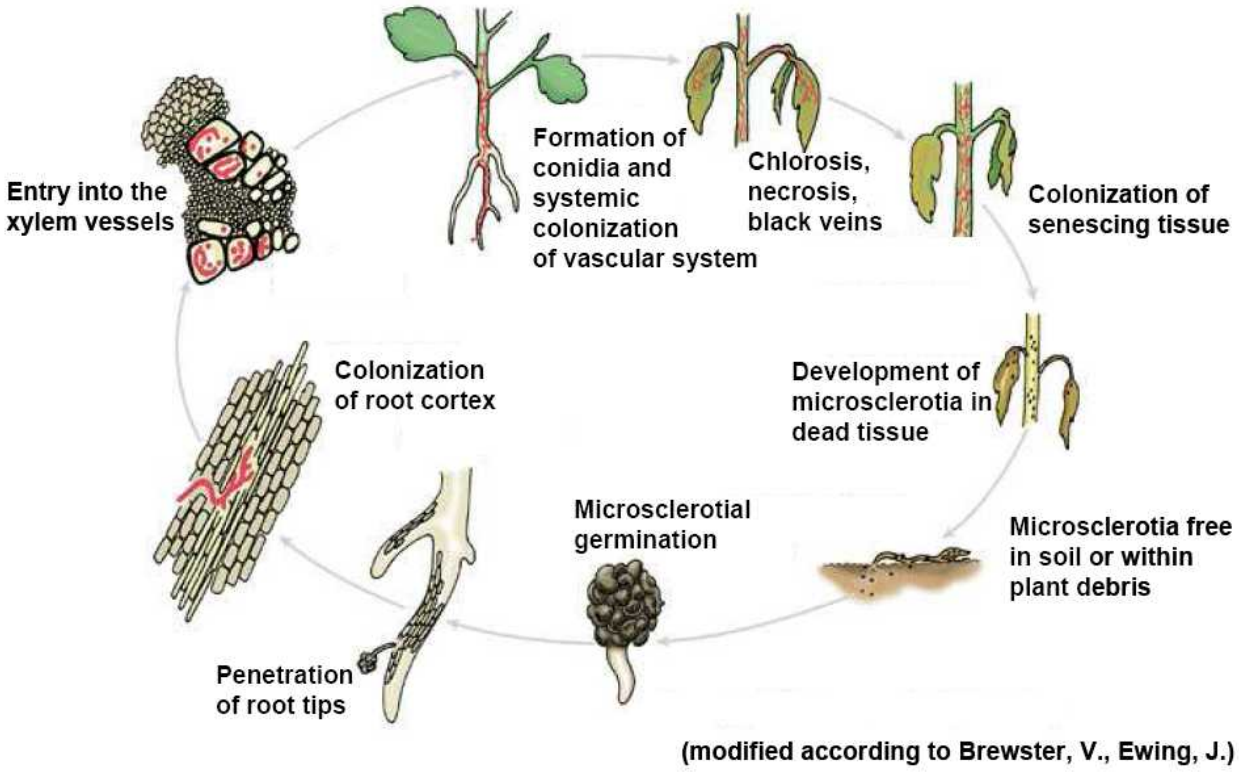

Figure 1.1: Monocyclic life style of microsclerotia-producing *Verticillium* spp. Modified according to Rowe and Powelson (35).

Infection symptoms such as the ones described above are triggered by diverse processes and mechanisms. Fungal mycelium clogs the xylem vessels, which can slow down the water and mineral supply into the upper plant compartments thus causing the typical wilting symptoms (36). Furthermore, *Verticillium* species can secrete cell-wall degrading enzymes like polygalacturonases and pectinases (pectin lyases, pectin esterases) (37, 38, 39) that soften and destabilize the structure of xylem vessel walls. Studies on pectinase deficient mutants showed slower symptom development and less severity, but the degree of colonization between wild-type and mutants was not changed, thus pectinases were classified as virulence factors but not as pathogenicity factors (40, 41). Oligomers released by this enzymatic attack to the plant cell walls sometimes act as elicitors triggering stress responses and plant defence reactions. Another mechanism contributing to symptom development is the release of phytotoxic fungal secondary metabolites. In the past, many experiments demonstrated that crude extracts of *in vitro* cultured *Verticillium* can induce symptoms, High molecular components of protein lipopolysaccharides or glycoproteins have been reported as potential effectors (42-45). The existence of such systemically acting, toxic compounds was confirmed by several 'root split' experiments (46, 47). For *B. napus*, typical stunting can be initiated by treatment with the sterile-filtered supernatant of *V. longisporum* cultures (48). The nature and chemical structure of phytotoxic metabolites synthesized by *Verticillium* during the infection process remain unknown with the exception of one example. Recently, a new family of small phytotoxic peptides, assigned NEPs (necrosis and ethylene inducing peptides), was described as being produced by plant pathogenic fungi like *Fusarium oxysporum*, *Botrytis cinerea* and also by *V. dahliae* (49, 50, 51). In *Verticillium*, the corresponding gene sequence of the VdNEP peptide was elucidated and cloned. The purified recombinant VdNEP causes wilting and chlorosis in treated leaves of the native host cotton as well as in *Arabidopsis* and tobacco leaves (51). In literature, NEPs are believed to act more as elicitors than as phytotoxins (51, 52).

During late senescence of the host plant, *Verticillium* switches to the saprophytic stage metabolising the nutrients released by the dying plant tissue. As the nutrients become depleted, the fungus produces its resting structures (micorsclerotia) and completes its life cycle.

Molecular mechanisms acting during all stages of *Verticillium* differentiation are hardly understood. The parasitic phase is of high interest because the fungus expresses its arsenal of

pathogenicity and virulence factors to approach infect and parasite its host specifically at this stage. Investigation of the parasitic stage of V*erticillium* by functional and comparative genomics, transcriptomics, proteomics and metabolomics presents an integrated approach to elucidate the interaction between the host and the pathogen, and may provide valuable resources to develop resistant plants in the future.

Verticillium as a model-system

Verticillium is on the way to become a model-system. In 2006, the whole mitochondrial genome sequence of *V. dahliae* was released (53), thus this information has been applied mainly (in combination with former molecular data) for the design of DNA markers (5, 54**)** and for phylogenetic analyses of the taxon *Verticillium* (9, 13, 55, 56). In 2004, a comparative genome sequencing project of *V. dahliae* and *V. albo-atrum* was initiated by the NSF/USDA Microbial Genome Sequencing Program in collaboration with the Broad Institute. Data stored in the data base and gene-annotated sequences are freely accessible at (57). On transcript level, a collection of in total 2626 individual ESTs (expressed sequence tag) of *V. dahliae* were compiled from two independent transcript sequencing projects (51, 58), the sequences were archived and are freely available within the NCBI GenBank database. Beside this comprehensive information on nucleic acid level projects have been initiated in the field of metabolomics and proteomics, thus *Verticillium* research is entering the –omics era. Functional analysis of genes related to pathogenesis or other developmental processes was initiated in conjunction with sequenced cDNA libraries of *V. dahliae*. Meanwhile individual genes and their products have been characterized using this resource, namely genes coding for a necrosis and ethylene inducing peptide (NEP) (51), a trypsin protease (59), a mitogen-activated MAP kinase (60), a hydrophobin (61) and a glyoxalase I (62).

Transcriptomic studies by cDNA-AFLP

The transcriptomic analysis of an organism, tissue or cell consists in measuring the abundance of all detectable mRNAs of transcribed genes at a defined time point or during a time course. Since the mid 90s several tools for differential transcriptomic studies have been established. SAGE (Serial Analysis of Gene Expression [63]) and MPSS (Massive Parallel Signature Sequencing [64]) are one-pass sequencing strategies on cloned cDNA populations feasible for the absolute quantification of distinct transcript molecules by counting transcript sequence frequencies. The SAGE protocol produces only short cDNA tags, rendering access

to existing gene sequences necessary for complete gene identification. By means of high transcriptome coverage, including transcripts of low abundance, these techniques involve huge amounts of sequencing. A second group of transcriptomic tools relies on DNA/DNA hybridization reactions. SSH libraries (Suppression Subtractive Hybridization [65]) are constructed after hybridization of a tester and a driver cDNA population in a subtractive manner to extract regulated transcripts within the tester population. A drawback of SSH is that it can only compare two transcript populations. A direct comparison between more than two mRNA samples (e.g. in time course experiments) is impossible. Hybridization of cDNA molecules to DNA probes fixed on a solid surface (chip) became generally known as microarray technology (66). The advantage of DNA chips lies in the high density-spotted DNA probes on a microarray slide which enables high sample throughput for whole genome analysis. Nowadays, commercial suppliers saturate scientists with products and services for model organisms such as *Arabidopsis*, the mouse or *Homo sapiens*. But this aspect points at the main drawback of microarray technology, which is its limitation to model only genome-sequenced systems. As a close-end technology, only the expression of known, sequenced genes represented on a microarray slide, can be profiled. A third group of technologies suitable for transcriptome studies based on fingerprints of cDNAs separated by electrophoresis are Differential Display (DD) (67) and cDNA-AFLP (68). Both offer an attractive alternative to microarrays when gene sequence information is limited. Without the requirement of having a sequenced genome, these tools are applicable to any species. Furthermore, they are very sensitive, because they include amplification of cDNA fragments via PCR, so that all transcripts present in an mRNA sample can potentially be detected. A second advantage is a clear distinction between transcripts comprising high homology in sequence (e.g. members of a gene family), which encounter difficulties in the microarray-based hybridization step. Finally, being open-end strategies, new genes of unknown sequence can be identified. Because of the latter two points, these techniques were used in the past predominantly as gene discovery tools. cDNA-AFLP is reported to be superior to DD because of a higher reproducibility and accuracy as well as less false positives (69). We used cDNA-AFLP to profile the transcriptome of *V. longisporum* because of these advantages. cDNA-AFLP is based on the ligation of DNA adaptors to enzymatically double-digested cDNAs. PCR with adaptor-specific primers guarantees robust and reliable amplification of transcripts, which are subsequently separated by electrophoresis. By the introduction of so-

called selective nucleotides (N) at the 3´ terminus of the cDNA-AFLP primers, transcripts are amplified in groups of 4^N PCR samples leading to sets of cDNA fragments which can be separated and analysed (70). A recent innovation in cDNA-AFLP comprises the immobilization of cDNA molecules onto a solid carrier at the digestion step (71, 72) which eliminates any redundant cDNA bands from the system. This has become generally known as the 'one gene - one tag' variant (Fig. 1.2).

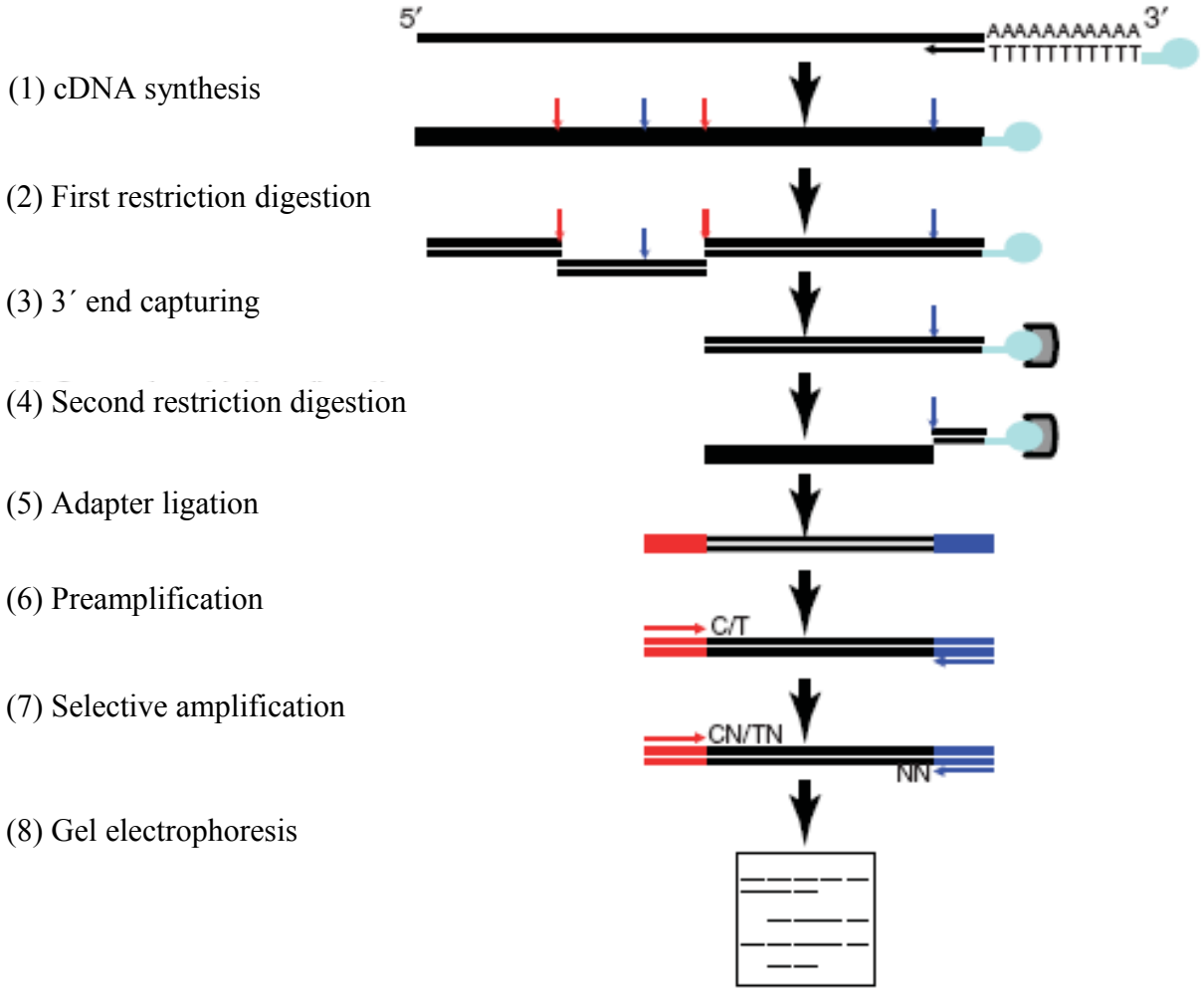

Figure 1.2: cDNA-AFLP protocol according to Breyne et al. (71) and Vujlsteke et al. (72).

In recent years, cDNA-AFLP has been improved in terms of increased sample throughput and providing realistic quantitative data of global gene expression. During PCR, cDNA fragments are labelled with fluorescent dyes in order to use modern capillary-based DNA sequencers for fragment separation and detection rather than using autoradiography platforms generating digital chromatograms of cDNA fragment patterns (73, 74). By the use of proper computer software for automatic processing of cDNA fragment patterns quantitative data of gene expression are produced by measuring band/peak intensities. Intensity values of quantified cDNA peaks have to be normalized (75) to allow for statistical analysis aiming at the identification of transcripts showing significant changes in their expression levels (71) to exclude false discovery rates (76).

There is a fast growing pool of information about genomic and gene-coding sequences of an increasing number of organisms archived in generally accessible bioinformatic databases (NCBI, EMBL, RIKEN, etc.). Although this information is incomplete, it can be used for computer-based (*in silico*) simulations of cDNA-AFLP to predict cDNA fragment patterns. In the last years, several computer programs have been published providing tools for DNA sequence-based simulations. The program GenEST (77) serves as a bidirectional link between predictions of cDNA fragment patterns generated by cDNA-AFLP and bioinformatic sequence databases including the annotation of separated cDNA-AFLP bands to known genes in a database. This supersedes time-consuming cloning and sequencing of cDNA-AFLP bands. Another software tool, published by Kivioja et al. (78), can help to reduce the sample usage in the PCR step of cDNA-AFLP by 25-50%. Other computer programs were used to perform pre-experimental cDNA-AFLP simulations for the calculation of statistical proportions like transcript coverage. Such *in silico* analyses can be helpful to design the optimal restriction enzyme combination for the organism under investigation. For the design of a convenient cDNA-AFLP protocol, transcript coverage and, cDNA fragment size are the most decisive issues. cDNA fragment size corresponds to the gene sequence information of cDNA-AFLP fragments that have been cloned once for sequencing. By *in silico* simulations for a single pair of restriction enzymes, transcript coverage not higher than ~60% was obtained. To increase the coverage, one might consider applying a second pair of enzymes on the same mRNA population, and calculations showed that this leads to an increased coverage of ~80% (71). But this step would also involve the introduction of transcript redundancy into cDNA-AFLP, which is successfully ruled out by

the immobilization of cDNA molecules (described above). These facts led us to design an improved cDNA-AFLP protocol with the aim to increase the transcript coverage, while keeping the redundancy at a low level.

Aim of this project

The aim of this work was (i) to investigate the global gene expression of *V. longisporum* under "infectious simulating conditions" by cDNA-AFLP and (ii) to improve the cDNA-AFLP method and optimize automated data processing.

In detail, the goal was to profile the transcriptome of *V. longisporum* under stimulated xylem sap conditions for the identification of pathogenesis-related genes involved in activities of *V. longisporum* functioning as an adaptation to growth in the vessel system. To discover any genes induced by xylem metabolites, cDNA-AFLP technique was applied in a differential display concept in order to perform a comparison of non-treated status vs. treatment with xylem sap metabolite. Emphasis was predominantly placed on genes specifically induced by secondary metabolites of *B. napus*, but not involved in general catabolic pathways turned on by sugars and amino acids, and not in genes induced by mineral ions. (i) Fungi were treated with metabolites while growing *in vitro* in a standardized liquid medium SXM (simulating xylem medium [59]) imitating the general nutritional environment prevalent in the xylem, and (ii) fungi were treated with only ethyl acetate extracts of xylem sap. Two different types of xylem sap were used for the treatment, originating either from *B. napus* plants, which had not been infected previously with *V. longisporum*, or from *V. longisporum*-infected plants. Changes in the fungal gene expression were expected due to newly synthesized metabolites of the plant as a response to *Verticillium*-infection, when using xylem sap preparations from infected plants.

Because only small changes in fungal gene expression profiles were expected due to the mild treatment with only ethyl acetate extracts of xylem sap, a high coverage of the fungal transcriptome was required for a quantitative cDNA-AFLP approach. For this purpose, (i) the classical cDNA-AFLP protocol was improved technically and (ii) data variability and error propagation in cDNA-AFLP was investigated to optimize automated data processing for a more reliable interpretation of transcriptomic data sets.

Former reports about simulated data showing only unsatisfying transcript coverage by classical cDNA-AFLP protocols caused us to develop and apply an improved cDNA-AFLP protocol based on a multiple digestion of immobilized cDNAs. Conventional protocols apply

a single pair of restriction enzymes to the cDNAs; hence a cDNA molecule is only captured by this method, if recognition motifs of both enzymes are present within the sequence. Our improved protocol aimed at increasing the coverage of transcripts, because cDNAs restricted by the first enzyme, but not by the second, are occasionally restricted by a third or forth, etc., and thus can be recovered by this improved protocol. Several software tools were programmed to simulate cDNA-AFLP experiments in preparation for the experimental part of this work to find the optimal restriction enzyme combination. None of the software tools available were able to simulate properly the issues of our proposed cDNA-AFLP strategy. To perform simulations of multiple digestions on immobilized cDNA sequences, we developed a new software tool, called MECS (**M**ultiple **E**nzyme **c**DNA-AFLP **S**imulation) which could demonstrate the superiority of the improved protocol in terms of enhanced transcript coverage, low transcript redundancy and reduced PCR sample effort.

Variance and statistical error are issues central to processing transcriptomic data. In microarray hybridization, variance has been thoroughly studied and partitioned into components assigned to single experimental steps (79, 80). This kind of analysis has been lacking for electrophoresis-based transcriptomics, limiting the quantitative interpretation of transcript profiles obtained by cDNA-AFLP, mRNA differential display and related techniques. Systematic errors can be excluded by experimental design and proper normalization, but random errors originating from variance inherent to biological systems, as well as technical variance introduced by various treatments, affects the results. False and missing assignments of matching cDNA bands are the source of most serious errors in electrophoresis-based transcriptomics. These errors do not occur in microarray hybridization and sequencing-based transcriptome analysis. Secondly, the total variance of the cDNA-AFLP procedure was partitioned into the following components of cDNA synthesis: adapter ligation, preamplification, amplification and electrophoresis. These are the stages which had to be targeted to optimize the reliance of this procedure.

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Chapter 2 - Improved coverage of cDNA-AFLP by sequential digestion of immobilized cDNA

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BMC Genomics

Methodology article

Improved coverage of cDNA-AFLP by sequential digestion of immobilized cDNA

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Abstract

Background: cDNA-AFLP is a transcriptomics technique which does not require prior sequence information and can therefore be used as a gene discovery tool. The method is based on selective amplification of cDNA fragments generated by restriction endonucleases, electrophoretic separation of the products and comparison of the band patterns between treated samples and controls. Unequal distribution of restriction sites used to generate cDNA fragments negatively affects the performance of cDNA-AFLP. Some transcripts are represented by more than one fragment while other escape detection, causing redundancy and reducing the coverage of the analysis, respectively.

Results: With the goal of improving the coverage of cDNA-AFLP without increasing its redundancy, we designed a modified cDNA-AFLP protocol. Immobilized cDNA is sequentially digested with several restriction endonucleases and the released DNA fragments are collected in mutually exclusive pools. To investigate the performance of the protocol, software tool MECS (Multiple Enzyme cDNA-AFLP Simulation) was written in Perl. cDNA-AFLP protocols described in the literatur and the new sequential digestion protocol were simulated on sets of cDNA sequences from mouse, human and *Arabidopsis thaliana*. The redundancy and coverage, the total number of PCR reactions, and the average fragment length were calculated for each protocol and cDNA set.

Conclusion: Simulation revealed that sequential digestion of immobilized cDNA followed by the partitioning of released fragments into mutually exclusive pools outperformed other cDNA-AFLP protocols in terms of coverage, redundancy, fragment length, and the total number of PCRs. Primers generating 30 to 70 amplicons per PCR provided the highest fraction of electrophoretically distinguishable fragments suitable for normalization. For *A. thaliana*, human and mice transcriptome, the use of two marking enzymes and three sequentially applied releasing enzymes for each of the marking enzymes is recommended.

Background

Transcriptome analysis is vital to all fields of biology concerned with spatial and temporal patterns of gene activity. Hybridization of labeled cDNA to oligonucleotides immobilized in two-dimensional arrays became the method of choice for fast access to the transcriptome of

Chapter 3 - Components of variance in transcriptomics based on electrophoretic separation of cDNA fragments (cDNA-AFLP)

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Research Article

Components of variance in transcriptomics based on electrophoretic separation of cDNA fragments (cDNA-AFLP)

The sources of variance and errors in transcriptomics based on the electrophoretic separation of amplified cDNA fragments were investigated using cDNA-amplified fragment length polymorphism (AFLP). Transcriptome profiles of the plant-pathogenic fungus *Verticillium longisporum* were generated by a standard cDNA-AFLP protocol followed by electrophoretic separation of amplified DNA fragments in flatbed polyacrylamide gels with fluorescence detection as well as by capillary electrophoresis (DNA sequencer). The total variance was partitioned into contributions of cDNA synthesis, adapter ligation, preamplification, amplification, and electrophoresis. Parameters of computer-aided peak recognition and matching were investigated and strategies improving matching success based on double passage with different signal intensity thresholds were developed. The overall quality of data was similar for cDNA-AFLP and microarray hybridization. Variance of cDNA-AFLP was independent of signal intensity, whereas microarray data showed higher variance for low-intensity signals. Capillary electrophoresis significantly reduced the number of wrongly matched and unmatched signals as compared with flatbed gels. These results are also likely to apply to related electrophoresis-based transcriptome analysis techniques such as mRNA differential display.

Keywords:
Band matching / cDNA-AFLP / Peak matching / Peak recognition / Variance components
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1 Introduction

The set of abundances of mRNA molecules in an organ, tissue, or microbial culture represents a snapshot of gene expression at the transcriptional level. Simultaneous analysis of these mRNA molecules, designated transcriptomics, is a fundamental concept of functional genomics, which seeks to unravel the roles of individual genes in biological functions and processes. As gene expression is regulated primarily at the transcription level, comparison of the transcriptome state under different physiological or developmental stages reveals stage-specific patterns of gene expression and facilitates the assignment of biological functions to genes.

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Abbreviations: AFLP, amplified fragment length polymorphism**; PT,** position tolerance

So-called close-end transcriptomic techniques, most prominent among them being microarray hybridization, require prior knowledge of gene sequences and are therefore unsuitable for organisms with limited availability of sequence data. Open-end techniques do not require prior sequence knowledge and can therefore be used as gene discovery tools. Among the latter methods, electrophoretic analysis of cDNA fragments amplified by randomly primed PCR (mRNA differential display [1]) or by PCR primed at oligonucleotide adapters attached to DNA by ligation (cDNA-amplified fragment length polymorphism (AFLP)) has gained the most popularity.

cDNA-AFLP is based on selective amplification of subsets of restriction fragments originating from double-stranded DNA complementary to the transcriptome. cDNA is digested with two restriction endonucleases, resulting fragments are ligated to DNA adapters and amplified by PCR with adapter-specific primers. Subsets of these fragments are then amplified with primers, which consist of sequences complementary to the adapters and of additional, so-called selective nucleotides at the 3′ terminus. For all combinations of N selective nucleotides, DNA fragments are partitioned into 4^N subsets, which are separately amplified and analyzed by electrophoresis [2]. A recent innovation of

Chapter 4: Adaptation of *Verticillium longisporum* to xylem sap environment as revealed by cDNA-AFLP analysis

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ABSTRACT

Verticillium longisporum is a soilborne plant pathogen which infects cruciferous host plants via the roots and colonizes their vascular system. Because of the growing demand for bio-fuel the area under oilseed rape cultivation is increasing and *V. longisporum* has become a serious problem. We analysed *V. longisporum* transcriptome after treatment with extracts of *Brassica napus* xylem sap by an improved cDNA-AFLP method with the goal to identify genes which are selectively induced during the colonization of xylem. Among over 13,000 cDNA-AFLP signals recorded, only 34 signals responded to the treatments. Reverse transcription real-time PCR was applied on 21 cloned transcript fragments to confirm their differential expression and 9 transcripts were verified. Further on we analysed the regulation of candidate transcripts *in planta* and found 8 transcripts to be regulated. Sequence analysis indicates that the corresponding gene products might be involved in regulation, sugar-uptake, adhesion, apoptosis and the synthesis of secondary metabolites.

