Adhesion of the rapeseed pathogen *Verticillium longisporum* to its host *Brassica napus*

Uncovering adhesion genes and the evolutionary origin of the fungus

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Van Tuan Tran

aus Namdinh / Vietnam

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Referent: Prof. Dr. Gerhard H. Braus

Korreferentin: Prof. Dr. Stefanie Pöggeler

Anleiterin: Dr. Susanna Braus-Stromeyer

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Contents

Abbreviations

AFLP	amplified fragment length polymorphism
ALS	agglutinin-like sequence
AmpR	ampicillin resistance
ATMT	*Agrobacterium tumefaciens*-mediated transformation
bp	base pair
CDM	Czapek-Dox medium
cDNA	complementary DNA
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DsRed	*Discosoma* sp. red fluorescent protein
E. coli	*Escherichia coli*
EAP	enhanced adherence to polystyrene
EDTA	ethylenediaminetetraacetate
EPA	epithelial adhesin
FLO	flocculin
GFP	green fluorescent protein
GPI	glycosyl phosphatidylinositol
h	hour
H_2O_2	hydrogen peroxide
HPH	hygromycin phosphotransferase
IGS	intergenic spacer
ITS	internal transcribed spacer
KanR	kanamycin resistance
kb	kilobase
LB	left border / Luria Bertani medium
LiAc	Lithium acetate
min	minute
ml	milliliter
mM	milimolar
MM	minimal medium
NAT	nourseothricin acetyltransferease

NES	nuclear export signal
NLS	nuclear localization signal
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose both
PEG	polyethylene glycol
PEST	proline (P), glutamic acid (E), serine (S), and threonine (T)
RB	right border
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
rRNA	ribosomal RNA
s	second
S	Svedberg unit
SC-Ura	synthetic complete minus uracil medium
SDS	sodium dodecyl sulphate
SXM	simulated xylem medium
TE	Tris-Cl and EDTA
UV	ultraviolet
Va	*Verticillium albo-atrum*
VCG	vegetative compatibility group
Vd	*Verticillium dahliae*
Vl	*Verticillium longisporum*
WT	wild type
YPD	yeast extract peptone dextrose
µl	microliter
µm	micrometer

Summary

Verticillium longisporum, a soil-borne plant pathogen, is an emerging problem for oilseed rape and other crucifers. The mechanism of infection through plant roots and the evolutionary origin of this fungus are still not known.

Twenty four different cDNA sequences of *V. longisporum* involved in adhesion were characterized using a yeast screening system. These candidate sequences encode proteins of four groups including regulatory proteins, cell wall and membrane proteins, proteins of transport and metabolism, and hypothetical proteins with unknown functions. Two of the regulatory proteins named VTA1 and VTA2 could activate the expression of yeast FLO1 adhesin known to promote cell-cell adhesion (flocculation) and adhesion of yeast to different surfaces. These regulators might also control the expression of homologues of *FLO1* in *Vertillium* species.

Two high-throughput systems, one for gene silencing in *V. longisporum* and the other for gene disruption in *V. dahliae* have been developed. The silencing system with gateway technology requires less time for generating the silencing constructs. The silencing efficiency of *VTA2* gene could reach 80-90% in *V. longisporum*. *VTA2* gene could be co-silenced together with the red fluorescent protein gene in order to speed up the screening for the best silenced mutants. In addition, with a new gene disruption system, the gene for VTA2 in *V. dahliae* could be knocked-out in 45-76% transformants. The *VTA2* deletion mutant of *V. dahliae* lost its ability to produce conidia. The surface hydrophobicity of the mutant was completely altered and aerial mycelium formation was markedly reduced. Moreover, this mutant was sensitive to oxidative stress and less virulent on plants.

Sequencing of *VTA1* and *VTA2* together with rDNA and two velvet genes revealed the evolutionary origin of *V. longisporum*. This rapeseed pathogen is an interspecies hybrid between *V. dahliae* and *V. albo-atrum* at the dawn of species formation. Characteristic single nucleotide polymorphisms suggest a single initial hybridization event. A further step of speciation is homogenization of repetitive rDNA clusters. Homogenization happened at least twice and caused confusion in taxonomy, because both rDNA types can still be isolated from nature corresponding either to *V. albo-atrum* or *V. dahliae*.

Zusammenfassung

Verticillium longisporum, ein im Boden lebendes Pflanzenpathogen, stellt ein wachsendes Problem für Raps und andere Kreuzblütler dar. Der Infektionsmechanismus über die Pflanzenwurzel sowie der evolutionäre Ursprung dieses Pilzes sind noch weitgehend unbekannt.

In einem Hefe-Screening konnten 24 verschiedene cDNA Sequenzen von *V. longisporum* identifiziert werden, die potentiellen an der Adhäsion beteiligten sein könnten. Die Sequenzen konnten in vier Gruppen unterteilt werden: Regulatorische Proteine, Zellwand- und Membranproteine, Transport und Stoffwechselproteine, sowie konservierte Proteine mit unbekannter Funktion. Zwei der regulatorischen Proteine *VTA1* und *VTA2* aktivierten die Expression des Adhäsins FLO1 in Hefe, welches für die Zell-Zell-Adhäsion (Flokkulation) und Adhäsion von Hefe auf verschiedenen Oberflächen verantwortlich ist. Diese potentiellen Regulatoren sind möglicherweise auch in der Lage die Expression von FLO1 Homologen in *Verticillium* zu kontrollieren.

Es wurden zwei Systeme für die Erstellung von Knock-downs und Knock-outs in *V. longisporum* und *V. dahliae* entwickelt. Mit dem neuen System für Gen-"silencing" konnte mittels der "gateway" Technologie die Zeit für das Erstellen von Konstrukten verkürzt werden und eine "silencing" Effizienz von 80-90 % für das Gen *VTA2* erreicht werden. *VTA2* konnte zusammen mit einem rot fluoreszierenden Protein co-"gesilenced" werden. Dadurch konnte die Suche nach Mutanten mit der höchsten "silencing" Effizienz verbessert und verkürzt werden. Zudem konnte in *V. dahliae* das Gen *VTA2* mit dem Gen-Deletionssystem in 45-76 % der Transformanten deletiert werden. Eine Deletion von *VTA2* in *V. dahliae* führt zu einem Verlust der Konidienbildung, die Oberflächenhydrophobizität und die Bildung von Luftmyzel waren stark verändert. Ebenso war die Mutante sensitiv für oxidativen Stress und die Virulenz *in planta* war reduziert.

Die Sequenzierung der Gene *VTA1*, *VTA*2, der Gene für rDNA und zwei Velvet-Gene legten den evolutionären Ursprung von *V. longisporum* offen. Dieser Rapsschädling ist ein Hybrid aus *V. dahliae* und *V. albo-atrum*. Charakteristische Einzelnukleotid-Polymorphismen lassen vermuten, dass es sich um ein einzelnes initiales Hybridisierungsereignis handelt. Ein weiterer Schritt der Artbildung ist die Homogenisierung der repetitiven rDNA-Cluster. Diese Homogenisierung hat mindestens zweimal stattgefunden und zu erheblicher Verwirrung in der Taxonomie geführt, weil beide rDNA Typen aus *V. longisporum* isoliert werden können und die Stämme dann entweder *V. dahliae* oder *V. albo-atrum* zugeordnet werden können.

Chapter 1. INTRODUCTION

1.1. *Verticillium* species - fungal pathogens of wilt disease

1.1.1 *Verticillium* pathogens from the past to present

The *Verticillium* species are soil-borne plant pathogens belonging to ascomycetes. They broadly distribute through the world and cause vascular wilting diseases and early senescence in a huge range of economically important crops including alfalfa, cotton, lettuce, hops, olive trees, oilseed rape, cabbages, potato, tomato, strawberries etc. The most dangerous species, which cause billions of dollars in annual crop losses worldwide, are *Verticillium dahliae*, *V. albo-atrum* and *V. longisporum* (Pegg and Brady, 2002; Zeise and von Tiedemann, 2002; Agrios, 2005; Johansson *et al.*, 2006). This fungal genus has been named *Verticillium* based on the arrangement of phialides in whorls (verticillate shape) around the conidiophores and branching of the conidiophores also occurs in whorls at some levels (Figure 1D). The fungal mycelium is hyaline, simple or branched, septate and multinucleate. Each phialide carries a number of ovoid to elongated conidia (Berlanger and Powelson, 2000; Fradin and Thomma, 2006).

Wilt symptoms of *Verticillium* and *Fusarium* are very similar and difficult to distinguish by normal observation (Babadoost *et al.*, 2004). The symptoms caused by *Verticillium* appear more slowly, and only on the lower or outer parts of plants. Because symptoms can vary among plant hosts, there is no absolute diagnostic method for *Verticillium* pathogens (Rowe and Powelson, 2002). However, premature foliar chlorosis and necrosis and vascular discoloration in stems and roots are characteristic of all hosts when infected with *Verticillium* species (Figure 1). In some hosts, *Verticillium* infected older plants are usually stunted in various degrees (Kim *et al.*, 2001; Pegg and Brady, 2002; Rowe and Powelson, 2002; Fradin and Thomma, 2006, Gradders *et al.*, 2011).

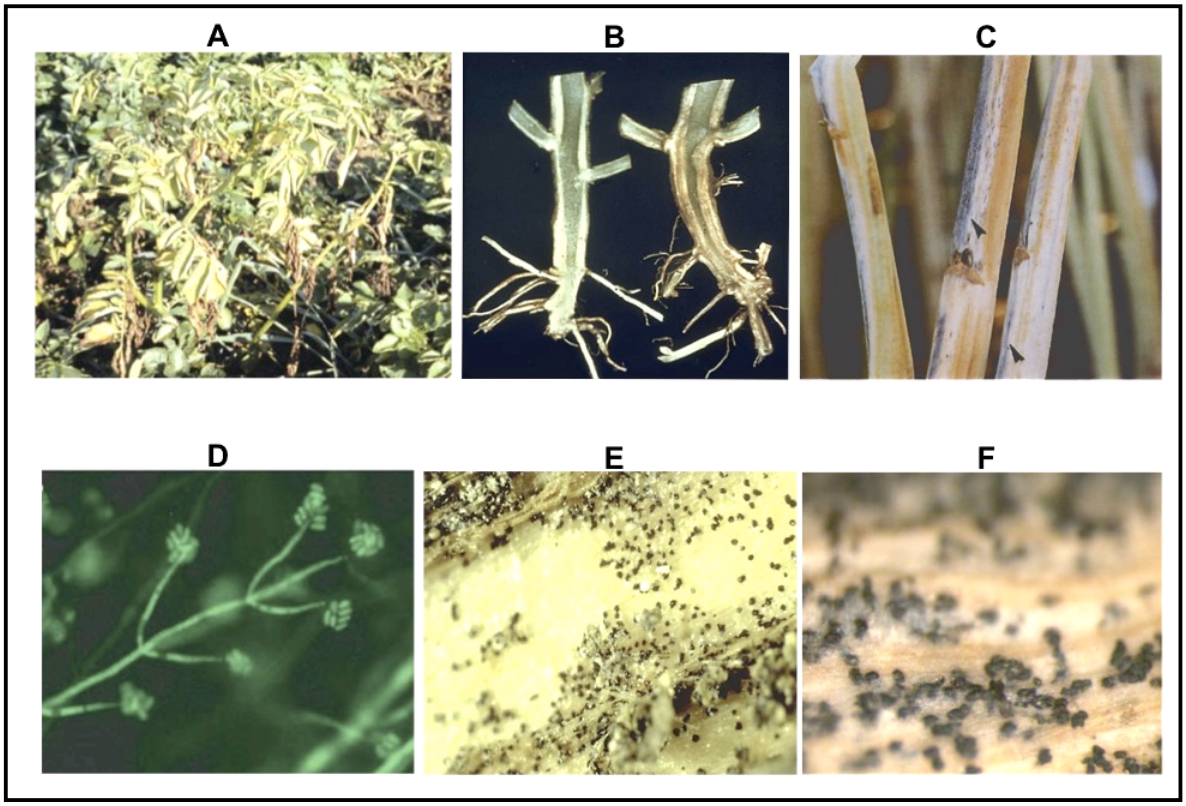


Figure 1. Wilt disease symptoms and morphological structures of *Verticillium* pathogens (adapted from Heale and Karapapa, 1999; Berlanger and Powelson, 2000; Andrie *et al.*, 2005; Eynck *et al.*, 2009). **(A)** Leaf necrosis and wilt in potato. **(B)** Vascular discoloration in potato stems (left: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

In 1879, Reinke and Berthold first described wilt on potato (*Solanum tuberosum*) at the botanic laboratory in the University of Göttingen and named the causal agent *Verticillium albo-atrum* (Reinke and Berthold, 1879; Hastie, 1973; Klosterman *et al.*, 2009). Until 1913 a second species causing wilt on dahlia (Asteraceae family) with morphological distinction was described by Klebahn, and named *V. dahliae* (Isaac, 1947; Hastie, 1973). In 1961, Stark isolated a *V. dahliae*-like fungus from horseradish in Hamburg, Germany (Stark, 1961). This fungus named *V. dahliae* var. *longisporum* produces microsclerotia like *V. dahliae* (Figures 1E, 1F) but conidia significantly longer than the typical *V. dahliae* strains. Therefore it was named *V. dahliae* var. *longisporum*. On the basis of the morphological differences and other characteristics of many similar strains,

Karapapa *et al*. suggested long-spored isolates as a new species, *V. longisporum* (Karapapa *et al.*, 1997). Currently, the genomes of *V. dahliae* and *V. albo-atrum* have been sequenced by Broad Institute, Havard-Massachusett, and are available at http://www.broad.mit.edu/annotation/genome/verticillium_dahliae/MultiHome.html. In addition, the sequencing of *V. longisporum* genome is in progress by the research group of Prof. Gerhard Braus at the University of Göttingen (Figure 2).

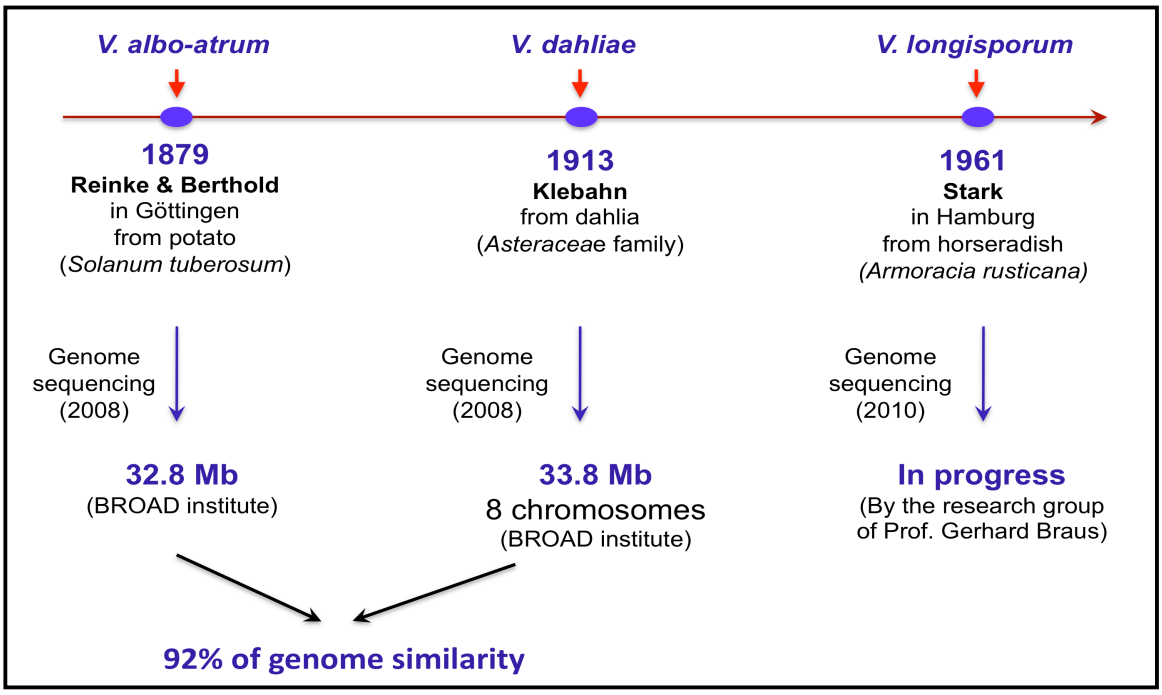


Figure 2. Timeline of discovery of three *Verticillium* plant pathogens. The genome sequencing revealed a very high similarity (>92%) in the DNA sequence between *V. dahliae* and *V. albo-atrum*. In addition, *V. longisporum* with a near diploid genome was suggested to be a hybrid between *V. dahliae* and *V. albo-atrum*.

1.1.2. Biology of *Verticillium* plant pathogens in nature

There is an overlap in host specificity between *V. dahliae* and *V. albo-atrum* (Kim *et al*., 2002; Zeise and von Tiedemann, 2002). *V. dahliae* has a significantly broader host range and is able to infect more than 200 plant species (Agrios, 2005). *V. albo-atrum* has in addition a specific host adaptation to lucerne and other leguminoses or to hops from the cannabaceae family (Pegg and Brady, 2002; Klosterman *et al*., 2009).

Vegetative compatibility has proved to be powerful in determining genetic diversity

of *V. dahliae* populations in nature (Puhalla, 1979; Joaquim and Rowe, 1990). Vegetative compatibility is the ability of hyphae from two strains of the same species to anastomose and form a viable heterokaryon. Fungal strains that anastomose and form heterokaryons with one another are considered to be vegetatively compatible and are assigned to the same group as a vegetative compatibility group (VCG). In contrast, strains that are incapable of anastomosing with one another and fail to establish heterokaryons are vegetatively incompatible (Elena, 1999). From different hosts and geographic origins, *V. dahliae* isolates have been classified into six groups (VCG1 to VCG6). Members of each group often share specific traits related to pathogenicity and aggressiveness (Bhat *et al.*, 2003; Jiménez-Díaz *et al.,* 2006; Dobinson *et al.*, 2000; Klosterman *et al.*, 2009). Among six vegetative compatibility groups, VCG1, VCG2 and VCG4 are popular and associated with a wide host range. These three groups were subdivided into VCG1A, VCG1B; VCG2A, VCG2B and VCG4A, VCG4B (Strausbaugh, 1993; Tsror *et al*., 2001; Zeise and von Tiedemann, 2001; Bhat *et al.*, 2003; Jiménez-Díaz *et al.,* 2006, Berbegala *et al.*, 2011).

On basis of virulence and aggressiveness to lucerne (*Medicago sativa*), *V. albo-atrum* isolates are divided into two groups, lucerne (alfalfa) and non-lucerne (hop, tomato, potato, pea and ornamental plants) (Morton *et al.*, 1995; Mahuku and Platt, 2002; Barbara and Clewes, 2003). However, VCG analyses failed in *V. longisporum* strains from crucifers, as the generation of nitrat-nonutilizing (*nit*) mutants might be prevented by the duplication of loci (Subbarao *et al.*, 1995; Zeise and von Tiedemann, 2001).

1.1.3. *Verticillium* disease cycle

Verticillium wilt is a monocyclic disease, because it has only one cycle of disease and inoculum production per season (Figures 3 and 4). *V. dahliae*, *V. longisporum* and *V. albo-atrum* survive in soil or in dead plant materials from season to season as resting structures including microsclerotia and melanized hyphae (Rowe and Powelson, 2002; Karapapa *et al.*, 1997, Eynck *et al.*, 2007). The survival of microsclerotia in the soil without hosts can reach 14 years (Wilhelm, 1955) but dark hyphae of *V. albo-atrum* are only viable for 2-5 years (Sewell *et al.*, 1964). Microsclerotia are stimulated to germinate in response to root exudates. Hyphae originating from germinating microsclerotia infect and colonize at root tips or following root hairs to the root surface. After penetration, fungal hyphae grow through cortical tissues toward developing vascular tissue. Once vascular tissues are infected, conidia are produced within the xylem vessels and move along with the transpiration stream. Conidia often become trapped at pit border members between vessels,

where they germinate, enter the neighboring vessels, and produce more conidia to repeat the process. Consequently, the fungus emerges from the xylem vessels to colonize neighboring vascular and cortical tissues, resulting in the development of disease symptoms such as wilting, chlorosis and necrosis. As the foliage begins to senesce, the fungus leaves the xylem elements and colonizes the surrounding non-vascular tissues. Microsclerotia are formed in the dying leaves and stems. Following incorporation of dead tissues into soil during subsequent cultivations, the microsclerotia are gradually released during decomposition of the tissues (Rowe and Powelson, 2002; Paul, 2003; Eynck *et al.*, 2007, Klosterman *et al.*, 2009).

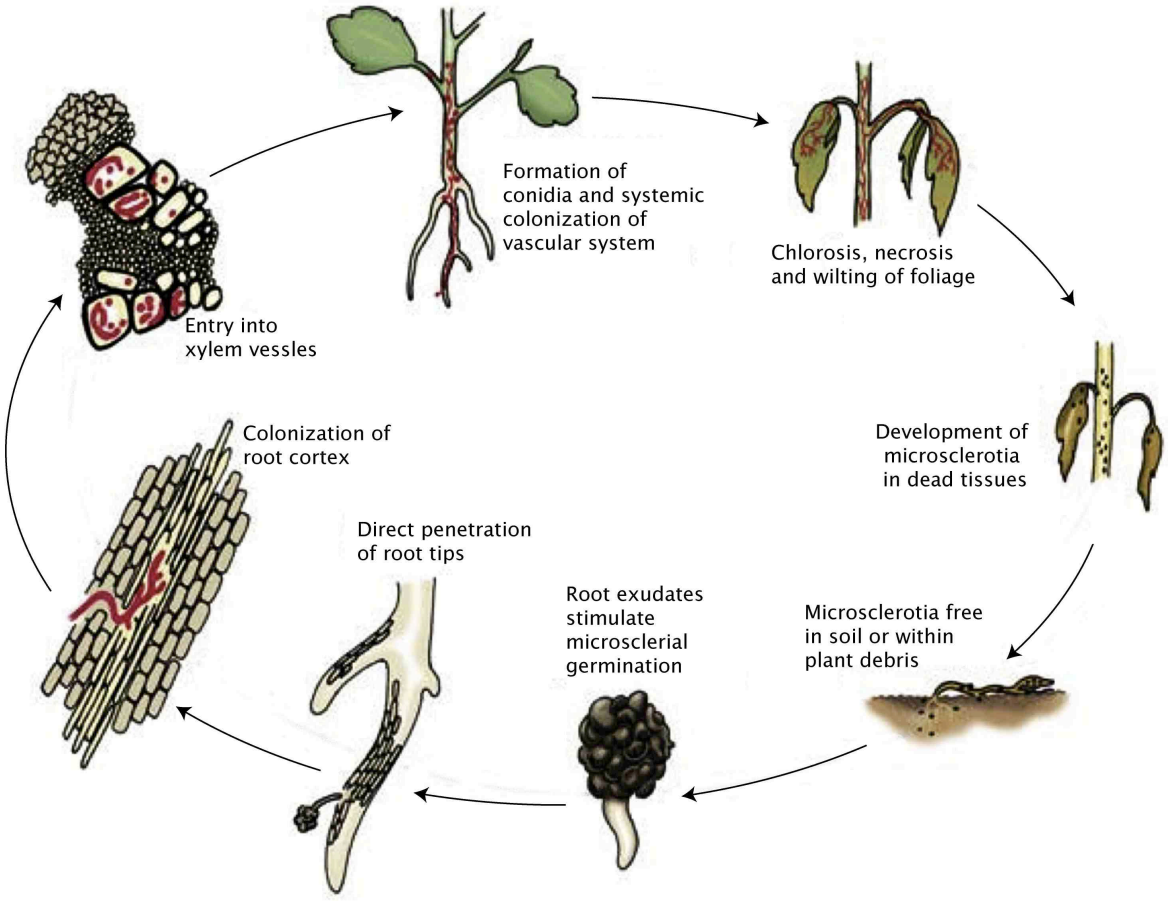


Figure 3. **The life cycle of *V. dahliae* on potato** (adapted from Rowe and Powelson, 2002). The cycle starts with resting structures (microsclerotia) in the soil. Germination of microsclerotia is probably stimulated by plant root exudates. Consequently, the fungus attaches to roots and penetrates the plants through the root tips. The fungus quickly occupies the xylem vessels, colonizes vascular systems and forms asexual conidia. The symptoms including chlorosis, necrosis and wilt of leaves appear early. When the plant is dead, the fungus produces microsclerotia for the next infection cycle.

1.1.4. Cruciferous crops are the favorite hosts of *V. longisporum*

V. longisporum is a soil-borne fungal pathogen and host-specific on the *Brassicaceae* family such as oilseed rape (*Brassica napus),* cabbage (*Brassica oleracea* var. *capitata*), horseradish (*Armoracia rusticana*), cauliflower (*Brassica oleracea* var. *botrytis*), etc. Presently, there is no effective fungicide for controlling this pathogen.

Oil production from rapeseed has been increasing over the last some decades and nowadays it becomes one of the most important vegetable oil sources only after soybean and cottonseed. Rapeseed oil is not only used for human and animal consumption, but also for industrial purposes as liquid fuels for diesel engines. Expansion of rapeseed cultivation has been facing to destruction by *V. longisporum* fungal pathogen (Heale and Karapapa, 1999; Pua and Douglas, 2004). This fungus was reported to be a vascular pathogen causing wilt diseases on oilseed rape *Brassica napus* in European countries including Sweden, Germany, France, England and Poland as well as in Canada (Karapapa *et al.*, 1997; Heale and Karapapa, 1999; Zeise and von Tiedemann, 2001; Steventon *et al.*, 2002; Johansson *et al.*, 2006; Qin *et al.*, 2006; Gladders *et al.*, 2011). The disease symptoms are hard to detect at early stages of growth process of rapeseed by observation. Although *V. longisporum* does not induce true wilting on oilseed rape plants, premature senescence and ripening are accompanied by systemic spread and extensive formation of microsclerotia on shoot tissue. This results in reduction of yields up to 50-70% (Dunker *et al.*, 2008). In greenhouse experiments, the most typical symptoms including chlorosis and stunting are often used to assess disease progression (Figure 4). However, stunting has never been observed in the field (Dunker *et al.*, 2008). Some greenhouse pathogenicity assays with *Brassica* crops showed that *V. longisporum* isolates are the most virulent, whereas *V. dahliae* strains are non-pathogenic or weakly virulent on these hosts (Zeise and von Tiedemann, 2002). *V. longisporum* infects mainly oilseed rape causing losses in plant fresh weight of 49% and killing about half of the plants at 42 days post inoculation (Zeise and von Tiedemann, 2002). This pathogen can also infect some other non-host plants (Bhat and Subbarao, 1999; Fahleson *et al*., 2004; Johansson *et al*., 2006) and conversely, other *Verticillium* species also weakly infect *B. napus* (Zeise and von Tiedemann, 2002; Collins *et al*., 2003).

In addition, recent analyses have shown that xylem sap of *B. napus* contains a number of organic acids and low concentrations of various amino acids such as glutamine, glutamic acid, aspartic acid, and γ-amino butyric acid (Singh *et al.*, 2010). Therefore,

rapeseed specificity of *V. longisporum* might be favored by the composition of the xylem sap.

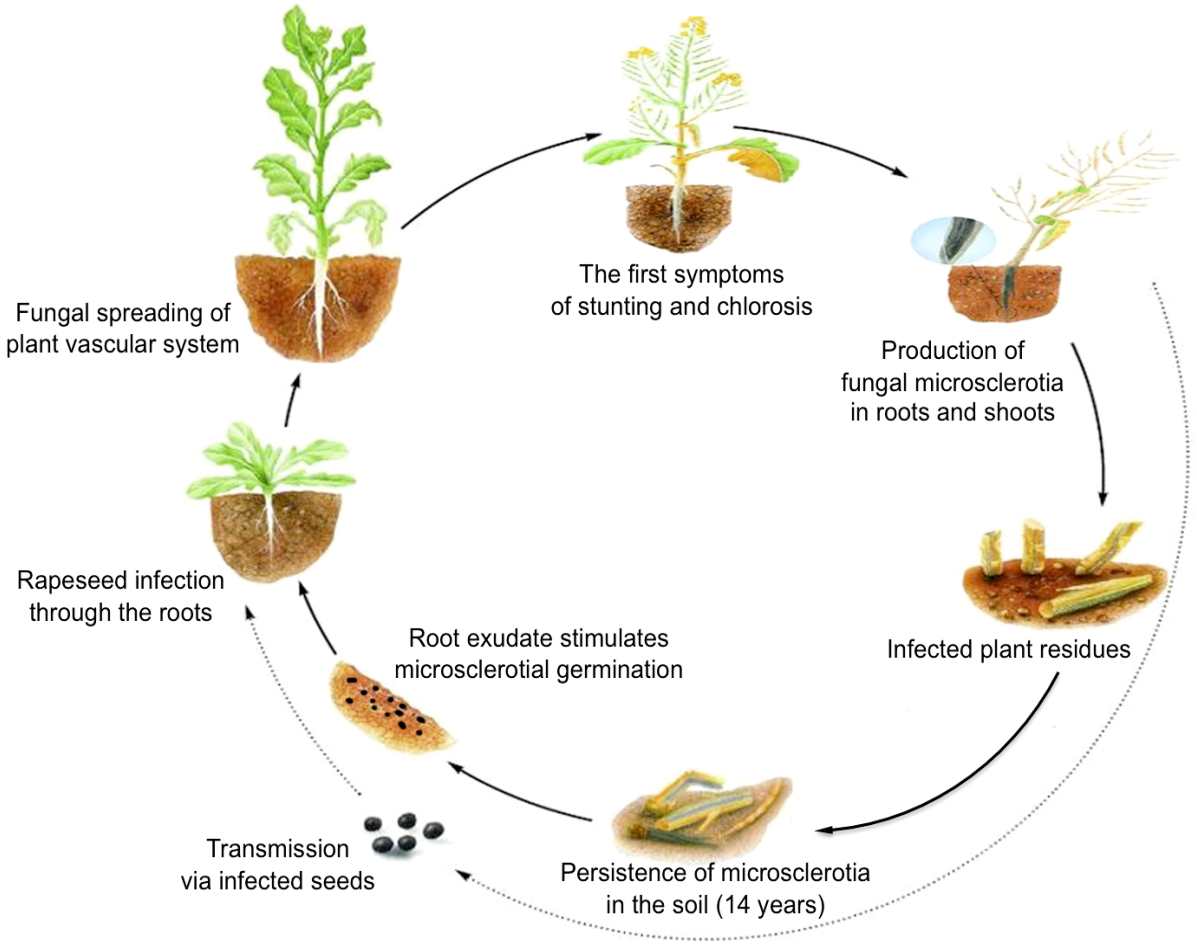


Figure 4. The life cycle of *V. longisporum* on oilseed rape (modified from Paul, 2003). Like *V. dahliae*, the infection cycle of *V. longisporum* also starts with germination of microsclerotia under the simulation of root exudates. The fungus enters the plant through the root hairs and grows in plant vascular system until the first symptoms of stunting and chlorosis can be observed. When the plant becomes old, the fungus produces microsclerotia in plant roots and shoots. These resting structures can be released from dead plant materials into the soil for the next cycle. Microsclerotia can survive in the soil for several years without rapeseed plants.

1.2. *Verticillium* taxonomy

Verticillium morphology includes a characteristic verticilliate arrangement of the three to five asexual spore carrying structures (phialides) forming branches at each node of

the conidiophores (Kim *et al.*, 2001). *V. dahliae* and *V. albo-atrum* are two closely related but distinct mature species. *V. albo-atrum* forms melanized resting mycelium, whereas the *V. dahliae* hyphae are not black. Instead *V. dahliae* forms resting black microsclerotia, which are melanized clumps formed by budding of mycelial cells (Goud *et al*., 2003). The formation of resting structures such as melanized microsclerotia by *V. dahliae*, and melanized hyphae but no microsclerotia by *V. albo-atrum* (Pegg and Brady, 2000) is the most distinctive feature for separation between *V. dahliae* and *V. albo-atrum*. In addition, *V. dahliae* grows and infects plant hosts at 30^{o}C, but *V. albo-atrum* fails to grow in culture or wilt plants at this temperature (Rowe and Powelson, 2002). On basis of morphological description and rDNA analysis, *V. albo-atrum* isolates were separated into two groups, Grp1 and Grp2. However, most of *V. albo-atrum* strains are referred to Grp1 (Robb *et al.*, 1993; Morton *et al.*, 1995; Barbara and Clewes, 2003; Robinson *et al.*, 2007; Klosterman *et al.*, 2009). Morphological analysis showed that Grp2 isolates produce resting structures in bundles of melanized hyphae, whereas Grp1 isolates form melanized singly hyphae (Mahuku and Platt, 2002). Sequence analysis of the ITS region showed that the ITS of Grp2 isolates contain 17 bases that are not found in the Grp1 isolates (Robb *et al.*, 1993; Mahuku and Platt, 2002). Although the differences are significant enough to classify Grp2 strains as a separate species, they are currently only recognized as a distinct taxonomic unit of *V. albo-atrum* (Mahuku and Platt, 2002).

Four other phytopathogenic species associated with the genus *Verticillium* are *V. tricorpus*, *V. nubilum, V. nigrescens* and *V. theobromae* (Barbara and Clewes, 2003). Unlike *V. dahliae* and *V. albo-atrum*, *V. tricorpus* and *V. nubilum* are soil saprophytes that can grow in the absence of a potential host (Isaac, 1967). *V. tricorpus* is considered to be a weak pathogen on many hosts and produces survival structures including chlamydospores, microsclerotia and melanized hyphae (Robinson *et al.*, 2007; Qin *et al.*, 2008). In contrast, *V. nubilum* produces only chlamydospores as the resting structure (Griffiths, 1982; Barbara and Clewes, 2003). Although *V. nigrescens* and *V. theobromae* are similar to other *Verticillium* species in morphology, they have been recently classified into the genera *Gibellulopsis* and *Musicillium*, respectively on basis of molecular evidences from rDNA (Zare *et al.*, 2007). In addition, *V. lecanii*, an entomopathogen and *V. fungicola*, a pathogen of mushrooms, were assigned to the genus *Lecanillium* (Zare and Gams, 2008).

V. longisporum isolated from the crucifer family of *Brassicaceae* (Stark, 1961) forms preferentially only three phialides per node and survives by means of black but compared

to *V. dahliae* elongated microsclerotia. *V. longisporum* produces twice (7-9 μm) as long asexual spores (conidia) in comparison to *V. dahliae* (3.5-5.5 μm) (Subbarao *et al.*, 1995; Karapapa *et al*. 1997; Zeise and von Tiedemann, 2001; Collins *et al.*, 2003). The numbers of *V. longisporum* isolates from crucifers are increasing and include horseradish from Illinois (Eastburn *et al.*, 1994), oilseed rapes from Europe and Canada (Heale and Karapapa, 1999; Zeise and von Tiedemann, 2001; Steventon *et al.*, 2002) or cauliflower from California (Koike *et al.*, 1994). However, the taxonomy of *V. longisporum* is under controversy due to lacking supportive evidences (Klosterman *et al*., 2009).

Currently, the sexual stage has not been found in *Verticillium* genus (Pegg and Brady, 2002; Klosterman *et al*., 2009).

1.3. Speciation and the fungal rapeseed pathogen *V. longisporum*

Speciation is a dynamic process of life on earth, which continuously generates independent lineages of novel organisms. Changes in ploidy, incompatibilities between genes or different alleles of a gene pair or chromosomal rearrangements can result in the development of reproductive barriers, which isolate different populations. This is a prerequisite for the evolution of diverging genetic elements, which convey an ecological separation. The analysis of the origin of a new species is a quest of more than 150 years of research since Charles Darwin (Darwin C., 1859). Besides the genomic comparison of mature species, which have already diverged significantly, the analysis of populations that are only partially separated or nascent species might reveal more insights into the process of species formation (Via S., 2009). Divergent selection which can be a driving force resulting in differentiation of genomes (Nosil *et al.*, 2009) can be achieved by the ecological separation of populations to different environments. An ecological speciation can be initiated by a subpopulation of a pathogen, which shifts to a different host, which allows speciation due to diverging evolution.

The list of hosts infected by *Verticillium* species is expanding and there is a continous increase in severity of disease outbreaks on known hosts (Vallad *et al.,* 2005, Klosterman *et al.,* 2009). Therefore *Verticillium* represents an interesting and relevant model to study speciation by comparing nascent to already significantly diverged mature species. Crucifers are hardly infected by *V. dahliae* or *V. albo-atrum*, whereas *V. longisporum* infects effectively these plants (Karapapa *et al.*, 1997; Zeise and von Tiedemann, 2002).

Presently, *V. longisporum* isolates are 'near-diploid' or amphihaploid fungi with higher nuclear DNA amounts (about 1.8 times) than those of *V. dahliae* or *V. albo-atrum* isolates (Karapapa *et al.*, 1997; Steventon *et al.*, 2002; Collins *et al.*, 2003). This almost diploid status might be the reason why numerous mutagenesis approaches have failed (Ingram, 1968; Hastie, 1973; Nagao *et al.*, 1994; Subbarao *et al.*, 1995; Zeise and von Tiedemann, 2001; Steventon *et al.*, 2002; Karapapa *et al.,* 1997; Collins *et al.*, 2003). Most filamentous ascomycetes are primarily haploid. Experimental studies with the model fungus *A. nidulans* suggest that during adaptation to a novel environment, haploids deriving from diploids by parasexual recombination reach a higher fitness than the original diploids (Schoustra *et al.*, 2007). *V. longisporum* might therefore represent a nascent species due to changes in ploidy where the subsequent reduction of the genome size has just started. Speciation might not even be accomplished in *V. longisporum*, because short-spored crucifer isolates might be haploid recombinants of long-spored isolates and distinct from non-crucifer isolates of *V. dahliae* (Collins *et al.*, 2003; Barbara and Clewes, 2003; Qin *et al.*, 2006; Clewes *et al.*, 2008; Klosterman *et al.*, 2009).

Increase in ploidy is achieved by a hybridization event between two haploid nuclei resulting in a diploid nucleus. Haploidization requires mitotic recombination and a gradual reduction of the genome by chromosome loss due to nondisjunction during mitosis. The fusion of two haploid nuclei can either happen in a homokaryon or in a heterokaryon. Heterokaryon formation is the result of parasexuality between two different compatible *Verticillium* species with different nuclei. Although fusion of nuclei of the same species is not totally excluded (Clewes *et al*., 2008), it seems likely that the fusion of different haploid nuclei to a heterozygous diploid interspecies hybrid had been the initiation event for *V. longisporum* formation that ultimately resulted in broadening the host range. Amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) support *V. longisporum* as interspecies hybrid between *V. dahliae* and *V. albo-atrum* (Karapapa *et al.*, 1997; Collins *et al.*, 2003; Steventon *et al.*, 2002). The internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA as well as mitochondrial genes suggest a closer relationship of *V. longisporum* to *V. albo-atrum* than to *V. dahliae* (Fahleson *et al.*, 2004), whereas sequence comparison of the beta-tubulin, histone 3 and 5S rRNA genes favours an interspecific hybrid between *V. dahliae* and one or even two yet unidentified species excluding *V. albo-atrum* as parents of *V. longisporum* (Clewes *et al.*, 2008; Collado-Romeo *et al.*, 2010; Inderbitzin *et al.*, 2011). In addition,

some artificial hybrids between *V. dahliae* and *V. albo-atrum* in laboratory also produced long spores and elongated microsclerotia like *V. longisporum* strains (Figure 5) (Hastie, 1973; Typas, 1983). For this reason, we have performed sequence comparisons of eleven *V. longisporum* isolates from Europe and America with *V. dahliae* and *V. albo-atrum* isolates to examine the initial steps in speciation of a plant pathogen with an 1.8 fold genome as a snapshot of speciation.

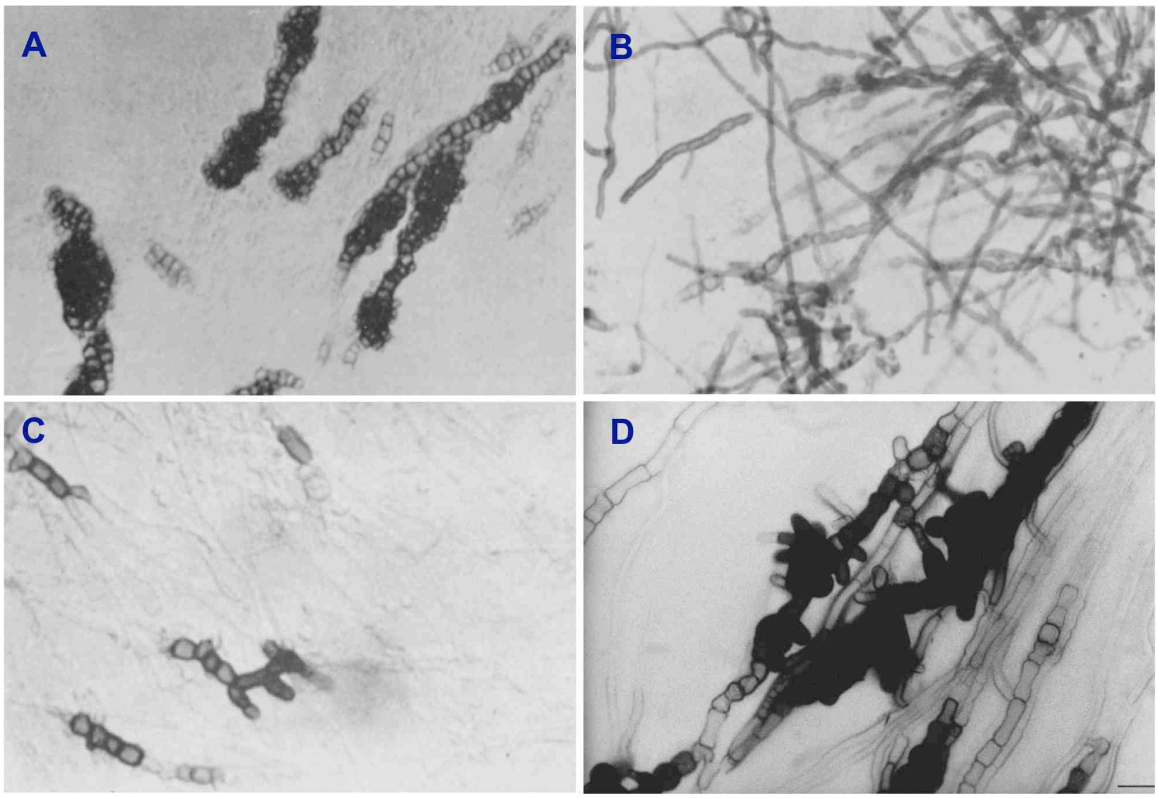


Figure 5. The resting structures of *Verticillium* species (adapted from Hastie, 1973 and Karapapa *et al.*, 1997). **(A)** Microsclerotia of *V. dahliae*. **(B)** Melanized hyphae of *V. albo-atrum*. **(C)** The microsclerotia of the artificial hybrid between *V. dahliae* and *V. albo-atrum*. **(D)** Microsclerotia of *V. longisporum*.

1.4. Molecular genetics of *Verticillium* plant pathogens

Although *Verticillium* species play important roles in agricultural production, there have been only few studies regarding the molecular mechanism involved in pathogenicity of this fungal genus (Dobinson *et al.*, 2004; Wang *et al.*, 2004; Rauyaree *et al.*, 2005; Klimes *et al.*, 2006a,b; Singh *et al.,* 2010; Tzima *et al.*, 2010a,b; Gao *et al.*, 2010). Such studies will promote rapid identification of molecular factors required for pathogenicity. In

addition, investigations for host-pathogen interaction via fluorescent tagging in *Verticillium* species were performed to understand infection stages of the fungi (Eynck *et al.*, 2007; Vallad and Subbarao, 2008). However, the molecular tools for such studies in *Verticillium* genus are limited.

1.5. Adhesion links fungal pathogens to host surfaces

During the two past decades, the importance of adhesion of fungi to host surfaces including both plant and animal before penetration has been clearly recognized (Hostetter, 2000). Fungal-substratum adhesion is usually mediated by glues (adhesins). This keeps the fungus from being blown or rinsed from a suitable environment. In addition, it also increases the surface area of contact with the host for fungal differentiation and penetration (Epstein and Nicholson, 2006). When a fungal adhesin is disrupted or blocked, the fungus may lose its virulence in killing the host cells (Brandhorst and Klein, 2000; Wang and St Leger, 2007). Therefore, anti-adhesins might provide an interesting disease control strategy because they can block the adhesins like an antigen/antibody model without uptake into fungal cells (Epstein and Nicholson, 2006).

1.5.1. Adhesion in yeasts

Adhesion of yeasts to hosts or environment surfaces is also mediated by adhesins. Most yeast adhesins are glycoproteins, and at least several are mannoproteins (Fukazawa and Kagaya, 1997; Dranginis *et al.*, 2007). They are usually located on the surface of the cell wall to perform the interaction of cell and the outside world. They are responsible for mating, colony morphology changes, biofilm formation, fruiting body development, and interactions with hosts (Dranginis *et al.*, 2007).

The budding yeast, *Saccharomyces cerevisiae*, possesses a family of flocculin genes that is divided into two groups. The first group includes FLO1, FLO5, FLO9 and FLO10 containing a PA14 conserved domain. The PA14 domain of adhesins is responsible for carbohydrate binding (Kobayashi *et al.*, 1998; Rigden *et al.*, 2004; Zupancic *et al.*, 2008). These flocculins promote cell-cell adhesion to lead to the formation of multicellular clumps (flocs), which sediment out of solution (Fichtner *et al.*, 2007; Linder and Gustafsson, 2008; Goossens and Willaert, 2010; Veelders *et al.*, 2010). The *FLO1*, *FLO5* and *FLO9* genes share high homology (more than 90%) in DNA sequence (Dranginis *et al.*, 2007; Goossens and Willaert, 2010). Flocculation plays an important role in the brewing industry, because yeast cells flocculate quickly at the end of fermentation and the

majority of cells can be separated easily from the culture medium (Verstrepen *et al.*, 2003). Many homologues of these adhesins were also found quite commonly in filamentous fungi (Linder and Gustafsson, 2008). The second group contains only FLO11 that is required for invasive growth and formation of pseudohyphae (Braus *et al.*, 2003; Fichtner *et al.*, 2007; Dranginis *et al.*, 2007).

The human pathogen *Candida albicans* adheres to polystyrene and medical devices as well as to human epithelial cells (Li and Palecek, 2003) mediated by some different adhesins such as ALS1-ALS9 (agglutinin-like sequence), EAP1 (enhanced adherence to polystyrene) or HPW1 (Hoyer, 2001; Dranginis *et al.*, 2007). The adhesin EAP1 was demonstrated to affect adhesion on both polystyrene and on epithelial cells when expressed in *S. cerevisae* and in a *C. albicans* mutant (Li and Palecek, 2003).

Another human pathogen of the genus *Candida*, *C. glabrata*, possesses a specific EPA (epithelial adhesin) family (EPA1-EPA7) with the PA14 conserved domain. These adhesins are required for binding of the fungus to host epithelia during infections (Dranginis *et al.*, 2007; de Groot and Klis, 2008; Zupancic *et al.*, 2008).

1.5.2. Adhesion in filamentous fungi

Only some proteins involved in adhesion of filamentous fungi have been investigated (Talbot *et al.*, 1996; Linder *et al.*, 2002; Wang and St Leger, 2007; Izumitsu *et al.*, 2010; Zhang *et al.*, 2011). Among them, hydrophobins are emerging to be potential candidates for fungal adhesion and pathogenesis as well as for medical and industrial applications (Kershaw *et al.*, 1998; Scholtmeijer *et al.*, 2001, Wang *et al.*, 2010).

Hydrophobins are small-secreted proteins that are produced only by filamentous fungi belonging to the ascomycetes and the basidiomycetes. They fulfil a broad spectrum of functions in fungal growth and development. Hydrophobins are moderately hydrophobic proteins of approximately 100 amino acids with eight conserved cysteine residues, but highly variable amino acid sequences (Wösten *et al.*, 1994; Kershaw *et al.*, 1998; Scholtmeijer *et al.*, 2001). They participate in the formation of various coatings and mediating adhesion of fungi to surfaces (Wang *et al.*, 2010; Izumitsu *et al.*, 2010; Zhang *et al.*, 2011). The mechanism of hydrophobin-mediated adhesion is an interesting aspect both for fungal biology and for many biotechnical immobilization applications (Linder *et al.*, 2005; Wang *et al.*, 2010). Hydrophobins act as adhesive components because their hydrophobic side binds to hydrophobic surfaces and the hydrophilic side binds to

hydrophilic surfaces. Consequently, a hydrophobin changes the polarity of its substratum to which a fungal pathogen can adhere (Wösten *et al*., 1994; Scholtmeijer *et al*., 2002; Epstein and Nicholson, 2006). Hydrophobins are multipurpose proteins (Wösten, 2001) that are involved in fungal adhesion to surfaces (Talbot *et al.*, 1996; Izumitsu *et al*., 2010; Zhang *et al*., 2011), toxicity to hosts (Temple *et al.*, 1997), fungal microsclerotial development and spore viability (Klimes *et al.*, 2006b; 2008) or fungal protection from recognition of host immune system (Aimanianda *et al*., 2009).

Recently two adhesins from *Metarhizium anisopliae*, MAD1 and MAD2, have been reported to be responsible for adhesion of the fungus to different surfaces to adapt to changes of living environment. The MAD1 adhesin allows the fungus to adhere to insect cuticle, whereas MAD2 adhesin is strongly induced by root exudates and promotes fungal adhesion to plant surface (Wang and St Leger, 2007; Pava-Ripoll *et al.*, 2011). When these genes were disrupted, the fungus lost about 90% of the adhesion ability to the corresponding surfaces. Moreover, the virulence of the fungus to the insect was also significantly reduced (Wang and St Leger, 2007).

In addition, some adhesins contain the arginine-glycine-aspartic (RGD) tripeptide motif that is a critical site for fungal pathogens to bind to plant and animal host receptors (Gale *et al.*, 1998; Hostetter, 2000). Plant roots also have potential carbohydrate receptors to which a fungal adhesin could bind, however molecular mechanism of adhesion to roots is poorly characterized (Recorbet and Alabouvette, 1997).

1.6. Aim of this work

Verticillium plant pathogens including *V. dahliae*, *V. longisporum* and *V. albo-atrum* cause wilt diseases in hundreds of different plants in over the world resulting in huge yield losses of agricultural production. The control of these *Verticillium* species is a serious problem because presently there is no fungicide against these pathogens available. *Verticillium longisporum* with near diploid state is one of the most dangerous pathogens for the oilseed crop, *Brassica napus*. The evolutionary origin of this fungus has been not known until now. If the origin of *V. longisporum* and molecular mechanisms of infection process of this fungus and two closely related species *V. dahliae* and *V. albo-atrum* are discovered, effective strategies of fungal control can be developed and applied.

The aim of this work was to characterize genes involved in a first contact between *V. longisporum* and its host *B. napus* using budding yeast as a new screening system. This

system also allows detecting different transcripts of a same gene in *V. longisporum*. The different transcripts of one gene together with genomic DNA sequencing of the corresponding gene in *V. longisporum* and two closely related species (*V. dahliae* and *V. albo-atrum*) might provide the direct evidences for evolutionary origin of this fungus. In addition, potential roles of adhesion-related genes in *V. longisporum* could be investigated using a new high-throughput gateway silencing system and confirmed by gene disruption in the 'parent' species *V. dahliae* using two new binary vectors.

Chapter 2. MATERIALS AND METHODS

2.1. Microbial strains and growth conditions

2.1.1. Bacterial strains

E. coli (*Escherichia coli*) strain DH5α, *ccd*B resistant *E. coli* strain (Invitrogen, Karlsruhe, Germany) and *Agrobacterium tumefaciens* AGL1 (Lazo *et al.*, 1991) were used for transformation procedures in this work. The *E. coli* strains were cultivated at 37°C, whereas the *A. tumefaciens* strain was grown at 25-28°C.

2.1.2. Yeast strains

The yeast strains derived from the S288C genetic background are non-adhesive (Table 1). The S288C background has a non-sense mutation in the open reading frame of the *FLO8* gene encoding a transcription regulator of *FLO1* and *FLO11* adhesin genes (Liu *et al*., 1998). Therefore, the expression of *FLO1* as well as *FLO11* is blocked.

The *kanMX4* cassette responsible for geneticin (G418) resistance was used for gene deletion in yeast. All strains were inoculated in YPD or SC-Ura medium (Guthrie and Fink, 2004) and incubated at 30°C.

Table 1. The yeast strains used in this study

Yeast strain	Genotype	Background	Source
BY4741	MATa: his3Δ1; leu2Δ0; met15Δ0; ura3Δ0	S288C	Euroscarf
Y06107 (*ΔFLO8)*	BY4741; *YER109C::kanMX4*	S288C	Euroscarf
Y06870 (*ΔFLO1*)	BY4741; *YAR050W::kanMX4*	S288C	Euroscarf
Y07106 (*ΔFLO10*)	BY4741; *YKR102W::kanMX4*	S288C	Euroscarf
Y05953 (*ΔFLO11*)	BY4741; *YIR019C::kanMX4*	S288C	Euroscarf
Y04072 (*ΔHPR1*)	BY4741; *YDR138W::kanMX4*	S288C	Euroscarf
Y02937 (*ΔTHO2*)	BY4741; *YNL139C::kanMX4*	S288C	Euroscarf

Yeast strain	Genotype	Background	Source
Y00508 (*ΔMFT1*)	BY4741; *YML062C::kanMX4*	S288C	Euroscarf
Y02861 (*ΔTHP2*)	BY4741; *YHR167W::kanMX4*	S288C	Euroscarf
Y8205 (*ΔCAN1*)	MATα; can1*Δ*::*STE2*pr-sp*HIS5*; *Δ*lyp1::*STE3*pr-*LEU2*; his3*Δ*1; leu2*Δ*0; ura3*Δ*0	S288C	Tong and Boone, 2007

2.1.3. *Verticillium* strains

Twenty-three strains (isolates) of *Verticillium* species from different hosts were used in this study (Table 2). Most of the *Verticillium* isolates were kindly provided by Prof. Andreas von Tiedemann, Georg-August University Göttingen (according to Zeise and Tiedemann, 2001; 2002) except that Vl-Bob70 isolate was derived from VdBob70 isolate (Qin *et al.*, 2006); Va-2, Va-3, Va-4 and Vtr-1 were purchased from the CBS-Fungal Biodiversiry Centre (Utrecht, Netherlands). The original names of some isolates are in parentheses; VCG means Vegetative Compatibility Groups and HSI stands for Heterokaryon Self-Incompatible. All strains were inoculated in potato dextrose broth (PDB) (Sigma-Aldrich Chemie GmbH, Munich, Germany) or in the liquid simulated xylem medium (SXM) (Neumann and Dobinson, 2003) and incubated for 7-10 days at 25^{o}C.

Spore preparation: the fungal isolates were grown separately in Czapek-Dox liquid medium (Smith, 1949) for 10 days, at 25^{o}C on a shaker, 120 rpm. Fungal conidia were harvested by filtering the culture through miracloth membrane (Calbiochem, Darmstadt, Germany), the filtrate was centrifuged at 5,000 rpm for 20 minutes at 4^{o}C. After a washing step with sterile tap water, the pellet was resuspended in sterile tap water. The number of spores was counted under microscope using a counting chamber and spore density was adjusted to 10^{7} spores/ml. Glycerin was added to the spore suspension at the final concentration of 20% and aliquots of the spore suspension were frozen in liquid nitrogen and stored at -80^{0}C.

Table 2. *Verticillium* isolates used in this study

Isolate	Species	Host	Geographic origin	VCG
Vd-2	*V. dahliae*	*Fragaria x ananassa* (strawberry)	Münterland/Germany	4B
Vd-8	*V. dahliae*	*Solanum tuberosum* (potato)	Münterland/Germany	4B
Vd-13	*V. dahliae*	*Gossypium hirsutum* (cotton)	Cordoba/Spain	HSI
Vd-39	*V. dahliae*	*Helianthus annuus* (sunflower)	Hessen/Germany	2B
Vd-52	*V. dahliae*	*Capsicum annuum* (pepper)	Burgenland/Austria	2B
Vd-73	*V. dahliae*	*Linum usitatissimum* (linseed)	Mecklenburg/Germany	2B
Vd-89	*V. dahliae*	*Lupinus luteus* (pea)	Mecklenburg/Germany	2B
Vl-18	*V. longisporum*	*Brassica napus* (rapeseed)	Mecklenburg/Germany	
Vl-19	*V. longisporum*	*Brassica napus* (rapeseed)	Mecklenburg/Germany	
Vl-32	*V. longisporum*	*Brassica napus* (rapeseed)	Mecklenburg/Germany	
Vl-40	*V. longisporum*	*Brassica napus* (rapeseed)	Mecklenburg/Germany	
Vl-43	*V. longisporum*	*Brassica napus* (rapeseed)	Mecklenburg/Germany	
Vl-59 (90-03)	*V. longisporum*	*Brassica oleracea* var. *botrytis* (cauliflower)	California/USA	
Vl-60 (90-10)	*V. longisporum*	*Brassica oleracea* var. *botrytis* (cauliflower)	California/USA	
Vl-Bob70	*V. longisporum*	*Brassica oleracea* var. *botrytis* (cauliflower)	California/USA	
Vl-82	*V. longisporum*	*Brassica napus* (rapeseed)	Mecklenburg/Germany	
Vl-83	*V. longisporum*	*Brassica napus* (rapeseed)	Mecklenburg/Germany	
Vl-84	*V. longisporum*	*Brassica napus* (rapeseed)	Mecklenburg/Germany	
Va-1	*V. albo-atrum*	*Solanum tuberosum* (potato)	Wiconsin/USA	
Va-2 (CBS453.51)	*V. albo-atrum*	*Medicago sativa* (alfalfa)	United Kingdom	
Va-3 (CBS393.91)	*V. albo-atrum*	*Humulus lupulus* (hop)	Belgium	
Va-4 (CBS322.91)	*V. albo-atrum*	*Lycopersicon esculentum* (tomato)	Naaldwijk/ Netherlands	
Vtr-1 (CBS101220)	*V. tricorpus*	*Brassica*	Nir-Itzhak/ Israel	

2.2. Bioinformatic methods

2.2.1. Primer designing

The primers in this study were designed using the Primer3 program (Rozen and Skaletsky, 2000) and purchased from Invitrogen (Karlsruhe, Germany) or from Eurofins-MWG (Ebersberg, Germany). The amplification efficiency of each primer pair was checked with Mastercycler Gradient (Eppendorf, Hamburg, Germany). For purposes of cloning or gateway recombination, the restriction sites or gateway recombination sequences were added to the 5' ends of the primers respectively (Table 3).

