Verticillium dahliae transcription factors Som1 and Vta3 control microsclerotia formation and sequential steps of plant root penetration and colonisation to induce disease

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Thuc Tri Bui
from Thai Nguyen, Vietnam

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Thesis Committee

Prof. Dr. Gerhard H. Braus
Department of Molecular Microbiology and Genetics
Institute of Microbiology and Genetics
Georg-August Universität Göttingen

Prof. Dr. Stefanie Pöggeler
Department of Genetics of Eukaryotic Microorganisms
Institute of Microbiology and Genetics
Georg-August Universität Göttingen

Members of the Examination Board

Reviewer I
Prof. Dr. Gerhard H. Braus
Department of Molecular Microbiology and Genetics
Institute of Microbiology and Genetics
Georg-August Universität Göttingen

Reviewer II
Prof. Dr. Stefanie Pöggeler
Department of Genetics of Eukaryotic Microorganisms
Institute of Microbiology and Genetics
Georg-August Universität Göttingen

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Albrecht-von-Haller-Institute of Plant Sciences

Prof. Dr. Kai Heimel
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Institute of Microbiology and Genetics

PD Dr. Michael Hoppert
Department General Microbiology
Institute of Microbiology and Genetics

Prof. Dr. Rolf Daniel
Department of Genomic and Applied Microbiology
Institute of Microbiology and Genetics

Date of oral examination: 21.11.2017

Affirmation

I hereby declare that this thesis was written independently and with no other sources and aids than quoted

Göttingen, 3.10.2017

Thuc Tri Bui

This work was accomplished in the group of Prof. Dr. Gerhard H. Braus, at the Department of Molecular Microbiology and Genetics at the Institute of Microbiology and Genetics, Georg-August Universität Göttingen.

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Summary

Verticillium dahliae belongs to the soil-borne ascomycete fungi. It causes wilt diseases and early senescence in more than 200 plant species including economically important crops. It can exist in the soil without a host for a decade by forming microsclerotia. Root exudates induce germination of microsclerotia. *V. dahliae* enters its hosts through root infection, colonises the root cortex and invades the xylem vessels. The host infection of pathogenic fungi requires penetration and colonisation processes. The penetration of the root surface needs adhesive proteins at several stages during the host-parasite interaction. Adhesion proteins are not well described in *V. dahliae* whereas they are well studied in *Saccharomyces cerevisiae*. *S. cerevisiae* Flo8 is a transcription factor of adhesion, which regulates the expression of flocculation genes such as *FLO1* and *FLO11*. The defective *FLO8* strain is unable to adhere to agar plates or to flocculate in liquid medium. *V. dahliae* nuclear transcription factors Som1 and Vta3 can rescue adhesion in a *FLO8*-deficient *S. cerevisiae* strain. Som1 and Vta3 induce the expression of *FLO1* and *FLO11* genes encoding adhesins. The *SOM1* and *VTA3* genes were deleted and their function in fungal induced plant pathogenesis was studied by genetic, cell biological, proteomic and plant pathogenicity experiments. *V. dahliae* Som1 and Vta3 are sequentially required for root penetration and colonisation of the plant host. Som1 supports fungal adhesion and root penetration and is required earlier than Vta3 in the colonisation of plant root surfaces and tomato plant infection. Som1 controls septa positioning, the size of vacuoles, and subsequently hyphal development including aerial hyphae formation and normal hyphal branching. Som1 and Vta3 control conidia and microsclerotia formation and antagonise in oxidative stress response. The molecular function of Som1 is conserved between the plant pathogen *V. dahliae* and the opportunistic human pathogen *Aspergillus fumigatus*. Som1 controls the expression of genes for adhesion and oxidative stress response. Som1, as well as Vta3, regulate a genetic network for conidia and microsclerotia formation and pathogenicity of *V. dahliae*.

Zusammenfassung

Verticillium dahliae gehört zu den bodenbürtigen Askomyceten. Dieser Pilz verursacht Welke-Erkrankungen und verfrühtes Altern in mehr als 200 verschiedenen, auch ökonomisch wichtigen Pflanzen. *Verticillium* kann im Boden ohne Wirtspflanze durch die Bildung von Mikrosklerotien bis zu 10 Jahre überleben. Wurzelexsudate induzieren die Auskeimung der Mikrosklerotien. *V. dahliae* infiziert seinen Wirt durch die Wurzel, besiedelt den Wurzelkortex und dringt dann in die Xylemgefäße ein. Die Infektion des Wirts durch pathogene Pilze erfordert Penetrations- und Kolonisierungsprozesse. Am Eindringen durch die Wurzeloberfläche sind adhäsive Proteine an verschiedenen Stellen der Wirt-Parasit-Interaktion beteiligt. Adhäsive Proteine sind in *S. cerevisiae* gut untersucht, während nur wenig über sie in *V. dahliae* bekannt ist. Der Adhäsions-Transkriptionsfaktor Flo8 aus Hefe reguliert die Expression der sogenannten „Flocculation"-Gene wie zum Beispiel *FLO1* und *FLO11*. Ein Stamm ohne *FLO8* ist nicht in der Lage an Agarmedium zu haften und in Flüssigmedium auszuflocken. Die im Zellkern lokalisierten Transkriptionsfaktoren Som1 und Vta3 können die Adhäsion in einem *S. cerevisiae* Stamm, welchem *FLO8* fehlt, wiederherstellen. Som1 und Vta3 induzieren die Expression von *FLO1* und *FLO11*, welche Adhäsine kodieren. Die *SOM1* und *VTA3* Gene wurden deletiert und ihre Funktion in der durch Pilze verursachten Pflanzenpathogenese wurde durch genetische, zellbiologische, Proteom- und Pflanzenpathogenitätsexperimente untersucht. *V. dahliae* Som1 und Vta3 sind sequenziell für die Penetration und Kolonisation des Pflanzenwirts erforderlich. Som1 unterstützt die pilzliche Adhäsion sowie das Eindringen in die Wurzel. Somit wird es früher für die Besiedlung der Pflanzenwurzeloberfläche und die Tomateninfektion benötigt als Vta3. Som1 kontrolliert darüber hinaus die Positionierung von Septen und die Größe von Vakuolen und folglich auch die Entwicklung von Hyphen inklusive der Bildung von Lufthyphen und normalen Hyphenverzweigungen. Som1 und Vta3 beeinflussen die Bildung von Konidien und Mikrosklerotien und wirken sich in der Antwort auf oxidativen Stress entgegen. Die molekulare Funktion von Som1 ist zwischen dem Pflanzenpathogen *V. dahliae* und dem opportunistischen Humanpathogen *Aspergillus fumigatus* konserviert. Som1 kontrolliert die Expression von Genen welche für Adhäsion und die Antwort auf oxidativen Stress benötigt werden. Sowohl Som1 als auch Vta3 regulieren ein genetisches Netzwerk für die Bildung von Konidien und Mikrosklerotien sowie die Pathogenität von *V. dahliae*.

1. Introduction

1.1 *Verticillium dahliae* – a pathogen of wilt diseases

Verticillium species are soil-borne plant pathogenic fungi which cause high losses of crops (Pegg & Brady, 2002; Berlanger & Powelson, 2005). The name *Verticillium* was given because of the phialide arrangement in verticillate shape around the conidiophores (Pegg & Brady, 2002; Berlanger & Powelson, 2005). There are three different species of *Verticillium* including *Verticillium albo-atrum*, *Verticillium dahliae*, and *Verticillium longisporum* (Pegg & Brady, 2002). However, the first *Verticillium* strain was detected in 1879 (Reinke & Berthold, 1879) until 1913 *V. dahliae* which causes wilt on dahlia (*Asteraceae* family) was first described (Isaac, 1947).

1.1.1 *V. dahliae* is a threatening plant pathogenic fungus

V. dahliae strains cause wilting diseases and early senescence in more than 200 plant species of economically important crops including tomato, potato, cotton, cabbages, and strawberries (Pegg & Brady, 2002). This species is causing significant loss in crop yield and are widely spread.

A *V. dahliae* strain can enter the root and develop resting structures not only in more than 200 plant host species but also in non-host plants (Pegg & Brady, 2002; Berlanger & Powelson, 2005). After entering the plant roots, this fungus produces conidia which are transported in the transpiration stream to any part of the plant. In which, conidia germinate and colonise the plant (Pegg & Brady, 2002; Berlanger & Powelson, 2005). *V. dahliae* produces resting structures which are called microsclerotia to survive in the soil without host plants. Microsclerotia are formed in dry tissues when the plant dies. Thick cell walls with dense melanin deposits of microsclerotia protect the fungus against extreme temperatures, enzymatic lysis, and UV light. They still can recover from animal feces after staying two days in the stomach (Pegg & Brady, 2002; Berlanger & Powelson, 2005). Therefore, microsclerotia can survive without hosts for a decade in the soil (Pegg & Brady, 2002). Additionally, *V. dahliae* can enter non-host plants and produce microsclerotia, which cause no symptoms or diseases (Pegg & Brady, 2002;

Berlanger & Powelson, 2005). Because of these reasons, it is not easy to treat *V. dahliae* even when using crop rotations or fungicides (Pegg & Brady, 2002). Therefore, *V. dahliae* causes a loss of billions of dollars in annual crops worldwide (Pegg & Brady, 2002; Berlanger & Powelson, 2005).

Conidia and microsclerotia of *V. dahliae* are easily transported worldwide by different ways. For instance, conidia are tiny cells which can be transported through xylem vessel systems with the transpiration stream from the root vessels into the shoot and thereby distributing the fungus to the whole plant. Therefore it is easy transported with crop products (Pegg & Brady, 2002; Berlanger & Powelson, 2005; Inderbitzin *et al.*, 2011b). Furthermore, they are easily transported by air for 20 feet or by water stream such as rivers and irrigation canals when the water was re-used. *V. dahliae* may also be distributed by contaminated seeds, insects, vegetative cutting, transplant, hand tools or farm machinery (Pegg & Brady, 2002; Berlanger & Powelson, 2005). Microsclerotia can also be found in seeds of infected plants, therefore, they are easily transported worldwide when crop products are exported or imported (Pegg & Brady, 2002). Nowadays, this fungus can be found worldwide in countries with cool or warm climate (Figure 1).

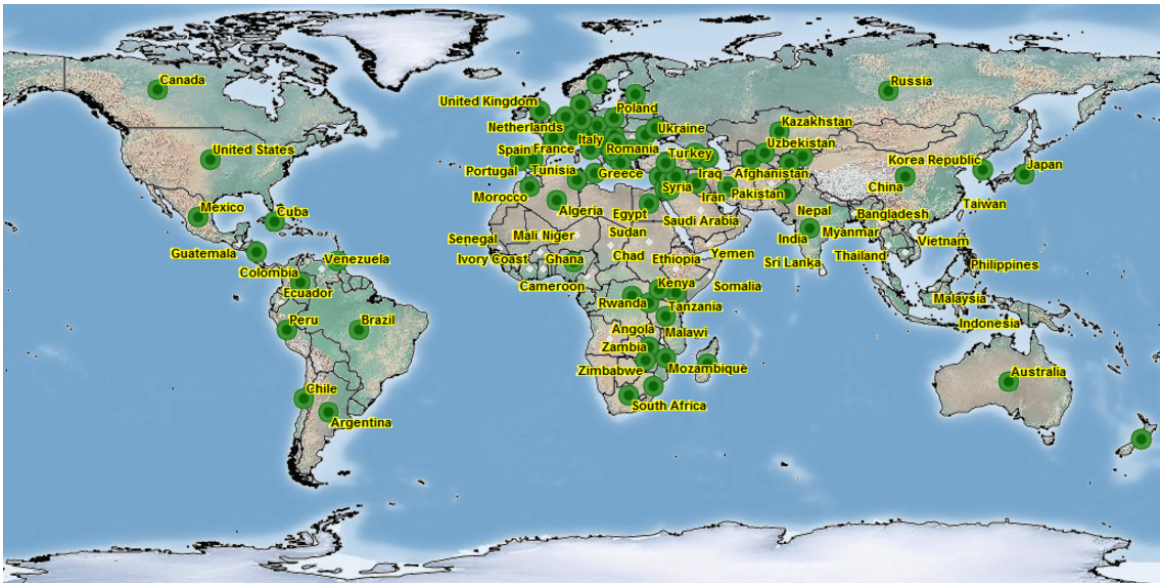


Figure 1. *V. dahliae* distribution. *V. dahliae* species were found worldwide from cool to warm climate. They are more common in Europe, America, and Africa than in Asia and Australia. Figure taken from http://www.plantwise.org/KnowledgeBank/Datasheet.aspx?dsid=56275.

1.1.2 *Verticillium* morphology

Hyphae of *V. dahliae* are mostly haploid, but hyphal tips may be multinucleate. They are hyaline, simple or branched, septated and multinucleated. Hyphal septa are perforated, but nuclei have not been reported to traverse the pore (Pegg & Brady, 2002; Berlanger & Powelson, 2005). The hyphal extension is directly proportional to the availability of growth capabilities. Diffusible morphogenic factors in *V. dahliae* inhibit hyphal elongation and conidiation and induce lateral branching (Brandt, 1967). Conidia are single cells which are born on phialides (Figure 2a, b, c). These phialides are arranged in whorls around conidiophores which are branched aerial hyphae(Pegg & Brady, 2002; Berlanger & Powelson, 2005). Each phialide carries a mass of conidia which are named conidiospore cluster in the following (Figure 2c). Conidia are hyaline and ovoid to an elongated shape. They have thin cell walls without melanin deposits (Figure 2d). Conidia are very small (3.5-5.5 µm) and are transported easily with the transpiration stream in plants (Pegg & Brady, 2002). Fungal materials such as microsclerotia can be therefore found in whole plants including seeds.

The resting structures, which are formed by hyphal welting, are usually found in the dead tissue of infected plants. They have thick cell walls with dense melanin layers. Black microsclerotia are found in the dead plant as black dots (Figure 2e, f, g) (Pegg & Brady, 2002; Berlanger & Powelson, 2005; Inderbitzin *et al.*, 2011b). These structures help *V. dahliae* to survive in the dead plants and the soil up to 15 years or after going through the animal stomach (Pegg & Brady, 2002).

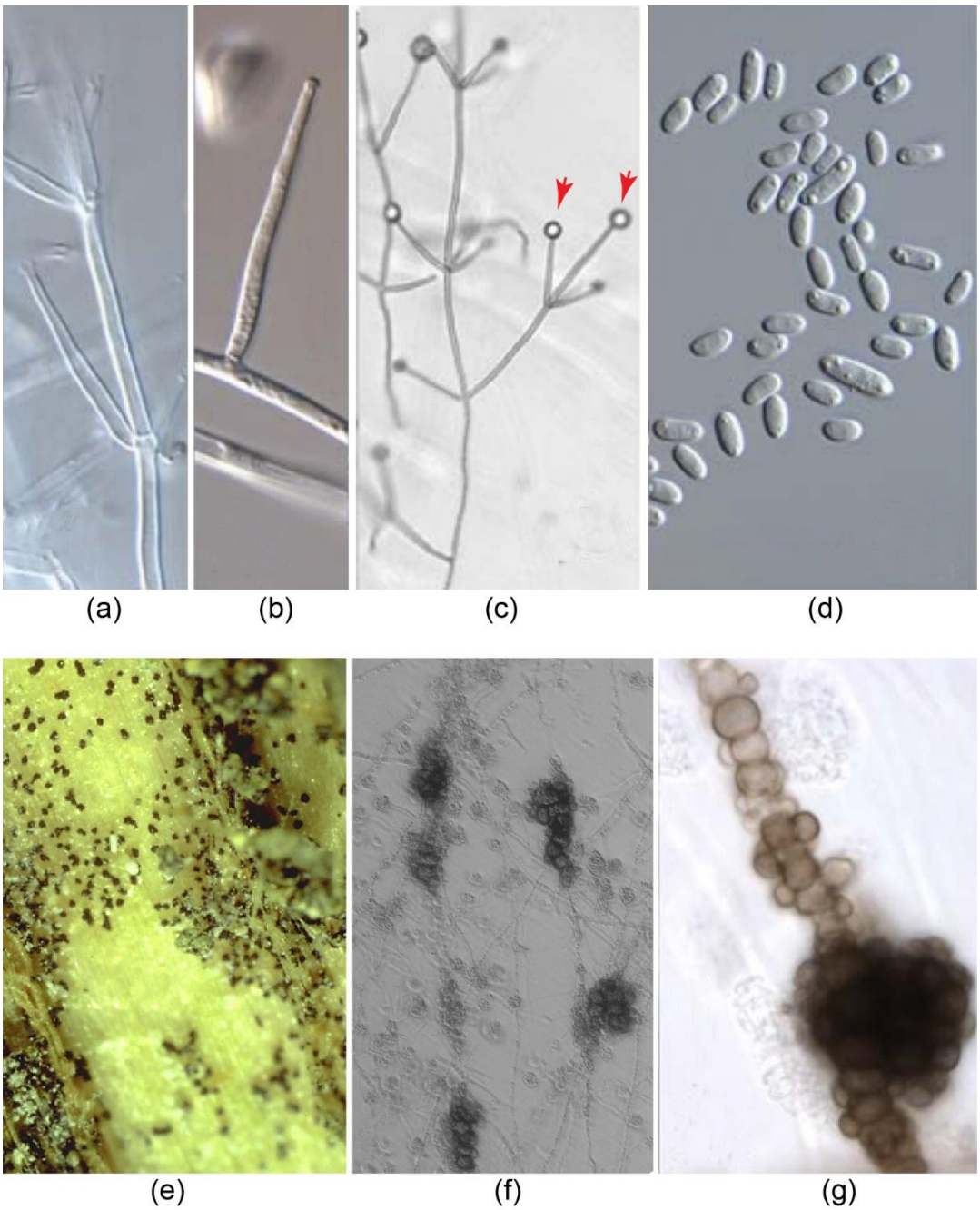


Figure 2. Conidia and microsclerotia of *V. dahliae.* (**a**) Whorl phialide. (**b**) Solitary phialide. (**c**) Branched conidiophore. (**d**) Conidia. (**e**) Microsclerotia in *planta*. (**f**) Microsclerotia on agar plates. (**g**) The structure of a single microsclerotium. The arrowhead shows a conidiospore cluster. This figure is modified from Inderbitzin *et al.* (2011a).

1.1.3 Disease symptoms of *V. dahliae* on tomatoes

Disease symptoms caused by *V. dahliae* are quite similar to *Fusarium* wilt symptoms which are difficult to distinguish in the field (Pegg & Brady, 2002; Berlanger & Powelson, 2005). Tomato infected stems have vascular discoloration consisting of dark-colored, elongated, necrotic tissue. The vascular discoloration may be accompanied by external symptoms such as wilting, yellowing, slow growth, abnormally heavy fruits or seeds, and death of leaves and plants (Pegg & Brady, 2002; Berlanger & Powelson, 2005). The lower and older leaves turn yellow earlier, wilt and dry later on. Infected plants are usually wilted in the midday and recovered in the evening. Most of the infected plants are stunted (Figure 3). Disease symptoms are more pronounced when plants are under drought stress (Pegg & Brady, 2002; Berlanger & Powelson, 2005).