BACKGROUND

Fungal genus *Verticillium* comprises a number of plant pathogenic species, which cause wilt disease in about 300 dicotyledonous plants resulting in economically relevant yield losses in vegetables, cotton, hop, sunflower, lucerne and other crops (1). *Verticillium* wilt fungi enter their hosts via roots and remain contained in the vascular tissue for most of their life cycle. Wilting symptoms caused by the infection are attributed to clogging of the vessel elements of the vascular tissue and to the effect of so far chemically not characterized phytotoxic substances designated as wilt toxins (2, 3). Since the 60s, a disease with symptoms slightly different from *Verticillium* wilt as known on other hosts was observed on oilseed rape and other crucifers (4). The intensification of oilseed rape production in Europe apparently contributed to the increase of the infection pressure by generating a soil reservoir of

microsclerotia in major growing areas. Since the 70s the disease became a significant threat to oilseed rape production. Promising *Verticillium*-resistant resynthesized genotypes have been recently identified in *B. oleracea* and *B. rapa* (5, 6), but oilseed rape breeding has so far not provided growers with *Verticillium*-resistant varieties preventing yield losses due to *Verticillium* infection.

Because the causal agent of *Verticillium* diseases in *Brassica spp*. is morphologically distinguishable from the wilt species *V. dahliae* by larger conidia, it was denoted *V. dahliae f.sp*. *longisporum* (4, 7). The elevation of the taxon to species level was suggested next to conidia morphology, vegetative compatibility, host specificity and genome fingerprints (8, 9, 10), while other authors argued that the pathogen should neither be regarded as *forma speciales* nor given a species status because *Verticillium* populations pathogenic on *Brassica spp*. comprise at least three kinds of hybrids of *V. dahliae* with other *Verticillium* species (11). We use the designation *V. longisporum* throughout this work while being aware that the taxonomical position of the pathogen has yet to be determined conclusively (12, 13).

We selected *V. longisporum* as a model for vascular plant pathogens, a unique group of plant-parasite microorganisms. The most distinctive feature of *V. longisporum* infection of crucifers as compared to other plant pathogenic *Verticillium* species is the lack of typical wilt symptoms. The pathogen instead causes stunting, chlorosis and anthocyanin accumulation, affects the flowering time and triggers early onset of senescence (14, 15). The long time period during which the fungus remains contained in xylem vessels offers an opportunity to study the interaction with the plant under compatible conditions and to identify physiological correlates and molecular basis of disease symptoms. In contrast to biotrophs, the fungus does not establish an interface to host cytoplasm. The entire exchange of chemical signals occurs in vascular elements, which consists of dead tissue but in contrast to the growth environment of nectrotrophs represents a functional plant organ. In common with biotrophs, the fungus has to avoid recognition which triggers defense responses. *V. longisporum* life cycle thus unifies features of both biotrophic and nectrotrophic pathogens. Because the fungus is confined to the vascular system, we decided to investigate the effects of xylem sap on the transcriptome of *V. longisporum*. Our goal was to identify genes involved in activities developed by *V. longisporum* as an adaptation to growth in the xylem.

MATERIALS AND METHODS

Plant material

The seed material of 'rapid cycle rape' (*Brassica napus* var. *napus*, Genom ACaacc [16]) was provided by the Department of Crop Sciences, Section Plant Pathology and Crop Protection, University of Goettingen. Contrary to common winter rape plants 'rapid cycle rape' does not need any vernalization, reaches only 80 cm height and develops into BBCH 61 stage within 55-60 days. In all experiments seeds were surface sterilized in 70% ethanol for 15 sec and rinsed three times in sterile tap water before sowing in sterile silica sand.

Inoculation method

Rape plants were carefully rinsed of silica substrate 7 days after sowing. The cleaned roots were dip inoculated for 45 min in a spore suspension (1 x 10^6 spores ml^{-1}) of *V. longisporum* isolate 43. Subsequently, plants were transferred into an earth/sand mixture (1/1 v/v) and raised under constant conditions in a climatic chamber with a day/night length of 16/8 h, 23°C at daylight and 20°C at night conditions.

Xylem sap sampling

Xylem sap was sampled using a Scholander type pressure bomb (17). Plants were carefully removed from their substrate at 14, 21, 28 or 35 dpi. The root system was cleaned by rinsing under tap water, dried and separated in root and shoot at the hypocotyl interface. The root system was spanned in the pressure chamber, with the cut end reaching above the level of the cover seal. By addition of N_2 the sealed closed chamber was pressurized to 4 bar. Xylem sap accumulating on the cut surface was collected over 15 min using a micropipette and stored on ice until sampling was finished. For gene expression analysis by cDNA-AFLP, xylem sap was extracted with 1 Vol. of ethyl acetate three times. The extracts were combined, portioned into sterile 25 ml Erlenmeyer flasks and dried.

Fungal cultures for cDNA-AFLP experiments

For all experiments *Verticillium longisporum* isolate 43 (10) was used and grown in 5 ml SXM liquid medium (18) stationary cultures at 23°C with a 12 h day/night cycle inoculated with 10 µl of 10^6 spores ml^{-1} glycerol spore solution. After 5 days cultures were transferred into Erlenmeyer flasks with dried xylem sap extracts. The amount of the extract used for each flask was adjusted in such a way that the concentration of metabolites in culture

medium equaled their original concentration in xylem sap. Cultures used as untreated controls were transferred to Erlenmeyer flasks containing dry residue of ethyl acetate extracts of water prepared in the same way as extracts of xylem sap in order to compensate for the effect of solvent impurities, softeners and other contaminants. The induction was stopped after 5 h by harvesting mycelium for RNA extraction. Each experiment consisted of three replicate with xylem sap extracts originated from different plant lots.

RNA extraction and modified cDNA-AFLP

Mycelium was crashed under liquid nitrogen and total RNA was extracted using a guanidinium isothiocyanate protocol with LiCl precipitation (19). cDNA-AFLP experiments were done according to Bachem et al. (20), while mRNA was immobilized on a column (Roche Applied Science, Penzberg, Germany) as described by Feron et al. (21). The standard cDNA-AFLP protocol was modified by using two sets of three restriction enzymes each, applied sequentially on immobilized cDNA. This procedure, increasing the coverage while keeping the redundancy low, was inspired by protocol published by Breyne et al. (22) and by Vuylsteke et al. (23). The sequences of adapters and primers are listed in Tab. 2.1. Four cDNA fragment pools were generated from two sets of immobilized cDNA. The first set was digested with TasI (Fermentas, St. Leon-Rot, Germany), this enzyme was called marking enzyme 1, and the released DNA fragments were discarded. After digestion with MaeII (synonym for HpyCH4IV New England Biolabs, Beverly, Mass), the released fragments were collected as the first fragment pool for preamplification. cDNA remaining on the column are digested with TaqI (Fermentas, St. Leon-Rot, Germany) and fragments released were collected as a second pool for preamplification. A second set of immobilized cDNA was digested with MboI (synonym for Bst143I Fermentas, St. Leon-Rot, Germany) used as marking enzyme 2. To reduce redundancy by excluding fragments containing TasI recognition site between Bst143I site and the polyadenylation site, immobilized DNA was also digested with TasI and fragments released were discarded. Truncated immobilized fragments were sequentially digested with HpyCH4IV (third pool) and TaqI (fourth pool).

Electrophoresis systems used for cDNA-AFLP

Collected cDNA fragments were labeled during selective cDNA-AFLP PCR with primers carrying either the fluorescent dye Cy5, Cy7 (Amersham Biosciences, Piscataway, USA), Dy682 or Dy752 (Dyomics, Jena, Germany). For cDNA fragment separation and detection

two different laser-based DNA electrophoresis systems were applied, a flat-bet gel electrophoresis (ALFExpress II, Amersham Biosciences, Piscataway, USA) and a capillary electrophoresis (CEQ 8000, Beckman Coulter, Fullerton, CA) to record DNA fragment electropherograms. When using ALFExpress II only Cy5 labeled cDNA fragments of single PCR primer combination samples could be analyzed per lane. The capillary electrophoresis were used for high throughput analysis of cDNA-AFLP fragment patterns, using four fluorescent emission detection channels for the reading of four different labeled PCR products in a unique capillary.

Quantitative evaluation of cDNA-AFLP signals

Raw data were imported and analysed with the GelCompar II software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Automated analysis of transcript fingerprints were done in a three step modus: (i) Migration time of bands in comparable lanes were normalised by selecting 5-10 endogenous peaks manually for internal standards. (ii) The intensity threshold for initial transcript signal detection was set to 1% relative to the highest peak intensity of the lane. In average 55 bands were detected per primer combination. (iii) For automated band matching a position tolerance of 0.3% of total length of gel image was set, which represents a position tolerance of a single nucleotide. A second band recognition was started with an intensity threshold of 1% of the total intensity in a lane or capillary and bands and unmatched after the first round not exceeding this threshold were discarded. Finally, GelCompar II determined densitometry intensity value corresponding to peak height for each detected transcript signal. Paired intensity values were then exported as sorted in a spreadsheet for the normalisation of peak intensities.

Normalization of densitometry intensity values

For the estimation of a normalization factor (NF), we calculated "uncorrected induction factors" (UIFs) for each pair of bands in corresponding lanes as the ratio of peaks heights corresponding to matched bands. NF was calculated as the mean of inner quartiles of sorted UIFs and applied to correct all absolute peak intensities (24). Induction factors were then calculated as the ratio of normalized peak intensities for matched bands in the treated sample to the intensity values in the control. cDNA-AFLP band patterns were analysed by single pairwise comparison of non treated vs. treated samples for the evaluation of differential expression by the 2.5 cut-off criterion.

Scatter plots

For the illustration of the cDNA-AFLP experiments the expression data of the first replicate including all 256 possible PCR primer combinations are depicted as scatter graphs (MA plots [25]), which were constructed by plotting the log(2) of the geometrical mean of normalized intensities of paired cDNA signals with $I_{control}$ and $I_{treatment}$ calculated by log(2) of square root (sqrt) of $I_{treatment}$ x $I_{control}$ on horizontal axis and the log(2) of ratios of intensities of paired cDNA signals ($I_{treatment}$ /$I_{control}$) on Y-axis.

Cloning of cDNA-AFLP fragments for sequencing

Flat-gel DNA sequencer ALFExpress II was used for preparative separation of cDNA-AFLP fragments. The pattern of bands labeled with Cy5 was recorded using a Typhoon 8600 laser scanner (Amersham Biosciences, Piscataway, USA) and printed. Polyacrylamide gel was aligned with the printed gel image and desired bands were located and excised. The DNA was purified out of the polyacrylamide piece following the protocol of Maxam and Gilbert (26). Purified DNA was used as template for re-amplification by PCR. Reamplified fragments were purified by agarose gel electrophoresis and ligated into pSK Bluescript KSII (Fermentas, St. Leon-Rot, Germany) for sequencing.

3´ RACE-PCR

With RACE PCR (rapid amplification of cDNA ends [27]), the same protocol for double-stranded cDNA synthesis as for real-time PCR was used, but instead of $(dT)_{20}$ primer an elongated oligoT nucleotide carrying a AGCCTAACCGTGAGAGAGCG G signature on its 5´ terminus was used. Gene-specific forward primers (Tab. S2) were paired with this universal primer. PCR products were separated on a 1.7% agarose gels, DNA was extracted, purified and used for direct sequencing with the same primers as for PCR.

Inverse PCR

Genomic DNA was extracted from *V. longisporum,* digested with restriction enzymes EcoRI, BamH, PstI, HindII, XbaI and SacI (enzymes from Fermentas, St. Leon-Rot, Germany) and self-ligated. Inverse gene-specific primers based on cDNA-AFLP fragment sequences were used for PCR. PCR products were separated on a 0.8% agarose gel, purified and either directly sequenced using PCR primers or cloned in standard plasmid vectors.

PCR-based gene extraction from a single clone genome library of V. longisporum

Genomic DNA was extracted from *V. longisporum* mycelium using CTAB protocol (28). For partial digestion 20 µg DNA was incubated with 0.4 U MboI for 30 minutes. Digested DNA was electrophoretic separated on a 0.6% agarose gel at 20 V over night. DNA fragments of 8–14 kb were excised and purified from the agarose gel using QIAEXII kit (Qiagen, Hilden, Germany) and ligated into pUC57 vector (Fermentas, St. Leon-Rot, Germany). For library construction cloned fragments were transformed into electrocompetent *E. coli* DH5α cells. The library contained a total number of 9,600 single clones in a 100 x 96 microtiter plate format. About 50% of the clones were determined by electrophoresis as containing circularized plasmid without any insert. For the screening of genes a strategy of stepwise of in total 40 PCRs with gene-specific primers (Tab. S2) were chosen to pinpoint single clones carrying an insert, which contained the searched gene sequence. In a first replicate of ten PCRs pooled clones of ten microtiter plates were used per reaction and analysed by electrophoresis on a 1.7% agarose gel. The positive clone pool showing a gene-specific PCR product were used for a dissecting second replicate of 10 PCRs, in which pooled clones of single microtiter plates were analysed. To pinpoint the single clone a third replicate of 20 PCRs dissected the positive tested microtiter plate by analysing pooled clones of each row and each line of the microtiter plate. This resulted in a single positive tested row and a single positive tested line and the crossing point localized the position of the positive single clone used to sequence the insert.

Determination of transcript levels by quantitative reverse transcription real-time PCR

RNA extracted for cDNA-AFLP was used for two-step qRT-PCR (quantitative reverse transcription real-time PCR) validation of cDNA-AFLP results (see above). For the first strand cDNA synthesis, 500 ng of total RNA was incubated with 100 pmol $(dT)_{20}$ primer, 1.0 mM dNTP mix, 20 U RiboLock RNAse inhibitor and 200 U MMLV-reverse transcriptase (enzymes from Fermentas, St. Leon-Rot, Germany) in 20 µl at 42°C for 90 min. cDNA was used as template for real-time PCR using SybrGreen Master Mix (Takara Bio Inc., Shiga, Japan). iCycler System (BioRad, Hercules, San Diego, CA) was used for amplification and melting curve analysis. Gene specific primers, derived from sequenced cDNA-AFLP fragments were used to amplify target fragments corresponding to the cDNA-AFLP fragments (Tab. S2). For amplification of constitutively expressed housekeeping gene, β-tubulin primers were used to amplify a 343 bp long fragment. During PCR, the detection of

fluorescence was carried out in the annealing step of each cycle. Following amplification, the melting curves were acquired by heating the samples to 95°C for 1 min, cooling to 55°C for 1 min and then slowly increasing the temperature from 65°C to 95°C at the rate of 1°C/min with a continuous measurement of the fluorescence. For quantification of transcript levels of target genes *in vitro* we selected β-tubulin as reference gene. Relative expression levels are given as mean ΔcT values. Analysis of significance of ΔcT values was done using unpaired t-test and two significance levels were set to $p < 0.1$ and to $p < 0.05$.

Quantification of fungal DNA in plant tissue by real-time PCR

200 mg fresh weight of plant hypocotyl tissue from *Verticillium*-infected plants was grinded under liquid nitrogen. Genomic DNA was extracted following the CTAB protocol (see above). To quantify fungal DNA within the extracted genomic DNA, real-time PCR with SybrGreen performed in iCycler (BioRad, Hercules, CA) was used. In PCR a 261 bp fragment of ITS region was amplified by the primer OLG 70 (CAGCGAAACGCGATATGTAG) and OLG 71 (GGCTTGTAGGGGGTTTAGA) and quantified (15). The absolute amount of fungal DNA in the plant matrix was derived from a calibration curve constructed from a dilution series of fungal DNA.

In planta expression analysis of V. longisporum genes by quantitative reverse transcription real-time PCR

Root and hypocotyl tissues of infected plants were harvested after 14, 21, 28, 35 dpi and used for total RNA extraction using a hot phenol extraction protocol. Total RNA from six infected plants were pooled and three pools of biological replicate were sampled at each time point. Polyadenyl-RNA was purified from total RNA extracts using Oligotex mRNA Purification Kit (Qiagen, Hilden, Germany). For first step RT reaction 500 ng mRNA was used with 50 pmol oligo-T primer, 1 mM dNTPs, 20 U RiboLock RNAse inhibitor and 200 U RevertAid reverse transcriptase (enzymes from Fermentas, St. Leon-Rot, Germany) in a 20 µl reaction volume. After RT reaction cDNA was purified using PCR Purification kit (Qiagen, Hilden, Germany) and 100 ng purified cDNA quantified using photo-spectrometer absorption measurement at 260 nm (GeneQuant, Cambridge, UK) was used as template for second-step real-time PCR using the same SybrGreen system described above. Two housekeeping genes, β-tubulin and ribosomal protein S17 (29), were used for normalization.

Means of ΔcT values were used to calculate change fold factors between *in vitro* and *in planta* samples according to the ΔΔcT method.

Table 4.1: Primers and adapters used in cDNA-AFLP.

No.	Sequence	Purpose
1	AATTCCTGCAGGGTCGTGCTAGTAGCT	TasI adapter forwards
2	TACAGCTACTAGCACGACCCTGCAGG	TasI adapter backwards MboI adapter backwards
3	GATCCCTGCAGGGTCGTGCTAGTAGCT	MboI adapter forwards
4	AGCTACTAGCACGACCCTGCA	TasI primer preamplification and MboI primer preamplification
5	GACCCTGCAGGAATTN	TasI primers amplification
6	ACCCTGCAGGGATCN	MboI primers amplification
7	CGGCGGCCGCTCAGTGTTAGTGTGACG	MaeII and TaqI adapter forwards
8	CATCGTCACACTAACACTG	MaeII adapter backwards and TaqI adapter backwards
9	CGTCACACTAACACTGAGCGGC	MaeII primer preamplification and TaqI primer preamplification
10	AGCGGCCGCCGTNN	MaeII primers amplifications
11	AGCGGCCGCCGANN	TaqI primers amplifications

RESULTS

Transcriptome analysis of V. longisporum by cDNA-AFLP

The methods currently dominating open-end transcriptomics are cDNA differential display (30) and cDNA-AFLP (20). We chose the latter for our work due to better reproducibility and a lower rate of false-positives in cDNA-AFLP as compared to differential display (31). A modification of the original cDNA-AFLP protocol allowed us to improve the coverage while keeping redundancy at a low level (see chapter 2).

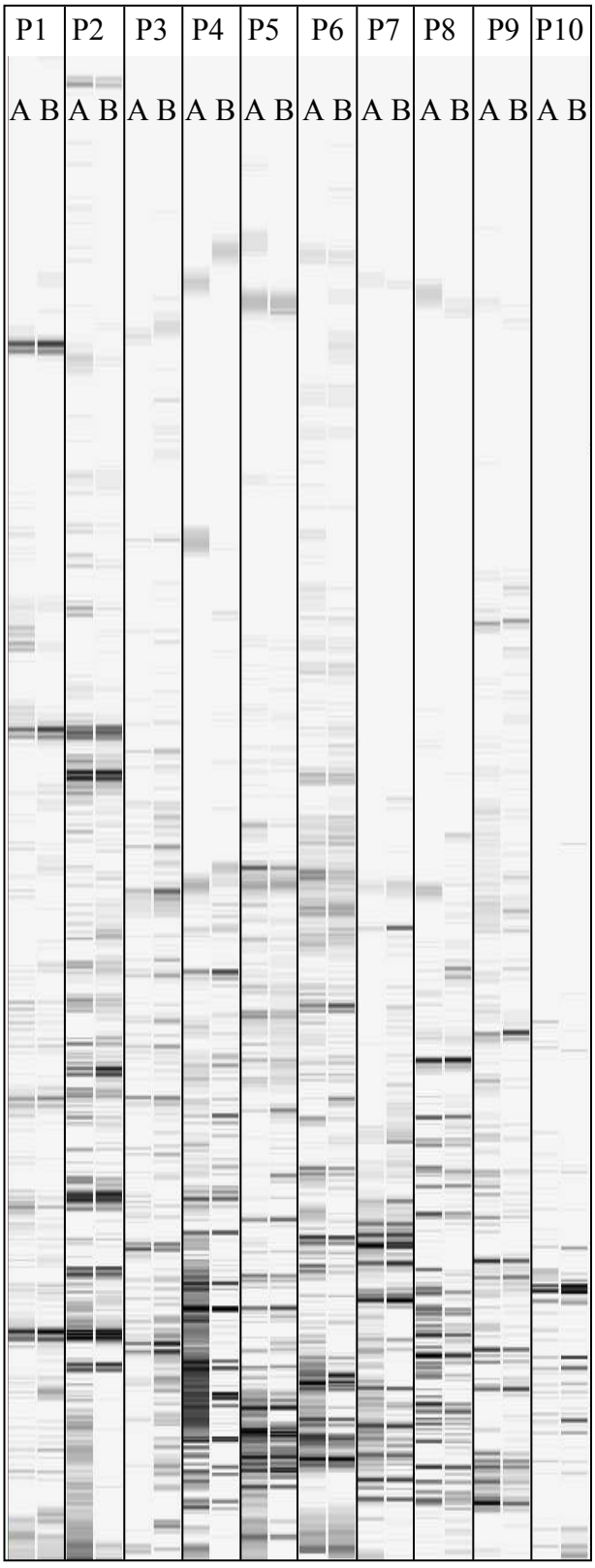

Figure 4.1: Virtual gel image of band patterns for pairwise cDNA-AFLP transcriptome profiling in *V. longisporum*. Pairwise band patterns of 256 different PCR primer combinations (on this image ten of them as P1-P10) were compared in each cDNA-AFLP experiment of non-treated versus xylem sap sample-treated versions. (A) Non-treated version, (B) treated version. Electrophoretic separation and detection of amplified DNA fragments was carried out using capillary electrophoresis (automated DNA sequencer) creating virtual gel images. Band pattern images were used for quantitative evaluation of differential gene expression profiling.

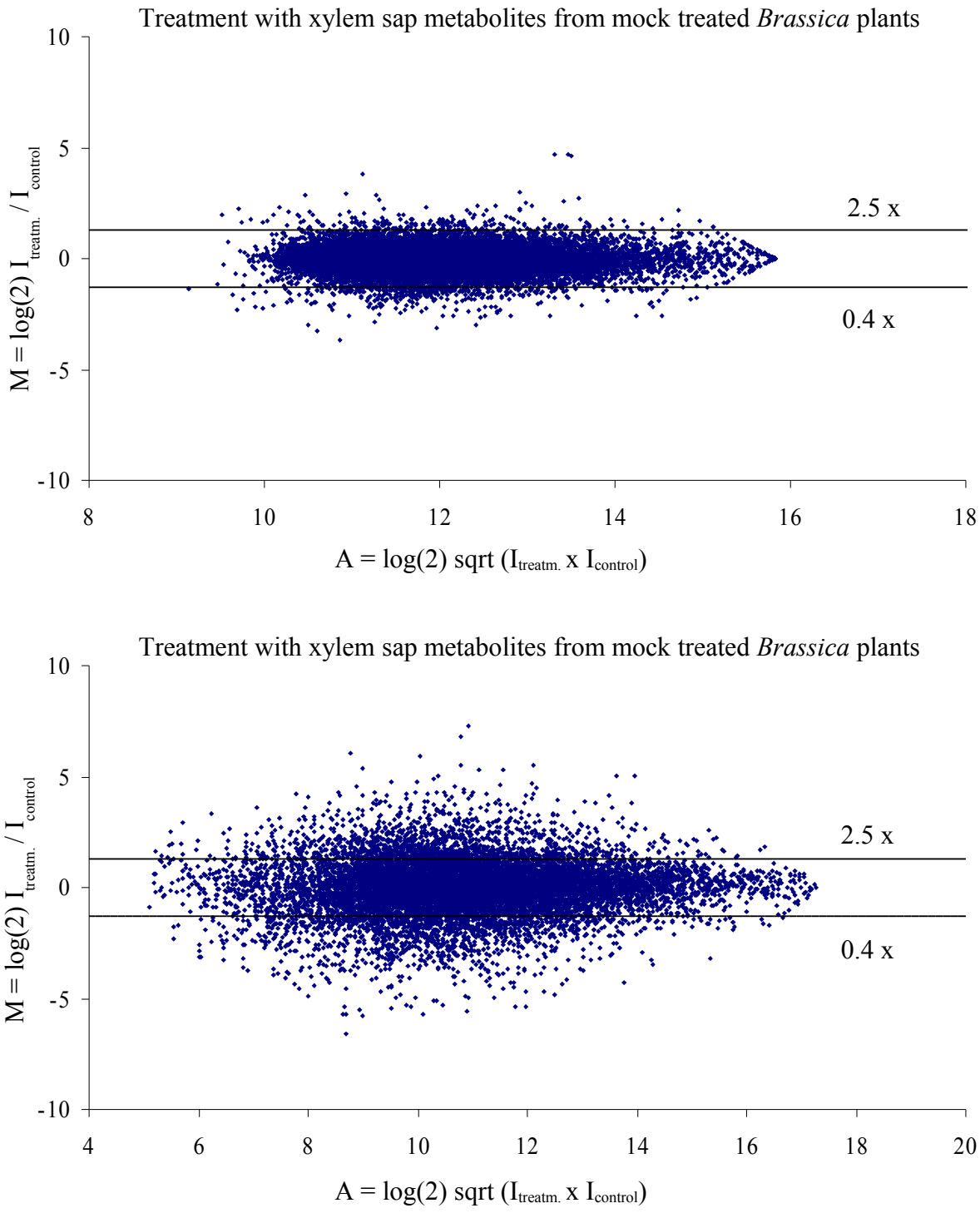

Figure 4.2: MA scatter plots of pairwise transcriptomic data of *V. longisporum* generated by cDNA-AFLP. Results of the 1st replicate of each cDNA-AFLP experiment are shown as MA scatter plots. Left: treatment with xylem sap from mock-treated plants. Right: treatment with xylem sap from *Verticillium*-infected plants. Horizontal lines show cut-off value of 2.5 for signal ratios, which was used to select primer combination for biological replicate.