Table 3. Primers used in this study

Name	**Sequence (5'-3')** (gateway recombination sequences, restriction enzyme sites are underlined and in parentheses)	**Product size** (kb)	**Description**
T7 (forward)	TAATACGACTCACTATAGGG		cDNA sequencing
CYC1 (reverse)	GCGTGAATGTAAGCGTGAC		cDNA sequencing
FLO8-A *FLO8*-B	AATAATGCAAACCCCACGAC AAGGTGATGCTCCATCCAAC	0.58	A part of *FLO8* transcription factor gene
FLO11-A *FLO11*-B	CGTTAATGGCTGTCCCAACT TGCATATTGAGCGGCACTAC	0.37	A part of *FLO11* adhesin gene
VTA1-fuseA *VTA1*-fuseB	GGGTCTAGAATGTCTTCAAGTTCCAAGACCC (*Xba*I) GGGCCCGGGGGCACGTTTCATTCCACCT (*Sma*I)	1.27	For fusion of *VTA1* gene to *GFP* gene under the control of *GAL1* promoter
VTA2-fuseA *VTA2*-fuseB	GGGTCTAGAATGTACCTGGTCCCCACG (*Xba*I) GGGCCCGGGCTGCGTCCCGTTGTGTTG (*Sma*I)	1.25	For fusion of *VTA2* gene to *GFP* gene under the control of *GAL1* promoter
ITS-F ITS-R	AGTAAGCGCAAGTCATCAGC AAGGAACCATAACTCGAAGCAT	0.85	ITS1-5.8S-ITS2
IGS-F IGS-R	ACGATCTGCTGAGGGTAAGC ATTCGCAGTTTCGCTTTGTAA	1.70-1.90	IGS region of the rDNA
18S-rRNA1 18S-rRNA2	GGGGATCGAAGACGATCAG TATTGCCTCAAACTTCCATCG	0.44-1.28	A part of 18S-rRNA gene

Name	**Sequence (5'-3')** (gateway recombination sequences, restriction enzyme sites are underlined and in parentheses)	**Product size** (kb)	**Description**
VEL1-F *VEL1*-612-R *VEL1*-R	ATGTCCGCCACCACCAT TCATGCGGAGGTAGAATCC TCATTTTGTGAAAATAGGCGTGTA	0.61-1.72	*Velvet*-like gene 1
VEL2-F *VEL2*-R	ATGAGCTACGACCAGCACC CTAATAATCGTCATCGTCGTCAT	1.62	*Velvet*-like gene 2
VTA1-F *VTA1*-R	ATGTCTTCAAGTTCCAAGACCC TCAGGCACGTTTCATTCCAC	1.27	$Zn(II)_2Cys_6$ zinc finger protein gene
VTA2-F *VTA2*-R	ATGTACCTGGTCCCCACGCAGC CTAGTGGCCCTGCCCAGGCT	1.70-1.76	*CON7* homologue gene
DsRed-F *DsRed*-R	ATGGCCTCCTCCGAGGAC CTACAGGAACAGGTGGTGGC	0.68	*DsRed* fluorescent gene
GWsense-F GWsense-R	AGCACAACCATGCAGAATGA GTTCCCTGGCTGTGTGTTTT	0.78	For verification of the gateway sense strand
GWanti-F GWanti-R	GCTGGAGGATACAGGTGAGC AGCACAACCATGCAGAATGA	0.79	For verification of the gateway antisense strand
Redi-gwF *Redi*-gwR	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCGAGGACGTCATCAAGGAG (*att*B1) GGGGACCACTTTGTACAAGAAAGCTGGGTCCCTCCCAGCCCATAGTCTT (*att*B2)	0.45	Gateway silencing fragment for *DsRed* gene
VTA2-gwF *VTA2*-gwR	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTACCTGGTCCCCACGC (*att*B1) GGGGACCACTTTGTACAAGAAAGCTGGGTCCGGCTGAGGATAGGCATGTTG (*att*B2)	0.56	Gateway silencing fragment for *VTA2* gene
HygCAS-F HygCAS-R	AATGAGCTCACTAGTCCGCGACGTTAACTGATATTG (*Sac*I, *Spe*I) AATTCTAGAGGGCCCAGGCCTCCGGTCGGCATCTACTCTATT (*Xba*I, *Apa*I, *Stu*I)	1.42	Hygromycin resistance gene with *TrpC* promoter
VTA2-P1 *VTA2*-P2	GGGGATATCTTCCCTGTCTCTGGGACTTG (*Eco*RV) GGTATGCCTGCATGTCGC	1.56	For sequencing and construction of *VTA2* deletion cassette
VTA2-P3 *VTA2*-P4	AACATGCCTATCCTCAGCCC GGGTCTAGACAGGAAGAGAAGCGAAGAGTG (*Xba*I)	1.54	

Name	**Sequence (5'-3')** (gateway recombination sequences, restriction enzyme sites are underlined and in parentheses)	**Product size** (kb)	**Description**
VTA2-comp-F *VTA2*-comp-R	GGGTCTAGATTCCCTGTCTCTGGGACTTG (*Xba*I) GGGAAGCTTCAGGAAGAGAAGCGAAGAG TG (*Hind*III)	3.54	*VTA2* cassette including the native promoter and terminator for *VTA2* gene recovery in the mutant
qVTA2-F *qVTA2*-R	TACTCCTTCGTTCCGATTCCTG TACCATACGCCTTCTCACAACC	0.12	Quantification of *VTA2* expression using qRT-PCR
qVHP1-F *qVHP1*-R	CTATTGCGACGATTGCTCTG GAACGGCCAGACCAAGAATA	0.15	Quantification of *VHP1* expression using qRT-PCR
qVHP2-F *qVHP2*-R	GTTGCCGATCTGGACTGC TTAACCAATGACGGGAGTGC	0.15	Quantification of *VHP2* expression using qRT-PCR
qVHP3-F *qVHP3*-R	AGTCCTTCACTGCCATCGTC GCAGCTCTGCTTGAAGTCGT	0.20	Quantification of *VHP3* expression using qRT-PCR
qVHP4-F *qVHP4*-R	AGGACCGCCAAGTCTACATC GAGGACACCCTGGTCAAGAA	0.20	Quantification of *VHP4* expression using qRT-PCR
qVHP5-F *qVHP5*-R	GCTGCGCTACTAACGTCCTC CACAGGACACCCTGGTTGA	0.15	Quantification of *VHP5* expression using qRT-PCR
qVAP1-F *qVAP1*-R	TTCAACCCGAACAACATCAC CCTGATAAATGGTGGGATCG	0.19	Quantification of *Verticillium FLO1* homolog expression
qH2A-F *qH2A*-R	CCCGTGACAACAAGAAGACTCG GCAGGAAAGAAAAGCCAAAACC	0.22	Quantification of the *H2A* histone gene expression

2.2.2. Tools for DNA sequence analysis

DNA sequences were verified by using the 4Peaks software (www.mekentosj.com) For molecular analyses, DNA sequences were collected either from the *Saccharomyces* genome database (http://www.yeastgenome.org) or from the *Verticillium* group database (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html). The open reading frame (ORF) of each gene was identified and translated into a protein sequence by using the ORF Finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/). The protein sequence was searched for a secretion signal using SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), for detection of GPI anchor using GPI modification site prediction (http://mendel.imp.ac.at/sat/gpi/gpi_server.html) or PredGPI prediction server (http://gpcr2.biocomp.unibo.it/predgpi/pred.htm), for conserved domains or motifs using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) or Pfam (http://pfam.sanger.ac.uk/) or Prosite (http://expasy.org/prosite/), for characterization of protein degradation signals using the Epestfind tool (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind), and for the analysis of protein repeats using the RADAR program (http://www.ebi.ac.uk/Tools/Radar/). The information of DNA sequences was determined by blasting the sequences in Genbank (http://blast.ncbi.nlm.nih.gov/).

Restriction sites of the DNA sequences were determined by using NEBcutter V2.0 program from New England Biolabs (http://tools.neb.com/NEBcutter2/) or EnzymX software (www.mekentosj.com). The nuclear localization signals (NLS) were predicted using the NLStradamus webtool (http://www.moseslab.csb.utoronto.ca/NLStradamus/). The nuclear export signals (NES) were identified using the NetNES server (http://www.cbs.dtu.dk/services/NetNES/). Hydrophobicity pattern of a protein was determined using the ProtScale (http://expasy.org/cgi-bin/protscale.pl).

2.2.3. DNA analysis and comparison

Characterization of 18S rRNA gene, ITS1, 5.8S rRNA, ITS2, 28S rRNA and IGS of the rDNA was based on Genbank accession number AF104926 (Pramateftaki *et al.*, 2000). For the other conserved genes, the introns and exons were determined by comparing the cDNA sequences with their genomic DNA sequences using the ClustalW program (Thompson *et al.*, 1994). Detection of repeated motifs of the genes was carried out with the UGENE v1.7.1 software (UniPro, Novosibirsk, Russia). For DNA analysis and comparison

in more details, the commercial software Geneious Pro 5.0.4 (Biomatter Ltd, Auckland, New Zealand) was used.

2.2.4. Phylogenetic analysis

The phylogenetic trees were constructed with the MEGA 4.0 software (Tamura *et al.*, 2007) based on the neighbor-joining method (Seitou N. and Nei M., 1987). The statistical reliabilities of the internal branches were assessed for all trees by using the bootstraps of 1,000 replicates.

2.2.5. Drawing of plasmid maps and models

The plasmid maps and models in this study were drawn using the Savvy program (http://www.bioinformatics.org/savvy/) and Adobe Illustrator CS4, respectively.

2.3. Genetic manipulations

2.3.1. *E. coli* transformation

Preparation of E. coli competent cells: 10 fresh colonies of *E. coli* were inoculated into 250 ml of SOB medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) in a 1 liter flask. The flask was incubated on a shaker (100 rpm) at 20°C until the OD_{600} of the culture was about 0.6-0.8. The culture was kept in ice for 10 min and centrifuged at 5,000 rpm for 10 minutes at 4°C. The pellet was then resuspended in 80 ml TB buffer (10 mM PIPES/HEPES, 15 mM $CaCl_2.2H_2O$, 250 mM KCl; 55 mM $MnCl_2$; pH6.7). This cell suspension was incubated on ice for 10 min followed by centrifugation at 4,000 rpm for 10 min at 4°C. The pellet was resuspended in 20 ml TB followed by the addition of DMSO by gently swirling to a final concentration of 7%. This cell suspension was further incubated for 10 min on ice and dispensed (400 µl) into 1 ml aliquots. The aliquots were frozen in liquid nitrogen and stored at -80°C for later use.

E. coli transformation: an aliquot of competent cells was first allowed to thaw on ice. About 5-10 µl of the ligation reaction or 50 ng of a plasmid was added to 100 µl of the competent cells and incubated for 20 min on ice. The cells were then subjected to heat shock at 42°C for 40 seconds and then kept on ice for 5 minutes. About 800 µl of SOC medium (SOB + 20 mM glucose) was added to the mixture followed by a recovery at 37°C with shaking at 180 rpm for 1 hour. The cells were collected by centrifugation at 10,000 rpm for 1 min and plated on a LB agar plate supplemented with an appropreate antibiotic

for selection (a final concentration of ampicillin or kanamycin is 100 μg/ml and 30 μg/ml for zeocin).

2.3.2. *Agrobaterium tumefaciens* transformation

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

Agrobacterium transformation: The freeze-thaw method (Jyothishwaran *et al*., 2007) was used for *A. tumefaciens* transformation. A tube of frozen competent cells (200 μl) was kept on ice for 10 min to thaw and 10 μl of a binary vector (100 ng/μl) was mixed to the competent cells. The tube was inverted gently for three times and incubated on ice for 5 min, then frozen in liquid nitrogen for 10 min and thawed at 37°C in a heat block for 5 min. The mixture was added 800 μl of SOC medium without antibiotics and incubated at 28°C for 1 hour in a shaker at 160 rpm. The cells collected by centrifugation at 5,000 rpm for 5 min were spread on a LB plate containing 100 μg/ml kanamycin and incubated at 25-28ºC or at room temperature for 48-72 hours to gain colonies. Colony PCR was employed to screen positive colonies using a specific primer pair.

2.3.3. *Agrobacterium*-mediated *Verticillium* transformation

For *A. tumefaciens*-mediated transformation (ATMT) of *Verticillium* species, a positive bacterial colony was grown 5 ml of LB medium supplemented with 100 μg/ml kanamycin (also with 50 μg/ml carbenicillin to avoid contamination of other bacteria if necessary) for overnight. About 0.5-1.0 ml of the culture was diluted with induction medium (IM) containing 200 μM acetosyringone (AS) to get an optical density of 0.2 at the wavelength at 600 nm (OD_{600} = 0.2), then the diluted culture was grown additionally at 28ºC for 4-5 hours at 160 rpm. A mixture of equal volumes (150 μl) of the bacterial culture

and the frozen spore suspension (10^7 spores/ml) was spread onto the filter paper of 85-mm diameter (Satorius, Göttingen, Germany) on a agar plate of induction medium containing 200 μM acetosyringone (IMAS) that is identical to liquid IM, except it contains 5 mM of glucose instead of 10mM glucose. Following co-cultivation at 25°C for 48-72h, the filter paper was transferred to a PDA (potato dextrose agar) plate added hygromycin B (50 μg/ml) or nourseothricin (50 μg/ml) as the selection agent for fungal transformants, and cefotaxime (50 μg/ml) to kill the *A. tumefaciens* cells. The plates were sealed with parafilm, inverted and incubated at 25°C for 8-10 days.

2.3.4. Yeast transformation

The yeast competent cells: a yeast strain was grown in 10 ml YPD (yeast extract peptone dextrose: 2% peptone, 1% yeast extract, 2% glucose) liquid medium on a rotatory shaker, at 30°C for overnight. In the morning of next day, 1 ml of the overnight culture was transferred to a new tube containing 10 ml YPD for 4 hours of additional growth to obtain young yeast cells. The cells were harvested by centrifugation at 2,500 rpm for 10 min. The pellet was washed for two times with 10 ml of 100 mM LiOAc/TE (5 ml of 1 M Tris-Cl pH8.0; 1 ml of 0.5 M EDTA pH 8.0; 100 mM LiOAc in a total volume of 50 ml H_2O). The competent cells were resuspended in 1ml of 100 mM LiOAc/TE for transformation.

Yeast transformation: 1 μg of plasmid DNA was mixed with 200 μl of competent yeast cells in a microfuge tube. About 20 μl of single-stranded carrier DNA (salmon sperm DNA) already incubated at 65°C for 10 min was added to the mixture. About 800 μl of 50% PEG 4000 in LiAc/TE was added to the tube. The tube was inverted for five times and incubated at 30°C for 1 hour. The cells were then subjected to heat shock at 42°C for 25 minutes and centrifuged at 2,500 rpm for 2 min. The supernatant was discarded, the pellet was resuspended in 1 ml YPD and incubated at 30°C for 1 hour on a shaker. Afterwards, the cells were harvested at 2,500 rpm for 1 min and spread on SC-Ura plates (synthetic complete medium lacking uracil) and incubated at 30°C for 4 days.

2.3.5. Yeast adhesion assays using the *V. longisporum* cDNA library

The whole cDNA library of *V. longisporum* (Singh *et al.*, 2010) in the yeast expression vector pYes-Dest52 (Invitrogen, Karlsruhe, Germany) was used to transform separately three non-adhesive yeast mutants (*ΔFLO8*, *ΔFLO11* and *ΔFLO1*) of the S288C genetic background. Yeast transformants were selected and induced on plates or flasks of

the SC-Ura+galactose medium. For screening of adhesion genes with agar surface, the plates were washed gently under a stream of tap water. Adhesive transformants on the agar plates were collected for further analyses. For screening with glass surface, microscopic glass slides were incubated with the mixture of transformed cells in the flasks for overnight. Afterwards, the slides were rinsed slowly with sterile water and remaining cells on the sides were colleted and spread on selective agar plates for next detailed analyses.

2.3.6. Genetic screening using a robot system

The yeast expression plasmid (pYes-Dest52) carrying the transcript of the *Verticillium* gene (*VTA1* or *VTA2*) and uracil gene for selection was transferred to each strain of the yeast collection using the synthetic genetic analysis (Tong *et al*., 2001; Tong and Boone, 2007; Jonikas *et al*., 2009). The first, the plasmid was transformed into the MATα strain Y8205 (Tong and Boone, 2007). This strain derived from the S288C genetic background is non-adhesive and deposited in our collection as another name YLF441. The Y8205 strain carries the *HIS3* gene for histidine biosynthesis under the control of *STE2* promoter that is only expressed in MATa cells. *STE2* gene found in MATa cells encodes a receptor for the α-factor pheromone secreted by MATα cells.

The *Verticillium* gene-carrying Y8205 strain was used for mating to about 5,000 strains from the MATa deletion collection (Euroscarf, Frankfurt, Germany), in which each strain has a non-essential gene replaced with the *KanMX4* cassette of geneticin resistance ($G418^R$). All strains of the collection derived from the S288C genetic background are non-adhesive. After the mating step, all diploids were sporulated and 5,000 MATa haploids were re-isolated using a medium lacking histidine. Finally, only MATa cells possess both the geneticin resistance cassette and the plasmid were selected using a medium supplemented with geneticin but lacking uracil. The *Verticillium* gene (*VTA1* or *VTA2*) from the plasmid in each strain of the collection was induced to express by galactose. All steps were performed with the TECAN robot system (TECAN, Crailsheim, Germany) and 384-well plates were used. The whole procedure was described in Figure 6. The plates were incubated for 4 days at 30^{o}C and washed under a slow stream of tap water. Strains that were washed away revealed a loss of adhesion ability linked to *VTA1* or *VTA2*. These strains were checked in the collection map (Euroscarf, Frankfurt, Germany) to find corresponding genes of deletion.

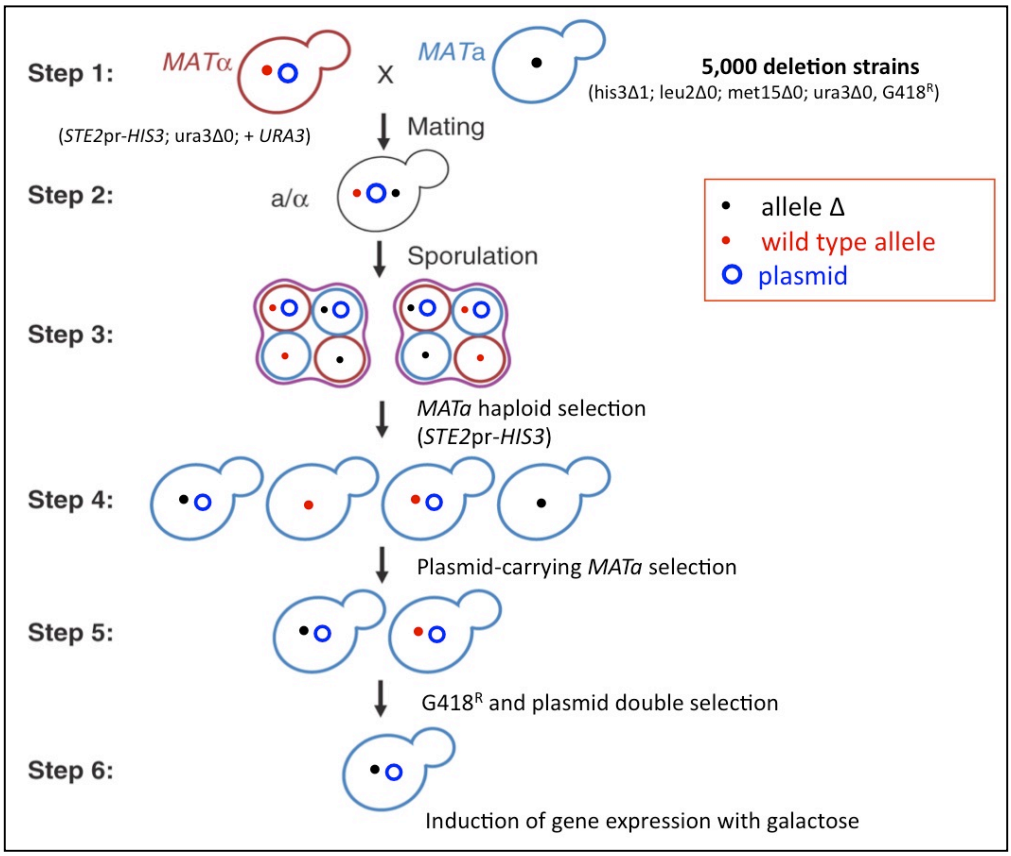


Figure 6. Synthetic genetic analysis method (modified from Tong and Boone, 2007). The MATα strain transformed with the yeast expression vector of *VTA1* or *VTA2* cDNA was mated to the MATa deletion collection. Each MATa haploid carrying a corresponding deleted gene and the vector was selected via sporulation and then by geneticin resistance and uracil prototrophy. Expresion of the *Verticillium* gene in all MATa strains was induced by galactose for screening potential partners for adhesion.

2.3.7. Plasmid isolation from *E. coli*

The plasmids carrying the gateway cassette(s) such as pYes-Dest52, pDONR/Zeo, pGS1, pGS2 must be maintained and propagated in the *ccd*B-resistant *E. coli* strain (Invitrogen, Karlsruhe, Germany). For the other plasmids, the *E. coli* strain DH5α was used instead. A single colony containing a plasmid of interest was inoculated in 5 ml of LB liquid medium with an appropriate antibiotic (100 μg/ml for ampicillin and kanamycin, 30 μg/ml for zeocin) and incubated at 37°C, 180 rpm for overnight. The plasmid was isolated using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

In addition, the boiling lysis method for plasmid isolation (Ehrt and Schnappinger, 2003) could be also used for some serial assays. The overnight culture (1 ml for high-copy plasmids, 5 ml for low-copy plasmids) was centrifuged at 5,000 rpm for 10 min. The pellet was resuspended in 400 µl STET buffer (8% sucrose; 50 mM Tris-HCl, pH8.0; 50 mM EDTA, pH8.0; autoclave; 5% Triton X-100). The tube was added 5 µl RNase and 25 µl lysozyme before being inverted for several times to mix. Then, the tube was kept in a boiling water pot for 1 min. The mixture was centrifuged at maximum speed (13,000 rpm) at 4^{o}C for 20 min. The viscous pellet was removed from the tube with a sterile toothstick and 600 µl of isopropanol was added. The tube was inverted by hand for 3-4 times and incubated at room temperature for 5 min. Afterwards, the tube was centrifuged at 13,000 rpm for 10 min and the supernatant was discarded. The white pellet was washed with 0.5 ml of 70% ethanol and havested again by centrifugation for 2 min at 13.000 rpm. The supernatant was discarded. The residual liquid was completely removed by pipetting after a centrifugation for 1 min. The precipitate was dried at room temperature for 20 min and dissolved in TE buffer. RNA in the sample was eliminated using 4 µl of RNase (Qiagen, Hilden, Germany) at 37^{o}C for 1 hour.

2.3.8. Isolation of nucleic acids from yeast and *Verticillium*

From yeast: Total DNA (genomic DNA and plasmid) extraction was performed according to the protocol from Rose *et al.* (1990): Yeast strain was inoculated in a 5-ml tube with SC-Ura liquid medium for overnight, on a rotatory shaker at 30^{o}C. The cells were collected by centrifugation of the whole culture at 3,000 rpm for 10 min. The pellet was resuspended in 300 µl Smash-Grab lysis buffer (10 mM Tris, pH 8.0; 1 mM EDTA; 100 mM NaCl; 1% SDS; 2% Triton X-100) and transferred to 2 ml eppendorf tube with a lid. Then, 250 mg of glassbead and 300 µl of phenol/chloroform were added to the tube. The cells were broken by vortexing at 13,000 rpm for 10 min. The tube was centrifuged at 13,000 rpm for 10 min again. The supernatant was transferred to new 1.5 ml tube containing 1 ml ice-cold absolute ethanol and kept at -30^{o}C for 1 hour. The pellet was harvested by centrifugation at 13,000 rpm for 10 min and washed in 1 ml ethanol of 75%. Finally, the pellet was dried at room temperature for 30 min and dissolved in 100 µl TE buffer. RNA was eliminated by 4 µl RNase (10 mg/ml) at 37^{o}C for 1 hour. The plasmid can be recovered by transformation of 5 µl total DNA into *E. coli* DH5α.

From Verticillium: The fungal isolates were grown in the potato dextrose broth (PDB) (Sigma-Aldrich Chemie GmbH, Munich, Germany) or in the liquid simulated xylem medium (SXM) (Neumann and Dobinson, 2003) for one week, at 25^{o}C. The fungal mycelium was harvested with miracloth (Calbiochem, Darmstadt, Germany) and ground to fine powder in liquid nitrogen using a pestle and mortar. The fungal powder was used directly for nucleic acid extraction or frozen in liquid nitrogen and preserved at -80^{o}C for later.

Genomic DNA was extracted from the fungal powder according to Kolar *et al.* (1988) with some modifications. In brief, about 0.8 g of the fungal powder was transferred to a 2.0 ml tube. One mililiter of a fresh lysis buffer (50 mM Tris-HCl, pH7.2; 50 mM EDTA; 3% SDS; 1% 2-Mercaptoethanol) was added to the tube. The tube was vortexed at maximum speed for 10 seconds and incubated at 65^{o}C for 1 hour. About 0.8 ml of phenol : chloroform (24:1) was added to the tube and the tube was inverted by hand for several times to mix. Centrifugation of the tube was performed at 13,000 rpm for 20 min and 300-400 µl of the supernatant phase was transferred carefully to a new 1.5 ml tube containing 0.6 ml isopropanol. The tube was centrifuged at maximum speed for 10 min. The pellet was dried at 37^{o}C for 20 min and dissolved in TE buffer. The DNA sample was treated with 4 µl of RNase A at 65^{o}C for 30 min to eliminate RNA. DNA concentration was measured using NanoDrop *ND*-1000 spectrophotometer (PEQLAB, Erlangen, Germany). The DNA quality was checked on 1% agarose gel using 5 µl genomic DNA.

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

2.3.9. PCR and DNA purification

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

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

2.3.10. Cloning and sequencing

About 250 ng of a PCR product and 500 ng of a plasmid were digested with appropriate restriction enzymes (Fermentas, St. Leon-Rot, Germany) for 2 hours. The 5' phosphate group of the restricted plasmid was removed to avoid false positive colonies resulting from an unexpected religation. This was done by adding 1 µl shrimp alkaline phosphatase (SAP) (Fermentas, St. Leon-Rot, Germany) directly to 20 µl of the digestion reaction and the reaction was incubated at 37°C for 30 min. The restricted DNA samples were analyzed on a 1% agarose gel and the expected bands were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the provided manual. For ligation, about 150 ng of the restricted insert DNA was mixed with 50 ng of the restricted plasmid (3:1) along with 1 µl of T4 DNA ligase (Fermentas, St. Leon-Rot, Germany) and 1X T4 DNA ligase buffer in a total volume of 10-20 µl. The ligation reaction was incubated at room temperature for 1 hour or at 16^{o}C in a heat block for overnight. The ligation mixture was used to transform *E. coli* competent cells.

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

2.3.11. Screening specific signatures for the hybrid origin of *V. longisporum*

The investigation of seven different genes/DNA regions with twenty-two fungal isolates by cloning and sequencing would not be an effective approach due to its time-consumption. Therefore, only some isolates were selected as representatives including one *V. dahliae* isolate (Vd-73) from linseed *Linum usitatissimum*, three *V. longisporum* isolates (Vl-19 and Vl-43 from oilseed rape *Brassica napus*, Vl-Bob70 from cauliflower *Brassica oleracea* var. *botrytis*) and one *V. albo-atrum* isolate (Va-1) from potato *Solanum tuberosum*. Firstly, the target genes from these isolates were amplified and sequenced directly for both strands, the chromatograms were checked carefully to detect different sequences in one sample. The next, mixed sequences were separated from each other by cloning and 10-15 transformants were sequenced. Lastly, the sequences of each gene from the representative isolates were compared to find specific signatures for detection of the hybrid origin. The genes carrying the specific signatures were then used as the standard markers to investigate all the remaining fungal isolates.

It is important to note that at least 10-15 positive transformants of each cloning procedure must be sequenced to avoid artefact sequences generated by PCR. Because each gene of *V. longisporum* near diploid hybrid is usually maintained in pair with two slightly different isogenes, PCR will fuse two isogenes of the gene to produce some hybrid sequences. These hybrid sequences are artificial and can be detected by DNA aligments.

2.3.12. Plasmid construction

All plasmids used in this study are listed in Table 4.

For yeast:

For GFP co-localization with *Verticillium* genes in yeast, the *GFP* (green fluorescent protein) gene from pME3459 plasmid (Padmanabhan *et al*., 2009) was isolated and cloned into p426*GAL1* plasmid (Mumberg *et al*., 1994) at *Sma*I/*Cla*I restriction sites to generate the plasmid p*GAL1-GFP*. The ORFs of *VTA1* and *VTA2* genes without the stop codon were fused to the N-terminal part of *GFP* gene at *Spe*I/*Sma*I sites of p*GAL1-GFP* to create the plasmids p*GAL1-VTA1GFP* and p*GAL1-VTA2GFP*, respectively. These constructs were verified by PCR and restriction digestion.

For Verticillium:

Fluorescent expression vectors

pHQ1 plasmid was constructed as follows: the plasmid pRHN1 (Janus *et al.*, 2007) was digested with *Sac*I and pPK2-hph (Convert *et al.*, 2001) with *Xba*I. Then, both plasmids were blunted by Klenow Fragment DNA polymerase (Fermentas, St. Leon-Rot, Germany). The *DsRed* expression cassette containing *gpdA* promoter, *DsRed* gene and *TrpC* terminator was released from the blunted pRHN1 with *Hind*III and ligated into the blunted pPK2-hph after this vector was treated with the same enzyme *Hind*III and purified.

pHQ2 plasmid was constructed for dual expression of *DsRed* and *GFP*. The *DsRed* cassette was isolated from pRHN1 using the enzymes *Sac*I and *Hind*III and ligated to the *Sac*I/*Hind*III-digested pCAMgfp binary vector (Sesma and Osbourn, 2004) to create pHQ2.

Gene disruption vectors

Two binary vectors, pKO1 and pKO2, were constructed for the purpose of gene disruption in the *Verticillium* species. The pPK2-hph vector (Covert *et al*., 2001) was digested with *Sac*I and *Xba*I to remove the full cassette for hygromycin resistance (3.93 kb). At the same time, the hygromycin resistance gene with *TrpC* promoter (1.42 kb) was amplified from the pSilent-1 vector using the primers HygCAS-F/HygCAS-R and high-fidelity Phusion DNA polymerase (Finnzyme, Espoo, Finland). The PCR product was then digested with *Sac*I and *Xba*I and ligated into the restricted pPK2 to create pKO1 vector. To generate pKO2 vector, the nourseothricin resistance cassette (1.45 kb) was isolated from pNAT1 vector (Janus *et al.*, 2007) with *Sac*I and *Apa*I. This resistance cassette was used to replace the hygromycin resistance cassette of the pKO1 vector at *Sac*I and *Apa*I sites.

These vectors were used to disrupt *VTA2* gene in *V. dahliae*. A fragment of 3.1 kb covering the entire *VTA2* gene from *V. dahliae* strain Vd-73 was amplified using the pairs *VTA2*-P1 (GGG**GATATC**TTCCCTGTCTCTGGGACTTG) and *VTA2*-P4 (GGG**TCTAG A**CAGGAAGAGAAGCGAAGAGTG). These primers contained the restriction sites, *Eco*RV and *Xba*I (bold, underlined) respectively at the 5' ends to facilitate cloning of the PCR product. The resulting 3.1-kb *VTA2* fragment was cloned into pKO1 at *Eco*RV and *Xba*I to create p*VTA2* vector without the resistance cassette. After that, this vector was digested with *Sac*I and *Apa*I to remove a 670-bp fragment located at 1,657 bp downstream from the start codon of *VTA2* gene. The hygromycin resistance cassette (1.42 kb) and

nourseothricin resistance cassette (1.45 kb) were excised from the original pKO1 and pKO2 with the same enzymes *Sac*I and *Apa*I. The resistance gene cassettes were cloned separately into the restricted p*VTA2* to generate pKO1*VTA2* and pKO2*VTA2*. These *VdVTA2* deletion constructs were introduced into *A. tumefaciens* AGL1 for fungal transformation. The resulting transformants were screened by PCR and verified by Southern hybridization.

For complementation, a 3.54-kb fragment including 1,000 bp upstream of the start codon, the *VTA2* coding sequence with introns and 932 bp downstream of the stop codon was amplified from genomic DNA of Vd-73 strain. The resulting PCR product from the primers *VTA2*-comp-F (GGG**TCTAGA**TTCCCTGTCTCTGGGACTTG) and *VTA2*-comp-R (GGG**AAGCTT**CAGGAAGAGAAGCGAAGAGTG) was digested with *Xba*I and *Hind*III (bold, underlined), and then cloned into pPK2-hph at the sites *Xba*I and *Hind*III to generate p*VTA2*comp+. The complementation vector p*VTA2*comp+ carrying the hygromycin resistance cassette followed by the full native cassette of *VTA2* gene was introduced to Vd73Δ*VTA2* mutants of nourseothricin resistance via ATMT method. Transformants resistant to hygromycin were chosen for single-spore isolation and for checking recovery of *VTA2* gene.

Gene silencing vectors

The construction of the gateway silencing plasmids was performed as follows: the cassette A from the Gateway® vector conversion kit (Invitrogen, Karlsruhe, Germany) was ligated to the pSilent-1 (Nakayashiki *et al.*, 2005) or pREDi (Janus *et al.*, 2007) at the *Sna*BI restriction site after being dephosphorylated by shrimp alkaline phosphatase (SAP). Similarly, this cassette was also introduced to the opposing direction at *Stu*I restriction site of the plasmids already carrying the sense gateway cassette. The resulting plasmids containing the cassette A in both directions (sense and antisense) were verified with the specific primer pairs (GWsense-F/GWsense-R, GWantisense-F/GWantisense-R) and digested with some appropriate restriction enzymes and partially sequenced to confirm. The fragment containing the *TrpC* promoter, sense-gateway cassette A, a cutinase intron of *Magnaporthe grisea*, antisense-gateway cassette A and *TrpC* terminator was isolated by *Xba*I enzyme and ligated into pPK2-hph or pPK2-nat to generate pGS1 gateway silencing vectors or pGS2-nat gateway co-silencing vector. All the gateway vectors were propagated and maintained in the *ccd*B resistant *E. coli* strain accompanied by the Gateway® vector conversion kit (Invitrogen, Karlsruhe, Germany).

pPK2-nat was constructed by replacing hygromycin resistance cassette of pPK2-hph (Covert *et al.*, 2001) with nourseothricin resistance cassette from pNAT1 (Janus *et al.*, 2007) isolated with *Avr*II and *Xba*I.

To test the gateway silencing vectors, *DsRed* fluorescence gene and *VTA2* gene of *V. longisporum* were employed to generate hairpin RNA-mediated silencing constructs using BP clonase and LR clonase enzyme mix together with pDONR/Zeo plasmid (Invitrogen GmbH, Karlsruhe, Germany) according to manufacturer's instructions with some modifications. In brief, two primer pairs were designed to adapt to gateway reactions, meaning that the *att*B1 sequence (ACAAGTTTGTACAAAAAAGCAGGCTTC) was added to 5' end of the forward primers and the *att*B2 sequence (ACCACTTTGT ACAAGAAAGCTGGGTC) to 5' end of the reverse primers. A fragment of 400-550 bp was amplified from cDNA of each gene using a corresponding primer pair (*Redi*-gwF/*Redi*-gwR or *VTA2*-gwF/*VTA2*-gwR). The purified PCR product (100 ng) and pDONR/Zeo plasmid (50 ng) were added to a 1.5 ml tube to get a total volume of 4 µl (1X TE buffer can be used if necessary). The tube was added 1 µl of the BP clonase enzyme mix and incubated at 25^{o}C or at room temperature for 1 hour. To terminate the reaction, the tube was added 0.5 µl of proteinase K and incubated at 37^{o}C for 10 minutes. The whole reaction was used to transform *E. coli* DH5α with the heat shock procedure. LB plates containing 30 µg/ml of zeocin (or phleomycin) antibiotic were used for selection of transformants. Ten colonies were checked with colony PCR using the specific primer pair. The recombinant plasmid was isolated using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany).

The recombinant plasmid (also called the entry vector) carrying a gene fragment of interest was mixed with the gateway silencing vector (pGS1 or pGS2) in presence of LR clonase enzyme mix. This reaction was performed like BP reaction but for selection kanamycin antibiotic (100 µg/ml) was used instead of zeocin. Ten transformants were checked by colony PCR using the specific silencing primers for *DsRed* or *VTA2* gene. Four positive *E. coli* transformants were cultured in tubes of 5-ml LB containing kanamycin antibiotic (100 µg/ml) for overnight and the plasmids were purified using QIAprep Spin Miniprep Kit. To confirm a correct construct for silencing, the gateway recombinant vectors carrying the target gene sequence in both sense and antisense directions were digested with some appropriate enzymes to check and partially sequenced afterwards.

Table 4. The plasmids used in this study

Name	Type	Description	Source
pDONR/Zeo	Gateway	Containing a gateway cassette, Zeocin^R	Invitrogen
pYes-Dest52	Gateway	Containing *GAL1* promoter, a gateway cassette, *CYC1* terminator, *URA3*, 2μ, Amp^R	Invitrogen
pJET1.2	Cloning	A dephosphorylated cloning vector with blunt ends	Fermentas
pHL135	Cloning	Containing the full native cassette for *FLO8* gene, *URA3*, 2 μ, Amp^R	Liu *et al.*, 1996
p426*GAL1* (pME2795)	Cloning	Containing *GAL1* promoter, *CYC1* terminator, *URA3*, 2 μ, Amp^R	Mumberg *et al.*, 1994
pME3459	Cloning	Containing *MET25* promoter, *GFP* gene and *CYC1* terminator	Padmanabhan *et al.*, 2009
p*GAL1-GFP*	Cloning	p426*GAL1* containing *GFP* gene	This study
p*GAL1-VTA1GFP*	Cloning	p*GAL1-GFP* containing *VTA1*	This study
p*GAL1-VTA2GFP*	Cloning	p*GAL1-GFP* containing *VTA2*	This study
pNAT1	Cloning	Containing the nourseothricin resistance gene (*nat1*) under *gpdA* promoter of *A. nidulans*	Janus *et al.*, 2007
pRHN1	Cloning	*DsRed* gene with *gpdA* promoter, nourseothricin resistance gene (*nat1*) from *Streptomyces noursei*	Janus *et al.*, 2007
pREDi	Cloning	Containing the *DsRed* silencing cassette with *TrpC* promoter, nourseothricin resistance gene (*nat1*)	Janus *et al.*, 2007
pSilent-1	Cloning	Silencing cassette with *TrpC* promoter of *A. nidulans*, hygromycin resistance	Nakayashiki *et al.*, 2005
pPK2-hph	Binary	ATMT, hygromycin resistance gene (*hph*) with the full *gpdA* promoter	Covert *et al.*, 2001
pPK2-nat	Binary	ATMT, nourseothricin resistance gene (*nat1*) with the full *gpdA* promoter	This study
pCAMgfp	Binary	*GFP* expression with *ToxA* promoter, hygromycin resistance	Sesma *et al.*, 2004
pHQ1	Binary	*DsRed* expression with *gpdA* promoter, hygromycin resistance	This study

Name	Type	Description	Source
pHQ2	Binary	*GFP* gene with *ToxA* promoter and *DsRed* gene with *gpdA* promoter, hygromycin resistance	This study
pGS1	Binary	Gateway silencing with *TrpC* promoter, hygromycin resistance or nourseothricin resistance	This study
pGS2	Binary	Gateway *DsRed* co-silencing with *TrpC* promoter and nourseothricin resistance	This study
pKO1	Binary	Gene disruption vector with hygromycin resistance cassette	This study
pKO2	Binary	Gene disruption vector with nourseothricin resistance cassette	This study

2.4. Yeast adhesion and flocculation tests

For adhesion, the yeast strains were grown on the SC-Ura plates containing 2% glucose or 2% galactose for *GAL1* promoter induction. After three days of incubation at 30ºC, the plates were washed under a water stream and photographed with GelDoc detector (Bio-Rad, Munich, Germany).

For flocculation, the strains were cultivated in test tubes with 10 ml of the liquid SC-Ura medium containing 2% glucose or galactose. The tubes were kept in a rotatory shaker at 30ºC for 24-48 hours. Then, the culture tubes were vortexed strongly and left in a rack for 1 min. The photography was performed with a digital camera.

2.5. Yeast flocculation assays

The flocculent ability of the yeast strains was performed following the method for Ca^{2+}-dependent flocculation assay (Bester *et al*., 2006). In brief, the clones were inoculated in test tubes containing 10 ml of the SC-Ura medium added 2% galactose as the sole carbon source for 48 hours at 30ºC with shaking. Then, the 0.5M EDTA solution (pH 8.0) was added to the yeast cultures to a final concentration of 50 mM. The tubes were mixed by vortexing at the maximum speed until the flocs were suspended completely. The optical density at the wavelength of 600 nm (OD_{600}) was determined immediately by using 100 µl of each resuspended culture and 900 µl of a 50mM EDTA solution (pH 8.0) followed by

spectrophotometric measurement (= A). Afterwards, 1 ml of each resuspended cultures was transferred to a microcentrifuge tube and spinned for 20 seconds at 10,000 rpm. The supernatants were discarded and the pellets were washed with 1 ml of 10 mM $CaCl_2$ solution to induce flocculation. The tubes were vortexed at maximum speed for 10 seconds and left in a rack for 60 seconds without any disturbance. Transfer 100 µl of the liquid phase (nearby the surface) to a microcentrifuge tube and add 900 µl of 50 mM EDTA solution. Then, mix well by vortexing and measure the OD_{600} (= B).

The formula for flocculation rate (%) = (1-B/A) x 100.

2.6. Southern hybridization

The Southern hybridization was performed following the recommendations in the provided manuals from GE Healthcare manufacturer. In brief, about 25-30 µg of genomic DNA was digested for overnight with 3 µl of an appropriate restriction enzyme that uncuts or cuts the target genes at a unique site outside the probe sequence. The digested mixture was analyzed on a 1% agarose gel, DNA was denaturated and transferred to the Amersham Hybond-N membrane (GE Healthcare, Munich, Germany) by blotting. DNA molecules and the membrane were cross-linked under UV light. A fragment (about 600 bp) of the target gene or the whole gene was amplified and labelled as probe using Amersham AlkPhos Direct labelling Reagent (GE Healthcare, Munich, Germany). The DNA on the membrane was hybridized to the specific probe in hybridization buffer at 60^oC for overnight. The membrane was treated carefully with washing buffers to eliminate background. Then, chemiluminescent signals were detected using the Amersham CDP-Star Detection reagent (GE Healthcare, Munich, Germany).

2.7. Quantitative real-time PCR

The total RNA was extracted from 0.1 g of fungal mycelium grown in SXM medium using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Concentration of RNA samples was measured using NanoDrop-*ND1000* spectrophotometer (PEQLAB, Erlangen, Germany). Then, 1 µg of total RNA was transcribed into cDNA in 20 µl using the QuantiTect® Reverse Transcription kit (Qiagen, Hilden, Germany). The expression level of a gene was measured by real-time PCR using a specific primer pair. Reactions contained 2 µl of each 0.5 pM primer (forward and reverse), 1 µl of the cDNA, and reverse transcriptase-grade PCR water to a final volume of 11 µl. The 5 PRIME MasterMix (5 PRIME GmbH, Hamburg, Germany) was added to obtain a final running volume of 20 µl

per reaction. Each reaction was run in triplicate for both the standard and unknown samples. Reactions were run under the following conditions using the Light Cycler 2.0 System (Roche, Manheim, Germany): 95°C denaturation for 3 min, 42 cycles at 95°C for 10s, 63°C for 15s and 72°C for 25s to calculate cycle threshold values, followed by 95°C for 1 min, 55°C for 1 min and 80 times of 55°C for 10s, increasing temperature by 0.5°C each cycle to obtain melting curves and to enable data analyses. Standard curves were produced with purified DNA products of 10 and 1 pg/µl and starting concentrations of 100, 10, and 1 fg/µl. A baseline subtracted curve fit was used to generate standard curve data. Absolute amounts of transcripts were calculated using a correlation coefficient formula generated from the standard curve in each run.

2.8. Microscopic analyses

Yeast cells were grown to log phase in SC-Ura medium containing 2% glucose. The cells were harvested by centrifugation and resuspended in SC-Ura containing 2% galactose for 4 hours to induce gene expression. Fluorescence of the GFP fusion proteins was visualized in unfixed cells by using a Axioplan fluorescence microscope (Carl Zeiss, Göttingen, Germany). Images were captured using a digital camera (Hamamatsu Orca-100, Hamamatsu, Japan) and Openlab software (Improvision, UK).

Screening of the fungal transformants for GFP/DsRed fluorescence signals, a small sample of mycelium at a random position in the plate was removed and placed in a small sterile water drop on a microscopic slide. The sample with a coverslip on the top was equally distributed by a slight force from a finger. GFP and DsRed signals were detected visually, and the isolates were scored on the fluorescence intensity.

For phenotypic analysis, amounts of about 10,000 spores of *VTA2*-knockout mutants and wild type were inoculated on different solid media including Czapek-Dox medium (Smith, 1949), minimal medium (Hill and Kafer, 2001), potato dextrose agar (Carl Roth GmbH, Karlsruhe, Germany), simulated xylem medium (Neumann and Dobinson, 2003) and oatmeal agar (Difco, Lawrence, USA) at 25°C. The experiment was repeated three times. The plates were observed and photographed daily using Olympus SZX12 microscope with camera (Olympus, Hamburg, Germany). For fungal growth rate, colony diameters were measured after 4, 6, 8, 10 and 12 days post inoculation.

2.9. Measurement of hydophobicity

About 500,000 spores of three *VTA2* knock-out mutants and the wild type Vd-73 were spread on the PDA plates without nourseothricin antibiotic using glass beads. The plates were incubated at 25^{o}C. After 3 weeks, 100 µl of sterile water was placed on the fungal mycelia on plates (at 3 positions). The plates were closed and kept at room temperature for 10 min, 30 min, 1 hour, 2 hours and 20 hours. The water amounts were recollected to check and the experiment was repeated three times.

2.10. Plant experiments

2.10.1. *Verticillium-Arabidopsis* interaction

Ten *Arabidopsis thaliana* seeds were sowed on a "vertical angular" plate containing 1/2 Murashige and Skoog medium (MS) with sugar, pH5.7 and 15g plant agar/liter. The plates were kept for 24 h in a 4°C chamber. Pre-growth of *Arabidopsis* for 10 days at 20-22°C, 60% humidity and longday (16 h light, 8 h dark). The plants were transferred to agarose plates (10-13g agarose/liter). The plates were incubated for 2 more days. After that, the roots were sprayed with a spore suspension of 50,000 spores/ml. The plant infection by the fungus was observed via the fluorescent signals of the fungus after 24, 48, 72, 96 hours post inoculation (hpi) by using a confocal microscope LSM Leica SP5 (Leica, Solms, Germany) (Michael Reusche's protocol).

2.10.2. Plant infection assays

Ten day old seedlings of rapeseed *Brassica napus*, *Arabidopsis thaliana* or tomato were inoculated with 10^7 fungal spores/ml (mutants or wild type), and mock with tap water for 40 min by root-dipping. The seedlings were then transferred in pots with a sterile sand:soil (1:2) mixture. The plants were allowed to grow in a climate chamber with 16 hours light at 23°C and 8 hours dark at 20°C. The height of plants or leaf size was measured to calculate disease scores at 7, 14, 21, 28 and 35 days post inoculation (dpi).

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

Chapter 3. RESULTS

3.1. *Verticillium longisporum* genes reprogram adhesion and flocculation in the budding yeast *Saccharomyces cerevisiae*

3.1.1. The budding yeast as a model for fungal adhesion assays

The budding yeast *Saccharomyces cerevisiae* was used successfully to isolate fungal adhesins and adhesion regulators from the human pathogen *Candida albicans* (Li and Palecek, 2005). In this study, we used three non-adherent yeast mutants derived from S288C background to screen genes involved in adhesion from the rapeseed pathogen *V. longisporum*. In fact, the S288C background is non-adhesive and non-flocculating due to a non-sense mutation in *FLO8* gene encoding a transcription factor for the expression of *FLO1* and *FLO11* adhesin genes. FLO1 and FLO11 adhesins are responsible for substrate adhesion and cell-cell adhesion causing flocculation (Kobayashi *et al*., 1996; Liu *et al*., 1998; Bester *et al*., 2006; Fichtner *et al*., 2007). Three yeast mutants used in this study include *ΔFLO8*, *ΔFLO11* and *ΔFLO1*. A mixed cDNA library of *V. longisporum* was constructed in the pYes-Dest52 vector expressing the cDNAs in yeast cells in the presence of galactose (Singh *et al*., 2010). We transformed separately the whole cDNA library into the non-adhesive yeast strains and screened about two million transformants for adhesion to agar and to glass surface. Most transformants were washed away from the plates or glass under a slow water stream. However, some transformants were still tightly attached to the agar surface (Figures 7A and 7B). As a result, 54 potential transformants of adhesion were detected. Consequently, to verify these cDNA sequences (expressed sequence tags - ESTs) whether they could really make non-adherent yeasts become adhesive, we re-isolated the plasmids from these yeast transformants and multiplied them in *E. coli* cells. Then the plasmids purified from *E. coli* were re-transformed into the non-adhesive yeasts. Finally, from 54 potential transformants we identified 44 candidates that were able to adhere again to the corresponding surfaces (agar or glass). The *Verticillium* candidate cDNAs for adhesion were sequenced using two specific primers including *T7* primer (5'-TAATACGACTCACTATAGGG-3') nearby the *GAL1* promoter and *CYC1* primer (5'-GCGTGAATGTAAGCGTGAC-3') in the *CYC1* terminator.

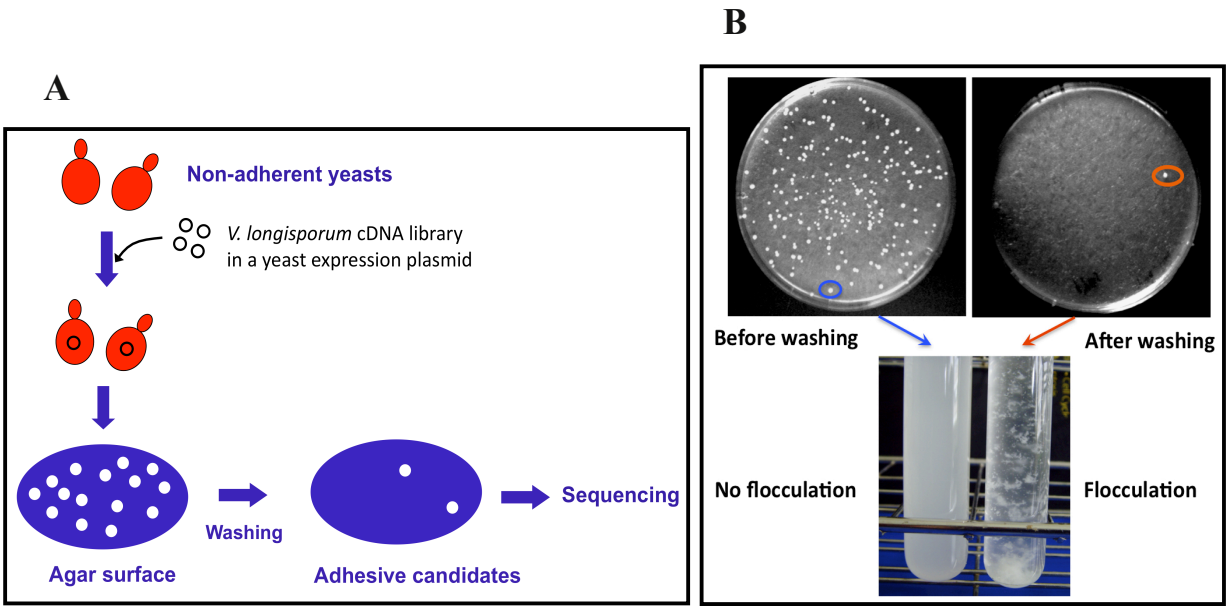


Figure 7. Screening adhesion genes of *V. longisporum* using the yeast *S. cerevisiae*. (A) *Model for screening adhesion genes of V. longisporum*. The whole cDNA library of the rapeseed pathogen *V. longisporum* was used to transform the non-adhesive yeast. The expression of *V. longisporum* cDNA molecules under the control of the yeast-specific *GAL1* promoter were induced by galactose. The plates were rinsed with water to find potential transformants of adhesion. Then, the plasmid from the corresponding candidates were isolated for sequencing. **(B)** *Screening of adhesion transformants using agar surface*. Most of the transformants were washed away under the water stream, but some were still adhered to the agar surface. These potential transformants were also tested for cell-cell adhesion (flocculation) in the liquid medium.

Sequencing of the corresponding ESTs from these 44 candidates revealed twenty-four different sequences. These sequences were analyzed using the bioinformatics tools and the databases from the National Center for Biotechnology Information (NCBI). In general, they were classified into four groups including 10 putative regulatory proteins (42%), 4 putative cell wall and membrane proteins (16.5%), 3 putative transport proteins (12.5%), 1 putative metabolism protein (4%), 6 hypothetical proteins with unknown functions (25%) (Figure 8). Interestingly, the data showed a dominance of the regulatory proteins that could reprogram adhesion of the non-adhesive yeast mutants and at least four putative cell wall proteins with conserved domains and signals for secretion and membrane attachment (GPI anchor) that promoted adhesion of yeast to the tested surfaces.

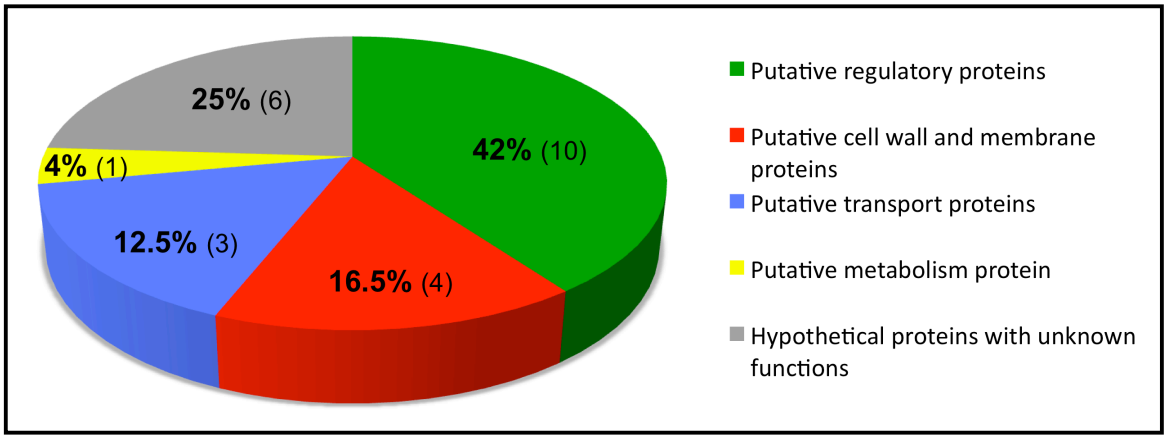


Figure 8. Distribution of the adhesion sequences to different function groups. Twenty-four cDNA sequences were classified into four groups including regulatory proteins (10), cell wall and membrane proteins (4), proteins of transport (3), protein of metabolism (1) and hypothetical proteins with unknown functions (6).

Ten putative regulatory proteins (R3H domain-containing protein, LsmAD domain-containing protein, RNA-binding protein with RRM motif, extensin protein, Homeobox transcription factor, CON7 transcription regulator, Cephalosporin C regulator, glucose repressor CRE1, C6 zinc transcription factor and ARID/BRIGHT domain-containing protein) involved in a broad range of different functions including transcription and post-transcription processes were detected from the adhesion assays (see details in Table 5A). We also found 4 putative cell wall and membrane protein-encoding genes. However, only one of them is known as the homologue of Mmc protein that is involved in microcycle conidiation of the entomopathogenic fungus *Metarhizium anisopliae* (Liu *et al.*, 2010). In addition, 3 putative proteins of transport (potassium channel transporter, Thi4 domain protein, Sec23/Sec24 trunk domain-containing protein) and 1 putative protein of metabolism (aldehyde dehydrogenase domain protein) were found and described in Table 5A. Interestingly, we found a sequence encoding Thi4 domain protein required for thiamine biosynthesis (Akiyama and Nakashima, 1996). In yeast, thiamin biosynthesis is induced under adhesion-inducing conditions in response to amino acid starvation (Kleinschmidt *at al.*, 2005).

Table 5A. Characterization of the adhesion sequences using the bioinformatics tools. All twenty-four sequences were searched for the information based on the NCBI databases. In parallel, they were also translated into protein sequences in order to characterize conserved domains, secretion signals, GPI anchors, etc. Candidates ***a*** were found in the adhesion screen using the *ΔFLO8* yeast strain on agar, candidates ***b*** were detected in the *ΔFLO11* mutant screen, the candidates ***c*** resulted from the screen with the *ΔFLO1* mutant and the candidate ***d1*** resulted from the *ΔFLO8* strain screened on glass surfaces.