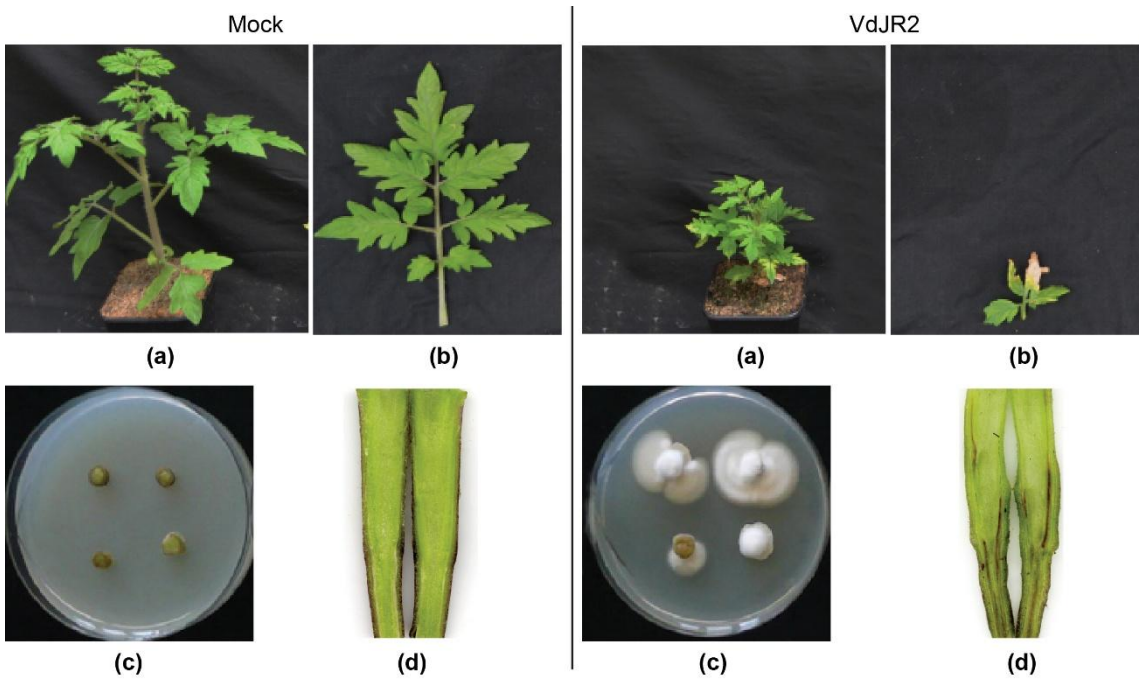


Figure 3. Wilt disease symptoms of *V. dahliae.* *V. dahliae* causes wilt diseases and early senescence in tomato plants (**a**) Height of tomato plants. (**b**) Length and disease symptoms in the first leaves. (**c**) Outgrowth of fungi from stems. (**d**) Disease symptoms in hypocotyls. Figure is modified from Tran *et al.* (2014).

1.1.4 *V. dahliae* disease cycle

The life cycle of *V. dahliae* is monocyclic. It has only one disease cycle as in other *Verticillium* species (Figure 4). The life cycle of *V. dahliae* can be divided into 2 steps root entering and developing in the plant. The root entering starts when microsclerotia are stimulated by root exudates or nutrients to germinate and grow in the soil toward the root surface (Berlanger & Powelson, 2005; Eynck *et al.*, 2007). The mycelium can reach up to one cm into the soil without nutrient supply before attaching to the plant root surface. Hyphae can infect and colonise root tips or follow root hairs to the root surface for penetration (Pegg & Brady, 2002; Berlanger & Powelson, 2005).

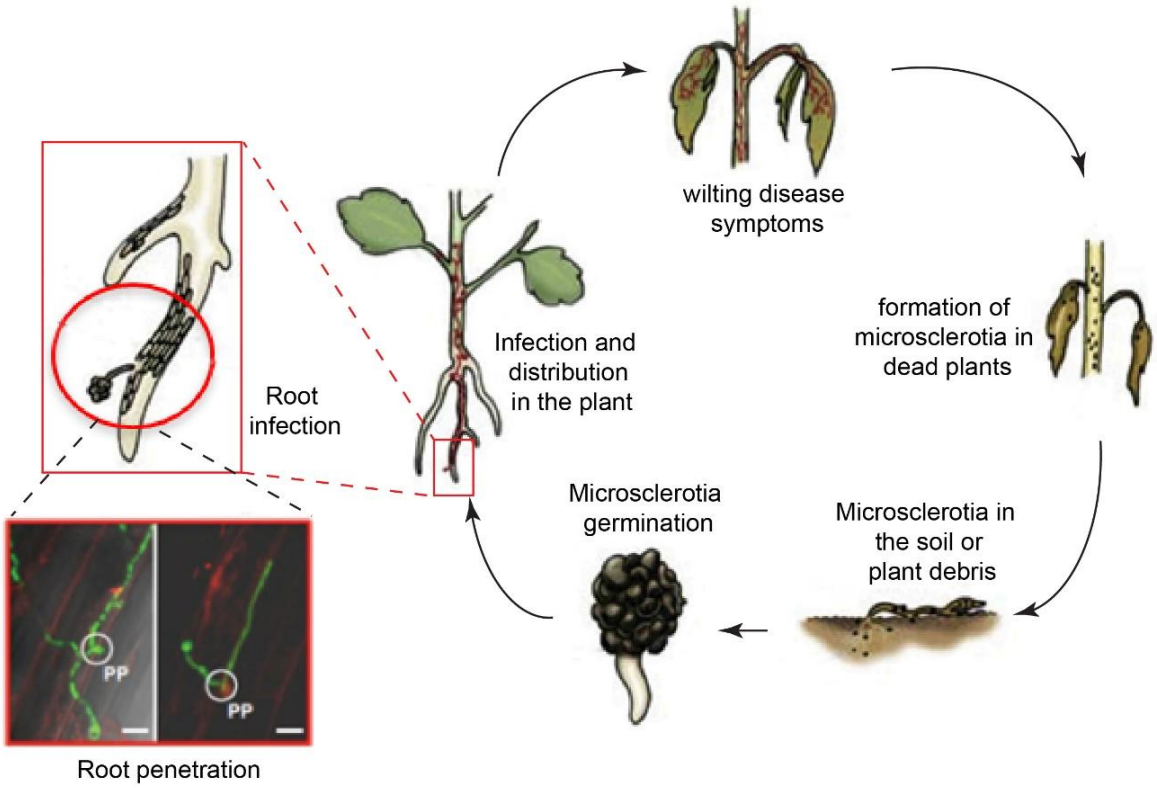


Figure 4. *V. dahliae* life cycle. The life cycle of *V. dahliae* starts when microsclerotia are stimulated by root exudates and germinate. Hyphae grow towards roots and penetrate root tips by forming penetration points (PP). Hyphae colonise the root cortex and enter the xylem vessels before forming asexual conidia and distributing in whole the plant. Symptoms like wilting, chlorosis and necrosis appear early in old leaves. Microsclerotia are formed in dead leaves, stems or in the soil until new plants are available. This figure is modified from Berlanger & Powelson (2005) and Tran *et al.* (2014).

The second step starts when the pathogenic fungi are already in the plant. Pathogenic fungal hyphae grow through cortical tissues toward vascular tissues and xylem vessels. The pathogen produces conidia which are transported from the root to the shoot with the xylem sap stream (Pegg & Brady, 2002; Berlanger & Powelson, 2005). During the transition, conidia germinate and develop inside the xylem vessels and enter the neighboring cells. Wherein, fungal hyphae produce more conidia to repeat this distribution process. Consequently, the pathogenic fungus occupies the cortical tissues, vascular cells, and the xylem vessels from roots to shoots. The disease symptoms such as wilting, chlorosis and necrosis start with appearing in lower and older parts of infected plants (Pegg & Brady, 2002; Berlanger & Powelson, 2005). Conidia have thin cell walls without melanin which cannot protect them against stress (Eynck *et al.*, 2007). Therefore, microsclerotia are formed in the whole dying plant including roots, leaves, seeds, branches, and stems. If dead plants are incorporated into the soil, microsclerotia are gradually released during decomposition of the tissues and new disease cycles will start when new plants are cropped (Pegg & Brady, 2002; Berlanger & Powelson, 2005).

1.2 Adhesion is essential for fungal pathogens

Adhesion of fungi to host surfaces is an important pathogenicity factor for both, plant and animal pathogenic fungi (Hostetter, 2000). Adhesive proteins are glues known as the substratum adhesion of fungi. They promote fungi to stay on surfaces under effects of blowing or washing (Yan *et al.*, 2011; Lin *et al.*, 2015; Epstein & Nicholson, 2016). Additionally, they increase the contact surface area between hosts and fungi which supports the penetration process of pathogens. Fungal mycelia require adhesion proteins for binding on root surfaces. Fungi may lose virulence if adhesion proteins are disrupted (Wang & St Leger, 2007; Yan *et al.*, 2011; Zhao *et al.*, 2016). Mad1 and Mad2 were shown to be required for adhesion. *Metarhizium anisopliae* strains lacking *MAD1* or *MAD2* are not able to infect insects (Wang & St Leger, 2007). Som1, a homolog of Flo8 in *Magnaporthe oryzae*, is essential for adhesion on rice leaves. The deletion strain of *SOM1* is avirulent (Wang & St Leger, 2007; Yan *et al.*, 2011). Therefore, anti-adhesions can block adhesion protein functions like an antigen/antibody model without uptake into cells (Epstein & Nicholson, 2016). Consequently, anti-adhesins might be used as a new strategy to control diseases.

1.2.1 Adhesion in yeasts

Yeasts such as *Saccharomyces cerevisiae* or *Candida albicans* are unicellular fungi and exist in single vegetative cells, which can change to multi-cellular growth with the promotion of adherent proteins (Dranginis *et al.*, 2007). Multi-cellular growth of yeasts can be found in flocculation, biofilm formation, and pseudohyphal processes (Braus *et al.*, 2003; Verstrepen *et al.*, 2003; Fichtner *et al.*, 2007). These procedures require expression of specific cell wall associated adhesins which regulate cell-cell or cell-surface adhesion (Kobayashi *et al.*, 1998; Braus *et al.*, 2003; Verstrepen *et al.*, 2003; Fichtner *et al.*, 2007). Adhesive proteins are usually located on the surface of cell walls to facilitate the interaction of cell-cell or cell-surface adhesion (Rigden *et al.*, 2004; Fichtner *et al.*, 2007; Wang & St Leger, 2007). Adhesive proteins in *S. cerevisiae* are well known. A family of flocculation genes (*FLOs*) was reported to play key roles in adherence (Kobayashi *et al.*, 1996). There are two groups of flocculation genes. One group harbors a PA14 conserved domain including *FLO1*, *FLO5*, *FLO9*, *and FLO10* (Kobayashi *et al.*, 1996; Kobayashi *et al.*, 1998). The PA14 domain sequence possesses a carbohydrate-binding function (Rigden *et al.*, 2004). The *FLO1*, *FLO5*, and *FLO9* genes share high sequence similarity (Dranginis *et al.*, 2007). Flo1 is required for both, adhesion and flocculation (Kobayashi *et al.*, 1998). Flocculation is useful in the brewing industry because flocculated cells are easily separated from the culture medium at the end of fermentation (Verstrepen *et al.*, 2003). Therefore, one does not need a complicated filter system to take out cells from the culture medium.

The other group of flocculation genes contains *FLO11* which is only responsible for initial surface adhesion providing only some cell layers (Fichtner *et al.*, 2007). There are several transcription factors which bind to the promoter of *FLO11* for activation or repression (Figure 5). Flo8 and Ste12 are found in the promoter region of *FLO11* and required for expression of *FLO11*. Lack of either *FLO8* or *STE12* causes inactivation of *FLO11*. Sfl1 was reported to repress the expression of flocculation genes. It was found in the promoter region of *FLO11*. The Sfl1 binding site overlaps with that of Flo8 (Figure 5) (Octavio, 2009).

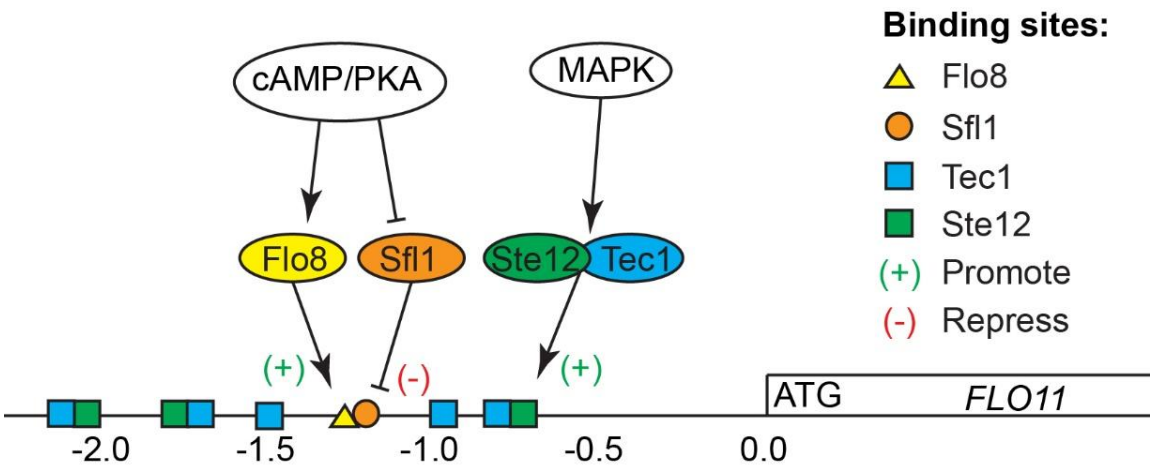


Figure 5. Expression of *FLO11* is controlled by cAMP/PKA and MAPK pathways. Mitogen activated protein kinase (MAPK) activates *FLO11* via the Ste12 and Tec1 complex, whereas the cyclic AMP dependent protein kinase A cascade (cAMP/PKA) controls *FLO11* through Flo8 and Sfl1. Flo8 activates expression of *FLO11*, whereas Sfl1 represses its expression. Flo8 activation and Sfl1 repressor binding sites are overlaping. This figure is modified from Octavio *et al.* (2009).

Flo8 is known as a transcription factor which is in downstream of the cyclic AMP dependent protein kinase A (cAMP/PKA) pathway, which was reported to control fungal development or pathogenicity in yeast *S. cerevisiae* or plant pathogenic fungus *M. oryzae*. The defect of *FLO8* causes an inactivation of *FLO1* and *FLO11* genes in yeast. The point mutant of *FLO8* gene shows a non-adhesive *S. cerevisiae* on agar plates and in liquid medium (Figure 6).

The primary colonisation of *Candida albicans* to host surfaces requires adherent proteins (Sundstrom, 2002). Several adhesive proteins are found such as enzymes, agglutinin-like sequence protein, integrins, and lectin-like protein (Cotter & Kavanagh, 2000; Sundstrom, 2002; Bonfim-Mendonca *et al.*, 2015). The outer cell wall layer is rich in glycosylated mannoproteins, which may contribute to host-fungus interactions. The protein mannosyltransferases (Pmt) can be found in the endoplasmic reticulum and mannosyltransferases (Mnt) in the Golgi (Timpel *et al.*, 2000; Munro *et al.*, 2005). Pmt is required for adherence to endothelial cells, whereas Mnt1 and Mnt2 are essential for human buccal epithelial cells. Both of them are involved in the glycosylation process (Timpel *et al.*, 2000; Munro *et al.*, 2005). A putative glycosidase (Csf4p) has a major role in adherence. Deletion of *CSF4* reduces adhesion to mammalian cells (Alberti-Segui *et al.*, 2004). *CAMP65* encodes a putative β-glucanase, which is essential for adhesion to plastics (Sandini *et al.*, 2007). Secreted aspartate proteinase such as Sap1, Sap2, Sap3, Sap4, and Sap6 may also contribute to adhesion (Naglik *et al.*, 2004).

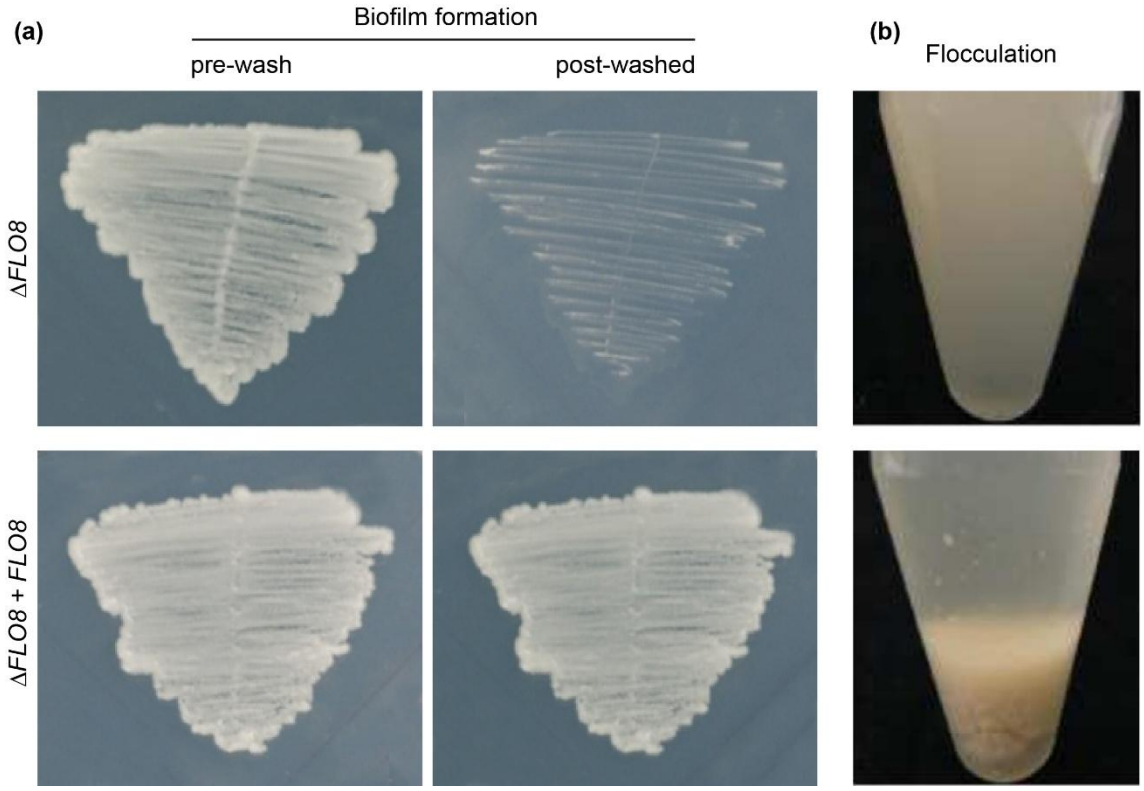


Figure 6. Adhesion of *S. cerevisiae* on agar plates and in a liquid medium. (**a**) Biofilm formation on agar plates. The strain expressing *FLO8* can produce biofilm on an agar plate, which is unable to be washed off, whereas the deletion strain of *FLO8* is removed by washing. (**b**) Flocculation of a strain expressing *FLO8* gene. This figure is modified from Tran *et al.* (2014) and from Lin *et al.* (2015).

The agglutinin-like sequence (Als) protein family was reported to be essential for adhesion to human epithelial cells (Fu *et al.*, 2002; Sundstrom, 2002). The *ALS* gene family encodes a group of GPI-anchored proteins which are necessary for adhesion (Hoyer *et al.*, 1998; Hoyer, 2001; Fu *et al.*, 2002). There are nine *ALS* genes (*ALS1-ALS9*) in the *C. albicans* genome. Among Als proteins Als1p, Als3p, and Als5p are essential for adherence (Hoyer *et al.*, 1998; Hoyer, 2001; Fu *et al.*, 2002; Sundstrom, 2002). Als1p and Als3p are important for binding to endothelial and epithelial cells, whereas Als5p binds to extracellular matrix proteins (Hoyer *et al.*, 1998; Fu *et al.*, 2002; Epstein & Nicholson, 2016). Structure and functions of Als1p are similar to Flo11 of *S. cerevisiae* (Fu *et al.*, 2002; Sundstrom, 2002). Expression of *ALS1* is controlled by the transcription factor Efg1p, which is a key regulator of filamentation in *C. albicans* (Fu *et al.*, 2002). Als1 and Hwp1 protein promote the biofilm formation process (Fan *et al.*, 2013).