Because we were interested in genes specifically induced by secondary metabolites of *B. napus* but not in catabolic pathways, which are turned on by sugars and amino acids, and because we wanted to exclude genes induced by mineral ions, we used ethyl acetate extracts of xylem sap for the treatment of fungal cultures. The amount of extract added to the medium was adjusted in such a way that concentration of putative metabolites reached the same level as the original xylem sap, provided extraction of these metabolites into ethyl acetate was complete. A short incubation time of 5 h was chosen to limit transcriptome changes caused by the depletion of common precursors and other secondary effects. Because the composition of xylem sap is likely to change due to infection, we generated two transcriptome profile sets, comparing the effects of xylem metabolites extracted from mock treated plants and from plants infected with *V. longisporum*. In each cDNA-AFLP experiment we applied 256 primer combinations and compared pairwise band patterns originated from the non-treated versus sample-treated versions (Fig. 4.1). The outcome of the two cDNA-AFLP experiments is shown in Fig. 4.2. Primer combinations generating patterns which contained signals with normalized intensities enhanced due to the treatment more than 2.5-fold or reduced to less than 40% as compared to the control were selected for a second replicate with independent cultures. A third replicate was analyzed using primer combinations which revealed induced or suppressed signals in the first and second replicate. A summary of cDNA-AFLP replicate, the number of transcripts generated by a number of PCR primer pairs and the fraction of differentials, which kept exceed the cut-off in each replicate, are shown for both experiments in Tab. 4.2. Because no transcript could be identified exceeding the 2.5 cut-off in all three replicate in the experiment treating *V. longisporum* with xylem sap extracts from mock treated plants, we, nevertheless, accepted 3 transcripts as differentially expressed exceeded the 2.5 cut-off in the first two replicate and showed normalized intensities changed due to the treatment more than 2.0 fold in the third replicate. In the experiment with xylem sap from infected plants 31 exceeded the 2.5 cut-off three times and were accepted as differentially expressed. Out of them, 23 signals occurred both in the control and treated sample in all three replicate, 7 signals occurred both in control and treatment in two replicate only (in one of the replicate the signal was detected only in the control or in the treated sample and were accepted as differentially expressed), 1 signal occurred in both control and treatment in one replicate only (in two replicate the signal was

detected only in control or treatment). The electropherograms of one of the most distinctly differentially expressed transcripts are shown in Fig. 4.3.

Table 4.2: Gene discovery strategy in cDNA-AFLP for the identification of regulated transcripts based on selective PCR replication.

	Treatment with xylem sap metabolites from mock treated plants			Treatment with xylem sap metabolites from *Verticillium*-infected plants		
RNA sample	**1st replicate**	**2nd replicate**	**3rd replicate**	**1st replicate**	**2nd replicate**	**3rd replicate**
cDNA signals	33,223	8,424	590	22,373	21,449	4,148
Primer pairs	256	169	11	256	237	84
Differentials	1,238	18	3	2,703	294	31

1st replicate of expression data were generated using all 256 PCR primer combinations. For the 2nd and 3rd replicate only those primer combination were performed including signals (differentials) exceeded the cut-off threshold.

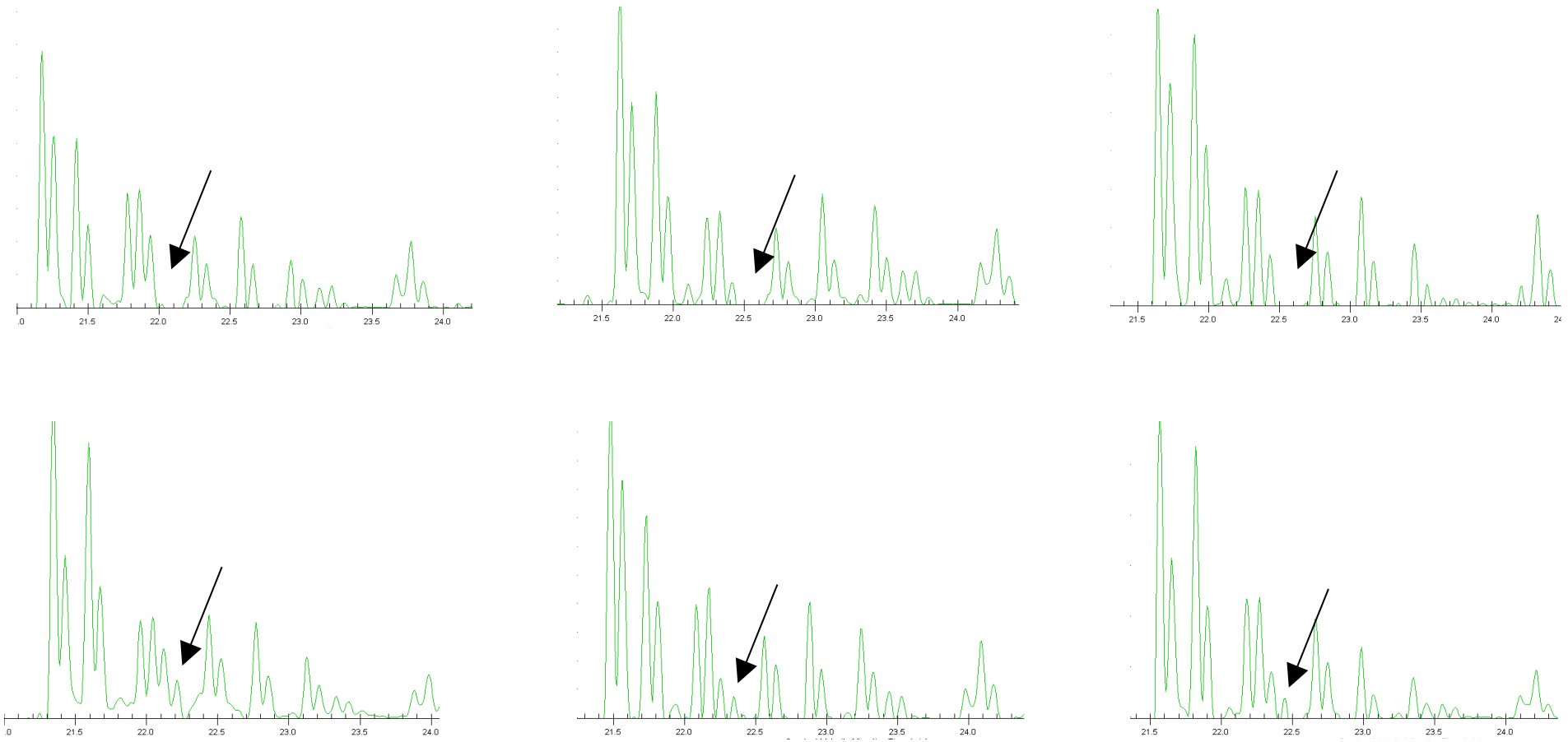

Figure 4.3: Differential expressed transcript detected by cDNA-AFLP. Electropherograms of three replicate of each non-treated (top) and treated experiments (bottom) with xylem sap extracts from *V. longisporum*-infected plants generated by a unique primer combination in which the candidate transcript TDF 12 was identified. The enhanced peak heights of differential transcript TDF 12.1 are labeled by arrows within the chromatograms.

Fragment sizes and primer combinations (site of restriction enzyme and selective nucleotides) of all transcript derived fragments (TDF) accepted as differentially expressed as well as the change fold factor calculated as the geometric mean of intensity ratios are given in Tab. 4.3. Based on the size and the flanking sequence expression data can be linked to DNA sequence libraries of *V. longisporum* (32).

Table 4.3: List of transcripts accepted as differentially expressed by cDNA-AFLP.

Transcript-ID	Primer combination		Fragment size (bp)	Change fold factor
Treatment with xylem sap metabolites from mock treated plants				
TDF 2	TasI-T	TaqI-GC	71	3.17
TDF 5	TasI-T	MaeII-GT	80	0.25
TDF 6	TasI-G	MaeII-AT	78	0.44
Treatment with xylem sap metabolites from *Verticillium*-infected plants				
TDF 7	TasI-A	MaeII-GC	71	3.03
TDF 8	TasI-C	TaqI-TA	65	3.23
TDF 9	TasI-C	TaqI-GT	77	4.31
TDF 10	TasI-T	MaeII-GC	68	3.06
TDF 11	TasI-T	TaqI-TT	72	2.51
TDF 12	TasI-C	MaeII-AA	172	10.32
TDF 13	TasI-T	MaeII-GA	44	6.17
TDF 14	TasI-A	TaqI-AT	102	0.32
TDF 15	TasI-C	MaeII-TA	71	3.35
TDF 16	TasI-A	TaqI-TG	40	3.88
TDF 17	TasI-C	TaqI-AG	63	6.34
TDF 19	TasI-C	TaqI-AA	86	3.29
TDF 21	TasI-A	TaqI-TA	92	12.06
TDF 22	TasI-A	TaqI-TA	93	3.86
TDF 24	TasI-T	TaqI-CA	56	3.37
TDF 25	TasI-T	TaqI-GC	135	10.23
TDF 26	TasI-C	MaeII-TA	104	3.62
TDF 27	TasI-C	TaqI-AA	75	3.07
TDF 29	TasI-A	MaeII-TC	74	0.04
TDF 30	TasI-G	TaqI-AA	75	2.51
TDF 31	TasI-A	MaeII-GC	182	4.49
TDF 32	MboI-A	MaeII-AA	79	5.39

TDF 33	MboI-A	MaeII-GG	97	9.39
TDF 34	MboI-G	MaeII-AA	76	6.56
TDF 35	TasI-C	MaeII-AA	91	3.27
TDF 36	TasI-C	MaeII-AA	92	3.95
TDF 37	TasI-G	TaqI-GC	59	0.18
TDF 38	TasI-G	MaeII-AC	80	0.32
TDF 39	TasI-C	MaeII-CA	199	0.31
TDF 40	MboI-A	MaeII-TA	82	0.24
TDF 41	TasI-G	TaqI-GC	86	0.15

Twenty-one cDNA-AFLP bands out of the 34 fragments accepted as differentially expressed towards all three replicates were selected for excision from PAA gels, re-amplification and cloning. 18 out these 21 fragments were successfully cloned and sequenced. cDNA bands selected from the pool for cloning had to be greater than 50 bp, signals had to be of high intensity for band detection on the fluorescence scanner and co-migrated cDNA-bands of also high intensity must be in distance to the candidate band of at least 10 bp (Fig. 4.4).

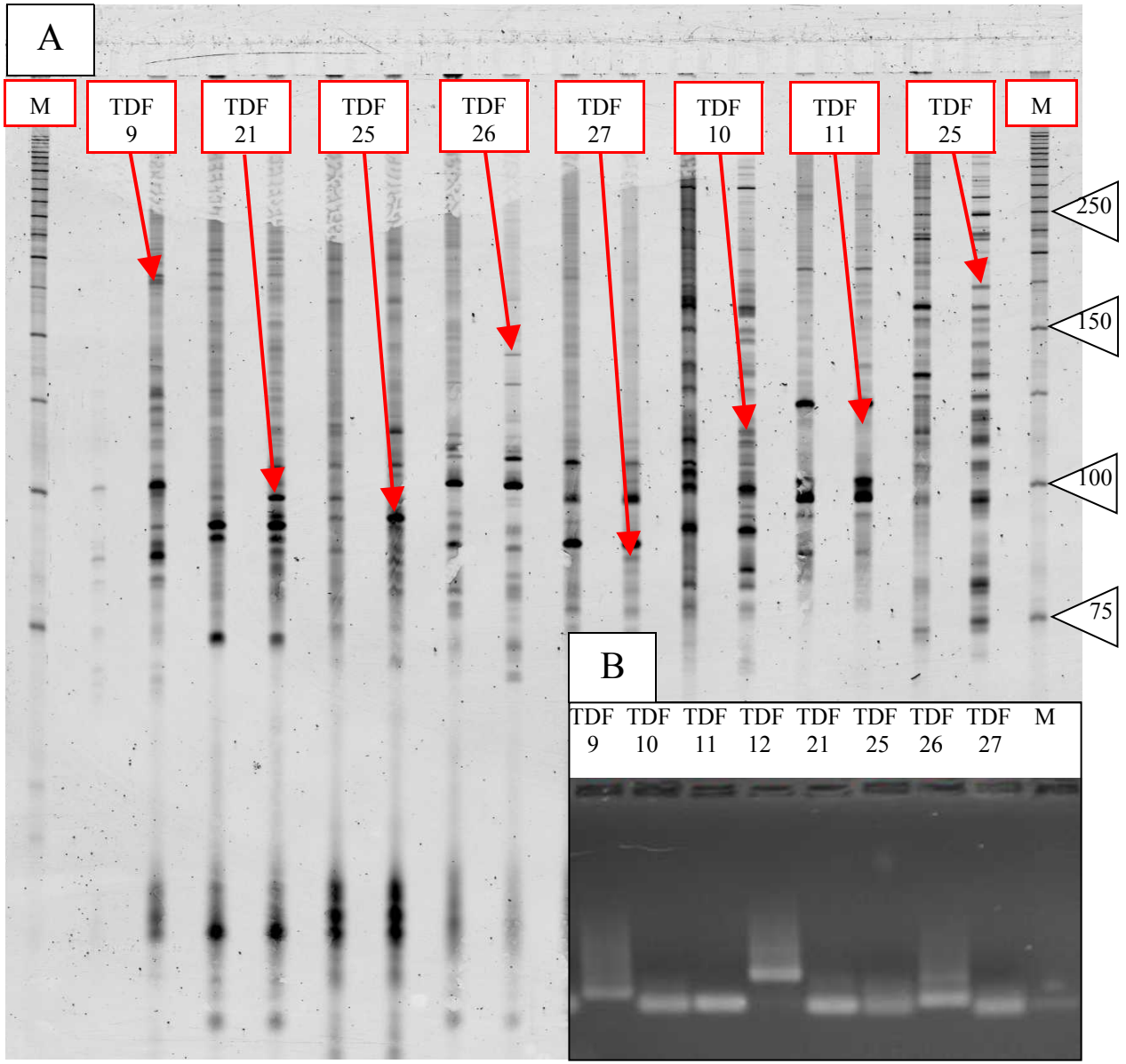

Figure 4.4: Polyacrylamide gel electrophoresis for cDNA fragment preparation and analytical agarose gel electrophoresis of re-amplified cDNA fragments.
Preparative electrophoresis and recognition of cDNA fragments selected for cloning and sequencing (TDFs) are marked with red arrows in (A).
The DNA was purified out of the polyacrylamide piece and used as template for re-amplification by PCR (B).
(M) indicates DNA size markers: in polyacrylamide-based gel electrophoresis band sizes of 50-500 bp in 25 bp steps; in agarose gel electrophoresis a 100 bp fragment.

Because most of the extracted fragments still consisted of mixtures of several sequences, up to 16 clones from each fragment were sequenced, resulting in a total of 124 sequences, 78 of which were unique (Tab. S1). The average length of cloned fragments was 82 bp. We extended 10 cDNA sequences, 4 to full-length using different strategies in order to improve the reliability of BLAST search and primer design for gene-specific PCR (Tab. 4.4).

Table 4.4: pBLAST results obtained for sequence-extended candidate cDNAs of *V. longisporum*.

Transcript-ID	TDF size (bp)	Type of sequence extension	cDNA size (bp)	Encoded putative protein
TDF 2.1	71	4	630	Isochorismate hydrolase
TDF 6.2	78	3	4540	WSC carbohydrate binding
TDF 7.1	71	2	1560	Unknown protein
TDF 10.2	68	4	1591	Zinc-finger transcription factor
TDF 12.1	204	1, 4		
TDF 11.1	72	4	2289	Catalase
TDF 22.2	68	2	752	Type II HAD hydrolase
TDF 24.1	56	2, 4	1403	Unknown protein
TDF 26.2	104	2	928	Unknown protein
TDF 34.2	76	2	588	Cell death regulator

Strategies for transcript sequence extension: 1 – RACE-PCR, 2 – inverse PCR, 3 – gene extraction from a *V. longisporum* genome library, 4 –amplification and sequencing of *V. longisporum* PCR products using gene specific primers according to *V. dahliae*VdLs17 genome sequence (33).

Among the 78 unique cDNA-AFLP fragment sequences we selected those to confirm differential expression, which sequence occurred more than once (see supplementary data, Tab. S1) or rather for which a high change fold factor was calculated. For 21 transcript fragments we succeeded in designing gene-specific primers (Tab. S2) to measure relative differences of expression by quantitative reverse transcription real-time PCR using the same RNA templates as in the cDNA-AFLP experiments. Nine transcripts were validated to be regulated showing a significant change in relative expression levels (Fig. 4.5).

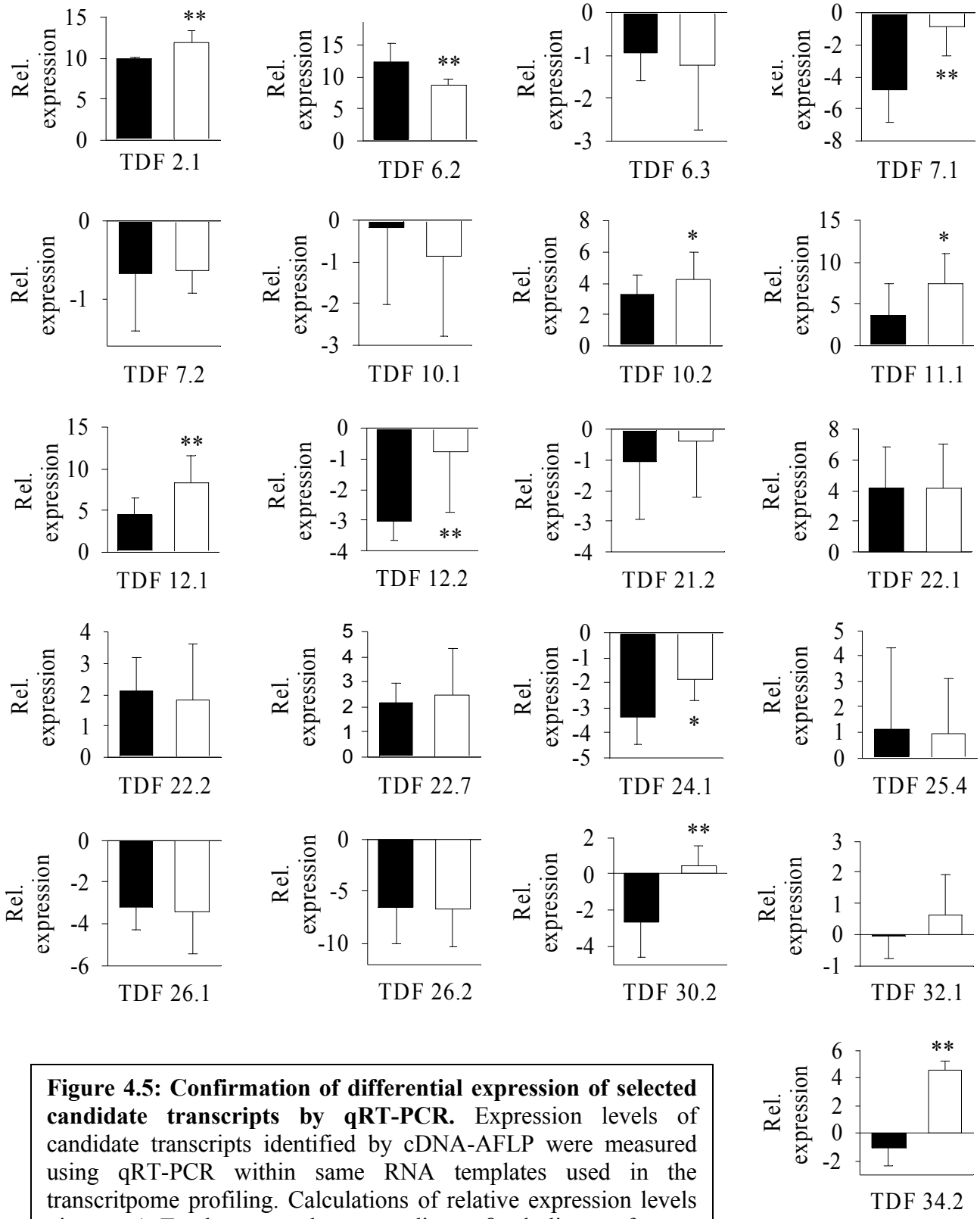

Figure 4.5: Confirmation of differential expression of selected candidate transcripts by qRT-PCR. Expression levels of candidate transcripts identified by cDNA-AFLP were measured using qRT-PCR within same RNA templates used in the transcritpome profiling. Calculations of relative expression levels given as ΔcT values were done according to β-tubulin as reference gene. Black bars given the geometric mean of expression levels of non-treated fungal samples and white bars expression levels of xylem sap induced samples. Error bars indicate the standard deviation. Significance levels in student's test were set to $p < 0.1$ (single asterisk) and $p < 0.05$ (double asterisks).

In planta transcript levels of selected V. longisporum genes

Verification of the effect of xylem sap on *V. longisporum* by qRT-PCR described above was carried out using RNA isolated from pure fungal cultures treated with xylem sap extracts. In order to see whether these effects occur *in situ* during the colonization of the plant we used qRT-PCR to quantify the levels of fungal transcripts within plant tissue. As negative control we compared transcript levels *in planta* with the levels in fungal liquid cultures in SXM medium (18), which simulates the composition of xylem sap concerning pectin, certain amino acids and mineral ions, but does not contain secondary metabolites of *Brassica* that have also been used as control samples in the cDNA-AFLP experiments. The expression of fungal transcripts was measured in root and hypocotyl tissue of *Verticillium*-infected *Brassica* plants after 14, 21, 28 and 35 dpi. During the time period from infection till 28 dpi (days past inoculation) the fungus remains confined to xylem of root and hypocotyl, while disease symptoms remain limited to stunting and chlorosis (34). At 35 dpi the fungus colonizes the stem and extended chlorosis and senescence develop, sometimes accompanied by premature flowering and necrosis.

Visible symptoms of *V. longisporum* infection of host plants are depicted in Fig. 4.6 and were assessed by scoring the disease according to Zeise (35) and by measuring the plant shoot height. Fungal propagation within the plants was determined as the amount of fungal DNA within hypocotyl tissue by quantitative real-time PCR (Tab. 4.5).

Table 4.5: Assessment of disease symptoms and fungal DNA in *V. longisporum*-infected *Brassica* plants

	Mock treated *B. napus*		*Vertcillium* infected *B. napus*		
Days post inoculation	**Disease score**	**Shoot length (cm)**	**Disease score**	**Shoot length (cm)**	**ng fungal DNA per mg fresh weight**
14	1.00±(0.00)	10.55±(1.31)	1.00±(0.00)	8.64±(1.08)	1.24±(0.21)
21	3.03±(0.60)	20.55±(7.22)	3.71±(0.66)	9.85±(1.04)	1.21±(0.29)
28	3.13±(0.42)	31.93±(4.86)	4.84±(0.67)	15.82±(6.94)	1.70±(0.41)
35	3.24±(0.39)	36.19±(6.40)	5.98±(0.73)	30.24±(7.28)	6.15±(2.00)

Standard deviation is given in brackets.

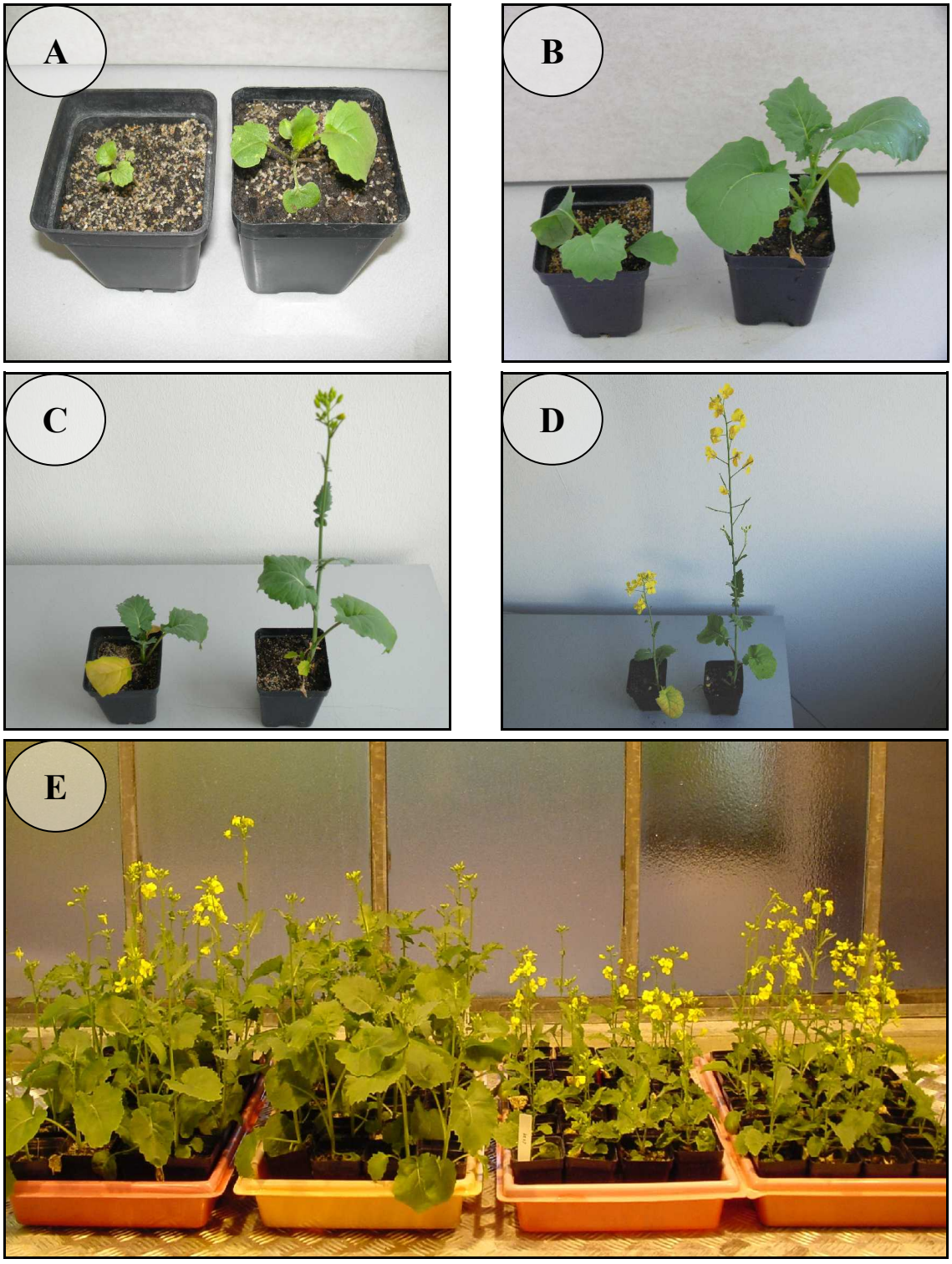

Figure 4.6: Visible disease symptoms on *B.napus* after infection with *V. longisporum*. Typical symptoms on *B. napus* after infection with *V. longisporum* under greenhouse conditions are shown. (A) After 14 dpi first symptoms of stunting effects become visible, which are more evident after 21 dpi (B). Till 28 dpi (C) the fungus remains confined to xylem of root and hypocotyl, while disease symptoms remain limited to stunting and chlorosis. At 35 dpi (D), during the mature stage of *Brassica*, the fungus colonizes the stem and extended chlorosis and senescence develop, sometimes accompanied by premature flowering and necrosis. (E) shows sets of mock treated (left site) and *V. longisporum*-infected *Brassica* plants (right site) after 28 dpi used for xylem sap preparation and extraction of xylem metabolites by ethyl acetate.