Candidate	**Description**	**Conserved domain and possible functions** (Using the bioinformatic tools: NCBI-BLAST, InterProScan, Pfam, ExPASy-ScanProsite and GPI anchor prediction)	**NCBI accession number**
a1, a3	R3H domain-containing protein (*Metarhizium anisopliae*): 59%	The name of the R3H domain comes from the characteristic spacing of the most conserved arginine and histidine residues. The function of the domain is predicted to bind ssDNA or ssRNA in a sequence-specific manner and involved in stress responses.	EFZ01698
a2	Hypothetical protein (*Metarhizium anisopliae*): 32%	This gene codes for a secreted protein with unknown function. The encoded protein is small (174 amino acids) with a secretion signal and a putative GPI anchor attachment site.	EFZ00960
a4, b18	Homeobox and C_2H_2 transcription factor (*Metarhizium anisopliae*): 76%	C_2H_2 zinc domain can bind to RNA and protein targets.	EFY95994
a6, a7, a15, b7, b10, b12, b14, c7	Transcriptional regulator CON7 (*Magnaporthe grisea*): 49%	C_2H_2 zinc domain, CON7 is a central regulator of infection-related morphogenesis in the rice blast fungus *Magnaporthe grisea*	ABI96241
a5, a8, a9, a10, a18, c1, c8, c15	Putative cephalosporin C regulator (*Metarhizium anisopliae*): 73%	The DNA-binding domain of RFX is the central domain of the protein and binds ssDNA as either a monomer or homodimer for gene expression.	EFY97581
a11, b2	LsmAD domain-containing protein (*Colletotrichum graminicola*): 65%	Lsm-associated domain (LsmAD), found in proteins that function in nuclear RNA processing and mRNA decay.	EFQ32946
a12	Putative potassium channel, voltage-dependent transporter (*Grosmannia clavigera*): 39%	This protein might be involved in transport of ions.	EFW98782
a13	Putative RNA-binding protein (*Metarhizium acridum*): 46%	RNA recognition motif (RRM) is probably diagnostic of an RNA binding protein	EFY89243
a14, a19, c6	Hypothetical protein (*Metarhizium acridum*): 64%	Prokar_Lipoprotein domain: prokaryotic membrane lipoprotein lipid attachment site profile.	EFY92745

Candidate	Description	Conserved domain and possible functions (Using the bioinformatic tools: NCBI-BLAST, InterProScan, Pfam, ExPASy-ScanProsite and GPI anchor prediction)	NCBI accession number
a16, a20	Thiazole biosynthetic enzyme (*Metarhizium anisopliae*): 80%	Thi4 domain is involved in the biosynthesis of thiazole.	EFY99491
a17	Hypothetical protein (*Sordaria macrospora*): 52%	Aldedh domain, aldehyde dehydrogenases are enzymes that oxidize a wide variety of aliphatic and aromatic aldehydes using NADP as a cofactor.	CBI51038
a21	Hypothetical protein (*Metarhizium acridum*): 56%	No conserved domain, unknown function	EFY88153
a22	The glucose repressor CRE1 (*Trichoderma harzianum*): 64%	Zn-finger domain with C_2H_2 type. This protein might be involved in glucose metabolism.	CAA64656
b1	Extensin (*Metarhizium anisopliae*): 57%	CASC3/Barentsz eIF4AIII binding. This domain is found on CASC3 (cancer susceptibility candidate gene 3 protein) which is also known as Barentsz (Btz). CASC3 is a component of the EJC (exon junction complex) which is a complex that is involved in post-transcriptional regulation of mRNA in metazoa. The complex is formed by the association of four proteins (eIF4AIII, Barentsz, Mago, and Y14), mRNA, and ATP required for mRNA localization and nonsense-mediated mRNA decay	EFY98452
b3	Hypothetical protein (*Metarhizium acridum*): 45%	No conserved domain, unknown function	EFY89994
b5	Hypothetical protein (*Verticillium albo-atrum*): 98%	No conserved domain, unknown function	XP_003007879
b4, b9, b17	Hypothetical protein (*Colletotrichum graminicola*): 58%	No conserved domain, unknown function	EFQ29487
b6, b8, b11, b15, b19	C6 zinc transcription factor (*Colletotrichum lagenarium*): 49%	Fungal $Zn(II)_2Cys_6$ binuclear cluster domain. The N-terminal region of a number of fungal transcriptional regulatory proteins contains a Cys-rich motif that is involved in zinc-dependent binding of DNA. The region forms a binuclear Zn cluster, in which two Zn atoms are bound by six Cys residues. A wide range of proteins are known to contain this domain. These include the proteins involved in amides catabolism; regulation of cellulase, xylanase and cutinase genes; aflatoxin biosynthesis; nitrate assimilation; conidiophore morphogenesis; fruiting body development; melanin biosynthesis ect.	BAE98094
b13	ARID/BRIGHT DNA binding domain-containing protein	Members of the recently discovered ARID (AT-rich interaction domain) family of DNA-binding proteins are found in fungi and	EFY99726

Candidate	Description	Conserved domain and possible functions (Using the bioinformatic tools: NCBI-BLAST, InterProScan, Pfam, ExPASy-ScanProsite and GPI anchor prediction)	NCBI accession number
	(*Metarhizium anisopliae*): 50%	invertebrate and vertebrate metazoans. ARID-encoding genes are involved in a variety of biological processes including cell growth, differentiation and development. They are also required for transcriptional activation of cyst wall protein and involved in the modification of chromatin structure.	
b16	Sec23/Sec24 trunk domain-containing protein (*Colletotrichum graminicola*): 58%	COPII (coat protein complex II)-coated vesicles carry proteins from the endoplasmic reticulum (ER) to the Golgi complex. COPII-coated vesicles form on the ER by the stepwise recruitment of three cytosolic components: Sar1-GTP to initiate coat formation, Sec23/24 heterodimer to select SNARE and cargo molecules, and Sec13/31 to induce coat polymerization and membrane deformation. Sec23 p and Sec24p are structurally related, folding into five distinct domains: a beta-barrel, a zinc-finger, an alpha/beta trunk domain, an all-helical region, and a C-terminal gelsolin-like domain.	EFQ34176
b20	Hypothetical protein (*Colletotrichum graminicola*): 56%	No conserved domain, unknown function	EFQ27603
b21	Hypothetical protein (*Colletotrichum graminicola*): 55%	DUF1837 domain of unknown function	EFQ31115
c3	Integral membrane protein (*Metarhizium anisopliae*): 62%	This protein contains seven transmembrane regions with unknown function.	EFY98086
d1	Mmc protein (*Metarhizium anisopliae*): 62%	A small secreted protein of 154 amino acids with a secretion signal and a GPI anchor attachment site is involved in fungal microcycle conidiation.	ACN92038

Furthermore, all these candidates were examined for adhesion to some different surfaces including agar, plastic, plant roots (rapeseed) and glass. Our data indicated that many of the candidates could adhere very tightly to all four surfaces, whereas the original *FLO8* deletion strain (S288C*ΔFLO8*) could not adhere to any of these surfaces (Figure 9). This proved that the genes from *V. longisporum* were able to switch the non-adhesive yeasts to a new adhesive state.

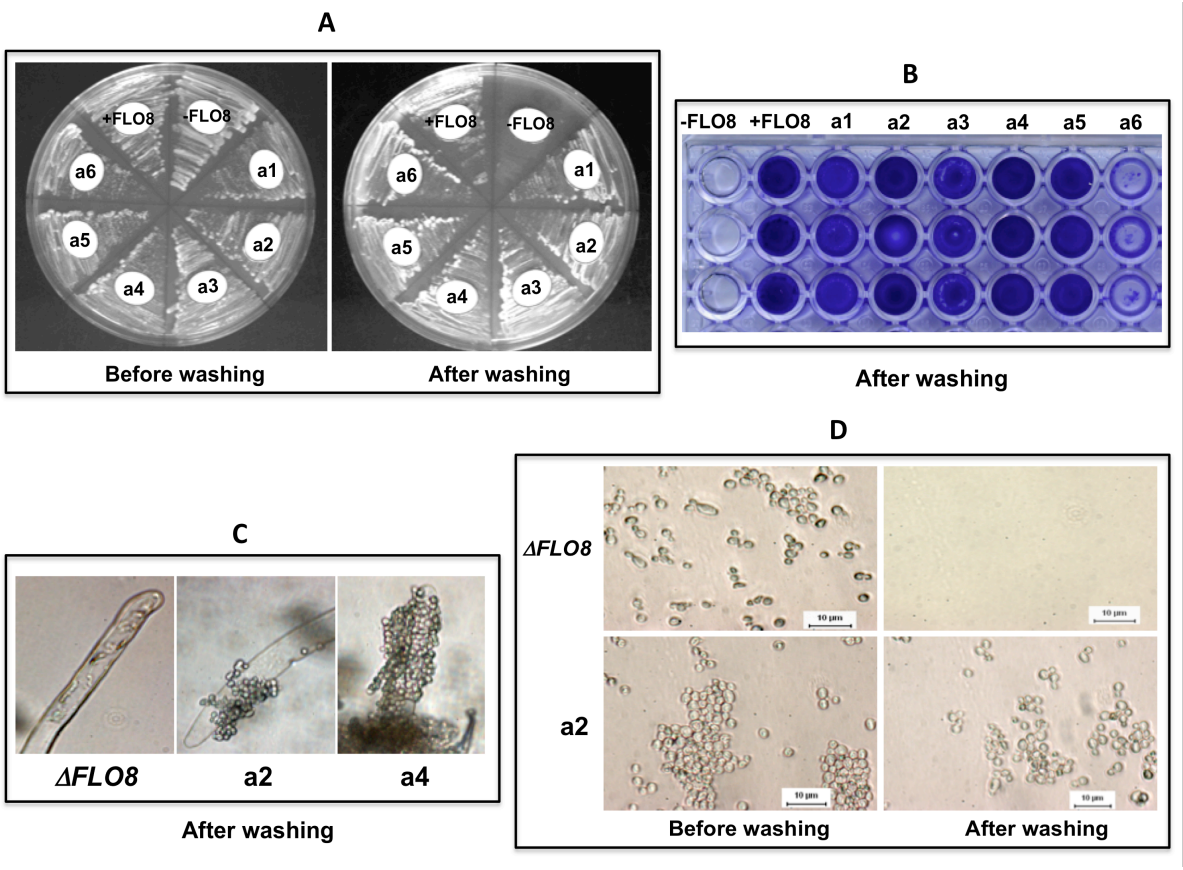


Figure 9. Adhesion of some representive candidates to different surfaces. (A) *Adhesion to agar surface*. The candidates were grown on SC-Ura+galactose plates. After 4 days of incubation at 30°C, the plates were washed with water to check. **(B)** *Adhesion to plastic surface*. The candidates were inoculated in SC-Ura+galactose liquid medium for 24-36 hours. The samples were distributed to holes of a 96-well plastic plate, three times for each sample. The plate was incubated at 30°C for overnight without shaking. The next day, the samples were stained with a drop of crystal violet solution and washed to check. **(C)** *Adhesion to plant roots*. The yeast cultures from (B) were added to root areas of rapeseed seedlings in Petri plates. The plates were kept at 30°C for 4-6 hours. The plant roots were rinsed gently with water and checked under a microscope. **(D)** *Adhesion to glass surface.* Similar to (C) but microscopic slides were used instead of plant roots.

The data also showed that under the induction of galactose in the liquid medium, the corresponding sequences from the candidates promoted cells to adhere to each other to form flocs consisting of many thousands of cells. Cell-cell adhesion between yeast cells is often called flocculation. Flocculation might protect the cells in the middle of the flocs from the environmental stress (Verstrepen and Klis, 2006). The results for flocculation ability and adhesion of all candidates to the surfaces are summarized in Table 5B.

Table 5B. Adhesion and flocculation of yeast expressing 24 candidate genes to different surfaces. Twenty-four adhesive candidates corresponding to the accession numbers from NCBI or BROAD *Verticillium* database were tested for their adhesion ability to the natural and artificial surfaces including rapeseed roots, agar, plastic and glass. The flocculation assays were also performed for the candidates. Flocculation is Ca^{2+}-dependent and represents adhesion of single cells mediated by flocculins to form flocs that quickly sediment to the bottom of tubes.

Candidate	***Verticillium* accession number**	**Surface adhesion**				**Flocculation (%)**
		Agar	Plastic	Glass	Rapseed roots	
a1, a3	XP_003006300 (NCBI)	+	+++	+	++	15.67
a2	XP_003001025 (NCBI)	+	+++	++	+++	10.20
a4, b18	XP_003007693 (NCBI)	+	+++	+++	+++	47.26
a6, a7, a15, b7, b10, b12, b14, c7	XP_003003070 (NCBI)	++	++	++	++	22.50
a5, a8, a9, a10, a18, c1, c8, c15	XP_003006218 (NCBI)	+++	+++	+++	+	48.28
a11, b2	XP_003007313 (NCBI)	++	+++	+++	++	58.29
a12	XP_003009125 (NCBI)	++	++	+++	+	1.36
a13	XP_003005634 (NCBI)	++	-	+++	++	0
a14, a19, c6	XP_003009401 (NCBI)	+++	+++	+	++	57.74
a16, a20	XP_003005955 (NCBI)	+	+++	++	++	63.50
a17	XP_003002810 (NCBI)	++	-	+++	-	3.10
a21	XP_003004676 (NCBI)	++	+++	-	+	25.84
a22	VDAG_00586 (BROAD)	+	+++	+	+++	71.36
b1	XP_003008985 (NCBI)	+++	+++	++	+++	49.08
b3	XP_003001258 (NCBI)	+++	+++	++	-	21.27
b5	XP_003007879 (NCBI)	+++	+++	++	-	77.60
b4, b9, b17	XP_003007782 (NCBI)	+++	+	++	++	22.31
b6, b8, b11, b15, b19	VDAG_00192 (BROAD)	+++	+++	++	++	77.74

Candidate	*Verticillium* accession number	Surface adhesion				Flocculation (%)
		Agar	Plastic	Glass	Rapseed roots	
b13	XP_003005481 (NCBI)	+++	+++	+++	-	48.58
b16	XP_003005496 (NCBI)	++	-	++	-	20.23
b20	XP_003009061 (NCBI)	+++	+++	+++	+++	53.23
b21	XP_003004340 (NCBI)	+++	+++	++	-	73.49
c3	VDAG_05461 (BROAD)	+	-	+	nd	nd
d1	XP_003008678 (NCBI)	-	-	++	nd	nd

+++ strong; ++ medium; + weak; - no adhesion; nd = not determined

3.1.2. *Verticillium* transcription regulators activate adhesion and flocculation in the non-adhesive yeasts

From the complementation assays, we found that three of twenty-four sequences are the most dominant, two of them belong to zinc family with specific domains of transcription activators and the other belongs to RFX-DNA binding domain family. All three induce strong adhesion and flocculation in the non-adhesive yeasts (Table 5B) and were named as *V*erticillium *T*ranscription *A*ctivator genes, *VTA1*, *VTA2* and *VTA3* respectively. It could be that these transcription activators might also play a similar role in regulating adhesion or biofilm formation in *Verticillium*. Recently, the roles of some artificial zinc finger proteins inducing expression of yeast FLO11 adhesin have been reported (Shieh *et al*., 2007). Therefore, we selected *VTA1* and *VTA2* genes for further analyses using the budding yeast *S. cerevisiae* as a model. The *VTA1* and *VTA2* genes have the coding sequences of 1,278 bp and 1,245 bp, respectively. The *VTA1* gene with $Zn(II)_2Cys_6$ conserved domain shows a low homology to AflR transcription factor that is required for aflatoxin biosynthesis in Aspergilli (Yu *et al.*, 1996). The *VTA2* gene with C_2H_2 zinc motif is highly similar to *CON7* gene that is responsible for regulation of a veriety of different genes in the rice blast fungus *Magnaporthe grisea* (Odenbach *et al*., 2007). Interestingly, we found two different cDNA molecules for *VTA2* gene with distinct signatures (Figure 10A). Both *VTA2* transcripts were able to induce a strong adhesion of

the non-adherent yeasts to agar surface (Figure 10B). However, flocculation caused by *VTA2* gene is weaker than by that of *VTA1* gene (Figures 10C-D)

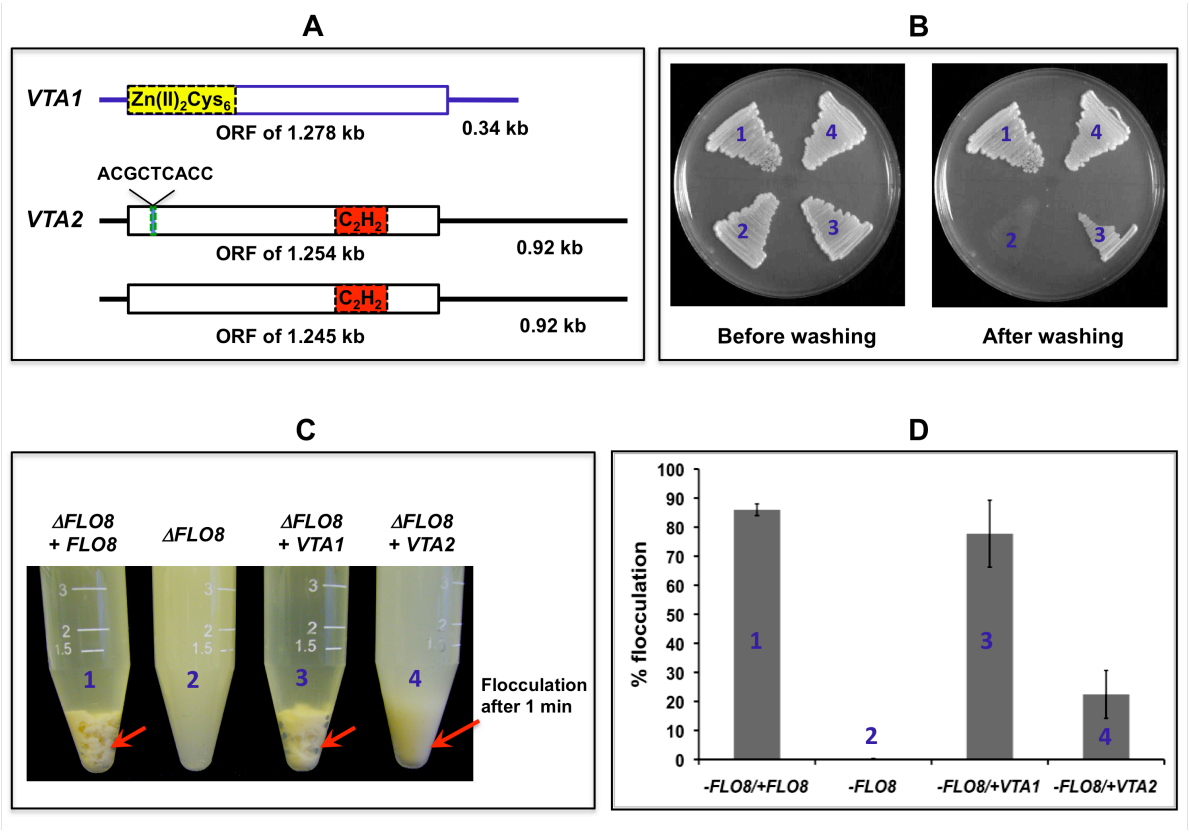


Figure 10. Expression of two *Verticillium* transcription regulators in the non-adhesive yeast. **(A)** *The structure of VTA1 and VTA2 transcripts*. *VTA1* contains $Zn(II)_2Cys_6$ conserved domain at the N-terminal end. In contrast, *VTA2* with two different transcripts carries C_2H_2 zinc motif at the C-terminal end. **(B)** *VTA1 and VTA2 as regulators of adhesion in yeast*. These transcription regulators recover adhesion ability of the non-adhesive yeast on agar. **(C)** *Flocculation in the non-adhesive yeast by VTA1 and VTA2*. Both *Verticillium* regulators are able to activate flocculation in yeast like FLO8 transcription factor. **(D)** *Measurement of Ca^{2+}-dependent flocculation*. Flocculation induced by both *Verticillium* regulators and FLO8 regulator requires the presence of Ca^{2+} ions in the medium. VTA1 and FLO8 exhibit similar flocculation percentages, whereas VTA2 displays a reduced flocculation.

3.1.3. Coordination of *Verticillium* transcription regulators and yeast THO complex in promoting adhesion and flocculation of the non-adhesive yeast

The *Verticillium* transcription regulators induced the adhesion and flocculation in the non-adhesive yeasts with unknown mechanisms. Therefore we performed a genetic screening for the whole yeast deletion collection of about 5,000 genes that are not essential

for the yeast survival (Euroscarf, Frankfurt am Main, Germany). The goal of this screening was to show which genes were activated by the *Verticillium* transcription regulators. We used a robot system to complement these 5,000 deletion mutants with *VTA1* or *VTA2* gene under the control of *GAL1* promoter. We found that some candidates could not adhere to the agar surface and also not flocculate when the expression of *VTA1* or *VTA2* gene was induced by galactose. This could be that the corresponding genes from these candidates coordinate with the *Vertcillium* genes to promote adhesion and flocculation in the non-adhesive yeasts. We probed these candidates in the deletion collection map and found that they belong to the THO complex comprising 4 subunits (HPR1, THO2, MFT1 and THP2). To verify this result, all four THO deletion mutants were picked from the yeast collection and transformed with the plasmid carrying *VTA1* or *VTA2*. Our data confirmed that all four THO subunits are required for adhesion and flocculation of the non-adhesive yeast regulated by these transcription regulators (Figure 11).

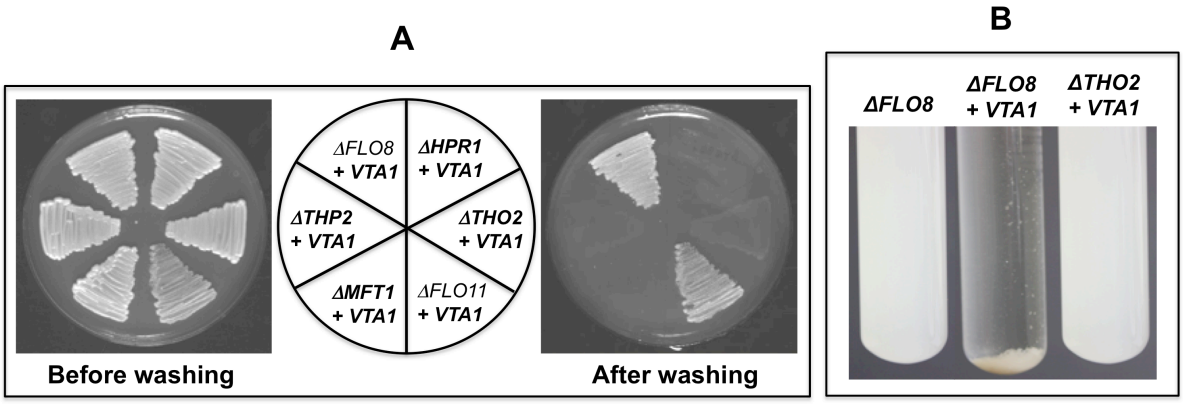


Figure 11. THO complex involved in yeast adhesion and flocculation induced by VTA1 transcription regulator. The VTA1 regulator was not able to activate adhesion **(A)** and flocculation **(B)** in the THO deletion mutants when compared with the deletion strains of *FLO8* and *FLO11*.

In yeast, THO complex is responsible for transcription elongation of the genes with internal repeats such as *FLO1* and *FLO11* and also involved in telomere maintenance (Voynov *et al*., 2006; Askree *et al.*, 2004). Therefore, we recruited these THO subunits including HPR1 (YDR138), THO2 (YNL139C), MFT1 (YML062C) and THP2 (YHR167W) from the *Saccharomyces* genome database (http://yeastgenome.org) and

analyzed them in the *Verticillium* genome database (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html). The results revealed that only THO2 subunit is conserved from yeast to *Verticillium*.

3.1.4. *VTA1* and *VTA2* induce the yeast FLO1 adhesin-mediated adhesion and flocculation

The S288C genetic background that we used mainly in this study does not express *FLO1* and *FLO11*, because the FLO8 regulator for these adhesin genes is defect by a non-sense in its open reading frame sequence. When this background is complemented with *FLO8* gene, both *FLO1* and *FLO11* adhesin genes are expressed (Liu *et al.*, 1996, Fichtner *et al.*, 2007). *FLO1* is responsible for main adhesion and flocculation, whereas *FLO11* is for the invasive growth, pseudohyphal formation and a weak adhesion (only a thin layer on the agar surface) (Verstrepen and Klis, 2006; Fichtner *et al.*, 2007). From the genetic screening assays with the robot system, we found that THO four-subunit complex is involved in adhesion activated by two *Verticillium* transcription factors. Furthermore, the THO complex controls expression of two adhesin genes *FLO1* and *FLO11* in yeast (Voynov *et al*., 2006). Therefore, we examined whether two *Verticillium* transcription regulators activated expression of the yeast adhesin genes. We transformed these *Verticillium* genes separately in three deletion strains including S288C*ΔFLO1*, *ΔFLO10* and *ΔFLO11*. We found that *FLO1* gene was involved in adhesion and flocculation and its expression was controlled by the *Verticillium* regulators (Figures 12A-B). The results showed that *FLO1* was only partially responsible for adhesion because a weak adhesion still appeared in the *FLO1* deletion strain (Figure 12A). However this gene was entirely accountable for flocculation of the transformants with *VTA1* and *VTA2* (Figure 12B). This suggests that *VTA1* and *VTA2* activate expression of *FLO1* adhesin gene for flocculation and for adhesion. These regulators might also induce expression of the other adhesin genes for the rest of adhesion (Figure 12A).

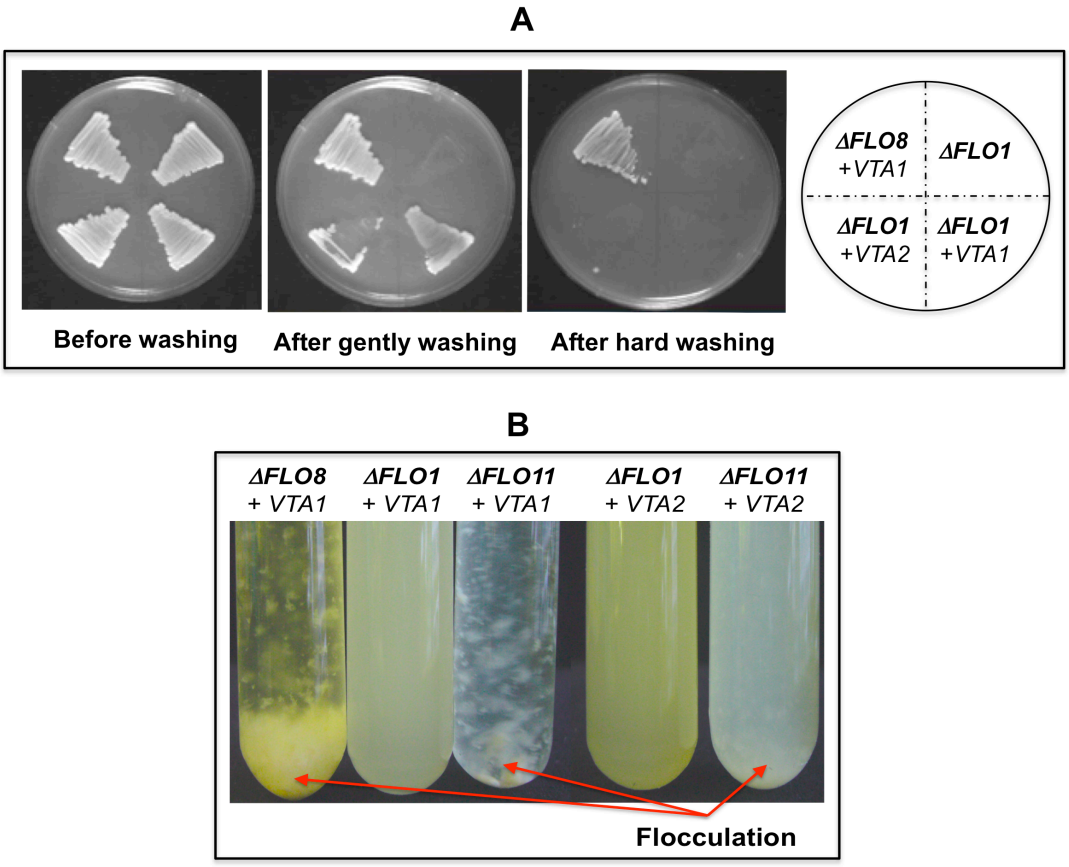


Figure 12. Adhesion and flocculation by the *Verticillium* regulators are *FLO1*-dependent. These regulators require *FLO1* adhesin gene for a partial adhesion to agar surface **(A)** and for a total flocculation in the liquid medium **(B)**. When *FLO1* gene is deleted, both *VTA1* and *VTA2* are not able to induce flocculation anymore, but still activate a weak adhesion to agar.

We additionally examined the expression of the *FLO10* and *FLO11* adhesin genes in the presence of *VTA1* and *VTA2*. Our data indicated that these adhesins were not involved in the yeast adhesion regulated by two the *Verticillium* transcription regulators (Figure 13). However we could not characterize whether these genes are involved in adhesion and flocculation regulated by the *Verticillium* genes or not, because the *FLO5* and *FLO9* deletion mutants were not available. Designing of gene-specific primers for *FLO1*, *FLO5* and *FLO9* was also not feasible due to the very high homology of these genes.

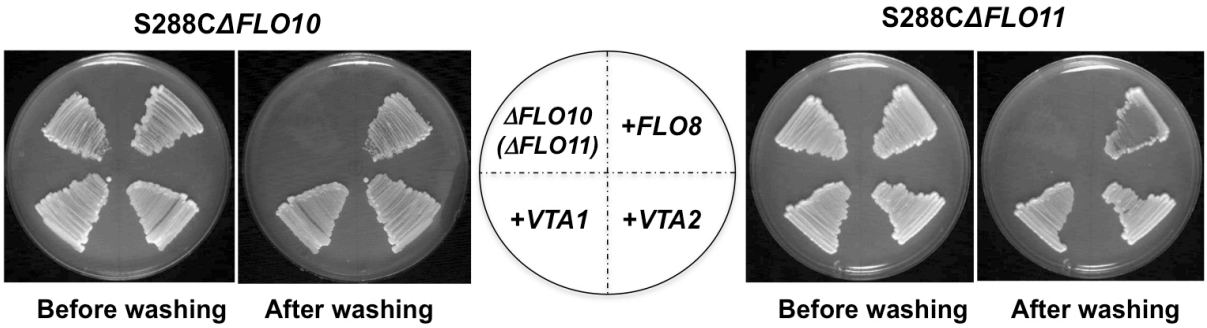


Figure 13. ***FLO10* and *FLO11* are not essential for adhesion and flocculation regulated by two *Verticillium* regulators**. The mutants of *FLO10* and *FLO11* expressing *VTA1* or *VTA2* gene resulted in a strong adhesion. Due to the defect of *FLO8* gene in S288C genetic background, this gene was used to recover its function in both *FLO10* and *FLO11* deletion strains.

3.1.5. VTA1 and VTA2 transcription factors are localized to the yeast nucleus

Both VTA1 and VTA2 carry specific conserved domains of zinc protein family. The *VTA1* gene encodes a deduced polypeptide of 424 amino acids with $Zn(II)_2Cys_6$ domain located at the N-terminal end, whereas the deduced polypeptide with 417 amino acids corresponding to *VTA2* sequence possesses C_2H_2 zinc domain at the C-terminal end. Because most of zinc cluster proteins are localized to the nucleus (MacPherson *et al.*, 2006), therefore we examined the structures of these two regulators in more details to find signals for localization, nuclear export and protein stability by using some bioinformatics tools (as described in Materials and Methods). We found that both contain nuclear localization signals (NLS), nuclear export signals (NES) and signals for protein degradation (PEST) (Figure 14). In the PEST regions, the amino acid sequences are rich in proline (P), glutamic acid (E), serine (S), and threonine (T). The presence of these regions can result in the rapid intracellular degradation of the corresponding proteins (Rogers *et al.*, 1986).

A

Zn(II)$_2$Cys$_6$ domain

MSSSSKTRQPRLRA**SCDGCFLAKVKCSKARPMCSRCLSCGLECNYSPSSR**AGKPKADHSSNSRLVHLQTPPTS

ISNTADENAAVLLRDIPVTHPLYKFDTDWDVTMDFADTFSHPYGRGPSNPCEANGLDTRDPGNVTSIYSNNLP

PEST

WTPPNDFASINYTDLSYTGTSLSGPQSRSVSMDDTTSQLNSWVDSVS**RDTQCFSTTAPALTPASMGSSYFPSP**

SSTPRNGPSAQRKVSSKHDGANFCTCFTVCLQSLQDMHSASSPDPPPFDVVLSLNRKAVESCAALLACAPCLS

RSGTHTTAMLLATIIGKITSFYQKATHSYFDGGIEDGIAGQGSMHGLSGTGASLGISLGAYTLGGEDGRWLEL

NES PEST NLS

EILA**REL**QKLEEVYAQF**RDVCGELTEDPEVSR**AMIGYLGHNLGTTLKVVSHR**KGGMKRA**

B

NES

MYLVPTQPHHFVGNHAPLLASSSPVSSPGSRHGNRPEATSSALLST**LTTL**NTSMERSAAEYSQSGLPSPYPSN

PEST

CGDTRSEGSSADHSSAAHYSSQQEV**RPSNYSTSATPTSEYSVYPPSAR**SGSFPEHIHRPYHPASNPSGGSGGM

AQQASNPSIAGPSPTYAYGQQSPYGPPPGDMQAYQHAYPQPRPDWTGYGQHSAGLTPATHHFPPTPSSAPPNG

C_2H_2 zinc domain

RPTQFGNQVYSFVPIPGAQQHKRPRRRYEEIERM**YKCGWQGCEKAYGTLNHLNAHVTMQSHGTKRT**PEEFKE**I**

NLS

RKEWKQRKKEEEAARKAEDEQRRAAAAAAAAQAAQNGGPDPQSGPDGGPPSGYGGGRLPPIGYSPSYPPNGPP

SAGVPQQQPLPEYNGTHMYQPANYQAQPPSPYGQPSQGMYSQHNGTQPGQGH

Figure 14. **The molecular structures of two *Verticillium* transcription regulators.** VTA1 protein consists of 424 amino acids **(A)** and VTA2 has 417 amino acids **(B)**. These proteins belong to the zinc finger family with the specific domains. Both possess the signals for nuclear localization (NLS), export (NES) and degradation (PEST).

To confirm these structures, we generated p246*GAL1-GFP* vector by cloning *GFP* gene into p246*GAL1* (Mumberg *et al.*, 1994). Consequently, the *VTA1* and *VTA2* transcripts without the stop codon were amplified and fused to the N-terminus of the *GFP* gene resulting in two fusion constructs, p246*GAL1-VTA1GFP* and p246*GAL1-VTA2GFP* (Figure 15A). All three constructs were used to transform the non-adhesive yeasts (S288C*ΔFLO8* and S288C*ΔFLO1*). Expression of *GFP* gene and the fused sequences (*VTA1GFP*, *VTA2GFP*) under the control of *GAL1* promoter was induced by galactose. Our data demonstrated that *VTA1* and *VTA2* encode the nuclear proteins that are localized to the yeast nucleus (Figure 15B). Moreover, these GFP-fused proteins could still activate flocculation and adhesion in the non-adhesive yeast *ΔFLO8* strain but not in the *ΔFLO1*

strain as shown in the Figure 15B with a group cells tightly adhering to each other and to the glass surface as well.

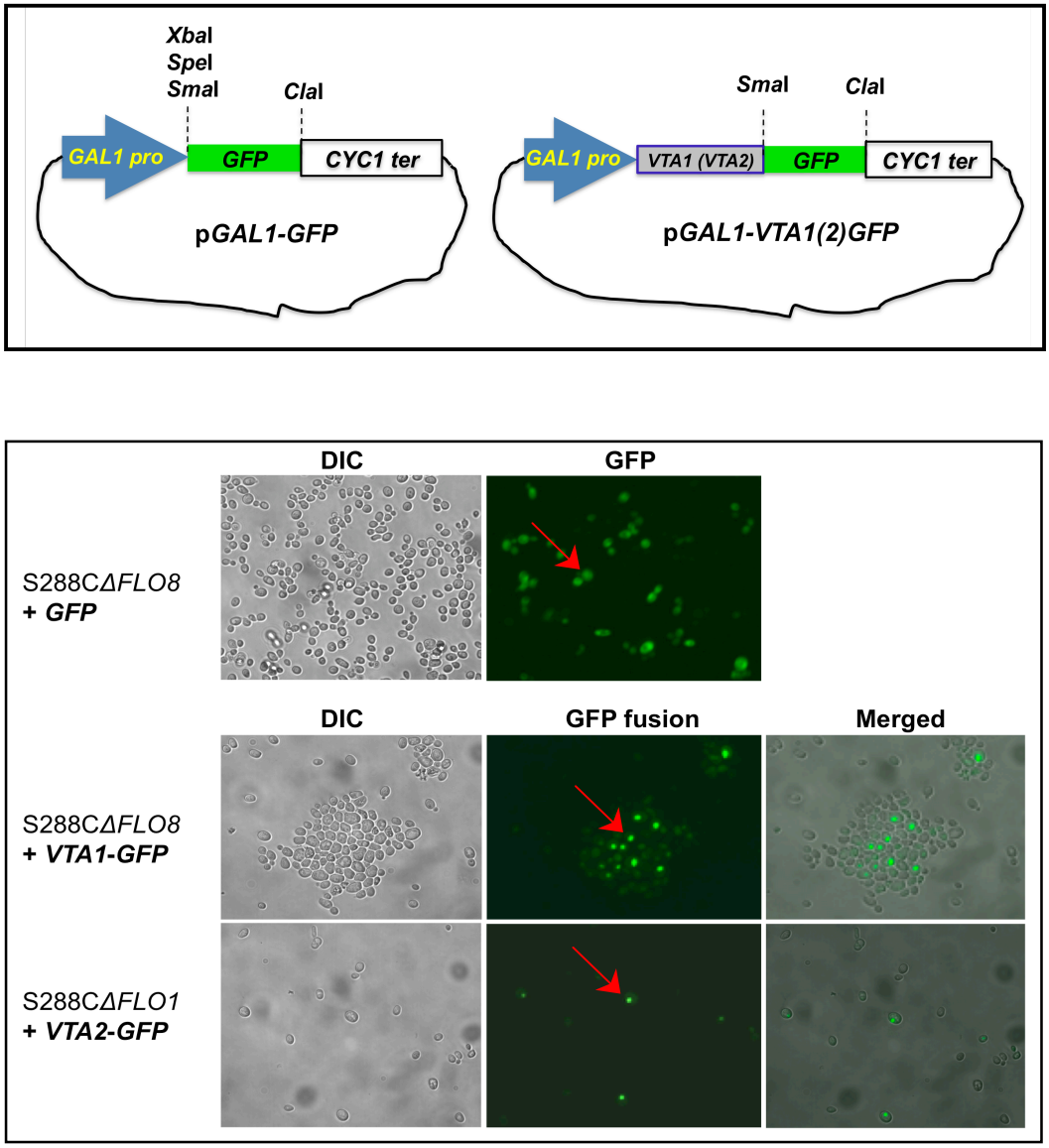


Figure 15. **Localization of the *Verticillium* transcription regulators, VTA1 and VTA2, in yeast. (A)** *The vectors of GFP fusion*. The coding sequences of *VTA1* and *VTA2* without the stop codons were amplified and fused to the N-terminal end of *GFP* gene. Expression of the hybrid sequences and of only *GFP* gene under the control of the yeast-specific *GAL1* promoter was induced by galactose. **(B)** *Localization of VTA1 and VTA2 in the non-adhesive yeast cells*. Both transcription regulators were localized to the yeast nucleus. The green fluorescent signal appeared only in some cells may be due to degradation of these proteins via the PEST motifs. The GFP fused proteins could still activate flocculation and adhesion in the non-adhesive yeasts but not in *FLO1* deletion mutant as indicated by red arrows.

3.1.6. Putative adhesins in *Verticillium* plant pathogens

From the yeast complementation assays for adhesion genes of *V. longisporum*, we did not identify any homologue of the yeast adhesins. Therefore we performed some comparative analyses using the adhesin genes from http//:yeastgenome.org to search in the *Verticillium* genome database from BROAD Institute and in the *V. longisporum* genome database from our project (unpublished). Interestingly, we found a homologue of FLO1 adhesin in all three species *V. dahliae* (BROAD accession number: VDAG_03514), *V. albo-atrum* (NCBI accession number: XP_003003925) and *V. longisporum* (contig09388). The FLO1 homologue in *Verticicillium* named VAP1 (*Verticillium* Adhesive Protein 1) is a quite big gene of 2.2 kb encoding a 740-amino acid polypeptide. This polypeptide possesses a secretion signal, a region of some repeats and an adhesin-specific PA14 domain at the C-terminal end, but lacks a GPI anchor (Figure 16A). The VAP1 also reveals a good homology to Mam3 adhesin of the fission yeast *Schizosaccharomyces pombe* (Linder and Gustafsson, 2008). This putative adhesin lacking a GPI anchor might be secreted into the external medium and subsequently attached the cell wall by non-covalent binding to chitin chains via its repeat region like the mechanism of the WI-1/BAD1 adhesin in the pathogenic fungus *Blastomyces dermatitidis* (Brandhorst and Klein, 2000).

In addition, we also characterized a homologue of EPA2 adhesin (VAP2) with the adhesin-specific PA14 domain (BROAD accession number: VDAG_09122). The EPA2 adhesin is responsible for adherence of *Candida glabrata* to host cells but not expressed in vitro (Peñas *et al*., 2003). The putative VAP2 adhesin gene in *Verticillium* encodes a polypeptide of 712 amino acids and possesses typical characteristics of an adhesin: a signal peptide for secretion, a conserved PA14 domain for lectin binding and a region containing repeats (Figure 16B).

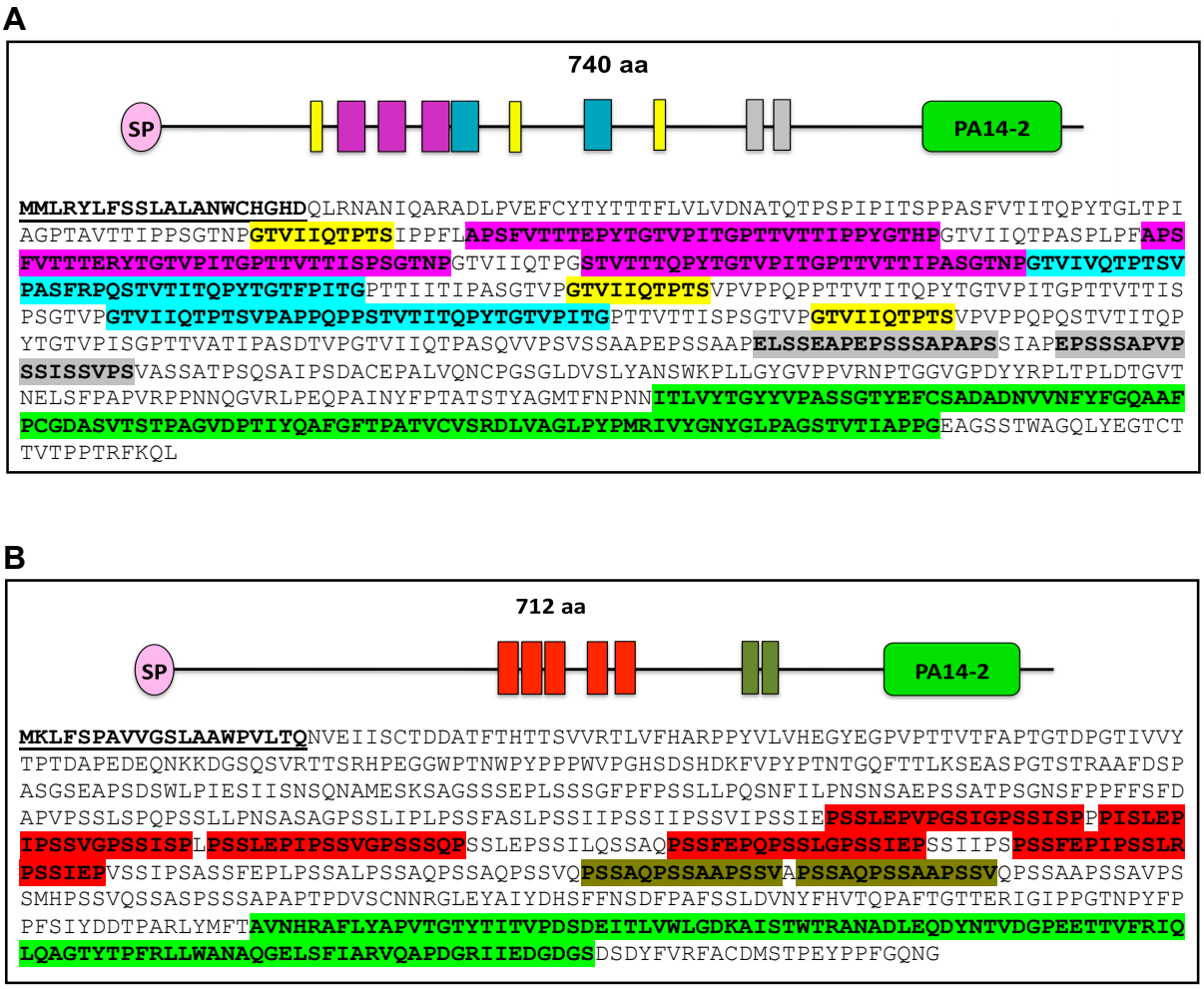


Figure 16. The structure of two putative adhesins in *Verticillium*. FLO1 homologue (VAP1) in *V. longisporum* **(A)**. EPA2 homologue (VAP2) in *V. dahliae* **(B)**. Both possess typical characteristics of an adhesin including a signal peptide (SP), a region of colored repeats and a carbohydrate-binding domain (PA14-2).

3.2. Development of high-throughput tools for *Verticillium* species

Presently, the genomes of *V. dahliae* and *V. albo-atrum* are publicly available at http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html. This is a big advantage for exploring pathogenic mechanisms at the molecular level of these *Verticillium* plant pathogens and the very closely related species *V. longisporum*. However, genetic tools efficient for *Verticillium* species are not available or too complicated to perform. Therefore in this study, we have developed high-throughput tools for *Verticillium* species including fluorescent tagging, RNA-mediated silencing and gene

disruption. The newly developed tools are not only used for *Verticillium*, but also for other filamentous fungi. These tools will allow characterizing quickly potential roles of putative adhesion genes from yeast complementation assays as well as putative *Verticillium* adhesin genes from the genome database analyses.

3.2.1. Fluorescent tagging of *Verticillium* species for assays of fungal adhesion to plant roots and for a new silencing system

3.2.1.1. Agrobacterium-mediated fungal transformation is simple and effective for gene transfer into genomes of Verticillium species

To facilitate genetic analysis of pathogenicity of *Verticillium* plant pathogens, two transformation methods including protoplast-mediated transformation and *Agrobacterium tumefaciens*-mediated transformation (ATMT) have been developed. Dobinson (1994) reported a DNA-mediated transformation system using protoplasts generated from *V. dahliae*. Transformation efficiency of this method was quite low, only between 3 and 5 hygromycin resistant transformants/μg vector DNA. The DNA was integrated into the *V. dahliae* genome with a single copy or multiple copies or even in tandem array (Dobinson, 1994). Therefore, this method is not used any longer for *Verticillium* transformation. Currently, the most preferred method for *Verticillium* species is ATMT. The ATMT method has been successfully used for gene disruption, RNA-mediated gene silencing and fluorescence tagging in *Verticillium* species (Dobinson *et al.*, 2004; Rauyaree *et al.*, 2005; Klimes *et al.*, 2006; Eynck *et al.*, 2007; Vallad and Subbarao, 2008; Singh *et al.,* 2010; Tzima *et al.*, 2010; Gao *et al.*, 2010; Knight *et al.*, 2010; Paz *et al.*, 2011).

In this work, we have improved the procedure for *Verticillium* transformation using *A. tumefaciens* as a DNA carrier. We used *A. tumefaciens* to transfer two fluorescent genes, *GFP* and *DsRed*, into two *Verticillium* pathogenic species (*V. dahliae* and *V. longisporum*). *A. tumefaciens* bacterial cells were treated with $MgCl_2$ and $CaCl_2$ to become competent (see details in Materials and Methods). The bacterial competent cells can be preserved in glycerol at -80^{o}C for 1 year without any problem for transformation efficency. The fluorescent gene-containing binary vectors were transformed separately into this bacterium by freezing the mixture (vector + bacterial cells) in liquid nitrogen, thawing it at 37^{o}C and recovering bacterial cells with antibiotic-free SOC medium. As a result, we could get about 100 positive colonies for 1 μg of each transforming vector. This method is

effective and much easier to perform than electroporation method that requires special equipments (Singh *et al.*, 2010).

To transfer the fluorescent gene (*GFP* or *DsRed*) from the binary vector in *Agrobacterium* to *Verticillium*, we mixed the fungal frozen spores with bacterial cells carrying the vector and spread the mixture on a filter paper of an induction medium plate containing acetosyringone (AS). During 72 hours of co-incubation at 25^{o}C, AS induces bacterial cells to deliver T-DNA (transfer DNA) fragment carrying the fluorescent gene from the binary vector to the fungal genome. Afterwards, the membrane was shifted to a selection plate for 10 days of additional incubation to favor the development of resistant transformants. On average, from about 1.5 million of the fungal frozen spores ($0.15x10^{7}$) as material for transformation we obtained 40-50 hygromycin resistant transformants per plate. This method is much more efficient than the protoplast-mediated transformation that was reported by Dobinson (1994). The quality of *Verticillium* frozen spores is still good enough for ATMT method after 12 months of preservation at -80^{o}C, therefore ATMT requires less time than the protoplast-mediated method for material preparation of transformation.

3.2.1.2. Expression of the fluorescent genes DsRed and GFP in Verticillium plant pathogens

More than a decade, green fluorescent protein has been used popularly to light up fungal biology for exploring mechanisms of infection and interaction between host plants and fungal pathogens (Lorang *et al.* 2001; Sesma and Osbourn, 2004; Andrie *et al.*, 2005; Eynck *et al.*, 2007; Vallad and Subbarao, 2008). In addition, the DsRed fluorescent protein is also a good reporter for ascomycetes fungi (Mikkelsen *at al.*, 2003; Janus *et al.*, 2007). However, it has not been yet used for investigations of plant-*Verticillium* interaction. Here, we report that DsRed protein can be used fruitfully for this aspect. It can be also used to replace GFP protein if the green fluorescent signal is interfered by backgrounds from plant hosts.

We found that expression of red fluorescent gene *DsRed* under the control of *gpdA* promoter from *Aspergillus nidulans* promotes the red pigment formation in *Verticillium* species on solid media after 2-3 weeks at 25^{o}C. However, the red pigment is only visualized clearly on agar plates (PDA medium) containing hygromycin. At concentration of 50-100 μg/ml, hygromycin suppresses the formation of melanized survival structures,

microsclerotia. This results in a reduction in black color of fungal mycelium and favours the clear appearance of the red color on agar plates (Figures 17A-C).

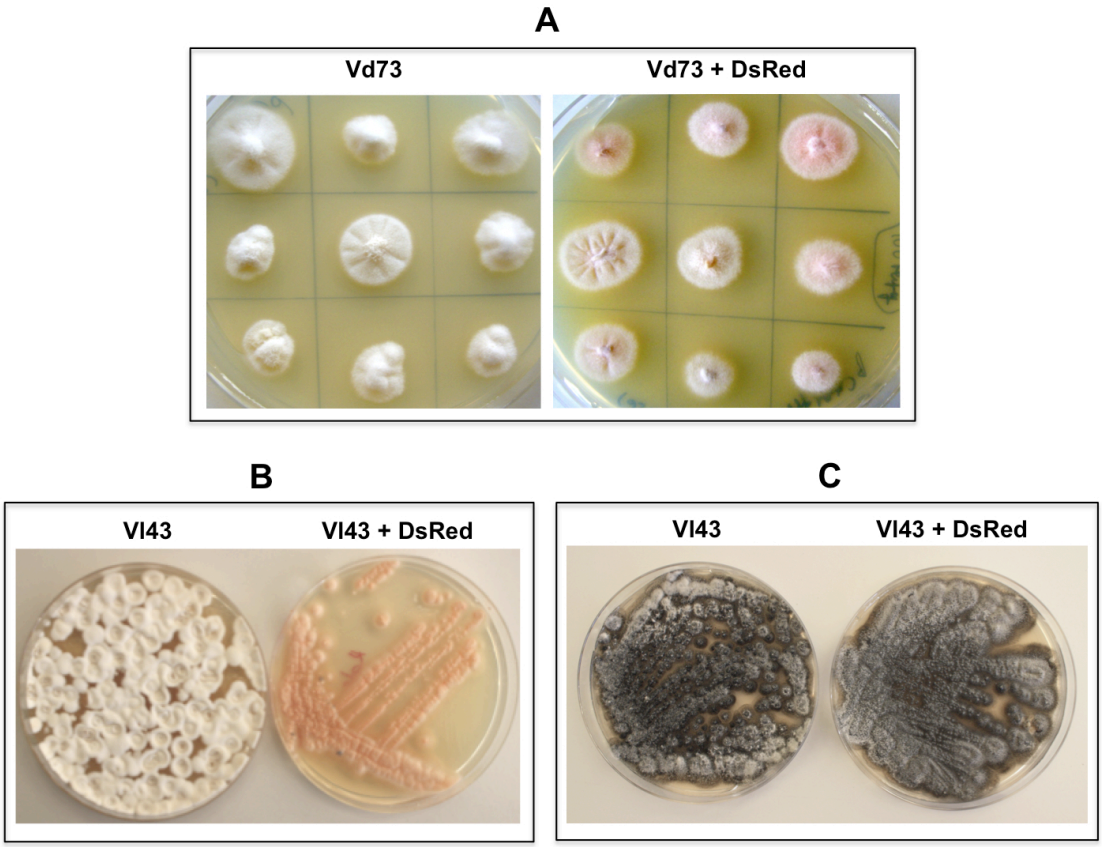


Figure 17. Expression of the *DsRed* fluorescent gene in *Verticillium*. (A) *V. dahliae* transformed with the empty vector pPK2-hph (left) and with the *DsRed* carrying vector pHQ1 (right). The transformants were grown on hygromycin-containing PDA plates for 2 weeks. **(B)** *V. longisporum* with empty vector pPK2-hph (left, white) and with *DsRed* carrying vector pHQ1 (right, red) after 4 weeks of growth on hygromycin-containing PDA medium. **(C)** Microsclerotia formation of *V. longisporum* transformed with empty vector pPK2-hph (left) and with *DsRed* carrying vector pHQ1 (right) after 4 weeks of growth on PDA medium lacking hygromycin.

Similarly, *GFP* fluorescent gene under the control of *ToxA* promoter from fungal plant pathogen *Pyrenophora tritici-repentis* (Sesma and Osbourn, 2004) was also expressed strongly and effectively in both *Verticillium* species. However, expression of the fluorescent genes are very various among the tagged transformants. Therefore we recruited Southern hybridization to examine if the T-DNA copy number decided the signal strength. The results showed that the fluorescent signal was not dependent of the DNA copy number integrated into the fungal genome (Figure 18).

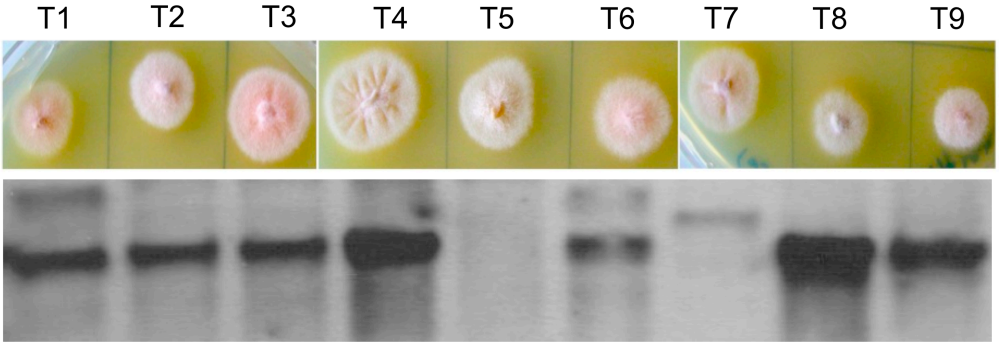


Figure 18. Correlation between the fluorescent signal and DNA copy number. Expression of *DsRed* fluorescent gene in *V. dahliae* strain Vd73 resulted in different accumulation of the red pigment in the fungal transformants. Southern hybridization with *DsRed* probe showed a single copy of T-DNA in most of transformants. The expression of the fluorescent genes seem to depend on position of T-DNA integration in the genome, but not on the DNA copy number.

3.2.1.3. Visualization of the early events of plant infection by Verticillium longisporum

Germination, attachment and plant surface colonization of *V. longisporum* are the initial steps for a successful infection on oilseed rape (Eynck *et al.*, 2007). We demonstrated that the early stages of plant infection by *V. longisporum* could be observed clearly using either the red fluorescent protein or green fluorescent protein (Figure 19).

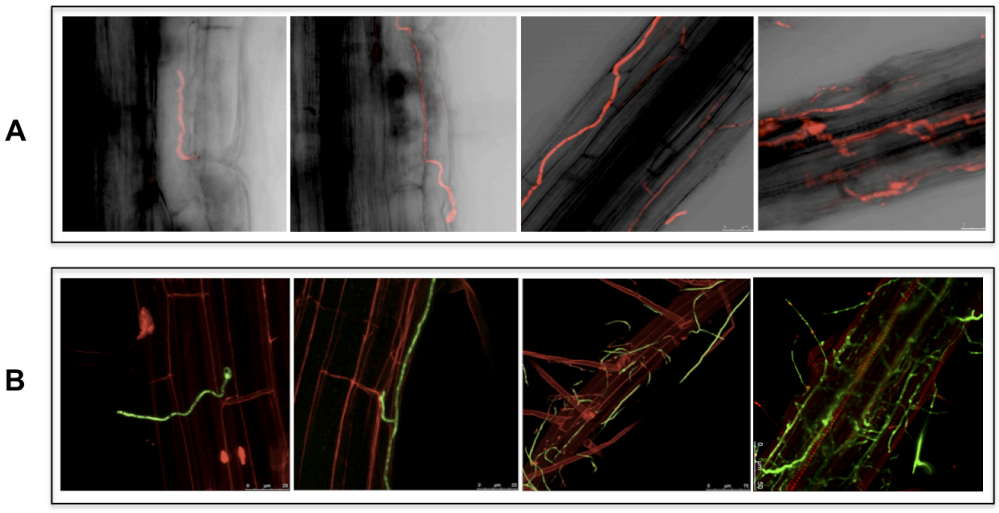


Figure 19. **The early infection stages of *Arabidopsis* through the roots by *V. longisporum***. The *DsRed* tagged *V. longisporum* **(A)** and *GFP* tagged *V. longisporum* **(B)** were used to infect *A. thaliana*. From left to right: fungal germination of the roots, growth and penetration into the xylem, colonization of root surface and fungal growth inside the xylem vessle (photos: Michael Reusche).

3.2.1.4. Dual-expression of GFP and DsRed in Verticillium species

Expression of two different fluorescent genes at the same time in *Verticillum* species using only one selection marker (hygromycin) would be helpful for testing new silencing (RNAi) systems. In these systems, one of the fluorescent genes can be co-silenced together with an endogenous gene, while the other is used as a marker to investigate the plant root infection ability of silenced mutants. We transformed the pHQ2 vector carrying *GFP* and *DsRed* genes into *V. dahliae* and *V. longisporum*. The resulting transformants resistant to hygromycin were selected for both the red and green fluorescent signals (Figure 20A). The stability of these fluorescent transformants were tested on the PDA medium lacking hygromycin for 5 successive generations using a single spore. Because silencing strategies are suitable for *V. longisporum*, a near diploid fungus, we performed some plant infection assays for a fluorescent version of this fungus (Vl43GR) using *Arabidopsis thaliana* and rapeseed *Brassica napus* (Figure 20B-C).