The hyphal wall protein 1 (Hwp1) is necessary for adhesion on the surface layer of the stratified squamous epithelium, which is found exclusively at the germ

tube surface (Sharkey *et al.*, 1999; Sundstrom, 2002). Hwp1 is required for biofilm formation in *C. albicans*. Deletion of *HWP1* was defective in biofilm formation and effective human infection (Sharkey *et al.*, 1999; Padovan *et al.*, 2009). *C. albicans* binds to extracellular matrix proteins which might need the presence of integrin-like receptors (Int1) at the host surfaces (Calderone, 1998). Expression of the *INT1* encoding integrin-like protein of *C. albicans* can reprogram non-adherent yeast *S. cerevisiae* to adhere to human epithelial cells (Calderone, 1998; Gale *et al.*, 1998). The deletion of *INT1* in *C. albicans* represses adhesion to human epithelial cells (Gale *et al.*, 1998).

The epithelial adhesion protein family (EPA) contains seven proteins (Epa1-Epa7) which harbor the PA14 conserved domain. Although there are seven members of the Epa family, only Epa1 is essential for binding to the epithelial cell during infection (Sundstrom, 2002; Dranginis *et al.*, 2007; Zupancic *et al.*, 2008). Epa1 is a Ca^{2+}-dependent lectin which shows homology to Flo1 of *S. cerevisiae* and binds to N-acetyllactosamine-containing glycoconjugates. Adhesion of the *EPA1* deletion strain was reduced by 95% compared to the wild-type strain (Sundstrom, 2002; Li & Palecek, 2003). Other *EPA* genes are low expressed. For instance, *EPA6* is not expressed *in vitro*, whereas its expression is enhanced during experimental urinary infection (Vitenshtein *et al.*, 2016; Zajac *et al.*, 2016).

1.2.2 Adhesion and virulence in filamentous fungi

Adhesion of filamentous fungi is not as well studied as adherence in yeast and is usually associated with virulence. Appressorium formation in filamentous fungi was reported to play important roles in adhesion and virulence (Figure 7) (Clergeot *et al.*, 2001; Xue *et al.*, 2002; Gourgues *et al.*, 2004; Jeong *et al.*, 2007; Yan *et al.*, 2011). There are several transcription factors and cell wall proteins which were reported to have important roles in appressoria formation and promotion of adhesion and virulence (Clergeot *et al.*, 2001; Xue *et al.*, 2002; Gourgues *et al.*, 2004; Jeong *et al.*, 2007; Yan *et al.*, 2011; Li *et al.*, 2015a). The cAMP/PKA pathway and Pmk1 MAPK cascade are required for appressoria development and pathogenicity in the rice blast fungus (Lee & Dean, 1993). The cAMP signaling pathway responds to an inductive signal from rice leaves such as hydrophobic surface and wax monomers, which is required for appressoria formation in *M. oryzae* (Zhou *et al.*, 2012; Li *et al.*, 2015a). Deletion of *MAC1*, a gene

encoding an adenylate cyclase, cannot form appressoria, aerial hyphae, and conidia (Zhou *et al.*, 2012; Li *et al.*, 2015a). *MoSOM1,* a homolog of *FLO8* in *M. oryzae*, is located downstream of the cAMP/PKA signaling pathway, which is regulated by MoMac1 (Yan *et al.*, 2011). MoSom1 physically interacts with MoCdf1. Disruptions of *MoSOM1* and *MoCDF1* show defects in adhesion, appressoria formation and virulence (Yan *et al.*, 2011). *MoMPG1*, a gene encoding a hydrophobin protein, is controlled by MoSom1, which is essential for fungal binding to plant leaves during plant infection (Beckerman & Ebbole, 1996; Pham *et al.*, 2016). *Aspergillus fumigatus* AfSomA, a homolog of Flo8, physically interacts with Ptab. Both of them play important roles in biofilm formation and adhesion (Lin *et al.*, 2015). AfSomA is essential for virulence, whereas AfPtab is not (Lin *et al.*, 2015). Adhesion of the fungi to the host surface and virulence require formation and function of appressoria (Braun & Howard, 1994; Beckerman & Ebbole, 1996; Xue *et al.*, 2002; Li *et al.*, 2015a).

Genes specifically expressed in appressoria of *M. grisea* were examined. There are 72 genes only expressed in mature appressoria including *GAS1* and *GAS2* (Lu *et al.*, 2005). Gas1 and Gas2 are not essential for appressoria formation, but they are required for appressorium penetration and lesion development (Xue *et al.*, 2002; Lu *et al.*, 2005). A main polysaccharide component of the cell wall, which was reported to play important roles in appressoria formation is chitosan. Chitosan can be formed from chitin under the catalysis of chitin deacetylases (CDAs) (Pochanavanich & Suntornsuk, 2002). Chitosan is reported to localise in the germ tube and appressoria. Deletion strains of *CDA* genes showed loss of chitin deacetylation, reduced adherence and appressoria formation on an artificial hydrophobic surface (Geoghegan & Gurr, 2016).

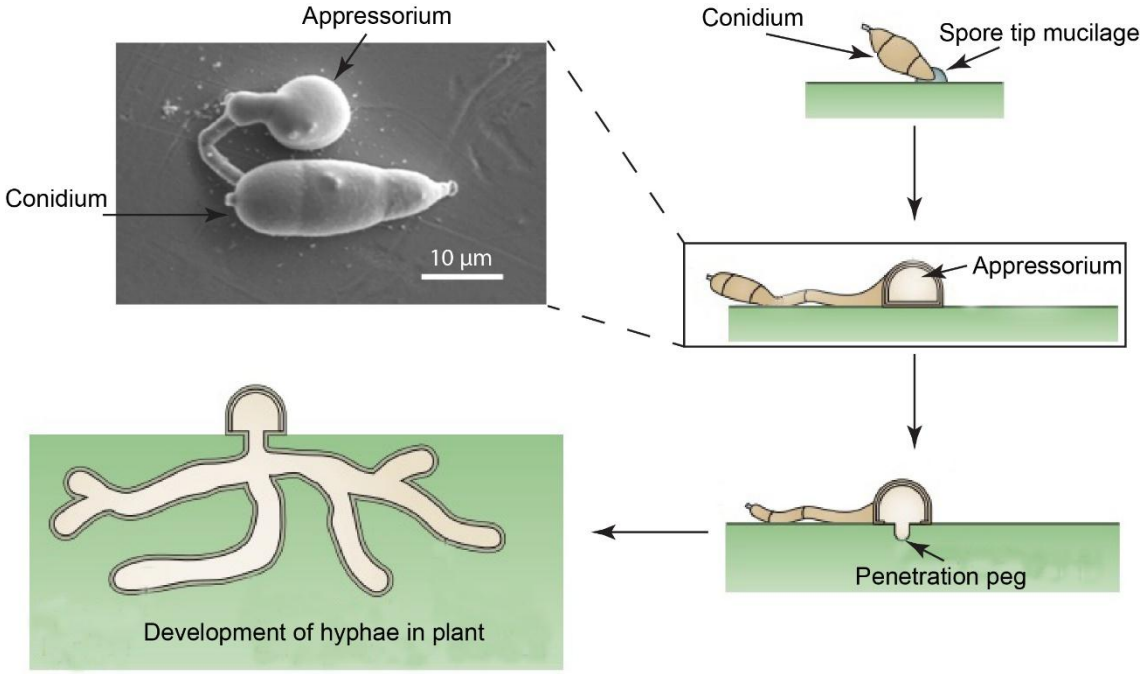


Figure 7. Appressoria are required for the rice blast fungus *Magnaporthe oryzae* infection. Spores attach to the hydrophobic cuticle by spore tip mucilage, germinate and produce a narrow germ tube. Appressoria are formed in the tip of the germ tube. Appressoria become melanised and substantial turgor before forming a narrow penetration peg at the base. The penetration peg punctures the cuticle and enters the rice epidermis. Hyphae continue developing, moving from cell to cell, and inducing disease symptoms in plant leaves. This figure is modified from Skamnioti & Gurr (2007) and from Wilson & Talbot (2009).

The tetraspanin protein family was reported to join membrane signalling complexes controlling cell differentiation, motility, and adhesion (Clergeot *et al.*, 2001). The *PLS1* gene encodes a putative integral membrane protein and is related to the tetraspanin family. It is localised in plasma membranes and vacuoles. Pls1 plays an appressorial function. This protein is essential for penetration of *M. grisea* into host leaves (Clergeot *et al.*, 2001). BcPls1, a homolog of Pls1 in *Botrytis cinerea*, was also reported to be involved in the penetration process into host plant leaves (Gourgues *et al.*, 2004). Hydrophobin proteins were shown to play crucial roles in cell morphogenesis and pathogenicity in several plant pathogenic fungi including *M. oryzae* (Linder *et al.*, 2005; Pham *et al.*, 2016). Mpg1 and Mhp1 are hydrophobin proteins, which are highly expressed during rice blast infection. They promote spore adhesion and penetration into the host plant (Beckerman & Ebbole, 1996; Pham *et al.*, 2016). The fasciclin family protein MoFlp1 contains a glycosylphosphatidylinositol (GPI) anchor and is localised on the vacuole membrane. The deletion strain of *MoFLP1* showed defects in adhesion,

appressorium turgor, and virulence (Liu *et al.*, 2009). Adhesion protein and appressoria formation are also studied in other pathogenic fungi (Hwang *et al.*, 1995; Wang & St Leger, 2007; Zhang *et al.*, 2009).

In *Colletotrichum gloeosporioides*, *CAP20* is known to be expressed during appressoria formation. The *CAP20* deletion strain does not show defects in appressoria formation but its function might be unfulfilled. The deletion strain showed a drastic decrease in virulence on avocado and tomato fruits (Hwang *et al.*, 1995). *Metarhizium anisopliae* adherence proteins Mad1 and Mad2 are produced on the conidial surface. Expression of *MAD1* and *MAD2* caused yeast to adhere to insect cuticle or a plant surface. Mad1 and Mad2 are required for adhesion in *M. anisoplia* (Voegele *et al.*, 2005). The disruption of *MAD1* caused delayed germination, suppressed blastospore formation, and greatly reduced virulence to the caterpillar. The deletion of Mad2 blocked adhesion of *M. anisopia* to plant epidermis (Wang & St Leger, 2007). BbHog1 encodes a functional homolog of yeast high-osmolarity glycerol (HOG), which regulates expression of hydrophobin genes such as *HYD1* and *HYD2* in *Beauveria bassiana* (Zhang *et al.*, 2009).

1.2.3 Adhesion and virulence in *V. dahliae*

The plant pathogen *V. dahliae* enters the plant roots without forming appressoria. Its hyphae can directly go into or form hyphopodia before entering the roots (Figure 8). There are several genes which have been reported to be related to adhesion and virulence in *V. dahliae*. The *VdPLS1* gene encoding a tetraspanin protein was specifically expressed in hyphopodia, which was shown to function as an adapter protein for the recruitment and activation of VdNoxB. Both, VdPls1 and VdNoxB, were required for hyphopodia peg formation (Figure 8). Deletion strains of *VdPLS1* and *VdNOXB* were unable to colonise cotton plant roots and to cause disease symptoms.

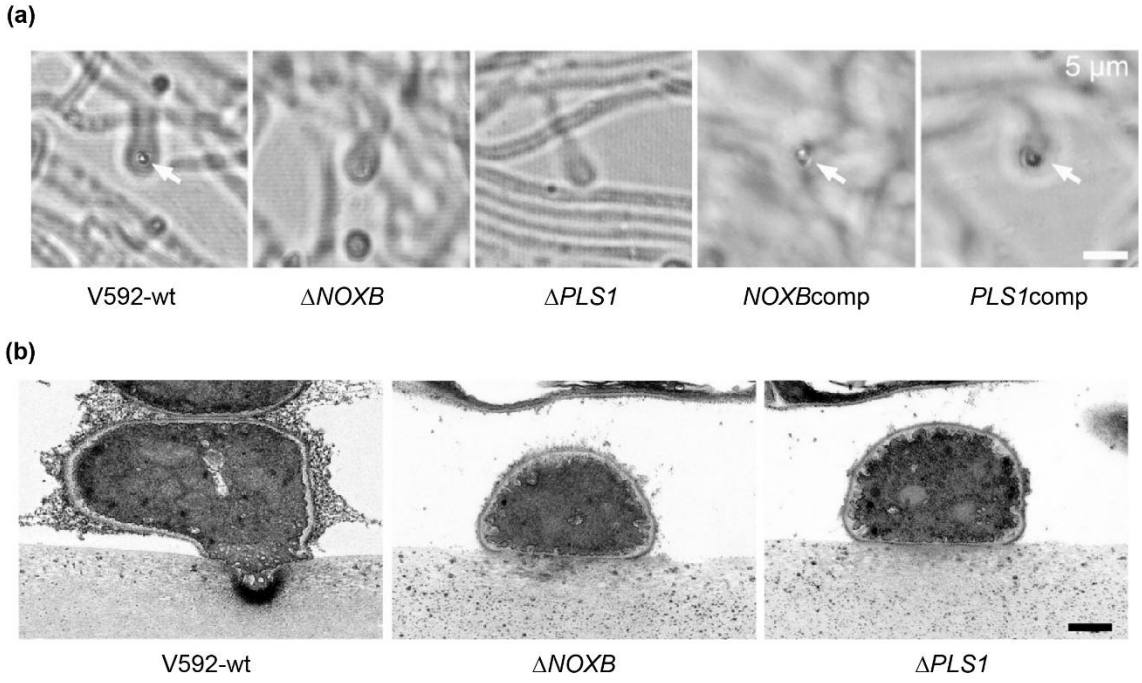
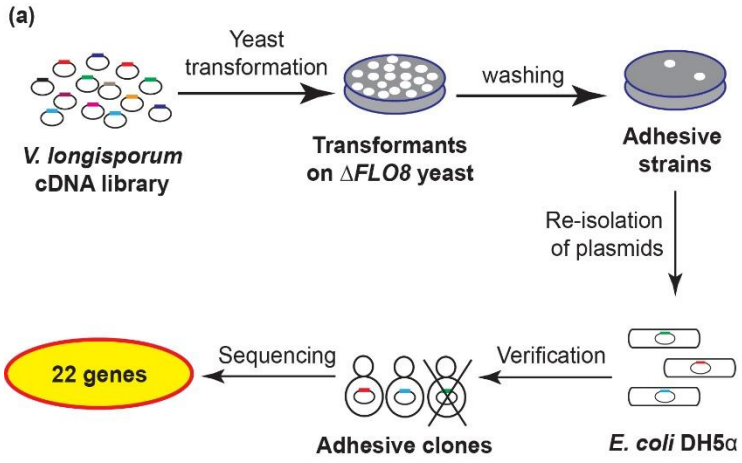


Figure 8. NoxB and Pls1 are essential for hyphopodia peg formation. (**a**) Hyphopodia peg development on cellophane membrane at 2 dpi. Hyphopodia pegs indicated by the arrowhead were only observed in V592-wt and complementation strains. They are not found in neither of the deletion strains of *NOXB* nor *PLS1*. (**b**) Cerium deposits were observed at the tip of hyphopodia peg in V592-wt, whereas they were not detected in both deletion strains of *NOXB* and *PLS1*. Scales bars are 5 µm. This figure is modified from Zhao *et al.* (2016).

Transcription factor Mcm1 belongs to the SRF subfamily of MADs-box family (Xiong *et al.*, 2016). Mcm1 is known as a key regulator of conidiation, microsclerotia formation, and virulence. Conidia production in the *MCM1* deletion strain was reduced by 80% compared with the wild-type strain. No microsclerotium was detected in the deletion strain of *MCM1*, whereas they were detectable in the wild-type strain. Deletion of Vd*MCM1* showed defects in adhesion of *V. dahliae* to abiotic surfaces and virulence on tomatoes. This deletion strain also failed to adhere to plant roots and to infect smoke trees. It promotes, however, hyphal growth and cell wall integrity (Xiong *et al.*, 2016). Putative adhesion genes from *Verticillium* were also screened using defective *FLO8 S. cerevisiae* (Tran *et al.*, 2014).

S. cerevisiae with a defect *FLO8* gene was used as a tool to screen for adhesion genes from *Verticillium longisporum*. In total twenty-two genes from *V. longisporum* were found to be able to reprogram the non-adhesive yeast to adherence (Figure 9a). However, only nineteen genes had predicted cellular functions and homologs to these genes could also been identified in *V. dahliae.* The predicted cellular

functions differ and include transcription regulation, signaling, protein trafficking, morphogenesis, pigmentation, and unknown functions (Figure 9b). None of them revealed any typical adhesion characteristics like GPI anchor or signal peptides (Tran *et al.*, 2014). Six out of the nine are putative transcription regulatory genes, which were named *Verticillium* transcription activator of adhesion (*VTA1* - *VTA6*). Vta2 encodes a C_2H_2 zinc finger protein, which is the homolog of the *M. grisea* Con-7 regulator. This gene is required for colonisation on plant roots and virulence of *V. dahliae* (Tran *et al.*, 2014). The deletion of *VTA2* resulted in a strain which was unable to colonise roots and cause disease symptoms. Additionally, it was defective in conidia formation in SXM (Tran *et al.*, 2014). Vta3 is a putative suppressor of A-kinase (Sak1), encoding a Winged helix-turn-helix DNA binding protein.



(b) Functional groups

Predicted functions	Number
Morphogenesis and pathogenicity	2
Pigmentation	1
Regulation of transcription and development	9
Signaling and protein trafficking	3
Nucleic acid binding	2
Unknown functions	5

Figure 9. A *FLO8* defective *S. cerevisiae* strain was used as a tool to screen for adhesion genes in *Verticillium*. (**a**) *V. longisporum* cDNA library under the control of the yeast specific galactokinase *GAL1* promoter was transferred into the non-adherent yeast deletion strain of *FLO8*. The transformants were grown on agar plates containing galactose and were gently washed with water. Plasmids from adhesive transformants were re-isolated, transformed into *E. coli*, and followed by verifying adhesion of yeast. The confirmed genes were sequenced and analysed. Twenty-two genes were found during the screen. (**b**) Predicted gene functions with Pfam/interProScan or BLAST search. Nine genes were putative transcription factors. This figure is modified from Tran *et al.* (2014).

1.2.4 Wing helix-turn-helix DNA binding proteins

Wing helix-turn-helix DNA binding proteins harbor a related winged helix-turn-helix DNA binding motif which consists of two wings (W1, W2), three alpha helices (H1, H2, H3) and three beta-sheets (S1, S2, S3) arranged in the order H1-S1-H2-H3-W1-S3-W2 (Figure 10) (Gajiwala & Burley, 2000). The wing W1 and helix H3 response DNA binding. Wing W1 of Rfx1 bind to major groove whereas helix H3 attach to minor groove of DNA. The wing helix-turn-helix DNA binding motif is highly conserved in fungi.