All 10 confirmed transcript fragments identified and confirmed as differentially expressed *in vitro* were chosen for measuring *in planta* expression. In addition two candidate genes not identified by cDNA-AFLP were selected, because we expected to be up-regulated within the plant tissue. The first one was assigned *VlNEP*, the product of which is a putative member of necrosis and ethylene inducing peptides (NEPs) shown to elicit plant responses and symptom development in different plant systems. *VlNEP* is a homologue of *V. dahliae VdNEP* (36), which was identified in the genome of *V. longisporum*. The second gene selected for *in planta* expression studies, assigned as *VlPKS1*, putatively encodes a type I polyketide synthase. A fragment of the gene was isolated based on its conserved L-ketoacylsynthase domain and extended by primer walking (Wolf et al. unpublished). Relative expression levels of fungal transcripts in hypocotyl samples and *in vitro* samples of SMX-growing mycelium as the control were used to calculate a change fold factor describing *in planta* vs. *in vitro* expression ratios. For 9 out of 11 candidate genes transcripts could be detected *in planta* by qRT-PCR. Seven of them showed significant change in expression levels *in planta* compared to *in vitro* (Fig. 4.7).

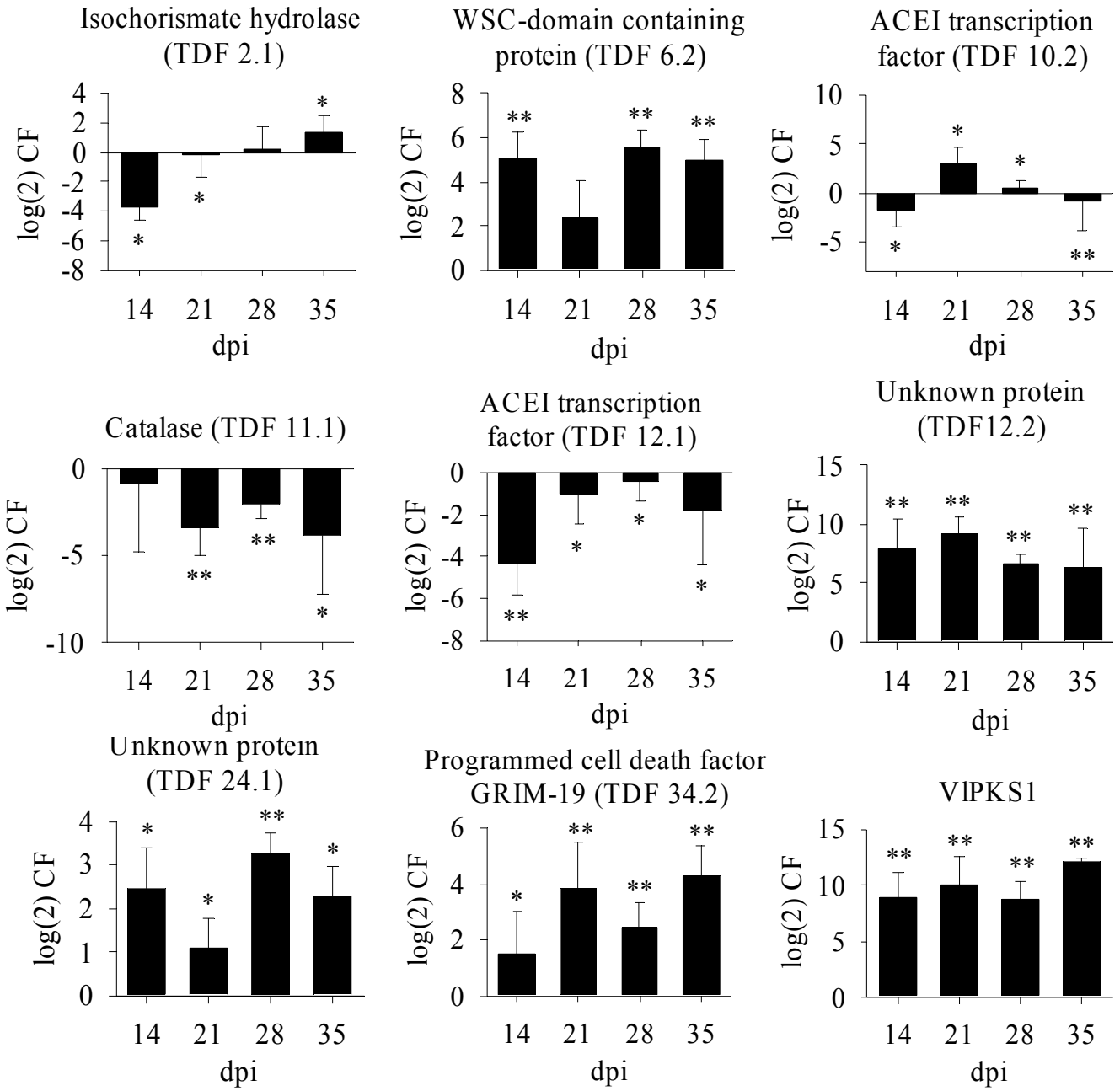

Figure 4.7: *In planta* gene expression analysis of selected *V. longisporum* target genes at different time points after infection. Transcript levels of selected *Verticillium*-genes were measured and normalized using two housekeeping genes, β-tubulin and ribosomal peptide S17. Ratios of relative expression levels between *Verticillium in vitro* samples (SXM) as control and *in planta* samples (root and hypocotyl tissue of *Vertcillium*-infected *Brassica* plants) were calculated as the mean of 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 both reference genes, respectively; single asterisk indicates significant change in the expression levels regarding to one of the reference genes and double asterisks regarding to both reference genes.

DISCUSSION

Transcriptome profiling of the effect of xylem sap extracts prepared from mock treated and V. longisporum-infected B. napus plants on gene expression in V. longisporum

V. longisporum is well adapted to *B. napus* varieties with high glucosinolate content (37) and grows well on agar plates containing glucosinolates (38). This ability appears to be *V. longisporum*-specific, because *V. dahliae* is inhibited by *Brassica spp*. extracts and the growth suppression correlates with glucosinolate content of the plant (39).Other metabolites like oxylipins can act as natural fungicides of *Brassica* towards different fungal pathogens. In contrast to other *Brassica*-specific pathogens *V. longisporum* showed no significant affects in hyphal growth rates by ten different epoxy and hydroxy fatty acids tested as biostatic against *Phoma lingam*, *Sclerotinia sclerotiorum* and *Alternaria brassicae* in an *in vitro* growth inhibition assay (40). We are particularly interested in learning, whether phytoalexins and other fungitoxic secondary metabolites of *B. napus* trigger specific responses in the fungus and therefore decided to use ethyl acetate extract of xylem sap rather than whole xylem fluid for the treatment of fungal cultures for cDNA-AFLP experiments. The intention was to exclude amino acids, sugars, polymers and mineral ions which are likely to affect gene expression in *V. longisporum*, too. Changes in the transcriptome due to treatment with xylem sap extracts are likely to be caused by inducible plant defense chemicals (phytoalexins), because we observed essentially no changes in cultures treated with xylem sap from mock treated plants and transcript profiles exhibited a clear difference between treatments with xylem metabolites from mock treated and *Verticillium*-infected plants (Fig. 4.1, Tab. 4.2). This indicated that plant metabolites induced by infection with *V. longisporum*, which accumulate in xylem and are extractable to ethyl acetate, exert specific effects on *V. longisporum* transcriptome. Chemical analysis of ethyl acetate extracts of xylem sap from *B. napus* plants infected with *V. longisporum* will be needed as a first step towards the clarification of the cause of the observed effects. The availability of sequences of genes induced by xylem sap extracts will facilitate the identification of the metabolites responsible for the effect. This might be achieved by fractionation guided by qRT-PCR or by assays with marker gene fusions.

The low number of differentially expressed genes identified in our transcriptome study indicated that *V. longisporum* is well adjusted to this environment, though the amplification step in cDNA-AFLP experiments facilitates the visualization of low-level transcripts. For example, identified candidate genes corresponding to transcripts appear to be transcription factors or other regulatory proteins (TDF 12.1 – a putative zinc-finger transcription regulator, TDF 30.2 – a putative transcription regulator, TDF 34.2 – a putative programmed cell death regulator). Because the

fungus is well adapted to vascular tissue, spending the largest part of its life cycle in xylem sap (except for the dormant stage), we assume that the majority of genes that *V. longisporum* required in xylem sap environment are active constitutively. Extensive re-programming of gene expression might occur at other stages of the life cycle, for example during the formation of microsclerotia and/or the induction of dormant propagules to germination. We expect that considerable changes in gene expression profiles will be found when these life phases are compared to the growth in xylem sap.

Although the false positive rate is reduced in cDNA-AFLP as compared to cDNA differential display, the number of hits not confirmed in replicate was high. This observation is common to all expression profiling technologies including microarrays. A disadvantage of cDNA-AFLP as compared to microarrays is that the verification of hits by full-scale replicate is too laborious. We therefore repeated merely primer combinations which generated hits in all previous replicates. This strategy leaves false negatives undisclosed and may thus underestimate the number of genes affected by the treatment. The identification of induced and suppressed transcripts was not biased towards low or high intensities, because signal variance was independent of intensity as indicated in the MA scatter plots (Fig. 4.1). This is a marked difference to microarray technology, which has been discussed in chapter 3, where the signal variability for low intensity values is so high that these signals are often discarded. We therefore assume that the low frequency of transcripts affected by xylem sap extracts found in our experiments reflected the adaptation of *V. longisporum* to xylem sap environment rather than a low sensitivity or incomplete coverage of cDNA-AFLP profiles.

V. longisporum transcripts affected by xylem sap metabolites in vitro and in planta

Because the cDNA fragments isolated from the cDNA-AFLP gel almost consisted in mixtures of several sequences within only one hold the true differential, qRT-PCR was used for their identification as well as for the confirmation of differential expression. Towards the 14 tested TDF groups, comprised 21 individual transcript fragment sequences, 10 TDFs within 9 groups could be confirmed as being differential expressed, whereas two tested transcripts from TDF 12, TDF 12.1 and TDF 12.2, were both validated as up-regulated. To characterize whether these nine genes are also be regulated during the infection process of *V. longisporum*, we measured the expression within hypocotyl tissue of infected plants. We also included two further genes within *in planta* expression analysis expected to be up-regulated during infection process. The first one putatively coding for a necrosis and ethylene-inducing peptide, we designated as VlNEP, and the other comprised high homology to a known polyketide synthase type I, thus we called VlPKS1. Among these 11 target genes 7 could be classified as significantly regulated *in planta* regarding

to two independent housekeeping genes. While two regulated transcripts of the cDNA-AFLP screening (TDF 12.2 and TDF 24.1) kept unknown in their putative function, we matched hits to known gene function by pBLAST alignment search for regulated genes (Tab. 4.4).

V. longisporum transcript corresponding to TDF 6.2 comprises an ORF of 4540 nucleotides and translated cDNA is predicted to code for a 1536 amino acids (aa) protein. Within the ORF putative domains of two different functions were identified, but a proper prediction of the whole gene is not been known, yet. At the C-terminus four-fold repetitive WSC (water-soluble carbohydrate binding) domains were found on the basis of sequence similarities. The involvement in response to heat shock and to other extra-cellular stress factors was demonstrated in the yeast *S. cerevisiae* WSC-domain containing genes (*wsc1, wsc2, wsc3*). Further on, functionality of these genes in cell wall integrity was reported for the same organism (41, 42, 43). Gene deletions (single, double and triple mutants in *wsc* genes*)* in *S. cerevisiae* indicated a role in sensing and modulating stress response by the PKC1-MPK1 and the RAS-cAMP pathways (41, 44, 45). Upstream of the repetitive WSC-domains a single fibronectin type III (FN3) motif was identified. FN3-modules are widely spread in animal proteins and occur mainly as part of extracellular, receptor-like proteins or in polypeptides involved in adhesion (46). Interestingly, FN3-domains were also identified in different bacteria in conjunction with amylase, endoglucanase and cellulase activities (47, 48). Differences between the genes of different bacterial species were not greater than between different animal species, this result suggests a horizontal gene transfer event acquired from an animal cell (46). Meanwhile, FN3-like sequences have also been found in saprophytic and plant pathogenic fungi (49, 50), and activities of peptides harbouring FN3-modules are comparable to those described above. Both functionalities let raise the suggestion of a putative role of this gene in sugar uptake and in adhesion to carbohydrate like surfaces.

The gene sequence corresponding to the cDNA-AFLP transcript derived fragment (TDF) 12.1 comprised high sequence homology to the Cys_2-His_2-type zinc-finger transcription factor (TF) ACEI , which was characterized in the yeast *H. jecorina* (identity of *V. longisporum* cDNA to ACEI was 49.8%). A multiple alignment of putative Vl-ACEI from *V. longisporum* with conserved protein sequences of other fungi is shown in Fig. 4.7. The nucleotide sequence identity within this gene between *V. longisporum* and *V. dahliae* was determined at a level of 95%, which was in the range of identities (95-99%) due to other gene sequences characterized in this work (data not shown). ACEI was elucidated as an effective repressor of cellulase and xylanase genes. This was confirmed by *aceI*Δ mutants, as these showed a better growth on cellulose-based medium than the wild type (51, 52, 53). In the last years, several zinc-finger proteins were identified and characterized as being transcriptional factors. It turned out that some

are key regulators for developmental processes and for plant pathogenesis, while in the last often responsive for the regulation of exocellular polysaccharolytic activities (54, 55, 56). Thus, Vl-ACEI might regulate further factors in pathogenesis and adaptation to xylem environment beside regulation of exocellular polysaccharolytic activities.

```
V.longisporum     ------------------------------------------------------------
V.dahliae         MSCQNPRRRSPVTRVGDASSNGLTSLKTNMTLRKGATFHSPTS-LDSSSIDAFIPPALGR 59
T.reesei          MSFSNPRRRTPVTRPGTDCEHGLS-LKTTMTLRKGATFHSPTSPSASSAAGDFVPPTLTR 59
F.graminearum     MSFSHPRRRTPVTRPDCDTENALS-FKNSSTLRKGATFHSPTSPSLTSDI-AFVPPTLPR 58
P.anserina        --MSSNPRRTPMTRPD---SRGLS-LKTN-TLQKGATFHSPTSPTSTTEN-VFRPPSLPR 52

V.longisporum     ------------------------------------------------------------
V.dahliae         -SQTNLEDVVGAHVRRMEMIVSGIETSLNLN-----DTPRPASKPSRDECLPRTNGFLGR 113
T.reesei          -SQSAFDDVVDASRRRIAMTLNDIDEALSK---ASLSDKSPRPKPLRDTSLPVPRGFLEP 115
F.graminearum     -AQSHLDDVVDANRRRVALTLNDIDEALAKTQELSLSSTS-KPMTLRDTGLPIPRGFLEG 116
P.anserina        RSQSNLDDVIDSHRRRAALTLDEFDRTLAGLSISDSPSSAAARKILREDSPPIPRGILN- 111

V.longisporum     ------------------------------------------------------------
V.dahliae         PTVDPAMAKDTKTSGERRVLRPRH-RRSSEQHASDSGLGTSLASSVEKQAPSITSKTS-- 170
T.reesei          PVVDPAMNKQEP---ERRVLRPRS-VRRTRNHASDSGIGSSVVSTNDKAGAADSTKKP-- 169
F.graminearum     PIVDPKMTKEE----ERRTLRPRGRTSRALEDHSDSGLGTSVASTNEKRGAVTASKEAKV 172
P.anserina        HTLDTVMAKGKE--VERKVLRPRT-RRTSRHHDSDSGLGTSIASTNEKIAAKEQTVAK-- 166

V.longisporum     ---------APVSQLLYQRS----------VWGDTYRSL---------------------- 20
V.dahliae         KASAITRSAAAPSNTMTKVSG-LSSKAVSRVHEHVLRPLRAKPELKDFEPIVLDIPRRIR 229
T.reesei          QASALTR-SAASSTTAMLPS--LSHRAVNRIREHTLRPLLEKPTLKEFEPIVLDVPRRIR 226
F.graminearum     QTRCLTRSAAAAAATGKLPS--LGSKAFSRIHEHTLRPLLAKPTLKEFKPIVLDIPRRIQ 230
P.anserina        -TTAVTRSAAATRTTTTTTTQVLGQRANNRICEHTLKPLLGKPEFKDFHPLLLECPKKIQ 225
                           *.        :          :  .. :.*

V.longisporum     -----------------MKERAKTAALYLDFCLTSIRCIQATVEYLSDREQIRPADRPYT 63
V.dahliae         DKEIICLRDLEKTLIFMAPERAKTAALYLDFCLTSIRCIQATVEYLSDREQIRPADRPYT 289
T.reesei          SKEIICLRDLEKTLIFMAPEKAKSAALYLDFCLTSVRCIQATVEYLTDREQVRPGDRPYT 286
F.graminearum     SKEIICLRDLEKTLIFMAPEKAKSATLYLDFCLTSVRCIQATVEYLSDREQIRPADRPYT 290
P.anserina        DKEIVCLRDLEKTLLLVAPERTKSAGLYLDFCLTTIQCIQATVEYLSDREQTRPRDVPYS 285
                             *::*:* ********:::*********:**** ** * **:

V.longisporum     NGYFIDLVEQIRQYAGQLATAKEAGVEGREMDVDPTDEVKLFGGISQNGRPAELVRVRKD 123
V.dahliae         NGYFIDLVEQIRQYAGQLATAKEAGVEGREMDVDPTDEVKLFGGISQNGRPAELVRVRKD 349
T.reesei          NGYFIDLKEQIYQYGKQLAAIKEKGSLADDMDIDPSDEVRLYGGVAENGRPAELIRVKKD 346
F.graminearum     NGYFLDLKDQILEYGKQLAAKNS----GDEMDIDASDEIKLVGGLSVNGRPAELVRVRKD 346
P.anserina        SGYFIDLVDQIRHYAQQLSEAKEKG-ENDEMDVDPTDEIKLHGGIHINGRPAELVRIKKN 344
                  .***:** :** .*. **:  :.      :**:*.:**::* **:  *******:*::*:

V.longisporum     GQAISMATGLPVDMDE-DGKDFPRLKRSLSQQLADDEEIMRSMARRKKNAAPEELAPKKC 182
V.dahliae         GQAISMATGLPVDMDE-DGKDFPRLKRSLSQQLADDEEIMRSMARRKKNATPEELAPKKC 408
T.reesei          GTAYSMATGKIVDMT--ESPTP--LKRSLSEQREDEEEIMRSMARRKKNATPEDVAPKKC 402
F.graminearum     GTYISLDTGKPVETDD-DAPMK--MKRSLSQQLEDEEEIQRSMARRKKNASPEELAPKKC 403
P.anserina        GKMISMATGEPIESIEEESSGAVRIKRSASEELEDEEEIMRSMARRKKNATPEELAPKKC 404
                  *   *: **  ::    :.     :*** *::  *:*** **********:**::*****

V.longisporum     REPGCNKEFKRPCDLTKHEKTHSRPWKCPVKTCKYHEYGWPTEKGMDRHHNDKHSSAPPM 242
V.dahliae         REPGCNKEFKRPCDLTKHEKTHSRPWKCPVKTCKYHEYGWPTEKEMDRHHNDKHSSAPPM 468
T.reesei          REPGCTKEFKRPCDLTKHEKTHSRPWKCPIPTCKYHEYGWPTEKEMDRHINDKHSDAPAM 462
F.graminearum     REPGCNKEFKRPCDLTKHEKTHSRPWKCPVSTCKYHTYGWPTEKEMDRHHNDKHSAAPAM 463
P.anserina        REHGCNKEFKRPCDLTKHEKTHSRPWKCPVTTCKYHEYGWPTEKEMDRHHNDKHSAAPPM 464
                  ** **.**********************: ***** ******* **** ***** **.*

V.longisporum     HECLFKPCPYKSKRESSCKQHMEKAHGWQYVRTKTNGGKKAPSVAGSSAQPTPQLGNMAT 302
V.dahliae         HECLFKPCPYKSKRESNCKQHMEKAHGWQYVRTKTNGGKKAPSVAGSSAQPTPQLGNMAT 528
T.reesei          YECLFKPCPYKSKRESNCKQHMEKAHGWTYVRTKTNG-KKAPSQNGSTAQQTPPLANVST 521
F.graminearum     YECSFKPCPYKSKRESNCKQHMEKAHGWTYVRTKTNG-KKLPSIAGSVQQQTPPLGNMST 522
P.anserina        FECYYKPCPYKSKRESNCKQHMEKAHGWTYVRTKTNGKKPGSSIAGGSTHPTPQLGHIST 524
                  .** :***********.********** ******** *  .*  *.  : ** *.:::*
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V.longisporum     PSS--SHSIATPPEESTSLFPPFNHDDFPHYVPAEEFADTCLGPMGQPPMTLEGIDFNDL 360
V.dahliae         PSS--SHSIATPPEESTSLFPPFNHDDFPHYVPAEEFADTCLGPMGQPPMTLEGIDFNDL 586
T.reesei          PSSTPSYSVPTPPQD------QVMSTDFPMYPADDDWLATYG----AQPNTIDAMDLGLE 571
F.graminearum     PSSIEYNSVPTPPQNDV---TQFVGNDFPLYPTDSDWMSVNN----IPAEAMN-IDLTLD 574
P.anserina        PSSDMSAGVATPPDDWS--HIYSSGLEFPTYMPDSDFG--------MIPQELHLEYSPVD 574
                  ***    .:.***::           :** * . .::          .  :.

V.longisporum     GVSPTDYNTPSTDTSYPYTSYQDGPEFVINNDDIYGARVQIPTPAWP----------EKM 410
V.dahliae         GVSPTDYNTPSTDTSYPYTSYQDGPEFVINNDDIYGAHVQIPTPAWP----------EKM 636
T.reesei          NLSP----ASAASSYEQYPPYQNGSTFIINDEDIYAAHVQIPAQLPTPEQVYT----KMM 623
F.graminearum     STSP----ASASS-YEQYAPYQNGSDFILDNEDLYAAHMQLPAHFPSPEQAVMYSNPKMM 629
P.anserina        NPTP-----STDSGMDHSSAYQDISTDFTLYEDIYSANVQLPTPMHAN------IYDKPM 623
                  . :*     .: .     ..**: .  .   :*:*.*.:*:*:   .          : *

V.longisporum     MAGMQNYAPVSACQPQMMPEPLAP-HISPIGQGNAMLFTPNSLAEVDEGFDD-------- 461
V.dahliae         MAGMQNYAPVSACQPQMMPEPLAP-HISPIGQGNAMLFTPNSLAEVDEGFDD-------- 687
T.reesei          PQQMPVYH-VQQEPCTTVPILGEP-QFSPNAQQNAVLYTPTSLREVDEGFDES------- 674
F.graminearum     QQQLPMYQQVPQQIPQPIPVQTAPSQFSPTGQETAMLFTPNSLRDVDEGFDDS------- 682
P.anserina        EPQFTPFTGAELCP--------LPAQLSPIGQANAMLFTPTSM--VDEGFDDQHELAAMT 673
                     :  :  .             * ::** .* .*:*:**.*:  ******:

V.longisporum     FGGCGDDFTLFP-VNGLDKDAQFQTLFGSEMPSSGLG--LSQGASQDFFGNG-MDWSSME 517
V.dahliae         FGGCGDDFTLFP-VNGLDKDAQFQTLFGSEMPSSGLG--LSQGASQDFFGNG-MDWSSME 743
T.reesei          YAADGADFQLFP--ATVDKTDVFQSLFT-DMPSANLG--FSQTTQPDIFNQ--IDWSNLD 727
F.graminearum     FGADGMDFPLFPGGNGMAKTNNYQPLFG-EIPSANVG--FSQNSQ-DPFQM--MDWSSGG 736
P.anserina        NMASGGDFILFPNQAGVSKPMYNDSLFATDLPLQGMGTGYSQPSTQDLINGFHVDWSAHD 733
                    . * ** ***    : *    :.**  ::*  .:*   ** :  * :    :***

V.longisporum     YHTYSQQPQHQQ 529
V.dahliae         YHTYSQQPQHQQ 755
T.reesei          YQGFQE------ 733
F.graminearum     FLANL------- 741
P.anserina        LSAYLPQ----- 740
```

Figure 4.7: Alignment of translated cDNA sequence Vl-ACEI from *V. longisporum* with homologous protein sequences of other fungi.

Multiple alignment of Vl-ACEI with homologous protein sequences of other fungi was constructed using the ClustalW2 algorithm. Identical amino acids (aa) are indicated in the consensus line with asterisks and conserved aa with dots. Red letters denote small and hydrophobic aa, blue letters acidic aa, mangenta letters basic aa and green letters hydroxyl-, amine and basic aa.