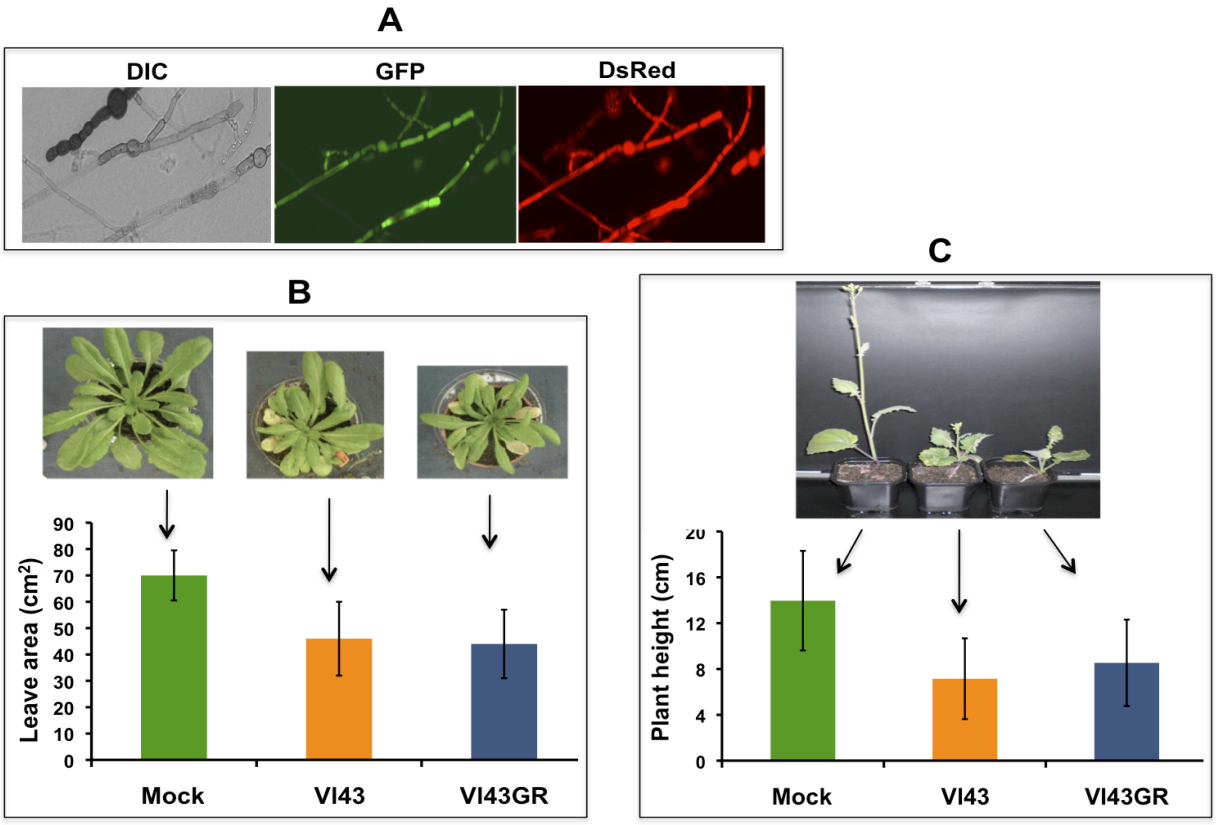


Figure 20. The dual-fluorescent tagging for *V. longisporum* and plant infection assays. **(A)** Co-expression of *GFP* and *DsRed* in *V. longisporum* Vl-43 resulting in the stable fluorescent version of this strain (Vl43GR). **(B)** The symptoms and the disease scores on *Arabidopsis thaliana* infected by *V. longisporum*. **(C)** The symptoms and the disease scores on rapeseed *Brassica napus*.

The data indicated that the fluorescent version of *V. longisporum* (Vl43GR) could infect both plants with the same disease scores like the original strain Vl43. Therefore, this fluorescent strain could be used for further investigations instead of the original one. In addition, we also employed a stable dual fluorescent transformant of *V. dahliae* Vd73 (Vd73GR) to test efficiency of the new silencing system in parallel with the fluorescent *V. longisporum* strain (Vl43GR).

3.2.2. New high-throughput silencing vectors for *Verticillium* plant pathogens

In this work, we have constructed successfully two new generation silencing vectors (Figure 21) for studying gene function in *Verticillium* species, especially for *V. longisporum*. These vectors contain a gateway cassette in both directions, sense and antisense, separated by a short intron of cutinase gene from the rice blast fungus *Magnaporthe grisea*.

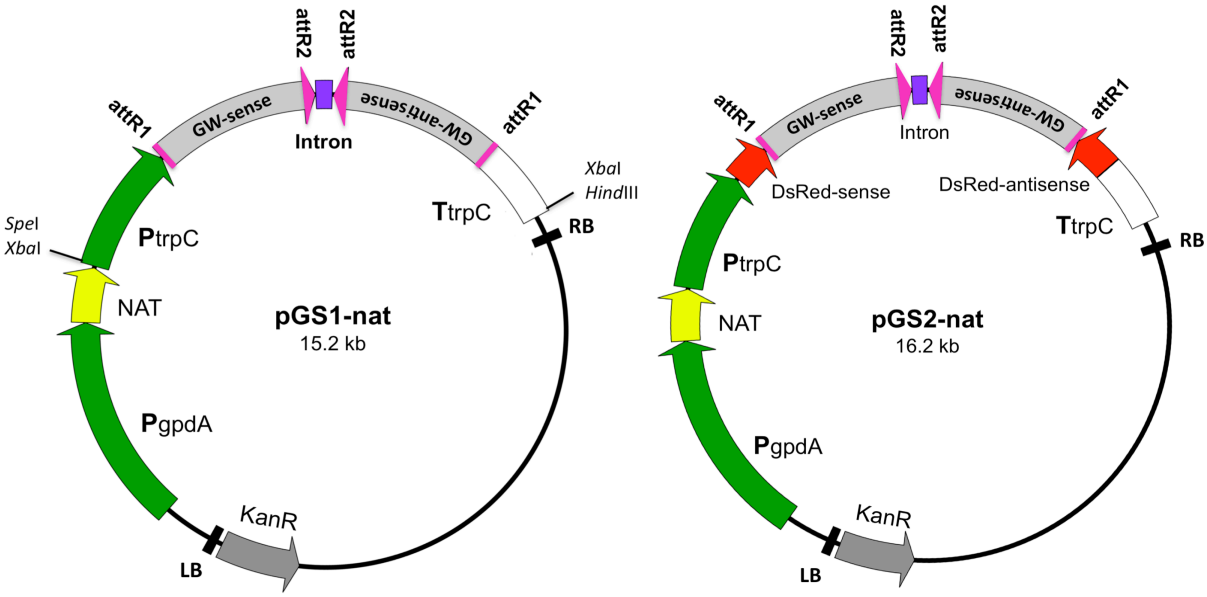


Figure 21. The structure of two new silencing vectors, pGS1-nat and pGS2-nat. Both vectors are integrated with the gateway cassette in sense and antisense direction for generating hairpin RNA silencing constructs. These vectors are almost the same except the *DsRed* silencing cassette integrated in pGS2-nat vector for co-silencing purposes. The same gateway cassettes in both directions contains two special recombination sequences (*att*R1 and *att*R2). They will be replaced automatically with a target gene fragment via two reactions using BP clonase and LR clonase.

The presence of the gateway cassettes in these vectors make the construction of silencing cassettes for target genes become much easier and faster than the conventional silencing strategy (Nakayashiki *et al.*, 2005; Janus *et al.*, 2007) that requires many restriction sites for cloning as well as it is time-consuming, especially when a binary vector is used for fungal transformation via *Agrobacterium* (Singh *et al.*, 2010). The new gateway silencing system uses only two enzymes (BP clonase and LR clonase) for all cases instead of many different restriction enzymes for conventional cloning. The gateway cassette harbors a *ccdB* toxin gene that its product, ccdB toxin, kills normal *E. coli* strains such as DH5α. If the recombination reaction (BP reaction or LR reaction) occurs correctly, this toxic gene will be replaced with a target gene fragment resulting in a non-toxic recombinant vector for the *E. coli* DH5α cells. Therefore screening to find a correct construct is quite simple. In this study, we used the *DsRed* gene as an indicator and the *VTA2* gene as an endogenous gene of the fungus to prove silencing efficiency of these vectors for *Vertcillium* species. We amplified a 430-bp fragment of *DsRed* and a 550-bp fragment of *VTA2* gene with gateway recombination sequences at the ends as *att*B1-430/550 bp-*att*B2. These PCR products were integrated into the gateway silencing vectors (the *DsRed* fragment into pGS1-nat for *DsRed* silencing and the *VTA2* fragment into pGS2-nat for co-silencing of *VTA2* gene and *DsRed*) to generate pGS1-Redi and pGS2-VTA2i respectively through two steps using BP clonase and LR clonase. Briefly, the *att*B sequences of a PCR product (*DsRed* or *VTA2*) were combined with the *att*P sequences of a mediated vector pDONR/Zeo by BP clonase to generate an entry vector containing the target gene fragment with the *att*L recombination sequences. This entry vector was used to provide the target gene fragment for the gateway silencing vector. In presence of LR clonase, the *att*L sequences of the entry vector were combined with the *att*R sequences of the gateway silencing vector to produce a corresponding gene silencing construct for downregulation of the target gene (Figure 22). The pGS1-Redi construct for silencing of *DsRed* and the pGS2-VTA2i for *VTA2* silencing were screened from *E. coli* transformants with colony PCR using the corresponding primer pair *DsRed*-gwF/*DsRed*-gwR and *VTA2*-gwF/*VTA2*-gwR. Consequently, these constructs were verified by enzymatic digestion before being partially sequenced using GWsense-R for the sense fragment and GWanti-F for the anti-sense fragment. These primers bind to the short intron in the silencing vectors.

We found that the correct recombination efficiency was very high for both steps (99% for BP clonase and 70-80% for LR clonase) (Figure 22).

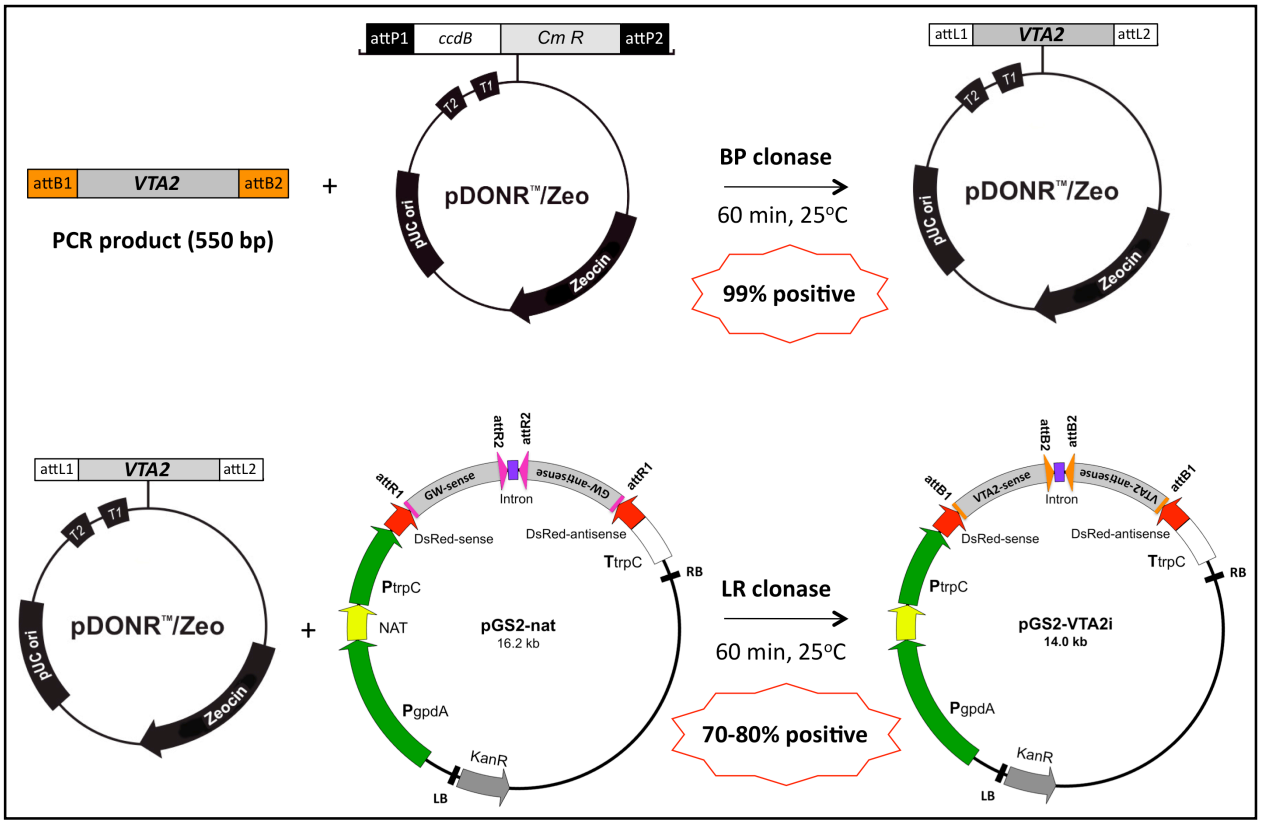


Figure 22. Two-step procedure to create the hairpin RNA-mediated silencing construct for *VTA2* gene. A fragment of 550 bp was amplified from the *VTA2* transcripts. The PCR product with *att*B recombination sequences was transferred to the donor vector (pDONR/Zeo) by BP clonase via a reaction between *att*B sequences and *att*P sequences of this vector. The reaction results in pDONR-VTA2 vector (entry vector) with *att*L recombination sequences. In presence of LR clonase, the entry vector exchanges the *VTA2* fragment with the gateway cassettes in pGS2-nat via a reaction between *att*L sequences and *att*R sequences. This reaction creates the silencing construct for *VTA2* gene (pGS2-VTA2i).

To verify correct silencing constructs using restriction enzymes, we developed a quick strategy in which a silencing construct is digested with a single enzyme that cuts the target gene sequence at a unique site. This means that the enzyme cuts both the sense sequence and antisense sequence at one site only. As a result, one corresponding fragment with a certain size is released. Therefore, the correct construct is confirmed. A single enzyme that cuts both the target sequence and the vector can be also used if corresponding

fragments are distinct from each other in size. For example, to confirm the silencing constructs for *VTA2* gene, we used *Bam*HI restriction enzyme. This enzyme cuts the *VTA2* sequence at one site and the vector sequence at two single sites resulting in three fragments with different sizes and the rest part of vector backbone on agarose gel (Figure 23). The sizes of these fragments were identical to the sequence-based sizes that we calculated. In addition, we sequenced partially the *VTA2* silencing construct and found that the enzymatic digestion pattern for this construct was completely correct.

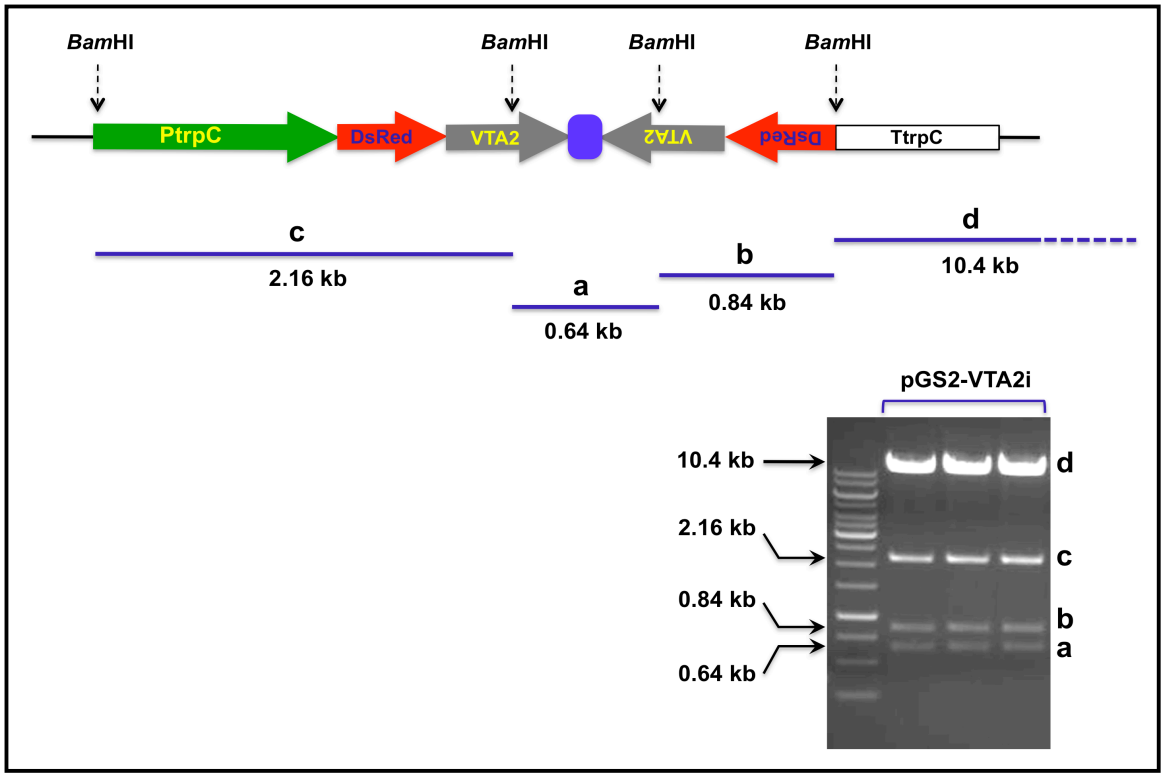


Figure 23. Confirmation of the correct silencing constructs for *VTA2* gene by enzymatic digestion. The silencing construct for *VTA2* gene (pGS2-VTA2i) was verified by digestion with *Bam*HI enzym. On the gel, four DNA fragments (a, b, c, d) were corresponding to the sizes (0.64 kb, 0.84 kb, 2.16 kb and 10.4 kb) from calculation based on the sequence of pGS2-VTA2i vector.

We transformed the pGS1-Redi carrying the *DsRed* silencing cassette and pGS2-VTA2i carrying both silencing cassettes for *DsRed* and *VTA2* into the green and red fluorescence-expressing *V. dahliae* strain (Vd73GR) and *V. longisporum* (Vl43GR). In this silencing approach, the expression of *GFP* was used as the reference signal that was constant in the silenced mutants when compared with reduction of the *DsRed* signal caused by DsRed silencing. We found that pGS1-Redi and pGS2-VTA2i could downregulate

expression of *DsRed* gene in both *Verticillium* species. However, the silencing of *DsRed* in *V. dahliae* was much stronger than that in *V. longisporum*. In *V. dahliae* the *DsRed* silencing could be classified into four levels (Figures 24, 25) including: about 20% transformants of level I (no color for both mycelium and conidia), 40% of level II (a very weak red color for mycelium and conidia reduced in red), 30% of level III (mycelium reduced in red color with very red conidia) and 10% of level IV (wild type-like red mycelium and conidia). In contrast, the silencing of *DsRed* in *V. longisporum* was much weaker with three levels: only about 6% of level II, 54% of III and 40% of IV.

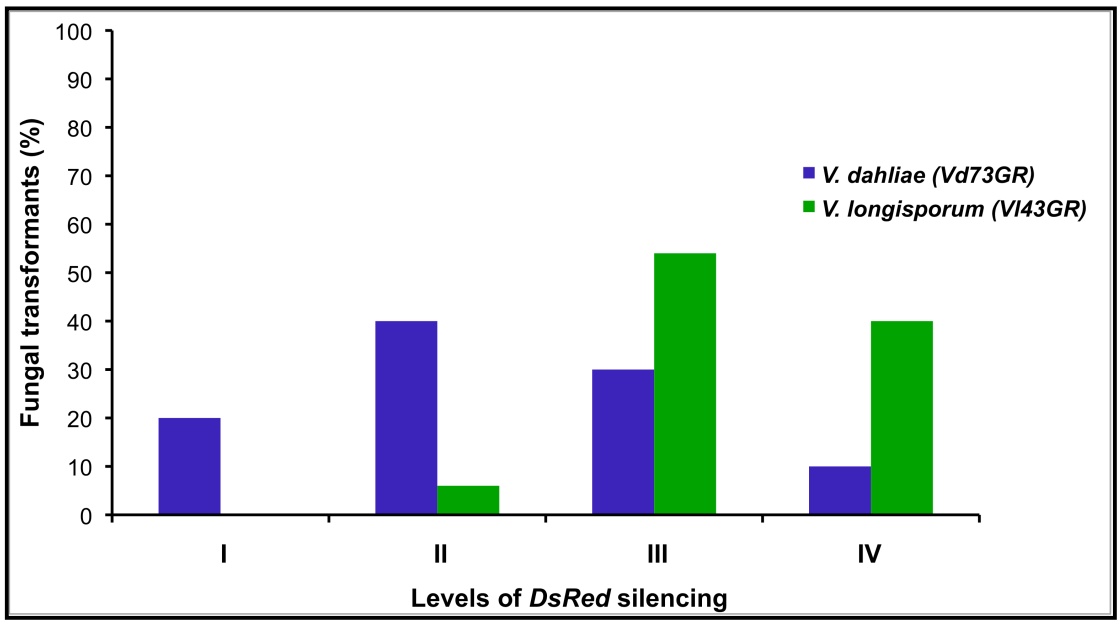


Figure 24. Gene silencing in *V. dahliae* and *V. longisporum* based on *DsRed* reporter gene. Both *GFP* and *DsRed*-expressing species (Vd73GR and Vl43GR) were transformed with the *DsRed* silencing construct (pGS1-Redi). The *DsRed* silenced mutants could be divided into 4 levels for *V. dahliae* and 3 levels for *V. longisporum* on basis of the DsRed signal. *DsRed* expression was suppressed completely in *V. dahliae* with about 20% of total transformants (level I), but not in *V. longisporum*.

We examined co-silencing efficiency of *VTA2* gene in the *DsRed* silenced mutants by using quantitative real-time PCR. The results showed that *VTA2* was silenced up to 80% in the *DsRed*-silenced mutants of the level I and less in the level II (50%) and III (20%) (Figure 25). This confirmed that *VTA2* and *DsRed* were silenced together. The reduction of

DsRed expression in co-silenced mutants seems to be the signal for downregulation of *VTA2*.

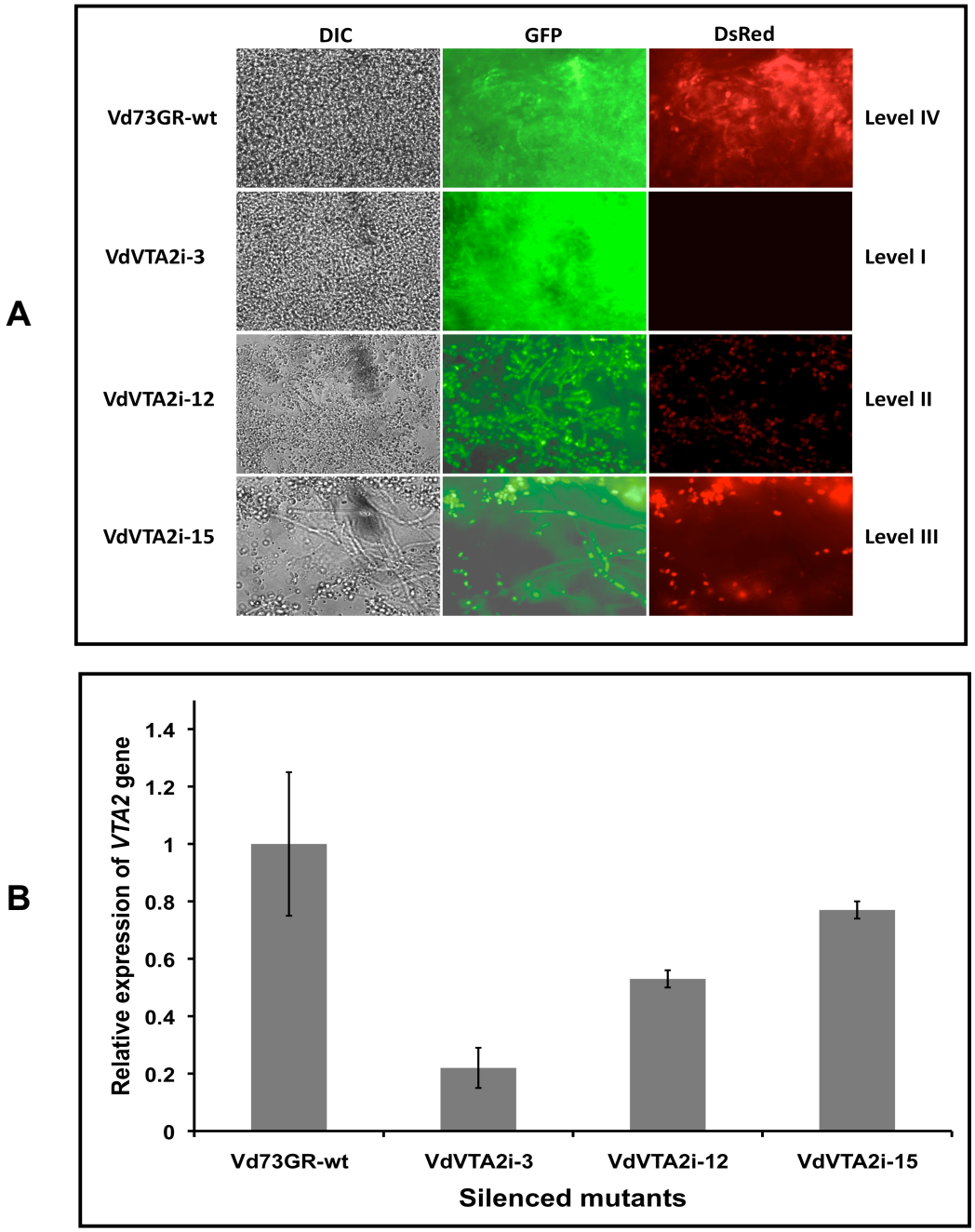


Figure 25. Co-silencing of *DsRed* and *VTA2* in *V. dahliae*. (A) Downregulation of *DsRed* by the co-silencing construct pGS2-VTA2i with four different levels (I - IV) representing no DsRed signal to wild type DsRed signal. **(B)** The correlation between the red fluorescent signal and expression of *VTA2* gene revealed co-silencing levels. Reduction of DsRed signal revealed silencing efficiency for *VTA2* gene: 80% for level I (VdVTA2i-3 silenced mutant), 50% for level II (VdVTA2i-12) and 20% for level III (VdVTA2i-15).

We focussed on the co-silencing efficiency of *DsRed* and *VTA2* in *V. longisporum*. The signal of *DsRed* reporter gene indicated that *DsRed* silencing in *V. longisporum* is weaker than in *V. dahliae.* Only about 6% of total transformants has a significant reduction in the red fluorescent signal. We checked expression of the endogenous gene *VTA2* in three silenced mutants with less DsRed signal (Figure 26A). The results showed that expression of *VTA2* gene in *V. longisporum* was silenced up to 80-90% (Figure 26B).

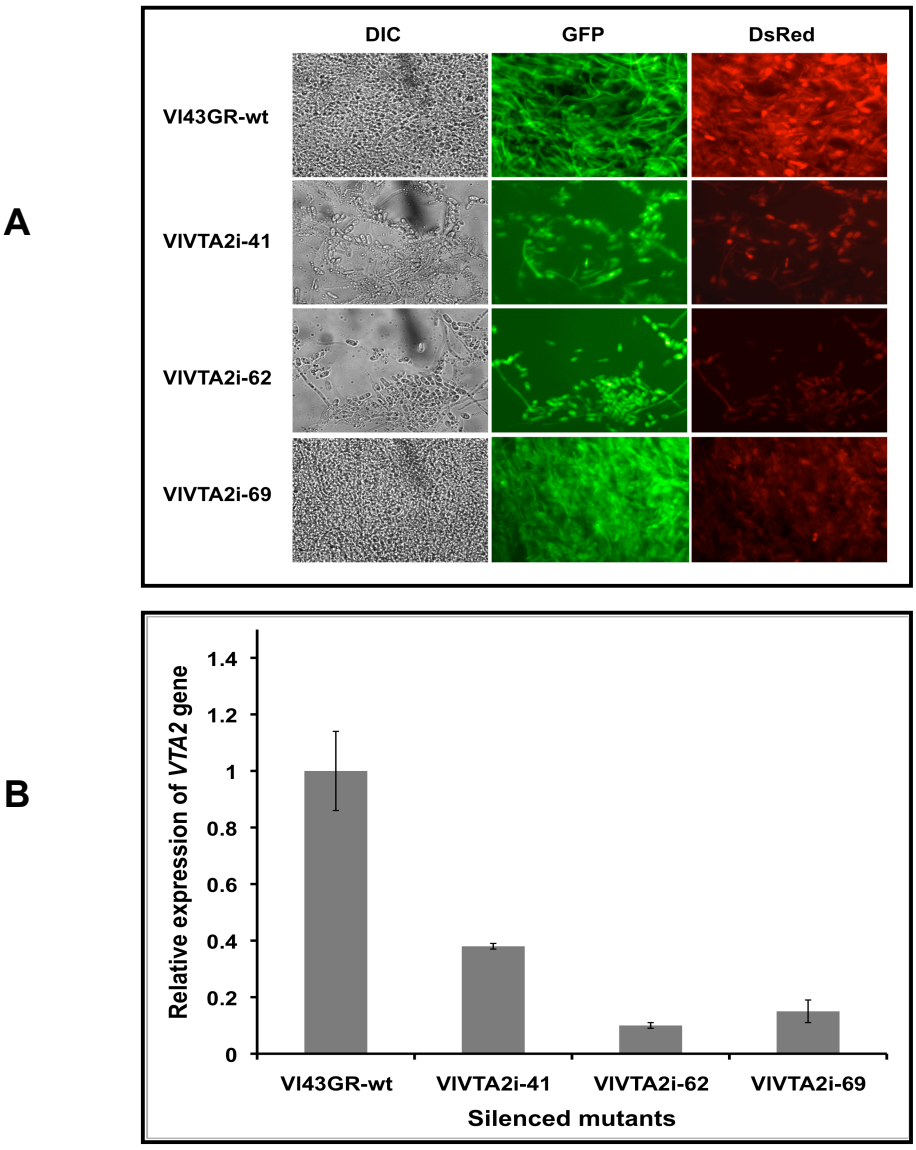


Figure 26. Co-silencing of *DsRed* and *VTA2* in *V. longisporum*. (A) Reduction of the DsRed signal by the co-silencing construct pGS2-VTA2i with three levels (II - IV) exhibitting less red color in fungal mycelium and conidia to wild type-like DsRed signal. **(B)** Reduction of DsRed signal revealed silencing efficiency for *VTA2* gene: 62% for VlVTA2i-41 silenced mutant, 90% for VlVTA2i-62 and 85% for VlVTA2i-69.

3.2.3. Development of new vectors for gene disruption in *Verticillium* species

Silencing strategy is perhaps the best option for studying genes of interest in fungal species that their genomes are not yet sequenced or for investigating lethal genes. However silencing suppresses maximally about 80-90% of gene expression in fungi (Nakayashiki *et al.*, 2005; Janus *et al.*, 2007; Singh *et al.*, 2010; this study). The rest activity of the gene (10-20%) might be still enough for its function in the fungus. In order to understand completely function of the gene in the fungus, the gene must be destroyed or removed from the fungal genome. Because up to now, the *Agrobacterium tumefaciens*-mediated transformation method is the most effective for gene transfer into *Verticillium* species, strategies of gene disruption in *Verticillium* require a binary vector as a deletion cassette carrier. Gene disruption in *V. dahliae* was performed successfully by inserting a resistant cassette into open reading frames of genes using transposons (Dobinson *et al.*, 2004; Rauyaree *et al.*, 2005; Klimes *et al.*, 2006) or by multi-step cloning using different plasmids (Tzima *et al.*, 2010). Both of these approaches are complicated and time-consuming. Therefore, we developed two binary vectors for gene disruption (pKO1 and pKO2) that can be used directly for making deletion constructs as well as for *Verticillium* transformation via *Agrobacterium*. These binary vectors possess either hygromycin or nourseothricin resistance gene as selection markers (Figure 27). Therefore they can be used to perform a single or double knockout in haploid species *V. dahliae* and *V. albo-atrum*, or even deleting both copies for each gene in the near diploid *V. longisporum*. As reported in the previous sections, *VTA2* gene could promote adhesion and flocculation in yeast by inducing expression of *FLO1* adhesin gene. In the rice blast fungus *Magnaporthe grisea*, the homologue of this gene (*CON7*) is required for fungal pathogenicity and growth *in planta*. The disruption of *CON7* gene caused changes in morphology of fungal spores (Odenbach *et al.*, 2007). In *Verticillium*, silencing of *VTA2* gene in *V. dahliae* Vd73 resulted in a slow growth phenotype, but no other changes could be observed when compared with the wild type phenotype. Moreover, it was not clear whether the delayed growth of the silenced mutants represented the final phenotype or only the intermediate one caused by the incomplete suppression of *VTA2* gene. Therefore we used these new knockout vectors to construct the cassettes for disruption of *VTA2* gene in *V. dahliae*.

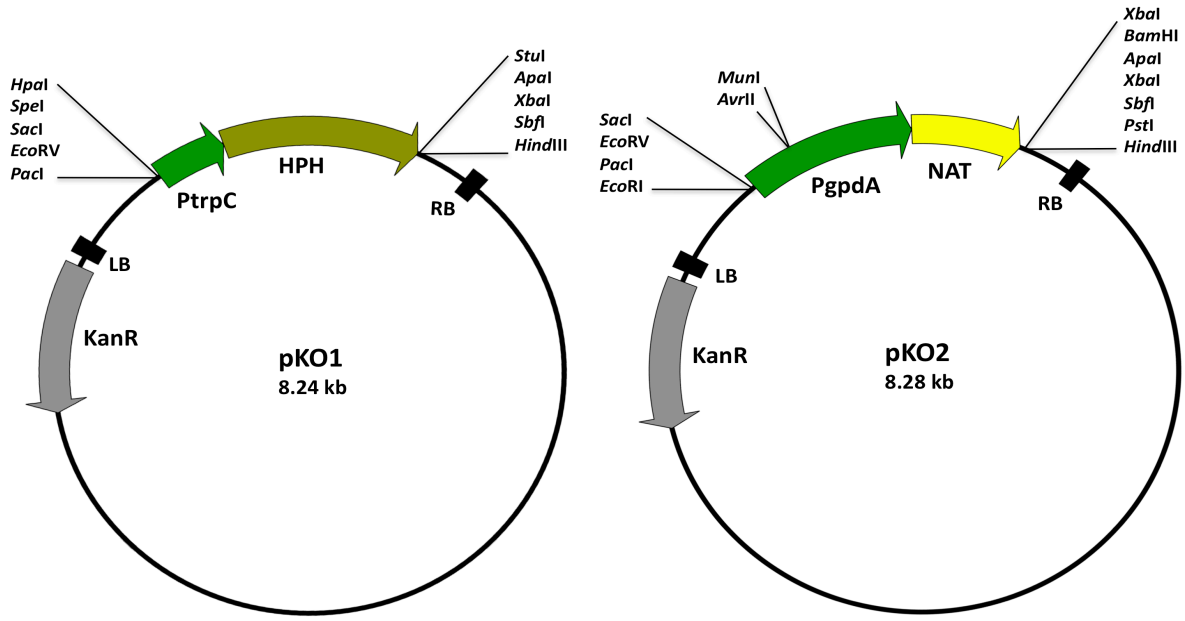


Figure 27. The structure of two new binary vectors for gene knockout. The pKO1 vector contains the hygromycin resistance cassette, whereas pKO2 carries the nourseothricin resistance cassette. Both possess the multiple cloning sites before and after the resistance cassettes that allow generating easily constructs for disrupting genes of interest in *Verticicillium*.

A big advantage for construction of the *VTA2* deletion cassettes was that the open reading frame of this gene contains two enzymes *Sac*I and *Apa*I that were also used to isolate hygromycin and nourseothricin resistance cassette from pKO1 and from pKO2 respectively. This allowed us to exchange easily the resistance cassettes with each other. We amplified a 3.1-kb fragment of *VTA2* gene using the primer pair P1/P4 (Figure 29) and cloned it into pKO1 at *Eco*RV and *Xba*I sites to generate p*VTA2* vector without any resistance cassette. A 670-bp fragment of the *VTA2* open reading frame from p*VTA2* was removed and replaced with hygromycin or nourseothricin resistance cassette using *Sac*I and *Apa*I. As a result, two deletion constructs pKO1*VTA2* and pKO2*VTA2* for *VTA2* disruption were generated. The *VTA2* deletion cassette contains 1.7 kb of 5'-region of the gene + hygromycin (nourseothricin) resistance cassette of 1.42 kb (1.45 kb) + 0.8 kb of 3'-region of the gene.

We transformed these deletion constructs of *VTA2* into *V. dahliae* strain Vd73 from linseed. Consequently, 100 transformants were selected randomly for each construct. On PDA plates, we found two different phenotypes for the transformants resulted from both

deletion constructs. The first group (24%) displayed the wild type-like phenotype, whereas the phenotype of the second group (76%) was distinct from the wild type strain. The surface of fungal mycelium of the second group as indicated by red markers was smooth and brittle during 1-2 weeks of growth and after 3 weeks it became strongly melanized (Figures 28A). Because *VTA2* gene is conserved fully among *V. dahliae* isolates (see Section 3.4), we transformed pKO2*VTA2* construct into another *V. dahliae* strain, Vd52 from pepper, to examine whether this deletion construct was able to disrupt *VTA2* gene in this strain or not. The results showed that the transformants also exhibited two distinct phenotypes identical to two groups from Vd73 strain (Figure 28B) with 55% of the first group and 45% of the second one. This proved that the same deletion construct could be used to disrupt *VTA2* gene in the different *V. dahliae* strains.

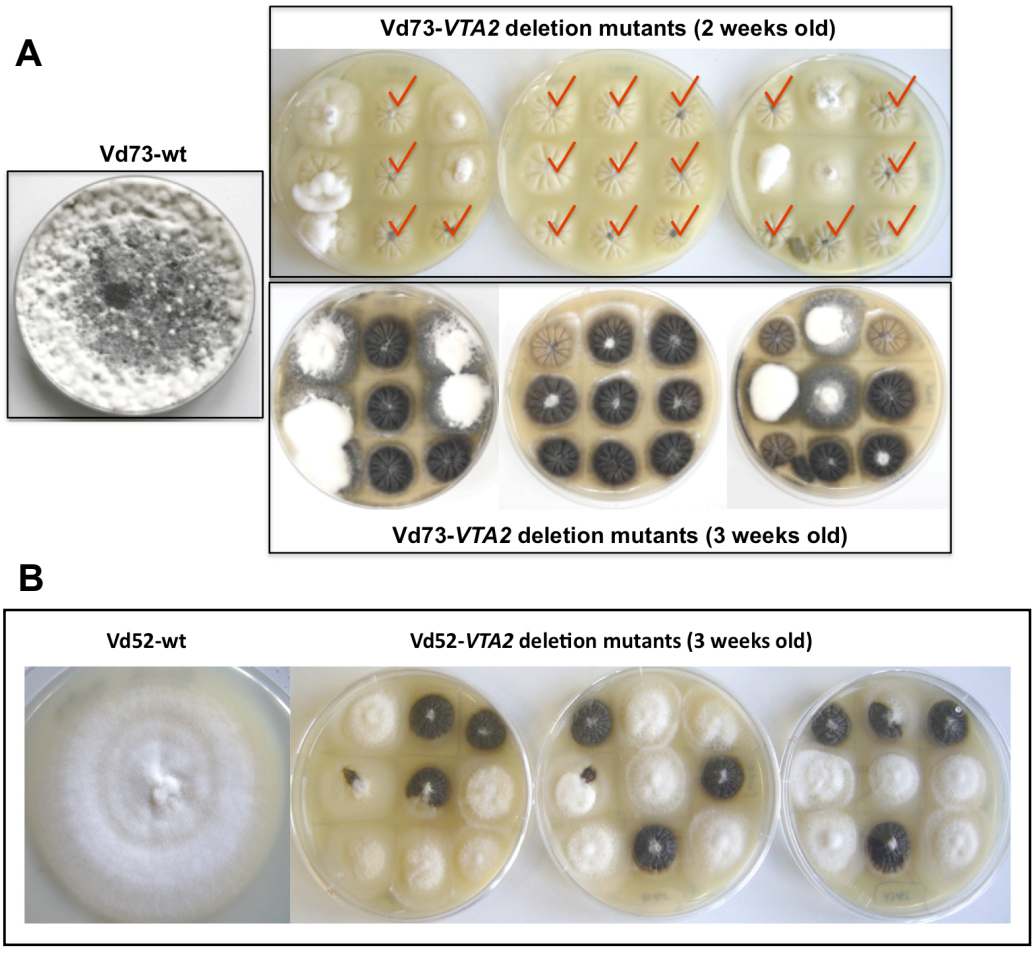


Figure 28. Disruption of *VTA2* gene in *Verticillium dahliae*. Deletion of *VTA2* gene in Vd73 strain from linseed **(A)** and in Vd52 strain from pepper **(B)** resulted in two different phenotypes, one was identical to the wild type, the other (marked in red) was completely distinct from the wild type. The second one was delayed in growth and quickly melanized by early microsclerotial formation.

We used PCR and Southern hybridization to examine the structure of *VTA2* gene for 4 deletion mutants (M1, M2, M3, M26) of the second group with the distinct phenotype derived from Vd73 strain. The results from PCR showed that the primer pair P3/P4 amplified a 1.5-kb fragment of *VTA2* gene from the genome of the wild type strain and a 2.5-kb fragment including the resistance cassette from the genome of the mutants (Figures 29A-B). From Southern blot, the 550-bp fragment of *VTA2* gene as probe detected only one band for *VTA2* gene in both wild type and mutants. The Southern band from the deletion mutants was 1 kb bigger than the band from the wild type strain explaining the successful exchange between the deletion cassette and locus of *VTA2* gene (Figure 29C).

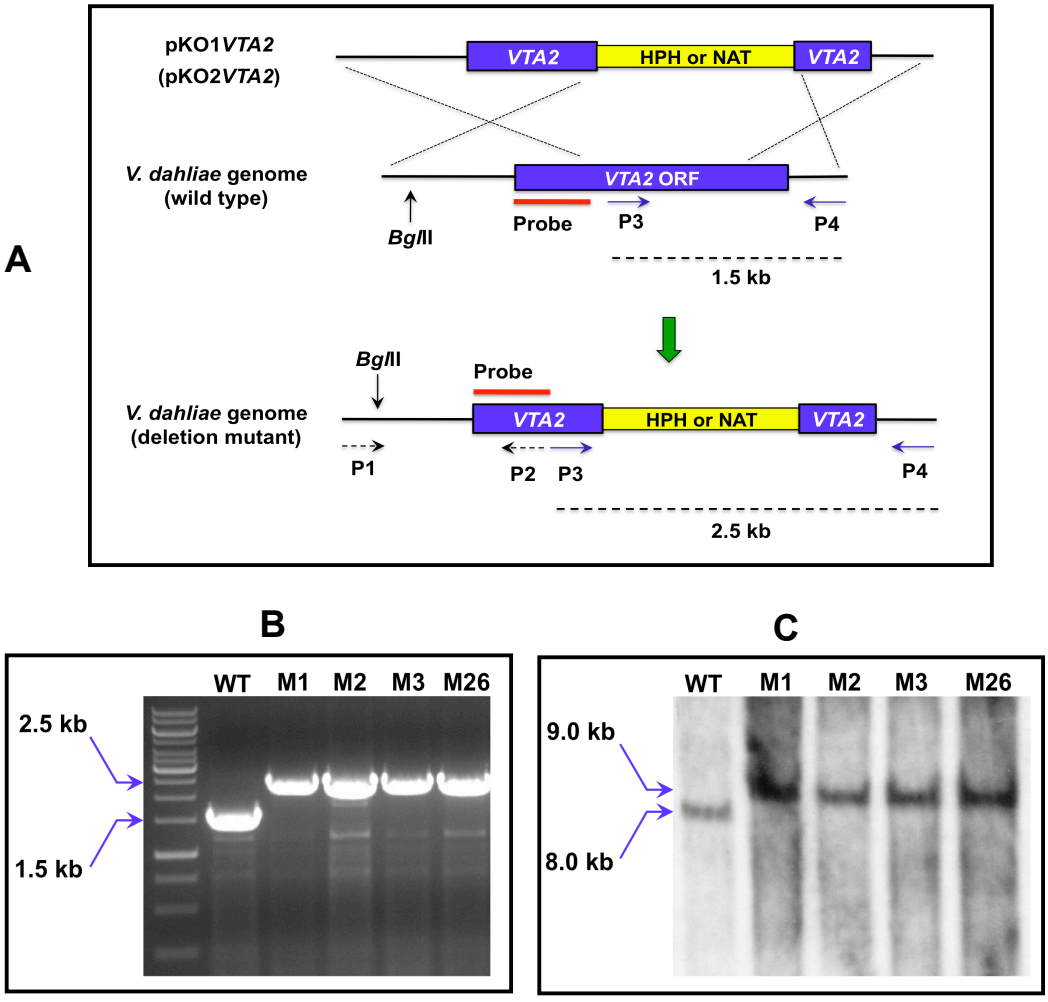


Figure 29. Strategies for deletion of *VTA2* gene in *V. dahliae* and confirmation of the corresponding deletion mutants. (A) Model for exchanging between the deletion construct and locus of *VTA2* gene in *V. dahliae*. **(B)** Screening *VTA2* deletion mutants using PCR with the primer pair P3/P4. **(C)** Confirmation of the *VTA2* deletion mutants by Southern using *Bgl*II for genome digestion. The same band (9 kb) in the mutants is 1 kb bigger than the one in the wild type Vd73.

In principle, if a gene is destroyed, fungal mutants will not be able to produce corresponding transcripts of this gene. Therefore, we measured expression of *VTA2* gene in three mutants (M1, M3, M26) comparing with the wild type Vd73 and one transformant possessing the wild type-like phenotype (ectopic transformant). The results indicated that production of *VTA2* transcripts was defect in all three mutants in contrast to the wild type strain and the ectopic transformant (Figure 30).

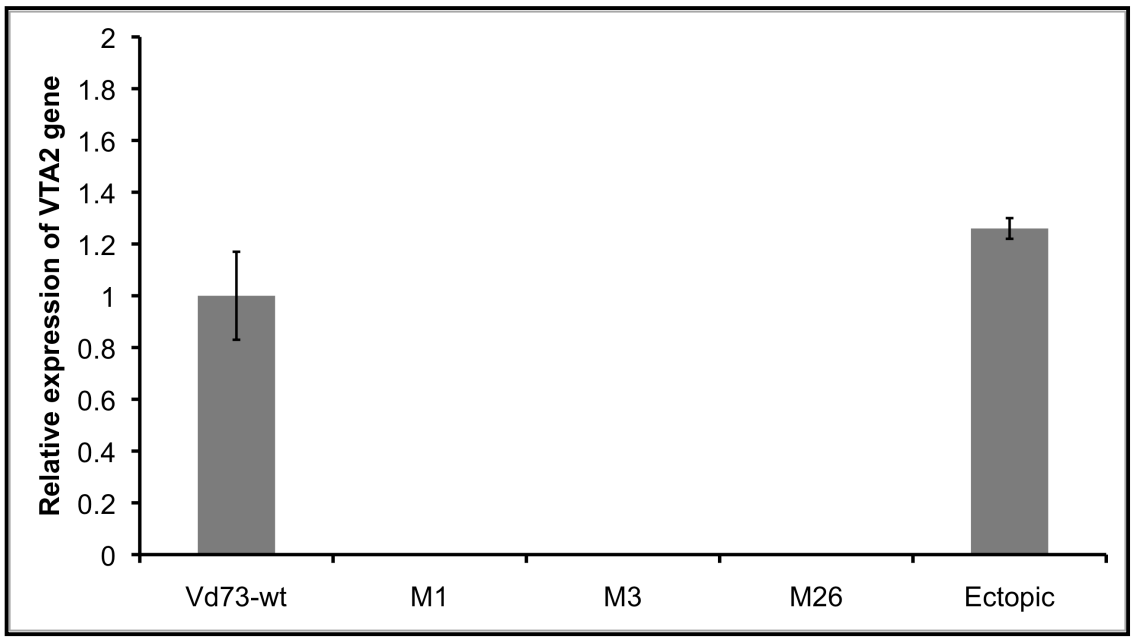


Figure 30. Confirmation of *VTA2* gene disruption in the mutants by quantitative real-time PCR. Expression of *VTA2* gene in the mutants (M1, M3, M26) compared with the wild type strain Vd73 and one ectopic transformant. Loss of *VTA2* gene expression represents a successful gene disruption in the mutants.

Our data demonstrated that the homologous recombination occurring between the deletion cassettes and locus of *VTA2* gene in fungal genome resulted in a disruption of *VTA2* gene in 76% transformants derived from Vd73 strain and in 45% transformants from Vd52 strain.

3.3. VTA2 transcription regulator controls fungal development, surface hydrophobicity and virulence

3.3.1. VTA2 is expressed during fungal conidiation process

We checked expression of *VTA2* gene during growth process of *V. longisporum*. The fungus was grown in the liquid simulated xylem medium (SMX) containing pectin as the sole carbon source. The expression of *VTA2* was measured at the different time points (0-7 days) by quantitative real-time PCR. The data showed that *VTA2* gene was expressed specifically in fungal spores (0 day) and in the stages of spore formation (4-7 days), but hardly in the early stages of fungal growth after germination of spores (1-3 days). The maximal expression of *VTA2* gene was at 4 days and reduced gradually by the time (Figure 31). This suggests that VTA2 transcription regulator is required for spore production of the fungus.

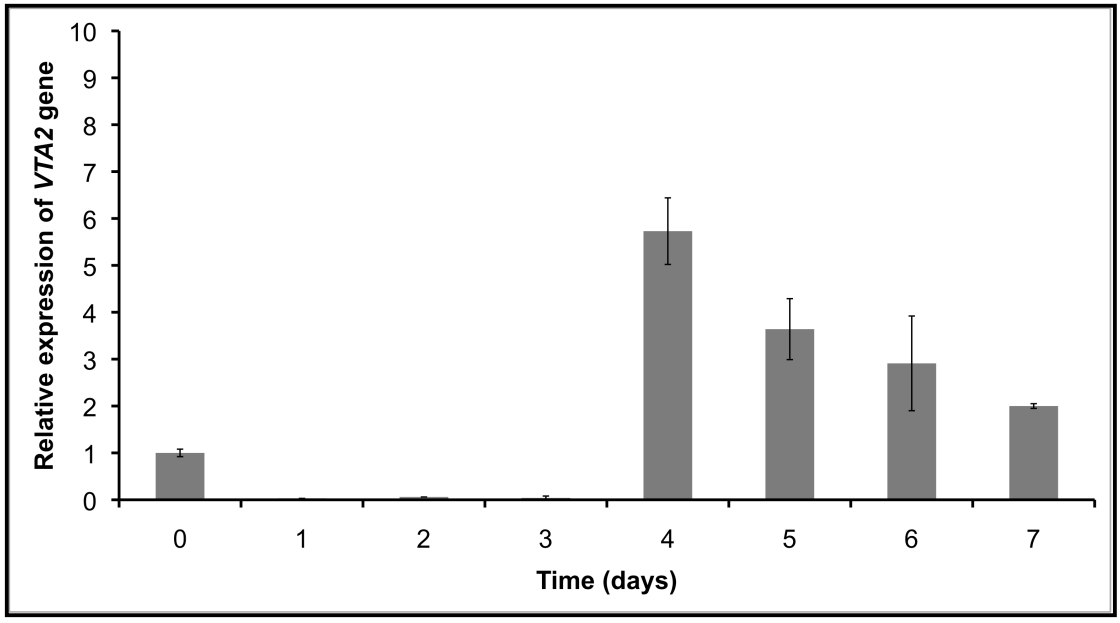


Figure 31. Expression of *VTA2* transcription regulator gene in *V. longisporum* at different time points. Fungal spores were used as the starting material (0 day) for this assay. Expression of the gene in the fungus was checked every 24 hours. The fungus was grown in SXM liquid medium at 25^{o}C, on a shaker of 100 rpm.

3.3.2. VTA2 controls fungal growth and conidia formation

As described above, the deletion of *VTA2* gene in *V. dahliae* resulted to changes in the phenotype. Therefore, we performed a closer look at growth rate and the morphology of the deletion mutants. The data showed that growth of the *VTA2* deletion mutants was about 30% slower than that of the wild type Vd73 (Figure 32).

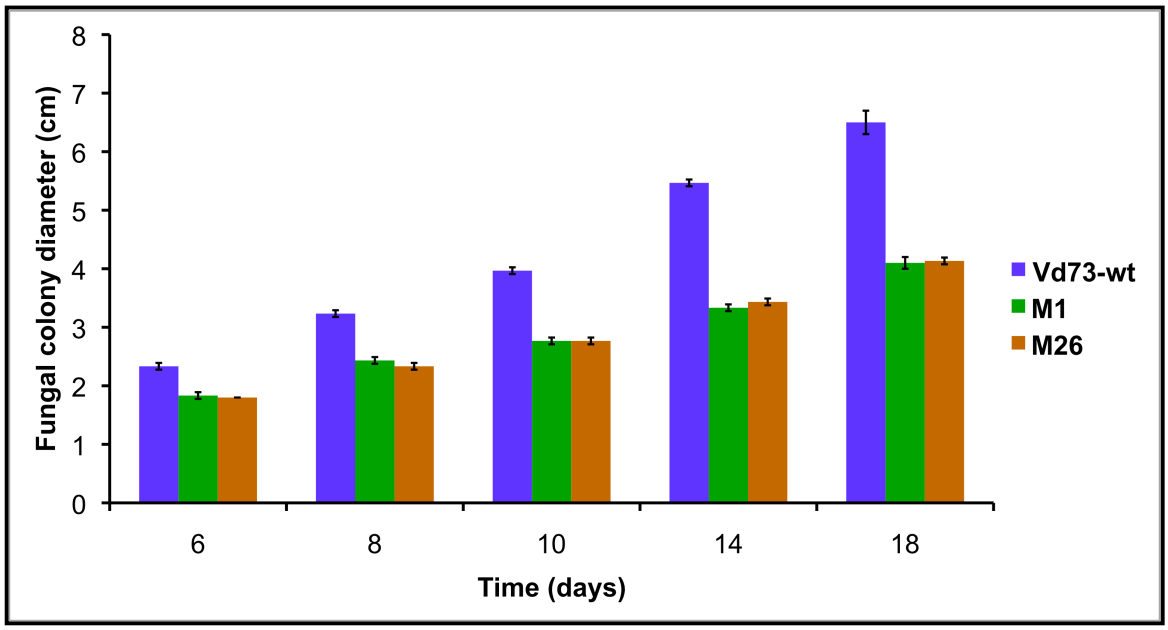


Figure 32. Growth rate of two *VTA2* deletion mutants (M1, M26) compared with the wild type strain Vd73. The mutants and wild type strain were grown on the rich medium (PDA) plates. The plates were incubated at 25°C and the diameter of fungal colonies was measured after 6, 8, 10, 14 and 18 days.

Furthermore, we found that the mutants were defect in formation of the aerial mycelia on solid media resulting in smooth surface of fungal colonies. In addition, we observed only a few conidiophores in the mutants in contrast to the wild type strain Vd73 that produced a lot of conidiophores (Figure 33A). As a result, only about 0.5% spores were produced in the mutants when compared with the wild type (Figure 33B).

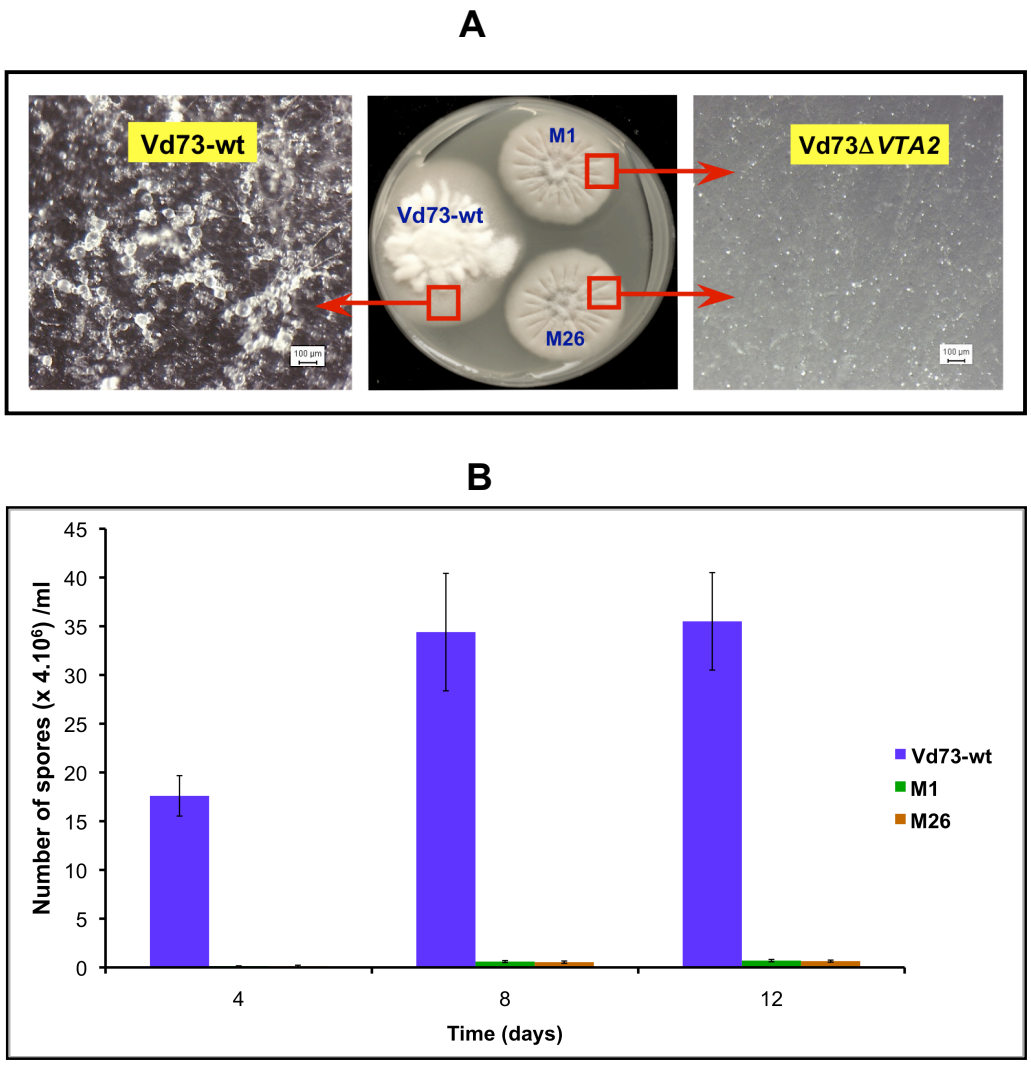


Figure 33. Requirement of VTA2 for aerial mycelium and conidia formation. **(A)** Formation of the aerial mycelia in the mutant and the wild type Vd73 after two weeks on PDA plates. The mutants lost ability of aerial mycelium production. **(B)** The mutants were almost defect in spore formation on solid as well as in liquid media. This resulted in production of a few spores (0.5%) compared with the wild type strain Vd73. The spore numbers were counted after 4, 8 and 12 days.