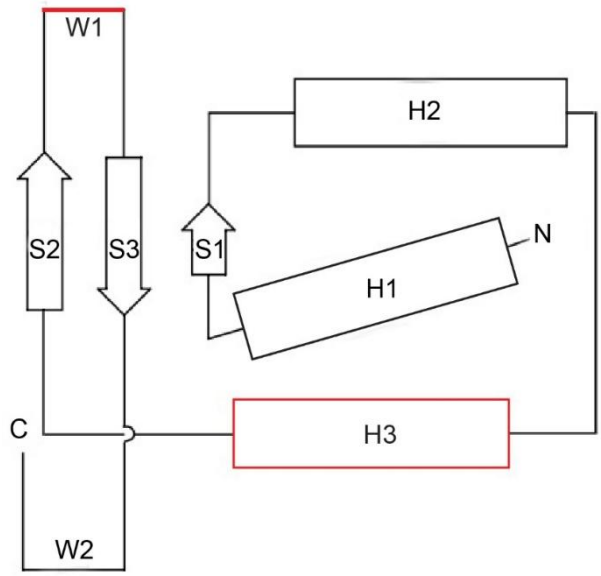


Figure 10. Topology of the winged helix fold. The winged helix motif contains two wings (W1 and W2), three α-helices (H1, H2, and H3), and three β-strands (S1, S2, and S3), which are arranged in order H1-S1-H2-H3-S2-W1-S3-W2. The wing W1 and helix H3 of Rfx1 are essential for DNA binding. The wing W1 binds to major groove whereas helix H3 overlies the minor groove of the X-box of DNA. The N-terminus is largely helical, whereas the C-terminus is composed of two strands and two large loops of wings. Red colour indicates the binding site of this motif to the X-box of DNA. This figure is modified from Gajiwala & Burley (2000).

The winged helix-turn-helix DNA binding domain is well studied in yeasts (Min *et al.*, 2014). In *S. cerevisiae*, the ScRfx1 transcription factor is reported to bind to the promoters of target genes and is involved in DNA repairing (Emery *et al.*, 1996). ScRfx1p recruits the repressors Tup1p and Cyc8p to inhibit the transcription of target genes (Emery *et al.*, 1996). This complex directly interacts with Sfl1, a suppressor gene for flocculation, which plays a major role in suppressing the expression of the flocculation gene *FLO11* (Emery *et al.*, 1996). The winged helix-

turn-helix DNA binding domain is also studied in filamentous fungi. In *Penicillium chrysogenum*, PcRfx1 binds to the promoter region of penicillin biosynthetic genes. Lack of *PcRFX1* caused a reduction of penicillin production (Bugeja *et al.*, 2010). In *Fusarium graminearum*, FgRfx1 is required for aerial hyphal formation, conidia formation, and virulence. In *V. dahliae*, the function of Vta3, a homolog of ScRfx1, has not been studied yet, whereas its putative interaction partner VdCyc8 was reported to play important roles in conidia and microsclerotia formation, as well as in virulence. Vta3 can rescue defective *FLO8* in *S. cerevisiae* (Tran *et al.*, 2014) and thereby might play important roles in *V. dahliae* adhesion.

1.3 Regulation of conidia and microsclerotia formation

Gene expression during microsclerotia formation was examined (Neumann & Dobinson, 2003; Duressa *et al.*, 2013), however, the mechanism of that process in *Verticillium* has not been described yet. Genes especially required for conidiation are well studied in *N. crassa*, *A. nidulans*, and *A. fumigatus* (Roberts & Yanofsky, 1989; Tao & Yu, 2011; Son *et al.*, 2013), whereas they have not been explained in *Verticillium* yet. Some factors of conidia and microsclerotia formation were described in *V. dahliae* (Klimes & Dobinson, 2006; Tran *et al.*, 2014; Zhao *et al.*, 2016).

1.3.1 Regulation of conidiation

Conidia play a role in distribution of the pathogen *in planta* or in the environment through water or the air (Pegg & Brady, 2002; Berlanger & Powelson, 2005). In *Neurospora crassa*, conidia formation requires the expression of *CON* family genes *CON-1* to *CON-13*. Con-6, Con-8, Con-10, Con-11, and Con-13 play a unique role during early conidia formation. Con-6 and Con-8 are expressed early during early conidia formation (Roberts & Yanofsky, 1989). Deletion of *MgCON-6* from *M. grisea* blocked conidia formation (Shi & Leung, 1995). MgCon-7 from *M. grisea* and Vta2, a homolog of Con-7 in *V. dahliae*, were shown to play an important role in conidia formation (Shi & Leung, 1995; Tran *et al.*, 2014).

Transcriptional factors BrlA, AbaA, and WetA are reported to play a central role in asexual development in *Aspergillus*. BrlA is essential for vesicle formation at the tip of aerial hyphae and controls the expression of *ABAA* and *WETA* (Tao and Yu, 2011). AbaA is needed for phialide differentiation from vegetative hyphae and it

activates *WETA* which is required for conidiophore maturation (Figure 11). The deletion strains of AbaA can produce metulae, but they were unable to generate phialides (Tao & Yu, 2011; Son *et al.*, 2013). AbaA and WetA promote *VOSA* which is required for trehalose accumulations and conidia maturation. VosA was reported to repress the expression of *BRLA* (Tao and Yu, 2011). In *Verticillium*, the homologs of BrlA, AbaA, and WetA are found. But their function in conidia formation has not been studied yet, but some proteins are known to be related to conidia formation such as Pls1, Vta2, Mcm1, Vdh1, and Vmk1 (Rauyaree *et al.*, 2005; Klimes & Dobinson, 2006; Tran *et al.*, 2014; Xiong *et al.*, 2016; Zhao *et al.*, 2016).

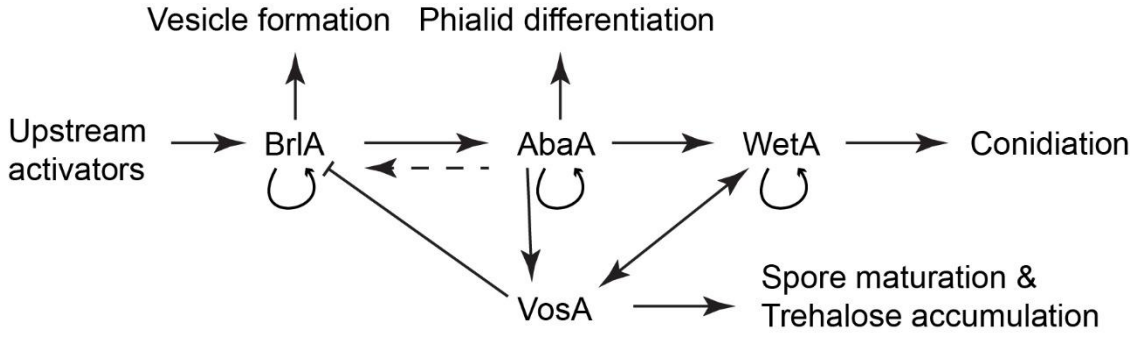


Figure 11. The central developmental pathway of conidia formation in *A. nidulans*. BrlA is essential for vesicle formation and regulates *ABAA*. AbaA is required for phialide differentiation and control *WETA* which is necessary for conidia formation. *VOSA* is regulated by AbaA and WetA and represses *BRLA* expression during vegetative growth. Arrow heads indicate activating effects. Dashed line shows proposed interaction. This figure is modified from Tao & Yu (2011).

1.3.2 Regulation of microsclerotia formation

Microsclerotia have thick cell walls with dense melanin layers, which can survive up to 15 years in the soil without the presence of the hosts (Pegg & Brady, 2002; Berlanger & Powelson, 2005; Fradin *et al.*, 2009). In previous studies, genes specifically expressed during microsclerotia formation were examined (Neumann & Dobinson, 2003; Duressa *et al.*, 2013). 153 genes were only expressed in induction medium for microsclerotia production. Approximately 50% of them were hypothetical proteins with unknown function (Duressa *et al.*, 2013). The others are putatively involved in pigmentation and transcription factors associated with pigment production, secondary metabolic enzymes, cell growth, morphogenesis, signaling, carbohydrate active enzymes and transport proteins (Neumann & Dobinson, 2003; Duressa *et al.*, 2013). Additionally, there are several genes which

were reported to control the formation of microsclerotia such as the Cyc8 glucose repression mediator protein (VdCyc8) (Li *et al.*, 2015b), the mitogen-activated protein kinase protein (VdVmk1) (Rauyaree *et al.*, 2005), the mitogen-activated protein kinase (VdPbs2) (Tian *et al.*, 2016), the transfer membrane mucin (VdMsb) (Tian *et al.*, 2014), the homolog of a high osmolarity glycerol (VdHog1) (Wang *et al.*, 2016), the calcineurin-responsive zinc finger transcription factor (VdCrz1) (Xiong *et al.*, 2015), the MADs-box transcription factor (VdMcm1) (Xiong *et al.*, 2016), and the hydrophobin protein (Vdh1) (Klimes & Dobinson, 2006). Especially, the hydrophobin protein Vdh1 is known as a key factor for microsclerotia formation. It is expressed during microsclerotia formation. Deletion of *VDH1* does not affect growth or virulence, but the deletion strain is unable to form microsclerotia (Klimes & Dobinson, 2006).

1.4 Aim of this work

The infection of the host by pathogenic fungi requires penetration and colonisation processes (Tran *et al.*, 2014; Zhao *et al.*, 2016). Defects in the penetration or colonisation step blocks plant infection by pathogenic fungi (Yan *et al.*, 2011; Tran *et al.*, 2014; Zhao *et al.*, 2016). To penetrate the root surface, adhesive proteins are needed at several stages during the host-parasite interaction (Braun & Howard, 1994; Hostetter, 2000). However, the understanding of the adhesion process of *Verticillium* is still limited. Adhesion is well studied in *S. cerevisiae.* The flocculation genes (*FLOs*) encode cell wall-associated adhesions. *FLO1* and *FLO11* promote flocculation or substrate adhesion, respectively (Liu *et al.*, 1996; Verstrepen & Klis, 2006; Fichtner *et al.*, 2007; Van Mulders *et al.*, 2009). Flo1 is required for both, adhesion and flocculation, whereas Flo11 is only responsible for initial surface adhesion providing only some cell layers (Fichtner *et al.*, 2007). Both of them are activated by Flo8 which is located downstream of the cAMP/PKA pathway (Rupp *et al.*, 1999; Fichtner *et al.*, 2007).

The *FLO8* deletion strain BY4742 (Euroscarf, Frankfurt, Germany) does not produce a functional Flo8 protein and is therefore unable to adhere to agar plates or between cells in liquid medium (Liu *et al.*, 1996; Kim *et al.*, 2014). This strain was used to dissect adhesion in *Verticillium* and to isolate specific genes required for adherence which might also control early plant infection. Twenty-two genes were reported by Tran *et al.* to be able to reprogram non-adhesive yeasts to adhere to

agar plates. Nine teen out of the twenty-two genes were also identified in *V. dahliae* (Tran *et al.*, 2014). Six of them are supposed to be transcription activator genes, which were named as *Verticillium* transcription activator of adhesion (*VTA*) (Tran *et al.*, 2014). The homolog of Vta3 in *Fusarium graminearum* is reported to play key roles in DNA damage repair, conidiation, fungal growth, and pathogenicity (Huang *et al.*, 1998; Min *et al.*, 2014), whereas the function in adhesion has not been studied yet. Low expression of *SOM1* and expression of *VTA3* can rescue adhesion in non-adhesive yeast strains.

The first aim of this study was to analyse the function of Som1 and Vta3 in adhesion of *S. cerevisiae* by testing the expression of related adhesion genes. The function of Som1 and Vta3 in adhesion and virulence in *V. dahliae* was the second part of this work. Genetical, cell biological, proteomic assay and plant pathogenicity approaches were applied. Gene deletion and complementation strains were generated. Som1 and Vta3 functions were investigated by comparing the deletion strain to the wild-type and complementation strains. The wild type and deletion strain were labelled with GFP to test root infection. Root infection was examined with scanning electron microscopy. Proteins, which are down regulated in the deletion of *SOM1* were compared by a proteomic assay between cell lysates of the wild-type and deletion strains. The expression of relevant genes for adhesion, conidiation, microsclerotia formation, oxidative stress response and virulence was tested by real-time PCR. Interaction partners of Som1 and Vta3 were identified by a pull-down assay using the GFP-trap. The AfSom1 in *A. fumigatus* is required for adhesion and virulence (Lin *et al.*, 2015). The third part of this study was to test whether AfSomA and Som1 share similar functions in plant and human pathogenic fungi. The functional complementation study by expressing *AfSOMA* in the *SOM1* deletion strain of *V. dahliae* was performed to examine the change of adhesion, growth, and the formation of aerial hyphae, conidia, and microsclerotia.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals used in this study were purchased from Sigma-Aldrich (Deisenheim, Germany), Merck (Darmstadt, Germany), Roche Diagnostics GmbH (Mannheim, Germany), Invitrogen GmbH (Karlsruhe, Germany), AppliChem GmbH (Darmstadt, Germany), and Serva Electrophoresis GmbH (Heidelberg, Germany).

Enzymes used in this study were supported by Thermo Fisher Scientific GmbH (St. Leon-Rot/Schwerte, Germany) and Biolab inc. (Massachusetts, USA).

2.1.2 Primers

Primers used in this study were synthesized by Eurofins MWG GmbH (Ebersberg, Germany) and are listed in Table 1.

Table 1. Primers used in this study

q: RT-PCR primers, F: forward primer, R: reverse primer, restriction sites and overhang were underlined.

Primer	5'-3' direction	Reference
For knockout		
gdpA-NAT-F (*Sac*I)	GGG GAG CTC ACT AGT GGT ACC AGC GC	This study
gdpA-NAT-R (*Apa*I)	GGG GGG CCC GGA TCC TCA GGG GCA GGG	
SOM1-P1 (*Pac*I)	GGG TTA ATT AAC GGA GGA TGA TTG AGT AGC C	This study
SOM1-P2 (*Spe*I)	GGG ACT AGT GAG ACA AGG TGG GAG TGG AA	
SOM1-P3 (*Xba*I)	GGG TCT AGA CAA TGT GCC ATG ACG CTA TC	This study
SOM1-P4 (*Sbf*I)	GGG CCT GCA GGT TGA GTT CGG CTG TCT ACC C	
VTA3-P1 (*Pac*I)	GGG TTA ATT AAT CAG CTG AAA TGT GGA ATC G	This study
VTA3-P2 (*Spe*I)	GGG ACT AGT CCG GCT TGT GAT GAA GCT AT	
VTA3-P3 (*Xba*I)	GGG TCT AGA CTT TTC GCT TTA GGG GGT TC	This study
VTA3-P4 (*Sbf*I)	GGG CCT GCA GGG CGA GTT TTC GGT AGG TGA G	

Table 1. (Continued)

q: RT-PCR primers, F: forward primer, R: reverse primer, restriction sites and overhang were underlined.

Primer	5'-3' direction	Reference
For complementation		
*SOM1*comp-F (*Xba*I)	GGG TCT AGA GGG GCA TTG TGT GTA CCT CT	This study
*SOM1*comp-R (*Sbf*I)	GGG CCT GCA GGG AAT GGC GGA GAC AAA GAA T	
SOM1-Y-F (*Xho*I)	GGG CTC GAG ATG AAC GTA AAC CCG AAC GT	This study
SOM1-Y-R (*Hind*III)	GGG AAG CTT TTA TTC GGC TCC AAT TTC TCC	
ScFLO8-F (*Xba*I)	GGG TCT AGA ATG AGT TAT AAA GTG AAT AGT TCG TAT	This study
ScFLO8-R (*Sbf*I)	GGG CCT GCA GGT CAG CCT TCC CAA TTA ATA A	
*VTA3*comp-F (*Xba*I)	GGG TCT AGA GCC ATG AAG TGA CCG ACT TT	This study
*VTA3*comp-R (*Sbf*I)	GGG CCT GCA GGG CCT GGA GTC TGG AGC ACT A	
For localisation		
SOM1GFP-F	ATC CTT CTT TCT AGC AGG GGC ATT GTG TGT ACC TCT	This study
SOM1GFP-R	GCC CTT GCT CAC CAT ACC ACC GCT ACC ACC TTC GGC TCC AAT TTC TCC	
FLO8GFP-F	TGA CAT CAC CCT CGA GAT GAG TTA TAA AGT GAA TAG TTC GTA TCC AG	This study
FLO8GFP-R	GCC CTT GCT CAC CAT ACC ACC GCT ACC ACC GCC TTC CCA ATT AAT AAA ATT GAA A	
SOMAGFP-F	TGA CAT CAC CCT CGA GAT GAA TCA GAT GAA TGT GAC GGG	This study
SOMAGFP-R	GCC CTT GCT CAC CAT ACC ACC GCT ACC ACC TAA GCC ATC TCC GGC GC	
VTA3GFP-F	ATC CTT CTT TCT AGC AGC CAT GAA GTG ACC GAC TTT	This study
VTA3GFP-R	GCC CTT GCT CAC CAT ACC ACC GCT ACC ACC TTG CTC GAT GCC CTG CC	

Table 1. (Continued)

q: RT-PCR primers, F: forward primer, R: reverse primer, restriction sites and overhang were underlined.

Primer	5'-3' direction	Reference
For sequencing		
*SOM1*seq 1	CCC AAA ACC TTC TCA AGC AA	This study
*SOM1*seq 2	CTG ATT CTG CTT TGC CTG GT	
*SOM1*seq 3	CAT GGC CAA CAA TCA GAA CA	This study
*SOM1*seq 4	AAA GTT TGG TGC CAT AGA AGG A	
*SOM1*seq 5	CAA TCT CGC GGC TCT CCT A	This study
*SOM1*seq 6	CCC GGA TTC TAG AAC TAG TGG A	
*VTA3*seq 1	CCC AAA CCT GTT CTG GAC AC	This study
*VTA3*seq 2	GAG CGA CAG CTC AAT GAC AA	
*VTA3*seq 3	TTC TCA ACC CAG CGA GTT TC	This study
*VTA3*seq 4	GGT CTT CAG TCC TGG GAA CA	
*VTA3*seq 5	GTC ATC CGT GCC AAA GAA G	This study
*VTA3*seq 6	ACG GGA ACC ATA GTG ATC CA	
For RT-PCR		
q*ABAA*-F	CGA GAA GAA CCT CAA CAA GGA G	This study
q*ABAA*-R	CTA CGG TGA AGA GAC GGG AAA C	
q*ALG9*-F	TTG CCG TTT TCA CTC AAC AA	This study
q*ALG9*-R	CCA AAG CCA CTA TCC GTG AC	
qCAP20-F	TAT CTC CAG AAG GCC GAC AC	This study
qCAP20-R	GCC ATT GCC AAG TTT CTT GT	
q*CON-6*-F	CGA GGA GAG CAA GCA GCA CT	This study
q*CON-6*-R	GAC GTT GCC AGG GTT CTT GT	
q*CON-8*-F	ACG CAG CTC CAA CAC CCT CT	This study
q*CON-8*-R	GTG AAA TTG CGC CAC ATC TTG	
q*CPX2*-F	ATG TCG CCT CGC TGA AGA AGA	This study
q*CPX2*-R	ACG CTT GTC TGA GTT GCG GTA G	
q*FAS1*-F	CTG CTG ACC AAC GAG ACG TA	This study
q*FAS1*-R	GCT AAT GTT GAG GGG CAG AG	

Table 1. (Continued)

q: RT-PCR primers, F: forward primer, R: reverse primer, restriction sites and overhang were underlined.