Full-length of gene sequence of the up-regulated transcript TDF 34.2 comprises an ORF of 588 bp including two introns and a translated sequence of 119 aa. TDF 34.2 translated sequence bears similarity to a small peptide, called GRIM-19, which was characterized as a cellular regulator of mitochondria-dependent programmed cell death in higher eukaryotes (57, 58, 59, 60). PCD machinery has been investigated and characterized in detail in higher multicellular eukaryotes. A couple of highly conserved genes with homology to PCD factors have been also identified in microbial unicellular eukaryotes including several fungi (61, 62). However, the function of homologous genes, their mode of action in regulation cascades and the role of PCD machinery in microbes in general is hardly understood. In plant pathology the most prominent form of PCD is the hypersensitive reaction (HR) of plants as a response to attack of a biotrohpic pathogen in a non-compatible situation (63). A hypothesis, how PCD might be involved in *V.*

longisporum/*B. napus* interaction, based upon the fact that *B. napus* is known to produce a variety of secondary metabolites with antifungal properties like phytoanticipins and phytoalexins (64). If one of these substances exerts PCD in *V. longisporum*, the fungus might up-regulate *in planta* the expression of a repressive regulator of its induced PCD machinery to escape apoptosis.

Polyketides belong to a large group of well-characterized fungal secondary metabolites. Among them two major groups dominate scientific literature in plant pathology. Mould fungi (*Aspergillus spp.*, *Penicillium spp.*, *Fusarium spp.*) produce a large pallet of chemically highly diverse polyketides belonging to the class of mycotoxins (fumonisins, aflatoxins, zearalenones) (65, 66, 67). Mycotoxins are harmful to endothermic animals. A second type of a non-secreted polyketide playing a role in plant pathology is melanin; a black pigment broadly distributed in the kingdom of fungi. Next to its general function as a protector against environmental stress (68), melanin was discovered to play an important role in appressoria-mediated infection processes of plant pathogens (69, 70). In *V. longisporum*, a type I polyketide synthase (PKS) gene was identified, designated as *VlPKS1*, and it turned out that gene expression was strongly up-regulated *in planta*. *VlPKS1* comprises high sequence homology to a PKS type I gene of *Colletotrichum lagenarium* (71), a fungal pathogen causing anthracnose on melons and other host plants. PKS type I are multi-domain genes involved in the production of melanin. *PKS1*$^{-}$ mutants of *C. lagenarium* morphologically changed to albinos (72). Possibly, *VlPKS1* is involved in the biosynthesis of melanin during the microsclerotia formation of *V. longisporum*, which is initiated *in planta* at the end of the parasitic life stage.

VlNEP is the homologous gene to *VdNEP* originating from *V. dahliae*, which was described for the first time by Wang et al. (36). By the infiltration of purified VdNEP into leaves of the natural host plant cotton as well as in tobacco and in *Arabidopsis* wilt and chlorosis symptoms were elicited (36). The plant response to several NEPs has been studied in some cases for both purified proteins and by genetically engineered fungal strains. For most NEPs it was observed that they are capable of inducing HR-like response in plants, but the mechanism of initiation of this response remains unrevealed, while the cellular role is not clear yet whether NEPs are true elicitors triggering plant defense or whether this induction is based on a different activity (73 74, 75). NEPs are also active as secondary signalling molecules. In concentrations of 20–30 pmol/g coca leaf, Nep1 induces ethylene production 6 hours post-application (76, 77). The same effect was found in parsley during NPP1 infiltrations (73). In *Arabidopsis*, up-regulation of *PR1* was observed 8 hours post application of NPP1 indicating a response in the salicylic acid (SA) pathway (72). A similar situation was decribed in Arabidopsis after application of Nep1. Marker genes for the JA/ET pathway were activated after in Arabidopsis seedlings 30 min after treatment

with Nep1 (78, 79). Ethylene production is supposed to play also an active role in *Verticillium* pathogenesis and in symptom development, which had been studied on ethylene-deficient mutants in *Arabidopsis*. *Etr1-1* mutants (Col-0 background) showed less severe senescence symptoms as revealed by higher levels of chlorophyll content than the wild-type (14). Several ethylene-deficient mutants (*ein2-1*, *ein4-1*, *ein6-1*, *etr1-1* [Col-0 background]) showed enhanced susceptibility towards *V. longisporum* and the expression of ethylene-dependent *PR-4* was increased 7 dpi (80).

Little is known about fungal NEP expression in vivo. By RT-PCR analysis *PsojNIP* transcripts (coding for NEP from *P.sojae*) were detected in infected soybean tissues only until 12 hpi (81). Bailey et al. (76) found out that *Nep1* disruption and over-expressing in *F. oxysporum* had no effect on pathogenicity in *Erythroxylum coca*. Entering the intercellular space, there is little known about the fate of NEPs. Based on their hydrophilic property it is quite unlikely that they cross the plasma membrane into the plant cell (74). It is more likely that NEPs react with a receptor molecule attached to the outer surface of plant cell walls (77, 79). Once the receptor of NEP detection on cellular level is characterized, knowledge of the mode of action of these proteins should lead to a better understanding of the basis of NEP–plant interactions.

Although high transcript levels of *VlNEP* were measured in hypocotyl tissue of *V. longisporum*-infected *B. napus* plants, reactions were dissimilar to those reported in context with NEPs. This discrepancy could be either explained by post-transcriptional regulation of the VlNEP peptide, by defective secretion or by insusceptibility of the plant. Contrary, VlNEP might actively function as elicitor, but might only affect parts of the actually expressed symptoms in *B. napus*, like the onset of early senescence.

We expect that the analysis of genes identified in this work, especially after treatment with xylem sap metabolites from infected plants, will deepen our understanding of chemical interactions between *V. longisporum* and its host occurring in xylem sap and shed light on the interaction of vascular pathogens with host plants in general. Quantitative gene expression profiling of *V. longisporum* by genome-wide cDNA-AFLP helped us to reveal that adaptation to the xylem environment during plant colonization seems to be achieved by just a small number of regulated fungal genes, which, however, might be important for interaction with the plant. Their role during pathogenesis can be elucidated by infection assays by gene inactivation and with over-expression mutants. Reporter gene fusions will be helpful in the elucidation of temporal and spatial expression patterns of candidate genes during the infection and colonization processes. We hope that the availability of sequences of genes induced during plant colonization will facilitate the elucidation of molecular adaptation of *V. longisporum* to parasitic lifestyle within the xylem environment of host plant *B. napus*.

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Chapter 5 - Final discussion

Functional analysis based on transcriptomics, proteomics and metabolomics are ultimate steps to characterize mechanisms, functions and processes of the pathogenic interaction between *V. longisporum* and its host plant *B. napus* on the molecular level. To our knowledge, this is the first report on functional profiling of the transcriptome of *V. longisporum*. Using an improved protocol we applied cDNA-AFLP for the quantitative analysis of global gene expression aiming to identify regulated genes coding for products involved in the infection process of *V. longisporum* on *B. napus*.

Improved coverage of cDNA-AFLP by sequential digestion of immobilized cDNA

cDNA-AFLP provides a well-established and a low investment alternative in transcriptomics compared to microarrays or cDNA sequencing strategies which does not require prior gene sequence information. To enhance the efficiency of cDNA-AFLP technique in terms of increasing the maximum transcript coverage while keeping the transcript signal redundancy at a low level, we developed modifying steps to classical protocols relying on sequential endonuclease digestion of immobilized cDNA molecules. We implemented a new software tool called MECS (**M**ultiple **E**nzyme **c**DNA-AFLP **S**imulation) to demonstrate the superiority of our modified protocol in comparison to standard cDNA-AFLP methods. We optimized the number and order of restriction enzymes by simulation with cDNA sequences from *A. thaliana*, *M. musculus* and *Homo sapiens*. MECS simulations revealed that 2 marking enzymes in combination with 2 to 3 releasing enzymes (terms for classes of restriction enzymes used in the improved cDNA-AFLP protocol, see chapter 2) were the optimal line-up. Transcript coverage was increased by 9-13% compared to classical protocols but transcript redundancy did not increase significantly. In general, redundancy should be kept low so as to reduce the total number of PCR reactions necessary to perform all possible primer combinations in whole-genome transcriptomics. The PCR effort is mainly regulated by the number of selective nucleotides incorporated into the PCR primers and, in turn, leads to a specific number of PCR products. Based on PCR simulations with ESTs we tested gradual primer selectivity and. We recommend setting selectivity so that on average, between 30 and 70 PCR products are amplified. This range guarantees a high level of cDNA signals which can be analyzed and reduces events of overlapping cDNA bands because of identical length as well as PCR reactions generating less than 20 products which would be insufficient for proper quantification. However, it turned out that even with the optimal number of PCR products only about 75% of the

fragments are analyzable. Further steps aiming at reducing redundancy in cDNA-AFLP should be verified carefully by simulations.

Minimization of redundancy by pre-restriction of the immobilized cDNA template caused a loss of transcript coverage and reduced the average fragment length. In multiple digestion protocols, the sequence of releasing enzymes affects the partition of fragments into several pools. Classical protocols, in which the cDNA template is digested with a pair of enzymes, generate a single fragment pool. Using more enzyme pairs enhances the coverage, but pools are prepared independently and thus cannot be controlled in their size. Based on simulations, our data confirmed that partitioning fragments into a higher number of pools can reduce the total PCR effort.

Nevertheless, an optimal strategy which brings out a compromise between redundancy suppression, maximum transcript coverage, minimal PCR effort and long cDNA fragments has to be found by simulations. MECS can help to find the protocol of preference for a particular experiment. High quality sequences must be used for simulations to predict authentic statistical values of sequence coverage, redundancy and fragment length.

Components of variance in transcriptomics based on electrophoretic separation of cDNA fragments (cDNA-AFLP)

The sources of variance and errors in transcriptomics based on the electrophoretic separation of amplified cDNA fragments were investigated using cDNA-AFLP. The overall quality of data generated by cDNA-AFLP was comparable with microarray hybridization. Variance of cDNA-AFLP was independent of signal intensity while microarray data showed higher variance for low intensity signals. Discarding signals with low intensity values in order to improve the quality of transcriptomics data, as is common practice with microarray-based data, is not recommended in for electrophoresis-based transcriptomics data. During the cDNA-AFLP transcriptome profiling of plant pathogenic fungus *V. longisporum*, a set of expression data was generated based on technical replicate originating from a single RNA preparation to characterized systemic errors in cDNA-AFLP in more detail. Total variance was partitioned into contributions of cDNA synthesis, adapter ligation, preamplification, amplification and electrophoresis. The highest contribution to variance originated from preamplification, indicating that this step should be the primary target of optimization. Data variance was also analysed duirng electrophoretic separation of amplified DNA fragments by a comparison of flatbed polyacrylamide gel electrophoresis including fluorescence detection with capillary electrophoresis (DNA sequencer). Higher reliability in data performance observed for capillary electrophoresis indicated its superiority to flat-gel-based electrophoresis. In electrophoresis-based transcriptomics band

matching is a crucial step in automated data processing. Parameters of computer-aided band recognition and matching were investigated and strategies improving matching success based on double passage with different signal intensity thresholds were developed. The analysis of variance components in transcriptomics based on electrophoretic separation of cDNA fragments and particular in cDNA-AFLP led to a more reliable interpretation of data sets of gene expression and improved the informational output of the *V. longisporum* transcriptome profiling.

V. longisporum as a model system

Verticillium can be used as a model for vascular plant pathogens, because it is an ascomycete growing well under standard lab-scaled condition and is easy to manipulate genetically via *Agrobacterium tumefaciens*-mediated transformation (1). Gene sequence information is available in public databases collected from a mitochondrial genome sequencing initiative published by Pantou et al. (2) and from two independent cDNA sequencing projects each elucidated on *V. dahliae* (3, 4). In 2006 a comparative genome sequencing project of *V. dahliae* and *V. albo-atrum* was started by the NSF/USDA Microbial Genome Sequencing Program. Genomic sequences as well as annotated gene sequence releases are available at (5). Through the present work presented here, new gene sequence information for *V. longisporum* is contributed as the result of a functional analysis of the transcriptome focussing on the identification and characterization of target genes induced by xylem secondary metabolites.

Adaptation of _V. longisporum_ to xylem sap environment as revealed by cDNA-AFLP analysis

A comparative transcriptome analysis of *V. longisporum* was performed applying an improved cDNA-AFLP protocol for a genome-wide quantitative cDNA-AFLP approach with the goal of discovering new genes induced by xylem metabolites of its host *B. napus*. Thus, expression profiles of fungal cultures after treatment with extracts of *B. napus* xylem sap were compared to water-treated controls. Transcript profiling was conducted either after treatment with extracts from mock treated plants or *Verticillium*-infected plants. Most of those genes that showed a significant change with regard to expression were identified in the set of treated with xylem sap metabolites of *V.longisporum*-infected plants. Changes in chemical composition of xylem sap during infection and the ensuing colonization of the *Brassica* by *V. longisporum* was determined by a comparative analysis of metabolic profiles of xylem sap extracted from healthy *B. napus* plants and plants infected with *V. longisporum*. Using HPLC with mass spectrometric detection, signals specifically affected by infection with *V. longisporum* were identified (6). These signals belonged to three categories: metabolites suppressed by infection, metabolites increased after infection and infection-specific metabolites. Differences in metabolic peaks were also observed

analysing only ethyl acetate extracts of xylem sap (6). Ethyl acetate extracts of xylem sap were also used for induction of fungal gene expression in the cDNA-AFLP transcriptome profiling. Fig. 5.1 shows a comparison of HPLC spectra of 20-fold up-concentrated ethyl acetate extracts of *Brassica* xylem sap prepared from healthy and from *V. longisporum*-infected plants 28 dpi, respectively. Induced or suppressed compounds are supposed to be related to plant response to the infection such as antifungal activities or other defence-related mechanisms, while compounds appeared only in infected xylem sap prepa their secretion into the intercellular space rations are supposed to represent fungal expressed metabolites *in planta*. (6). For example, the metabolic peaks m/z 214 and m/z 193 are supposed to reflect a reduction of the *Brassica* phytoalexin Brassicanal A (7) after *V. longisporum* infection. The signal of m/z 163 seems to appear only after infection with *V. longisporum*. These preliminary results of the analysis of changes in the chemical composition of *Brassica* xylem sap after infection with *V. longisporum* seem to explain, why treatments with xylem sap metabolites of *V. longisporum*-infected plants displayed a slight increase in the number of fungal transcripts changes in expression as revealed in cDNA-AFLP analysis.

We accomplished a mild induction of fungal gene expression by using only extracts of xylem sap, resulting in a small set of differentially expressed genes. Among >13 thousand individual cDNA-AFLP signals recorded in each profiling set, in total only 34 signals in total responded to one of the treatments. The ethyl acetate extraction was chosen to rule out any inducible effects of sugars or amino acids on metabolic pathways in *V. longisporum* or the induction of genes responding to mineral ions. In order to determine whether the identified regulated genes are also induced *in situ* during the colonization of *B. napus* we measured fungal transcript levels in hypocotyl tissue of infected plants. We identified ten transcript fragments where expression changed significantly in relation to when cultures were grown in liquid SXM medium. This supports the assumption that these genes are involved in pathogenesis of *V. longisporum* and the adaptation to xylem sap environment.

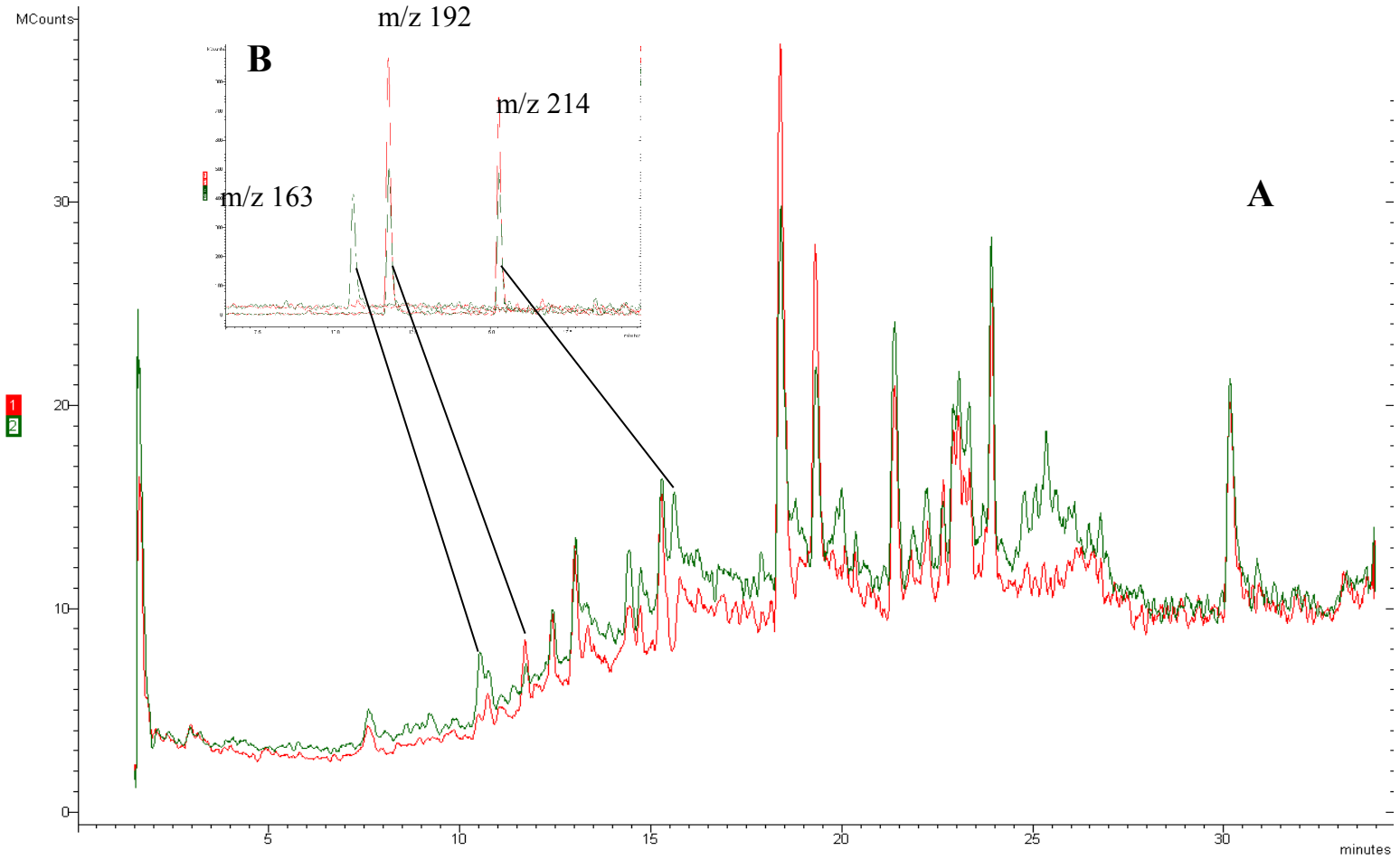

Figure 5.1: Comparative analysis of ethyl acetate extracts of xylem sap from healthy and *V. longisporum*-infected *Brassica* plants using HPLC-coupled mass-spectrometry. Ethyl acetate extracts prepared from healthy Brassica plants (red chromatograms) and of *V. longisporum*-infected plants (green chromatograms) were concentrated 20-fold for HPLC-MS analysis. Chromatogram A shows records of full ion scans in positive modus of xylem sap extracts in range of 100-1000 Da. Candidate peaks of chemical compounds changed due to *V. longisporum* infection were filtered and are shown exclusively in chromatogram B.

Inducible genes in *V. longisporum* and their putative role in pathogenesis

Pathogenicity factors expressed by *Verticillium* during the infection process and compounds causing symptom expression in host plants were basically linked to polysaccharolytic activities of exocellular polypeptides (pectinases, galacturonases), which are responsible for degradation of the plant cell wall (8). While fungus uses the released monomers as nutrients, it is supposed that hydrolysed cell wall compounds can also act as elicitors triggering plant defence response and symptom development (9). Further on, fungal protein lipopolysaccharides or glycoproteins are reported to bear phytotoxic properties on host plant cells or also act as elicitors (10). In this work, new genes were identified as the outcome of a comparative transcriptome profiling in *V. longisporum* under infection simulating conditions. These genes were characterized as being regulated in their expression under the influence of *B. napus* xylem secondary metabolites and *in situ* during the colonization of the vessel system. There is no doubt that further studies are necessary to confirm whether and to what extent these target genes are in fact involved in pathogenesis. For this purpose, concepts for the experimental investigation of gene functionality are proposed as a brief outlook at the end of this discussion. By means of the putative function of candidate genes elaborated by pBLAST analysis, new aspects of the *B. napus*/*V. longisporum* interaction are hypothesized. As soon as the hypothesized functionality is proven to be true, a better understanding in fungal xylem parasitism in general and in individual features of the *V. longisporum* pathogenicity will follow. From this study, the most promising genes active in virulence putatively code for functions of regulation of polysaccharolytic activities, adhesion and stress response, for regulating in programmed cell death and of the biosynthesis of secondary metabolites.

Transcription factor for extracellular polysaccharolytic enzymes

The translated gene sequence corresponding to the cDNA-AFLP transcript derived fragment (TDF) 12.1 comprised high sequence homology to the Cys_2-His_2-type zinc-finger transcription factor (TF) ACEI, which was characterized in the yeast *T. reesei*. This fungus has a long tradition for industrial-scaled cellulase production and in biotechnology (11). ACEI was elucidated as an effective repressor of cellulase and xylanase genes. This was confirmed by *aceI*Δ mutants, as these showed a better growth on cellulose-based medium than the wild type (12, 13, 14). In the last years, several zinc-finger proteins were identified

and characterized as being transcriptional factors. It turned out that some are key regulators for developmental processes and for plant pathogenesis. In *F. oxysporum*, another vascular wilt pathogen, the inducible zinc-finger protein XlnR was shown to regulate the expression of xylanase genes. (15). In the same species, another zinc-finger protein, Ctf1, regulates the expression of cutinases. In contrast to the stem pathogen *F. solani*, which requires a Ctf1-regulated expression of the cutinase gene *cut1* for penetration in pea (16), in a study with the root pathogen *F. oxysporum* infecting tomato plants hyphal infection was not dependent on cutinase gene induction (17). Another gene coding for a zinc-finger protein in *F. oxysporum f.sp. melonis* is called *FOW2*. Mutagenesis of this gene led to a complete loss of pathogenicity, the mutant was unable to penetrate plant roots (18). Thus, it is very likely that the inducible transcription factor in *V. longisporum*, designated as Vl-ACEI, regulates the gene expression of exocellular polysaccharolytic enzymes, presumably being an important factor in pathogenesis of *V. longisporum*. However, it is commonly known that TFs stand at the axis of regulation having influences on different developmental processes. Thus Vl-ACEI might regulate further factors in pathogenesis and adaptation to xylem environment.

Regulator of stress response and adhesion

Within the ORF corresponding to TDF 6.2 putative domains of two different functions and properties were identified (Fig. 5.2), but a proper prediction of the functionality of the whole gene is not known yet. At the C-terminus, fourfold repetitive WSC (water-soluble carbohydrate binding) domains were found on the basis of sequence similarities. The involvement in response to heat shock and to other extracellular stress factors was demonstrated in the yeast *S. cerevisiae*, for three WSC-domain containing genes (*wsc1, wsc2, wsc3*). Further on, functionality of these genes in cell wall integrity was reported in the same organism (19, 20, 21). Gene deletions (single, double and triple mutants of all *wsc* genes*)* in *S. cerevisiae* indicated a role in sensing and modulating stress response by the PKC1-MPK1 and the RAS-cAMP pathways (19, 22, 23). MAP kinase-mediated signaling pathways are also involed in pathogenicity of *Verticillium*. Disruption of a MAP-kinase I gene in *V. dahliae* (*VMK1*) severely reduced virulence in diverse host plants (24). A link of TDF 6.2 in *V. longisporum* to signalling pathways is supported by sequence similarities to a slime mould cAMP receptor (pBLAST E-value: E-46) upstream of the WSC-motifs containing ORF. Beside their potential function in response to environmental stress, the WSC-domains containing gene in *V. longisporum* may also be involved in the degradation

of plant xylem cell walls, although we did not find any evidence for a catalytic domain within this ORF, as this was found in *wsc* genes of other filamentous fungi (Fig. 5.2). For the degradation process of plant cell walls non-catalytic, carbohydrate binding modules are supposed to be involved in microbial cell adhesion, because in order to facilitate hydrolysis, catabolic enzymes that attack the plant cell wall need to be in close and prolonged contact with this composite substrate (25). Such a function is supported by the identification of a type III fibronectin (FN3) domain next to the WSC-domains within the same ORF. Fibronectins are collagen-like fibre polypeptides. FN3-modules are widely spread in animal proteins and occur mainly as part of extracellular, receptor-like proteins or in polypeptides involved in adhesion (26).