3.3.3. VTA2 affects microsclerotial formation and is required for oxidative stress

Microsclerotia are important survival structures of *V. dahliae* and *V. longisporum* (Karapapa *et al.*, 1997; Klosterman *et al.*, 2009). In the *VTA*2 deletion mutants, we found that the deletion of *VTA2* gene caused induction of early production of melanized resting structures in the mutants on the different nutrient media (Figure 34A), suggesting that lacking of *VTA2* the fungus quickly switched to resting phase by producing microsclerotia to resist environmental stress. Interestingly, the mutants preferred to grow inside the agar medium. This seemed to be a way to escape from oxygen. The early formation of

microsclerotia in the mutants might be a reason to explain for this situation. We extended to examine anti-stress ability of the mutants using PDA medium added different concentrations of H_2O_2. The results showed that the mutants were more sensitive to oxidative stress than the fungal wild type Vd73 (Figure 34B). Therefore we suggest that the mutants induce the early formation of microsclerotia to prevent oxidative stress from environment.

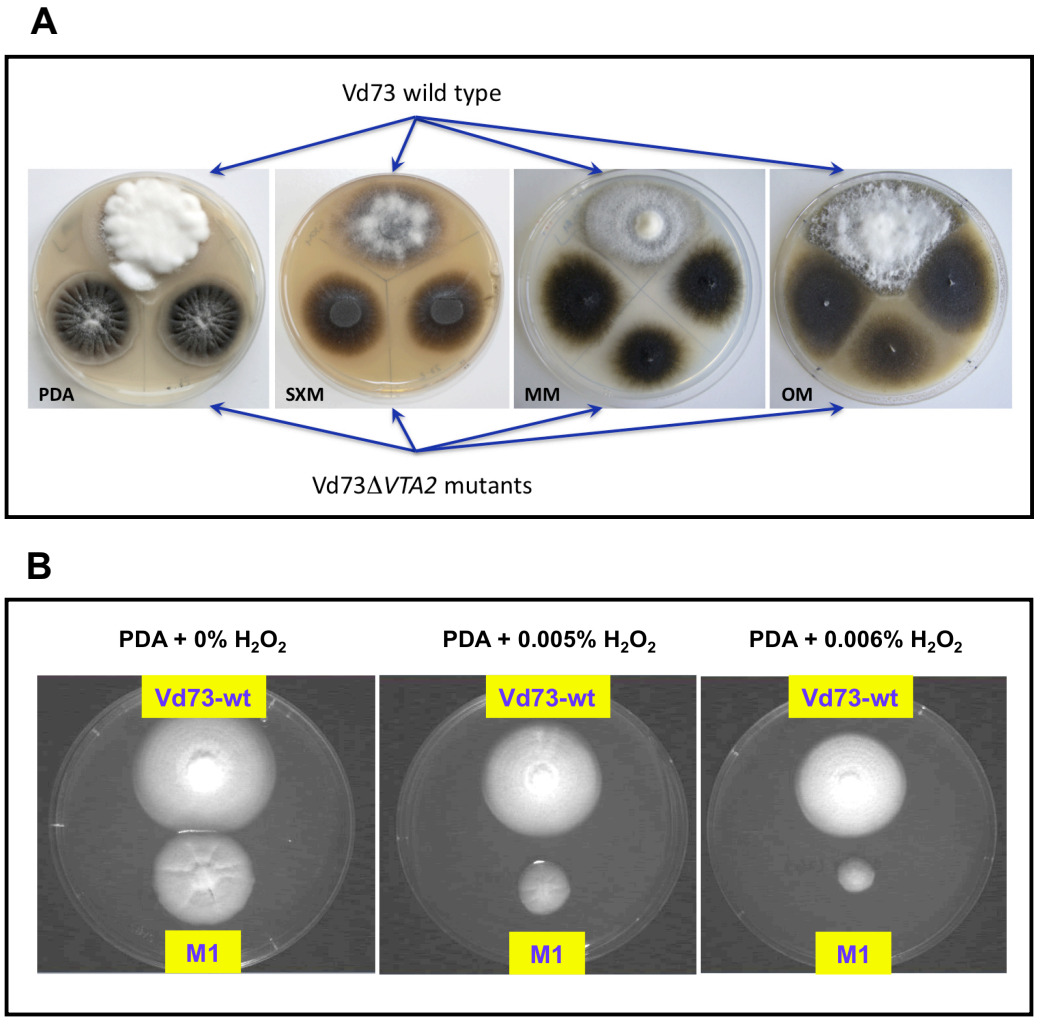


Figure 34. Early microsclerotial development and oxidative stress response in *VTA2* deletion mutants. (A) The wild type Vd73 and the mutants were grown on four different nutrient media including PDA (potato dextrose agar), SXM (simulated xylem medium), MM (minimal medium) and OM (oatmeal). After three weeks at 25°C on all four media, the mutants switched the whole mycelia to microslerotia. **(B)** The *VTA2* deletion mutant (M1) was much more sensitive to H_2O_2 than the wild type strain Vd73. The mutant and the wild type were grown on PDA plates with and without H_2O_2

3.3.4. VTA2 is required for surface hydrophobicity

The *VTA2* deletion mutants exhibited the smooth phenotype due to lacking aerial mycelium. To test surface hydrophobicity, we placed water drops of 100µl on the fungal mycelia of the wild type and the mutant. We found that the mycelium surface of the mutant was wettable and water could easily pass through. In contrast, the wild type produced a thick layer of aerial mycelium preventing water to penetrate (Figure 35).

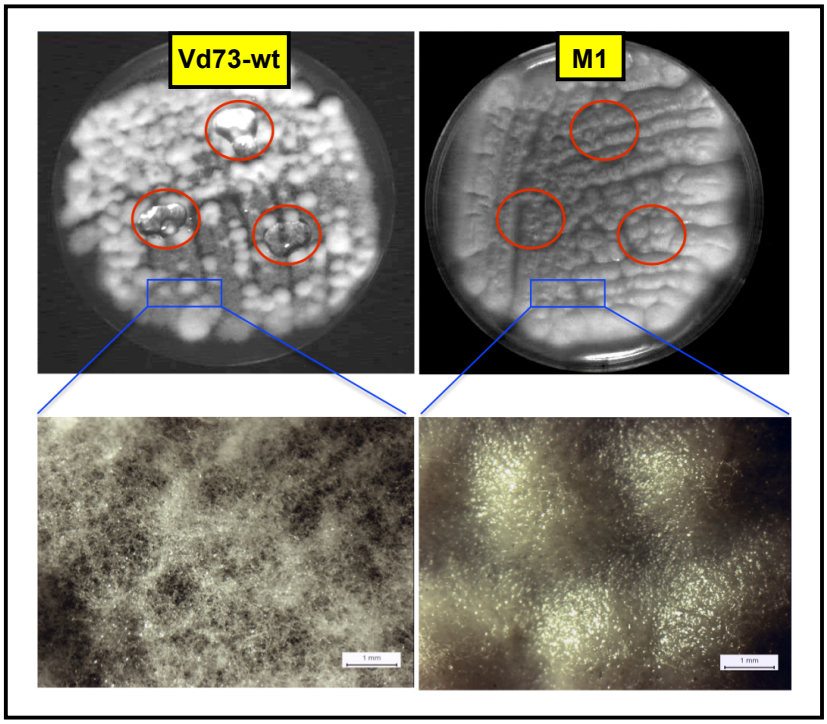


Figure 35. Lost of surface hydrophobicity in the *VTA2* deletion mutant. The mycelium of the mutant M1 lost completely surface hydrophobicity when compare with wild type strain Vd73. The water drops could not penetrate the mycelium surface after 24 hours, whereas water drops fully passed through the surface of the mutant after 10 min. The red rings indicate the positions of water drops.

3.3.5. *VTA2* gene is required for filamentous growth

The *VTA2* deletion mutants lost ability of the filamentous growth, typically in the minimal medium when compared with the wild type strain Vd73 and with the *VTA2* rescued transformant. The mutants exhibited yeast-like growth as clumps at the bottom of the flasks (tubes). This phenomenon is similar to flocculation in the budding yeast that we mentioned at the beginning of this work.

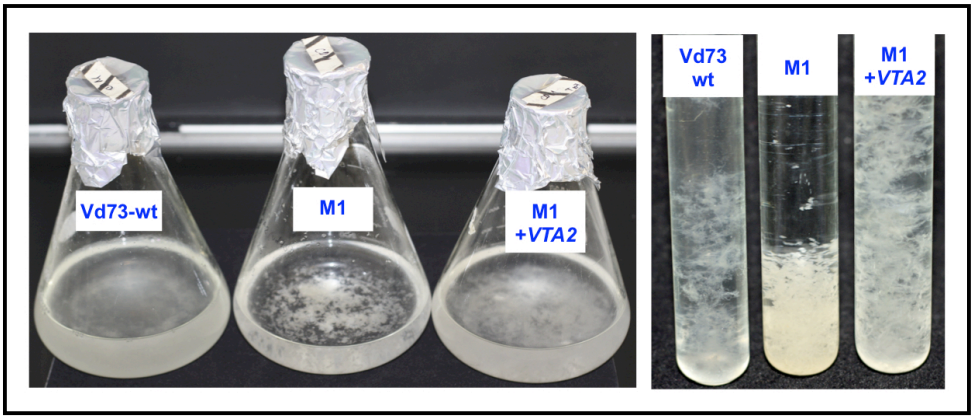


Figure 36. Defect in filamentous growth of the *VTA2* deletion mutants. The mutants represented yeast-like growth in the liquid minimal medium in contrast to the wild type and the rescued transformant. The rescued transformant was generated by the ectopic integration of the full *VTA2* gene into the genome of the mutant M1.

3.3.6. *VTA2* gene is involved in virulence of the fungus on the plant

We performed the infection assays of the *VTA2* deletion mutant (M1) and the wild type strain Vd73 on tomato plants by root-dipping method. The results showed that both the mutant and the wild type were not able to infect the plants. Although the Vd73 strain from linseed was reported to belong to VCG2B that can infect a broad range of different hosts (Zeise and von Tiedemann, 2002), this strain could not infect tomato plants in this experiment. However, when we injected spore suspension from the mutants and the wild type strain into the plants, disease symptoms appeared for the wild type strain. Perhaps this strain could not penetrate the tomato plants through the roots. By injecting fungal spores into the plants, we found that the wild type Vd73 caused the typical symptoms on tomato including stunting and chlorosis, whereas the *VTA2* deletion mutant only exhibited a slight symptom of chlorosis as indicated by red arrows (Figure 38). This suggests that *VTA2* gene is involved in virulence of the fungus. However, with the injection method, it is not possible to identify whether the *VTA2* deletion mutant still adhere and penetrate plant roots or not.

Currently we are performing some infection assays for *VTA2* silenced mutants from Vl43 strain on rapeseed *Brassica napus* and for *VTA2* deletion mutants from Vd52 strain on tomato. *Verticillium dahliae* strain Vd52 is able to infect strongly tomato (Zeise and von Tiedemann, 2002).

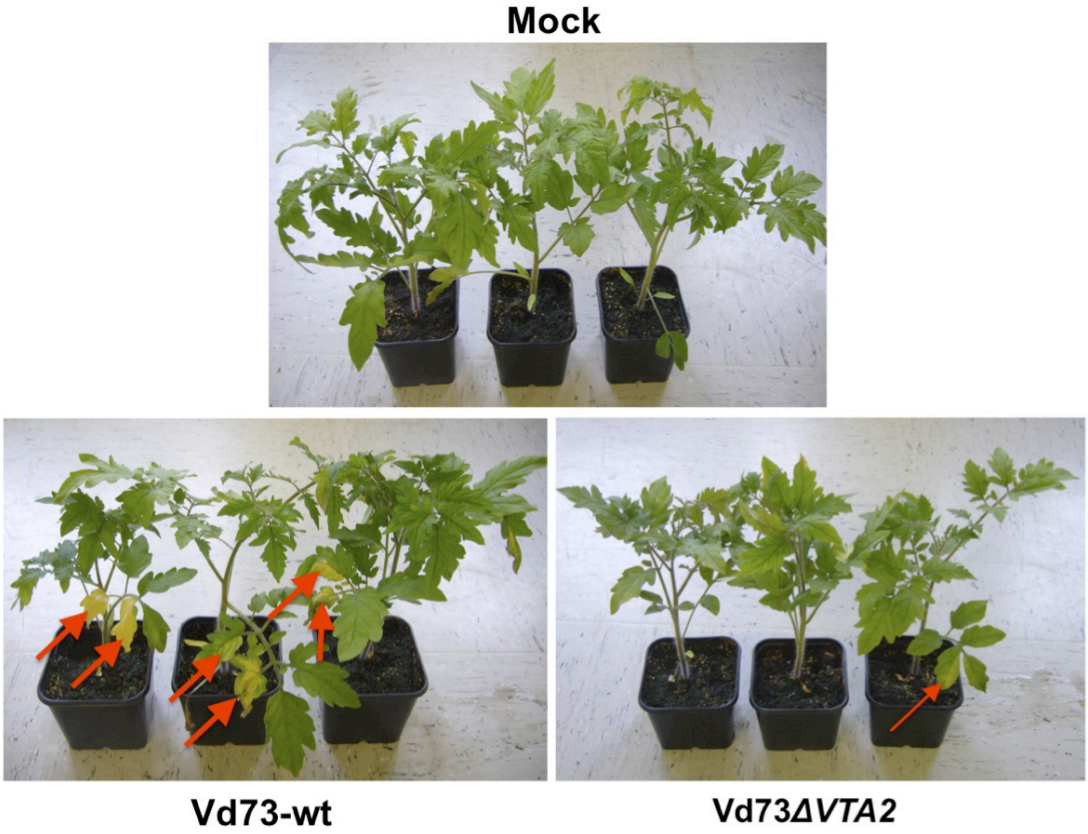


Figure 38. *VTA2* gene is involved in virulence of the fungus on the tomato plants. Spores from the *VTA2* mutant and from the wild type Vd73 were injected into stems of the plant using sterile needles. The symptoms by infection with the wild type Vd73 such as stunting and chlorosis (red arrows) appeared after 2 weeks of the injection, but only the weak chlorosis symptom (the red arrow) by the mutant.

3.4. Snapshot of speciation: the origin of the recently evolved fungal rapeseed pathogen *Verticillium longisporum*

From the yeast adhesion assays using the *V. longisporum* cDNA library, we found two distinct transcripts for *VTA2* gene in *V. longisporum*. These transcripts carry the specific signatures that might support recognition of the evolutionary origin of this fungus. Therefore, we sequenced completely *VTA2* gene together with some other conserved genes including *VTA1*, two *velvet* genes and rDNA in *V. longisporum* and in two very closely related species *V. dahliae* and *V. albo-atrum* as putative parents. We compared *V. longisporum* isolates from Europe and California to examine the initial steps in speciation of a plant pathogen with a 1.8 fold genome as a putative snapshot of speciation.

Polymorphism of rDNA repeats in mature species is normally remarkably low in higher fungi as a consequence of concerted evolution (Ganley and Kobayashi, 2007). However, within a species rDNA repeats can carry high amounts of single nucleotide polymorphisms (Simon and Weiß, 2008). We found that one initial step of speciation of *V. longisporum* is the homogenisation of the repetitive rDNA clusters. rDNA of either parent results in different *V. longisporum* isolates in two distinct rDNA types corresponding to either *V. albo-atrum* or *V. dahliae*. *V. longisporum* rDNA homogenization might be the committing step in speciation prior to the homogenization of single gene pairs. Single genes of both rDNA types still come in isogenic pairs which can be clearly assigned to *V. dahliae* or *V. albo-atrum* as parents. Characteristic single nucleotide polymorphisms resulting in different Southern patterns suggest that both different *V. longisporum* rDNA types are probably the result of a single original hybridization event.

3.4.1. Different *V. longisporum* isolates carry either *V. dahliae* or *V. albo-atrum* rDNA

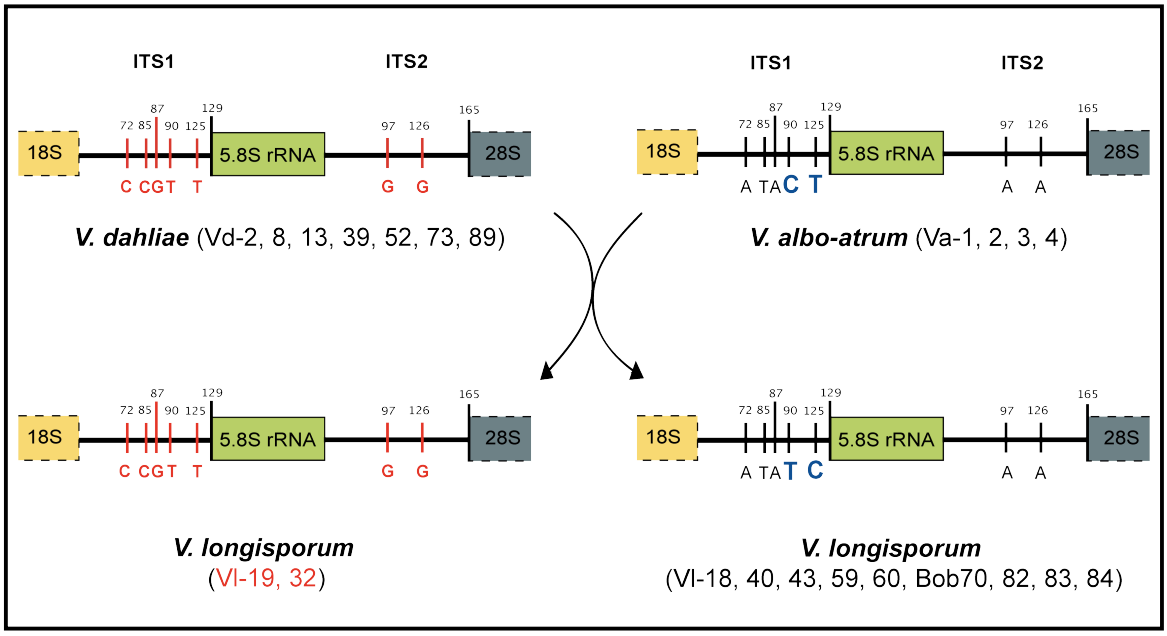
V. longisporum is a near diploid fungus which might be an interspecific hybrid of two different parents. rDNA is a common taxonomic marker with copy numbers varying between 30 and 30,000 copies in most eukaryotes (Rooney and Ward, 2005; Ganley and Kobayashi, 2007). We have analyzed and compared the rDNA from 22 *Verticillium* isolates (11 *V. longisporum*, 7 *V. dahliae*, 4 *V. albo-atrum*) originating from different regions (Table 2) for a more comprehensive picture of the evolution of *V. longisporum*. Ribosomal DNA repeats including the 18S rRNA gene, 5.8S rRNA gene, two internal transcribed spacers (ITS1 and ITS2) and a large intergenic region (IGS) were analyzed. Polymerase chain reaction (PCR) amplification using various primer pairs (ITS-F/ITS-R, IGS-F/IGS-R, Table 3) resulted for each primer pair in only one single sequence characteristic for each analyzed strain of the three species. The ITS-F and ITS-R primers resulted in amplified DNA including a part of the 18S rRNA gene (212 bp), ITS1 sequence (129bp), 5.8S rRNA gene (158 bp), ITS2 sequence (165 bp) and a part of 28S rRNA (178 bp). The 5.8S rRNA gene located between ITS1 and ITS2 is completely conserved within all three species. All seven *V. dahliae* isolates are separated from all four *V. albo-atrum* isolates in the ITS1 and the ITS2 region. However, there are two different ITS types (ITS-typ-1 and ITS-typ-2) within the 11 *V. longisporum* isolates. The ITS-typ-1 of the two European isolates Vl-19 and Vl-32 is identical to *V. dahliae*, whereas the ITS-typ-2 including six European isolates (Vl-18, Vl-40, Vl-43, Vl-82, Vl-83, Vl-84) and three

American isolates (Vl-59, Vl-60, Vl-Bob70) is identical to *V. albo-atrum* except of two pyrimidine exchanges in ITS1 (Figure 39A).

A closer inspection of the intergenic spacer (IGS) separating rDNA repeats revealed PCR products ranging from 1.7 to 1.9 kb using the primer pair IGS-F/IGS-R. The IGS region is highly polymorphic within all three species and can be divided into two parts based on the sequence conservation. The variable part of approximately one kilo base starts at the end of the 28S rRNA gene and contains some repeats and many singly nucleotide polymorphisms. In contrast, the conserved part of about 0.7-0.9 kb is situated adjacent to the 18S rRNA gene and reveals high similarity within all three species (Figure S1A). In agreement to the data obtained with the internal transcribed spacers (ITS), there are two different IGS sequence types (IGS-typ-1 and IGS-typ-2) within the 11 *V. longisporum* isolates. The IGS-typ-1 (two European isolates Vl-19 and Vl-32) is highly similar to the IGS region of *V. dahliae* containing a tandemly repeated signature of 81 nucleotides (AGCTACCCGGGA ATTGGACCAGTTTTGAGGCTGGCAGCTACCCGGGAGTTGCTGAAAAACGACCA AGTCGGACACCTTGG). On the contrary, the IGS-typ-2 (9 European and 3 American *V. longisporum* isolates Vl-18, Vl-40, Vl-43, Vl-59, Vl-60, Vl-Bob70, Vl-82, Vl-83, Vl-84) is closer to the IGS region of *V. albo-atrum* but has accumulated a tandemly repeated signature of 39 nucleotides (ATCTGGGAGCTACCCGGGAGTTGGAAATTTGGAGA ACGG) and more single nucleotide polymorphisms (Figures 39B, S1A).

Similarly, the 18S rRNA gene-specific primer pair (18S-rRNA1/18S-rRNA2) resulting in a 0.44-1.28 kb fragment revealed two types of 18S rRNA genes for different *V. longisporum* isolates. Both types differ in an intron of 839 nucleotides, which is present in 9 *V. longisporum* isolates carrying *V. albo-atrum* rDNA (Vl-18, Vl-40, Vl-43, Vl-59, Vl-60, Vl-Bob70, Vl-82, Vl-83, Vl-84). This 839-bp intron has been previously proposed to be the result of a horizontal gene transfer provided by another organism like a host plant (Karapapa and Typas, 2001). This intron is not present in the 18S rRNA gene of the two *V. longisporum* isolates carrying the *V. dahliae* rDNA (Vl-19, Vl-32). This intron is also absent in the 18S rRNA gene of the analysed *V. albo-atrum* isolates. However, the 18S rRNA exon regions of *V. albo-atrum* result in identical 18S rRNAs as the 9 *V. longisporum* isolates of the *V. albo-atrum* rDNA type (Figure S1B).

A



B

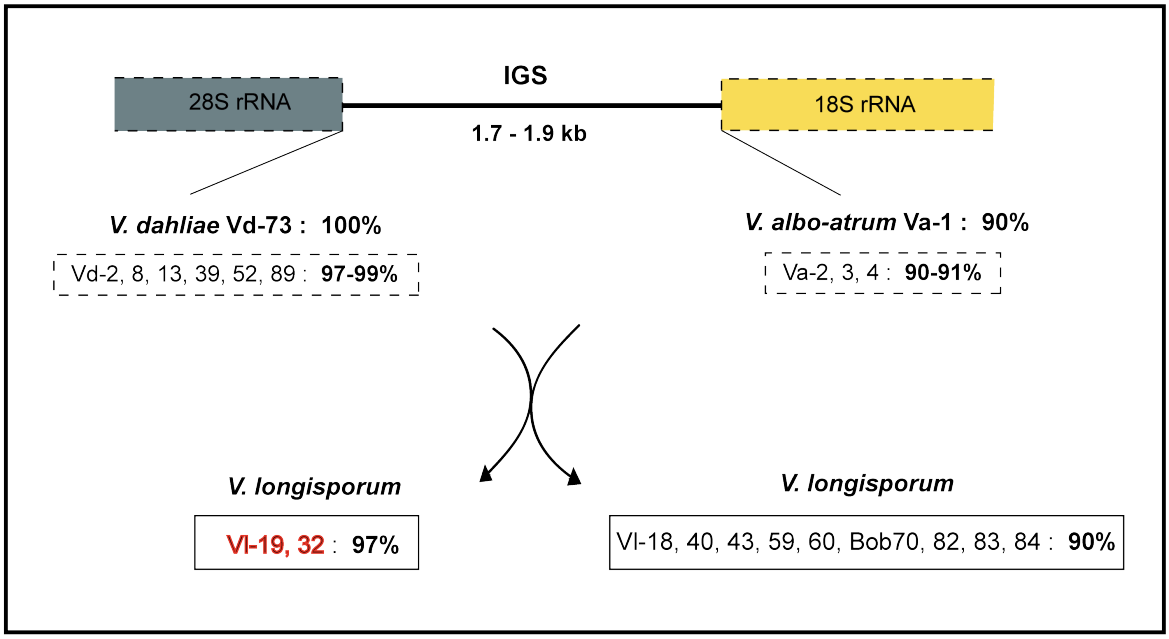


Figure 39. Analysis of *Verticillium* ribosomal DNA repeats. *V. longisporum* isolates carry only one type of rDNA deriving either from *V. dahliae* or from *V. albo-atrum*. The internal transcribed spacer (ITS) comprises 129 nucleotides for ITS1 and 165 nucleotides for ITS2. Differences between the indicated *V. dahliae*, *V. albo-atrum* strains described in Table 2 are indicated. *V. longisporum* separates into the two strains Vl-19 and Vl-32 isolated from rapeseed and identical to *V. dahliae* from Northern Germany. The larger group is highly similar to *V. albo-atrum* except two pyrimidine exchanges at positions 90 and 125 (indicated by bold). These strains were isolated from

Europe and California and were isolated from rapeseed or cauliflower (details in Table 2), the intergenic spacer (IGS) located between 28S rRNA and 18S rRNA shows a high variation among the three species with the length ranging from 1.7 to 1.9 kb.. The IGS region of *V. dahliae* Vd-73 isolated from linseed (Vd-73) served as standard for the calculation of sequence similarity. The 18S rRNA gene reveals an intron of 839 nucleotides only present in *V. longisporum* isolates carrying *V. albo-atrum* rDNA. **(A)** ITS, (**B)** IGS.

Both different rDNA types of *V. longisporum* can be isolated from the same geographical region (Mecklenburg-Vorpommern) in Northern Germany. This suggests that there might be different generic pathways resulting in isolates described as *V. longisporum*.

3.4.2. Both rDNA types of *Verticillium longisporum* carry a pair of identical isogenes for regulators which are conserved in ascomycetes

The molecular analysis of single genes is yet limited in *V. longisporum.* We examined two *velvet* genes (*VeA* and *VelB*), which are conserved in ascomycetes and control secondary metabolism and fungal development including hyphal morphogenesis or the formation of the conidiophore producing the asexual spores (Bayram *et al.*, 2008; Hoff *et al.*, 2010).

The *VeA* orthologues *VEL1* were amplified from genomic DNA of all seven *V. dahliae* isolates using *VEL1*-F/*VEL1*-R primers designed specifically according to the *V. dahliae* DNA sequence (http://www.broadinstitute.org/annotation/genome/verticillium_ dahliae/GeneDetails.html?sp=S7000001884992543) and resulted in a 1.7 kb product. A product of the same size could be amplified with the same primers from all 11 *V. longisporum* isolates, but not from the four *V. albo-atrum* isolates. Determination of the DNA sequences revealed that the *VEL1* genes from *V. longisporum* and from *V. dahliae* are 99% identical (accession numbers: FR839676, FR839677). The primer set *VEL1*-F/*VEL1*-612-R present in *V. dahliae* as well as in *V. albo-atrum* http://www.broad institute.org/annotation/genome/verticillium_dahliae/GeneDetails.html?sp=S70000021353 27296) resulted in the amplification of a 612-bp fragment in all three species, *V. albo-atrum, V. dahliae* and *V. longisporum.* Genomic DNA sequencing resulted in a single *VdVEL1* sequence in *V. dahliae* which is also part of the 1.718 kb fragment and a similar but distinct single *VaVEL1* sequence in *V. albo-atrum*. The *V. dahliae VdVEL1* sequence is

also present in *V. longisporum (VlVEL1-1,* Figure S2). Only the *V. longisporum* isolates carry a second sequence *VlVEL1-2* which carries several single nucleotide polymorphisms resulting in a nucleotide sequence with 95% identities to both *V. dahliae* and *V. albo-atrum* (Figure S2). This suggests that there are two similar isogenes for *VEL1* in *V. longisporum* but only one corresponding gene in *V. dahliae* or *V. albo-atrum*. Southern hybridization was performed to verify the two paralogues and to compare the environment of the two gene loci to the putative parents *V. dahliae* and *V. albo-atrum*. We selected one representative of each rDNA types of *V. longisporum*, Vl-19 with the *V. dahliae* rDNA and Vl-43 as *V. albo-atrum* rDNA type for the analysis. The results from Southern hybridization are identical for both rDNA types and support the presence of two *VEL1* isogenes in both rDNA types which are in both cases derivatives of *V. dahliae* and *V. albo-atrum*, respectively (Figure 40A).

Amplification of the *VelB* homologues *VdVEL2* of *V. dahliae* (Vd-73) and *VaVEL2* of *V. albo-atrum* (Va-1) using the primers *VEL2*-F/*VEL2*-R designed according to the *V. dahliae* and *V. albo-atrum* DNA sequences (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/GeneDetails.html?sp=S7000001884952965 and http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/TranscriptDetails.html?sp=S70 00002135337572) resulted in a 1.6 kb fragment with 94% identities between the two species. Amplification of *V. longisporum* (Vl-43) genomic DNA resulted in two similar but distinct sequences for *VEL2* differing from each other by several single nucleotide polymorphisms (SNPs). Two these sequences share 95% identity to each other and 94-98% identity to the sequences of *V. albo-atrum* and *V. dahliae* (Figure S3). These data suggest that similar to *VEL1* there are also two isogenes for *VEL2* in the *V. longisporum* genome. Comparison of the Southern hybridization pattern of both rDNA types revealed two loci for *VEL2* gene in *V. longisporum* and corroborated that one of two these loci is derived from *V. dahliae.* The other Southern band is identical in both *V. longisporum* rDNA types but distinct to both *V. dahliae* and *V. albo-atrum.* This suggests a common origin for both types of rDNA types of *V. longisporum.* The different second band might either reflect that a different strain or a close relative of *V. albo-atrum* is the second parent or that a point mutation in the intergenic region has happened after hybrid formation (Figure 40B).

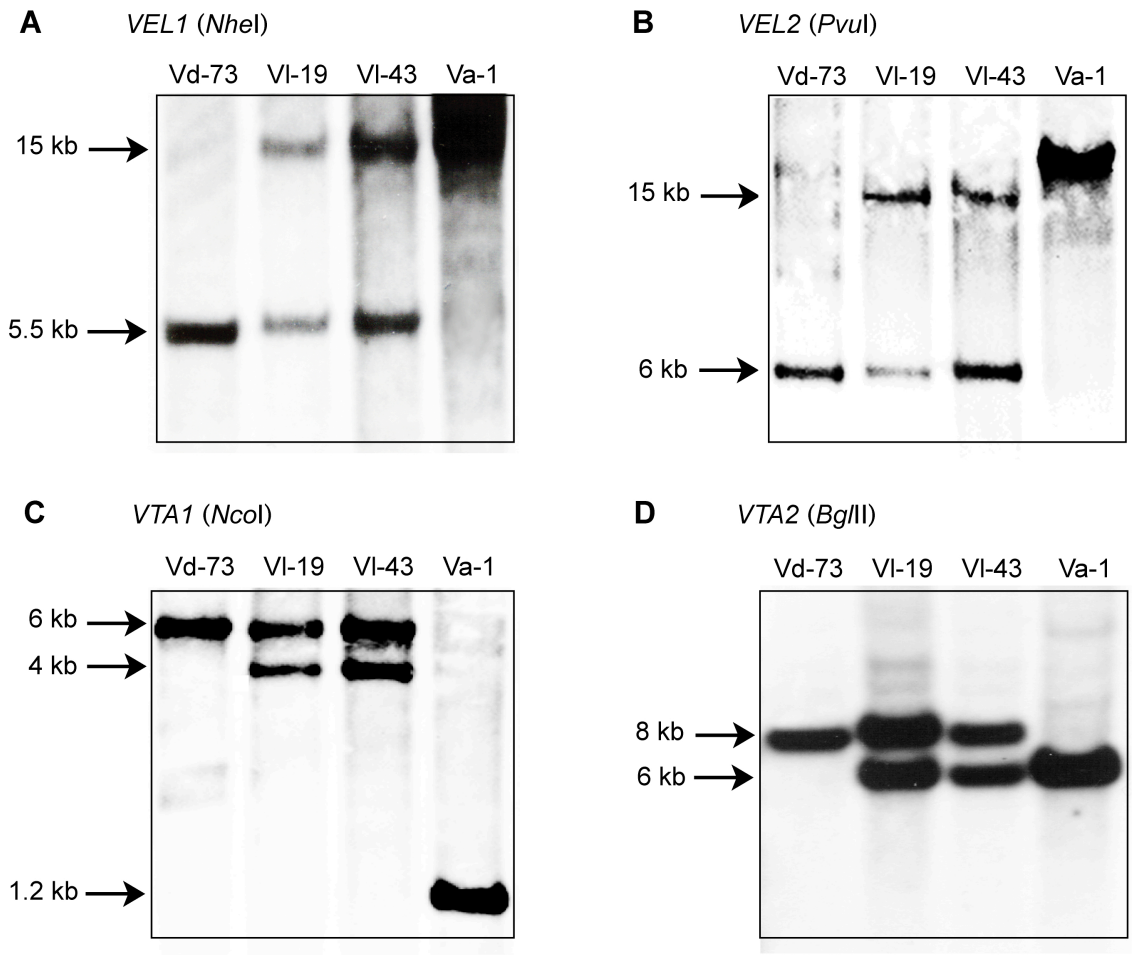


Figure 40. Comparative Southern hybridization for four single genes of *V. dahliae* and *V. albo-atrum* in comparison to two isogenes of two *V. longisporum* strains with different rDNA repeats. (**A)** Genomic DNA of all indicated strains was digested with *Nhe*I and probed with labeled *VEL1* DNA. The same DNAs were also treated with *Pvu*I and hybridized to a *VEL2* probe **(B)**, digested with *Nco*I with *VTA1* probe **(C)** and digested with *Bgl*II and probed to *VTA2* (**D**).

Conclusively, our data demonstrate that in contrast to the rDNA repetitive genes, which are uniform, there are two isogenes for *VEL1* and *VEL2* in *V. longisporum* with high identities (94-99%) to each other and to the corresponding but single genes of *V. dahliae* or *V. albo-atrum.*

3.4.3. *V. dahliae* and *V. albo-atrum* are the parental genomes for two paralogue transcription factor isogene pairs of *V. longisporum*

We extended our analysis by examining two orthologues of transcriptional regulatory genes *VTA1* and *VTA2*, which are expressed in the cDNA library of *V. longisporum* and identified from the yeast complementation assays. They belong to the zinc finger family and are conserved within filamentous ascomycetes (Figure S4B, S5B). The *VTA1* gene encodes a protein containing the $Zn(II)_2Cys_6$ conserved domain. This domain is similar to the conserved domain of the AflR transcription factor in Aspergilli required for the biosynthesis of the cancerogenic aflatoxin (Yu *et al.*, 1996). The *VTA2* gene containing a C_2H_2 conserved motif is an orthologue of the *CON7* gene in the rice blast fungus *Magnaporthe grisea*. The CON7 protein controls approximately 100 genes and is essential for appressorium formation and growth *in planta* (Odenbach *et al.*, 2007).

V. longisporum carries two isogenes for *VTA1* without introns. They correspond to single genes in *V. dahliae* or *V. albo-atrum.* The two 1.275-1.278 kb *VTA1* sequences display a 95 to 99% identity to each other and to the corresponding genes of the corresponding putative parents with a significant different signature: a three-nucleotide sequence in the longer *VTA1* sequence (*VTA1-1*) of *V. longisporum* is only present in *V. dahliae*. The *V. albo-atrum* as well as the shorter *VTA1* sequence (*VTA1-2*) of *V. longisporum* carry a small deletion of this sequence (Figures 41, S4A). Comparison of the Southern hybridization pattern revealed two loci for the *VTA1* genes in both rDNA types of *V. longisporum.* One locus corresponds to *V. dahliae.* The other gene which carries the deletion of the corresponding *V. albo-atrum* gene shows a different pattern. This could be due to a point mutation in the *V. albo-atrum* genome acquired during speciation after hybridization or could reflect another *V. albo-atrum* strain carrying this point mutation as parent (Figure 40C). The data corroborating *V. longisporum* as an interspecies hybrid between *V. dahliae* and *V. albo-atrum* (or a close relative) support that the different rDNA types of *V. longisporum* might share a common original hybridization event.

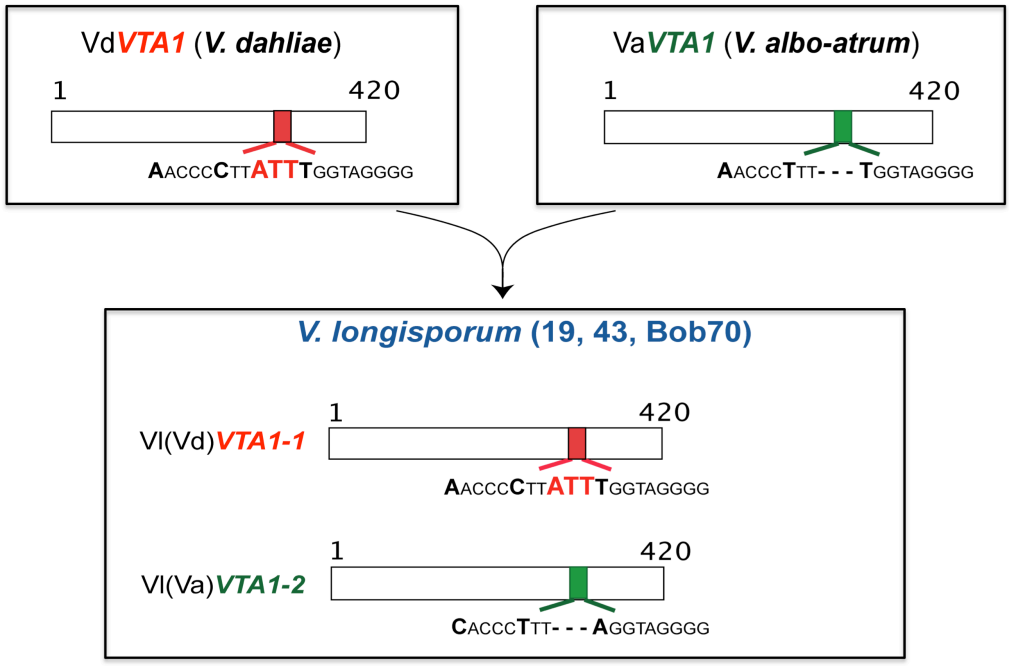


Figure 41. Two *VTA1* isogenes of *V. longisporum* (*VTA1-1*, *VTA1-2*) in comparison to the corresponding single genes of *V. dahliae* and *V. albo.atrum*. *VTA1-1* shares with the *V. dahliae* orthologue the same signature consisting of a three-nucleotide insertion in the coding region, whereas *VTA1-2* and the *V. albo-atrum* orthologue *VTA1* lacks this insertion.

The origin of *V. longisporum* as interspecies hybrid from a *V. dahliae* and a *V. albo-atrum* parent was further confirmed when we compared the *VTA2* genes of all three species. *VTA2* genes are broadly conserved within filamentous fungi and the *Verticillium* isogenes are related to fungal plant pathogens such as *Fusarium* species and *Magnaporthe grisea* (Figure S5C). Comparison of the expressed sequence tags to the genomic sequences amplified by PCR revealed a complex gene structure for *VTA2*, which includes four introns and five exons. The *V. dahliae* and *V. albo-atrum VTA2* orthologues vary slightly in size with 1.701 kb and 1.754-1.759 kb, respectively (Figures S5A and S5D). The first exon of *V. dahliae VTA2* has an insertion of nine nucleotides (ACGCTCACC) when compared with *V. albo-atrum VTA2*. In addition, the third intron (I3) of the *V. dahliae* gene (217 bp) is 68 nucleotides shorter than *V. albo-atrum* I3 (285 bp). In contrast to the single parental genes, both types of *V. longisporum* rDNA types carry again two paralogues which carry two distinct gene signatures which classify them either as *V. dahliae* or *V. albo-atrum* genes (Figures 42, S5A).

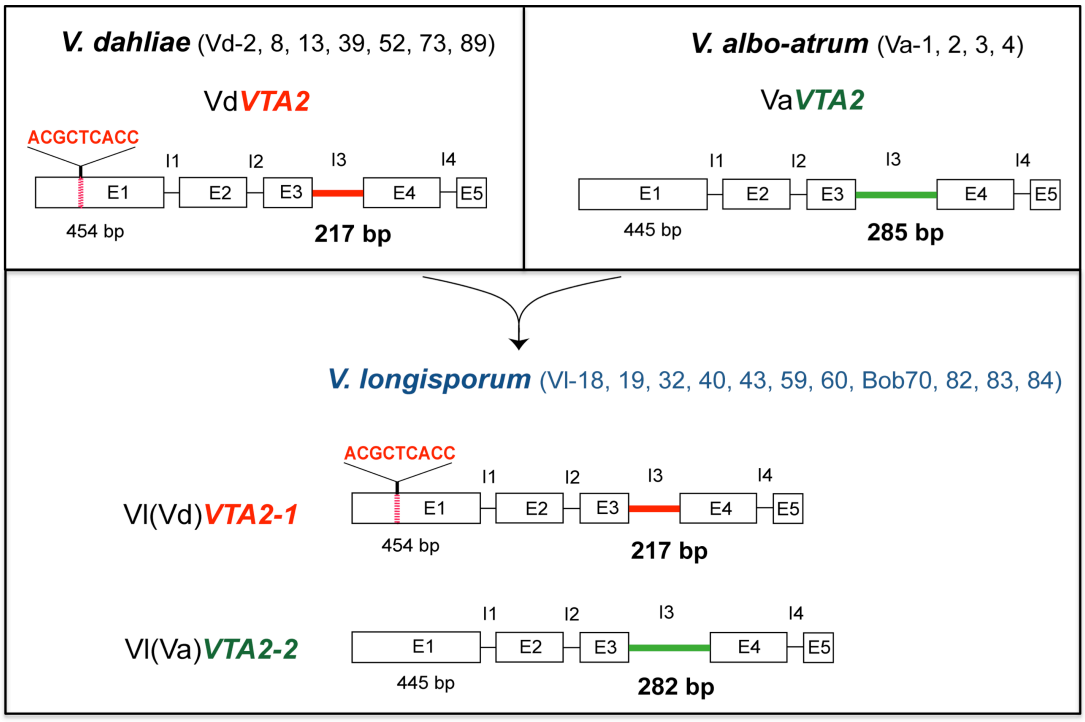


Figure 42. Comparative scheme of *VTA2* homologues from all three *Verticillium* species. *VTA2-1* and *VTA2-2* isogenes of *V. longisporum* are derivatives from *V. dahliae* and *V. albo-atrum,* respectively. Characteristic signatures include a nine-nucleotide insertion (ACGCTCACC) in the first exon (E1) and the length of the third intron (I3). *VTA2-1* and the *V. dahliae* orthologue carries the nine-nucleotide insertion in the first exon and a shortened third intron (217 bp), whereas *VTA2-2* corresponds to the *V. albo-atrum* orthologue lacking the insertion in the first exon and carries and extended third intron (282 bp).

We compared the two *VTA2* isogenes of the eight European and the three American *V. longisporum* isolates (Table 2) to further analyze a nascent species during geographic distribution. The two *V. longisporum VTA2* isogenes *VTA2-1* and *VTA2-2* varied in length slightly between 1.701 and 1.755 kb. *VTA2-1* is identical in all 11 *V. longisporum* isolates and differs only slightly from the corresponding *V. dahliae* gene (99% identity). It carries the characteristic ACTCTCACC insertion in the first exon and the shortened third intron of 217 bp as *V. dahliae VTA2*. The other *VTA2-2* isogene is also identical in all 11 *V. longisporum* isolates and resembles *VTA2* of *V. albo-atrum* (97% identity). It carries the characteristic first exon of *V. albo-atrum* lacking the nine-nucleotide insertion as well as the longer third intron of 282 bp. Southern hybridization confirmed that the two *V.*

longisporum VTA2 isogenes are either a derivative of *V. dahliae* or of *V. albo-atrum* (Figure 40D).

The *VTA2* gene analyses corroborated that the two isogenes *VTA2-1* and *VTA2-2* are completely conserved within all 11 *V. longisporum* isolates derived from Europe and America and have not (yet) been changed during geographical separation. In contrast, these isolates represent two different rDNA types of *V. longisporum*. Sequencing of the corresponding *VTA2* genes from seven *V. dahliae* isolates from different geographical regions also reveals an absolute conservation of this gene (100% identity). In contrast, *V. albo-atrum* isolates display minor changes in *VTA2* genes depending on hosts. The *VTA2* genes from the Va-1 isolate deriving from potato in Wisconsin and from the Va-2 isolate from alfalfa from the United Kingdom exhibit a slight reduction of 98% identity to each other (Figure S5D) but still carry the same signatures for the first exon and the third intron. This variation in small nucleotide polymorphisms in *V. albo-atrum* might be the reason for the differences in the hybrid *V. longisporum* where the *VTA2-2* gene from *V. albo-atrum* carries more SNPs than *VTA2-1* from *V. dahliae* (Figure S5A).

In total, our data show that single genes in the *V. dahliae* or *V. albo-atrum* genome are present as isogene pairs in the hybrid *V. longisporum*. Both rDNA types of *V. longisporum* carry identical isogene pairs suggesting a single hybridization event and subsequent homogenization of rDNA where either parental rDNA could be lost.

Chapter 4. DISCUSSION

4.1. Reprograming of yeast adhesion by *Vertillium longisporum* genes

From the yeast adhesion assays, ten putative regulatory proteins covering a broad range of different functions were detected. Four of them are involved in post-transcription processes: R3H domain-containing proteins binding to ssDNA or ssRNA are required for stress reponses and involved in mRNA decay (Saleh *et al*., 2006; Liu *et al.*, 2007). LsmAD domain-containing proteins are functioning in nuclear RNA processing and mRNA decay. The LsmAD domain is found in proteins that interact physically with poly(A)-binding protein (PABP), a key regulator of mRNA translation (Satterfield and Pallanck, 2006). RNA-binding proteins with RNA recognition motif (RRM) are involved in the regulation of the morphology as well as in pathogenic development in fungi (Becht *et al.*, 2005). Extensin proteins are CASC3/Barentsz eIF4AIII binding complexes required for mRNA localization and nonsense-mediated mRNA decay (Palacios *et al.*, 2004).

In contrast, six other regulators may be involved in regulating different trancription processes: Homeobox transcription factors with C_2H_2 zinc finger motifs are required for conidiation, appressorium development and pathogenicity in the rice blast fungus *Magnaporthe grisea* (Kim *et al.*, 2009). The CON7 transcription regulator of this fungus is essential for appressorium formation of infection and fungal growth *in planta* (Shi and Leung, 1995). This regulator controls expression of approximately 100 genes which are involved in cell wall-associated proteins including a chitinase, a chitin synthase, chitin-binding proteins, a β-glucanase and a β-1,3-glucanosyl transferase, as well as the pathogenicity factor-encoding gene *PTH11* and several other genes encoding G protein-coupled receptors (Odenbach *et al.*, 2007). The cephalosporin C regulator, a member of the RFX transcription factor family that regulates cephalosporin C biosynthesis, and controls morphological development in the β-lactam producer *Acremonium chrysogenum* (Hoff *et al.*, 2005). The glucose repressor CRE1 carrying the two zinc fingers similar to its homologue, Mig1p in *Saccharomyces cerevisiae*, is required for repressing several genes encoding cell wall-degrading enzymes and other genes of assimilation of non-sugar carbon sources (Jonkers and Rep, 2009). In yeast, presence of glucose inactivates the central Snf1 protein kinase. This allows the regulatory protein Mig1p to bind to the *FLO11* promoter and recruit the general repressors Tup1 and Ssn6, resulting in repression of *FLO11*

(Verstrepen and Klis, 2006). C6 zinc transcription factors possess a $Zn(II)_2Cys_6$ binuclear cluster domain that is exclusive in fungi. They cover a broad range of functions such as amides catabolism; regulation of cellulase, xylanase and cutinase genes; aflatoxin biosynthesis; nitrate assimilation; fruiting body development; switch between biotrophy and necrotrophy during infection; melanin biosynthesis; conidiophore morphogenesis and so on (MacPherson *et al.*, 2006). ARID/BRIGHT domain-containing proteins are involved in a variety of biological processes including cell growth, differentiation, and development (Wilsker *et al.*, 2002). They are also required for transcriptional activation of cyst wall protein and involved in the modification of chromatin structure (Dallas *et al.*, 2000; Wang *et al.*, 2007).

Our data showed that the *Verticillium* transcription regulators (VTA1 and VTA2) induced the FLO1-mediated adhesion and flocculation in the non-adhesive yeasts. However, other adhesins such as FLO5 and FLO9 might be also involved in the observed adhesive behavior. We were not able to exclude the involvement of these proteins. We also showed that the VTA1 and VTA2 transcription regulators from *V. longisporum* are expressed in the nucleus of the yeast cells and promote the cells sticking together to form the flocs (flocculating phenomenon) as well as adhering to the agar surfaces. Both the regulators have the PEST sequences (two for VTA1 and one for VTA2). The PEST sequences are responsible for a rapid intracellular degradation of the proteins containing them (Rogers *et al.*, 1986). This might be the reason to explain for the existence of the fluorescent signals only in some cells, but not in all after 4 hours of the galactose induction (Figure 8B).

We characterized THO complex is required for flocculation and adhesion in yeast with the *Verticillium* transcription regulator genes. This complex might affect directly the transcription elongation of the *Verticillium* genes or yeast flocculin genes resulting in a loss or reduction of gene function. We also showed that the yeast THO2 homologue is present in *Verticillium*. In yeast, this complex is required for transcription elongation of the genes with internal repeats such as *FLO1* and *FLO11* and for telomere maintenance (Voynov *et al*., 2006; Askree *et al.*, 2004). Null mutations of any of the four THO complex genes are viable and display similar phenotypes including a defect in transcription elongation, an increase in mitotic recombination between direct repeats (Chavez *et al.*, 2000), and defects in mRNA export (Strässer *et al.*, 2002; Jimeno *et al.*, 2002). Because

THO complex controls the expression of genes with internal repeats such as *FLO1*, *FLO11* in yeast, it might also do a similar function in *Verticillium* species.

In addition, two these putative adhesins (VAP1, VAP2) were not found from the yeast complementation assays. In this work, we used the *V. longisporum* cDNA library generated from the fungal cultures of artificially normal growth (Singh *et al*., 2010). Therefore these adhesin genes might not be expressed in vitro like the EPA2 adhesin of *Candida glabrata* (Peñas *et al*., 2003) or their RNA molecules (more than 2 kb) were quite long to be converted fully into the corresponding cDNAs by the cDNA biosynthesis kit. It could also be that these adhesins are only responsible for adhesion of *Verticillium* to plant surfaces, but not to the artificial surfaces such as agar, plastic or glass. Those might be the reasons why we could not find them from yeast adhesion assays.

In summary, we used successfully the budding yeast *S. cerevisiae* as a model for screening genes of adhesion from *V. longisporum*. These genes could make the non-adhesive yeasts become adhesive and flocculating. However, many genes with unknown functions from the assays remain to be characterized. In this study, we demonstrated that at least two *Verticillium* regulators (VTA1 and VTA2) could promote adhesion and flocculation of the yeast by inducing the expression of FLO1 adhesin gene. In addition, a homologue of FLO1 adhesin was also found in all three *Verticillium* species, suggesting that two these regulators might regulate expression of FLO1 homologue gene in *Verticillium* in the same manner.

4.2. New high-throughput molecular tools for investigating gene function in *Verticillium* plant pathogens.

In this work, we have simplified and optimized the ATMT method for *Verticillium* species. The procedure is easy to carry out, saving time and highly efficent to get transformants. In addition, we also show the T-DNA is integrated into the fungal genome with only a single copy in most of transformants. This will play an important role for gene disruption in *Verticillium* species without unexpected by-products of ectopic integration. We showed that two promoters *gpdA* and *ToxA* from *A. nidulans* and *P. tritici-repentis* respectively can be used effectively for gene expression in *Verticillium*. A successful transformation of *DsRed* gene into *Verticillium* species can be easily recognized by red

pigment accumulation of the fungal mycelium on solid media containing the hygromycin selective agent.

Furthermore, both *DsRed* and *GFP* have been expressed successfully in *V. dahliae* and *V. longisporum*. We also showed that the dual-fluorescent tagged *V. longisporum* strain infected the host *Brassica napus* and the non-host *Arabidopsis thaliana* with the same disease scores when compared with the original *V. longisporum* strain. Therefore the fluorescent version of *V. longisporum* can be used for further investigations as a wild type (wt) strain.

The rapeseed pathogen *V. longisporum* is a near diploid fungus with a complex genome. The nuclear DNA content of this pathogen is almost double (Karapapa *et al*., 1997; Collins *et al.*, 2003). Therefore, gene silencing seems to be the optimal approach for investigating gene function. This method has been proved to be functional in *V. longisporum* (Singh *et al.*, 2010). However the efficiency of this method is not yet fully investigated for this fungus. In addition, generation of silencing constructs based on cloning techniques is very time-consuming and not feasible for broad investigation of several genes at the same time. Therefore, we introduced a new high-throughput silencing system for *Verticillium* pathogenic species. This system including two binary silencing vectors integrated the gateway technology allows creating quickly silencing constructs for genes of interest. The first generation silencing vector (pGS1-nat) was proved to be effective in downregulate gene expression (*DsRed* reporter gene) in *V. dahliae*, but less effective in *V. longisporum*. Therefore if this vector is used to investigate gene function in *V. longisporum*, it will require more time for screening the best silenced mutants with realtime PCR. To overcome this obstacle, we developed the second generation silencing vector (pGS2-nat) for co-silencing using *DsRed* reporter gene. This vector can be used to silence expression of both *DsRed* and an endogenous gene in *Verticillium* at the same time. Therefore the best silenced mutants can be easily detected via the reduction of the red fluorescent signal. This is especially useful for studies of gene function in *V. longisporum* because of low efficiency of gene silencing in this fungus.

We also showed that two newly developed knockout vectors are effective to remove target genes from the fungal genome. The disruption of *VTA2* gene in *V. dahliae* resulted in an obvious phenotype that was never observed clearly by the silencing strategy. It implies that the rest activity (10-20%) of *VTA2* gene from the silencing approach may be still enough to maintain normal morphology of this fungus (Figure 43). Therefore the total

function of *VTA2* gene can only be uncovered by the gene disruption approach. Because both vectors are functional for gene disruption in *V. dahliae*, we suggest that they can be also applied for the near diploid hybrid *V. longisporum* to explore potential contributions of single copies of each gene to growth and pathogenicity.

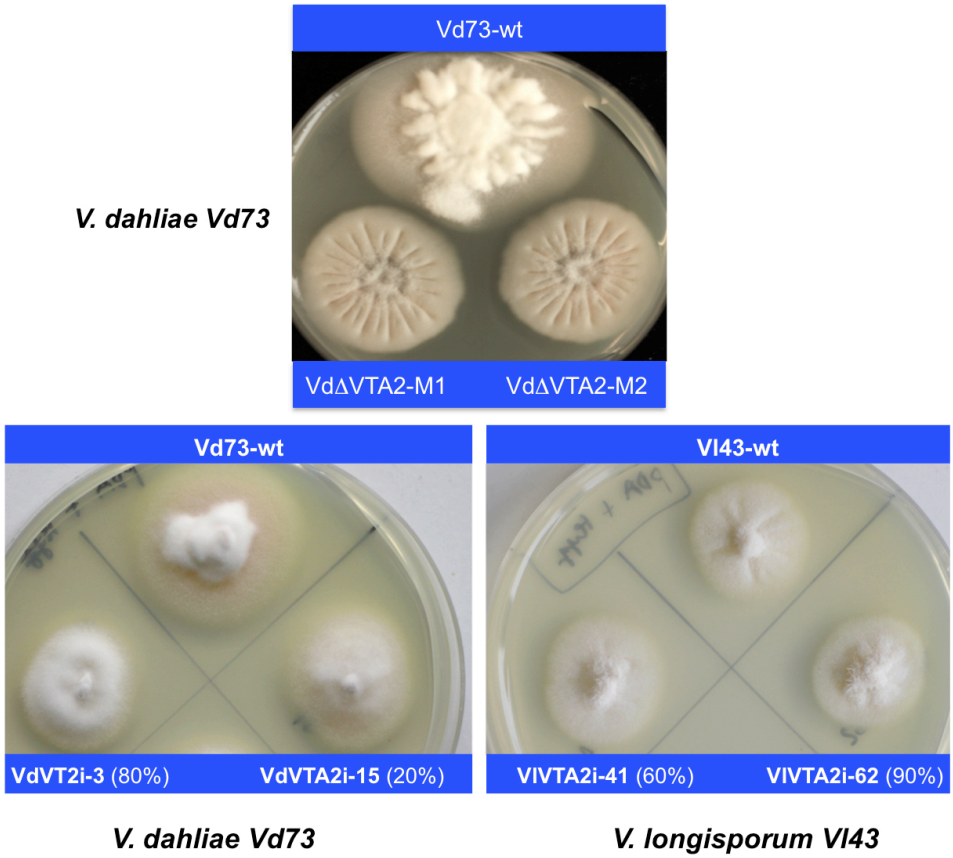


Figure 43. Changes in morphology of the *VTA2* silenced mutants in comparison with the *VTA2* deletion mutants. The *VTA2* silenced mutants in *V. dahliae* and *V. longisporum* exhibit the delayed growth when compared with the wild type strains (Vd73 and Vl43), however the rest activity (10-20%) of the gene is enough to maintain their wild type-like phenotypes. In contrast, the *VTA2* deletion mutants in *V. dahliae* are completely altered in the phenotype including loss of aerial mycelium and the smooth surface of the colonies when compared with the corresponding wild type strain.

Furthermore, roles of all the candidate genes from the yeast adhesion assays could be investigated using the gateway silencing system developed in this work. This system can be used as a quick test to screen genes for adhesion in *V. longisporum* or in *V. dahliae*.

Afterwards, the real functions of these genes can be confirmed in *V. dahliae* by using the gene disruption system.

4.3. VTA2 as a central regulator for fungal development, surface hydophobicity and virulence

VTA2 gene plays important roles in the growth, development and virulence of the fungus. It controls the growth rate, surface hydrophobicity, aerial mycelium development and conidia formation as a positive regulator.