Primer	5'-3' direction	Reference
q*GAPDH*-F	AAC GTC TCC GTT GTT GAC CTG A	This study
q*GAPDH-R*	GTC CTC GGT GTA GGC CAG AAT G	
q*INO1*-F	GAC ACC AAG ACC AGG AAG GA	This study
q*INO1*-R	GAA GGC AGA GGC ACA ATC TG	
q*MAD1*-F	CGA CCT CGA GTT CCA CTA CG	This study
q*MAD1*-R	CGA AGC CGA TCT TCC AGA TA	
q*MAD2*-F	GGA GGA TGA TGA CTG GCA CT	This study
q*MAD2*-R	GGC AGA TAG TGG TGG TCT GG	
q*NLP2*-F	CCG TCT CTC ATC AGC ATC GT	This study
q*NLP2*-R	CGT TGA ACA CCT TGA GGT ACG	
q*OLG 70*	CAG CGA AAC GCG ATA TGT AG	Eynck *et al.* (2007)
q*OLG 71*	GGC TTG TAG GGG GTT TAG A	
q*PLS1*-F	GGC TGC GAG AAA CTT GAT CT	This study
q*PLS1*-R	AGG TAG ACA CCC ACG CAA AG	
q*PP2A1*-F	CCC GAG AAG GAG GTT GAA GT	This study
q*PP2A1*-R	CAT CCG TCT CCT TAC GCA TT	
q*PP2A2*-F	ACA TTC GCT TCA ACG TAG CC	This study
q*PP2A2*-R	AAC ATC CAC ATC GTC GTC CT	
q*PRY1*-F	GCT GCC ATT CTC ACA ACA CA	This study
q*PRY1*-R	TCC AAT GGC CAG ATT CTC TC	
q*ScFLO11*-F	GGT GTC ACT GGT CCA AAA GG	This study
q*ScFLO11*-R	TTG CAT ATT GAG CGG CAC TA	
q*ScFLO1*-F	GAA CGC TGT TTC TTG GGG TA	This study
q*ScFLO1*-R	TGA AAG TAC CGG TCC ATG GTT	
q*ScFLO8*-F	CAG CAG CCT TTG CTC AAG AT	This study
q*ScFLO8*-R	CTC TGA GCC ACC TCT GGA AG	
q*SFL1*-F	CGA ATC GCT ACA CGA CTT GA	This study
q*SFL1*-R	TTA GCG TCG TTG CTG CTA TG	

Table 1. (Continued)

q: RT-PCR primers, F: forward primer, R: reverse primer, restriction sites and overhang were underlined.

Primer	5'-3' direction	Reference
q*SNOD1*-F	CCC AAA AGC AGG TCA AGA AG	This study
q*SNOD1*-R	ATG GCG AGG ACA TTG ATG GT	
q*SOD3*-F	GAA AAC ACG GCT TCG TTG AGT C	This study
q*SOD3*-R	GAG GTT GCT GCT GAA GTG AAG G	
q*SOM1*-F	CCA ACA AGC AGA TGC CTA ATG C	This study
q*SOM1*-R	CTT GCA GCG TGG TTA CTT C	This study
q*SOMA*-F	AAT GCA GTT GAT GCT GCT TG	This study
q*SOMA*-R	CCT CGT TTC ATC TGC TCG TT	This study
q*TAF10*-F	CCA GGA TCA GGT CTT CCG TA	This study
q*TAF10*-R	TCA GCA ACA GCG CTA CTG AG	This study
q*VDH1*-F	ACG ATT GCT CTG TTT GCT GGA G	This study
q*VDH1*-R	CCT GGC ACT CTT TGG GGT AGA	This study
q*VTA1*-F	CAC AGG GGC GAG TCT AGG TA	This study
q*VTA1*-R	CCC GAG GTA CCC GAT CAT AG	This study
q*VTA2*-F	TAC TCC TTC GTT CCG ATT CCT G	This study
q*VTA2*-R	GCG CAT TGA GAT GGT TCA GAG T	This study
q*VTA3*-F:	GGA TGG CAA AGT CAA CGT CT	This study
q*VTA3*-R:	CGA ACA GAC CGA ATT GAT CC	This study
q*VTA4*-F	ATC CCA AGC TCC AGG CTA CT	This study
q*VTA4*-R	GTA CGA GGC GGT CTG TGA AG	This study
q*VTA5*-F	CCC CTC ATC CCA GTC TCA AT	This study
q*VTA5*-R	CAA CGC TCG TGG AAC TCT TC	This study
q*VTA6*-F	CAA GAA GTT CTC CCG CTC TG	This study
q*VTA6*-R	GAG AGA CGT TGG GCG AAG AC	This study

2.1.3 Plasmids

Plasmids used in this study are listed in Table 2.

Table 2. Plasmids used in this study

p: promoter, t: terminator, R: resistance, *NAT*: nourseothricin, *HPH*: hygromycin.

Plasmid	Description	Reference
pJET1.2/blunt	Cloning vector with blunt ends	(Fermentas)
pME4548	$^{p}gpdA::NAT1^{R}$	This study
pME4549	$^{p}SOM1::^{p}gpdA::NAT1^{R}::SOM1^{t}$	This study
pPK2	$^{p}gpdA::HPH^{R}::trpC^{t}$	Covert (2001)
pME4550	$^{p}SOM1:SOM1::SOM1^{t}$, $^{p}gpdA:::HPH::trpC^{t}$	This study
pGreen2	$^{p}gpdA::GFP::trpC^{t}$	Tran (2014)
pME4551	$^{p}SOM1::SOM1::GFP::trpCt$	This study
pME4552	$^{p}gpdA::ScFLO8::GFP::trpC^{t}$	This study
pME4553	$^{p}gpdA::AfSOMA::GFP::trpC^{t}$	This study
pME4554	$^{p}VTA3::^{p}gpdA::NAT1^{R}::VTA3^{t}$	This study
pME4555	$^{p}VTA3:VTA3::VTA3^{t}$, $^{p}gpdA:::HPH^{R}::trpC^{t}$	This study
pME4556	$^{p}VTA3::VTA3::GFP::trpC^{t}$	This study
pME4557	$^{p}gpdA::SOM1::GFP::trpC^{t}$	This study
pME2795	$^{p}GAL1$, $CYC1^{t}$, $URA3$, Amp^{R}	Mumberg (1994)
pME4558	$^{p}GAL1::ScFLO8::$ $CYC1^{t}$, $URA3$, Amp^{R}	This study
pME4559	$^{p}GAL1::SOM1::$ $CYC1^{t}$, $URA3$, Amp^{R}	This study
pME2787	$^{p}MET25$, $CYC1^{t}$, $URA3$, Amp^{R}	Mumberg (1994)
pME4560	$^{p}MET25::ScFLO8::CYC1^{t}$, $URA3$, Amp^{R}	This study
pME4561	$^{p}MET25::SOM1::CYC1^{t}$, $URA3$, Amp^{R}	This study
pME4561	$^{p}GAL1::VTA3::CYC1^{t}$, $URA3$, Amp^{R}	This study
pNAT1	$^{p}gpdA::NAT1$	Janus (2007)

2.1.4 Organisms

2.1.4.1 Bacterial strains

Escherichia coli strain DH5α (Invitrogen, Karlsruhe, Germany) and *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al*., 1991) were used for transformation procedures in this study. They were cultivated in Luria-Bertani (LB) medium (0.5% yeast extract, 1% bacto-tryptone and 1% NaCl) at 37^{o}C for *E. coli* or 25^{o}C for *A. tumefaciens.*

2.1.4.2 Fungal strains

Saccharomyces cerevisiae strains are derived from BY4741 and BY4742, the non-adhesive S288c genetic background, carrying a truncated *FLO8* gene. The S288c background has a nonsense mutation in the open reading frame (ORF) of the *FLO8* gene encoding a transcriptional regulator of *FLO1* and *FLO11* adherent genes (Liu *et al.*, 1996). Therefore, the expression of *FLO1,* as well as *FLO11,* is blocked. All *S. cerevisiae* strains were incubated in YPD or SC-Ura medium at 30^{o}C.

Verticillium dahliae strain JR2 wild-type was provided by Bart Thomma, Laboratory of Phytopathology in Wageningen, Netherlands (Fradin *et al.*, 2009). All strains were inoculated at 25^{o}C in potato dextrose broth (PDA) (Sigma-Aldrich Chemie GmbH, Munich, Germany), Minimal medium (MM) (Bennett & Lasure, 1991), Czapek-Dox medium (CDM) (Smith, 1948) or a modified simulated xylem medium (SXM) (Neumann & Dobinson, 2003) which is composed of 0.2% pectin from citrus peel (Sigma-Aldrich Chemie GmbH, Munich, Germany), 0.4% casein hydrolysate (OXOID Ltd, Basingstoke, Hampshire, England), 2 mM $MgSO_4$, 1x ASPA, and 1x trace elements.

The *Verticillium* strains were grown in SXM on a shaker at 120 rpm at 25^{o}C for seven days. Conidia were harvested by filtration of the culture through a miracloth membrane (Calbiochem, Darmstadt, Germany), the filtrate was washed twice with sterile water before resuspending in the solution containing 0.96% NaCl and 0.05% Tween 80. The number of spores was counted in a counting chamber under a binocular microscope and the spore density was adjusted to 10^7 spores/ml. Aliquots of spore suspension containing 25% of glycerol were frozen in liquid nitrogen and stored at -80^{o}C. Fungal strains used in this study are listed in Table 3.

Table 3. Fungal strains used in this study

Amp: Ampicillin, p: promoter, t: terminator, R: resistance, *NAT*: nourseothricin, *HPH*: hygromycin.

Strain	Description	Reference
Verticillium		
VGB0001	Δ*SOM1::NAT1*^{R}	This study
VGB0002	Δ*SOM1::NAT1*^{R}	This study
VGB0003	Δ*SOM1::NAT1*^{R}, ^{p}*SOM1:SOM1::SOM1*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0004	Δ*SOM1::NAT1*^{R}, ^{p}*SOM1:SOM1::SOM1*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0009	Δ*VTA3::NAT1*^{R}	This study
VGB0010	Δ*VTA3::NAT1*^{R}	This study
	JR2-wt	Fradin *et al.* (2009)
VGB0074	Δ*SOM1::NAT1*^{R}, ^{p}*gpdA:GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0075	Δ*SOM1::NAT1*^{R}, ^{p}*gpdA::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0079	Δ*VTA3::NAT1*^{R}, ^{p}*VTA3:VTA3::VTA3*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0080	Δ*VTA3::NAT1*^{R}, ^{p}*VTA3:VTA3::VTA3*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0084	Δ*SOM1::NAT1*^{R}, ^{p}*SOM1::SOM1::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0085	Δ*SOM1::NAT1*^{R}, ^{p}*SOM1::SOM1::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0090	JR2, ^{p}*gpdA::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0172	Δ*SOM1::NAT1*^{R}, ^{p}*gpdA::AfSOMA::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0173	Δ*SOM1::NAT1*^{R}, ^{p}*gpdA::AfSOMA::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study

Table 3. (Continued)

Amp: Ampicillin, p: promoter, t: terminator, R: resistance, *NAT*: nourseothricin, *HPH*: hygromycin.

Strain	Description	Reference
VGB0174	JR2, ^{p}*gpdA::SOM1::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0175	JR2, ^{p}*gpdA::SOM1::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0178	Δ*SOM1::NAT1*^{R}, ^{p}*gpdA::ScFLO8::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0179	Δ*SOM1::NAT1*^{R}, ^{p}*gpdA::ScFLO8::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0184	Δ*VTA3::NAT1*^{R}, ^{p}*gpdA:GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0185	Δ*VTA3::NAT1*^{R}, ^{p}*gpdA:GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0281	Δ*VTA3::NAT1*^{R}, ^{p}*VTA3::VTA3::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
VGB0282	Δ*VTA3::NAT1*^{R}, ^{p}*VTA3::VTA3::GFP::trpC*^{t}, ^{p}*gpdA:::HPH::trpC*^{t}	This study
S. cerevisiae		
BY4742	*MATα,* Δ*FLO8,* Δ*HIS3,* Δ*LEU2,* Δ*URA3*	Euroscarf
BY4741	*MATα,* Δ*FLO8,* Δ*HIS3,* Δ*LEU2,* Δ*MET25,* Δ*URA3*	Euroscarf
RH3647	BY4742, ^{p}*MET25, CYC1*^{t}*, URA3, Amp*^{R}	This study
RH3648	BY4741, ^{p}*GAL1, CYC1*^{t}, *URA3, Amp*^{R}	This study
RH3649	BY4742, ^{p}*MET25::ScFLO8::CYC1*^{t}, *URA3, Amp*^{R}	This study
RH3650	BY4741, ^{p}*GAL1::ScFLO8::CYC1*^{t}, *URA3, Amp*^{R}	This study
RH3651	BY4742, ^{p}*MET25::SOM1::CYC1*^{t}, *URA3, Amp*^{R}	This study
RH3652	BY4741, ^{p}*GAL1::SOM1::CYC1*^{t}, *URA3, Amp*^{R}	This study
RH3653	BY4741, ^{p}*GAL1::VTA3::CYC1*^{t}, *URA3, Amp*^{R}	This study

2.2 Methods

2.2.1 Bioinformatic analysis

Predicted functions of deduced proteins and conserved domains/motifs were examined with InterProScan (Mulder & Apweiler, 2007), Pfam (Finn *et al.*, 2016), and BLAST searches at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) (Mittler *et al.*, 2010). Nuclear localisation signals (NLS) were predicted at SeqNLS: Nuclear localisation signal prediction based on frequent pattern mining and linear motif scoring (http://mleg.cse.sc.edu/seqNLS/) (Lin & Hu, 2013). Gene numbers and gene sequences of *V. dahlia* are according to the VertiBase (http://www.vertibase.org). Protein alignments were performed by Clustal Omega at European Molecular Biology Laboratory-European Bioinformatics Institute (http://www.ebi.ac.uk) (Sievers & Higgins, 2014).

2.2.2 Gene deletion, complementation, and overexpression

2.2.2.1 Gene deletion

The vector pPK2 (Covert *et al.*, 2001) was digested with enzymes *Sac*I and *Apa*I. In parallel, the nourseothricin resistance cassette (1.45 kb) was amplified by PCR from the pNAT1 vector (Janus *et al.*, 2007) with the primer pair *gdpA-NAT*-F/ *gdpA-NAT*-R (Table 1). This cassette was digested with *Sac*I and *Apa*I and ligated into pPK2 to generate the pME4548 vector (Table 2).

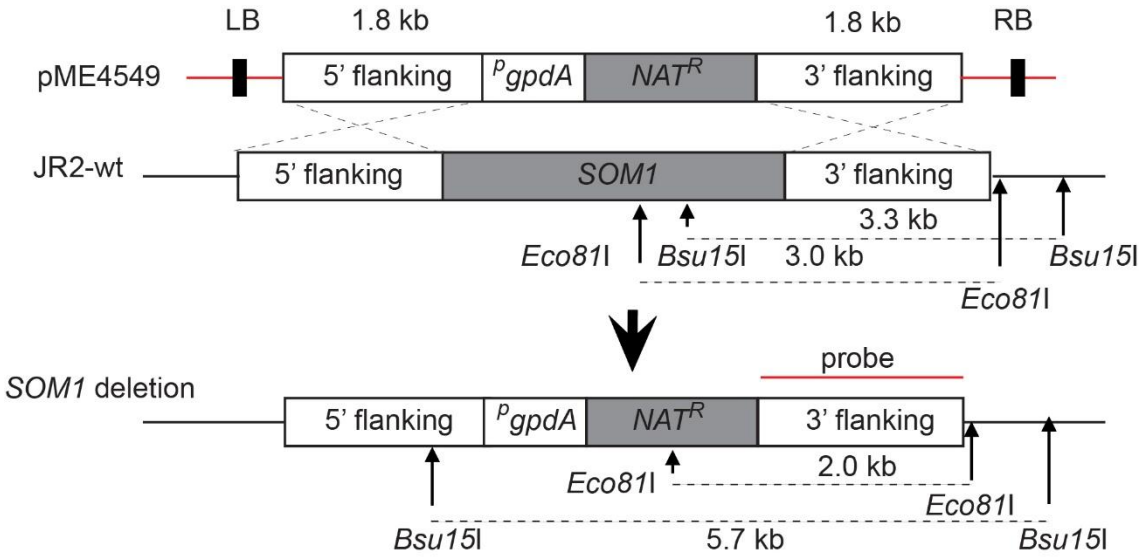


Figure 12. Strategies of *SOM1* deletion and confirmation in *V. dahliae.* *SOM1* was knocked out by using a strategy via homologous recombination between the deletion construct and wild-type gene locus. 5' and 3' flanking region (FR) are around 1.8 kb; p: promoter; R: resistance. The restriction enzyme site and predicted sizes are indicated.

Fragments of 1.9 kb upstream and downstream of *SOM1* (Figure 12) or the 1.1 kb of *VTA3* (Figure 13) were amplified by Phusion™ high-fidelity DNA polymerase (Thermo Fisher Scientific GmbH, St. Leon-Rot, Germany) with primer pairs *SOM1-P1/SOM1-P2*, *SOM1-P3/SOM1-P4*, *VTA3-P1/VTA3-P2*, or *VTA3-P3/ VTA3-P4* (Table 1). The fragments were processed by restriction enzymes (Table 1). Finally, they were individually cloned into pME4548 digested with the same enzymes to produce pME4549 and pME4554 (Figure 12, 13, Table 2). The results were confirmed by PCR and digestion with restriction enzymes before introducing into JR2-wt strain via *A. tumefaciens* AGL-1 strain (Lazo *et al.*, 1991; Timpner *et al.*, 2013) to generate VGB0001, VGB0002, VGB0009, and VGB0010 (Table 3). The fungal transformants were selected on potato dextrose agar (PDA) containing nourseothricin (50 µg/ml) and cefotaxime (100 µg/ml). The deletion strains were confirmed by Southern hybridization.

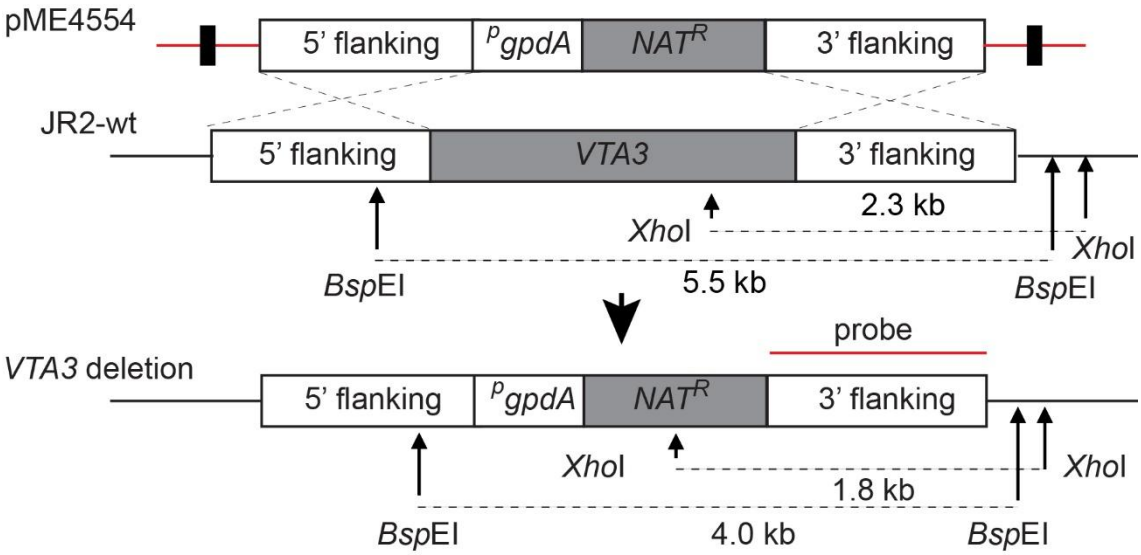


Figure 13. Deletion and confirmation strategies of *VTA3* gene in *V. dahliae*. *VTA3* was knocked-out via homologous recombination between the deletion construct and wild-type gene locus. 5' and 3' flanking region are around 1.0 kb; p: promoter; R: resistance. The restriction enzyme site and predicted sizes are indicated.