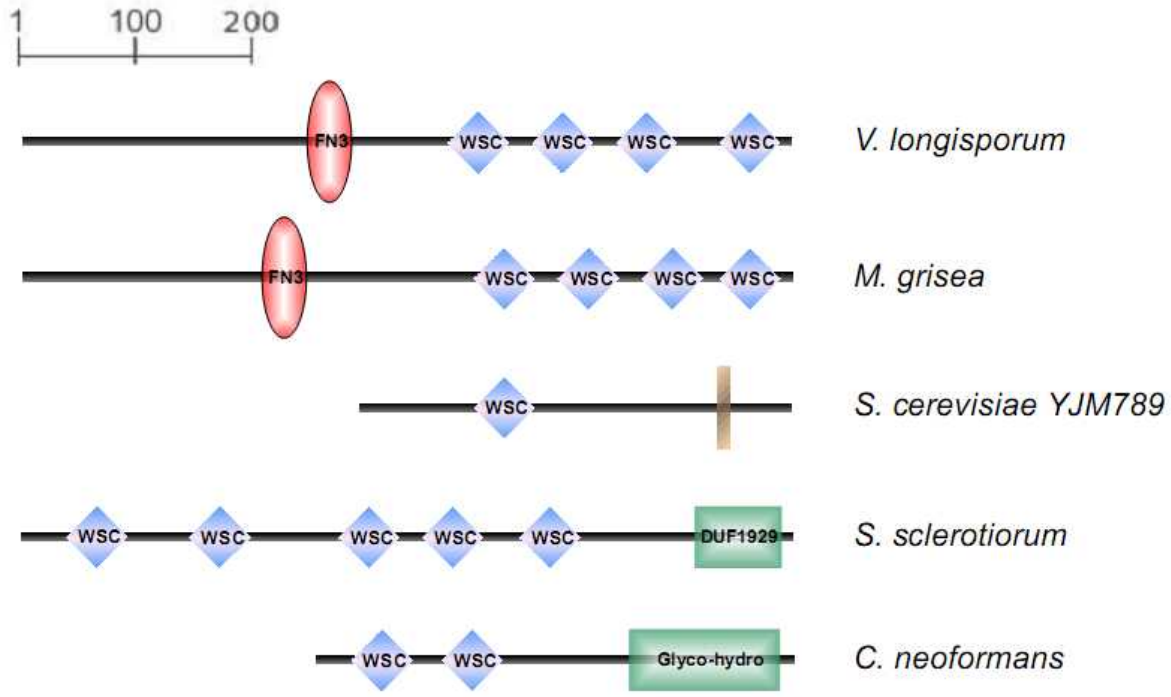

Figure 5.2: Predicted protein architecture of gene corresponding to TDF 6.2. The prediction of the protein architecture, the SMART on-line program (http://smart.embl-heidelberg.de/) was used. WSC-domain containing gene found in *V. longisporum* is shown in the line with WSC-motif containing genes of other fungi. Blue diamonds: WSC (water-soluble carbohydrate) binding domain, green squares: FN3 (fibronectin type III) domain. Grey squares are catalytic domains: Glyco-hydro (α-1,3-glucanase), DUF1929 (domain of unknown function) are mostly found in sugar-utilising enzymes, such as galactose oxidase (27). Yellow square in the yeast protein: transmembrane helix.

Interestingly, FN3-domains were also identified in different bacteria in conjunction with amylase, endoglucanase and cellulase activities (28, 29). Differences between the genes of different bacterial species were not greater than between different animal species; this result suggests a horizontal gene transfer event from an animal to a bacterial cell (26). Meanwhile, FN3-like sequences have also been found in saprophytic and plant pathogenic fungi (30, 31), and activities of peptides harbouring FN3-modules are comparable to those described above. Cellular adhesion in general is one of the predominant features in pathogenicity in several microbes including fungal-host plant interactions (32). In *V. longisporum*, adhesion of hyphae to the root hairs of host plant *B. napus* prior of infection was illustrated by Eynck et al. (33), implicating that proteins modulating adhesion in *V. longisporum* are crucial factors of pathogenicity.

Regulator of programmed cell death

The translated sequence of the *V. longisporum* gene corresponding to TDF 34.2 bears similarity to a small peptide which has been characterized as a cellular regulator, called GRIM-19, in higher eukaryotes (34-37). In mammalian cells, GRIM-19 was described to play a regulative role in mitochondria-dependent programmed cell death (PCD). Two types of PCD are described in literature, both leading to degradation of cell compartments and finally to cell lysis. On the one hand, autophagy as a "self-eating" process is based on lysosome activity of damaged cells, cell recycling and as a strategy for survival during nutritional starvation. On the other hand, apoptosis features externalization of phosphatidylserine, accumulation of DNA strand breaks and the release of mitochondrial cytochrome c into the cytosol (38). PCD machinery has been investigated and characterized in detail in higher multicellular eukaryotes. Due to the release of vast genome sequence information during the last few years, a couple of highly conserved genes with homology to PCD factors have also been identified in microbial unicellular eukaryotes including several fungi (39, 40). However, the function of homologue genes, their mode of action in regulation cascades and the role of PCD machinery in microbes in general is hardly understood. For fungal organisms several studies showed that PCD is involved in developmental processes (41, 42, 43), during the interaction with other organisms including pathogenic interrelationships (44, 45), and in aging (46, 47). In plant pathogenic interaction the most prominent role of PCD is the hypersensitive reaction (HR) of plants as a response to pathogen attack. By fast release of reactive oxygen species (ROS) in terms of an oxidative

burst, PCD is rapidly induced within the attacked plant cell to isolate the pathogen locally. HR is often triggered by the recognition of a secreted fungal compound which elicits the plant interception system (48). When this genetic resource of disease resistance is missing in plants, elicitors sometimes shape up as virulence factors. On the other hand, some necrotrophic fungi intend to induce PCD by toxins as part of their virulence (e.g. *Alternaria spp*., *Botrytis spp*.) and plant cells that are killed serve as a nutrient source (44, 49, 50). The accumulation of enhanced H_2O_2 levels at the intercellular site of contact between host plant and pathogen can cause cell wall damages for both partners. And it was shown that H_2O_2 exposed by the host can also induce PCD in an attacking pathogen. In *A. fumigatus*, an opportunistic human pathogen, an oxidative respiratory burst exerted by neutrophils (type of phagocytosing white blood cells of humans) kills germinating conidia through the induction of PCD (51). In plant pathology, fungal ROS-detoxification via enzymes (peroxidase, superoxide dismutase) or non-enzymatically (GSH, flavonoids) are, therefore, pathogenicity factors, because they avoid fungal cell wall damage and silence plant defence responses (52). However, indications for an oxidative burst - as a key event in several plant pathogen interactions - were not detected during the infection of *B. napus* by *V. longisporum* (53). Initiation of PCD within an attacking pathogen by the release of an inducing signal (so called pro-apoptotic substance) other than ROS has also been described. For example, tomato plants produce the saponin α-tomatine as an effective phytoanticipin, toxic to a broad range of fungi. It was demonstrated that α-tomatine is a pro-apoptotic substance triggering PCD in tomatine-sensitive *F. oxysporum* strains (54). Insensitive isolates showed high virulence towards α-tomatine-producing tomato cultivars, because they were able to detoxify α-tomatine by the enzyme tomatinase (55). In an opposed function of PCD the plant pathogenic fungus *Pyricularia oryzae* (teleomorph *Magnaporthe oryzae*), the causal agent of rice blast, requires conidia autophagy for successful appressoria formation and host cell penetration. Deletion mutants in the *MgATG8* gene locus which is homologous to the *ATG8* autophagy gene in the yeast *S. cerevisiae* (56) showed impairment in PCD. Conidial cell death was arrested and the mutant turned out to be apathogenic (57).

Up-regulation of gene expression of the GRIM-19 homolog of *V. longisporum in planta* indicates that PCD is somehow involved in the xylem colonization of *V. longisporum*. Considering the two known mechanisms of PCD described in plant pathogenic fungi, in *V. longisporum* PCD might play a role in penetration of plant cell walls. No specialized cell

structures like appressoria or hyphopodia during the infection process of *V. longisporum*. Instead, hyphal swelling was reported to be a characteristic feature at sites of fungal penetration of solid surfaces like xylem cell walls (33) as a reaction to mechanical resistance given by the plant cell wall. Another possibility of how PCD might be involved in the *V. longisporum*/*B. napus* interaction is based on the fact that *B. napus* is known to produce a variety of secondary metabolites with antifungal properties like phytoanticipins or phytoalexins (7, 58). If one of these substances exerts a pro-apoptotic effect on *V. longisporum*, fungal PCD machinery might be activated. As a kind of counteracting, *V. longisporum in planta* might express a repressive regulator of its induced PCD machinery to escape apoptosis.

Secondary metabolites

Secondary metabolites are defined as compounds not strictly needed for the survival and reproduction of their producer (59). The nature of secondary metabolites is characterized by a high diversity of chemical structures and classifications; each chemical group is produced only by a small number of species or even by only one species. Some secondary metabolites are secreted, sometimes only at very low concentrations, some are not secreted. While secreted secondary metabolites can be regarded as messengers in chemical communication between organisms (competition, symbiosis, pathogenesis), non-secreted secondary metabolites are often involved in cellular differentiation processes.

Polyketides belong to a large group of fungal secondary metabolites (59). Among them, two major groups dominate scientific literature in plant pathology. Mould fungi (*Aspergillus spp.*, *Penicillium spp.*, *Fusarium spp.*) produce a large pallet of chemically highly diverse polyketides belonging to the class of mycotoxins (fumonisins [60], aflatoxins [61], zearalenones [62]). Mycotoxins are harmful to endothermic animals. As mycotoxins are mostly secreted to the confines of their environment by their producer, the ecological role(s) of mycotoxins are still under extensive discussion. Suggestions of the benefits of producing mycotoxins rank from competition factors bearing inhibitory effects against other fungal species and microbes (63), protection of nutritional substrates from ingestion by animals (64) to poisonous effects of mycotoxins on plants, thus being defined as virulence factors in plant pathology (65, 66). A second type of a non-secreted polyketide playing a role in plant pathology is melanin; a black pigment broadly distributed in the kingdom of fungi. Next to its general function as a protector against environmental stress (67), melanin was discovered

to play an important role in appressoria-mediated infection processes of plant pathogens. In *P. oryzae*, it was demonstrated that the accumulation of melanin is required to elevate an enhanced tugor pressure within the appressorial cell for fungal penetration (68, 69). In *V. longisporum*, a type I polyketide synthase (PKS) gene was identified, designated as *Vl-PKS1*, and it turned out that gene expression was strongly up-regulated *in planta*. Vl-PKS1 showed high sequence homology to a PKS type I of *C. lagenarium* (70), a fungal pathogen causing anthracnose on melons and other host plants. PKS type I are multi-domain genes involved in the production of melanin. *PKS1*$^{-}$ mutants of *C. lagenarium* morphologically changed to albinos, formed non-melanized appressoria and showed a reduction of penetration potential (71). During late stages of pathogenesis, *V. longisporum* starts to produce microsclerotia; structures which are strongly melanized. In a comparison of EST collections from *V. dahliae* cultured in SXM (simulating xylem fluid) and DMS (developing microsclerotia) media genes for melanin biosynthesis were exclusively found in the DMS collection (3). The *PKS1* gene of *V. longisporum* characterized in this work is supposedly involved in this kind of developmental differentiation.

A second class of secondary metabolites supposed to be involved in plant pathogenesis comprises small peptides (24-30 kDa) which are described to be excreted by diverse pathogens and to induce ethylene production and necrosis in plants. In literature, this class of peptides is designated as NEP, NIP, NLP or NPP by different authors (72-75) however, in this work the term NEP (necrosis- and ethylene inducing peptide) is used. Genes putatively coding for NEPs comprise high sequence conservation across a variety of taxonomically unrelated microbes; not only in different plant pathogenic ascomycetes, but also in oomycetes (*Phytophthora spp.*, *Pythium spp.* [73, 77, 78]) and in Gram-positive as well as in Gram-negative bacteria (76). *VlNEP* is the homologue gene to *VdNEP* originating from *V. dahliae* which was for the first described by Wang et al. (4). The segregation of NEP sequences elucidated by phylogenetic analysis turned out to be in agreement with the evolutionary history of the organisms (Fig. 5.3). This leads to the implication that the distribution of NEP genes was not due to a horizontal gene transfer, as this was discussed by Pemberton and Salmond in their 2004 review (75). Conserved sequence structures putatively coding for functionality of NEP are the GHRHDWE motif in the centre and cysteine residues at the N-terminus (75, 79, 80). Both features are also present in the *Verticillium* NEPs, indicating an intact functionality which was confirmed by the fact that VdNEP was

shown to elicit wilt and chlorosis symptoms on leaves of different plant species (4). In general, NEP-encoding genes are found in single copy, in two and four copies; the highest numbers of copies were identified in *Pythium aphanidermatum* and in *Phytophthora parasitica*, respectively (73, 77). In this context, NEPs are supposed to reflect the spectrum of hosts of *P. parasitica*, as NEPs are common to all *P. parasitica* strains and each come into action in a different host (75). The same conclusion was made by Staats et al. (80) in *B. cinerea*. The presents of two copies of NEP genes may have adaptive significance to hosts. Plant pathogenic species of *Verticillium* (*V. longisporum*, *V. dahliae*, *V. albo-atrum*) seem to have only a one or two copies (*V. longisporum* possesses two copies regarding to its near-diploid status) of one NEP gene. The host range of *V. longisporum* is limited to crucifers (81, 82), in contrast to that, *V. dahliae* and *V. albo-atrum* both show broad host ranges. This indicates that *Verticillium*-NEP does not determine any host specificity.

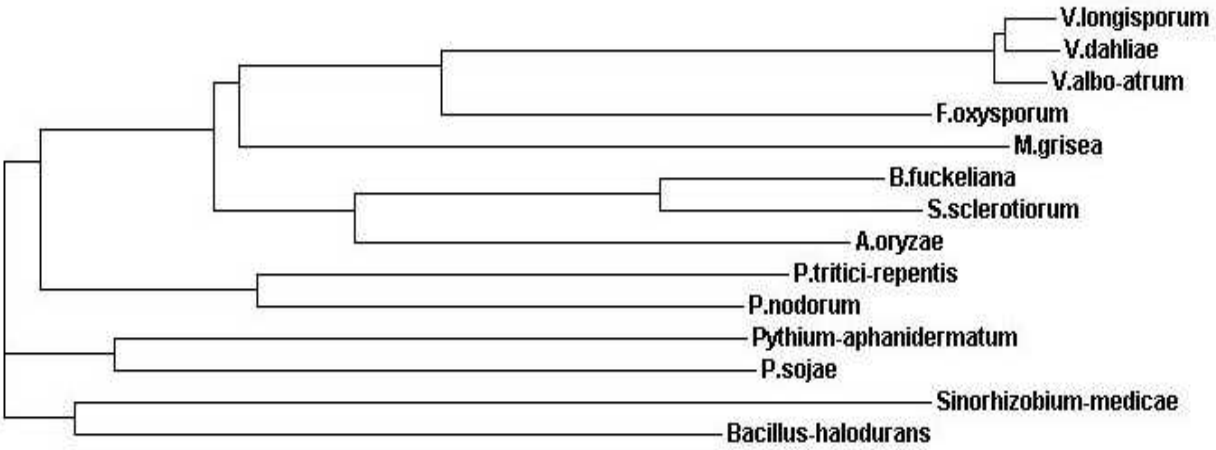

Figure 5.3: Phylogenetic tree based on NEP protein sequences. Phylogeny tree showing evolutionary relationships was constructed from an alignment of NEP protein sequences using the multiple alignment program ClustalW2 in combination with Neighbour-joining algorithm.

For most NEPs it was observed that they are capable of inducing a HR-like response in plants (83), but the mechanism of initiation of this response remains unknown. The cellular

role of NEPs is also under discussion and it is not clear yet whether NEPs are true elicitors triggering plant defense or whether this induction is based on a different activity (75, 79). Recognition of NEP induces signal transduction cascades mediated by calcium and MAP kinases (73). In spotted knapweed, a plant response linked to a calmodulin-like protein was reported. *Arabidopsis* reacts to Nep1 (NEP from *F. oxysporum*) with the activation of AtWRKY-18, a member of the WRKY family of transcription factors and of protein kinases (84). The treatment with NPP1 (NEP from *Phytophthora spp*.) resulted in the induction of typical HR-like symptoms due to the rapid accumulation of ROS and in biosynthesis of phytoalexin in parsley and in *Arabidopsis* (73). In *Arabidopsis*, callose deposition at the site of PaNie (NEP from *P. aphanidermatum*) application was observed within 24 hours after treatment (77). The activation of the phenylpropanoid pathway which is responsible for lignin and phytoalexin synthesis has also been characterized. Thus, PaNie treatments in carrot cells resulted in maximum transcription levels of a phenyl-ammonia-lyase gene at 4 hours post-application (83). Additionally, at 48 hours after PaNie application, the synthesis of 4-hydroxybenzoic acid was also detected in carrot cells, as this is a common compound for the reinforcement of plant cell walls (83). NEPs are also active as secondary signalling molecules. In concentrations of 20–30 pmol/g coca leaf, Nep1 induces ethylene production 6 hours post-application (73, 85). The same effect was found in parsley during NPP1 infiltrations (73). In *Arabidopsis* up-regulation of *PR1* was observed 8 hours post application of NPP1, indicating a response mediated by the salicylic acid (SA) pathway (73). Plant defence reaction in *B. napus* to an infection by *V. longisporum* was only in parts concordant to those related to NEP as described above, although it was demonstrated in this work that *V. longisporum* transcribes high levels of the *VlNEP in planta*. Thus, HR-like symptoms provoked by an oxidative burst is not typical for a *V. longisporum* infection in *Brassica* (53). Histological studies of the infection processes of *V. longisporum* gave no hint for any callose deposition as part of a penetration resistance in *B. napus* (34). It was further shown that *B. napus* plants infected with *V. longisporum* were significantly changed in jasmonic acid (JA) and in SA levels, but only at 14 and 35 dpi. SA levels increased significantly in shoot samples at these time points. In plant/pathogen interactions an increase of SA is an indicator for the attack of a biotrophic, incompatible pathogen usually inducing systemic acquired resistance (SAR). But the induction of SAR was not clearly observed in *V. longisporum*-infected *B. napus* (53, 86). In *Arabidopsis*, experimental data are controversial regarding the

role of particular signaling pathways during the infection with *V. longisporum*. Veronese et al. (87) found no induction of the SA-dependent reference genes *PR1* and *PDF1.2* in the ecotypes Columbia (Col-0) and C24 at 9 dpi. Contrary to that, Johannson et al. (88) reported an induction of the SA-dependent reference genes *BGL2* and *PR1* in Col-0 as early as 5 dpi. Quantification of phytohormones in different ecotypes including Col-0 and C24 using the same *V. longisporum* isolate as in the present work showed a similar up-regulation of JA at 28 dpi and SA at 35 dpi, thus only at late stages of infection (89). The phenylpropanoid pathway, which is induced by SA, is responsible for biosynthesis of phenolic secondary metabolites, e.g. lignin. In histological studies, a higher degree of vascular occlusion (tyloses, gels) as well as lignin deposition was found in *B. napus* as a response to *V. longisporum* infection, suggesting that this pathway is part of the mechanisms involved in plant defence (90). The decrease of JA levels in shoot samples, antagonistic to the accumulation of SA, supports the conclusion that the activation of the JA/ethylene signals transduction and thus the defense of necrotrophic pathogens are mainly suppressed. On the other hand, first analyses of the ethylene precursor 1-aminocyclopropan-1-caboxylic acid indicate that ethylene possibly influences symptom development, too (53). Although ethylene production in *Brassica* after *V. longisporum* infection has not been quantified in detail yet, it is supposed to take part actively in symptom development. In *Arabidopsis*, ethylene production is supposed to play an active role in *Verticillium* pathogenesis and in symptom development. This has been studied on ethylene-deficient mutants. *Etr1-1* mutants (Col-0 background) showed less severe senescence symptoms as revealed by higher levels of chlorophyll content than the wild-type (87). Several ethylene-deficient mutants (*ein2-1*, *ein4-1*, *ein6-1*, *etr1-1* [Col-0 background]) showed enhanced susceptibility in contrast to JA- or SA-deficient mutants and the expression of ethylene-dependent *PR-4* was increased 7 dpi with *V. longisporum* (88). The contradictory observations regarding the paradigm of the antagonism in SA and JA/ethylene signal pathways (91) gives rise to the hypothesis of the existence of an unknown ethylene-associated pathway (88). Beside this, theses findings support the proposal to describe *V. longisporum* to be neither a typical biotrophic nor a typical necrothrophic pathogen.

Little is known about fungal NEP expression in vivo. By RT-PCR analysis *PsojNIP* transcripts (NEP from *P. sojae*) were detected in infected soy bean tissues only until 12 hpi. *PsojNIP* transcription was initiated at the shift from non-harmful, biotrophic lifestyle to the

destructive, necrotrophic phase (74). In addition to evolving virulence factors, NEPs might also play a role in changing pathogenic lifestyle. In *F. oxysporum*, different aspects of NEPs are revealed. Bailey et al. (72) found out that *Nep1* disruption had no effect on pathogenicity in *Erythroxylum coca*. Nep1 over-expressing strains were also not altered in pathogenicity, however, transcripts were not detectable *in situ* neither in the wild-type nor in over-expression strains. In this work, *VlNEP* gene expression in *V. longisporum* was characterized as being strongly induced *in planta* and transcripts were detected from 14 to 35 dpi. There is little known about the fate of NEPs, when secreted into the intercellular space. Based on their hydrophilic property it is quite unlikely that they cross the plasma membrane into the plant cell (75, 92). It is more likely that NEPs react with a receptor molecule attached to the outer surface of plant cell walls. Jennings et al. (90) figured out that NEP necrosis patterns follow the paths of leaf veins, and thus concluded that the proteins may be soluble and translocated systemically. Once the receptor of NEP detection on cellular level is characterized, knowledge of the mode of action of these proteins will lead to a better understanding of the basis of NEP–plant interactions.

The plant response to several NEPs has been studied in some cases for both purified proteins and by the use of genetically engineered fungal strains (see above). Although high transcript levels of *VlNEP* were measured in hypocotyl tissue of *V. longisporum*-infected *B. napus* plants, reactions were dissimilar to those reported in context with NEPs. This discrepancy could be either explained by post-transcriptional regulation of the VlNEP peptide, by defective secretion or by insusceptibility of the plant. Contrary, VlNEP might actively function as elicitor, but might only affect parts of the actually expressed symptoms in *B. napus*, like the onset of early senescence. The typical symptoms of stunting and leave yellowing are supposedly triggered by *V. longisporum* elicitors other than VlNEP. Thus, by fractionation as to molecular size using the supernatant of a SXM-cultured *V. longisporum*, fractions >5000 Da induced stunting effects on 7 days-old seedlings of *B. napus* (53). The induction of plant responses and symptom development induced by high molecular extracellular compounds of *Vertcillium* has been often described (10); however, their nature is still unknown.

Outlook

Functional analysis of the *V. longisporum* transcriptome under infection-simulating conditions and confirmed elucidation of the regulation of target genes on transcriptional level *in planta* revealed a set of gene determinants most likely to play a role in the pathogenesis of this xylem parasitic fungus. To accelerate the progress in disclosing molecular mechanisms in the *V. longisporum* / *B. napus* interaction, two concepts for experimental investigations are proposed:

- Functional investigation of target genes and gene products by genetically manipulated strains of *V. longisporum*
- Integrated analysis of *V. longisporum* target genes (present work) and changes detected in metabolic profiles in the xylem sap of *V. longisporum*-infected *B. napus* plants (6)

Among strategies for gene inactivation post-transcriptional silencing by transformational introduction of dsRNA has been applied successfully for many targets in different organisms (93, 94). To date, siRNA-mediated gene silencing is the most promising strategy for *V. longisporum* due to its double haploidy (95). Additionally, mutants should be constructed which over-express target genes; particularly those which are found to be down-regulated *in planta*. Using those strains in plant infection assays, both strategies of gene manipulation should provide precious information about the role of these genes in pathogenesis. Other strategies of genetic engineering in *V. longisporum* beside gene inactivation should also be carried out. Reporter-fused constructs could be used for local and spatial expression profiles of gene products and their cellular localization which would give evidence of their functionality. Furthermore, reporter constructs will be of great impact especially for studying those fungal peptides that are secreted into the intercellular space. For example, depicting the fate of *in planta* expressed VlNEP within the *Brassica* system would be very valuable to understanding the site of molecular recognition and elicitation on cellular levels.

The know-how of monitoring changes in transcript levels of target genes being regulated by xylem sap metabolites and of construction of promoter-fused reporter strains of *V. longisporum* will serve as very valuable tools with regards to the integrated analyses of fungal target genes and corresponding xylem sap marker metabolites. By a comparative metabolomic profiling of the xylem sap collected from mock-treated and *V. longisporum*-infected *B. napus* plants, a set of marker compounds were identified as being either induced,

repressed or newly detectable in infected plants, the last class includes fungal expressed metabolites *in planta* (6). Fractionation of xylem sap guided by qRT-PCR or by assays with marker gene fusions help to find the link between fungal target genes and marker xylem sap metabolites, thus reviling information about chemical interactions in association with the identified genetic markers in *V. longisporum* pathogenesis.

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Summary

Functional transcriptomics is vital in biology. Comparative analysis of the transcriptome in different physiological or developmental stages facilitates the assignment of biological functions to genes. In this work, we chose cDNA-AFLP (cDNA-amplified fragment length polymorphism) differential display to investigate the genes involved in the pathogenicity of *Verticillium longisporum* infecting oilseed rape (*Brassica napus*).

cDNA-AFLP transcriptomics does not require prior sequence information and can therefore be used as a gene discovery tool. Unequal distribution of restriction sites used to generate cDNA fragments negatively affects the performance of cDNA-AFLP. Some transcripts are represented by more than one fragment while other escape detection, causing redundancy and reducing the coverage of the analysis. With the goal of improving the coverage of cDNA-AFLP without increasing its redundancy, we designed a modified cDNA-AFLP protocol. Immobilized cDNA is sequentially digested with several restriction endonucleases and the released DNA fragments are collected in mutually exclusive pools. To investigate the performance of the protocol, software tool MECS (**M**ultiple **E**nzyme **c**DNA-AFLP **S**imulation) was written in Perl. Simulation based on EST collections of *Arabidopsis*, mouse and human revealed that the sequential digestion strategy outperformed other cDNA-AFLP protocols in terms of coverage, redundancy, fragment length, and the total number of PCRs while the use of two marking and three sequentially applied releasing enzymes for each of the marking enzymes is recommended.

The sources of variance and errors in electrophoresis-based transcriptomics (cDNA-AFLP) were investigated including a comparison of separation and detection of amplified cDNA fragments in flatbed polyacrylamide gel-based as well as capillary electrophoresis (DNA sequencer). Capillary electrophoresis showed a significant reduction in errors and data variability. The total variance was partitioned into contributions of cDNA synthesis, adapter ligation, preamplification, amplification and electrophoresis. The highest contribution to variance originated from preamplification, indicating that this step should be the primary target of optimization. Parameters of computer-aided band recognition and matching were investigated and strategies improving matching success based on double passage with different signal intensity thresholds were developed. The overall quality of data generated by cDNA-AFLP was comparable with microarray hybridisation. Variance of cDNA-AFLP was

independent of signal intensity while microarray data showed higher variance for low intensity signals.