Surface hydrophobicity of fungal mycelium is usually involved in hydrophobins (Wösten at al., 1994; van Wetter at al., 1996; Wösten, 2001). Therefore the loss of the surface hydrophobicity in the *VTA2* deletion mutants may be also caused by defects in expression of hydrophobin genes in *Verticillium*. We characterized five hydrophobins (VHP1-VHP5) from *Verticillium* genome databases. These hydrophobins possess a typical conserved motif with 8 cysteins (Figure 44A) and display hydrophobic patterns (Figure 44B). Currently we are investigating potential contributions of these hydrophobins to the surface hydrophobicity as well as pathogenicity of the *Verticillium* species using the *VTA2* mutants comparing with the corresponding wild type strains.

```
VHP1     MRFSIATIALFAGAAMAHPANLETSLETRELRTACSG-LLHGTPLCCSTSILGLAVLDCST 59
VHP2     MQ-FTIVAALFASVAMAAPATLHAR------ATVCPTGLLYGVAQCCATSVLGVADLDCSV 54
VHP3     MKSFTAIVALFAGLAMATPATLDTR------GSVCSG--LYGNPQCCATDVLGVASLDCQN 52
VHP4     MQFFTVTTALFASLALAAPV-VEDRQVYIP----CSG--LYGSPQCCATDVLGVANLDCGE 54
VHP5     ----------------------------------------MTPVCCATNVLGLLALNCDA 20
                                                    ** *  **   * *

VHP1     P-KSARNGEDMRRNCN--GKQPQCCTLGISEIALLCQRPIGA-- 99
VHP2     PSSTPSDGADLKRICAESGAAAMCCSIPLAGQGVLCTPVIG--- 95
VHP3     P-KSANNANDFKQSCASTGKSAFCCTLPVAGQAVLCNKPVGV-- 94
VHP4     PPAVPTNASEFQATCATIGQRARCCVLPILDQGVLCNNPAGVDE 98
VHP5     PSKTPTSAKDFQKICADAGTAAKCCTLNLLNQGVLCQVPVGVAA 64
         *             *   *    **         **    *
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Figure 44A. The putative hydrophobins in *Verticillium* plant pathogens. Alignment of five hydrophobins shows the conserved motif 8 cysteins. The hydrophobins are small proteins with the secretion signals.

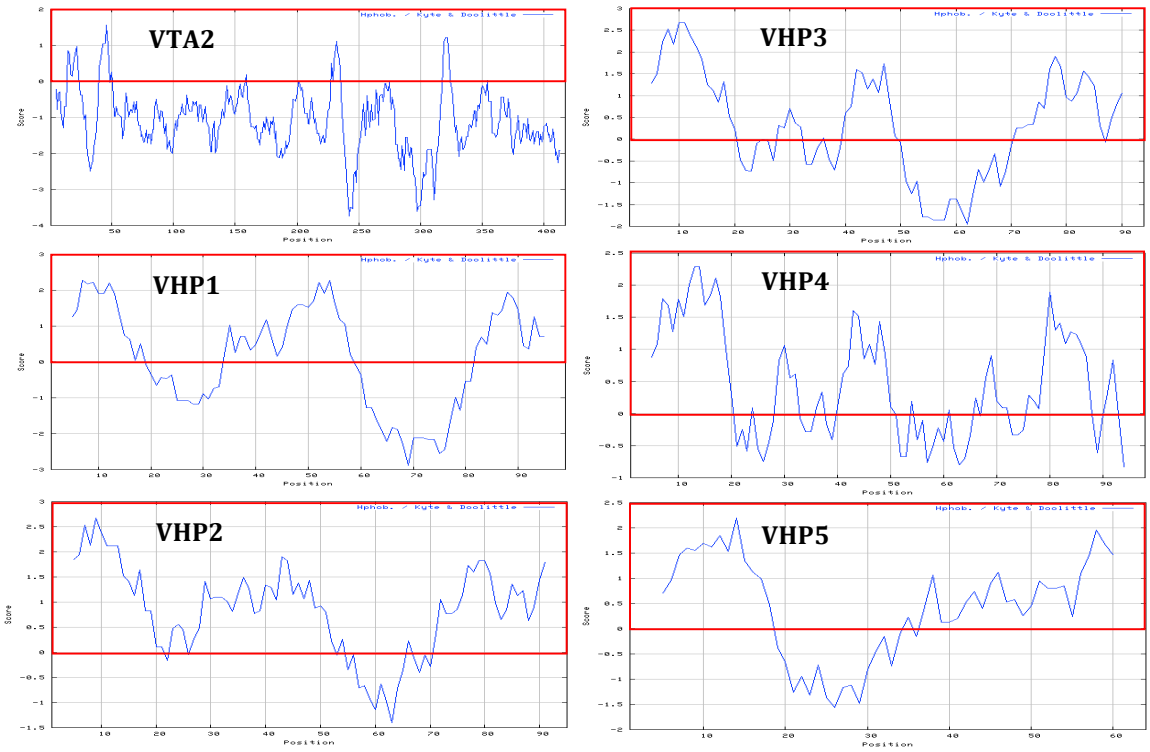

Figure 44B. Hydrophobic patterns of five hydrophobins. All five hydrophobins are highly hydrophobic when compared with VTA2 transcription regulator as indicated by the red frames.

In addition, VTA2 regulator may also control expression of *FLO1* adhesin homologue gene (*VAP1*) in *Verticillium*. Therefore the expression of this gene in the *VTA2* deletion mutants is under investigation.

4.4. Rapeseed pathogen *V. longisporum* is the interspecific hybrid between *V. dahliae* and *V. albo-atrum*

We show here the present state of species formation for a plant pathogen where the loss of parental rDNA accounts for the reduction from the diploid to the 1.8 fold ploidy state of the *V. longisporum* hybrid. Both rDNA types representing either the *V. dahliae* parent or the *V. albo-atrum* parent were found in isolates from a specific region of Northern Germany. Other *V. longisporum* isolates from Europe or California only represent the *V. albo-atrum* rDNA type. Since all other analyzed isogene pairs are conserved from both parents in both rDNA types, we assume that there was only a rare and maybe single original hybridization event prior to the homogenization of rDNA. It is tempting to speculate that this might have happened in Northern Germany.

4.4.1. Linking speciation by natural selection to fungal plant pathogens

Speciation by natural selection includes several general mechanisms. Ecological speciation can favour alleles at different loci in a sub-population in a specific environment distinct from the environment of another sub-population. Similar selection might result in mutation-based speciation if sub-populations accumulate a different series of mutations which is subsequently separated by ecological speciation (Schluter and Conte, 2009). Fungal plant pathogens adapt rapidly to new hosts and are therefore interesting models for ecological speciation (Giraud *et al.*, 2010). The hybrid *V. longisporum* infects in nature only crops of the crucifer *Brassicaeae* family (Karapapa *et al.*, 1997; Heale and Karapapa, 1999; Zeise and von Tiedemann, 2001; Collins *et al.*, 2003, Qin *et al.*, 2006; Gladders *et al.*, 2011), whereas the parental *V. dahliae* and *V. albo-atrum* strains infect more than 200 different plants except crucifers (Pegg and Brady, 2002; Agrios, 2005). The specialized inhabitation on crucifer hosts for *V. longisporum* might represent an ecologically dependent natural selection, where an interspecies hybrid is favoured instead of its parents. At present, the hybrid can colonize ecological niches (crucifer crops) unavailable to the parents. It is currently unclear whether one parent had already accumulated some suitable specific mutations prior to hybridization which supported the interaction to the new host or ecological speciation has started only after hybridization. The ecological separation of the parental habitat disconnects gene flow between hybrid and both parents and the speciation of the hybrid becomes more feasible (Mallett, 2007).

4.4.2. New species formation by increasing the ploidy and interspecific hybridization

Polyploidy increases the number of duplicated sequences in the genome resulting in homologous recombination or gene conversion mechanisms that may lead to novel intergenic interactions (Wendel, 2000). Organisms with a higher ploidy level have a higher potential for distinct beneficial alleles. The rate of adaptive evolution seems to increase with the ploidy level resulting in more beneficial mutations in polyploid populations (Otto and Whitton, 2000). The ploidy state can vary between chromosomal doublings and whole genome duplication and can also include different genomes as a result of interspecies hybridization. Interspecific hybridization might start with one genome from each parent (50:50), but recombination and gene conversion may eventually lead to unequal contributions (Mallet, 2007). Interspecies hybrids can lead to dominant phenotypes

resulting from the combination of the parental genomes. Hybridizations between closely-related fungal species play major roles in the generation of new species to invade new host plants (Brasier *et al.*, 1999, Staats *et al.*, 2004). Increasing the ploidy of *V. longisporum* by hybridization between *V. dahliae* and *V. albo-atrum* might already change the spore size. This is supported by laboratory interspecies hybridization between *V. dahliae* and *V. albo-atrum* (Typas, 1983). Some of these artificial diploid hybrids produced long spores (8.2 ± 0.2 µm) similar to the spores of *V. longisporum*. It is currently unclear whether the increased spore size represents a trait which facilitates infection of cruciferous hosts.

4.4.3. Loss of duplicated genes and accumulation of mutations during speciation

Polyploidy results in duplicated genes where not necessarily both genes have to remain active. Both transcription factors encoding isogene pairs that we have analyzed seem to be expressed because we found for each a corresponding cDNA derived from the transcripts. Diploidization allows that one gene could retain its original function whereas the other copy may become epigenetically silenced or inactivated by mutations. Duplicated genes might also encounter recombination, which changes the genetic locus in a concerted evolution (Wendel, 2000). Physical elimination of a parental genome-specific repeated DNA occurred in wheat in amphiploids in early generations. This may play a role in the initial stabilization of the nascent amphiploid plant (Han *et al.*, 2005).

Repeated genes might homogenize and the rDNA repeats are prominent examples where one variant of can become dominant via mutations. Homogenization is caused by deletion events or continuous multiplication during unequal recombination. Single nucleotide polymorphisms are present in ribosomal genes with an unexpectedly high amount (Simon and Weiß, 2008). rDNA repeats may accumulate point mutations to avoid homogenization to a certain degree (Ganley and Kobayashi, 2007). Interspecific hybrids of cotton, show changes in the number of rDNA repeats within a locus and little heterogeneity in the internal transcribed spacer regions (Wendel, 2000). Our analysis of *V. longisporum* showed that homogenization of rDNA genes can shift into both directions in nature further supporting that present *V. longisporum* isolates represent an early state of species formation.

4.4.4. Role of horizontal gene transfer

Horizontal gene transfer plays an important role during evolution. All *V. longisporum* of the *V. albo-atrum* rDNA type possess a group I intron of 839 bp in the 18S

rRNA gene. This intron has not been found in other *Verticillium* isolates and might represent a foreign DNA provided by horizontal gene transfer. Such horizontal transfers between fungal species have been proposed for group I introns based on their phylogenetic distribution. Introns are highly dynamic in fungal genomes with characteristics reminiscent to transposable elements. Fungi carry retrotransposable elements in the form of group I or II introns with a specific RNA secondary structure providing a self-splicing pathway (Aguileta *et al.*, 2009).

We conclude from our data that the interspecies hybrid *V. longisporum* which specifically infects crops of the *Brassicaceae* represents a snapshot for speciation (Figure 43). The hybridization event between *V. dahliae* and *V. albo-atrum* generated a diploid interspecific hybrid (2n). We cannot discriminate whether a specific *V. albo-atrum* strain has accumulated some mutations prior to hybridization or whether these changes have happened after hybridization. These minor deviations which are present in all *V. longisporum* isolates support that the original hybridization was rare or might even have happened only once. Interestingly, repeated DNA homogenization represents a distinct second step and can shift in both directions. Isolates lost either the ribosomal DNA set derived from *V. dahliae* or from the *V. albo-atrum* parent. Speciation is a highly dynamic process and might end in rehaploidization. Two natural isolates of *V. longisporum* from rape and sugarbeet were forced to generate haploid segregants by treatment with the haploidizing agent *p*-fluorophenylalanine. These haploid segregants produced relatively short spores and their nuclear DNA content was halved resulting in similar values as *V. dahliae* or *V. albo-atrum* (Jackson and Heale, 1985). Haploid *Verticillium* isolates were described from crucifers which were shown to be distinct from *V. dahliae* as well as from *V. albo-atrum* (Collins *et al*., 2003; Qin *et al*., 2006). It will be interesting to monitor the future development of *V. longipsorum* to determine the kinetics of species formation which might correlate with a reduction of the genome size. In addition, it is still an open question which factors changed host specificity of the hybrid: this might include changes in gene dosage of specific genes, the accumulation of specific mutations in one copy of distinct isogene pairs or combinatory effects resulting from the combination of the *V. albo-atrum* and the *V. dahliae* genomes. A further reduction of the genome size might disclose these host-specificity factors which are still hidden in the 1.8 fold genome size of *V. longisporum*.

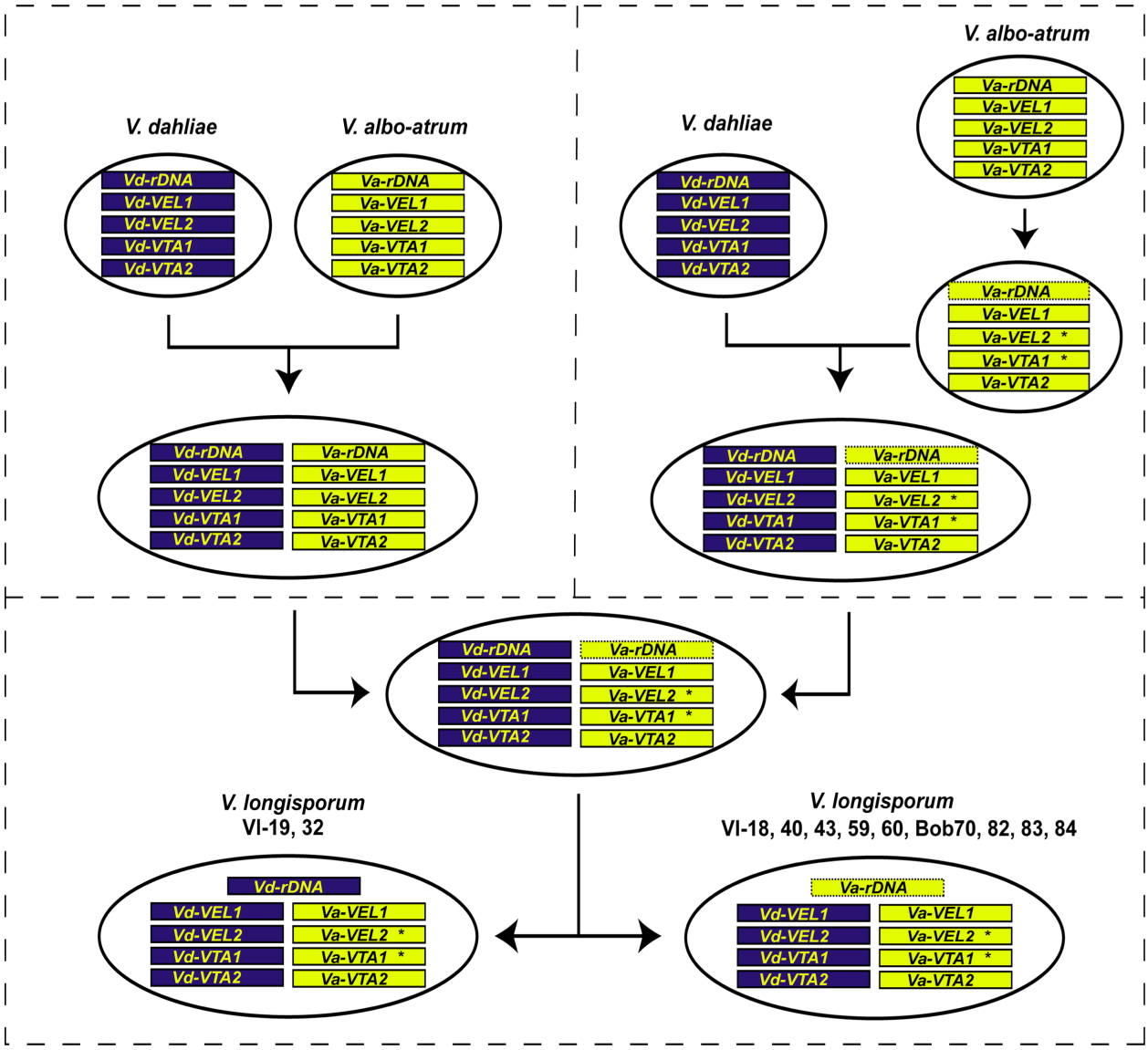

Figure 45. Current status of the evolution of the crucifer pathogen *V. longisporum* species. The interspecies hybridization event has occurred between *V. dahliae* and *V. albo-atrum* or a variant of *V. albo-atrum* with some characteristic mutations. These mutations which could also be accumulated after hybridization suggest a single initial fusion event. Homogenisation of rDNA represents a later step in speciation which has happened at least twice resulting in a *V. dahliae* or *V. albo-atrum* rDNA type. Asterisks indicate characteristic point mutations, the dashed box symbolizes the changes in rDNA compared to isolated *V. albo-atrum* strains.

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Curriculum vitae

Van Tuan Tran

Born on January 12, 1978 in Namdinh, Vietnam

Education

1984-1993	Primary education in Namdinh, Vietnam
1993-1996	Secondary education in Namdinh, Vietnam

Scientific background

1996-2000	Bachelor of Science (B.Sc.) in Biotechnology at Hanoi University of Science, Vietnam.
2001-2003	Master of Science (M.Sc.) in Microbiology at Hanoi University of Science, Vietnam.
2000-2006	Lecturer of Microbiology at Hanoi University of Science, Vietnam.
10/2006-4/2011	PhD thesis at the Department of Molecular Microbiology and Genetics, Institute of Microbiology and Genetics, Georg-August University of Göttingen, Germany.

SUPPLEMENTARY DATA

For the Section 3.4: Snapshot of speciation: the origin of the recently evolved fungal rapeseed pathogen *Verticillium longisporum*

A (IGS)

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Vd-2        -----------------CGGCACCTAGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 42
Vd-8        -----------------CGGCACCTAGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 42
Vd-13       -------------------GCACCTAGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 40
Vd-39       ---------------CCCGGCACCTAGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 44
Vd-52       -----------------CGGCACCTAGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 42
Vd-73       ------CCTCGATAACCCGGCACCTAGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 53
Vd-89       -------------------GCACCTAGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 40
Vl-19       -------------------GGCACCTAGTGCCGCGATATGGAGGGGTTGAGATGGGGTAGA 42
Vl-32       -------------------GCACCTAGTGCCGCGATATGGAGGGGTTGAGATGGGGTAGA 41
Vl-18       -----------------CTGGTACCTGGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 43
Vl-40       ------CCTCAATAACCTGGTACCTGGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 53
Vl-43       ------CCTCAATAACCTGGTACCTGGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 53
Vl-60       ------TCTCAT--ACCTGGTACCTGGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 51
Vl-Bob70    ------CCTCAATAACCTGGTACCTGGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 53
Vl-82       -------CTCAT--ACCTGGTACCTGGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 50
Vl-83       TCCGATTCTCAT--ACCTGGTACCTGGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 57
Vl-84       ----ATTCTCAT--ACCTGGTACCTGGTGCCGCGATATGGAGGGGTTGAGATGGG-TAGA 53
Va-1        ------CCTCAATAACCTGGCACCTGGTGCTGCGATATAGAGGCGTTGAGATGGG-TAGA 53
Va-2        ----------------TTGGCACCTGGTGCTGCGATATAGAGGCGTTGAGATGGG-TAGA 43
Va-3        -----------------TGGCACCTGGTGCTGCGATATAGAGGCGTTGAGATGGG-TAGA 42
Va-4        ---------------CCTGGCACCTGGTGCTGCGATATAGAGGCGTTGAGATGGG-TAGA 44
                           * **** **** ******* **** *********** ****

Vd-2        G---------GGGAAGGCGGAGGAGGG-------------GGGGAAGAGAGAGCGAGAGT 80
Vd-8        G---------GGGAAGGCGGAGGAGGG-------------GGGGAAGAGAGAGCGAGAGT 80
Vd-13       G---------GGGAAGGCGGAGGAGGG-------------GGGGAAGAGAGAGCGAGAGT 78
Vd-39       G---------GGGAAGGCGGAGGAGGG-------------GGGGAAGAGAGAGCGAGAGT 82
Vd-52       G---------GGGAAGACGGAGGAGGG-------------GGGGAAGAGAGAGCGAGAGT 80
Vd-73       G---------GGGAAGGCGGAGGAGGG-------------GGGGAAGAGAGAGCGAGAGT 91
Vd-89       G---------GGGAAGACGGAGGAGGG-------------GGGGAAGAGAGAGCGAGAGT 78
Vl-19       G---------GGGAAGGCGGAGGAGGG-------------GGGGAAGAGAGAGCGAGAGT 80
Vl-32       G---------GGGAAGGCGGAGGAGGG-------------GGGGAAGAGAGAGCGAGAGT 79
Vl-18       GAGG-AGAGGGGGAGAGTGGAAGGCGGAGGA----GGGAGGGAGAGAAGGAAGAGCGAGT 98
Vl-40       GAGG-AGAGGGGGAGAGTGGAAGGCGGAGGA----GGGAGGGAGAGAAGGAAGAGCGAGT 108
Vl-43       GAGG-AGAGGGGGAGAGTGGAAGGCGGAGGA----GGGAGGGAGAGAAGGAAGAGCGAGT 108
Vl-60       GAGG-AGAGGGGGAGAGTGGAAGGCGGAGGA----GGGAGGGAGAGAAGGAAGAGCGAGT 106
Vl-Bob70    GAGG-AGAGGGGGAGAGTGGAAGGCGGAGGA----GGGAGGGAGAGAAGGAAGAGCGAGT 108
Vl-82       GAGG-AGAGGGGGAGAGTGGAAGGCGGAGGA----GGGAGGGAGAGAAGGAAGAGCGAGT 105
Vl-83       GAGG-AGAGGGGGAGAGTGGAAGGCGGAGGA----GGGAGGGAGAGAAGGAAGAGCGAGT 112
Vl-84       GAGG-AGAGGGGGAGAGTGGAAGGCGGAGGA----GGGAGGGAGAGAAGGAAGAGCGAGT 108
Va-1        GAGGGAGAAGGGGAAGGCGGAGGAGGGAAGAAGAGGGGGGGGGGGAGATGGAAGAGCGAGT 113
Va-2        GAGG-AGAAGGGGAAGGCGGAAGAGGG-------------GGGGAGATGGAAGAGCGAGT 89
Va-3        GAGGGAGAAGGGGAAGGCGGAGGAGGGAAGAAGAGGGGGGGGGGGAGATGGAAGAGCGAGT 102
Va-4        GAGGGAGAAGGGGAAGGCGGAGGAGGGAAGAAGAGGGGGGGGGGGAGATGGAAGAGCGAGT 104
            *         ****    *** *  **             ** **   *  ** * ****

Vd-2        CCGCACTC--AGCGCCTTGC-TCTAGC-GACCTCGCCGCTATGTCGCCGCGTTTTCGCTG 136
Vd-8        CCGCACTC--AGCGCCTTGC-TCTAGC-GACCTCGCCGCTATGTCGCCGCGTTTTCGCTG 136
Vd-13       CCGCACTC--AGCGCCTTGC-TCTAGC-GACCTCGCCGCTATGTCGCCGCGTTTTCGCTG 134
Vd-39       CCGCACTC--AGCGCCTTGC-TCTAGC-GACCTCGCCGCTATGTCGCCGCGTTTTCGCTG 138
Vd-73       CCGCACTC--AGCGCCTTGC-TCTAGC-GACCTCGCCGCTATGTCGCCGCGTTTTCGCTG 147
Vd-52       CCGCATTC--AGCGCCTTGC-TCTAGC-GACCTCGCCGCTATGTCGCCGCGTTTTCGCTG 136
Vd-89       CCGCATTC--AGCGCCTTGC-TCTAGC-GACCTCGCCGCTATGTCGCCGCGTTTTCGCTG 134
Vl-19       CCGCACTC--AGCGCCTTGC-TCTAGC-GACCTCGCCGCTATGTCGCCGCGTTTTCGCTG 136
Vl-32       CCGCACTC--AGCGCCTTGC-TCTAGC-GACCTCGCCGCTATGTCGCCGCGTTTTCGCTG 135
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Vl-18       CCACACC---GGCACCTTGC-TCTGGC-GACCT---TGCTGTCTCGCCGCGTTTTCGCTG 150
Vl-40       CCACACC---GGCACCTTGC-TCTGGC-GACCT---TGCTGTCTCGCCGCGTTTTCGCTG 160
Vl-43       CCACACC---GGCACCTTGC-TCTGGC-GACCT---TGCTGTCTCGCCGCGTTTTCGCTG 160
Vl-60       CCACACC---GGCACCTTGC-TCTGGC-GACCT---TGCTGTCTCGCCGCGTTTTCGCTG 158
Vl-Bob70    CCACACC---GGCACCTTGC-TCTGGC-GACCT---TGCTGTCTCGCCGCGTTTTCGCTG 160
Vl-82       CCACACC---GGCACCTTGC-TCTGGC-GACCT---TGCTGTCTCGCCGCGTTTTCGCTG 157
Vl-83       CCACACC---GGCACCTTGC-TCTGGC-GACCT---TGCTGTCTCGCCGCGTTTTCGCTG 164
Vl-84       CCACACC---GGCACCTTGC-TCTGGC-GACCT---TGCTGTCTCGCCGCGTTTTCGCTG 160
Va-1        CCATGCCCTGGGCACCTTGCTTCTTGCAGACCTCTTTGTCGTCTGGCCGCGTCTTCGCTG 173
Va-2        CCATGCC-TGGGCACCTTGACTCTTGCAGACCTCCTTGTCGTCTCGCCGCGTCTTCGCTG 148
Va-3        CCATGCCCTGGGCACCTTGCTTCTTGCAGACCTCTTTGTCGTCTGGCCGCGTCTTCGCTG 162
Va-4        CCATGCCCTGGGCACCTTGCTTCTTGCAGACCTCTTTGTCGTCTGGCCGCGTCTTCGCTG 164
            **         ** *****  *** ** *****    *   * * ******* *******

Vd-2        TGCGTTGCAAGGCAGACGCCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 196
Vd-8        TGCGTTGCAAGGCAGACGCCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 196
Vd-13       TGCGTTGCAAGGCAGACGCCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 194
Vd-39       TGCGTTGCAAGGCAGACGCCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 198
Vd-52       TGCGTTGCAAGGCAGACGCCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 196
Vd-73       TGCGTTGCAAGGCAGACGCCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 207
Vd-89       TGCGTTGCAAGGCAGACGCCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 194
Vl-19       TGCGTTGCAAGGCAGACGCCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 196
Vl-32       TGCGTTGCAAGGCAGACGCCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 195
Vl-18       TGCGTTGCAAGGCAAACACCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 210
Vl-40       TGCGTTGCAAGGCAAACACCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 220
Vl-43       TGCGTTGCAAGGCAAACACCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 220
Vl-60       TGCGTTGCAAGGCAAACACCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 218
Vl-Bob70    TGCGTTGCAAGGCAAACACCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 220
Vl-82       TGCGTTGCAAGGCAAACACCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 217
Vl-83       TGCGTTGCAAGGCAAACACCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 224
Vl-84       TGCGTTGCAAGGCAAACACCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 220
Va-1        TGCGTTGCAAGGCAAACACCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 233
Va-2        TGCGTTGCAAGGCAAACAC---------GGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 199
Va-3        TGCGTTGCAAGGCAAACACCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 222
Va-4        TGCGTTGCAAGGCAAACACCGCTGTCACGGCCGCCTACGGCGTCCGCTGCCGCGGACTAC 224
            ************** ** *         ********************************

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Vd-8        GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCCGGGGCCCGAAAGATTTTTTTTCTC 256
Vd-13       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCCGGGGCCCGAAAGATTTTTTTTCTC 254
Vd-39       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCCGGGGCCCGAAAGATTTTTTTTCTC 258
Vd-52       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCCGGGGCCCGAAAGATTTTTTTTCTC 256
Vd-73       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCCGGGGCCCGAAAGATTTTTTTTCTC 267
Vd-89       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCCGGGGCCCGAAAGATTTTTTTTCTC 254
Vl-19       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCCGGGGCCCGAAAGATTTTTTTTCTC 256
Vl-32       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCCGGGGCCCGAAAGATTTTTTTTCTC 255
Vl-18       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCAGAGGGGCCCGAAAGATTTTTTTTCTC 270
Vl-40       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCAGAGGGGCCCGAAAGATTTTTTTTCTC 280
Vl-43       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCAGAGGGGCCCGAAAGATTTTTTTTCTC 280
Vl-60       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCAGAGGGGCCCGAAAGATTTTTTTTCTC 278
Vl-Bob70    GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCAGAGGGGCCCGAAAGATTTTTTTTCTC 280
Vl-82       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCAGAGGGGCCCGAAAGATTTTTTTTCTC 277
Vl-83       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCAGAGGGGCCCGAAAGATTTTTTTTCTC 284
Vl-84       GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCAGAGGGGCCCGAAAGATTTTTTTTCTC 280
Va-1        GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCTGGGGCCCGAGAGATTTTTTTTCTC 293
Va-2        GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCTGGGGCCCGAGAGATTTTTTTTCTC 259
Va-3        GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCTGGGGCCCGAGAGATTTTTTTTCTC 282
Va-4        GCGCCTGCGGCGCGGGATCTTTCAGGGGGCCCCTCTGGGGCCCGAGAGATTTTTTTTCTC 284
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Vd-2        AATTCCCGGGTAGGTGGTCTCTGAGAGTGGCCAGAGACAGCCCCAAGTCCTACCATCTAT 316
Vd-8        AATTCCCGGGTAGGTGGTCTCTGAGAGTGGCCAGAGACAGCCCCAAGTCCTACCATCTAT 316
Vd-13       AATTCCCGGGTAGGTGGTCTCTGAGAGTGGCCAGAGACAGCCCCAAGTCCTACCATCTAT 314
Vd-39       AATTCCCGGGTAGGTGGTCTCTGAGAGTGGCCAGAGACAGCCCCAAGTCCTACCATCTAT 318
Vd-52       AATTCCCGGGTAGGTGGTCTCTGAGAGTGGCCAGAGACAACCCCAAGTCCTACCATCTAT 316
Vd-73       AATTCCCGGGTAGGTGGTCTCTGAGAGTGGCCAGAGACAGCCCCAAGTCCTACCATCTAT 327
Vd-89       AATTCCCGGGTAGGTGGTCTCTGAGAGTGGCCAGAGACAACCCCAAGTCCTACCATCTAT 314
Vl-19       AATTCCCGGGTAGGTGGTCTCTGAGAGTGGCCAGAGACAGCCCCAAGTCCTACCATCTAT 316
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Vl-32       AATTCCCGGGTAGGTGGTCTCTGAGAGTGGCCAGAGACAGCCCCAAGTCCTACCATCTAT 315
Vl-18       AATTCCCGGGTAGCTGGTCTCTGGAAGTGGCCAGAGACAGCCC-AAGTCCTACCATCTAT 329
Vl-40       AATTCCCGGGTAGCTGGTCTCTGGAAGTGGCCAGAGACAGCCC-AAGTCCTACCATCTAT 339
Vl-43       AATTCCCGGGTAGCTGGTCTCTGGAAGTGGCCAGAGACAGCCC-AAGTCCTACCATCTAT 339
Vl-60       AATTCCCGGGTAGCTGGTCTCTGGAAGTGGCCAGAGACAGCCC-AAGTCCTACCATCTAT 337
Vl-Bob70    AATTCCCGGGTAGCTGGTCTCTGGAAGTGGCCAGAGACAGCCC-AAGTCCTACCATCTAT 339
Vl-82       AATTCCCGGGTAGCTGGTCTCTGGAAGTGGCCAGAGACAGCCC-AAGTCCTACCATCTAT 336
Vl-83       AATTCCCGGGTAGCTGGTCTCTGGAAGTGGCCAGAGACAGCCC-AAGTCCTACCATCTAT 343
Vl-84       AATTCCCGGGTAGCTGGTCTCTGGAAGTGGCCAGAGACAGCCC-AAGTCCTACCATCTAT 339
Va-1        AATTCCCGGGTAGGTGGTGTCTGGAAGTGGCCAGAGA----------TCCTACCATCTAT 343
Va-2        AATTCCCGGGTAGGAGGTGTCTGGAAGTGGCCAGAGACGACCC-AAGTCCTACCATCTAT 318
Va-3        AATTCCCGGGTAGGTGGTGTCTGGAAGTGGCCAGAGA----------TCCTACCATCTAT 332
Va-4        AATTCCCGGGTAGGTGGTGTCTGGAAGTGGCCAGAGA----------TCCTACCATCTAT 334
            *************  *** ****  ************          *************

Vd-2        GGAGGTGGTGGGGTTTTT-GGCGTCAAGGTGGAAAGCTACCCGGGAATTGGA-------- 367
Vd-8        GGAGGTGGTGGGGTTTTT-GGCGTCAAGGTGGAAAGCTACCCGGGAATTGGA-------- 367
Vd-13       GGAGGTGGTGGGGTTTTT-GGCGTCAAGGTGGAAAGCTACCCGGGAATTGGA-------- 365
Vd-39       GGAGGTGGTGGGGTTTTT-GGCGTCAAGGTGGAAAGCTACCCGGGAATTGGA-------- 369
Vd-52       GGAGGTGGTGGGGTTTTT-GGCGTCAAGGTGGAAAGCTACCCGGGAATTGGA-------- 367
Vd-73       GGAGGTGGTGGGGTTTTT-GGCGTCAAGGTGGAAAGCTACCCGGGAATTGGA-------- 378
Vd-89       GGAGGTGGTGGGGTTTTT-GGCGTCAAGGTGGAAAGCTACCCGGGAATTGGA-------- 365
Vl-19       GGAGGTGGTGGGGTTTTT-GGCGTCAAGGTGGAAAGCTACCCGGGAATTGGA-------- 367
Vl-32       GGAGGTGGTGGGGTTTTT-GGCGTCAAGGTGGAAAGCTACCCGGGAATTGGA-------- 366
Vl-18       GGAGGTGGTGGGATTTTTTGGCGTCAAGGTGGTCAGCTACCCGGGAGTTGGG--TGAAAA 387
Vl-40       GGAGGTGGTGGGATTTTTTGGCGTCAAGGTGGTCAGCTACCCGGGAGTTGGG--TGAAAA 397
Vl-43       GGAGGTGGTGGGATTTTTTGGCGTCAAGGTGGTCAGCTACCCGGGAGTTGGG--TGAAAA 397
Vl-60       GGAGGTGGTGGGATTTTTTGGCGTCAAGGTGGTCAGCTACCCGGGAGTTGGG--TGAAAA 395
Vl-Bob70    GGAGGTGGTGGGATTTTTTGGCGTCAAGGTGGTCAGCTACCCGGGAGTTGGG--TGAAAA 397
Vl-82       GGAGGTGGTGGGATTTTTTGGCGTCAAGGTGGTCAGCTACCCGGGAGTTGGG--TGAAAA 394
Vl-83       GGAGGTGGTGGGATTTTTTGGCGTCAAGGTGGTCAGCTACCCGGGAGTTGGG--TGAAAA 401
Vl-84       GGAGGTGGTGGGATTTTTTGGCGTCAAGGTGGTCAGCTACCCGGGAGTTGGG--TGAAAA 397
Va-1        GGAGGTGGTGGGATTTTTTGGCGTCAAGGTGGACAGCTACCCGGGAATTGGACCTGAAAA 403
Va-2        GGAGGTGGTGGGATTTTTTGGCGTCAAGGTGGACAGCTACCCGGGAATTGGACCTGAAAA 378
Va-3        GGAGGTGGGGGGATTTTTTGGCGTCAAGGTGGACAGCTACCCGGGAATTGGACCTGAAAA 392
Va-4        GGAGGTGGTGGGATTTTTTGGCGTCAAGGTGGACAGCTACCCGGGAATTGGACCTGAAAA 394
            ******** *** ***** *************  ************ ****

Vd-2        ----CCAGTTTTGA-GGCT-----------------------------GGCAGCTACCCG 393
Vd-8        ----CCAGTTTTGA-GGCT-----------------------------GGCAGCTACCCG 393
Vd-13       ----CCAGTTTTGA-GGCT-----------------------------GGCAGCTACCCG 391
Vd-39       ----CCAGTTTTGA-GGCT-----------------------------GGCAGCTACCCG 395
Vd-52       ----CCAGTTTCGA-GGCT-----------------------------GGCAGCTACCCG 393
Vd-73       ----CCAGTTTTGA-GGCT-----------------------------GGCAGCTACCCG 404
Vd-89       ----CCAGTTTCGA-GGCT-----------------------------GGCAGCTACCCG 391
Vl-19       ----CCAGTTTTGA-GGCT-----------------------------GGCAGCTACCCG 393
Vl-32       ----CCAGTTTTGA-GGCT-----------------------------GGCAGCTACCCG 392
Vl-18       ACGACCAAGTCAGACATCT-----------------------------GGGAGCTACCCG 418
Vl-40       ACGACCAAGTCAGACATCT-----------------------------GGGAGCTACCCG 428
Vl-43       ACGACCAAGTCAGACATCT-----------------------------GGGAGCTACCCG 428
Vl-60       ACGACCAAGTCAGACATCT-----------------------------GGGAGCTACCCG 426
Vl-Bob70    ACGACCAAGTCAGACATCT-----------------------------GGGAGCTACCCG 428
Vl-82       ACGACCAAGTCAGACATCT-----------------------------GGGAGCTACCCG 425
Vl-83       ACGACCAAGTCAGACATCT-----------------------------GGGAGCTACCCG 432
Vl-84       ACGACCAAGTCAGACATCT-----------------------------GGGAGCTACCCG 428
Va-1        CTGGCCAAGTCGGACAGCTACCCGGGAATTGGAACCAGTGCTGACGCTGGCAGCTACCCG 463
Va-2        CTGGCCAAGTCGGACAGCTACCCGGGAATTGGAACCAATGGTGACGCTGGCAGCTACCCG 438
Va-3        CTGGCCAAGTCGGACAGCTACCCGGGAATTGGAACCAGTGCTGACGCTGGCAGCTACCCG 452
Va-4        CTGGCCAAGTCGGACAGCTACCCGGGAATTGGAACCAGTGCTGACGCTGGCAGCTACCCG 454
                ***  *  **   **                                ** *********

Vd-2        GGA-------------------------------------GTTGGCTGAAAAACGACCAAG 417
Vd-8        GGA-------------------------------------GTTGGCTGAAAAACGACCAAG 417
Vd-13       GGA-------------------------------------GTTGGCTGAAAAACGACCAAG 415
Vd-39       GGA-------------------------------------GTTGGCTGAAAAACGACCAAG 419
Vd-52       GGA-------------------------------------GTTGGCTGAAAAACGACCAAG 417
Vd-73       GGA-------------------------------------GTTGGCTGAAAAACGACCAAG 428
Vd-89       GGA-------------------------------------GTTGGCTGAAAAACGACCAAG 415
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Vl-19       GGA-----------------------------------GTTGGCTGAAAAACGACCAAG 417
Vl-32       GGA-----------------------------------GTTGGCTGAAAAACGACCAAG 416
Vl-18       GGA-----------------------------------GTTGG-----AAAT------- 430
Vl-40       GGA-----------------------------------GTTGG-----AAAT------- 440
Vl-43       GGA-----------------------------------GTTGG-----AAAT------- 440
Vl-60       GGA-----------------------------------GTTGG-----AAAT------- 438
Vl-Bob70    GGA-----------------------------------GTTGG-----AAAT------- 440
Vl-82       GGA-----------------------------------GTTGG-----AAAT------- 437
Vl-83       GGA-----------------------------------GTTGG-----AAAT------- 444
Vl-84       GGA-----------------------------------GTTGG-----AAAT------- 440
Va-1        GGA-----------------------------------GTTGGCCGGAAAACGACCAAG 487
Va-2        GGAATTGGAACCAATGGTGACGCTGGCAGCTACCCGGGAGTTGGCCGAAAAGCGACCAAG 498
Va-3        GGA-----------------------------------GTTGGCCGGAAAACGACCAAG 476
Va-4        GGA-----------------------------------GTTGGCCGGAAAACGACCAAG 478
            ***                                     *****     **

Vd-2        TCGGACACC----------TTG-GAGCTACCCGGGAATTGG-----------------A 448
Vd-8        TCGGACACC----------TTG-GAGCTACCCGGGAATTGG-----------------A 448
Vd-13       TCGGACACC----------TTG-GAGCTACCCGGGAATTGGGAGCTACCCGGGAATTGGA 464
Vd-39       TCGGACACC----------TTG-GAGCTACCCGGGAATTGGGAGCT-------------A 455
Vd-52       TCGGACACC----------CTG-GAGCTACCCGGGAATTGG-----------------A 448
Vd-73       TCGGACACC----------TTG-GAGCTACCCGGGAATTGG-----------------A 459
Vd-89       TCGGACACC----------CTG-GAGCTACCCGGGAATTGG-----------------A 446
Vl-19       TCGGACACC----------TTG-GAGCTACCCGGGAATTGGGAGCT-------------A 453
Vl-32       TCGGACACC----------TTG-GAGCTACCCGGGAATTGGGAGCT-------------A 452
Vl-18       TTGGAGAAC------GGATCTGGGAGCTACCCGGGAGTTGGA------------------ 466
Vl-40       TTGGAGAAC------GGATCTGGGAGCTACCCGGGAGTTGGA------------------ 476
Vl-43       TTGGAGAAC------GGATCTGGGAGCTACCCGGGAGTTGGA------------------ 476
Vl-60       TTGGAGAAC------GGATCTGGGAGCTACCCGGGAGTTGGA------------------ 474
Vl-Bob70    TTGGAGAAC------GGATCTGGGAGCTACCCGGGAGTTGGA------------------ 476
Vl-82       TTGGAGAAC------GGATCTGGGAGCTACCCGGGAGTTGGA------------------ 473
Vl-83       TTGGAGAAC------GGATCTGGGAGCTACCCGGGAGTTGGA------------------ 480
Vl-84       TTGGAGAAC------GGATCTGGGAGCTACCCGGGAGTTGGA------------------ 476
Va-1        TCGGACAGCTACCCTGGAATTGCGGGCTACCCGGGAATTGGG------------------ 529
Va-2        TCGGACAGCTACCCGGGAATTGCGAGCTACCCGGGAATTGGG------------------ 540
Va-3        TCGGACAGCTACCCTGGAATTGCGGGCTACCCGGGAATTGGG------------------ 518
Va-4        TCGGACAGCTACCCTGGAATTGCGGGCTACCCGGGAATTGGG------------------ 520
            * *** * *           ** * *********** ****

Vd-2        CC----AGTTTTGAGGCTGG----CAGCTACCCGGGAGTTGGCTGAAAAA-CGACCAAGT 499
Vd-8        CC----AGTTTTGAGGCTGG----CAGCTACCCGGGAGTTGGCTGAAAAA-CGACCAAGT 499
Vd-13       CC----AGTTTTGAGGCTGG----CAGCTACCCGGGAGTTGGCTGAAAAA-CGACCAAGT 515
Vd-39       CCCGGGAATTGGAAATTTGGAGAACGGATTTTGGTGAGTTGGCTGAAAAAACGACCAAGT 515
Vd-52       CC----AGTTTCGAGGCTGG----CAGCTACCCGGGAGTTGGCTGAAAAA-CGACCAAGT 499
Vd-73       CC----AGTTTTGAGGCTGG----CAGCTACCCGGGAGTTGGCTGAAAAA-CGACCAAGT 510
Vd-89       CC----AGTTTCGAGGCTGG----CAGCTACCCGGGAGTTGGCTGAAAAA-CGACCAAGT 497
Vl-19       CCCGGGAATTGGAAATTTGGAGAACGGATTTTGGTGAGTTGGCTGAAAAAACGACCAAGT 513
Vl-32       CCCGGGAATTGGAAATTTGGAGAACGGATTTTGGTGAGTTGGCTGAAAAAACGACCAAGT 512
Vl-Bob70    ------AATTTGGAGAACGG-------AT-------------------------------- 492
Vl-60       ------AATTTGGAGAACGG-------AT-------------------------------- 490
Vl-43       ------AATTTGGAGAACGG-------AT-------------------------------- 492
Vl-40       ------AATTTGGAGAACGG-------AT-------------------------------- 492
Vl-82       ------AATTTGGAGAACGG-------AT-------------------------------- 489
Vl-83       ------AATTTGGAGAACGG-------AT-------------------------------- 496
Vl-84       ------AATTTGGAGAACGG-------AT-------------------------------- 492
Vl-18       ------AATTTGGAGAACGG-------AT-------------------------------- 482
Va-1        ------AATCTGGAGAACGG-------ATTTTGGGTAGTTGGCTGCAAGA-CGGCCAAGT 575
Va-2        ------AATCTGGAGAACGG-------ATTTTGGGTAGTTGGCTGCAAGA-CGGCCAAGT 586
Va-3        ------AATCTGGAGAACGG-------ATTTTGGGTAGTTGGCTGCAAGA-CGGCCAAGT 564
Va-4        ------AATCTGGAGAACGG-------ATTTTGGGTAGTTGGCTGCAAGA-CGGCCAAGT 566
                  * *    *    **        *

Vd-2        CGGACACC----------------------------------------------------- 507
Vd-8        CGGACACC----------------------------------------------------- 507
Vd-13       CGGACACC----------------------------------------------------- 523
Vd-39       CAGACATC----------------------------------------------------- 523
Vd-52       CGGACACC----------------------------------------------------- 507
Vd-73       CGGACACC----------------------------------------------------- 518
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Vd-89       CGGACACCCTGGAGCTACCCGGGAATTGGACCAGTTTCGAGGCTGGCAGCTACCCGGGAG 557
Vl-19       CAGACATC------------------------------------------------------ 521
Vl-32       CAGACATC------------------------------------------------------ 520
Vl-18       -------C------------------------------------------------------ 483
Vl-40       -------C------------------------------------------------------ 493
Vl-43       -------C------------------------------------------------------ 493
Vl-60       -------C------------------------------------------------------ 491
Vl-Bob70    -------C------------------------------------------------------ 493
Vl-82       -------C------------------------------------------------------ 490
Vl-83       -------C------------------------------------------------------ 497
Vl-84       -------C------------------------------------------------------ 493
Va-1        CAGACACC------------------------------------------------------ 583
Va-2        CAGACACC------------------------------------------------------ 594
Va-3        CAGACACC------------------------------------------------------ 572
Va-4        CAGACACC------------------------------------------------------ 574
                   *

Vd-2        ----------------------------TTGGAGCTACCCGGGAATTGGACCAGTTTTG 538
Vd-8        ----------------------------TTGGAGCTACCCGGGAATTGG---------- 528
Vd-13       ----------------------------TTGGAGCTACCCGGGAATTGGG--------- 545
Vd-39       ----------------------------TGGGAGCTACCCTGGAATTGGGA-------- 546
Vd-52       ----------------------------TTGGAGCTACCCGGGAATTGGGA-------- 530
Vd-89       TTGGCTGAAAAACGACCAAGTCGGACACCTTGGAGCTACCCGGGAATTGGGA-------- 609
Vd-73       ----------------------------TTGGAGCTACCCGGGAATTGG---------- 539
Vl-19       ----------------------------TGGGAGCTACCCTGGAATTGGGA-------- 544
Vl-32       ----------------------------TGGGAGCTACCCTGGAATTGGGA-------- 543
Vl-18       ----------------------------TGGGAGCTACCCGGGAGTTGGAA-------- 506
Vl-40       ----------------------------TGGGAGCTACCCGGGAGTTGGAA-------- 516
Vl-43       ----------------------------TGGGAGCTACCCGGGAGTTGGAA-------- 516
Vl-60       ----------------------------TGGGAGCTACCCGGGAGTTGGAA-------- 514
Vl-Bob70    ----------------------------TGGGAGCTACCCGGGAGTTGGAA-------- 516
Vl-82       ----------------------------TGGGAGCTACCCGGGAGTTGGAA-------- 513
Vl-83       ----------------------------TGGGAGCTACCCGGGAGTTGGAA-------- 520
Vl-84       ----------------------------TGGGAGCTACCCGGGAGTTGGAA-------- 516
Va-1        ----------------------------TGGGAGCTACCCGGGAATTGGGA-------- 606
Va-2        ----------------------------TGGGAGCTACCCGGGAGTTGAGG-------- 617
Va-3        ----------------------------TGGGAGCTACCCGGGAATTGGGA-------- 595
Va-4        ----------------------------TGGGAGCTACCCGGGAATTGGGA-------- 597
                                        * ********** *** ***