2.2.2.2 Gene complementation

For complementation, the fragments including 1.2 kb upstream, the ORF and 0.8 kb downstream of *SOM1* and *VTA3* were amplified from genomic DNA of JR2-wt using the primer pairs *SOM1*comp-F/*SOM1*comp-R or *VTA3*comp-F/*VTA3*comp-R (Table 1). They were individually cloned into pPK2 (Covert *et al*., 2001) between *Xba*I and *Sbf*I to generate pME4550 and pME4555 (Figure 14, Table 2). This construct was transformed into the VGB0001 or VGB0009 strain, respectively, via *A. tumefaciens* to generate VGB0003, VGB0004, VGB0079, and VGB0080 (Table 3). The transformants were selected on PDA plates containing both, hygromycin (50 µg/ml), and nourseothricin (50 µg/ml) and confirmed by Southern hybridization.

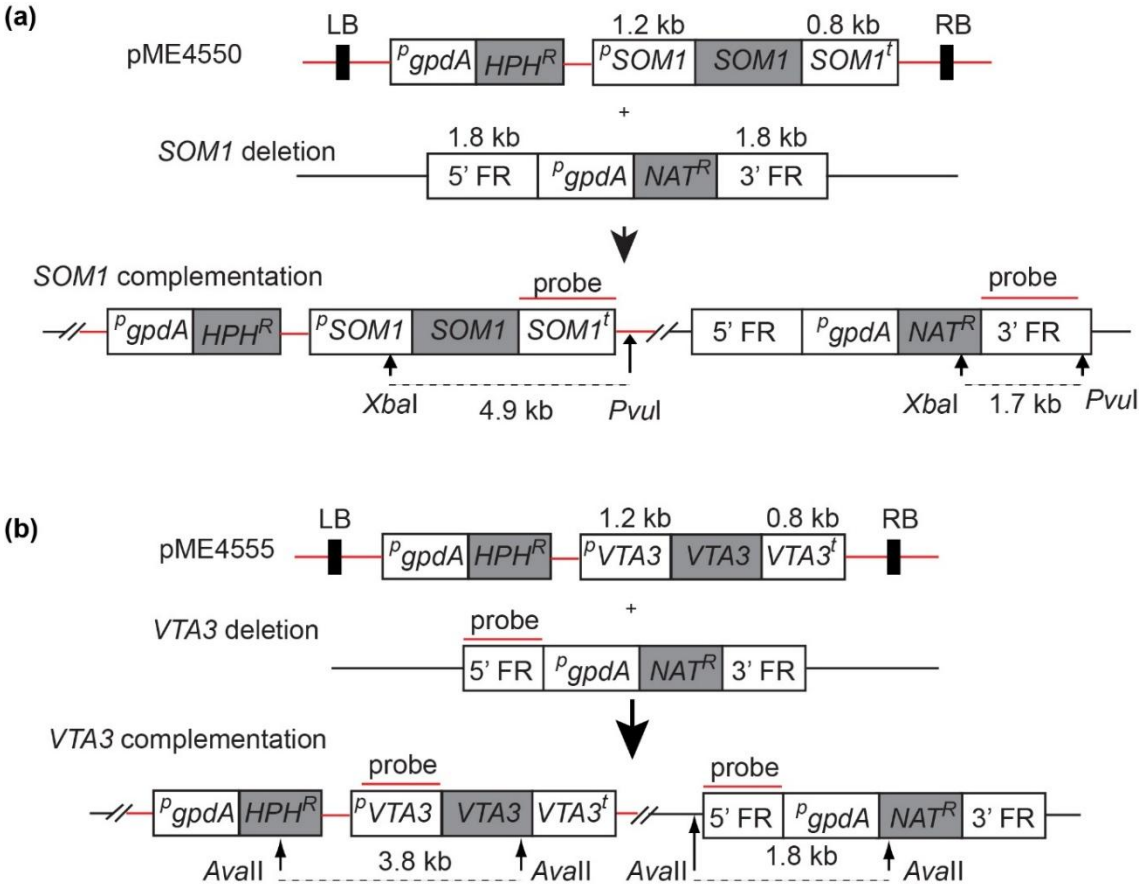


Figure 14. Complementation of *SOM1* and *VTA3* in *V. dahliae* and confirmation strategies. The construct for creating complementation strains contains the selection marker of hygromycin under the supervision of the *gpdA* promoter and a gene under its native promoter (1.2 kb) and the native terminator (0.8 kb). This construct was ectopically transformed into the deletion strain to generate the complementation strains. (**a**) Complementation of *SOM1* and verification strategies. (**b**) Complementation of *VTA3* and confirmation strategies. p: promoter; R: resistance. The name of strains, restriction enzyme site, and predicted sizes are indicated.

2.2.2.3 Gene overexpression

The ORF of *SOM1* without the stop codon was fused to the N-terminal end of the *GFP* gene in the pGreen2 backbone between *Xho*I and *Kpn*I to generate the pME4557 plasmid (Table 2). This combination gene is under the control of *gpdA* promoter. This construct was transformed into the *V. dahliae* JR2 wild-type to create VGB0174 and VGB0175 (Table 3) via *A. tumefaciens* (Timpner *et al.*, 2013). The transformed strains were selected on PDA plates and under a fluorescence microscope. The *SOM1* transcript levels was examined by RT- PCR. The growth, conidiation, and microsclerotia formation were tested.

2.2.3 Genetic manipulations

2.2.3.1 *E. coli* transformation

Preparation of *E. coli* competent cells using DMSO and TB buffer and transformation methods were modified from (Inoue *et al.*, 1990).

Preparation of E. coli competent cells: One fresh colony of *E. coli* was incubated in 5 ml LB medium (0.5% yeast extract, 1% bacto-tryptone, and 1% NaCl) at 37^{o}C on a rotator overnight. 500 µl of overnight cultures was added into 5 ml LB and grown for 4-5 h in the same condition as above. 1 ml of this culture was added to 250 ml SOB medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 10 mM $MgSO_4$) in a one liter flask and incubated on a shaker at 120 rpm at 20^{o}C overnight until the OD_{600} of culture is around 0.6. The culture was kept in ice for 10 min and centrifuged at 2500 rpm for 10 min at 4^{o}C. The pellet was washed with 80 ml of cool TB buffer (10 mM HEPES, 15 mM $CaCl_2.2H_2O$, 250 mM KCl, 55 mM $MnCl_2$, pH 6.7) for 10 min. The pellet was collected by centrifugation at the same condition as above and resuspended in 20 ml of TB buffer. DMSO was slowly added to suspension by gently swirling to a final concentration of 7%. This mixture was incubated for 10 min on ice before distributing 400 µl aliquot into 1.5 ml eppendorf tubes, frozen in liquid nitrogen and stored at -80^{o}C.

E. coli transformation: 10 µl of a ligation or 50 ng of a plasmid was added into an eppendorf tube containing 200 µl of thawed competent cells. This tube was kept on ice for 20 min and at 42^{o}C on a heating block for 40 s. 800 µl of SOC medium, SOB addition 20 mM glucose, was added into suspension. This mixture was incubated at 37^{o}C on a shaker for 1 h. The cell pellet was collected by centrifugation at 10000 rpm for 1 min and spread on LB agar plates containing an appropriate antibiotic for selection (ampicillin or kanamycin 100 µg/ml). The positive colonies were confirmed by colony PCR with specific primer pairs and digested with suitable restriction enzymes.

2.2.3.2 *A. tumefaciens* transformation

Preparation of *A. tumefaciens* competent cells and transformation were modified from (Jyothishwaran *et al.*, 2007).

Preparation of competent cells: *A. tumefaciens* cells were grown in 100 ml LB medium containing 50 µg/ml carbercillin in a one liter flask at 25ºC on a shaker overnight until the OD_{600} is around 0.6. Overnight cultures were kept on ice for 15 min and cell pellets were collected by centrifugation at 5000 rpm for 10 min at 4ºC. Pellets were sequentially washed with 20 ml $MgCl_2$ 100 mM for 1 h and dissolved with 20 ml $CaCl_2$ 20 mM for 4 h. Glycerol was added into resuspension to a final concentration of 25%. The mixture was distributed 200 µl aliquot to 1.5 ml eppendorf tubes, frozen in liquid nitrogen and store at -80ºC.

A. tumefaciens transformation: 1000 ng of a binary vector was added into an eppendorf tube containing 200 µl of thawed competent cells. The tube was incubated 10 min on ice, 10 min in liquid nitrogen, and 10 min at 37ºC in a heating block. The tube was added with 800 µl of SOC medium and incubate at 28ºC on a shaker at 120 rpm for one hour. Cell pellets were collected by centrifugation at 13000 rpm for 1 min and spread on LB plates containing 100 µg/ml kanamycin. Plates were grown at 25ºC for 72 h to gain colonies. Positive colonies were confirmed by PCR reaction with specific primer pairs.

2.2.3.3 *S. cerevisiae* transformation

The ORFs of *SOM1* and *VTA3* were cloned into pME2795 or pME2787 (Mumberg *et al.*, 1994) plasmids between *Xho*I and *Hind*III to create pME4559, pME4561, and pME4562 (Table 2). The recombinant plasmids were verified by PCR and enzymatic digestion and transformed into a non-adhesive yeast strain BY4741 or BY4742 to generate RH3651, RH3652, and RH3653 strains (Table 3). The yeast transformation was performed following the lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/SS carrier DNA/PEG) method (Gietz & Schiestl, 2007). Adhesion and flocculation tests were previously described (Tran *et al.*, 2014; Lin *et al.*, 2015).

2.2.3.4 *V. dahliae* transformation

Verticillium transformation method was modified from (Timpner *et al.*, 2013; Tran *et al.*, 2014). A positive colony of *A. tumefaciens* was grown in 5 ml of LB medium containing appropriate antibiotic overnight. 500 µl of overnight cultures were added to 5 ml of induction medium including acetosyringone (IMAS) (1X MM salts, 0.5% glycerol and 10 mM glucose) and grown at 25^{o}C for 5 h on a shaker at 120 rpm. This culture was mixed with the same volume of the spore solution (10^6 spores/ml). This mixture was spread onto the filter paper (Sartorius, Göttingen, Germany) on an agar plate of the IMAS medium that is similar to liquid medium excepting to contain 5 mM glucose instead of 10 mM. The plate was co-cultivated in the dark at 25^{o}C for 72 h. The filter paper was transferred to a PDA (potato dextrose agar) plate supplied with suitable antibiotics to select transformation and cefotaxime to kill all agrobacteria. Deletion strains were selected by nourseothricin (50 µg/ml) and complementation by hygromycin (50 µg/ml). The plate was grown for two weeks. Colonies were separately cultivated on selection medium and confirmed by Southern hybridization.

2.2.4 Confirmation of transformation

2.2.4.1 DNA purification

Plasmid purification from E. coli: A single colony of *E. coli* containing a plasmid of interest was incubated in 5 ml LB medium supplemented suitable antibiotic on a rotator at 37^{o}C overnight. The plasmid was isolated using Plasmid Miniprep Kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

DNA isolation from yeast: Total genomic DNA isolation method was modified from (Amberg *et al.*, 2005). The yeast strain was grown in 10 ml of YEP medium (1% yeast extract, 2% peptone, and 2% glucose) at 30^{o}C on a rotatory shaker overnight. The cell pellet was collected by centrifuging at 3000 rpm for 10 min. The pellet was ground to fine powder by a Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) using two small marble in a 2 ml centrifugation tube in liquid nitrogen. 300 µl of Smash-Grab lysis buffer (10 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% SDS, 2% Triton X-100) was added to a fine powder and mixed well by vortexing for 2 min. 300 µl of phenol/chloroform was added to the tube and inverted 10 times. The tube was centrifuged at 13000 rpm for 10 min.

The supernatant was transferred to a new 1.5 ml tube containing the same volume of isopropanol and kept in -20 for 30 min. The pellet of DNA was harvested by centrifugation at 13000 rpm for 10 min and washed twice with ethanol 75%. The pellet was dried at 37ºC for 30 min and dissolved in 100 µl TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA). The RNA was eliminated by 4 µl RNase (10 mg/ml) at 65ºC for 30 min. The quality of isolated DNA was checked by running a gel and the concentration of DNA was measured by a Nanodrop ND 1000 spectrophotometer (Peqlab Biotechnologies GmbH, Erlangen, Germany).

DNA isolation from Verticillium: Fungal spores (2 ml of 10^7 spores/ml) were added into 50 ml PDB (potato dextrose broth) medium and grown for seven days. Mycelia were harvested by filtering with a miracloth membrane (Calbiochem, Darmstadt, Germany) and ground to fine powder in liquid nitrogen using a mortar and pestle. The fungal powder was used directly for genomic DNA extraction. The extraction method was modified from (Kolar *et al.*, 1988). About 1.0 g of fungal powder was transferred into 2 ml centrifugation tube. One milliliter of pre-warmed lysis buffer (50 mM Tris pH 7.2, 50 mM EDTA, 3% SDS, and 1% 2-Mercaptoethanol) was added into fungal powder, mixed at maximum speed for 30 seconds, and incubated at 65ºC for 1 h. The mixture was centrifuged at maximum speed for 5 min. 800 µl of the supernatant was transferred to a new 2.0 ml tube. 800 µl of phenol:chloroform (24:1) was added to the supernatant and mixed well by inverting ten times. This tube was centrifuged at 13000 rpm for 20 min. 400 µl of supernatant was transferred to a new 1.5 ml tube containing 0.6 ml isopropanol and inverted ten times. The tube was centrifuged at maximum speed for 10 min. The pellet was dried at 37ºC for 30 min and dissolved in TE buffer. The RNA was eliminated by adding 4 µl of RNase A (10 mg/ml) at 65ºC for 30 min. DNA quality and concentration were checked by running a gel and measuring with a Nanodrop spectrophotometer.

Total DNA isolation from tomato materials: Plant roots and hypocotyls were separately ground to fine powder in liquid nitrogen using a mortar and pestle. The fine powder was directly used for DNA extraction by using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with some modification. 1 g of fine powder was added to a 2 ml tube (two tubes for each repetition). 1000 µl of pre-warmed AP1 buffer at 65ºC and 4 µl RNase A were added to the fine powder and mixed at maximum speed for 20 seconds. The sample was incubated at 65ºC for 15 min and inverted

two to three times during incubation. 250 µl of P3 buffer was added to the lysate and mixed well by inverting the tube. The sample was further incubated for 5 min on ice and centrifuged at the maximum speed for 5 min. The lysate was transferred into a Qiagen Shredder Spin column and centrifuged for 2 min. The flow-through of two tubes of the same sample was collected into a 10 ml falcon. A 1.5 volume of the AW1 buffer was added in that tube and mixed by pipetting. 650 µl of the mixture was transferred to a DNeasy Mini Spin column and centrifuged at 8000 rpm for 15 seconds. This step was repeated with the remaining sample. The column was dried by centrifuging for 1 min and washed twice with 500 µl of the AW2 buffer. The column was dried again by centrifuging for 1 min at 13000 rpm before transferring to a new 1.5 ml tube. The total DNA was eluted by adding 30 µl of AE buffer into the column and collected by centrifuging at maximum speed for 1 min. The concentration of DNA was measured by a Nanodrop spectrophotometer.

RNA isolation: Yeast strains were grown in 10 ml of an activation or part repression media on a rotatory shaker at 30^{o}C overnight. The pellet was harvested by centrifugation. 2 ml of spore solution (10^{7} spores/ml) of *V. dahliae* strains was added in 50 ml SXM and grown on a shaker at 120 rpm at 25^{o}C for three days. Mycelium was harvested using a miracloth membrane. The cell pellet and the mycelium were directly used for RNA isolation. The cell pellet and the mycelium were ground by a Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) with two iron marbles in a 2 ml tube in liquid nitrogen. RNA was extracted by using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

2.2.4.2 PCR amplification

The Phusion DNA polymerase (Thermo Fisher Scientific GmbH, St. Leon-Rot, Germany) was used for cloning, checking and confirmation purposes. PCR cycling including an initial denaturation at 98^{o}C for 30 seconds - 1 min followed by 40 cycles of denaturation at 98^{o}C for 10 seconds, annealing at 55^{o}C - 68^{o}C for 20 seconds, and extension at 72^{o}C for 15 seconds/kb; a final extension at 72^{o}C for 5 min and storage at 8^{o}C until used. PCR products were run on a 1% agarose gel. DNA fragments were isolated and purified with a Qiagen quick gel extraction kit (Qiagen, Hilden, Germany).

2.2.4.3 Southern hybridization

All deletion and complementation strains were confirmed by Southern hybridization. Fungal genomic DNA was isolated from seven days old mycelium which was grown in liquid PDM on a shaker at 25^{o}C. The genomic DNA was digested overnight with specific restriction enzymes. Digested DNAs were loaded and processed on 1% agarose gel for 2 h at 90 voltage (V). The DNA was denatured before being transferred to an Amersham hybond-N membrane (GE Healthcare, Braunschweig, Germany) by blotting overnight. DNA molecules and the membrane were cross-linked under ultraviolet (UV) light for 3 min for each side. The probes were prepared using the Amersham Alkphos Direct labeling Reagent Kit (GE Healthcare) according to the user's manual and hybridized to the denatured DNA on the membrane at 60^{o}C overnight. The probes were detected with the CDP-Star Detection reagent (GE Healthcare) following the user's manual.

2.2.5 Phenotypical analyses

2.2.5.1 Microsclerotia counting

The spores of the JR2-wt, the deletion, and complementation strains were dropped on CDM plates containing 3% cellulose (w/v) and grown at 25^{o}C for seven days. The number of microsclerotia on the surface and in agar was counted under a binocular microscope (Leica M165 FC). The mean value was calculated from three independent replicates.

2.2.5.2 Conidia examination

The formation of spores in liquid medium and conidiospore clusters on agar plates was examined. The same number of spores of indicated strains was grown in 50 ml SXM on a shaker at 120 rpm to test spore formation. The number of spores in culture media was counted at seven dpi using a Thoma counting chamber (Paul Marienfeld GmbH&Co.KG, Lauda-Königshofen, Germany). The experiment was performed in triplicate. The formation of conidiospore clusters was observed on CDM plates. The same number of spores of indicated strains was dropped on CDM plates and grown in the dark for three days. The formation of conidiospore cluster was observed with a binocular microscope**.**

2.2.5.3 Hyphal branching test

The same number of spores of the indicated strains was dropped on agar plates and grown in the dark at 25°C for two days. The hyphal branching was observed under the microscope, and the number of branches was further quantified by multiple micro channels (Stanley *et al.*, 2014). A piece of agar medium containing fungal mycelium was cut and placed next to the device opening. The growth direction of the hyphal tips was orientated toward the micro channels. In order to count the number of hyphal branches, the fungi were grown in the dark for two days. The number of hyphal branches was counted in 750 µm length of hyphae from the tip. The experiments were performed in triplicate with 20 hyphae per each repetition.