We analysed *V. longisporum* transcriptome after treatment with extracts of *Brassica napus* xylem sap by an improved cDNA-AFLP method with the goal to identify genes which are selectively induced during the colonization of xylem. Among over 13,000 individual cDNA-AFLP signals recorded, only 34 responded to the treatments. Reverse transcription real-time PCR was applied on 21 cloned transcript fragments to confirm their differential expression and 9 transcripts were verified. Further on we analysed the expression of candidate transcripts *in planta* and found 8 transcripts to be regulated in comparison to *in vitro* conditions. We expect that the analysis of genes identified in this work, especially after treatment with xylem sap metabolites from infected plants, will deepen our understanding of chemical interactions between *V. longisporum* and its host occurring in xylem sap and shed light on the interaction of vascular pathogens with host plants in general. Quantitative gene expression profiling of *V. longisporum* by genome-wide cDNA-AFLP helped us to reveal that adaptation to the xylem environment during plant colonization seems to be achieved by just a small number of regulated fungal genes, which, however, might be important for interaction with the plant. Sequence analysis indicates that the corresponding gene products might be involved in regulation, sugar-uptake, adhesion, apoptosis and the synthesis of secondary metabolites. Their role during pathogenesis can be elucidated by infection assays by gene inactivation and with over-expression mutants. Reporter gene fusions will be helpful in the elucidation of temporal and spatial expression patterns of candidate genes during the infection and colonization processes. We hope that the availability of sequences of genes induced during plant colonization will facilitate the elucidation of molecular adaptation of *V. longisporum* to parasitic lifestyle within the xylem environment of host plant *B. napus*.

Chapter 6: Supplementary data

Table S1: ESTs of *V. longisporum* affected by xylem sap extracts of *B. napus*.

pBLAST analysis was done with full gene sequences of *V. dahliae* VdLs17 (available at: http://www.broad.mit.edu/annotation/genome/verticillium_dahliae/MultiHome.html.), if matching to a *V. longisporum* EST.

EST ID	Number of clones	Sequence	pBLAST hit	E-value
TDF 2.1	2	AGTCCTCATTCCGCTCCATGCTCGGCGTGCCGCCGTCCA CGGCCTCCACCAGGACAGTGTCCTCGTCATCATCGACGC CCAGGGCGAATACGCCGAGGGCAAGCTCAAGATTTCCA ACATTGAGGCGTCGCGCCCCAACATCTCTTCCCTGCTGG AGAAGTACCGCGCCGCCAACGCGCCCATCGTCCACGTCG TCCACGAGACGCCCGCCGGCGCCCCTCTCTTCACGCAGG GCACGAAGCTCGCTGAGATCTTCGACGAGCTCACGCCCG AGGAAGGCGAGGCTGTCGTGACGAAGCACCACCCCGGT TCATTCGCCGACACCAACCTTCAGGAGATCCTGGAGAAG TCCGGCAAGAAGAAGATTGTGCTCGTCGGGTACATGGTG AGTCTTATCAACGAGATGGAAAGCTCGTCGTGGAACGTT GCTGACACATTGCAGGCTCACGTCTGCGTCTCGACGACG GCCAGGCAGGGCGCGCAGAGGGGGTGGGATGTCATCGT TGCCGAGGACGCTGTCGGTGACAGGGACATTCCCGGCGT GGATGCTGCGCAGCTTGTGAAGGTTGCTCTGGCTGAGAT TGCTGATGTCTTTGGCACTCTTGTCTCGAGCAAGGATATC AACTAG	Isochorismate hydrolase	E-44
TDF 6.1	1	AATTGATGCGTGTTGGGGGCAAAGCTGAACACCATGGGC TTGATGCAAAAACGCGAATACGT	P-type ATPase	E-56
TDF 6.2	1	ATGGACCCAGCTGTCGTCCGTAGTTCTCAATTCGATCGG	WSC-domains containing gene	E-0

		ATCTTCCAAACCACACTTCCCGGTCGATACGGCGGCCGT GCCGAGCAGATCTTCTCTCAGCCCCTCGTTTACACTCCCG ACGACAAGCAGTACGTCTATCTTGCGACCACGCAGAATA ATGTCTACAAACTCGATGCGCAGACGGGCGAAATCCTTG CCTCACGCAATCTCCACATTCCTTTCCTCTCGACAGACCT CGACGGATGCTACGATATTCAACCCCACGTCGGTGTCAC TGGTACTGGTGTCATCGATCCTGACACCGGCACCTACTA CCTCTTGGCCAAAACCTACGAGAACCAGGAGCTTGTCGA CGTCGCCCAAGGTCGCCCTGCCGGACGCTACTACCTGCA CGCTCTTGATGTGAACGATTTGAGCGAGCGTCCCAACTT CCCTGTCGGCCTCGAGGGTACCGTCGCCAGGAATAACCC CGATCGAAGCTTCAATGGCGGCATCCATCTTCAACGCCC TGCGCTGCTGCATGTGGGCCAGCATATTTACGCCGGCCT CGGCTCCCATTGCGTCAAATTCAACTTCACCGGCTGGGT CATGGGCTGGGATAAAACTACCGGAGAACAGGTCGAGC GCTTTGCGACGCAGGGCGAGGGCGTACCCCAGAACACC GAGGGCGGCGGCCTGTGGATGGCCGGTGGTGGCCTTGCT TCCGATGACCAGGGCTCCATCTTCTTCGCGACAGGCAAC GGATACGCTGGACAGCTTGCTGAGATTCCTGTTAACGGC CGCAACCCCCCGACTTCGCTTGAGGAGGCTGCTGTTCAC ATGACGATCCAAGAAGACGGAAGCTTAGATCTGGTCGA CTTCTTCATACCATGGGATAAGCGCGCTATGGATGGAGA CGACAAGGATCTTGGATCAAGCCCTCTTCAAATTCTACC CAGCGAATTTTCTTGCGGCAGCATCAGGCGTATCGGAGT CGTGACGGGCAAGAACAAGAAGACCTACTTCATCAATCT TGATGACATGGGTGGCTACCGCAACGGCGAGGATCGCTT CGACAATATCATCCAGACTTATGAACACGAGAACTCGGT TTATGCTGGTGCTGGCGTCTACCCTGGAGAGGGCGGTTA CATTTACATCAACGGTGCGTTTGGTTCAGGTCTGCGTCTC ACCCCCAACATTATTGCTAACTTGCACAAGTCGTACAAT ATCCTACCATCGTCTTCCGCTTCTCTTGCGCCAACGGTGT GCCTTCGTTCAATAAAGAGGCCGAGACCCCAGAGTCCAA		

		CGGCTACACCCTTGGCGTGAGCCACGGCACTGTCACCTC TCTCAACGGAGAGCCTGGCACTGCCATGCTCTGGACCAC TGACGTACAGAACCCGCCCGGCCAGCTTCGCATCTACGA TGCAGTCCCTCGCGACGGCGAGCTTGCCCTCCTCCGAAA GTGGGAGATTGCCGGCGTTACCAAATTCAGCCGCGCTGT ATTCGGCGACGGTATTATGTACCTGGGCACGACTACTGG CTTGTTCTACGGATTCGGCGCTCCCATCAACCGCCCCATC GAGTGCACTTCGCCTCTCGAATTCGGCGCCGTCAGTCTC GAAGCCAGCGCAGAGACCCGTACTTTGACTTGCACTGCT CTGATCAACACTGTTGTGAATGACATTTCTCTCCGCGAG GTAACCGATTTCAGCATTTCAGGACTGCCAACACTGCCC TTGACACTGGCTGTGGGCGCCACATTCACAATCGAGGCT GTCTTCGCGCCTACCGACCTCGGCCTTCTCTCCACCGATG TCAACATTGAGACGGAGAACAGCGTCGCCGGATACCGT ACCACCACATCTGCCCGACTCACCGGCACCGGCGAGACA GACAACCCAAGACTTTCTGTCAGCCCCAGAGAAATCGAA TTCGACAACGTCATCACAGCTGGCGCAGCGCCACCTGCT AACAATGTCGTGCTGTCGAACCAGGGCAACTCTGTTCTG ACAGTCAACGAGATCCGATACTCTGAGACGATCAACAG CACTCTGCAAACCTGGGATCCAGCCAGCGGCGCCCTAGT CATTGGGCCTTTCACAATCAGGAACATTCCTTCCACCATT GATGCCAATTCGGGCGCCACGGTCTCCGTATCCCTGAGC CCCGCGAATGGAGGCACATTCAGCGGGCACGTTCGATTC ATTACTGATGGTGGTAATACCGATGTGACCATGNCCGCT CACGTAGGTGCGGCTCCTGTGCTCCTCGTCGAGTTCGAG CGCCCNGACGGCGAGGGATGGACCACATATCNAGAAGG GACCGCATTCTCGTTTGGNGAGGTCACCCAGAACAACGT GAGAAACCTCAGGATGCGCATCACCAACACCGCCCCTGC TGGTGGTGTCAGACTATCTCTTACGGTCTCCAAACCTCCT CATGGCGGCTCCGGAATCATCAGGGCCAACAATGCTGTC GATCTTGGTGAGGGTACAAACCTGGGTCCTGGCCTGAGT GAAACTGCCGTGCTTTACTGTGCTGTTCCTAAGAGGCAG		

		TGGAACATGGAGCCCTACCAAGGCGAGGCTACCTGGAG TCTCAACACCAACGATCCCAAGGTGGCTTACCAGAACAT TCAATTTGAGTGTACTGCTGTCTNNNNNCAGTCTGCTCCT CTCCTGGAGAATGGGCTGTCGCAGTATCAGTATGTCGGT TGCTTCAGGGAAAACACCCCTGGCCGGCAATTGGCCAAC CAGCTCTACGGAAACGACGAGAACACCATTGCCATGTGC GTGGAGGCATGCGCTGCTGGCAACTACGTATTCTGCGGA ACGCAGTACCACCGAGAGTGTTGGGCCGGCCCGACCATC CCTAGGGAGCGTGTTGCGGACGTCAACTGCAACTTTGAC TGTGATGGGGATCTCAATCAGATCTGCGGTGGCAACGGT ATTGCGACTGGACCTGGTGGCGCTTACATTTCTCTCTTCG CCGATACTCTGCGATTCGATGGCAACGAGACCAACATCC CTGAGCCTGAGGAGCCAGTGGAACCTACTGATCCCATTG TTAACCCAGGCGTTGATGGATACATAAGCATTGGCTGCT ACACCGAAGCACCGGGGAGCCGCGCGCTGCCTTTCTTCT TCGCCACCGAGGATCAAACTGTCGCCATGTGCGTGGACG CCTGCTCGCTTCGTGACTACGAGTACGCTGGTGTCCAAT ATGGCGGAGAGTGCTGGTGCGCCAACGCTATCACGACG GGTGCCGTTCCGGCGCCTGACGCGGAATGCAACATGGCC TGCAATGACAACGCTGCCGAGTACTGCGGTGGCGGCTCT CGGCTCAACGTCTACCAACGCCAATCTGGAGGTTCTTTC CCTTCTGCTAGTGTCTCTTTGAATGGCACTATCCCGGCGC CGACTTCGTCTTTGATCCCCGCCTCTTCCGTGCCTGTCTC TTCTGCACCGATCATTTCAAGCATCGTGACGTCCGCCGC CCCGCCAGTGCCGACACCAGGAGAGGATCACTTTATTGG CGACTGGTCGTTCGAAGGCTGCTACACCGAAGGCGATGG CGTGCGGGCCTTGAACGGTCGCTTCTACGCCGACGACGA GTTGACGCTTGAAAAGTGTGCCGCCTTCTGCGAGGGTTT CGTTTACTTCGGTGCCGAATACGGGCGCGAGTGTGGTGC GGCGACGTTCATGGCACTGGCAGCGTGTTGGCGTCCAAT CAAGGCGACTGCAACTTTCCTTGTGGCGGCGACGGCTCA CAGTTTTGTGGTGCGGGCAACAGGCTTCAGATGTATCGC		

		TTCGGTGGTGCGGACGCGCCTTCGGGGGCTGTGAGCAGC TCTCTTGTGGTGCCCACGTCTGCGGTCGTCAGTTCTACTG TTGCTCTTGTTAGTTCAACTGCCGCAGAAGTCAGCTCTGC CGTTGCGGAGGTTAGCTCTCAGATAGCCATCGAGGCTAG CACTGCCCCTGCTTCCAGCTCCGTCGTTGAAGAATCCAG CACATCAGTCGTCGAGACCAGCTCGACGGTTGCTGTGGA GTCAAGCGCTGTTCAGTCCAGTTCTGCCCAACCCAGCTC CGCAGAGGCCAGCTCGGTCATCGAGATTATGTCTTCCGC CGTTGCATCTTCGAGCTTTGTTCCTAGCTCTGGGATCCCA TCATCCACGACCCCGACCTCGACGCCGTCACCCTCGGTC TATCCAGGCAACGATCTCTGGAACTACACGGGCTGCTAC TCTGAGCCCTCCCCTGGACGCCTTCTCCCCAGTCAGCTCC TGAACGACGGCGATGAGATGGACATTGAGCTCTGTCTCG ACGTCTGCGCTGGGTACAACTATGCAGGTGTCGAGTATG GTCGTGAGTGCTGGTGCGGTGACCGCCTCAACGCCGAGG GTGACGTCCCCAGCGAGGGCACAGCAGTGCCTGGCGAG CTGGTGGACGACGATGAGTGTGCCTTCCTGTGCCCTGGC AACAGGCTGAATTACTGCGGTGCAGGTGTCAGAATGAG CGTGTATATCCTGAGAGAGCGCGAGGAGGCGCTGGCTG AGGCGTCCTG		
TDF 6.3	1	AATTGTCCCCGATCGAAAAGCAAACTCAAAGGTGAAAAC AAACATTTTAGTGCTCCAGCAGACGCAAGCTTCCCTGGA CAAATACGT	UTP-glucose-1-phosphate uridylyltransferase	E-12
TDF 7.1	2	AAGCTTCTATAAATAGTAACCCTAACCCTCTAATAGTAT TCCCCCTTCGGAAGCTACTAGGTCGTAATACGCTATTAC TATAAACGAGGTTAGGAGTCGACTTCGACCTATTAAAGT CTTAATTAGAATCGAGTAGCTCTAGATTAAAATCTTAAG AAATAATTTAGTAGAGAATATATATATTAAAAAGAAGG AAACTTATTATTGAAATAACTATACTATACCTTAGTCTCC GACTATTATACCATTTAAACGCTATTCTACGTAGTCGCA AGCACTTACATTATATAAGGTAGCTAGCCGAGTTATTAC	No hit	

		TTAGGTTAGCTCGCGACTATCGGATCCCAATATATAATA GTAGAAGGCGTAACGTTAAGTAGCCGGCATAGTAATAA TAGTATAATAATTGTTATAATTAGGCACCGCGATCAACG AGCGCATAGTCTAGAGAGGATGCAGTTTCCATTTAATAG CCTAGGCACGTTTTCGTGGGTTATATGCCCTGCTTATGCG CTATAAATAATCTGCGAGGTCCCCTCATATAATGTGGAC TCGGGCACTAATGCAGGAAGCGTGCGACATTTTTAGGGG CGGAGAGATCGGGTCGAGATCGGGTCGAGATTGGGGGG AGAGATTAGGGGTTAAGGGGAGAGTTCGAGGGGAGAAT CTACGGGAGATAGTCCTATCCCCTATAGATATCCAAAAG TCTCGCTATTAGGCTATCCCTAGTAAGGAGGGGCAATAT CGCGCAAGTAGTAGCTAAGATACTAGCGGCCTTAATATC GCTAGCAAGGATAGCTACTAATAAATAGGTTCTATTCTT TCGAGGCTCATTTATAATACTTAAGAAGGTCACGATTAA TTAATATCTAATAACCTAGTTATTAAGGAATCTTATATA ACGAGTAAAGGGATCAACTACTAGTCTCTTAGGGCTCTC TAGGAAGATAGAATAATCGATGCCCTTCTCTAAATCTTA ATCTATTAACCTTTCTTATAACCCTTCCCTTACTATGCTC GGTCCTCTCTCCTTAATCCCTACGATTAAATAATAGGTA ATCCCCTATTAATCTTATACCCCTTCTTAGGTCTTAATAT AATACCTCGTCTCCCTTTTTATTTCGTCCCGAAGTATAAT ATATTCTAGGTTAAAGGTCCCTATTAAGTCCTAAGTAAT TCTCTTTAAGACTATTAGGCCCCTTCTTAGGTCTTAATAT AATACCTCGTCTCCCTNTNTATTTCGTCCCGAAGTATAAT ATATTCTAGGTTAAAGGTCCCNANTAAGTCNTAAGTAAT TNTCTTTAAGNCTANNGGGTATTATCTAGNCNAGAGNCG TCTANATNTTCTAGGTTTCTATNTAGTTCNNNNNNNNNN NTATCTTAAAGGGACCCTCCTATTATATAGCTAAGGTTC TTATATTAGTTTCTTCTAATCTAATAATTACCGCTTTACT ACTTCCGTTTCTCGTCTTAATTCTATTTCTTATTCTTTTAT TATTCTTAATTAATAATTCTAGAAGCTCTCTTCTATAGTT AATTAGCTAATAATCGCTTATTAATATAAGCTT		

TDF 7.2	3	AATTAGGCTCGCCGCCGGCAGTCGATATCAACAAACCGG TACTCTCTGTACCTGCCTCGCGCGCACGT	Clock-controlled protein 6	E-28
TDF 7.3	1	AATTACTCCTCGCCGTCCTTTCGGCGGATCTTTCTCCGAC GCAGAAGCTCTGCCAGGGGCACGT	Myosin class I	E-155
TDF 7.4	1	AATTATAGCACTGTCCGACATCGGTGAACTAACGATCATA AGAAGGATTGGCAGCACCACCAACCTTTGCACGT	Glycosyl hydrolase	E-0
TDF 7.5	1	AATTAAAGTGGTGGGTTCAACAAACTCTGCATGTTCGGC CATCTTCATAGACATTGGGGCTGTCTTGCACGT	AIG-2 like superfamily	E-27
TDF 7.6	1	AATTACCCCTCCCTCCCCTGCTATTCACAACTCATGCCCTG CACATCCGGCCATCGGCATCGACGGACCAACTCTCACTG CCAACGCCCTTGAGCACGT	No hit	
TDF 10.1	1	AATTTCACGGCACGCAGCAGTCGGAGAGACAAGTCTATG ATGTGCAGGTGGAAATCAAGCACGT	Vacuolar import protein	E-06
TDF 10.2	2	AATTTGTTCCGCCCACTTCCATCCGCATCTTCGCTTTTCGC CAGCGCGTCTTACGCCAGCCAGCACGT	ACEI transcription factor	E-0
TDF 10.3	1	AATTTGCGTCGCGTCGGTCGGCGTATAGGCGTCGGCTTCG ATCCGCACGACATGGTCCAGCACGT	No hit	
TDF 10.4	1	AATTTGGGCGGTCGATTTTTTAAAAGATGAATAGGAAAGC TTGAAGAGCTCAATGAATGAAGAGCACGT	No hit	
TDF 10.6	1	AATTTGGCTCGCCAGCCGGCAGTCGATATCAACAAATCCG GTACATTCTTGTACCTGCTTCGCGCGCACGT	No hit	
TDF 10.7	1	AATTTCGCACATTGCATCTGGATTCCCACGAAAGAAGGAT GCCAGGGCCCCTGCGACCATCGATCGTTGCATTACCTTCA GGTTGCAAAGTTGATACAGCACGT	Mitochondrial carrier	E-70
TDF 11.1	3	ATGGCCGAGTCGGAGAGCAAGTGCCCCGTCCACCAACTC AACAACGTCGGCGGCGGCGGCACCCGCAACCGTGACTG GTGGCCCAATGCTCTGAAGCTCAACATTCTCCGTCAGCA	Peroxidase	E-0

		CACAGATGTCACGAACCCCCTCGGCAACGACTTTGACTA CGCTGCTGCCTTCAACAGCCTCGACTACAATGCCGTCAA GAAGGACCTCAAGGACCTCATGACCGACTCTCAGGACTG GTGGCCCGCCGACTTTGGTCACTACGGAGGTCTCTTCGT TCGCATGGCGTGGCACAGCGCCGGTACCTACCGCGTCTT CGACGGCCGCGGTGGTGGCGGTCAGGGCCAGCAGCGTT TTGCTCCCCTGAACAGCTGGCCCGACAATGTCTCGCTCG ACAAGGCTCGTCGCCTGTTGTGGCCCATCAAGCAGAAGT ACGGCAACAAGATCTCCTGGGCTGATCTGCTGCTCCTCA CCGGCAACGTCGCCCTCGAGTCGATGGACTTCAAGACCT TTGGCTTCGCCGGTGGTCGGGCCGACGTCTGGGAGGCCG ACGAGTCGGTCTACTGGGGTGGCGAGACCACTTGGCTCG GCAACGACGTCCGCTACTCTGGTGGCAACAAGGGCGAC AAGGGTCCGGGCAGCCTCGTCACCGACGAGGGTCATGA TAAGAGCACCCACACCCGTGGCTTGCAGAAGCCTCTCGG TGCTGCCCATATGGGTCTGATCTACGTCAACCCCGAGGG CCCCGATGGCAACCCCGACCCTGTTGCTGCTGCCCACGA CATATAGGGCGATTGGGTACCGGGCCCCCCCTCGAGGTC GACGGTATCGATAAGCTTGATATCGAATTCCTGCAGGTA CCTCTTCAAGTACGAGTGGGAGCTCACCAAGAGCCCTGC TGGCGCCAACCAGTGGGTGGCCAAGACCGACGACGAGA TCATCCCCGATGCCTACGACTCGTCCAAGAAGCACCGCC CTACAATGCTCACCACCGATCTGTCCATGCGCTTCGATC CCGAGTACGAGAAGATCTCTCGCCGTTTCCTCGAGAACC CAGACCAGTTTGCCGATGTTTTCGCTCGCGCCTGGTTCA AGCTCCTTCACCGTGACCTTGGCCCCAAGGCTCGCTACC TCGGCCCTGAAATCCCTGCCGAAGACCTCCTGTGGCAGG ACCCCATCCCCGCTGTTGACCACCCTCTGATCGACGAGA GCGACATTGCCGCTCTCAAGAAGGAGATCCTCTCCTCCG GACCTGAGCCCTCGCAGTTCATCTCCGTTGCCTGGGGAG CGTCTTCTTCCTTCCGTGGCAGCGACAAGCGCGGTGGTG CCAACGGCGCCCGTATCCGCCTCGCCCCCCAGAAGGACT		

		GGGAGGTCAACAACCCCGCCCAGCTGGCCAAGGTTCTGC AGGCCCTTGAGGGTGTGCAGAAGAGCTTCAACGATGCTC AGCAGGGTGGCAAGAAGGTCTCTCTCGCCGACTTGATCG TCCTGGCCGGTAACGCCGCTGTTGGGAAGGCCGCGTCGG CCGCCGGCCACAGCGTCACCGTCCCCTTCACTCCTGGCC GTGGCGACGCCACTCACGAGCAGACCGACATTGAGTCC GTCAGCCACCTCGAGCCTTTTGCCGACGGCTTCCGCAAC TACGGCCACTCGACCGACCGCGTCAAGACGGAGCAGTT		
TDF 11.2	1	AATTTAGGCGCGTCAAATGAATATGAGAAGAATCCAAGA CGAGAACCGTACGGTCACCCTTCAATATAATCGA	Putative cyclase	E-76
TDF 11.3	1	AATTTCCCTTTTGGGAATGACATCAGCAAAGCCTCGCGCT CGCATGACCGAGCTCTTTAAAGAAAGACCAATCGA	No hit	
TDF 12.1	2	GGCACCGGTAAGTCAACTCTTGTAT- CAACGCAGCGTTTGGGGAGATACTTATCGGTCGTTGAT- GAAGGAGAGGGCCAAGACCGCCGCCT- TGTACCTCGATTTCTGCCTGACGTCCATTCGATGC- ATTCAAGCCACCGTCGAATACCTCAGCGAC- CGCGAACAAATTAGACCGGCCGACCGAC- CATACACTAACGGATACTTCATTG- ATCTCGTCGAACAGATTCGCCAATACGCCGGACAACTG- GCCACTGCCAAGGAAGCCGGAGTCGAGGGGCGTGAGAT- GGACGTCGACCCCACCGACGAGGTTAAGCTGTTCGGTG- GCATCTCGCAGAACGGCCGCCCCGCCGAGCT- TGTCCGCGTCAGAAAGGACGGTCAAGCCATCTCCATG- GCCACTGGCCTCCCCGTTGACATGGATGAGGACG- GCAAGGATTTCCCCAGACTGAAGCGCTCCCTGAGC- CAGCAGCTGGCAGACGACGAGGAGATCATGCGGTCCAT- GGCTCGCAGGAAGAAGAACGCTGCGCCGGAG- GAGCTCGCGCCCAAGAAATGCCGCGAGCCTGGCT- GCAACAAGGAGTTCAAGCGTCCCTGTGACCTGAC-	ACEI transcription factor	E-0