Vd-2        AGGCTGGCAGCTACCCGGGAGTTGGCTGAAAAACGACCAAGTCGGACACCTTGGAGCTAC 598
Vd-8        ------------------------------------------------------------
Vd-13       ------------------------------------------------------------
Vd-39       ------------------------------------------------------------
Vd-52       ----------------------------------------------------------AT 532
Vd-73       ------------------------------------------------------------
Vd-89       ----------------------------------------------------------AT 611
Vl-19       ------------------------------------------------------------
Vl-32       ------------------------------------------------------------
Vl-18       ---------ATT----TGGA------------------------------------------ 513
Vl-40       ---------ATT----TGGA------------------------------------------ 523
Vl-43       ---------ATT----TGGA------------------------------------------ 523
Vl-60       ---------ATT----TGGAGAACGG----------------------ATCTGGGAGCTAC 540
Vl-Bob70    ---------ATT----TGGAGAACGG----------------------ATCTGGGAGCTAC 542
Vl-82       ---------ATT----TGGA------------------------------------------ 520
Vl-83       ---------ATT----TGGA------------------------------------------ 527
Vl-84       ---------ATT----TGGA------------------------------------------ 523
Va-1        ---------GCTACCCTGGAA----------------------------TTGCGGGCTAC 629
Va-2        ---------GCTACCCGGGAA----------------------------TTGGGGGCTAC 640
Va-3        ---------GCTACCCTGGAA----------------------------TTGCGGGCTAC 618
Va-4        ---------GCTACCC---------------------------------------------- 604



Vd-2        CCGGGAATTGGACCATTTTTGAGGCTGGCAGCTACACGGGAGTTGGCTGAAAAACGACCA 658
Vd-8        ------ACCAGA-----TTTGAG------------------GCTGGC------------- 546
Vd-13       ------------------------------------------------------------
Vd-39       ---GCTACCCGG--------------------------GAATTGG--------------- 562
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Vd-52       TTGGAGAACGGA-----TTTTGGT---------------GAGTTGGCTGAAAAACGACCA 572
Vd-73       ------------------------------------------------------------
Vd-89       TTGGAGAACGGA-----TTTTGGT---------------GAGTTGGCTGAAAAACGACCA 651
Vl-19       ---GCTACCCGG-----------------------------GAATTGG-------------- 560
Vl-32       ---GCTACCCGG-----------------------------GAATTGG-------------- 559
Vl-18       --------------------GAAC--------------GGATTTTG--------------- 525
Vl-40       --------------------GAAC--------------GGATTTTG--------------- 535
Vl-43       --------------------GAAC--------------GGATTTTG--------------- 535
Vl-60       CCGGGAGTTGGAAATTTGGAGAAC--------------GGATTTTG--------------- 572
Vl-Bob70    CCGGGAGTTGGAAATTTGGAGAAC--------------GGATTTTG--------------- 574
Vl-82       --------------------GAAC--------------GGATTTTG--------------- 532
Vl-83       --------------------GAAC--------------GGATTTTG--------------- 539
Vl-84       --------------------GAAC--------------GGATTTTG--------------- 535
Va-1        CCGGGAATTGG---------GAGCT--------ACCCGGGAATTGG--------------- 658
Va-2        CCGGGAGTTGG---------GAGCT--------ACCCGGGAGTTGG--------------- 669
Va-3        CCGGGAATTGG---------GAGCT--------ACCCGGGAATTGG--------------- 647
Va-4        ------------------------------------GGGAATTGG--------------- 613


Vd-2        AGTCGGACACCTTGGAGCTACCCGGGAATTGGGAG---CTACCCGGGAATTGGGAG---- 711
Vd-8        ----------------------------------AG---CTACCCGGGAATTGGGAG---- 566
Vd-13       ----------------------------------AG---CTACCCGGGAATTGGGAG---- 565
Vd-39       ---------------------------------GAG---CTACCCGGGAATTGGGAG---- 583
Vd-52       AGTCAGACATCT-----------------GGGAG---CTACCCGGGAATTGGGAG---- 607
Vd-73       ---------------------------------GAG---CTACCCGGGAATTGGGAG---- 560
Vd-89       AGTCAGACATCT-----------------GGGAG---CTACCCGGGAATTGGGAGCTAC 690
Vl-19       ---------------------------------GAG---CTACCCGGGAATTGGGAGCTAC 585
Vl-32       ---------------------------------GAG---CTACCCGGGAATTGGGAGCTAC 584
Vl-18       ---------------------------------GAG---CTACCCGGGAGTTGGAAG---- 546
Vl-40       ---------------------------------GAG---CTACCCGGGAGTTGGAAG---- 556
Vl-43       ---------------------------------GAG---CTACCCGGGAGTTGGAAG---- 556
Vl-60       ---------------------------------GAG---CTACCCGGGAGTTGGAAG---- 593
Vl-Bob70    ---------------------------------GAG---CTACCCGGGAGTTGGAAG---- 595
Vl-82       ---------------------------------GAG---CTACCCGGGAGTTGGAAG---- 553
Vl-83       ---------------------------------GAG---CTACCCGGGAGTTGGAAG---- 560
Vl-84       ---------------------------------GAG---CTACCCGGGAGTTGGAAG---- 556
Va-1        ---------------------------------GAGGTGCTACCCGGGAATTGGGGG---- 682
Va-2        ---------------------------------GAGGTGCTACCCGGGAATTGGGGG---- 693
Va-3        ---------------------------------GAGGTGCTACCCGGGAATTGGGGG---- 671
Va-4        ---------------------------------GAGGTGCTACCCGGGAATTGGGGG---- 637
                                              **   ********** ****  *

Vd-2        --------------------------------ACGAATTCGCCGATTCCTCCACCACCAG 739
Vd-8        --------------------------------ACGAATTCGCCGATTCCTCCACCACCAG 594
Vd-13       --------------------------------ACGAATTCGCCGATTCCTCCACCACCAG 593
Vd-39       --------------------------------ACGAATTCGCCGATTCCTCCACCACCAG 611
Vd-52       --------------------------------ACGAATTCGCCGATTCCTCCACCACCAG 635
Vd-73       --------------------------------ACGAATTCGCCGATTCCTCCACCACCAG 588
Vd-89       CCGGGAATTGGGAG-----------------ACGAATTCGCCGATTCCTCCACCACCAG 732
Vl-19       CCGGGAATTGGGAG-----------------ACGAATTCGCCGATTCCTCCACCACCAG 627
Vl-32       CCGGGAATTGGGAGCTACCCGGGAATTGGGAGACGAATTCGCCGATTCCTCCACCACCAG 644
Vl-18       --------------------------------ACGAATTCGCCGATTCCTCCACCACTAG 574
Vl-40       --------------------------------ACGAATTCGCCGATTCCTCCACCACTAG 584
Vl-43       --------------------------------ACGAATTCGCCGATTCCTCCACCACTAG 584
Vl-60       --------------------------------ACGAATTCGCCGATTCCTCCACCACTAG 621
Vl-Bob70    --------------------------------ACGAATTCGCCGATTCCTCCACCACTAG 623
Vl-82       --------------------------------ACGAATTCGCCGATTCCTCCACCACTAG 581
Vl-83       --------------------------------ACGAATTCGCCGATTCCTCCACCACTAG 588
Vl-84       --------------------------------ACGAATTCGCCGATTCCTCCACCACTAG 584
Va-1        --------------------------------ACGAATTCGCCGATTCCTCCACCACCTG 710
Va-2        --------------------------------ACGAATTCGCCGATTCCTCCACCACCTG 721
Va-3        --------------------------------ACGAATTCGCCGATTCCTCCACCACCTG 699
Va-4        --------------------------------ACGAATTCGCCGATTCCTCCACCACCTG 665
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Vd-2        GCCGTTTCCCGTTACTCTTCTTAGTGCACTGGAAAGAACGTATCGTCCTTAGTATATTTC 799
Vd-8        GCCGTTTCCCGTTACTCTTCTTAGTGCACTGGAAAGAACGTATCGTCCTTAGTATATTTC 654
Vd-13       GCCGTTTCCCGTTACTCTTCTTAGTGCACTGGAAAGAACGTATCGTCCTTAGTATATTTC 653
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Vd-39       GCCGTTTCCCGTTACTCTTCTTAGTGCACTGGAAAGAGCGTATCGTCCTTAGTATATTTC 671
Vd-52       GCCGTTTCCCGTTACTCTTCTTAGTGCACTGGAAAGAGCGTATTGTCCTTAGTATATTTC 695
Vd-73       GCCGTTTCCCGTTACTCTTCTTAGTGCACTGGAAAGAACGTATCGTCCTTAGTATATTTC 648
Vd-89       GCCGTTTCCCGTTACTCTTCTTAGTGCACTGGAAAGAGCGTATTGTCCTTAGTATATTTC 792
Vl-19       GCCGTTTCCCGTTACTCTTCTTAGTGCACTGGAAAGAGCGTATCGTCCTTAGTATATTTC 687
Vl-32       GCCGTTTCCCGTTACTCTTCTTAGTGCACTGGAAAGAGCGTATCGTCCTTAGTATATTTC 704
Vl-18       GCCGTTTCTCGCTATTCTTTCTAGTGCATTAGAAAGAGCGCATCGTCTTTAGTATATTTC 634
Vl-40       GCCGTTTCTCGCTATTCTTTCTAGTGCATTAGAAAGAGCGCATCGTCTTTAGTATATTTC 644
Vl-43       GCCGTTTCTCGCTATTCTTTCTAGTGCATTAGAAAGAGCGCATCGTCTTTAGTATATTTC 644
Vl-60       GCCGTTTCTCGCTATTCTTTCTAGTGCATTAGAAAGAGCGCATCGTCTTTAGTATATTTC 681
Vl-Bob70    GCCGTTTCTCGCTATTCTTTCTAGTGCATTAGAAAGAGCGCATCGTCTTTAGTATATTTC 683
Vl-82       GCCGTTTCTCGCTATTCTTTCTAGTGCATTAGAAAGAGCGCATCGTCTTTAGTATATTTC 641
Vl-83       GCCGTTTCTCGCTATTCTTTCTAGTGCATTAGAAAGAGCGCATCGTCTTTAGTATATTTC 648
Vl-84       GCCGTTTCTCGCTATTCTTTCTAGTGCATTAGAAAGAGCGCATCGTCTTTAGTATATTTC 644
Va-1        GCCGTTTCACGCTATCTTTTCTAGTGTACTGGAAAGAGCGTATCGTCTTTAGTATATTTG 770
Va-2        GCCGTTTCCCGCTATTCTTTCTAGCGTACTGGAAAGAGCGTATCGTGTTTAGTGTATTTC 781
Va-3        GCCGTTTCACGCTATCTTTTCTAGTGTACTGGAAAGAGCGTATCGTCTTTAGTATATTTG 759
Va-4        GCCGTTTCACGCTATCTTTTCTAGTGTACTGGAAAGAGCGTATCGTCTTTAGTATATTTG 725
            ******** ** **   **  *** * * * ****** ** ** **  ***** *****

Vd-2        ACTCTTAAAAGTACTATATATACTTGAACGCTATTTG--TTTT-GGCTTTCAAGAGTCTG 856
Vd-8        ACTCTTAAAAGTACTATATATACTTGAACGCTATTTG--TTTT-GGCTTTCAAGAGTCTG 711
Vd-13       ACTCTTAAAAGTACTATATATACTTGAACGCTATTTG--TTTT-GGCTTTCAAGAGTCTG 710
Vd-39       ACTCTTAAAAGTACTATATATACTTGAACGCTATTTG--TTTT-GGCTTTCAAGAGTCTG 728
Vd-52       ACTCTTAAAAGTACTATATATACTTGAACGCTATTTTGTTTTT-GGCTTTCAAGAGTCTG 754
Vd-73       ACTCTTAAAAGTACTATATATACTTGAACGCTATTTG--TTTT-GGCTTTCAAGAGTCTG 705
Vd-89       ACTCTTAAAAGTACTATATATACTTGAACGCTATTTG--TTTT-GGCTTTCAAGAGTCTG 849
Vl-19       ACTCTTAAAAGTACTATATATACTTGAACGCTATTTG--TTTT-GGCTTTCAAGAGTCTG 744
Vl-32       ACTCTTAAAAGTACTATATATACTTGAACGCTATTTG--TTTT-GGCTTTCAAGAGTCTG 761
Vl-18       ACCCTTAAAAGTACTGTATGCACTCTACTACTATTTG--TTTT-GGCTTTAGAAAGTCTG 691
Vl-40       ACCCTTAAAAGTACTGTATGCACTCTACTACTATTTG--TTTT-GGCTTTAGAAAGTCTG 701
Vl-43       ACCCTTAAAAGTACTGTATGCACTCTACTACTATTTG--TTTT-GGCTTTAGAAAGTCTG 701
Vl-60       ACCCTTAAAAGTACTGTATGCACTCTACTACTATTTG--TTTT-GGCTTTAGAAAGTCTG 738
Vl-Bob70    ACCCTTAAAAGTACTGTATGCACTCTACTACTATTTG--TTTT-GGCTTTAGAAAGTCTG 740
Vl-82       ACCCTTAAAAGTACTGTATGCACTCTACTACTATTTG--TTTT-GGCTTTAGAAAGTCTG 698
Vl-83       ACCCTTAAAAGTACTGTATGCACTCTACTACTATTTG--TTTT-GGCTTTAGAAAGTCTG 705
Vl-84       ACCCTTAAAAGTACTGTATGCACTCTACTACTATTTG--TTTT-GGCTTTAGAAAGTCTG 701
Va-1        ACCATTGAAAATATTATATGTACTTTAGTATTATTTG--TTTT-AGGTCTCGAAAGTCTG 827
Va-2        ACCCTTGAAAATATTATATGTACTTTAGTATTATTTG--TTTT-AGGTTTTGAAAGTCTG 838
Va-3        ACCATTGAAAATATTATATGTACTTTAGTATTATTTG--TTTTTAGGTCTCGAAAGTCTC 817
Va-4        ACCATTGAAAATATTATATGTACTTTAGTATTATTTG--TTTT-AGGTCTCGAAAGTCTG 782
            **  ** *** ** * ***  ***  *    *****   ****  * * *  * *****

Vd-2        CTTGCG-GTGCACCAGTATTCCTATTGAGTTTCTGGGCCGAAATCCTACCACGCCACC-T 914
Vd-8        CTTGCG-GTGCACCAGTATTCCTATTGAGTTTCTGGGCCGAAATCCTACCACGCCACC-T 769
Vd-13       CTTGCG-GTGCACCAGTATTCCTATTGAGTTTCTGGGCCGAAATCCTACCACGCCACC-T 768
Vd-39       CTTGCG-GTGCACTAGTATTCCTATTGAGTTTCTGGGCCGAAATCCTACCACGCCACC-T 786
Vd-52       CTTGCG-GTGCACTAGTATTCCTATTGAGTTTCTGGGCCGAAATCCTACCACGCCACCCT 813
Vd-73       CTTGCG-GTGCACCAGTATTCCTATTGAGTTTCTGGGCCGAAATCCTACCACGCCACC-T 763
Vd-89       CTTGCG-GTGCACTAGTATTCCTATTGAGTTTCTGGGCCGAAATCCTACCACGCCACC-T 907
Vl-19       CTTGCG-GTGCACTAGTATTCCTATTGAGTCTCTGGGCCGAAATCCTACCACGCCACC-T 802
Vl-32       CTTGCG-GTGCACTAGTATTCCTATTGAGTTTCTGGGCCGAAATCCTACCACGCCACC-T 819
Vl-18       CCTATA-GTGTAATTGTATTCTTATTGGGTTTCTGGTGAGAATTCCCACCACTCCACT-C 749
Vl-40       CCTATA-GTGTAATTGTATTCTTATTGGGTTTCTGGTGAGAATTCCCACCACTCCACT-C 759
Vl-43       CCTATA-GTGTAATTGTATTCTTATTGGGTTTCTGGTGAGAATTCCCACCACTCCACT-C 759
Vl-60       CCTATA-GTGTAATTGTATTCTTATTGGGTTTCTGGTGAGAATTCCCACCACTCCACT-C 796
Vl-Bob70    CCTATA-GTGTAATTGTATTCTTATTGGGTTTCTGGTGAGAATTCCCACCACTCCACT-C 798
Vl-82       CCTATA-GTGTAATTGTATTCTTATTGGGTTTCTGGTGAGAATTCCCACCACTCCACT-C 756
Vl-83       CCTATA-GTGTAATTGTATTCTTATTGGGTTTCTGGTGAGAATTCCCACCACTCCACT-C 763
Vl-84       CCTATA-GTGTAATTGTATTCTTATTGGGTTTCTGGTGAGAATTCCCACCACTCCACT-C 759
Va-1        CTTATC-ATGTAATGATATTCTTGTTGGGTTTCTGGTGAGAAGTCGTATTGCTCCGCA-A 885
Va-2        CTTGTA-ATGTACTGGTATTCTTATTGGGTTTCTGGTGAGAAATCGTACTACTCTGCG-A 896
Va-3        CTTATCCATGTAATGATATTCTTGTTGGGTTTCTGGTGAGAAGTCGTATTGCTCCGCA-A 876
Va-4        CTTATC-ATGTAATGATATTCTTGTTGGGTTTCTGGTGAGAAGTCGTATTGCTCCGCA-A 840
            * *     ** *    ***** * *** ** *****   *** **  *   * *  *

Vd-2        AAACGTTAGAAGGCC-TAGCGAAATACATTGCAATCGAATAGGCACACTCAGATTACCCT 973
Vd-8        AAACGTTAGAAGGCC-TAGCGAAATACATTGCAATCGAATAGGCACACTCAGATTACCCT 828
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Vd-13       AAACGTTAGAAGGCC-TAGCGAAATACATTGCAATCGAATAGGCACACTCAGATTACCCT 827
Vd-39       AAACGTCAGAAGGCC-TAGCGAAATACATTGCAATCGAATAGGCACACTCAGATTACCCT 845
Vd-52       AAACGTCAGAAGGCC-TAGCGAAATACATTGCAATCGAATAGGCACACTCAGATTACCCT 872
Vd-89       AAACGTCAGAAGGCC-TAGCGAAATACATTGCAATCGAATAGGCACACTCAGATTACCCT 966
Vd-73       AAACGTTAGAAGGCC-TAGCGAAATACATTGCAATCGAATAGGCACACTCAGATTACCCT 822
Vl-19       AAACGTCAGAAGGCC-TAGCGAAATACATTGCAATCGAATAGGCACACTCAGATTACCCT 861
Vl-32       AAACGTCAGAAGGCCCTAGCGAAATACATTGCAATCGAATAGGCACACTCAGATTACCCT 879
Vl-18       AAACGTCAGAACACT-AGAAGAAATGTATTGCAATCGAATAGGCACACTTAGATTACCCT 808
Vl-40       AAACGTCAGAACACT-AGAAGAAATGTATTGCAATCGAATAGGCACACTTAGATTACCCT 818
Vl-43       AAACGTCAGAACACT-AGAAGAAATGTATTGCAATCGAATAGGCACACTTAGATTACCCT 818
Vl-60       AAACGTCAGAACACT-AGAAGAAATGTATTGCAATCGAATAGGCACACTTAGATTACCCT 855
Vl-Bob70    AAACGTCAGAACACT-AGAAGAAATGTATTGCAATCGAATAGGCACACTTAGATTACCCT 857
Vl-82       AAACGTCAGAACACT-AGAAGAAATGTATTGCAATCGAATAGGCACACTTAGATTACCCT 815
Vl-83       AAACGTCAGAACACT-AGAAGAAATGTATTGCAATCGAATAGGCACACTTAGATTACCCT 822
Vl-84       AAACGTCAGAACACT-AGAAGAAATGTATTGCAATCGAATAGGCACACTTAGATTACCCT 818
Va-1        AAACGGTAGAACGCT-AAAAGAAATGCATCGCAACCGAACAAGCATACTCGGATTACCCC 944
Va-2        AAACGGTAGAACGCT-AAA-CAAATGCATTGCAATCGAACAGGCATACTCGGATTACCCT 954
Va-3        AAMCGGTAGAACGCT-AAA-GAAATGCATCGCA-CCGAACA-GCATACTCGGATTACCCC 932
Va-4        AAACGGTAGAACGCT-AAAAGAAATGCATCGCAACCGAACAAGCATACTCGGATTACCCC 899
            ** **  ****  *       ****  ** ***  **** * *** ***  ********

Vd-2        ATGGCC-GGTCGTGCATCGCTGCAGCAGCGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 1032
Vd-8        ATGCCC-GGTCGTGCATCGCTGCAGCAGCGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 887
Vd-13       ATGGCC-GGTCGTGCATCGCTGCAGCAGCGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 886
Vd-39       ATGGTC-GGTCGTGCATCGCTGCAGCAGCGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 904
Vd-52       ATGGCC-GGTCGTGCATCGCTGCAGCAGCGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 931
Vd-73       ATGGCC-GGTCGTGCATCGCTGCAGCAGCGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 881
Vd-89       ATGGCC-GGTCGTGCATCGCTGCAGCAGCGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 1025
Vl-19       ATGGCCCGGTCGTGCATCGCTGCAGCAGCGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 921
Vl-32       ATGGCCCGGTCGTGCATCGCTGCAGCAGCGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 939
Vl-18       GTGGCC-GGTCGTGCATCGCTGCAGCAGTGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 867
Vl-40       GTGGCC-GGTCGTGCATCGCTGCAGCAGTGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 877
Vl-43       GTGGCC-GGTCGTGCATCGCTGCAGCAGTGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 877
Vl-60       GTGGCC-GGTCGTGCATCGCTGCAGCAGTGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 914
Vl-Bob70    GTGGCC-GGTCGTGCATCGCTGCAGCAGTGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 916
Vl-82       GTGGCC-GGTCGTGCATCGCTGCAGCAGTGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 874
Vl-83       GTGGCC-GGTCGTGCATCGCTGCAGCAGTGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 881
Vl-84       GTGGCC-GGTCGTGCATCGCTGCAGCAGTGTTGGGGCGGCCGTGGTGGTGGAATCGAGGT 877
Va-1        ATAACC-GGTCGTGCATCGCCGCAGCAGCGTTGTGGCGGCCGTAGTAGTGGAATTGAGGT 1003
Va-2        ATGACT-AGTCGTGCATCGCTGCAGCAGCGTTGCGGCGGCCGTGGTGGTGGAATTGAGGT 1013
Va-3        ATAACC-GGTCGTGCATCGCCGCAGCAGCGTTGTGGCGGCCGTAGTAGTGGAATTGAGGT 991
Va-4        ATAACC-GGTCGTGCATCGCCGCAGCAGCGTTGTGGCGGCCGTAGTAGTGGAATTGAGGT 958
             *      ************ ******* **** ********* ** ******* *****

Vd-2        TGTGGCGAGCTACCCGGGAA-TTGGGAGCTACCCGGGAATCGGG--AATCTGGAGGCGCC 1089
Vd-8        TGTGGCGAGCTACCCGGGAA-TTGGGAGCTACCCGGGAATCGGG--AATCTGGAGGCGCC 944
Vd-13       TGTGGCGAGCTACCCGGGAA-TTGGGAGCTACCCGGGAATCGGG--AATCTGGAGGCGCC 943
Vd-39       TGTGGCGAGCTACCCGGGAA-TTGGGAGCTACCCGGGAATTGGG--AATCTGGAGGCGCC 961
Vd-52       TGTGGCGAGCTACCCGGGAA-TTGGGAGCTACCCGGGAATTGGG--AATCTGGAGGCGCC 988
Vd-73       TGTGGCGAGCTACCCGGGAA-TTGGGAGCTACCCGGGAATCGGG--AATCTGGAGGCGCC 938
Vd-89       TGTGGCGAGCTACCCGGGAA-TTGGGAGCTACCCGGGAATTGGG--AATCTGGAGGCGCC 1082
Vl-19       TGTGGCGAGCTACCCGGGAA-TTGGGAGCTACCCGGGAATTGGG--AATCTGGAGGCGCC 978
Vl-32       TGTGGCGAGCTACCCGGGAA-TTGGGAGCTACCCGGGAATTGGG--AATCTGGAGGCGCC 996
Vl-18       TGTGGCGAGCTACCCGGGAA-TTGGAAGCTACCCGGCATTTGGGCCGACATCGAGGCGCC 926
Vl-40       TGTGGCGAGCTACCCGGGAA-TTGGAAGCTACCCGGCATTTGGGCCGACATCGAGGCGCC 936
Vl-43       TGTGGCGAGCTACCCGGGAA-TTGGAAGCTACCCGGCATTTGGGCCGACATCGAGGCGCC 936
Vl-60       TGTGGCGAGCTACCCGGGAA-TTGGAAGCTACCCGGCATTTGGGCCGACATCGAGGCGCC 973
Vl-Bob70    TGTGGCGAGCTACCCGGGAA-TTGGAAGCTACCCGGCATTTGGGCCGACATCGAGGCGCC 975
Vl-82       TGTGGCGAGCTACCCGGGAA-TTGGAAGCTACCCGGCATTTGGGCCGACATCGAGGCGCC 933
Vl-83       TGTGGCGAGCTACCCGGGAA-TTGGAAGCTACCCGGCATTTGGGCCGACATCGAGGCGCC 940
Vl-84       TGTGGCGAGCTACCCGGGAA-TTGGAAGCTACCCGGCATTTGGGCCGACATCGAGGCGCC 936
Va-1        TGTGGCGAGCTACCCGGGAA-TTGGGGGCTACCCGGGAATTGGGC-AACATCGAGGCGCC 1061
Va-2        TGTGGCGAGCTACCCGGGAA-TTGGGGGCTACCCGGGAATTGGGC-AATATCGAGGCGCC 1071
Va-3        TGTGGCGAGCTACCCGGGAAATTGGGGGCTACCCGGGAATTGGGC-AACATCGAGGCGCC 1050
Va-4        TGTGGCGAGCTACCCGGGAA-TTGGGGGCTACCCGGGAATTGGGC-AACATCGAGGCGCC 1016
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Vd-2        GACGACCCTCAGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCATGCGTGGGCACCTGT 1149
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Vd-8        GACGACCCTCAGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCATGCGTGGGCACCTGT 1004
Vd-13       GACGACCCTCAGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCATGCGTGGGCACCTGT 1003
Vd-39       GACGACCCTCAGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCATGCGTGGGCACCTGT 1021
Vd-52       GACGACCCTCAGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCATGCGTGGGCACCTGT 1048
Vd-73       GACGACCCTCAGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCATGCGTGGGCACCTGT 998
Vd-89       GACGACCCTCAGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCATGCGTGGGCACCTGT 1142
Vl-19       GACGACCCTCAGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCATGCGTGGGCACCTGT 1038
Vl-32       GACGACCCTCAGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCATGCGTGGGCACCTGT 1056
Vl-18       GACGACCCTCAGAACGGC----------TGCAGCGCACTGGGGCGTGCGTGGGCACCTGT 976
Vl-40       GACGACCCTCAGAACGGC----------TGCAGCGCACTGGGGCGTGCGTGGGCACCTGT 986
Vl-43       GACGACCCTCAGAACGGC----------TGCAGCGCACTGGGGCGTGCGTGGGCACCTGT 986
Vl-60       GACGACCCTCAGAACGGC----------TGCAGCGCACTGGGGCGTGCGTGGGCACCTGT 1023
Vl-Bob70    GACGACCCTCAGAACGGC----------TGCAGCGCACTGGGGCGTGCGTGGGCACCTGT 1025
Vl-82       GACGACCCTCAGAACGGC----------TGCAGCGCACTGGGGCGTGCGTGGGCACCTGT 983
Vl-83       GACGACCCTCAGAACGGC----------TGCAGCGCACTGGGGCGTGCGTGGGCACCTGT 990
Vl-84       GACGACCCTCAGAACGGC----------TGCAGCGCACTGGGGCGTGCGTGGGCACCTGT 986
Va-1        GACGACCCTCGGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCGTGCGTGGGCACCTGT 1121
Va-2        GACGACCCTCAGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCGTGCGTGGGCACCTGT 1131
Va-3        GACGACCCTCGGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCGTGCGTGGGCACCTGT 1110
Va-4        GACGACCCTCGGAACGGCGCAGGGCGGCTGCAGCCCACTGGGGCGTGCGTGGGCACCTGT 1076
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Vd-2        GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCACGAAAAAAGTTGCCAAAATCCGTTCTC 1209
Vd-8        GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCACGAAAAAAGTTGCCAAAATCCGTTCTC 1064
Vd-13       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCACGAAAAAAGTTGCCAAAATCCGTTCTC 1063
Vd-39       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCACGAAAAAAGTTGCCAAAATCCGTTCTC 1081
Vd-52       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCACGAAAAAAGTTGCCAAAATCCGTTCTC 1108
Vd-73       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCACGAAAAAAGTTGCCAAAATCCGTTCTC 1058
Vd-89       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCACGAAAAAAGTTGCCAAAATCCGTTCTC 1202
Vl-19       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCACGAAAAAAGTTGCCAAAATCCGTTCTC 1098
Vl-32       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCACGAAAAAAGTTGCCAAAATCCGTTCTC 1116
Vl-18       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCATGGAAAAAGTTGTCAAAATCCGTTCTC 1036
Vl-40       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCATGGAAAAAGTTGTCAAAATCCGTTCTC 1046
Vl-43       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCATGGAAAAAGTTGTCAAAATCCGTTCTC 1046
Vl-60       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCATGGAAAAAGTTGTCAAAATCCGTTCTC 1083
Vl-Bob70    GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCATGGAAAAAGTTGTCAAAATCCGTTCTC 1085
Vl-82       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCATGGAAAAAGTTGTCAAAATCCGTTCTC 1043
Vl-83       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCATGGAAAAAGTTGTCAAAATCCGTTCTC 1050
Vl-84       GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCATGGAAAAAGTTGTCAAAATCCGTTCTC 1046
Va-1        GGCCGTTTGGCAGAGTTGGTTCCGTGTGAGCCATGAAAAAAGTTGTCAAAATCCGTTCTC 1181
Va-2        GGCCATTTGGCAGAGTTGGTTCCGTGTGAGCCATGAAAAAAGTTGTCAAAATCCGTTCTC 1191
Va-3        GGCCGTTTGGCAGAGTTGGTTCCGTGTGAGCCATGAAAAAAGTTGTCAAAATCCGTTCTC 1170
Va-4        GGCCGTTTGGCAGAGTTGGTTCCGTGTGAGCCATGAAAAAAGTTGTCAAAATCCGTTCTC 1136
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Vd-2        CAAATTTTCCCATAGTCAGAGTGGGGCCCCCACCAAAAAAGATCAACGACTTTCGGCTGC 1269
Vd-8        CAAATTTTCCCATAGTCAGAGTGGGGCCCCCACCAAAAAAGATCAACGACTTTCGGCTGC 1124
Vd-13       CAAATTTTCCCATAGTCAGAGTGGGGCCCCCACCAAAAAAGATCAACGACTTTCGGCTGC 1123
Vd-39       CAAATTTTCCCATAGTCAGAGTGGGGCCCCCACCAAAAAAGATCAACGACTTTCGGCTGC 1141
Vd-52       CAAATTTTCCCATAGTCAGAGTGGGGCCCCCACCAAAAAAGATCAACGACTTTCGGCTGC 1168
Vd-73       CAAATTTTCCCATAGTCAGAGTGGGGCCCCCACCAAAAAAGATCAACGACTTTCGGCTGC 1118
Vd-89       CAAATTTTCCCATAGTCAGAGTGGGGCCCCCACCAAAAAAGATCAACGACTTTCGGCTGC 1262
Vl-19       CAAATTTTCCCATAGTCAGAGTGGGGCCCCCACCAAAAAAGATCAACGACTTTCGGCTGC 1158
Vl-32       CAAATTTTCCCATAGTCAGAGTGGGGCCCCCACCAAAAAAGATCAACGACTTTCGGCTGC 1176
Vl-18       CAAATTTTCCCATAGTCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1096
Vl-40       CAAATTTTCCCATAGTCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1106
Vl-43       CAAATTTTCCCATAGTCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1106
Vl-60       CAAATTTTCCCATAGTCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1143
Vl-Bob70    CAAATTTTCCCATAGTCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1145
Vl-82       CAAATTTTCCCATAGTCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1103
Vl-83       CAAATTTTCCCATAGTCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1110
Vl-84       CAAATTTTCCCATAGTCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1106
Va-1        CAAATTTTCCCATAGTCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1241
Va-2        CAAATTTTCCCATAATCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1251
Va-3        CAAATTTTCCCATAGTCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1230
Va-4        CAAATTTTCCCATAGTCAGAGTGGGGCCCTCACCAAAAAAGATCAACGACTTTCGGCTGC 1196
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Vd-2        AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1329
Vd-8        AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1184
Vd-13       AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1183
Vd-39       AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1201
Vd-52       AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1228
Vd-73       AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1178
Vd-89       AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1322
Vl-19       AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1218
Vl-32       AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1236
Vl-18       AGCGGCGTGCCTACCAAAAACAGGACCCAGGGTATGTAAGAAGGGCAAAGCGAAAGCAGA 1156
Vl-40       AGCGGCGTGCCTACCAAAAACAGGACCCAGGGTATGTAAGAAGGGCAAAGCGAAAGCAGA 1166
Vl-43       AGCGGCGTGCCTACCAAAAACAGGACCCAGGGTATGTAAGAAGGGCAAAGCGAAAGCAGA 1166
Vl-60       AGCGGCGTGCCTACCAAAAACAGGACCCAGGGTATGTAAGAAGGGCAAAGCGAAAGCAGA 1203
Vl-Bob70    AGCGGCGTGCCTACCAAAAACAGGACCCAGGGTATGTAAGAAGGGCAAAGCGAAAGCAGA 1205
Vl-82       AGCGGCGTGCCTACCAAAAACAGGACCCAGGGTATGTAAGAAGGGCAAAGCGAAAGCAGA 1163
Vl-83       AGCGGCGTGCCTACCAAAAACAGGACCCAGGGTATGTAAGAAGGGCAAAGCGAAAGCAGA 1170
Vl-84       AGCGGCGTGCCTACCAAAAACAGGACCCAGGGTATGTAAGAAGGGCAAAGCGAAAGCAGA 1166
Va-1        AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1301
Va-2        AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1311
Va-3        AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1290
Va-4        AGCGGCGTGCCCACCAAAAACAGGACCCAGGGTATGTAAGAAGGGGAAAGCGAAAGCAGA 1256
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Vd-2        ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1389
Vd-8        ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1244
Vd-13       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1243
Vd-39       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1261
Vd-52       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1288
Vd-73       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1238
Vd-89       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1382
Vl-19       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1278
Vl-32       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1296
Vl-18       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1216
Vl-40       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1226
Vl-43       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1226
Vl-60       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1263
Vl-Bob70    ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1265
Vl-82       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1223
Vl-83       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1230
Vl-84       ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1226
Va-1        ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1361
Va-2        ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1371
Va-3        ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1350
Va-4        ACCCGGGCGGGCGCCTCAGTGCGTCTTTCAGTAATGAGAATCCGCCGAACACTGTTCCAT 1316
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Vd-2        ATCATATCGAACGCTACTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1449
Vd-8        ATCATATCGAACGCTACTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1304
Vd-13       ATCATATCGAACGCTACTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1303
Vd-39       ATCATATCGAACGCTACTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1321
Vd-52       ATCATATCGAACGCTACTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1348
Vd-73       ATCATATCGAACGCTACTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1298
Vd-89       ATCATATCGAACGCTACTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1442
Vl-19       ATCATATCGAACGCTACTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1338
Vl-32       ATCATATCGAACGCTACTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1356
Vl-18       ATCATATCGAACGCTGTTTAGTGCCCCTGTTACGAGCCTACTGGTTCAAGCGGCTACCGA 1276
Vl-40       ATCATATCGAACGCTGTTTAGTGCCCCTGTTACGAGCCTACTGGTTCAAGCGGCTACCGA 1286
Vl-43       ATCATATCGAACGCTGTTTAGTGCCCCTGTTACGAGCCTACTGGTTCAAGCGGCTACCGA 1286
Vl-60       ATCATATCGAACGCTGTTTAGTGCCCCTGTTACGAGCCTACTGGTTCAAGCGGCTACCGA 1323
Vl-Bob70    ATCATATCGAACGCTGTTTAGTGCCCCTGTTACGAGCCTACTGGTTCAAGCGGCTACCGA 1325
Vl-82       ATCATATCGAACGCTGTTTAGTGCCCCTGTTACGAGCCTACTGGTTCAAGCGGCTACCGA 1283
Vl-83       ATCATATCGAACGCTGTTTAGTGCCCCTGTTACGAGCCTACTGGTTCAAGCGGCTACCGA 1290
Vl-84       ATCATATCGAACGCTGTTTAGTGCCCCTGTTACGAGCCTACTGGTTCAAGCGGCTACCGA 1286
Va-1        ATCATATCGAACGCTATTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1421
Va-2        ATCATATCGAACGCTATTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1431
Va-3        ATCATATCGAACGCTATTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1410
Va-4        ATCATATCGAACGCTATTTAGTGCCCCTGTTACGAGCCTACCGGTTCAAGGGGCTACCGA 1376
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Vd-2        GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1509
Vd-8        GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1364
Vd-13       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1363
Vd-39       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1381
Vd-52       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1408
Vd-73       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1358
Vd-89       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1502
Vl-19       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1398
Vl-32       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1416
Vl-18       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1336
Vl-40       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1346
Vl-43       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1346
Vl-60       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1383
Vl-Bob70    GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1385
Vl-82       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1343
Vl-83       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1350
Vl-84       GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1346
Va-1        GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1481
Va-2        GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1491
Va-3        GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1470
Va-4        GCAGCGTTCAACCTCCACGGAGGTAGCCGCCCAGGCGGTCTACCAAACTGGAGTTGCCTT 1436
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Vd-2        TCGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1569
Vd-8        TCGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1424
Vd-13       TCGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1423
Vd-39       TCGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1441
Vd-52       TCGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1468
Vd-73       TCGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1418
Vd-89       TCGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1562
Vl-19       TCGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1458
Vl-32       TCGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1476
Vl-18       ACGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1396
Vl-40       ACGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1406
Vl-43       ACGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1406
Vl-60       ACGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1443
Vl-Bob70    ACGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1445
Vl-82       ACGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1403
Vl-83       ACGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1410
Vl-84       ACGGCGCTGAGAGGTCCGCCTCTACAGCGACGCAAGATGGGCTCTGTGGGTGACTGGCCG 1406
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Vl-19       TTGGCCAAACCTACTTCTAGAGCAGCGGGAGACGACGCGCGTGGTGGAAGCCGACCAGCT 1518
Vl-32       TTGGCCAAACCTACTTCTAGAGCAGCGGGAGACGACGCGCGTGGTGGAAGCCGACCAGCT 1536
Vl-18       TTGGCCAAACCTACTTCTAGAGCAGCGGGAGACGACGCGCGTGGTGGAAGCCGACCAGCT 1456
Vl-40       TTGGCCAAACCTACTTCTAGAGCAGCGGGAGACGACGCGCGTGGTGGAAGCCGACCAGCT 1466
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Vl-83       TTGGCCAAACCTACTTCTAGAGCAGCGGGAGACGACGCGCGTGGTGGAAGCCGACCAGCT 1470
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Va-4        TTGGCCAAACCTACTTCTAGAGCAGCGGGAGACGACGCGCGTGGTGGAAGCCGACCAGCT 1556
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Vl-32       GGATGCTGCGTGGTGAACGGTGCCCTGGGGAATTCAGAGGGGTAAGGTGGATGCCGTTAG 1656
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Vl-40       GGATGCTGCGTGGTGAACGGTGCCCTGGGGAATTCAGAGGGGTAAGGTGGATGCCGTTAG 1586
Vl-43       GGATGCTGCGTGGTGAACGGTGCCCTGGGGAATTCAGAGGGGTAAGGTGGATGCCGTTAG 1586
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Vl-82       GGATGCTGCGTGGTGAACGGTGCCCTGGGGAATTCAGAGGGGTAAGGTGGATGCCGTTAG 1583
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Va-3        GGATGCTGCGTGGTGAACGGTGCCCTGGGGAATTCAGAGGGGTAAGGTGGATGCCGTTAG 1710
Va-4        GGATGCTGCGTGGTGAACGGTGCCCTGGGGAATTCAGAGGGGTAAGGTGGATGCCGTTAG 1676
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Vl-84       CGGGCCTCGCGGGCTATGCCTGAGTTGCCTGCCGGCGGTGCGTTCCCAGCGAACGTGACA 1646
Va-1        CGGGCCTCGCGGGCTATGCCTGAGTTGCCTGCCGGCGGTGCGTTCCTAGCGAACGTGACA 1781
Va-2        CGGGCCTCGCGGGCTATGCCTGAGTTGCCTGCCGGCGGTGCGTTCCTAGCGAACGTGACA 1791
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Vd-2        CCGGTGACTAAATATCTCTTCGGTGGTGCTTCGGCGCCCCGATGGCCATCCCCGGCGCAC 1869
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Vd-73       CCGGTGACTAAATATCTCTTCGGTGGTGCTTCGGCGCCCCGATGGCCATCCCCGGCGCAC 1718
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Va-1        CCGGTGACTAAATATCTCTTCGGTGGTGCTTCGGCGCCCCGATGGCCATCCCCGGCGCAC 1841
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Va-3        CCGGTGACTAAATATCTCTTCGGTGGTGCTTCGGCGCCCCGATGGCCATCCCCGGCGCAC 1830
Va-4        CCGGTGACTAAATATCTCTTCGGTGGTGCTTCGGCGCCCCGATGGCCATCCCCGGCGCAC 1796
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Vd-2        GATAGTT 1876
Vd-8        GATAGTT 1731
Vd-13       GATAGTT 1730
Vd-39       GATAGTT 1748
Vd-52       GATAGTT 1775
Vd-73       GATAGTT 1725
Vd-89       GATAGTT 1869
Vl-19       GATAGTT 1765
Vl-32       GATAGTT 1783
Vl-18       GATAGTT 1703
Vl-40       GATAGTT 1713
Vl-43       GATAGTT 1713
Vl-60       GATAGTT 1750
Vl-Bob70    GATAGTT 1752
Vl-82       GATAGTT 1710
Vl-83       GATAGTT 1717
Vl-84       GATAGTT 1713
Va-1        GATAGTT 1848
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Va-3        GATAGTT 1837
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B (18S rRNA)

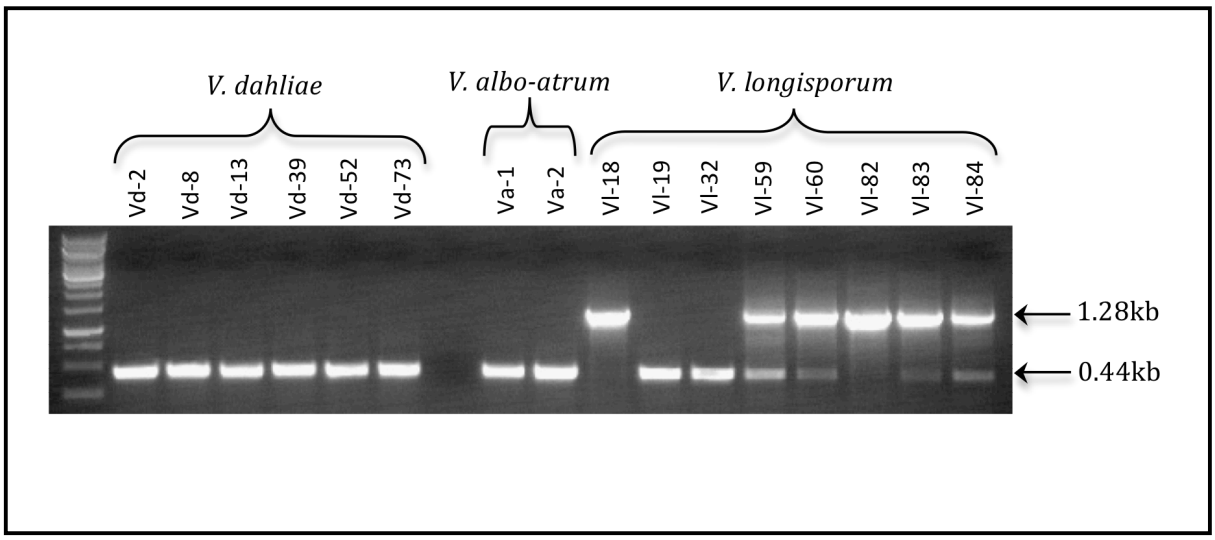

Figure S1. Differences of the intergenic regions (IGS) and 18S rRNA gene within three *Verticillium* species.

(A) Alignment of IGS regions from 11 *V. longisporum*, 7 *V. dahliae* and 4 *V. albo-atrum* isolates. The variable part (about 1 kb) of the IGS closed to 28S rRNA carries many short repeats and SNPs, whereas the part adjacent to the 18S rRNA displays a high conservation within all three species. Asterisks indicate identical nucleotides.

(B) Comparative analysis of 18S-rRNA within three *Verticillium* species. The sequences of 18S rRNA genes reveal two different groups for *V. longisporum*. The first group (9 *V. longisporum* isolates of the *V. albo-atrum* rDNA type) carries 18S rRNA gene with a large intron of 839 bp, whereas the second group (Vl-19, Vl-32) lacks any intron in this gene.

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VdVEL1          ATGTCCGCCACCACCATGGTTGCCGCCGACGACAGTCCCTCCGATGCCACTCGTTCAGTT 60
VlVEL1-1        ATGTCCGCCACCACCATGGTTGCCGCCGACGACAGTCCCTCCGATGCCACTCGTTCAGTT 60
VlVEL1-2        ATGTCCGCCACCACCATGGTTGCCGCCGACGACAGTCCCTCCGATGCCACTCGTTCAGTT 60
VaVEL1          ATGTCCGCCACCACCATGGTTGCCGCCGACGACAGCCCCTCCGATGCCACTCGTTCAGTT 60
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VdVEL1          CACACCCGCAACACCAAGGGCGGTCGACGACTTCATTATGAATTGACCTGCCTCCAACAG 120
VlVEL1-1        CACACCCGCAACACCAAGGGCGGTCGACGACTTCATTATGAATTGACCTGCCTCCAACAG 120
VlVEL1-2        CACACCCGCAACACCAAGGGCGGTCGACGACTTCATTACGAATTGACCTGCCTCCAACAG 120
VaVEL1          CACACCCGCAACACCAAGGGCGGTCGACGACTTCATTATGAATTGACCTGCCTCCAACAG 120
                ************************************ ********************

VdVEL1          CCCGAGCGCGCGAGAGCCTGTGGCTCCGGACAGAAGTGTAAGTTGGACATTCGGTGCGTT 180
VlVEL1-1        CCCGAGCGCGCGAGAGCCTGTGGCTCCGGACAGAAGTGTAAGTTGGACATTCGGTGCGTT 180
VlVEL1-2        CCCGAGCGCGCGAGAGCCTGTGGCTCCGGACAGAAGTGTAAGTTG-ACATGCGGTGCGTT 180
VaVEL1          CCCGAGCGCGCGAGAGCCTGCGGCTCCGGACAGAAGTGTAAGTTG-ACATGCGGTGCGTT 180
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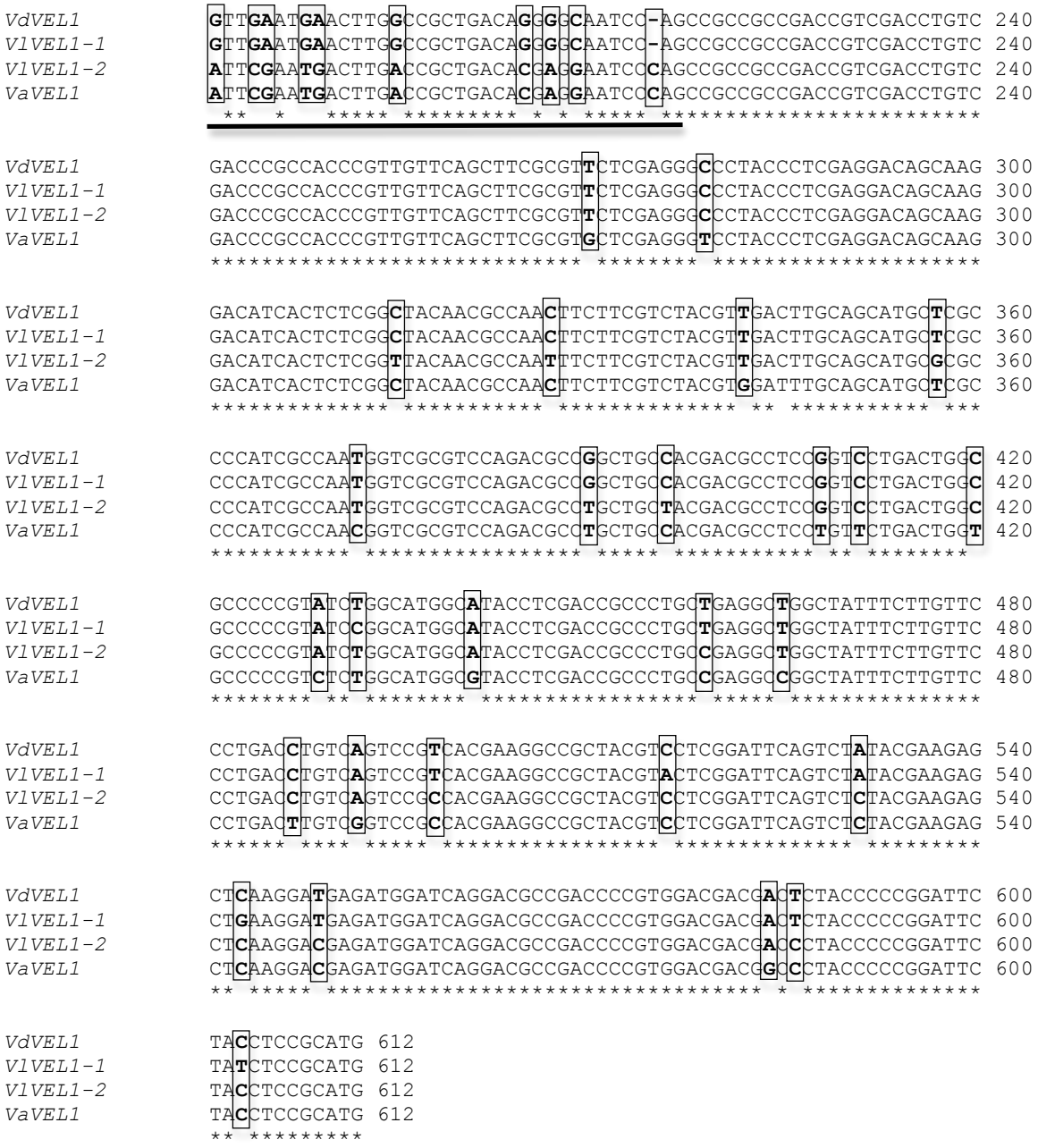