2.2.5.4 Localisation study

Fragments harboring 1.2 kb upstream and the ORF without stop codon of *SOM1* and *VTA3* were fused to the N-terminal of a *GFP* gene in the pGreen2 (Tran *et al.*, 2014) background between *Spe*I and *Kpn*I to generate pME4551 and pME4556 plasmids. These plasmids were transformed into the deletion strain VGB0001 or VGB0009 to produce VGB0084, VGB0085, VGB0284, and VGB0285 via the *A. tumefaciens* (Timpner *et al.*, 2013). The transformants were selected on PDA plates containing hygromycin (50 µg/ml) and cefotaxime (100 µg/ml) and screened under a fluorescent microscope with the green filter. The positive clones were further examined by Southern hybridisation. Localisation was examined by fluorescence microscopy. Fungal nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

2.2.5.5 Oxidative stress test

One volume of 100 µl of spore suspension (10^7 spores/ ml) of JR2-wt, the deletion or complementation strains was spread on PDA plates containing cefotaxime (100 µg/ml) with glass beads. A paper disc with a diameter of 6-8 mm containing 10 µl of 10% H_2O_2 or menadione (100 mg/ml) was placed in the center of each plate. Plates were incubated in the dark at 25°C for four days to show growth inhibition effects. These experiments were performed in triplicate.

2.2.5.6 Adhesion examination

Yeast adhesion test

The *GAL1* promoter is activated in SC-Uracil containing 2% galactose and the *MET25* promoter is part repressed in SC-Uracil medium containing 2% glucose. Yeast strains were grown on induction or part repression medium for three days at 30ºC. The plates were washed under water stream until negative strain was washed off. The plates were photographed before and after washing.

For flocculation in liquid medium, *S. cerevisiae* strains were cultivated in 10 ml induction or part repression media on a rotator for one day at 30ºC. The flocculation part and non-flocculation part were separately dried and measured. The experiment was performed in triplicate.

Verticillium adhesion test

Adhesion of *V. dahliae* on abiotic surfaces was examined with two different surfaces polystyrene and hydrophobic GelBond film. The JR2-wt, *SOM1* deletion, and *SOM1* complementation strains were grown on a polystyrene plate containing SXM for seven days at 25ºC. The wells were washed with a water stream. The polystyrene plate was photographed before and after washing. On hydrophobic GelBond film surfaces, the same number of spores of indicated strains in 1/10 liquid minimal medium (MM) were placed on the surface and incubated at 25ºC in the dark for 24 h. The GelBond film surfaces were washed with water and further processed under a microscope. All experiments were performed in triplicate.

2.2.6 Plant infection test

2.2.6.1 Tomato infection study

The tomato infection assay was modified from (Tran *et al.*, 2014). Ten-day-old tomato seedlings “MoneyMaker” (Bruno, Nebelung GmbH, Everswinkel, Germany) were infected with 10 ml spore solution ($2x10^6$ spores/ml) of the wild-type, deletion, and complementation strains or non-infected with tap water for 30 min by root dipping. Then, they were transferred into pots containing a mixture of sand and soil (1:1). Tomato plants were kept in the dark for 24 h to reduce stress effects. The infected plants were cultivated in a climate chamber with 16 h light and 8 h dark

at 22-25°C. The soil humidity was controlled at a minimum level to increase pathogenic symptoms. The plant heights were measured weekly until 35 days post infection (dpi). The fungus re-isolation from infected plants was modified from (Tran *et al.*, 2014). Hypocotyls of infected plants were sterilized in the solution containing 6% hypochlorite and 0.05% Tween 80 for 7 min, ethanol 70% for 5 min and washed twice with distilled water for 5 min. The sterilized hypocotyl was placed on PDA plates containing chloramphenicol (100 µg/ml) and cefotaxime (100 µg/ml) and cultivated at 25°C for seven days. The total DNA was extracted from roots and hypocotyls. The fungal DNA in total DNA was quantified by real-time PCR with OLG primers (Eynck *et al.*, 2007; Timpner *et al.*, 2013). The experiments were repeated twice with 16 plants per each treatment.

2.2.6.2 *Arabidopsis* root infection test

Root infection test was modified from (Tran *et al.*, 2014). The *Arabidopsis thaliana* seeds were sterilized in a solution containing 70% EtOH and 0.1% Tween 80 for 5 min and in EtOH 99% for 5 min and dried at 37°C for 1 h. The sterilized seeds were grown on Murashige and Skoog plates (Duchefa, Haarlem, Netherlands), which include 1% sucrose, 0.05% MES (Carl Roth, Karlsruhe, Germany), and 1.5% plant agar (Duchefa, Haarlem, Netherlands). The pH was adjusted to 5.7. *Arabidopsis* plants were cultivated in a climate chamber under 16 h light and 8 h dark at 22-25°C. Three week old plants were transferred to 1% agarose plates and incubated for 24 h. The plant roots were dipped in the spore solution (10^5 spores/ml) of the wild-type or the deletion strain expressing *GFP* for 35 min. Infected plants were put back to agarose plates and co-inoculated in a climate chamber. The root area was covered with aluminum foil for shading. Before microscopy, plant roots were stained with a solution containing propidium iodide 0.0025% and silvet 0.0005%. Confocal laser scanning microscope (Leica SP5) pictures were taken at different time points.

2.2.6.3 Scan electron microscopy

After a fixation step (2.5% glutaraldehyde, 2% paraformaldehyde, 1 mM $CaCl_2$, 1 mM $MgCl_2$, Tween 20, 50 mM NaN_3 in 100 mM HEPES pH 7.5), samples were treated with amino acid-sucrose-solution (2% arginine, 2% glycine, 2% glutamate, 2% sucrose) for one hour, guanidine-tannin-solution (2% guanidine, 2% tannin) for 30 min, and 1% osmium tetroxide in cacodylate buffer (pH 7; supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM $NaNO_3$) for 30 min at room temperature with

washing steps in between. The samples were dehydrated in a graded series of aqueous ethanol solutions (10-100%) and then critical point-dried. Finally, samples were mounted on aluminum stubs, sputtered with gold/palladium and examined with a scanning electron microscope EVO LS10 (Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

2.2.7 Protein methods

2.2.7.1 Protein isolation

The cell lysate proteins were isolated from three days old mycelium which was grown in 100 ml liquid MM on a shaker at 120 rpm at 25^{o}C. The mycelium was ground with liquid nitrogen and proteins were extracted with extraction buffer B (Schinke *et al.*, 2016).

2.2.7.2 Proteomic analysis

The same amount of cell lysate proteins (40 µg) of JR2-wt, *SOM1* deletion, and *SOM1* complementation strains were loaded in a 12% SDS-PAGE and run at 150 V for 30 min. Proteins in gels were stained with coomassie blue following the staining method (Neuhoff *et al.*, 1985; Neuhoff *et al.*, 1988). Each lane was cut into eight pieces. They were individually digested with trypsin in gels and peptides were treated with C18 stage tips as described by (Rappsilber *et al.*, 2007). Peptide from each piece was independently analysed by mass spectrometry and examined by the MaxQuant program. The list of proteins was filtered by the following workflow: (1) remove all contaminant including “only identified by site”, “reverse”, and “potential contaminant”; (2) transfer LFQ intensity to Log2 (LFQ intensity) and replace missing values by 1; (3) create group 1 base on Log2 (LFQ intensity) containing +*SOM1* (JR2-wt and *SOM1* complementation strains) and –*SOM1* (*SOM1* deletion strain) groups; (4) run two-sample tests between +*SOM1* and –*SOM1* groups. Test type: student’s t-test, use for truncation: permutation base FDR, FDA = 0.05; (5) filter to remove all non-significant different; (6) filter and keep all protein with three times Log2 (LFQ +*SOM1*) > 20; (7) filter and keep all protein with t-test different >10.

2.2.7.3 Western hybridization

Extracted proteins (40 µg) in the SDS loading dye were added to a 12% SDS-PAGE after heating at 95°C for 5 min, run for one hour at 150 V and transferred to a nitrocellulose membrane (GE Healthcare Europe GmbH, Freiburg, Germany). The GFP signal was detected by a mouse α-GFP antibody (Santa Cruz Biotechnology, Cat no: Sc-9996) (1:1000) and HRP conjugated goat mouse secondary antibody (Jackson Immunoresearch, Cat no: 115-035-003) (1:2000). Antibodies were diluted in TBST with 5% (w/v) non-fat dry milk.

2.2.7.4 GFP trap assay

Proteins of the fungal strains VGB0084, VGB0085, VGB0281, and VGB0282 were isolated as described above. Total protein extract was processed with 25 µl GFP-Trap beads (Chromo Tek GmbH, Planegg-Martinsried, Germany) according to user's manual. Eluted proteins were applied to a 12% SDS-PAGE and run for 13 min to check the quality of purified proteins. Gel pieces were isolated and digested with trypsin in gels according to Shevchenko's protocol (Shevchenko *et al.*, 1996). Peptides digested by trypsin were treated with C18 StageTips (Rappsilber *et al.*, 2007) before performing Mass spectrometry analysis (Tran *et al.*, 2014). Orbitrap raw files were analysed with the MaxQuant program (version 1.4.1.2) and protein list was filtered by Perseus (version 1.5.5.3).

The list of protein was sequentially filtered by the following workflow: (1) remove all contaminant including "only identified by site", "reverse", and "potential contaminant"; (2) transfer LFQ intensity to Log2 (LFQ intensity) and replace missing values by 1; (3) create group 1 containing of free GFP strain and group 2 comprising log2 (LFQ intensity) of *SOM1:GFP* strains; (4) remove rows if the group 1 is higher than 1; and (5) remove rows if 3 times of group 2 smaller than 10. The free GFP in VdJR2 wild-type was used as a negative control. The experiments were performed with three biological and three technical replicates in two independent strains.

2.2.8 Gene expression quantification

The gene expression was quantified with qPCR MasterMix (5 PRIMER, Hamburg, Germany) using the CFX Connect Real-Time PCR system (Bio-Rad Laboratories GmbH, München, Germany). Total RNAs were extracted from mycelium of *V. dahliae* which is grown in liquid SXM for three days or cells of *S. cerevisiae*

incubated overnight using the RNeasy Plant Mini Kit (Qiagen). The cDNA was synthesized using 0.8 µg of RNA with the Quantitech^{R} Reverse Transcription Kit (Qiagen, Hilden, Germany). The relative expression of the genes was calculated using the ΔΔCq method (Haimes & Kelley, 2014). *GAPDH*, *PP2A1*, and *PP2A2* were used as housekeeping genes for *Verticillium* or *TAF10* and *ALG9* for *S. cerevisiae*. Real-time PCR reactions contained 4 µl of the 2.5x MasterMix included SYBR, 5 pmol for forward and reversed primers, 100 ng cDNA and water in a final volume of 10 µl. All primers used for quantification are listed in Table 1. The experiment was performed with two independent deletion and complementation strains in triplicates.

3 Results

3.1 The transcription factors *SOM1* and *VTA3* can reprogram non-adhesive yeast strain

Vta3 is an ortholog of transcriptional repressor Rfx1, whereas Som1 is a potential homolog of Flo8 in *S. cerevisiae* (Tran *et al.*, 2014). In this study, the effect of Som1 and Vta3 in adhesion was examined in a non-adhesive yeast strain. The mechanism of Som1 and Vta3 in adhesion in yeast model were investigated. The genomic DNA and amino acid sequences of transcription factors *SOM1* and *VTA3* were analysed by bioinformatics programs and encoded protein domains were compared to the homologs in other fungi. The expression of relevant genes for adhesion in yeast was tested by real-time PCR.

3.1.1 *SOM1* and *VTA3* genes encode proteins comprising a LisH or a wing helix-turn-helix DNA binding domain

The *SOM1* and *VTA3* sequences were analysed with NCBI blast, Pfam, 9aaTAD, and SeqNLS to find homologs in other fungi and to predict the domains and the nuclear localisation signal (Figure 15, 16). *V. dahliae* Som1 (XP_009650808) shows similarity to *S. cerevisiae* Flo8 (23%), AfSomA from *A. fumigatus* (40%), and MoSom1 from *M. oryzae* (51%).

Alignments of the cDNA and the gDNA sequence of *SOM1* shows that the gene structure consists of six exons and five introns resulting in an open reading frame of 822 codons for a protein with a predicted molecular weight of 86.6 kDa (Figure 15). The results of the bioinformatic analysis indicated that the Som1 sequence harbors a LisH domain at its N-terminus, a SnAPC domain at the C-terminus, an SSDP domain in the middle and two putative nuclear localisation signals (NLS). Orthologs to the LisH domain were found in *Aspergillus fumigatus*, *Aspergillus nidulans,* and *Magnaporthe oryzae* with 50%, 54%, and 60% amino acid similarity, respectively. Moreover, it also displayed high comparability to orthologs in pathogens *Colletotrichum gloeosporioides*, *Diaporthe ampelina*, *Ustilaginoidea virens*, *Fusarium oxysporum*, and *Metarhizium rileyi:* 71%, 73%, 79%, 79%, and 74% amino acid similarity, respectively (Figure 15).

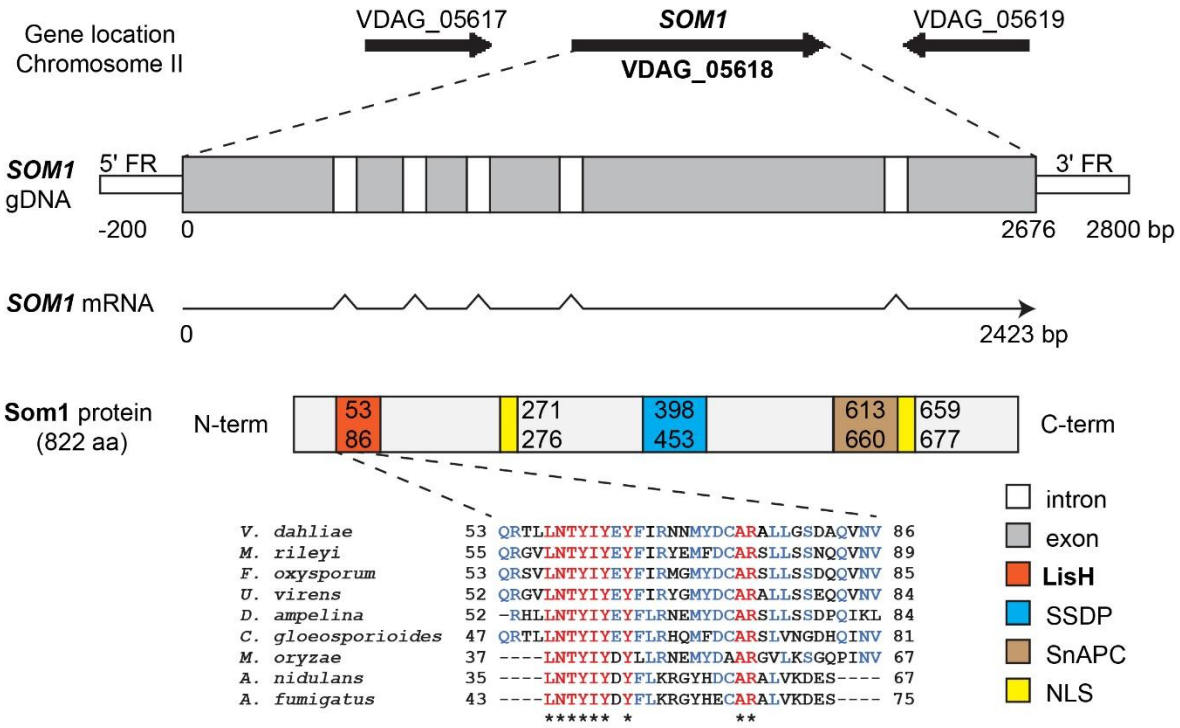


Figure 15. Gene locus and structure of *SOM1*. Genomic DNAs were compared with cDNA using MultAlin. Introns and exons were marked. The nuclear localisation signals (NLS) predicted by the SeqNLS software are displayed. The protein domains were predicted by three different programs: Pfam, NCBI blast, and 9aaTAD. Sequence alignments of the LisH domain of Som1 and related proteins from other organisms including *Metarhizium rileyi* (OAA39058), *Fusarium oxysporum* (XP_018248456), *Ustilaginoidea virens* (KDB12219), *Diaporthe ampelina* (KKY33496), *Colletotrichum gloeosporioides* ((XP_007286727), *Magnaporthe oryzae* (XP_003710835), *Aspergillus nidulans* (XP_682356), and *Aspergillus fumigatus* (XP_746706) are shown. Asterisks: identical residues; Red: high (90%), blue: low (50%) consensus values; FR: flanking region; N-term: N-terminus; C-term: C-terminus.

The *VTA3* gene contains two exons and one intron; the ORF encodes a 795 aa protein with predicted molecular weight of 87.4 kDa (Figure 16). The Vta3 protein sequence was analysed by bioinformatics programs and the data showed that it contains a wing helix-turn-helix DNA binding domain (WDBD), three different nine amino acid transactivator domains (9aaTAD), and one putative NLS (Figure 16). The WDBD domain showed little similarities to the ortholog in *Candida albicans* (48%), but similarities to the orthologs in *A. fumigatus*, *A. nidulans*, *Penicillium chrysogenum*, and *Acremonium chrysogenum:* 64%, 64%, 64% and 71%, respectively. It also showed high similarity to the orthologs in *F. graminearum*, *U. virens*, and *Metarhizium brunneum:* 95%, 99%, and 99%, respectively (Figure 16).

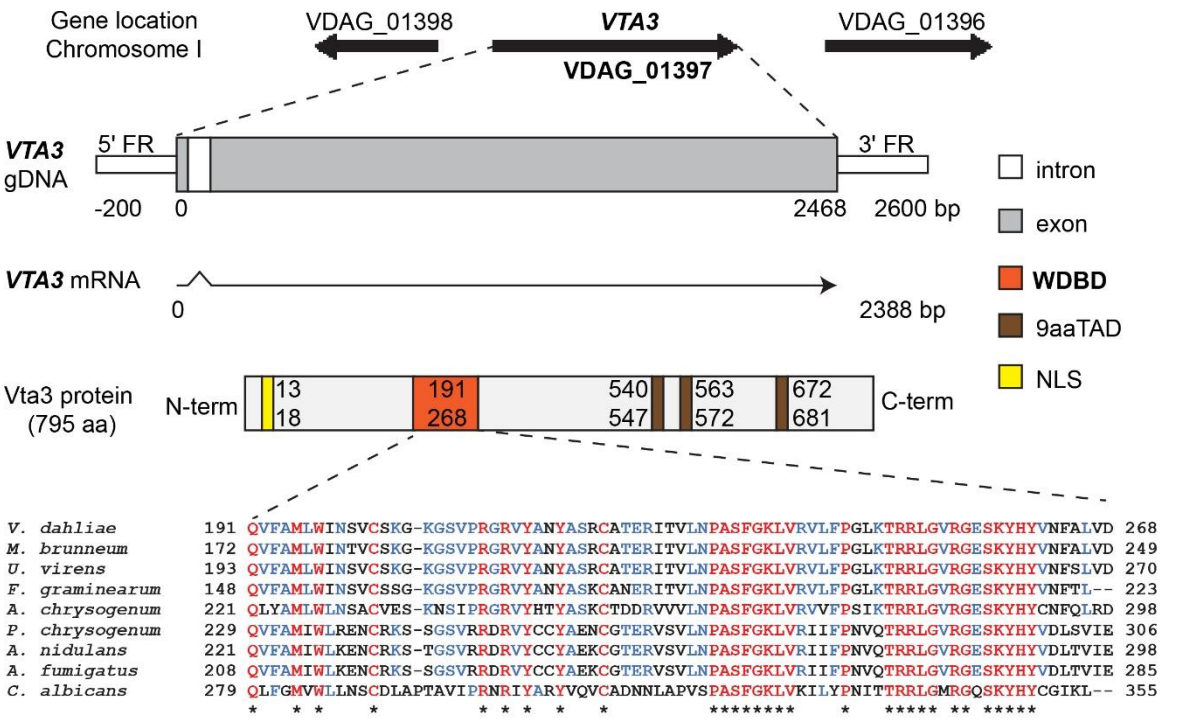


Figure 16. Gene locus and structure of *VTA3*. Introns and exons are marked. The predicted NLS and putative protein domains are indicated. Multiple alignments of (WDBD) of Vta3 and the corresponding proteins from other fungi include *Metarhizium brunneum* (XP_014546736), *Ustilaginoidea virens* (GAO17021), *Fusarium graminearum* (XP_011327190), *Acremonium chrysogenum* (CAB85587), *Penicillium chrysogenum* (CAB85619), *Aspergillus nidulans* (XP_659900), *Aspergillus fumigatus* (XP_754020), and *Candida albicans* (XP_713492). Asterisks: identical residues; Red: high (90%), blue: low (50%) consensus values; FR: flanking region; N-term: N-terminus; C-term: C-terminus.