		CAAGCACGAGAAGACTCACTCTCGTCCCTGGAAGTGC- CCTGTCAAGACGTGCAAGTACCACGAGTACGGCTGGC- CCACCGAGAAGGGGATGGACCGCCAT- CACAACGACAAGCACTCCTCAGCGCCCCCCATGCAC- GAGTGCCTGTTTAAGCCTTGCCCTTACAAGTCGAAGCGC- GAGTCAAGCTGCAAGCAGCACATGGAGAAGGCCCACG- GATGGCAGTACGTCCGCACCAAGACCAACGGCG- GCAAGAAGGCGCCCAGCGTTGCTGGAAGCTCG- GCACAGCCGACCCCTCAGCTTGGCAACATGGCAACGC- CCTCGAGCAGCCACAGTATTGCTACGCCGCCCGAGGA- GAGCACCAGCCTCTTCCCGCCTTTTAACCACGAT- GACTTCCCTCACTACGTCCCGGCCGAGGAGTTTGCT- GACACCTGCCTCGGGCCCATGGGACAGCCGCCCAT- GACGCTCGAGGGTATCGACTTTAACGACCTTGGCGT- GTCTCCCACTGATTACAACACCCCTTCTACCGACA- CATCCTACCCATACACCTCTTACCAGGATGGAC- CCGAGTTTGTCATCAACAACGATGACATTTACGGCGC- CCGTGTCCAGATCCCGACACCGGCGTGGCCCGAAAAG- ATGATGGCTGGCATGCAGAACTACGCCCCAGTGTCTGC- ATGCCAACCTCAGATGATGCCCGAGCCGCTCGC- CCCACACATCTCCCCGATAGGTCAGGGGAACGCCAT- GCTCTTCACGCCCAACTCGTTGGCCGAGGTTGACGAAG- GCTTTGATGATTTCGGCGGCTGTGGTGATGATTTCACCT- TGTTCCCCGTCAACGGGCTCGACAAG- GACGCACAATTCCAGACTCTGTTCGGCAGCGAGATGC- CCAGCAGCGGCCTCGGCTTGTCTCAGGGCGCCTCCCAG- GACTTCTTTGGGAACGGCATGGACTGGTCCAGCATG- GAATACCACACCTACTCCCAGCAGCCCCAGCAC- CAGCAGTAG		
TDF 12.2	1	AATTCGTCCTCGGGCCTCAAGGCAGAGTGTTGGGAGGCA CTTGAGATGGACAAGTACATCAACGACTGGATGGCAACC	Unknown protein	E-137

		AACAGCGCCGCTGCCGGGTGTGACACTCTTGGTTTTGCC CAGTGCTACCTCCAGTTCAACGGCCTGACCACTCTGACC TGTGATGACATTACGT		
TDF 12.3	1	AATTCGCAAAGTACCAACACCCGTTTCCAGAGCGTTGGC GTTCTTTGCCTGCTAAGACGCTAAGCCAAAGGCCTATTCG TGTCCATCTATACGGCGGCCGCTCATCGTCGTGGAGGAGG GCGGCGAGGGTGATTTTGCGGAGGGAGAGGGCCGGGTT GCTGGTTGCGGTGGTGGGGTCGTTGTAGATCTTGTGGGC GAGAGCGAGGACGCGGAGGATGTGCGACCAGTCGTGCG AGGCGTCGTAGTTGGACATGTAGTCTTTTACGT	Unknown protein	E-13
TDF 14.1	3	AATTAGAAACGGGTCATTATTTGTCAGCACCCGCCAATCA AGAGACGCTTGTCGCGTAACGCGGCTAAGCGTATGCCAC TAACGCGATCGCTCCTGGATTCGA	Proteasome regulatory particle subunit Rpt4	E-21
TDF 14.2	1	AATTAGCATAGAGTGTGTTATCCGAAGGCTACCGAGCCAA AGTTGATCAAGCAATATCTTGGTTTGCGTACACACGATTT ATGTCACTTGTTCTACATTCGA	Glycosyl hydrolase	E-0
TDF 21.1	2	AATTAGACAACCACCCTTTTGAGCTATTCTTGAGATGGCA TATAAGGGAGATCTATCTAGTGCACTATCGA	No hit	
TDF 21.2	7	AATTAGTGTGTACAGCGAAGAGCGATTGGGGGTTTCAAA GTCAGTTATTTGCTTTTTCGCTTTTATATCGA	No hit	
TDF 21.3	1	AATTAGAAGGTTATCGTTTATATATAGCTATAAAAGATAATA TTATCGGGAAAAGATAAAATATCTATCGA	No hit	
TDF 21.4	1	AATTATTCGTTCAATTTTCCACATAGCGTCACTGATGCTGG ATAACTGCTGCTGGGCTTCTATCGA	No hit	
TDF 22.1	2	AATTAGGTGCCCATCATGGCGGCGGAGCACCACTGCCGC TCTCGGATGACGGCAGGGGCTTCTTGTTCGCATAGGTGTC GTGTGGACGGTATCGA	Unknown protein	E-24
TDF 22.2	4	GCCACAATCCAGCCCCAGATTGCGACCAAGCTTGGGCTT	Type II:HAD-superfamily hydrolase	E-51

		GCAGCCCCGAATGCGGCCGAGAGCGAGGCTTTCGGCAA CTCTGTCAGCTCTTGGCCCGCTTTTCCCGATACTGTCGAC GCCNTGCGACGACTCTCGTCCCACTACAAGCTCGTCGTC TTGTCCAATGTCGACCGCGAATCGTTCAAGGGGTCAAAT GAGGGACCACTGCAGCGCGTGCCTTTCGACCTTATCATC ACCGCTCAGGATGTTGGTAGTTACAAGCCTAACCTGCAA AACTTCGTGCATATGCTGCGGGAAGTCAAAGAAAGGTTT GGGGTGGACAAGGAACATGTTATTCAGACTGCACAGAG CCAATTCCATGACCACCATCCGGCAAAGGAGATAGGCAT CAAATCATCAAATTAGTACTACTGTTACTGGTCGCCACA ACACTCGCCTAGTATAGTATGCTAGCTCGTTTCTATCGA ATACTGAACGTTGGCCACGATCGATAGAACAAGAGCCG CAGCAGACAGGCCCGTAATCACTGAGAATCCAGTCCTGT AAAGAGGGGCATCCTCAGAGCGGAACAGCTGCTTGCCA GCGCTGCCGTpCAATTCAATCAATTCAAATATCGGGGTT ATATTTAACTATTTGATATATTTACGGCTATAGATTTGAT TAATTGAATTGTTTAATTGGATTTAATAGTATTTAGTTAA ATATTAGAGGTTCTATAAGATTTTTCTAAGGAGATTTNA AAGATTTTAAAGC		
TDF 22.3	1	AATTATATGGCGATGTATACCAATCAGCTTCCCAATGTATC GCCGATTACCGTGATCTGGTCTTTGTGTATCGA	No hit	
TDF 22.4	1	AATTACCCTCTAGACTTACTATAACAAATAGCTTATATAGG CTATAATAGCGCTATAGGATAGCTAGGTATCGA	ABC ion transporter Ptype ATPase	E-81
TDF 22.5	1	AATTAGACGTTGTTCAACTGGACCTGATCCCCTTTGT-CAAGTTGAGCTGCGGGAATGTATCGA	F0/F1 ATPase	E-11
TDF 22.6	1	AATTAGCGAGCGTTGGGCTCTCCGCGCGACGAAAGGCTT CACAGGCTGGACTCGGCGGATCCTCGTTCGCTATCG	Unknown protein	E-08
TDF 22.7	1	AATTAGTGTGTACAGCGAAGAGCGATTGGGGGTTTCAAA GTCAGTTATTTGCTTTTTCGCTTTTATATCGA	No hit	

TDF 22.8	1	AATTAGTACTACTGTTACTGGTCGCCACAACACTCGCCTA GTATAGTATGCTAGCTCGTTTCTATCGA	No hit	
TDF 24.1	2	ATGGCTGCCTCACAGCTCGACCTTCCCCTTCCCGGTGCCT CCTTCTTCGATATTCACC*ATGATG*GAACGACCACCCGCAG TCCTGTCCCGTTGCCCG*ATG*GGGGCTGCAACTTTGTCGAC CTGACGCCCGGTGCCAACGGAGCCAAGTGTGG*ATG*TCGC CGCTTCTGGAGTCGCACTGTTTCCGGGAGAGGATTCGCA GATACCGTAGGATCGGACCACACGGCCTGGTGC*ATG*TGC ACTCACCACGCTTGTTACCACGACCACACCCGCGACGCA GAAGCCGCCACCCCCGTAATAGGATTCGTTCCCGGACAG GAGAATACAAAACTCAAGGGACATCGTGGGCCCCTAAG CCCCGTCGTTCAGG*ATG*CTTCGTTCCGCCTGCCTTCTGGA TTCTCTACTTCATTGGATCTT*ATG*AATCTGGACGCCGCG*A TG*CTGCTGCCC*ATG*AGCAAGCCCGAGG*ATG*CCAGGAACC CGCGCGCTGGACCCCTCAGCCAGCAGCTCGAATCTACAC TACAGGATACGTTAAGTTGGGGCGAGTTTGTCCAGTCTC AGTCAGCCAACACGACCACACTGCCCCCGATCCCCCCTC A*ATG*CCTT*ATG*CCCTCTCAACCCAGTTCCACCACATCATC TAGCCAAGCTCGTTACTTGCGCCCTTTTGCCGGGAAAGG ATTAAACACGCTCAGTGGTGTGCACCAGCCCGACCCTCG TTCTCCTCGCCAGGAAAAGCCGCACGACCTGGAGCCC*AT G*GACATTCAGCCCACAAGATCTGGA*ATG*GAAGTCGTTAC CGCGGGTGCCGCATCTGCTGTTGGAA*ATG*GTTCAGACAC	Unknown protein	E-0
TDF 24.2	1	AATTTCTTCAGGTAGTACCCGGCATGTTCCCGATTCGTGA CATTGAACACGTTGTCGA	No hit	
TDF 24.3	1	AATTTATCCCTTCTTCCCCTTTGCCCCGCACGCCTGGCGC TGTTGTCGA	No hit	
TDF 24.4	1	AATTTGAAACGCTGATCTAGAATTTGAGCAATCGCTCCAC CCAATTCTGTCGA	No hit	
TDF 24.5	1	AATTTGGCGGCGAAAAGCAAGTGGCATGGCTGGCAGGA	DNA polymerase III, translocase	E-81

		CTGCACCATATGCCCAGAATGCTACGGAACAGCTGTCGA		
TDF 24.6	1	AATTTCGGGCAAAAAAAGGGGAGCACCTGCCCCCCCGAGGATTAAACGGTGGTGTCGA	No hit	
TDF 25.1	4	AATTTGCTCCCTGCCGCCAGTTCTGGCTCGCCTACCGATCTCTGTGCCCCTCCGGCTGGTACACTCGCTGGGACGAGCAGCGCGAGGCTGGCTCTTTCCCGTCAAGCTCGA	Cytochrome C oxidase subunit Vib	E-46
TDF 25.2	3	AATTTCCGGAAAGAGATGCGAGCGCTGGACGCGAATGGGTATGTGGGGAGCTGCGAAGGCTCGA	No hit	
TDF 25.3	1	AATTTTTGTCGCTGATAGCTCCAGCTGTGTTAATAAGTAGTCTCTGCTCGTTATTTGCGCTCGA	No hit	
TDF 25.4	3	AATTTGGCGTTGGCAAGACCACGGCAGATGAACGCATCGGCGGCTTCTACTTGTTGCTCGA	No hit	
TDF 25.5	1	AATTTCGCTGACAAACACAAGGGTTCGCCAACTCGGCTGAGGACATCTGGTTTGAGCTCGA	CVNH domain	E-12
TDF 25.6	1	AATTTCGTCGTCGCTGACACCCAGGTCATCGCGAGGAATCCAGACAGTCGGCATGCGGGCTCGA	Unknown protein, putative DNA binding domain	E-100
TDF 25.7	1	AATTTGGCAGCCCCCTTGCCGGCGCAAGTCGAGCCGTTGGGGTCTTTGTCCAGCGCCAGAAGGAGGAGGAGCTGGAGGAATCGCACGGGAGGCTCCCGGCTCGA	Zinc-finger protein of unknown function	E-148
TDF 25.8	1	AATTTACGAGATGGCATGGCGCGGCTGCACCAGTCTAAGATCAATGTCTTGCGCGACCGGCAGGCCAAGCGCATGGAAGAGCTGCTCGA	IBR domain [in between ring fingers], indirect TF	E-89
TDF 25.9	1	AATTTGCCTAACAACACCATGTATATATGTAGATGCCCAGCCTTTGTTTAAACGCCGAGGCTCGA	CT1B_FUSSO cutinase transcription factor	E-0
TDF 26.1	2	AATTCCAGGGCCGCTCAGGCTGTCGCTATGCTAAAATGATGATCCAATGCGCCTCATGCATTACCCTCCTCGCATCTCTTCCTACATCTCCATCGTCGTAACGT	DNA topoisomerase I	E-164

TDF 26.2	2	CACGCATCGANTCACCGCAATCTCCTGTCGCTTCATATA CGCTCAGCTAATTCCANGGGTGTAGCACGCGCGGCGGAC CNTCGGTCCTCGTCNTCTGTCACCTAGCTGGCCGGATTTT ACCAATGTTGAGCCGGGCGGATCAAATCCCATCCTTGAC CTAAAAGTGAGGCTGGCAAGTACCCTGGTCCGGAATAC ACAAACACAACTAGCCTGTGATCCATGGAGATGGAGAC GAAAGCCGTTAAAGCACAAGTAGTTGCGTCGAAACGCG CTTCTGACATGCAGCGAGGGCATGTACCTAGGGGGGTTC GGTTCAGCCGGGCAACGTTGCTGCATATCCCCCCTCCCC CCCCTGTAGCCCCTCGCAGCTCCCCGGAAGACCAAGACG CATATCCCTGCCCTGGCTTGTATTATAATGGTGCTGCAGC AGGCTTCGAACGCCGTGTACCACAGCATACCATCCCTGG TCCCCCGCTCGAAGCCTGAAATGGGCGGGGATGACATCC ATTTGTGCTACCAGGCTTTCCCCCCGATCAGTTTTGGTCC CCCACATCGTGTCGCAACCTTTACTAACCAGAATACACA TAGTAAGATGGATCGTCCGACTGTCGGTGTAGGCTGCGG CGTGGCCATTTGCGTCGTCCCTGTTTCAAGGGGAAAAGA GGAAAGCAGACTGCCTGTGGCAGCGCGATGCAACGCCA CCTTAGGTACCTAGTCCACCTAAGTAGGTAGCTAGGTGC AGCCAGCAGGAGACCCAGGGCTTGCGGCTGAGTTGTAA GCCGAGCGGTCGCTGGGGCTGGGTTGACCCCCGTTACTG GATCGCTGGTAATGTACGTTATAACGTCTGGGGGCAGGT ACCTCAAGTACCTGAACTCAGGCAGGGGTAAAGCCAAG CTGACATTCGCACGAAGCGTGGAACGGAAGGACCCCTCT TCTCGGGCTATCCAAGTTTCTGACCCGTTGTCAGCTTGTC AGATGTCAGATGTTTCTTCAGAGTAACGTTATTTCGCCTG TCAGTGTACCCAAGGTAGCATGAGTTGCAGGGTACCTAC ATACTTAGGTGCAAGGGGGAGGGCGGCACTGCAAAGGC CCATGAGACAGCCTGCGGGCGGTGTGGAAGCAGCGGGC AGTTCGCCATAAGCCGAGGCTGCCCTCCCATCGAATGGC ACACGACCTGCTTCTCTGTGTGCATGGCCCGGTGTGTGT GTTTCGAGTTGCTCATGGGTCAGCGTGCAATGAATTCCT	No hit	

		TTCGGATGCAAGCAGTTCGCAGCCAGGACAGCCAACGA GCAAAAACCTTCAACGTGGTCGGCGCAGAGAGGGGCGA GGGCTCGCCTGGATTCTGGACGCCTGACTCCTGTGGTTC GTTGTTTTATCATTTTTCTGGCGAGCAACCAGCGATTGCG GCCCGTAACCCTAGCTGTCGAGCGAACACCTGACCACCT AGATTAGGCGGCGAGTGTGGGGTCCTCATGCCCCGTGGG GATTGCCTGATTAAGGACCTTCGCGATCCTGACCACATG CGATGAGCTTCAGAGCGGTCAAAATCTCCATCACAGCCT GTGAACACTCAAGACCACGAGAATATGCACATTGAGTTT CATTAAATTAAGGTACGTATCAAAGGGGGGTATGGGTAT GTTGCGCAATCTGATCGAGCCTTGGGCTGAATGCCCTGT GATCAGAGGCGCCCTTCAGTCCGTGAAAACTTGCCCCCA AGACAAATGTGAGTGAGGTATTTGGATAGGTAAAATCG GTCAACGAAAATACCACCCTCGTGGCCAGCCTCTAGAAC CTTCAAGGCCGAGGANTTTATGGGTCTGTTAATCTCATG TCAGCCCTGGTCGCTGANGCGGTAGANTGGATAAGANA TCTCACCATCAAAGAACCTATTGATCANATGTCGAGGCA GCTTGGCGTGTCCAGATGCTC		
TDF 27.1	3	AATTCCTTTCCATTTCATTTCAGTCCATTCGCTTCCTTTCC TTTCGA	No hit	
TDF 27.2	1	AATTCCGGTGCACGACCCGCATTCGGAGATCCAAACCGT TTTTTCGA	No hit	
TDF 27.3	1	AATTCTGGCCAGGGCGATTGCGCGTCTCGGGCTGAGCTA CATTTCGA	No hit	
TDF 27.4	1	AATTCAAGCCCCGTAATCCTTTGTCCACATAAAGAAAGGA AATCAGTGGTTTTCGA	No hit	
TDF 29.1	1	AATTAGCACATGATGACGCTTAGGCGGTCGGGGGGCATG GACGCTACATCATCAGCGTGAACGT	No hit	
TDF 29.2	2	AATTACAGGACCTAGGAGTATGGCTGGCTCGAAAGGTTG	Methyltransferase	E-172

		GGGTTCTTGACGAACGT		
TDF 29.3	2	AATTAGCATTGGTGAAATCGCAACAGTTGTTGGTCGCTACTACGCCATGGACCGTGATAAGCGTTGGGAACGT	Phosphoglycerat mutase	E-52
TDF 29.5	1	AATTACAGATGACCGATCTGTGGATGATCCAGCTGTCATGCTGAGAAGATGTTGAACGT	Phosphoglucomutase/phosphomannomutase, N-acetylglucosamine-phosphate mutase	E-142
TDF 29.6	1	AATTAGGTGGACGGCGTGTATCATACACGCACGCGATTTTTGGGGCCATTTATGCGGATCATCAGTGTGAACGT	No hit	
TDF 30.1	1	AATTGAGGATATAAAGATTCGGTATTTATCAAGAAAATGAAGCACAAGGTTGCCTCCCAAGGTAGCCTCATTTCGA	Nucleotide/sugar transporter, integral membrane protein	E-96
TDF 30.2	1	AATTGGGCGGGGAGACCCTCCATTACCAGGCAACATAAGGCAGTGAAAGGTACATTCACAAGAAGACACTTTCGA	No hit	
TDF 30.3	1	AATTGGCACCTCAGCTGATGCGGCGTAGCTGCCCTTTTTTTTTTTTTTCTTTCCTTCTTTTCCCTCACTCTTTCGA	No hit	
TDF 30.4	1	AATTGTGGGAGAAGGTATGATGATTGGTCTTTATAGGTGAAGTGCCACACATGGCTCCAGGGACATTGTGTTTCGA	No hit	
TDF 30.5	1	AATTGCTTGCTCAGAACTTGAGTGGGGGAGGAGCGTCAAGGATTGGCGGAGGGAGGTTTCGCTCATATTTCGA	Histone	E-54
TDF 32.1	3	GATCATACATAGCTCGGTAGCTTGGAGGTGAAGATGCATCGTATGCAACTATTTGTTTGTTCATGTCACCGCATTACGT	No hit	
TDF 33.1	1	GATCAAGGTGAGTTCACCGCATCTGCCGGTCTCTTCCATGAGCGGCGGCAACCAGCAGAAGGTGGTGCTGGCGCGCTGCCTCTCGACCCAGCCACGT		
TDF 33.2		GATCAGGCGCTGACCGAAGCCCAGCATGAGAACATGCACGGCTGCGTGATGTTCATCGACCTGAACCGCTTCAAGCCCATCAACGACACGCTGGGCCACGT		

TDF 33.3	1	GATCATCTTGTTACGACTTTCAACCACATATTCATAACATC CTCTGTCATCGTGGTTCAGCGCCTCTCCGGTGGACCACAC CTTCCATCTGCCACGT	SNF7 family protein transport into lysosomes/vacuoles	E-30
TDF 33.4	1	GATCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGGGA GGGGGGAGAAAGAGAACAGGAAGAAAAAAAACACTGT TCATCTTAGGATGNCCNNNNNCCACGT	No hit	
TDF 33.5	1	GATCAGTGGTATTGGCACACTACTAAGATATCACGCGCAT CGTGCGGTTTCTAGACCAGCTGACGACGAATCCGTGTAA CCGTCCGCGCACCATCTCCACGT	Peptidyl-prolyl cis-trans isomerase	E-08
TDF 33.6	1	GATCAGCTCGGTCATGCTGGCGAAGGTCTTGCCGTTGAG TGCCACCGGACGGCCTTCCTCGAAGCGCACGGTGACGGT CTCGGCCTTCACTTCCACGT	No hit	
TDF 34.1	1	GATCAGGACCTTGGCCATGGGCTTGTGCACGCGGCCGTG GAAGTCCACGCCGATACCCACGT	No hit	
TDF 34.2	1	ATGCCCCAGGACATGCCGCCCGTGGGCGGCTATA*ATG*CCGTTC AATACAAGGTGGGTTTACCGATCGATCCCGTCGTGGCCGACA GCTAGGCCAATTGGGCTCAATACAGTGCATCA*ATG*CCTCTTCA GCACCGATAAACTTCAACGAGACTCCGCATCAGGCCCCAGGC GCCCATACAGACTTCTCCCCGAGCAACGCCGGCTAACGCTTCT CCTAGCGTAACCTCCCCGCCCGCGGCTTCCGCCCTGGAATCCT CCTTCTCGGA*ATG*GGCGCCGTC*ATG*GGCTACGGCTGGTACAAA CTGATCAAGGGCATCCGCGAGGCCAAGTACGTCCTCGTTCCCC CCTCCCGCTTTGTCTCGTCTTGCTTCGGCTGCGTAGCTTGGAGC CGTTGACCAATCCTCCCTCGAACAAAGAACTGACCACC*ATG*CC AGCGAGCTTGCCCGTGAGAAG*ATG*TGGGCGCGCATCCACCTC ATTCCTCTCCTCCAGGCCGAGGAGGACCGCGACCAGATCCGC CG*ATG*GTACGCCGATCAGGCGCGCGAGAAGGAGCTGCTGGGC GAGAACACGCGTGTGTACCACACGGACAGGTAGGACTGACTG GACGGCTTGCTGTAGAGGGCCGTTTGCTAACGAGGTGCAGGG TTTGTCCGACCCACGTTCGCTGTTGCGCCGGAGAAGACGAAAT AG	GRIM-19 programmed cell death regulator	E-07

TDF 34.4	1	GATCATGAGACTGGGCGAGATGTCGTCAATAGCTAGCAG AGTGACTTGGCAATACCCACGT	Pre-ribosomal particle constituent	E-17
TDF 34.5	1	GATCAGGTCCTCGACACGACGAACTACACACTCGCGGGT TGGGAATCGGATGCGTCCACGT	No hit	

Table S2: Gene-specific primers for *V. longisporum*.

cDNA	Primers
β-tubulin	CTACCTGACCTGCTCCGCCATCT GCTGGTACTCCGAAACGAGATCG
Ribosomal protein S17	GCATCTGCGATGAGATCGCCA TCGGAGTTCTGGGTAAAGTCGAGA
TDF 2.1	AATTTGGTCAAATATTTATTGATCATAACGGT AGGATATCAACTAGGACGTCAC
TDF 6.2	AATTGGCCAACCAGCTCTACG ACGTATTCGCCAGCAGCG
TDF 6.3	AATTGTCCCCGATCGAAAAGCA ACGTATTTGTCCAGGGAAGCT
TDF 7.1	AATTAGGCACCGCGATCAACG ACGTGCCTAGGCTATTAAATGGAAAC
TDF 7.2	TAGGCTCGCCGCCGGCA ACGTGCGCGCGAGGCAGGTAC
TDF 10.1	AATTTCACGGCACGCAGCAGTCGGAGAGA ACGTGCTTGATTTCCACCTGCACATCA
TDF 10.2	AATTTGTTCCGCCCACTTCCATCCG ACGTGCTGGCTGGCGTAAGAC
TDF 11.1	AATTTGCCCCTACCATGCGAG TCGATTTCTTGTCTGTGTCGCC
TDF 12.1	AATTCCAGACTCTGTTCGGCAG ACGTAAAAGAAACAGGCCATCCC
TDF 12.2	AATTCGTCCTCGGGCCTCAAGGCAGA ACGTAATGTCATCACAGGTCAGAGTGG
TDF 21.2	GTGTGTACAGCGAAGAGCGATTGG GCGAAAAAGCAAATAACTGACTTTG
TDF 22.1	AATTAGGTGCCCATCATGGC TCGATACCGTCCACACGACAC
TDF 22.2	TTAGTACTACTGTTACTGGTCGCCA

	TCGATAGAAACGAGCTAGCATACT
TDF 22.7	AATTCCTTTCCATTTCATTTC TCGAAAGGAAAGGAAGCGAAT
TDF 24.1	AATTTGACAAAGCGACCGAAGCTA AGACTGAAGCCGGGCCAGA
TDF 25.4	AATTTGGCGTTGGCAAGACCACGG TCGAGCAACAAGTAGAAGCCGCCG
TDF 26.1	CTCAGCCGGTCGGTATGC ACGTTACGACGATGGAGATGTAG
TDF 26.2	AATTCGCACGAAGCGTGGAAC ACGTTACTCTGAAGAAACATCTGAC
TDF 30.2	AATTGGGCGGGGAGACCCTCCA TCGAAAGTGTCTTCTTGTGAATGTA
TDF 32.1	GATCATACATAGCTCGGTAGCTTGGA ACGTAATGCGGTGACATGAACA
TDF 34.2	ATCAGGCGCGCGAGAAGGAGCTG ACGTGGGTCGGACAAACCTGTC
VlNEP	CGGTGAGGTCAAGTAAGACACGC TGACGACATTCTCCCAGTCG
VlPKS1	ACATGTCGCCGCGCGAGCCTAC CAGGCTGTATCGATGTTCAGACTAGGTCCAC

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Publications of this work

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Presentations at national and international conferences:

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Eidesstattliche Erklärung

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

Göttingen, im September 2008.