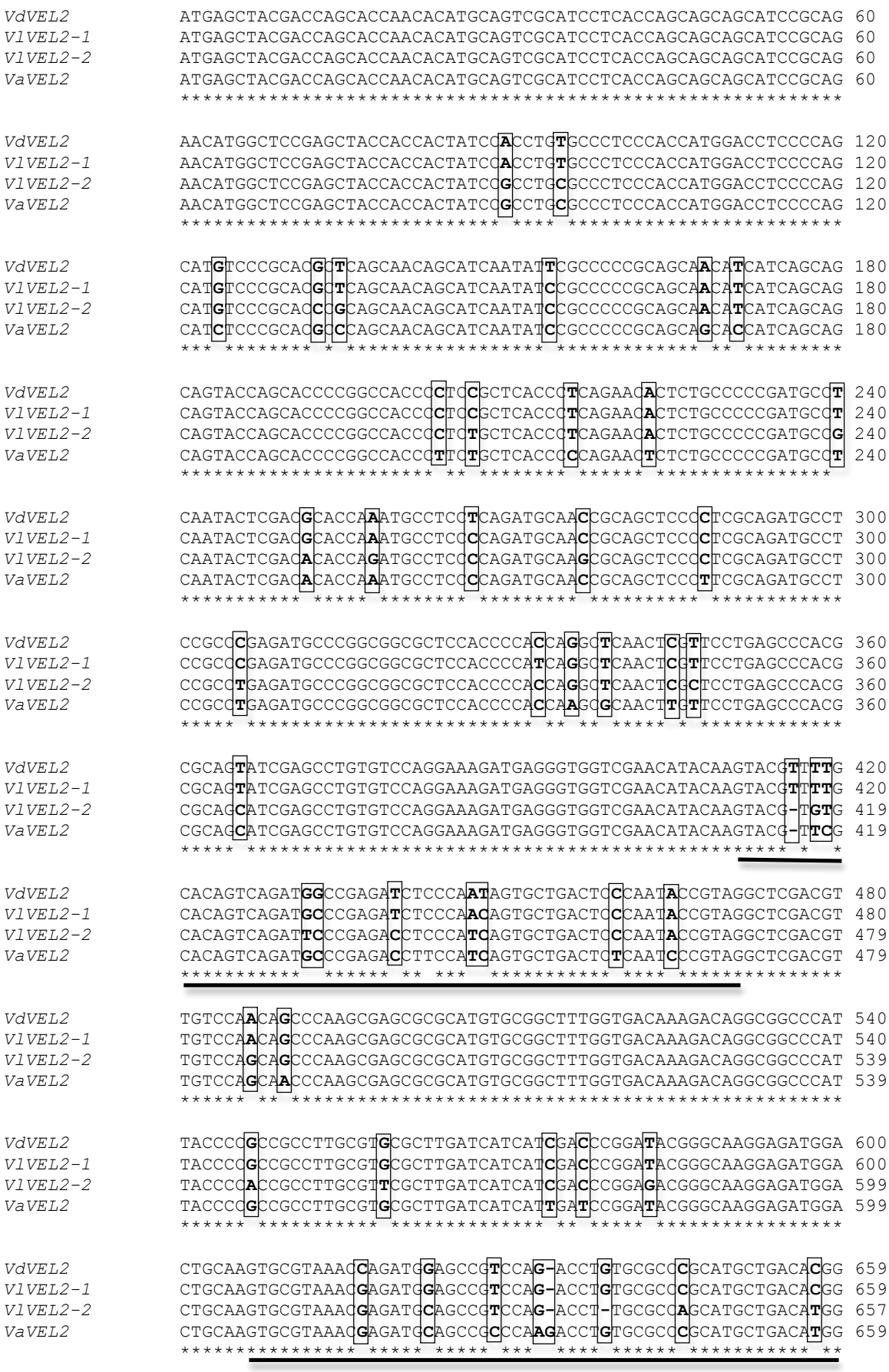


Figure S2. The 612 bp fragments of *V. longisporum VlVEL1-1* and *VlVEL1-2* isogenes compared with *VEL1* orthologues from *V. dahliae* and *V. albo-atrum*. Asterisks indicate identical nucleotides, the intron is underlined and nucleotide changes are framed in bold.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VlVEL2-1 ATGAGCTACGACCAGCACCAACACATGCAGTCGCATCCTCACCAGCAGCAGCATCCGCAG 60
VlVEL2-2 ATGAGCTACGACCAGCACCAACACATGCAGTCGCATCCTCACCAGCAGCAGCATCCGCAG 60
VaVEL2 ATGAGCTACGACCAGCACCAACACATGCAGTCGCATCCTCACCAGCAGCAGCATCCGCAG 60
VdVEL2 AACATGGCTCCGAGCTACCACCACTATCCACCTGTGCCCCTCCCACCATGGACCTCCCCAG 120
VlVEL2-1 AACATGGCTCCGAGCTACCACCACTATCCACCTGTGCCCCTCCCACCATGGACCTCCCCAG 120
VlVEL2-2 AACATGGCTCCGAGCTACCACCACTATCCGCCTGCGCCCCTCCCACCATGGACCTCCCCAG 120
VaVEL2 AACATGGCTCCGAGCTACCACCACTATCCGCCTGCGCCCCTCCCACCATGGACCTCCCCAG 120
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VlVEL2-2 CATGTCCCGCACCGCAGCAACAGCATCAATATCCGCCCCCGCAGCAACATCATCAGCAG 180
VaVEL2 CATCTCCCGCACGCCAGCAACAGCATCAATATCCGCCCCCGCAGCAGCACCATCAGCAG 180
VdVEL2 CAGTACCAGCACCCCGGCCACCCCTCCGCTCACCCTCAGAACACTCTGCCCCCGATGCCT 240
VlVEL2-1 CAGTACCAGCACCCCGGCCACCCCTCCGCTCACCCTCAGAACACTCTGCCCCCGATGCCT 240
VlVEL2-2 CAGTACCAGCACCCCGGCCACCCCTCTGCTCACCCTCAGAACACTCTGCCCCCGATGCCG 240
VaVEL2 CAGTACCAGCACCCCGGCCACCCTTCTGCTCACCCCCAGAACTCTCTGCCCCCGATGCCT 240
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VlVEL2-2 CCGCCTGAGATGCCCGGCGGCGCTCCACCCCACCAGGCTCAACTCGCTCCTGAGCCCACG 360
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VaVEL2 CACAGTCAGATGCCCGAGACCTTCCATCAGTGCTGACTCTCAATCCCGTAGGCTCGACGT 479
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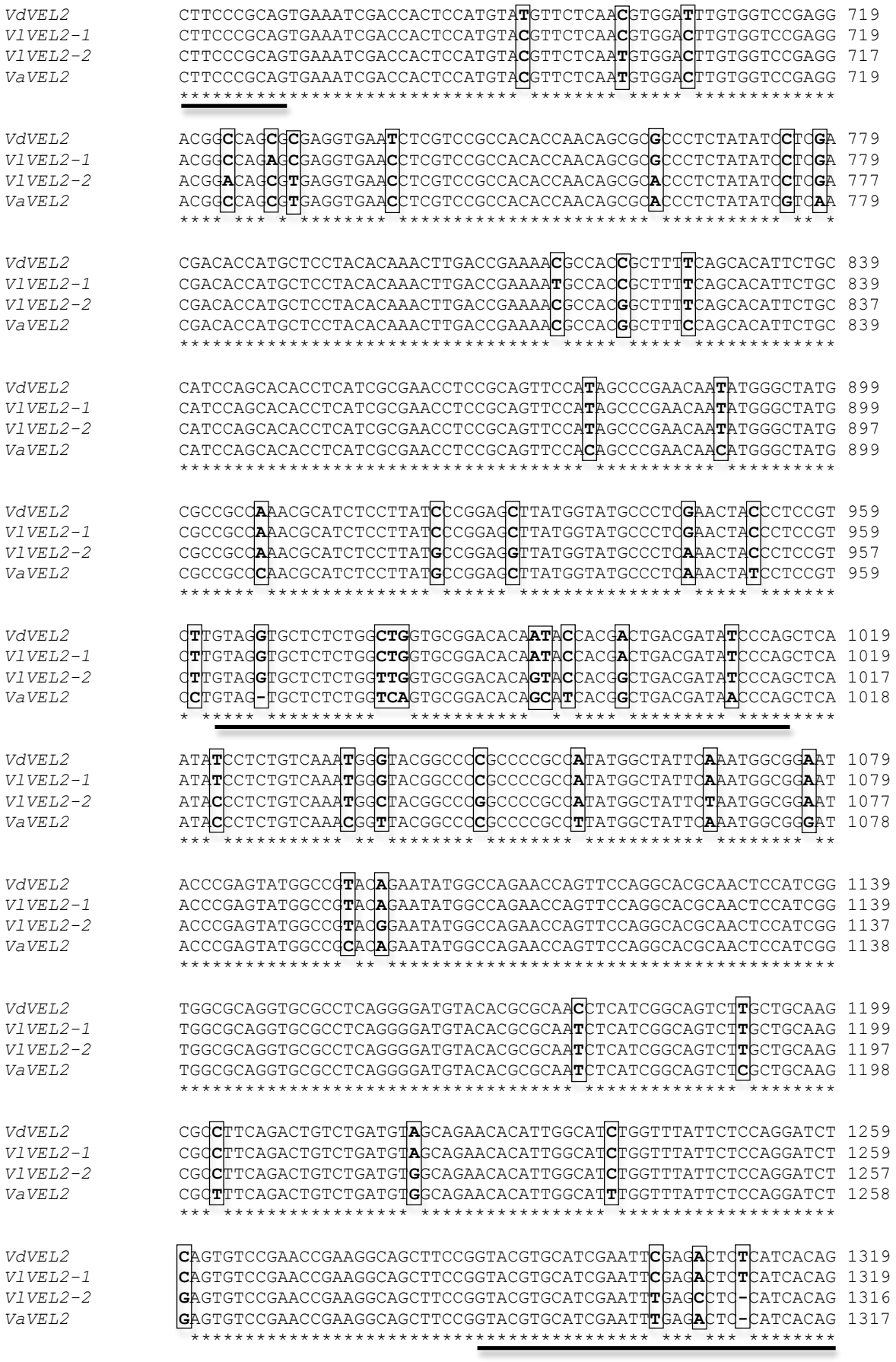
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VaVEL2 CCTGTAG-TGCTCTCTGGTCAGTGCGGACACAGCATCACGGCTGACGATAACCCAGCTCA 1018
VdVEL2 ATATCCTCTGTCAAATGGGTACGGCCCCGCCCCGCCATATGGCTATTCAAATGGCGGAAT 1079
VlVEL2-1 ATATCCTCTGTCAAATGGGTACGGCCCCGCCCCGCCATATGGCTATTCAAATGGCGGAAT 1079
VlVEL2-2 ATACCCTCTGTCAAATGGCTACGGCCCGGCCCCGCCATATGGCTATTCTAATGGCGGAAT 1077
VaVEL2 ATACCCTCTGTCAAACGGTTACGGCCCCGCCCCGCCTTATGGCTATTCAAATGGCGGGAT 1078
VdVEL2 ACCCGAGTATGGCCGTACAGAATATGGCCAGAACCAGTTCCAGGCACGCAACTCCATCGG 1139
VlVEL2-1 ACCCGAGTATGGCCGTACAGAATATGGCCAGAACCAGTTCCAGGCACGCAACTCCATCGG 1139
VlVEL2-2 ACCCGAGTATGGCCGTACGGAATATGGCCAGAACCAGTTCCAGGCACGCAACTCCATCGG 1137
VaVEL2 ACCCGAGTATGGCCGCACAGAATATGGCCAGAACCAGTTCCAGGCACGCAACTCCATCGG 1138
VdVEL2 TGGCGCAGGTGCGCCTCAGGGGATGTACACGCGCAACCTCATCGGCAGTCTTGCTGCAAG 1199
VlVEL2-1 TGGCGCAGGTGCGCCTCAGGGGATGTACACGCGCAATCTCATCGGCAGTCTTGCTGCAAG 1199
VlVEL2-2 TGGCGCAGGTGCGCCTCAGGGGATGTACACGCGCAATCTCATCGGCAGTCTTGCTGCAAG 1197
VaVEL2 TGGCGCAGGTGCGCCTCAGGGGATGTACACGCGCAATCTCATCGGCAGTCTCGCTGCAAG 1198
VdVEL2 CGCCTTCAGACTGTCTGATGTAGCAGAACACATTGGCATCTGGTTTATTCTCCAGGATCT 1259
VlVEL2-1 CGCCTTCAGACTGTCTGATGTAGCAGAACACATTGGCATCTGGTTTATTCTCCAGGATCT 1259
VlVEL2-2 CGCCTTCAGACTGTCTGATGTGGCAGAACACATTGGCATCTGGTTTATTCTCCAGGATCT 1257
VaVEL2 CGCTTTCAGACTGTCTGATGTGGCAGAACACATTGGCATTTGGTTTATTCTCCAGGATCT 1258
VdVEL2 CAGTGTCCGAACCGAAGGCAGCTTCCGGTACGTGCATCGAATTCGAGACTCTCATCACAG 1319
VlVEL2-1 CAGTGTCCGAACCGAAGGCAGCTTCCGGTACGTGCATCGAATTCGAGACTCTCATCACAG 1319
VlVEL2-2 GAGTGTCCGAACCGAAGGCAGCTTCCGGTACGTGCATCGAATTTGAGCCTC-CATCACAG 1316
VaVEL2 GAGTGTCCGAACCGAAGGCAGCTTCCGGTACGTGCATCGAATTTGAGACTC-CATCACAG 1317

Figure S3. Comparison of *V. longisporum VlVEL2-1* and *VlVEL2-2* isogenes and *VEL2* orthologues of *V. dahliae* (*Vd*) and *V. albo-atrum (Va)*. The full open reading frames (ORFs) of the genes are used for the comparison. Asterisks indicate identical nucleotides, four introns are underlined and nucleotide changes are framed in bold characters.

A

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VdVTA1          AACAGCCGCCTCGTACATCTGCAGACCCCTCCGACCAGCATCTCAACCACTGCGGACGAA 240
VlVTA1-1        AACAGCCGCCTCGTACATCTGCAGACCCCTCCGACCAGCATCTCAACCACTGCGGACGAA 240
VlVTA1-2        AACAGCCGCCTTGTACATCTGCAGACCCCTCCGACCAGCATCTCAAACACTGCGGACGAA 240
VaVTA1          AACAGCCGCCTCGTAAATCTGCAGACCCCTCCGACCAGTATCTCAACCACTGCGGACGAA 240
                *********** *** ********************** ******* ************


VdVTA1          AACGCTGCCGCTCTTCTGCGTGATATTCCCGTCACCCACCCTCTTTACAAGATCGACACA 300
VlVTA1-1        AACGCTGCCGCTCTTCTGCGTGATATTCCCGTCACCCACCCTCTTTACAAGATCGACACA 300
VlVTA1-2        AACGCTGCCGTTCTGTTGCGTGATATTCCAGTCACCCACCCTCTTTACAAGTTTGACACA 300
VaVTA1          AATGCTGCCTCTCTTCTGCGTGATATTCCAGTCACCCACCCTCTTTACAAGATCGACACA 300
                ** ******  ***  ************* ******************** * ******


VdVTA1          GATTGGGACGTTGCAATGGACTTTGCCGATACTTTCTCGAACCCCTTATTTGGTAGGGGC 360
VlVTA1-1        GATTGGGACGTTGCAATGGACTTTGCCGATACTTTCTCGAACCCCTTATTTGGTAGGGGC 360
VlVTA1-2        GATTGGGACGTTACAATGGACTTTGCCGATACTTTCTCGCACCCTT---ATGGTAGGGGC 357
VaVTA1          AATTGGGACGTTACAATGGACTTTGCCGATACTTTCTCGAACCCTT---TTGGTAGGGGC 357
                 *********** ************************** **** *   **********


VdVTA1          CCATTAAACCCTTGCGAGGCAAATGGCCTCGACACTCGGGACCCTGGCAACGTCACGAGT 420
VlVTA1-1        CCATTAAACCCTTGCGAGGCAAATGGCCTCGACACTTTGGACCCTGGCAACGTCACGAGT 420
VlVTA1-2        CCATCAAACCCTTGCGAGGCAAATGGCCTCGACACTCGGGACCCTGGCAACGTCACGAGT 417
VaVTA1          CCATCAAACCCTTGCGAGGCAAATGGCCTCGATACTCGGGACCCTGGCAACGTCACGAGT 417
                **** *************************** ***  ***********************


VdVTA1          ATATACAGCAACAATCTGCCGTGGACGCCTCCCAACGACTTTGCGTCGATCAACTACACC 480
VlVTA1-1        ATATACAGCAACAATCTGCCGTGGACGCCTCCCAACGACTTTGCGTCGATCAACTACACC 480
VlVTA1-2        ATATACAGCAATAATCTGCCGTGGACGCCTCCCAACGACTTTGCATCGATCAACTACACC 477
VaVTA1          ATATACAGCAACAATCTGCCGTGGACGCCTCCCAACGACTTTGCGTCGATCAACTACACC 477
                *********** ******************************** ***************


VdVTA1          GATCTTTCCTATACGGGCACAAACCTGTCAGGACCTCAAAGCTGCTCCGTATCGATGGAT 540
VlVTA1-1        GATCTTTCCTATACGGGCACAAACCTGTCAGGACCTCAAAGCTGCTCCGTATCGATGGAT 540
VlVTA1-2        GATCTTTCCTATACGGGCACAAGCCTGTCAGGACCTCAAAGTCGCTCCGTATCGATGGAT 537
VaVTA1          GATCTTTCCTATACGGGCACAAACCTGTCAGGACCTCAAAGCCGCTCCGTATCGATGGAC 537
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VdVTA1          GACACAACCTCCCAGCTTAACTCGTGGGTGGATTCAGTATCTCGCGACACACAATGTTTT 600
VlVTA1-1        GACACAACCTCCCAGCTTAACTCGTGGGTGGATTCAGTATCTCGCGACACACAATGTTTT 600
VlVTA1-2        GACACAACCTCCCAGCTTAACTCGTGGGTGGATTCAGTATCGCGCGACACACAATGTTTT 597
VaVTA1          GACACAACCTCCCAGCTTAATTCGTGGGTGGATTCAGTATCGCGCGATACACAATGTTTT 597
                ******************** ******************** ***** ************


VdVTA1          GGTGCAACTGCTCCAGCGTTGACGCCCGCAAGTATGAGCTCAAGCTATTTCCCAAGCCCC 660
VlVTA1-1        GGTGCAACTGCTCCAGCGTTGACGCCCGCAAGTATGAGCTCAAGCTATTTCCCAAGCCCC 660
VlVTA1-2        AGTACAACCGCTCCAGCGTTGACGCCCGCAAGTATGGGCTCAAGCTATTTCCCAAGCCCC 657
VaVTA1          AGTACAACCGCTCCGGCGTTGACGCCCGCAAGTATGAGCTCAAGCTATTTCCCTAGCCCC 657
                 ** **** ***** ********************** ***************** ******


VdVTA1          TCCTCAACACCACGGAACGGACCAAGCGCTCAAAGAAAGGTCAGCTCCAAACACGATGGT 720
VlVTA1-1        TCCTCAACACCACCGAACGGACCAAGCGCTCAAAGAAAGGTCAGCTCCAAACACGATGGT 720
VlVTA1-2        TCCTCAACACCACGGAACGGACCAAGCGCTCAAAGAAAGGTCAGCTCCAAACATGACGGT 717
VaVTA1          TCCTCAACGCCACGAAACGGACCAAGCGCTCAAAGAAAGGTCAGCTCCAAACATGATGGT 717
                ******** ****  ************************************ ** ***


VdVTA1          GCAAATTTCTGCACTTGTTTTACGGTATGCCTTCAGTCGCTCCAGGACATGCACAGTGCA 780
VlVTA1-1        GCAAATTTCTGCACTTGTTTTACGGTATGCCTTCAGTCGCTCCAGGACATGCACAGCGCA 780
VlVTA1-2        GCAAATTTCTGCACTTGTTTTACAGTCTGCCTTCAGTCGCTCCAGGACATGCACAGCGCA 777
VaVTA1          GCAAACTTCTGCACTTGTTTTACAGTCTGCCTTCAGTCGCTCCAGGACATGCACAGCGCA 77
                ***** ***************** ** ***************************** ***


VdVTA1          TCTTCACCCGATCCACCGCCATTCGACGTTGTTCTCTCATTAAATCGCCGAGCTGTGGAT 840
VlVTA1-1        TCTTCACCCGATCCACCGCCATTCGACGTTGTTCTCTCATTAAATCGCCGAGCTGTGGAT 840
VlVTA1-2        TCTTCACCCGATCCACCGCCATTCGACGTTGTTCTCTCATTAAATCGCAAAGCTGTGGAG 837
VaVTA1          TCTTCACCCGATCCACCGCCATTCGACGTTGTTCTCTCATTAAACCGCCGAGCTGTGGAT 837
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VdVTA1          AGCTGTGCTGCTCTGCTTGCATGCGCACCATGCCTAAGCCGTTCAGGAACACACACAACT 900
VlVTA1-1        AGCTGTGCTGCTCTGCTTGCATGCGCACCATGCCTAAGCCGTTCAGGAACACACACAACT 900
VlVTA1-2        AGCTGTGCTGCTCTGCTCGCATGCGCACCATGCCTAAGCCGTTCAGGAACACACACAACT 897
VaVTA1          AGCTGTGCTGCTCTGCTCGCATGCGCACCATGCCTAAGCCGTTCAGGAACACACACAACT 897
                ***************** ****************************************

VdVTA1          GCTATGCTTCTTGCGACTATCATCGGCAAGATCACGAGCTTCTACCAGAAGGCAACACAT 960
VlVTA1-1        GCTATGCTTCTTGCGACTATCATCGGCAAGATCACGAGCTTCTACCAGAAGGCAACACAT 960
VlVTA1-2        GCTATGCTTCTTGCGACTATCATCGGCAAGATCACGAGCTTCTACCAGAAGGCAACACAT 957
VaVTA1          GCCATGCTTCTTGCGACTATCATCGGCAAGATCACGAGCTTTTACCAGAAGGCAACACAT 957
                ** ************************************ ******************

VdVTA1          TCTTACTTCGATGCAGGCGTTGAAGACGGAATTGCCGGACAAGGCAATATGCATGGTCTG 1020
VlVTA1-1        TCTTACTTCGATGGAGGCGTTGAAGACGGAATTGCCGGACAAGGCAATATGCATGGTCTG 1020
VlVTA1-2        TCTTATTTCGATGGAGGCATTGAAGACGGAATTGCCGGACAAGGCAGTATGCATGGTCTG 1017
VaVTA1          TCTTATTTCGATGGAGGCATTGAAGATCGAATTGCCGGACAAGGCAGTATGCATGGTCTG 1017
                ***** ******* **** *******  ****************** *************

VdVTA1          AGCAGCACAGGGGCGAGTCTAGGTATCAGTCTTGGTGCCTATACATTGGGGGGCGAAGAC 1080
VlVTA1-1        AGCAGCACAGGGGCGAGTCTAGGTATCAGTCTTGGTGCCTATACATTGGGGGGCGAAGAC 1080
VlVTA1-2        AGTGGCACAGGGGCGAGTCTAGGTATCAGCCTTGGTGCCTATACCTTGGGGGGCGAAGAC 1077
VaVTA1          AGCGGCACAGGGGCGAGTCTAGGTATCAGTCTTGGTGCCTATACATTGGGGGGCGAAGAC 1077
                **  ************************* ************* ***************

VdVTA1          GGTCGCTGGCTTGAACTCGAGGTTCTCGCTCGCGAGCTACACAAGCTGGAAGAGGTATAC 1140
VlVTA1-1        GGTCGCTGGCTTGAACTCGAGATACTCGCTCGCGAGCTACACAAGCTGGAAGAGGTATAC 1140
VlVTA1-2        GGTCGTTGGCTTGAACTCGAGATTCTCGCTCGCGAGCTACAAAAGCTGGAAGAGGTATAC 1137
VaVTA1          GGTCGCTGGCTTGAGCTTGAGATTCTCGCTCGCGAGCTACACAAGTTGGAAGAGGTATAC 1137
                ***** ******** ** *** * ***************** *** **************

VdVTA1          GCTCAGTTCCGAGATGTGTGCGGTGAGCTCACCGAAGACCCGGAAGTCAGTCGAGCTATG 1200
VlVTA1-1        GCTCAGTTCCGAGATGTGTGCGGTGAGCTCACCGAAGACCCGGAAGTCAGTCGAGCTATG 1200
VlVTA1-2        GCTCAGTTCCGAGACGTGTGCGGTGAGCTCACCGAAGACCCGGAAGTCAGTCGAGCTATG 1197
VaVTA1          GCTCAGTTCCGAGACGTATGCGGTGAGCTCACCGACGACGCGGAAGTCAGTCGAGCTATG 1197
                ************** ** ***************** *** ********************

VdVTA1          ATCGGGTACCTCGGGCACAACTTGGGTACGACTCTCAAGGTCGTAAGCCATCGGAAGGGT 1260
VlVTA1-1        ATCGGGTACCTCGGGCACAACTTGGGTACGACTCTCAAGGTCGTAAGCCATCGGAAAGGT 1260
VlVTA1-2        ATCGGGTATCTCGGGCACAACTTGGGTACGACTCTCAAGGTCGTAAGCCATCGGAAAGGT 1257
VaVTA1          ATCGGGTACCTCGGGCACAACTTGGGTACGACTCTCAAGGTCGTAAGCCATCAGAAGGGT 1257
                ******** ****************************************** *** ***

VdVTA1          GGAATGAAACGTGCCTGA 1278
VlVTA1-1        GGAATGAAACGTGCCTGA 1278
VlVTA1-2        GGAATGAAACGTGCCTGA 1275
VaVTA1          GGAATGAAACGTGCCTGA 1275
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B

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Vl(Vd)VTA1-1      MSSSSKTRQPRLRASCDGCFLAKVKCSKARPMCSRCLSCGLECNYSPSSRAGKPKADHSS 60
Vl(Va)VTA1-2      MSSSSKTRQPRLRASCDGCFLAKVKCSKARPMCSRCLSCGLECNYSPSSRAGKPKADHSS 60
ClVTA1            MSS-SKPRQPRLRASCDGCFLAKVKCSKARPICSRCLACGLECRYSPSSRAGKPKSDNTA 59
CgVTA1            MSSNSKPRQPRLRASCDGCFLAKVKCSKARPICSRCLACGLECRYSPSSRAGKPKSDHSA 60
MoVTA1            MSASAKPRQPRLRASCDGCFLAKVKCSKARPICSRCLACGIECRYSPSTRAGKPKSGHNL 60
                  **   * ********************** ***** ** ** **** ******

Vl(Vd)VTA1-1      NSRLVHLQTPPTSISTTADENAAALLRDIPVTHPLYKIDTDWDVAMDFADTFSNPLFGRG 120
Vl(Va)VTA1-2      NSRLVHLQTPPTSISNTADENAAVLLRDIPVTHPLYKFDTDWDVTMDFADTFSHP-YGRG 119
ClVTA1            PSHANTVHMDMTDLSPIMDE-KNLMFSQH---PGMYKMESGWHTPTSMEGAMTRNPSISS 115
CgVTA1            PSHANTVHMDMTDLSPIMDE-KNLMFGQH---PGMYKMEPGWHTPTSMEGAMSRNPSISS 116
MoVTA1            NQHQNSSANDISGLSPVGDD-KTMMFNTHVHGPGLYRIDTGWNTPPGVMNGLT------- 112
                              *   *               *     *
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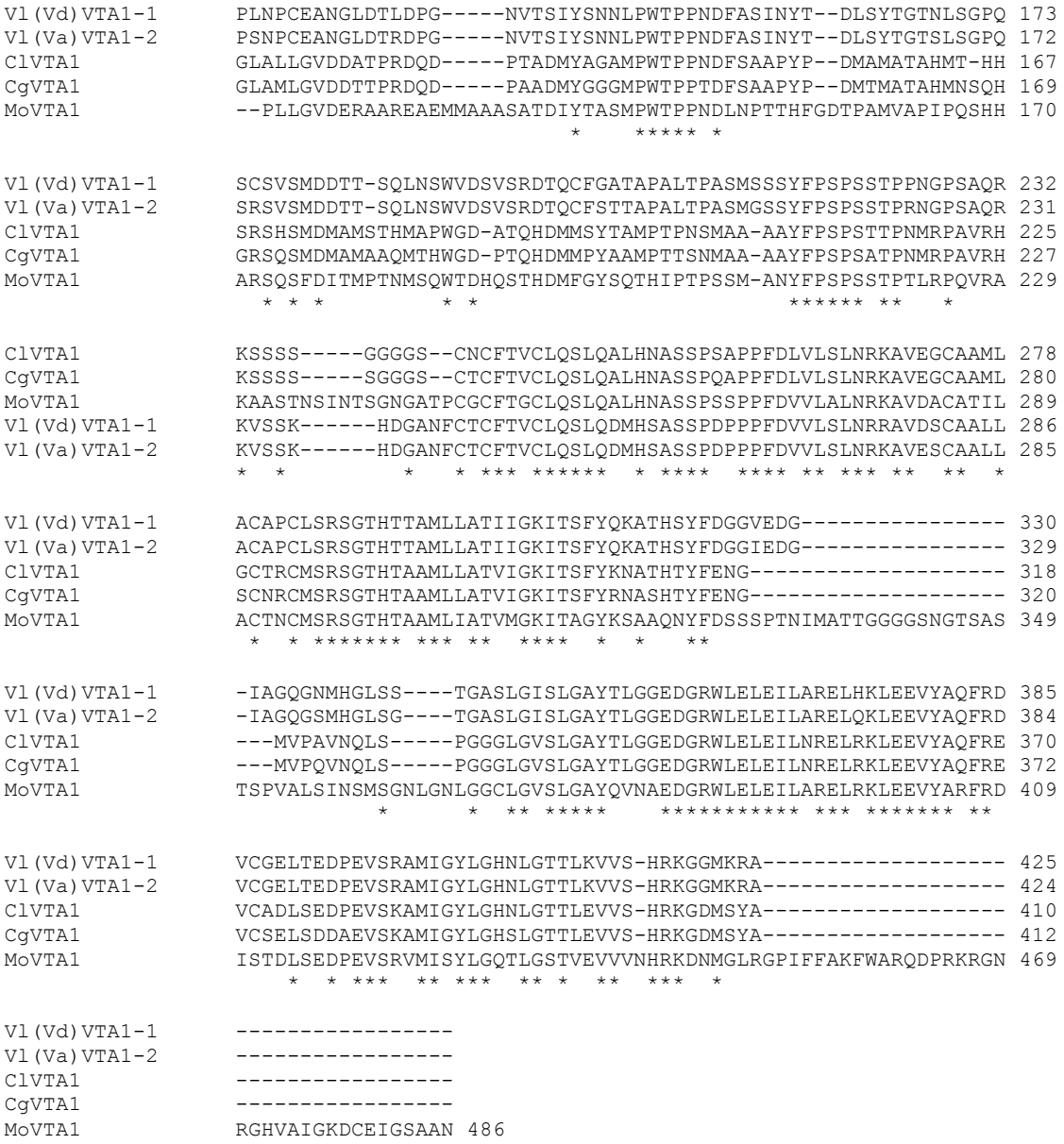

Figure S4. Conservation of the *VTA1* gene family within filamentous fungi.

(A) *VlVTA1-1* and *VlVTA1-2* of *V. longisporum* are derivatives from *V. dahliae* and *V. albo-atrum* respectively, with an increased number of small nucleotide polymorphisms in *VlVTA1-2*.

(B) Similarity of *Vl(Vd)VTA1-1* and *Vl(Va)VTA1-2* isogenes from *V. longisporum* to the *VTA1*-like genes from Cl = *Colletotrichum lagenarium* (BAE98094), Cg = *Colletotrichum graminicola* (EFQ29058), Mo = *Magnaporthe oryzae* (XP_367293). The DNA coding sequences are translated into deduced protein sequences and the asterisks indicate identical amino acids.

A

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VdVTA2          ATGTACCTGGTCCCCACGCAGCCTCATCACTTCGTTGGCAACCACGCACCGCTCCTTGCA 60
VlVTA2-1        ATGTACCTGGTCCCCACGCAGCCTCATCACTTCGTTGGCAACCACGCACCGCTCCTTGCA 60
VlVTA2-2        ATGTACCTGGTCCCCACGCAGCCTCATCACTTCGTTGGCAACCACGCACCGCTCCTTGCA 60
VaVTA2          ATGTACCTGGTCCCCACGCAGCCTCATCACTTCGTTGGCAACCACGCATCGCTCCTTGCA 60
                ************************************************ ***********

VdVTA2          TCCTCCTCCCCTGTCTCCTCGCCCGGCTCTTGTCACGGGAACCGCCCAGAAGCGACCTCC 120
VlVTA2-1        TCCTCCTCCCCTGTCTCCTCGCCCGGCTCTCGTCACGGGAACCGCCCAGAAGCGACCTCC 120
VlVTA2-2        TCCTCCTCCCCTGTCTCCTCGCCCGGCTCTCGTCACGGGAACCGCCCAGAAGCGACCTCC 120
VaVTA2          TCCTCCTCCCCTGTATCCTCGCCCGGCTCTCGTCACGGGAACCGCCCAGAAGCGACCTCC 120
                ************** *************** ****************************

VdVTA2          AGCGCGCTTCTGTCCACGCTCACCACGCTCAACACCTCTATGGAACGCAGTGCTGCCGAG 180
VlVTA2-1        AGCGCGCTTCTGTCCACGCTCACCACGCTCAACACCTCTATGGAACGCAGTGCTGCCGAG 180
VlVTA2-2        AGCGCGCTTCTGTCC---------ACGCTCAACACCTCTATGGAACGCAGTGCTGCCGAG 171
VaVTA2          AGCGCGCTTCTGTCC---------ACGCTCAACACCTCTATGGAACGCAGTGCTGCCGAG 171
                *************** ********************************

VdVTA2          TACTCGCAGTCAGGTTTGCCTTCGCCTTACCCAAGCAACTGCGGCGACACCCGTTCTGAA 240
VlVTA2-1        TACTCGCAGTCAGGTTTGCCTTCGCCTTACCCAAGCAACTGCGGCGACACCCGTTCTGAA 240
VlVTA2-2        TACTCGCAGTCAGGTTTGCCTTCGCCTTACCCAAGCAACTGCGGCGACACCCGTTCTGAA 231
VaVTA2          TACTCGCAGTCAGGTTTGCCTTCGCCTTACCCAAGCCACTGCGGCGACACCCGTTCTGAA 231
                ********************************** ************************

VdVTA2          GGATCATCTGCAGACCACTCGTCTGCTGCCCACTACTCTTCGCAACAGGAGGTTCGACCC 300
VlVTA2-1        GGATCATCTGCAGACCACTCGTCTGCTGCCCACTACTCTTCGCAACAGGAGGTTCGACCC 300
VlVTA2-2        GGATCATCTGCAGACCACTCGTCTGCTGCCCACTACTCTTCGCAACAGGAGGTTCGACCC 291
VaVTA2          GGATCATCTGCAGACCACTCGTCTGCTGCCCACTACTCTTCGCAACAGGAGGTTCGACCC 291
                ************************************************************

VdVTA2          AGCAACTACTCCACCTCGGCCACTCCCACCTCGGAGTACAGCGTCTACCCGCCTTCTGCG 360
VlVTA2-1        AGCAACTACTCCACCTCGGCCACTCCCACCTCGGAGTACAGCGTCTACCCGCCTTCTGCG 360
VlVTA2-2        AGCAACTACTCCACCTCGGCCACGCCCACCTCGGAGTACAGCGTCTACCCGCCCTCTGCG 351
VaVTA2          AGCAACTACTCTACCTCGGCCACTCCCACCTCGGAGTACAGCGTCTACCCGCCCTCTGCG 351
                *********** *********** **************************** ******

VdVTA2          CGGTCAGGATCCTTCCCTGAGCATATCCACAGACCATACCATCCAGCTAGCAACCCAAGC 420
VlVTA2-1        CGGTCAGGATCCTTCCCTGAGCATATCCACAGACCATACCATCCAGCTAGCAACCCAAGC 420
VlVTA2-2        CGGTCAGGATCCTTCCCTGAGCATATCCACAGACCATACCATCCAGCTACCAACCCAAGC 411
VaVTA2          CGGTCAGGATCCTTCCCTGAGCATATCCACAGACCATACCATCCAGCTACCAACCCAAGC 411
                ********************************************** **********

VdVTA2          GGTGGCAGCGGAGGCATGGCGCAACAAGCAAGCAGTCCGTCTTTGCCCCAGCAAGATGGA 480
VlVTA2-1        GGTGGCAGCGGAGGCATGGCGCAACAAGCAAGCAGTCCGTCTTTGCCCCAGCAAGATGGA 480
VlVTA2-2        GGTGGCAGCGGAGGCATGGCGCAACAAGCAAGCAGTCCGTCTTTGCCCCAGCAAGATGGA 471
VaVTA2          GGTGGCAGCGGAGGCATGTCGCAACAAGCAAGCAGTCCGTCTTTGCCCCAGCAAGATGGA 471
                ****************** ****************************************

VdVTA2          CGAAACCATCAACCCAACCAGCATCCCAAGTCTGACAACGATGTACCTATAGATCCCTCC 540
VlVTA2-1        CGAAACCATCAACCCAACCAGCATCACAAGTCTGACAACGATGTACCTATAGATCCCTCC 540
VlVTA2-2        CGAAACCATCAACCCAACCAGCATCCCAAGTCTGACAACGATGTACCTATAGATCCCTCC 531
VaVTA2          CGAAACCATCAACCCAACCAGCATCCCAAGTCTGACAACGATGTACCTATAGATCCCTCC 531
                ************************* ********************************

VdVTA2          ATCGCGGGACCCAGTCCTACCTACGCTTACGGACAGCAATCGCCGTACGGCCCGCCGCCC 600
VlVTA2-1        ATCGCGGGACCCAGTCCTACCTACGCTTACGGACAGCAATCGCCGTACGGCCCGCCGCCC 600
VlVTA2-2        ATCGCGGGACCCAGTCCTACCTACGCTTACGGACAGCAATCGCCGTACGGTCCGCCGCCC 591
VaVTA2          ATCGCGGGACCCAGTCCTACCTACGCTTACGGACAGCAATCGCCGTACGGCCCGCCGCCC 591
                ************************************************ *********

VdVTA2          GGCGACATGCAGGCATACCAACATGCCTATCCTCAGCCCCGTCCCGACTGGACGGGATAC 660
VlVTA2-1        GGCGACATGCAGGCATACCAACATGCCTATCCTCAGCCCCGTCCCGACTGGACGGGATAC 660
VlVTA2-2        GGCGACATGCAGGCATACCAACATGCCTATCCCCAGCCCCGTCCCGACTGGACGGGATAC 651
VaVTA2          GGCGACATGCAGGCATACCAACATGCCTATCCCCAGCCCCGTCCCGACTGGACGGGATAC 651
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VdVTA2          GGCCAGCACAGCGCTGGCCTGACGCCCGCCACCCATCACTTCCCGCCGACCCCGAGCTCT 720
VlVTA2-1        GGCCAGCACAGCGCTGGCCTCACGCCCGCCACCCATCACTTCCCGCCGACCCCGAGCTCT 72
VlVTA2-2        GGCCAGCACAGCGCTGGCCTGACGCCCGCCACCCATCACTTCCCGCCGACCCCGAGCTCC 711
VaVTA2          GGCCAGCACAGCGCTGGCCTGACGCCCGCCACCCACCACTTCCCGCCGACACCGAGCTCC 711
                ******************** ************** ************** ********

VdVTA2          GCGCCCCCGAACGGCAGGCCGACTCAGGTAAGTGTTGACTCGCACAACATGGCGCGCGCC 780
VlVTA2-1        GCGCCCCCGAACGGCAGGCCGACTCAGGTAAGTGTTGACTCGCACAACATGGCGCGCGCC 780
VlVTA2-2        GCGCCCCCGAACGGCAGGCCGACTCAGGTAAGTGTTGACTCGC-CAAGATGGCGCGCGCC 770
VaVTA2          GCGCCCCCGAACGGCAGGCCGACTCAGGTAAGTGTTGACTCGCACAAGATGGCGCGCGCC 771
                ****************************************** *** ************

VdVTA2          CCTCCCGCCCCCGCCGCATCGAGCTGGCCTTGCTAACCAAACAGTTTGGCAACCAGGTAT 840
VlVTA2-1        CCTCCCGCCCCCGCCGCATCGAGCTGGCCTTGCTAACCAAACAGTTTGGCAACCAGGTAT 840
VlVTA2-2        CCTCCCG-CCCCGCCGCATCGAGCTGGCCTTGCTAACCAAACAGTTTGGCAACCAGGTAT 829
VaVTA2          CCTCCCG-CCCCGCCGCATCGAGCTGGCCTTGCTAACCAAACAGTTTGGCAACCAGGTAT 830
                ******* ************************************************

VdVTA2          ACTCCTTCGTTCCGATTCCTGGCGCGCAGCAGCACAAGCGGCCGAGACGACGATACGAGG 900
VlVTA2-1        ACTCCTTCGTTCCGATTCCTGGCGCGCAGCAGCACAAGCGGCCGAGACGACGATACGAGG 900
VlVTA2-2        ACTCTTTCGTTCCGATTCCTGGCGCGCAGCAGCACAAGCGGCCGAGACGACGATACGAGG 889
VaVTA2          ACTCTTTCGTTCCGATTCCTGGTGCGCAGCAACACAAGCGGCCGAGACGACGATACGAGG 890
                **** ***************** ******** ****************************

VdVTA2          AGATTGAGCGCATGTACAAGTGTGGCTGGCAGGGTTGTGAGAAGGCGTATGGTACTCTGA 960
VlVTA2-1        AGATTGAGCGCATGTACAAGTGTGGCTGGCAGGGTTGTGAGAAGGCGTATGGTACTCTGA 960
VlVTA2-2        AGATTGAGCGCATGTACAAGTGTGGCTGGCAGGGTTGTGAGAAGGCGTATGGTACTCTGA 949
VaVTA2          AGATTGAGCGTATGTACAAGTGTGGCTGGCAGGGTTGCGAGAAGGCGTATGGTACTCTGA 950
                ********** ************************** ***********************

VdVTA2          ACCATCTCAATGCGCACGTCACCATGCAGTCGCACGGCACGAAGCGAACTCCTGAAGGTA 1020
VlVTA2-1        ACCATCTCAATGCGCACGTCACCATGCAGTCGCACGGCACGAAGCGAACTCCTGAAGGTA 1020
VlVTA2-2        ACCATCTCAATGCGCACGTCACCATGCAGTCGCACGGCACGAAGCGGACTCCTGAAGGTA 1009
VaVTA2          ACCATCTCAATGCGCACGTCACCATGCAGTCGCACGGCACGAAGCGGACTCCTGAAGGTA 1010
                ******************************************** ************

VdVTA2          TGCACCTACAAC-CTCCATCTTTGCCCTGTTCTTCACACCATCCCTGTCGCTGTTCTATG 1079
VlVTA2-1        TGCACCTACAAC-CTCCATCTTTGCCCTGTTCTTCACACCATCCCTGTCGCTGTTCTATG 1079
VlVTA2-2        TGCACCTACAACCTCCCATCTTTGCCCTGTTCTTCAATCCATCCCTGTCGCTGTTCTATG 1069
VaVTA2          TGCACTTACCAC-TCCCGTCTTTGCCCTGTTCCTCAACCCATCCATGTCGCTGCTCTATG 1069
                ***** *** **   ** ************** ***  ****** ******** ******

VdVTA2          CGGGACGCG--CAATGTGGGGGCTTTCCTGTTG---GGC---------------------- 1113
VlVTA2-1        CGCGACGCG--CAATGTGGGGGCTTTCCTGTTG---GGC---------------------- 1113
VlVTA2-2        CGCGACGCGACAAATGTGGTGGCTTTCCTGTTGGAGAGCGCGCCGAGTGGCGTGTACTTT 1129
VaVTA2          TGCGACACGATGAATGTGGTGGCTTTCCTGTGGGAGAGCGCGCCGAGTGGCGTACACTTG 1129
                 * *** **   ******* *********** *    **

VdVTA2          CCCTGTTGAAG--------AGCGCG-----------CCGAG----TAGTGTGTCCAGGCG 1150
VlVTA2-1        CCCTGTTGAAG--------AGCGCG-----------CCGAG----TAGTGTGTCCAGGCG 1150
VlVTA2-2        CCGTGTTTTTAGCCTCGCCACTACGCTCACACAGCTTCGTGGGTGCCGTGGAACTGGGCG 1189
VaVTA2          CCGTGTTTTTAGCCTCACCAGCACGACCACACAGCTCCGTGGGTGCTGTGGGACTGGGCG 1189
                ** ****            *   **            ** *      ***   *  ****

VdVTA2          TAAAATTGAGACTAG---CTTCTATGG---------------GCGACCCGAGCTACGGGG 1192
VlVTA2-1        TAAAATTGAGACTAG---CTTCTATGG---------------GCGACCCGAGCTACGGGG 1192
VlVTA2-2        TAAAATTGAGACTAG---CTTCTATCGGCGACCCGACTAGGGGCGACCCGAGCTACGGGG 1246
VaVTA2          TAAAATTGAGACTAGCAGCTTCTATGGGCGACCCGACTAGGGGCGACCCGAGCTACGGGG 1249
                ***************   ******* *               ******************

VdVTA2          -ACTTGGGTGATGAAAAGGCCAGAAGCTAATGCGTGACTGCAGAATTCAAAGAGATTCGT 1251
VlVTA2-1        -ACTTGGGTGATAAAAAGGCCAGAAGCTAATGCGTGACTGCAGAATTCAAAGAGATTCGT 1251
VlVTA2-2        -ACTTGGGTGATGAAAAGGCCGGAAGCTAATGCGTGACTGCAGAATTCAAAGAGATTCGT 1305
VaVTA2          GACTTGTGTGATGAAACGGCCTGAAGCTAATCCGTGACTGCAGAGTTCAAAGAGATTCGT 1309
                 ***** ***** *** **** ********* ************ ***************
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VdVTA2          AAAGAATGGAAGCAGCGCAAGAAGGAGGAAGAAGCTGCTCGCAAGGCCGAGGACGAGCAA 1311
VlVTA2-1        AAAGAATGGAAGCAGCGCAAGAAGGAGGAAGAAGCTGCTCGCAAGGCCGAGGACGAGCAA 1311
VlVTA2-2        AAAGAATGGAAGCAGCGCAAGAAGGAGGAAGAAGCTGCTCGCAAGGCCGAGGACGAGCAA 1365
VaVTA2          AAAGAATGGAAGCAGCGCAAGAAGGAGGAAGAAGCTGCTCGCAAGGCCGAGGACGAGCAA 1369
                ************************************************************

VdVTA2          CGTCGTGCGGCTGCTGCTGCTGCGGCAGCGCAAGCCGCCCAAAACGGTGGCCCAGACCCT 1371
VlVTA2-1        CGTCGTGCGGCTGCTGCTGCTGCGGCAGCGCAAGCCGCCCAAAACGGTGGCCCAGACCCT 1371
VlVTA2-2        CGTCGTGCGGCTGCTGCTGCAGCGGCAGCGCAAGCCGCCCAAAACGGTGGCCCAGACCCT 1425
VaVTA2          CGTCGTGCGGCTGCTGCTGCAGCGGCAGCGCAAGCCGCCCAAAACGGTGGCCCAGACCCT 1429
                ******************* ***************************************

VdVTA2          CAGTCCGGGCCCGACGGTGGCCCTCCTTCGGGCTATGGCGGTGGCCGTCTGCCCCCGATC 1431
VlVTA2-1        CAGTCCGGGCCCGACGGCGGCCCTCCTTCGGGCTATGGCGGTGGCCGTCTGCCCCCGATC 1431
VlVTA2-2        CAATCCGGGCCCGATGGCGGTCCTCCTTCTGGCTATGGTGGCGGTCGTCTGCCCCCGATC 1485
VaVTA2          CAATCCGGGCCCGACGGCGGTCCTCCTTCTGGCTATGGCGGCGGTCGTCTGCCCCCGATC 1489
                ** *********** ** ** ******** ******** ** ** ***************

VdVTA2          GGCTACAGCCCCTCCTATCCTCCCAACGGTCCTCCCTCCGCCGGCGTCCCTCAGCAGCAA 1491
VlVTA2-1        GGCTACAGCCCCTCCTATCCTCCCAACGGTCCTCCCTCCGCCGGCGTCCCTCAGCAGCAA 1491
VlVTA2-2        GGCTACAGCCCCTCCTATCCTCCCAACGGTCCTCCCTCCGCCGGCGTCCCTCAGCAGCAA 1545
VaVTA2          GGCTACAGCCCCTCCTACCCTCCCAACGGTCCTCCCTCCGCCGGCGTTCCTCAGCAGCAA 1549
                ***************** **************************** ************

VdVTA2          CCGCTGCCAGAGTACAACGGCACACACATGTACCAACCCGCCAACTACCAGGCGCAGCCT 1551
VlVTA2-1        CCGCTGCCAGAGTACAACGGCACACACATGTACCAACCCGCCAACTACCAGGCGCAGCCT 1551
VlVTA2-2        CCGCTGCCAGAGTACAACGGCACACACATGTACCAACCCGCCAATTACCAGGCGCAGCCT 1605
VaVTA2          CCGCTGCCAGAGTACAACGGCACACACATGTACCAACCCGCCAACTACCAGGCGCAGCCT 1609
                ****************************************** ***************

VdVTA2          CCGTCGCCATATGGCCAGCCGAGCCAGGGCATGTACTCTCAACGTAAGTTGGTTGCAGCC 1611
VlVTA2-1        CCGTCGCCATATGGCCAGCCGAGCCAGGGCATGTACTCTCAACGTAAGTTGGTTGCAGCC 1611
VlVTA2-2        CCGTCGCCGTATGGCCAGCCGAGCCAGGGCATGTACTCTCAACGTAAGTTGGTCGCAGCC 1665
VaVTA2          CCGTCGCCGTATGGCCAGCCGAGCCAGGGCATGTACTCTCAACGTAAGTTGGTTGCAGCC 1669
                ******** ******************************************* ******

VdVTA2          ACATCCCCTTCCCATGAGCAAGGAGGCGCACGACCGAGAAGGCTAACCGTGCAGCCAGAC 1671
VlVTA2-1        ACATCCCCTTCCCATGAGCAAGGAGGCGCACGACCGAGAAGGCTAACCGTGCAGCCAGAC 1671
VlVTA2-2        ACATCCCCTTCCCATGAGCAAGGAGGCACACGACCGAGAAGGCTAACCGTGCAGCCAGAC 1725
VaVTA2          ACGTCCCCTTCCCATGAGCAAGGAGGCACACGACCGAGAAGGCTAACCGTGCAGCCAGAC 1729
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VdVTA2          AACGGGACGCAGCCTGGGCAGGGCCACTAG 1701
VlVTA2-1        AACGGGACGCAGCCTGGGCAGGGCCACTAG 1701
VlVTA2-2        AACGGGACGCAGCCTGGGCAGGGCCACTAG 1755
VaVTA2          AACGGGACGCAGCCTGGGCAGGGCCACTAG 1759
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B

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Vl(Vd)VTA2-1      MYLVPTQP-HHFVGNHAPLLASSSPVSSPGSRHGNRPEATS-SALLSTLTTLNTSMERSA 58
Vl(Va)VTA2-2      MYLVPTQP-HHFVGNHAPLLASSSPVSSPGSRHGNRPEATS-SALLS---TLNTSMERSA 55
CgVTA2            MFVVPTQ--HQFVDNHASLLSSSSPQLG-----GSRPEAAAISSLLT---SLSTSMERSA 50
FgVTA2            MSLVPTQQPHTFVDNRASLHTA-----SNIAKLGSEATLDT---LLS----SPNSMERSA 48
FsVTA2            MFLVPTQQPHTFVDHRASLHSSPSPNNSHHPKLGSEVTVDA---LLS----SPISMERSA 53
MgCON7            MLASSRQPRHAFVEHHHQLSSSTLHRSGSPQTGTLRQDATTPTLATS----VGPSMDRSA 56
                  *     *  * **     *                                  ** ***

Vl(Vd)VTA2-1      AEYSQSGLPSPYPSNCGDTRSEGS--SADHSSAAHY--SSQQEVR---PSNYSTSATPTS 111
Vl(Va)VTA2-2      AEYSQSGLPSPYPSNCGDTRSEGS--SADHSSAAHY--SSQQEVR---PSNYSTSATPTS 108
CgVTA2            TEYSQSGLPSPYPSNCGDTRSEGS--SADHASAAQY--ATQQEVR---SSNYSTSATPTS 103
FgVTA2            TEYSQSGLPSPYPSNFGDTNSEGS--RADHASAAQY--PVKQEV------NYSTSATPTS 98
FsVTA2            PEYSQSGLPSPYPSNFGDTNSEGS--TADHASAAQY--PVKQEA------NYSTSGTPTS 103
MgCON7            SDYSQSGLPSPYPSNCGDNQSEAQSVTVDTSSAAQYNASAQQEVRSNNPGNYSASATPTS 116
                    ************* **  **      *  *** *     **       *** * ****

Vl(Vd)VTA2-1      EYSVYPPSARSGSFPEHIH-RPYHPASNPSGGSGGMAQQAS------------------- 151
Vl(Va)VTA2-2      EYSVYPPSARSGSFPEHIH-RPYHPATNPSGGSGGMAQQAS------------------- 148
CgVTA2            EYSVYPPSARSGSFPEHIQ-RSYHPASNHNGSSGGMAQQASSPSLPQQDGRNHQVPMPKS 162
FgVTA2            EYGVYPQTARSGSFPEHVQ-RSYHPASSAS--SGSMAQQQNSPSMPQQDGRSHQTHPVKS 155
FsVTA2            EYGVYPQSARSGSFPDHIQ-RSYHPASTPS--SGGMAQQQNSPSIPHQDGRNHQAHPVKS 160
MgCON7            EYGVYPASARSSSFPDHLQQRSYHPASNHSGSSG-------------------------- 150
                  ** ***  *** *** *   * ****      **

Vl(Vd)VTA2-1      ------NPSIAGPSPTYA-YGQQSPYGPPPGDM-QAYQH----AYPQPRPDWTGYGQHSA 199
Vl(Va)VTA2-2      ------NPSIAGPSPTYA-YGQQSPYGPPPGDM-QAYQH----AYPQPRPDWTGYGQHSA 196
CgVTA2            DHDVPIDPSIAAPSPTYA-YGQHSPYGPPPGDMSHSYQHP---AYAQPRPDWTGYGQHSA 218
FgVTA2            DNDVPIDPSIAAPSPTYASYGQHSPYAPNP-DMTHSYSHPGGGMYAQPRPDWAGYGQHGG 214
FsVTA2            DNDVPIDPSIAAPSPTYASYGQQSPYAPAP-DMTHSYSHPSSGMYAQPRPDWTGYGHHGG 219
MgCON7            ------DPSIAAPSPTYGAPAQYSPYGPPSQDMSHGYAHPGSNLYAQPRPDWSGYGQQHG 204
                        **** *****    * *** *   **   * *     * ****** ***

Vl(Vd)VTA2-1      G-LTPATHHFPPTPSSAPPNGRPTQVFGNQVYSFVPIPGAQQHKRPRRRYEEIERMYKCG 258
Vl(Va)VTA2-2      G-LTPATHHFPPTPSSAPPNGRPTQVFGNQVYSFVPIPGAQQHKRPRRRYEEIERMYKCG 255
CgVTA2            GPLTPASHVFPPTPSSAPPQGRP-----NQVYSFVPIPGAQQHKRPRRRYEEIERMYKCG 273
FgVTA2            APLTPGHPVYAQNAASAQPPQRP-----NQVYSFVPIPGAQQHKRPRRRYEEIERMYKCG 269
FsVTA2            APLTPGHPVYAQNPASAPPPARP-----NQVYSFVPIPGAQQHKRPRRRYEEIERMYKCG 274
MgCON7            APLTPGHHVFPQTPTSAPPQARP-----NQVYSFVPIPGAQQHKRPRRRYEEIERMYKCG 259
                    ***          ** *  **     ******************************

Vl(Vd)VTA2-1      WQGCEKAYGTLNHLNAHVTMQSHGTKRTPEEFKEIRKEWKQRKKEEEAARKAEDEQRRAA 318
Vl(Va)VTA2-2      WQGCEKAYGTLNHLNAHVTMQSHGTKRTPEEFKEIRKEWKQRKKEEEAARKAEDEQRRAA 315
CgVTA2            WNGCEKAYGTLNHLNAHVTMQSHGQKRTPEEFKEIRKEWKQRKKEEEAQRKAEDERQRQA 333
FgVTA2            WNGCEKAYGTLNHLNAHVTMQSHGQKRTPEEFKEIRKEWKQRKKEEEANRKAEEERQRQA 329
FsVTA2            WNGCEKAYGTLNHLNAHVTMQSHGQKRTPEEFKEIRKEWKQRKKEEEAARKAEEERQR-V 333
MgCON7            WQGCEKAYGTLNHLNAHVTMQSHGQKRTPEEFKEIRKEWKARKKEEEAARKADEERQR-- 317
                  * ********************** *************** ******* ***  *  *

Vl(Vd)VTA2-1      AAAAAAQAAQNGGPDPQSGPDGGP-PSGYGGGR----LPPIGYSPSYPPNGPPSAGVPQQ 373
Vl(Va)VTA2-2      AAAAAAQAAQNGGPDPQSGPDGGP-PSGYGGGR----LPPIGYSPSYPPNGPPSAGVPQQ 370
CgVTA2            AAAAAA-AAQNGSGDPQSGPDGSSSASAYASGRQ-VQLPPIGYQPSQYP-APPSAGGVAQ 390
FgVTA2            AAAAAA---QNGGPDPQ-GPDGTP-TSSYPGSRP-VQLPPIGYQPAQYP--PPPS-AVPQ 380
FsVTA2            AAAAQV---QNGGPDPQ-GPDGTP-TSSYPGSRP-VQLPPIGYQPAQYP--PPPGGSVPQ 385
MgCON7            -QAAQS---QGGSTEGQAGSDVSQSSNGYAGARGAVQLPPIGYQAGQYP--AATSTSVQQ 371
                    **     * *    * * *       *   *    ******     *          *

Vl(Vd)VTA2-1      QPLPEYNGTHMYQPANYQAQPPSPYGQPSQGMYSQHNGTQPGQGH 418
Vl(Va)VTA2-2      QPLPEYNGTHMYQPANYQAQPPSPYGQPSQGMYSQHNGTQPGQGH 415
CgVTA2            QPLPEYNSSHMYS-ANYQPQ--SPYGQPNQSMYSSRELKTPGAC- 431
FgVTA2            QPLPDYNNSHMYS--NYQPH--SPYAQPSQGIPYQSNGAQPPSH- 420
FsVTA2            QPLPDYNN-HMYS--NYQPH--SPYAQPSQGISYQSNGGQPPSH- 424
MgCON7            QPLPDYNASYMQG---YQPA--SPYGGSNQAMYNQR--------- 402
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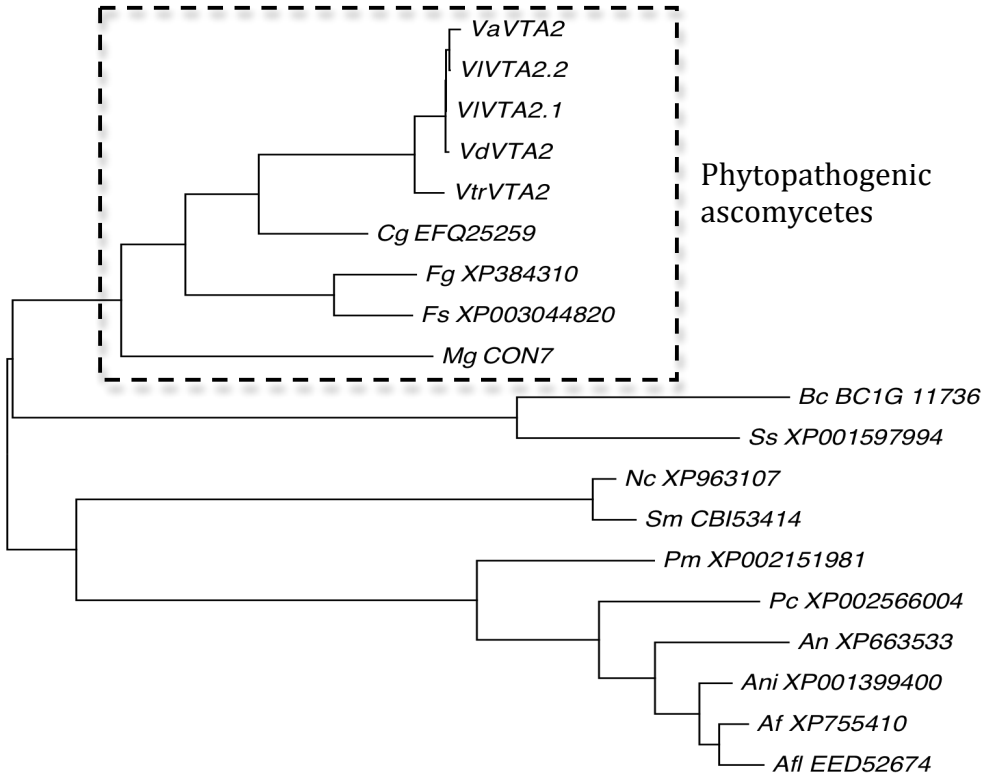

D

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Va-1.VTA2       ATGTACCTGGTCCCCACGCAGCCTCATCACTTCGTTGGCAACCACGCATCGCTCCTTGCA 60
Va-2.VTA2       ATGTACCTGGTCCCCACGCAGCCTCATCACTTCGTTGGCAACCACGCATCGCTCCTTGCA 60
                ************************************************************

Va-1.VTA2       TCCTCCTCCCCTGTATCCTCGCCCGGCTCTCGTCACGGGAACCGCCCAGAAGCGACCTCC 120
Va-2.VTA2       TCCTCCTCCCCTGTATCCTCGCCCGGCTCTCGTCACGGGAACCGCCCAGAAGCGACCTCC 120
                ************************************************************

Va-1.VTA2       AGCGCGCTTCTGTCCACGCTCAACACCTCTATGGAACGCAGTGCTGCCGAGTACTCGCAG 180
Va-2.VTA2       AGCGCGCTTCTGTCCACGCTCAACACCTCTATGGAACGCAGTGCTGCCGAGTACTCGCAG 180
                ************************************************************

Va-1.VTA2       TCAGGTTTGCCTTCGCCTTACCCAAGCCACTGCGGCGACACCCGTTCTGAAGGATCATCT 240
Va-2.VTA2       TCAGGTTTGCCTTCGCCTTACCCAAGCCACTGCGGCGACACCCGTTCTGAAGGATCATCT 240
                ************************************************************

Va-1.VTA2       GCAGACCACTCGTCTGCTGCCCACTACTCTTCGCAACAGGAGGTTCGACCCAGCAACTAC 300
Va-2.VTA2       GCAGACCACTCGTCTGCTGCCCACTACTCTTCGCAACAGGAGGTTCGACCCAGCAACTAC 300
                ************************************************************

Va-1.VTA2       TCCACCTCGGCCACTCCCACCTCGGAGTACAGCGTCTACCCGCCCTCTGCGCGATCAGGA 360
Va-2.VTA2       TCTACCTCGGCCACTCCCACCTCGGAGTACAGCGTCTACCCGCCCTCTGCGCGGTCAGGA 360
                ** ********************************************** ******

Va-1.VTA2       TCCTTCCCTGAGCATATCCACAGACCATACCATCCAGCTACCAACCCAAGCGGTGGCAGC 420
Va-2.VTA2       TCCTTCCCTGAGCATATCCACAGACCATACCATCCAGCTACCAACCCAAGCGGTGGCAGC 420
                ************************************************************

Va-1.VTA2       GGAGGCATGGCGCAACAAGCAAGCAGTCCGTCTTTGCCCCAGCAAGATGGACGAAACCAT 480
Va-2.VTA2       GGAGGCATGTCGCAACAAGCAAGCAGTCCGTCTTTGCCCCAGCAAGATGGACGAAACCAT 480
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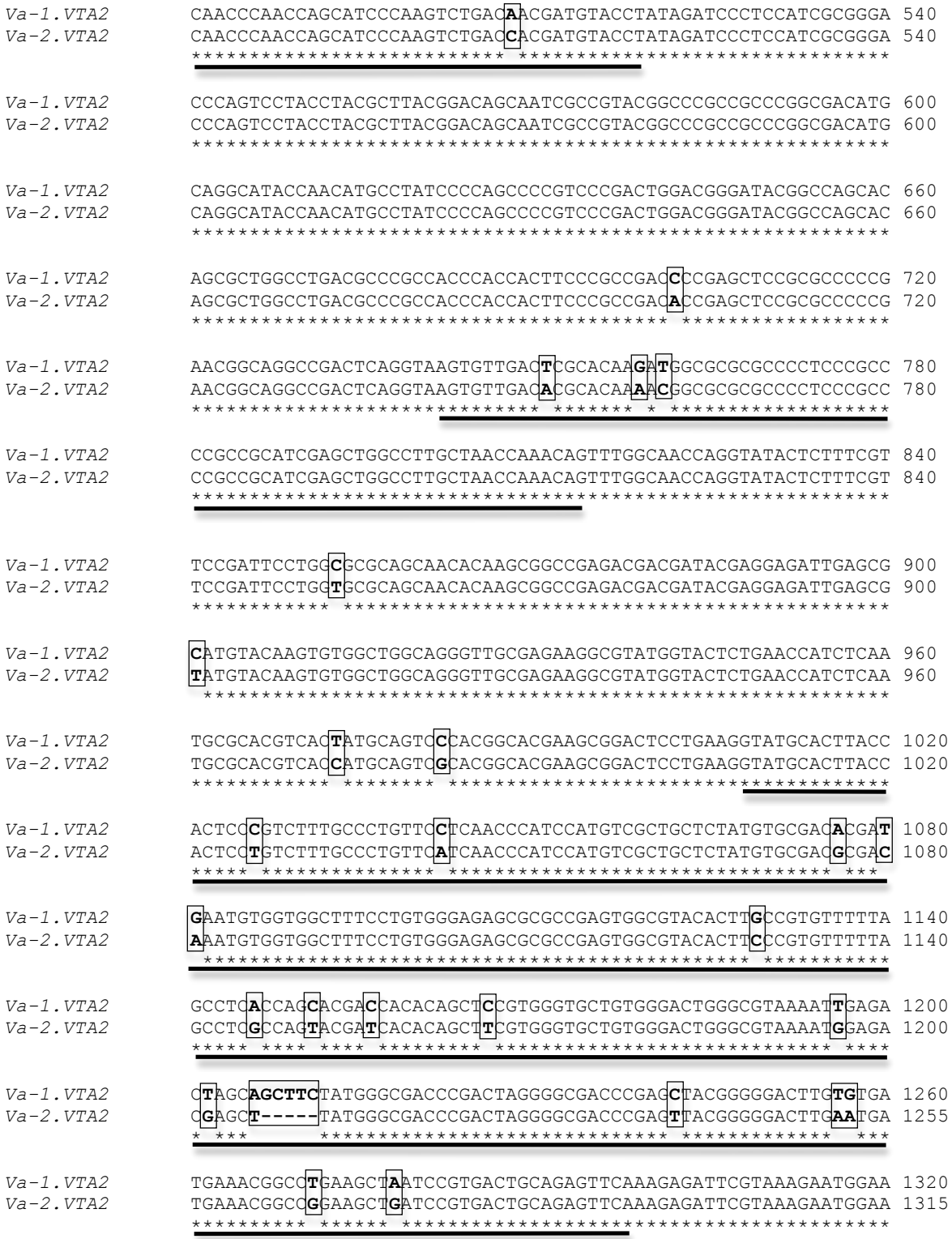
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Va-2.VTA2 CAACCCAACCAGCATCCCAAGTCTGACCACGATGTACCTATAGATCCCTCCATCGCGGGA 540
Va-1.VTA2 CCCAGTCCTACCTACGCTTACGGACAGCAATCGCCGTACGGCCCGCCGCCCGGCGACATG 600
Va-2.VTA2 CCCAGTCCTACCTACGCTTACGGACAGCAATCGCCGTACGGCCCGCCGCCCGGCGACATG 600
Va-1.VTA2 CAGGCATACCAACATGCCTATCCCCAGCCCCGTCCCGACTGGACGGGATACGGCCAGCAC 660
Va-2.VTA2 CAGGCATACCAACATGCCTATCCCCAGCCCCGTCCCGACTGGACGGGATACGGCCAGCAC 660
Va-1.VTA2 AGCGCTGGCCTGACGCCCGCCACCCACCACTTCCCGCCGACCCCGAGCTCCGCGCCCCCG 720
Va-2.VTA2 AGCGCTGGCCTGACGCCCGCCACCCACCACTTCCCGCCGACACCGAGCTCCGCGCCCCCG 720
Va-1.VTA2 AACGGCAGGCCGACTCAGGTAAGTGTTGACTTCGCACAAGATGGCGCGCGCCCCTCCCGCC 780
Va-2.VTA2 AACGGCAGGCCGACTCAGGTAAGTGTTGACACGCACAAAACGGCGCGCGCCCCTCCCGCC 780
Va-1.VTA2 CCGCCGCATCGAGCTGGCCTTGCTAACCAAACAGTTTGGCAACCAGGTATACTCTTTCGT 840
Va-2.VTA2 CCGCCGCATCGAGCTGGCCTTGCTAACCAAACAGTTTGGCAACCAGGTATACTCTTTCGT 840
Va-1.VTA2 TCCGATTCCTGGCGCGCAGCAACACAAGCGGCCGAGACGACGATACGAGGAGATTGAGCG 900
Va-2.VTA2 TCCGATTCCTGGTGCGCAGCAACACAAGCGGCCGAGACGACGATACGAGGAGATTGAGCG 900
Va-1.VTA2 CATGTACAAGTGTGGCTGGCAGGGTTGCGAGAAGGCGTATGGTACTCTGAACCATCTCAA 960
Va-2.VTA2 TATGTACAAGTGTGGCTGGCAGGGTTGCGAGAAGGCGTATGGTACTCTGAACCATCTCAA 960
Va-1.VTA2 TGCGCACGTCACTATGCAGTCCCACGGCACGAAGCGGACTCCTGAAGGTATGCACTTACC 1020
Va-2.VTA2 TGCGCACGTCACCATGCAGTCGCACGGCACGAAGCGGACTCCTGAAGGTATGCACTTACC 1020
Va-1.VTA2 ACTCCCGTCTTTGCCCTGTTCCTCAACCCATCCATGTCGCTGCTCTATGTGCGACACGATT 1080
Va-2.VTA2 ACTCCTGTCTTTGCCCTGTTCATCAACCCATCCATGTCGCTGCTCTATGTGCGACGCGACC 1080
Va-1.VTA2 GAATGTGGTGGCTTTCCTGTGGGAGAGCGCGCCGAGTGGCGTACACTTGCCGTGTTTTTA 1140
Va-2.VTA2 AAATGTGGTGGCTTTCCTGTGGGAGAGCGCGCCGAGTGGCGTACACTTCCCGTGTTTTTA 1140
Va-1.VTA2 GCCTCACCAGCACGACCACACAGCTCCGTGGGTGCTGTGGGACTGGGCGTAAAATTGAGA 1200
Va-2.VTA2 GCCTCGCCAGTACGATCACACAGCTTCGTGGGTGCTGTGGGACTGGGCGTAAAATGGAGA 1200
Va-1.VTA2 CTAGCAGCTTCTATGGGCGACCCGACTAGGGGCGACCCGAGCCTACGGGGGACTTGTGTGA 1260
Va-2.VTA2 CGAGCT-----TATGGGCGACCCGACTAGGGGCGACCCGAGTTACGGGGGACTTGAATGA 1255
Va-1.VTA2 TGAAACGGCCTGAAGCTAATCCGTGACTGCAGAGTTCAAAGAGATTCGTAAAGAATGGAA 1320
Va-2.VTA2 TGAAACGGCCGGAAGCTGATCCGTGACTGCAGAGTTCAAAGAGATTCGTAAAGAATGGAA 1315
Va-1.VTA2 GCAGCGCAAGAAGGAGGAAGAAGCTGCTCGCAAGGCCGAGGACGAGCAACGTCGTGCGGC 1380
Va-2.VTA2 GCAGCGCAAGAAGGAGGAAGAAGCTGCTCGCAAGGCCGAGGACGAGCAACGTCGTGCGGC 1375
Va-1.VTA2 TGCTGCTGCAGCGGCAGCGCAAGCCGCCCAAAACGGTGGCCCAGACCCTCAATCCGGGCC 1440
Va-2.VTA2 TGCTGCTGCAGCGGCAGCGCAAGCCGCCCAAAACGGTGGCCCAGACCCTCAATCCGGGCC 1435

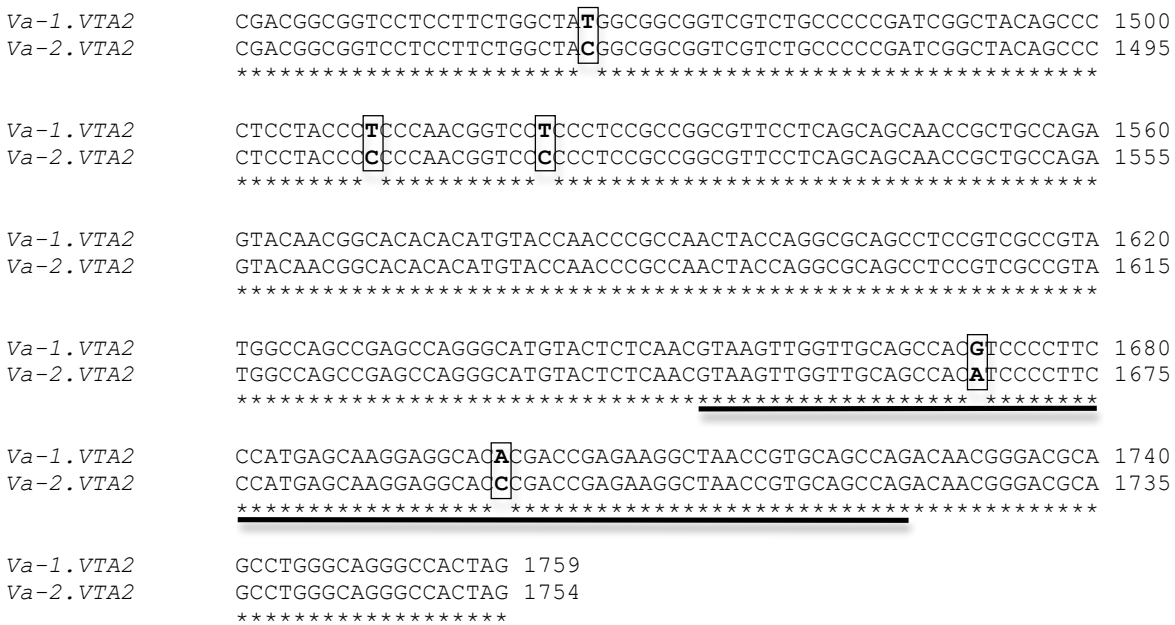

Figure S5. The *VTA2* gene is conserved within filamentous fungi.

(A) *VTA2* isogenes (*VTA2-1*, *VTA2-2*) from *V. longisporum* are derivatives from *V. dahliae* and *V. albo-atrum*, respectively. Asterisks indicate identical nucleotides and four introns are underlined.

(B) Similarity of *Vl(Vd)VTA2-1* and *Vl(Va)VTA2-2* isogenes from *V. longisporum* to the *VTA2*-like genes from Mg = *Magnaporthe grisea* (*CON7*, ABI96241), Cg = *Colletotrichum graminicola* (EFQ25259), Fg = *Fusarium graminearum* (XP_384310) and Fs = *Fusarium solani* (XP_003044820). The DNA coding sequences are translated into deduced protein sequences and the asterisks indicate identical amino acids.

(C) Phylogenetic analysis based on *VTA2* genes from *Verticillium* species (Va: *V. albo-atrum*, Vd: *V. dahliae*, Vl: *V. longisporum*, Vtr: *V. tricorpus*) and the homologues from other filamentous fungi including Cg: *Colletotrichum graminicola* (EFQ25259), Fs: *Fusarium solani* (XP_003044820), Fg: *Fusarium graminearum* (XP_384310), Mg: *Magnaporthe grisea* (*CON7*, ABI96241), Bc: *Botrytis cinerea* (BC1G_11736), Ss: *Sclerotinia sclerotiorum* (XP_001597994), Sm: *Sordaria macrospora* (CBI53414), Nc: *Neurospora crassa* (XP_963107), Pm: *Penicillium marneffei* (XP_002151981), Pc: *Penicillium chrysogenum* (XP_002566004), An: *Aspergillus nidulans* (XP_663533), Ani: *A. niger* (XP_001399400), Afl: *A. flavus* (EED52674) and Af: *A. fumigatus* (XP_755410). *VTA2/CON7* genes are highly conserved within filamentous fungi and show a close relationship among fungal plant pathogens (indicated by the dashed frame).

(**D)** The structure of the *VTA2* gene has slightly been changed within *V. albo-atrum* species. Va-1 isolate from potato and Va-2 from alfalfa. The asterisks indicate identical nucleotides, nucleotide changes are framed in bold and the introns underlined.