3.1.2 Som1 and Vta3 are nuclear proteins

Both, *SOM1* and *VTA3,* sequences contain a putative NLS, therefore, the localisation of these proteins in the cell was studied. Consequently, *SOM1* and *VTA3* genes under the control of their native promoter were fused to the *GFP* gene in pGreen2 (Tran *et al.*, 2014) to examine the subcellular localisation of Som1 and Vta3. Each construct was transferred into the corresponding deletion strain of *SOM1* and *VTA3,* respectively. The GFP signal of the transformation strains was observed by fluorescence microscopy after 24 h growth in SXM. Fungal nuclei were stained by 4',6-diamidin-2-phenylindol (DAPI).

The GFP signal was strongly detected in nuclei of the strains expressing *SOM1::GFP* or *VTA3::GFP* as shown in Figure 17. The GFP signal co-localised with the DAPI stained nuclei (Figure 17). Contrastingly, the GFP signal was identified throughout the whole hyphae in control cells expressing only free GFP (JR2-GFP) and it was not found in the wild-type strain (JR2-wt). The results indicate that both, Som1 and Vta3, are nuclear proteins.

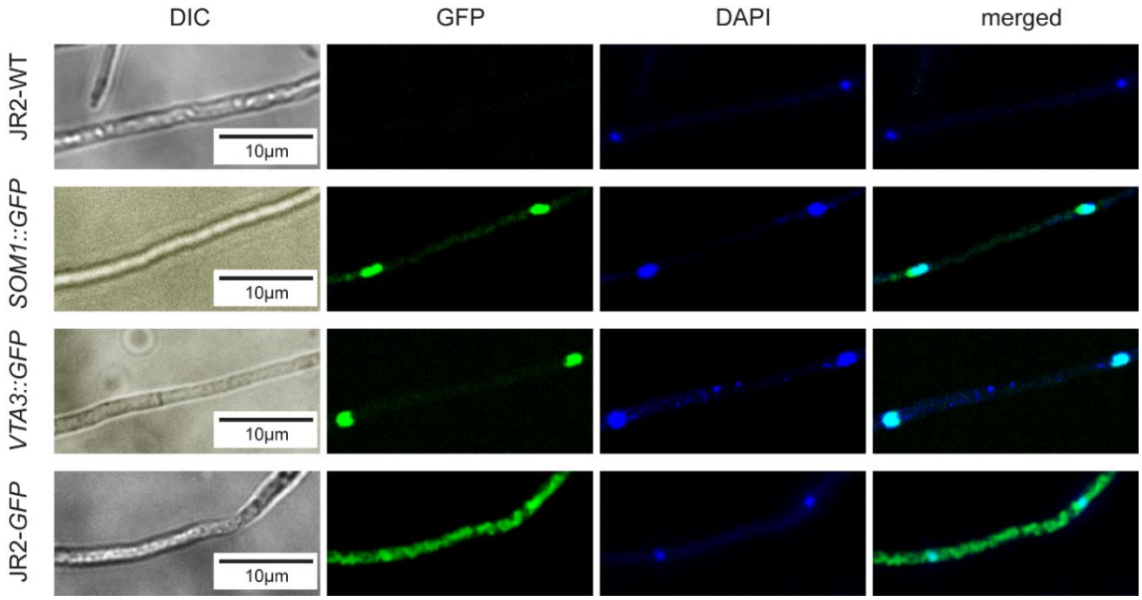


Figure 17. Som1 and Vta3 are nuclear proteins. The *SOM1* and *VTA3* genes under the control of the native promoter were fused to *GFP* separated by the GGSGG linker and ectopically transformed into the respective strains. Transformants were screened on selective medium containing hygromycin 50 µg/ml and examined by fluorescence microscopy. The positive clones with a strong GFP signal were grown in liquid SXM for 24 h. The localisation of proteins was observed by green fluorescence. Fungal nuclei were stained with 4',6-diamidin-2-phenylindol (DAPI). The strain names and filter are shown. Scale bars are represented.

3.1.3 Som1 and Vta3 can rescue *FLO8*-defective *S. cerevisiae* strains

To examine whether *SOM1* and *VTA3* can complement the *FLO8* deletion strain, adhesion experiments were performed on agar plates and in liquid medium. Strains harbouring the respective ORFs of *SOM1* and *VTA3* were under the control of either the *GAL1* promoter or *MET25*, and subsequently transformed to a yeast *FLO8*-deletion strain. The low expression of *SOM1* was achieved in SC-uracil medium where the *MET25* promoter is partially repressed whereas overexpression of *SOM1* was attained in SC-uracil + galactose or SC-uracil-methionine where the *GAL1* and the *MET25* promoters are induced. The adherence test to agar plates shown in Figure 18 clearly demonstrates that the yeast strain expressing *SOM1* under the control of either the *GAL1* or the *MET25* promoter is unable to bind to agar plates. It was easily washed off as seen also for the negative control ((-) control). However, yeast strains with low expression of *SOM1* under the control of the *MET25* promoter in SC-uracil medium or high expression of *VTA3* under the control of the *GAL1* promoter can reprogram non-adhesive *FLO8*-defective yeasts to adhesion. They still bind on agar plates after washing (Figure 18).

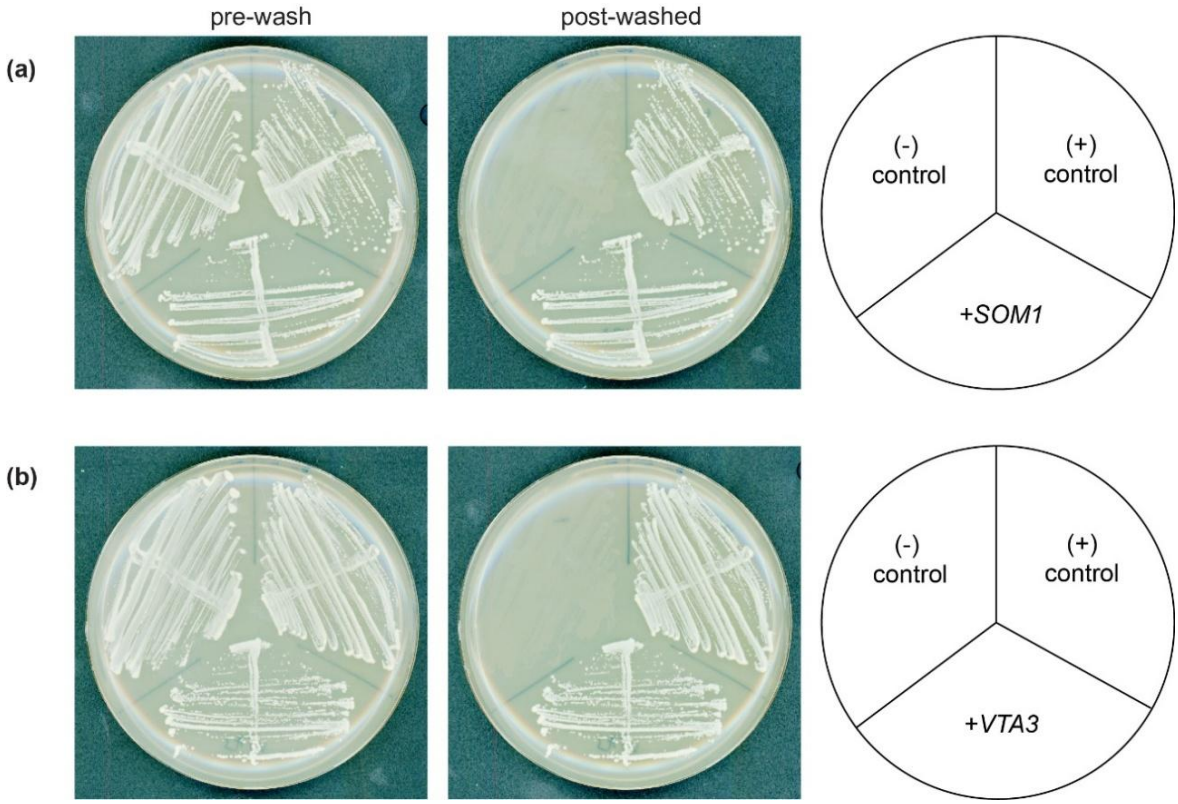


Figure 18. *SOM1* and *VTA3* can reprogram non-adhesive *FLO8*-deficient *S. cerevisiae* strains to adhesion on agar plates. Haploid *S. cerevisiae* strain BY4741 expressing *SOM1* or BY4742 expressing *VTA3*. The *S. cerevisiae FLO8* mutant strain with empty vector served as negative (-) control and the strain with a vector harboring *FLO8* was used as a positive (+) control. (**a**) Adhesion of the BY4741 strain with low expression of *V. dahliae SOM1* in SC-Ura+glucose medium. (**b**) Adherence of the BY4742 strain with high expression of *V. dahliae VTA3* in the SC-Ura + galactose medium were examined. The adhesion test was performed after two days of growth on agar plates. The plates were washed with water in the sink until the negative control was washed off. All experiments were performed in triplicates.

The flocculation experiment in liquid medium indicates that the negative control and the strains overexpressing *SOM1* are unable to flocculate, whereas the positive control and strains with low expression of *SOM1* or high expression of *VTA3* can flocculate well (Figure 19b). The ratio of flocculation of low expression of *SOM1* and high expression of *VTA3* is 0.42 and 0.35, respectively, whereas that of positive and negative controls is 0.55 and 0.00, respectively (Figure 19a). The expression of genes related to adhesion was further examined.

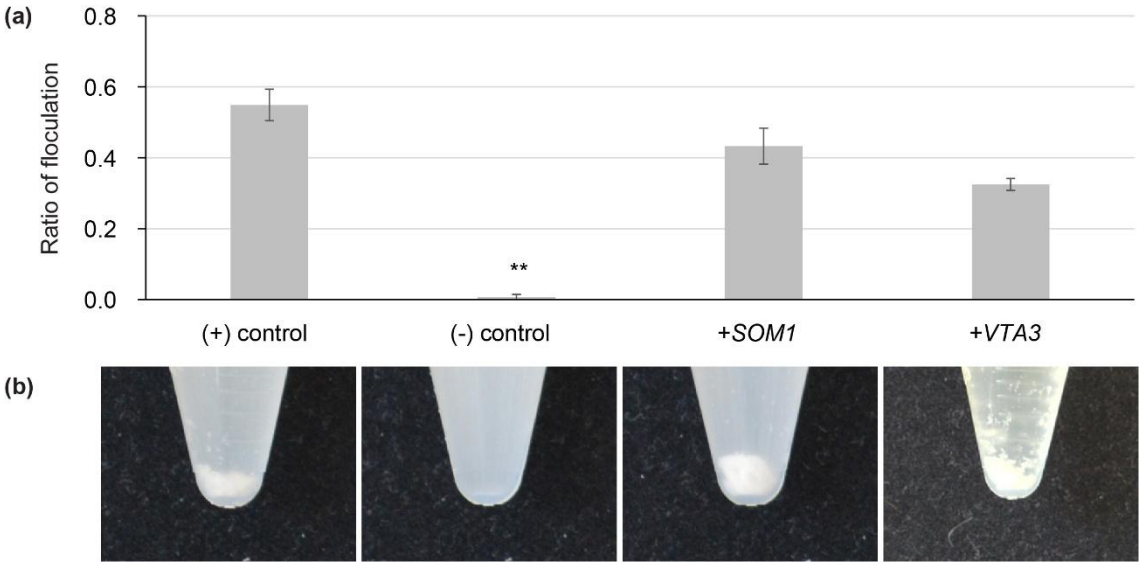


Figure 19. Som1 and Vta3 can activate flocculation of *FLO8*-defective *S. cerevisiae* in liquid medium. The BY4741 strain with low expression of *V. dahliae SOM1* in SC-Ura + glucose medium and the BY4742 strain with high expression of *V. dahliae VTA3* in the SC-Ura + galactose medium were examined. The *S. cerevisiae FLO8* mutant strain with empty vector served as negative (-) control and the strain with a vector harboring *FLO8* was used as a positive (+) control. Flocculation tests were carried out by growing indicated strains in 10 ml medium on a rotator for one day at 30°C. (**a**) Diagram of ratio of flocculation. The flocculation part and non-flocculation were separately dried and measured. Ratio of flocculation was calculated based on dry weight of flocculation part in total dry mass. (**b**) Flocculation in liquid medium. Pictures of the cultures with precipitated cells are represented. All experiments were performed in triplicates. Mean values and standard deviation are shown. The asterisk (**) indicates a significant decrease when compared to a positive control (Student's t-test, $p<0.01$).

3.1.4 Low expression of *SOM1* can activate flocculation genes

Expression of relevant genes for adhesion in yeast strains expressing *SOM1* was further analysed by real-time PCR. The results show that *FLO1* and *FLO11* are not activated neither in the strain overexpressing *SOM1* nor in the negative control, whereas their expression is significantly increased either by the expression of *FLO8* or by low expression of *SOM1* (Figure 20). Additionally, the overexpression of *SOM1* in *S. cerevisiae* causes the repressive expression of *STE12*. Expression of *STE12* is significantly decreased in this case (Figure 20). Taken together, low expression of *SOM1* can activate *FLO1* and *FLO11,* whereas high expression does not.

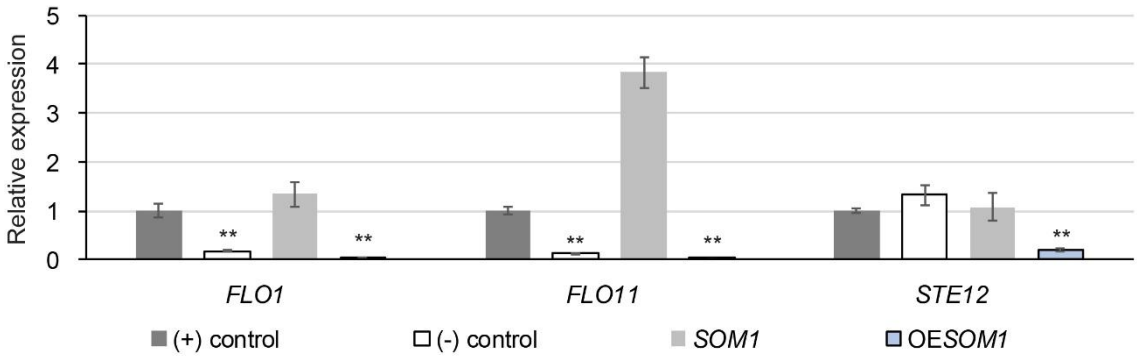


Figure 20. Som1 promotes the expression of *FLO1* and *FLO11* in *FLO8*-deficient *S. cerevisiae*. The RNAs of indicated strains were isolated from the cells which were cultivated in liquid medium for 24 h. The relative expression of genes related to adhesion was normalized to expression of the *TAF10* reference gene and the positive control. All experiments were performed in triplicates. Mean values and standard deviation are shown. The asterisk (**) indicates a significant decrease when compared to a positive control (Student's t-test, $p<0.01$).

3.1.5 Activation of *VTA3* can stimulate expression of flocculation genes

As expression of *VTA3* under the control of the *GAL1* promoter can rescue adhesion of non-adhesive yeasts (Figure 18), the effect of Vta3 on the expression of relevant genes for adherence was also examined by RT-PCR (Figure 21). The expression of *VTA3* under the control of the *GAL1* promoter stimulates the expression of *FLO1* and *FLO11*. Their expression is significantly increased compared to the negative control (Figure 21). Interestingly, the expression of other adhesion-related genes such as STE12, TEC1, PHD1, FLO10, and TPK2 does not change (data not shown), whereas the expression of gene for the repressor Sfl1 is significantly reduced (Figure 21). This means, Vta3 represses the expression of *SFL1* to de-repress the expression of flocculation genes including *FLO1* and *FLO11* and the yeast can adhere on a surface.

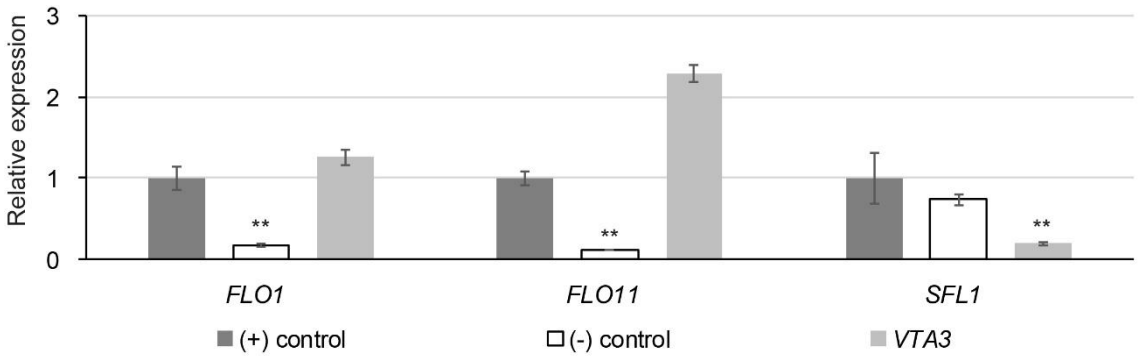


Figure 21. Vta3 stimulates the expression of *FLO1* and *FLO11* in *FLO8*-defective *S. cerevisiae*. The RNAs of indicated strains were isolated from the cells which were cultivated in liquid medium for 24 h. The relative expression of genes related to adhesion was normalized to the expression of the *TAF10* reference gene and the positive control. All experiments were performed in triplicates. Mean values and standard deviation are shown. The asterisk (**) indicates a significant decrease when compared to a positive control (Student's t-test, $p<0.01$).

3.2 Transcription factors *SOM1* and *VTA3* are required for morphology and virulence in *V. dahliae*

The functions of Som1 and Vta3 in *V. dahliae* were examined by using genetics, cell biology, proteomics, and plant infection experiments. The single deletion of *SOM1* and *VTA3*, and ectopic complementation strains were generated. The phenotypes of deletion strain were compared to that of the wild-type and complementation to address the function of genes.

3.2.1 Deletion and complementation of *SOM1* and *VTA3* in *V. dahliae*

Deletion and complementation strains of *SOM1* and *VTA3* were confirmed by Southern hybridization before functional analysis. The deletion of *SOM1* was confirmed with two different restriction enzymes, *Bsu15*I and *Eco81*I. The wild-type genomic DNA was used as a control. The expected bands of 5.7 kb and 1.9 kb were observed in the deletion strain, and 3.0 and 3.3 kb for the wild-type, respectively (Figure 22). The ectopic complementation of *SOM1* was confirmed with *Xba*I/*Pvu*I enzymes, using the genomic DNA of the *SOM1* deletion strain as a control. The expected bands for the complementation (1.7 kb and 4.9 kb) and the control strain 1.7 kb were observed as seen in Figure 22.

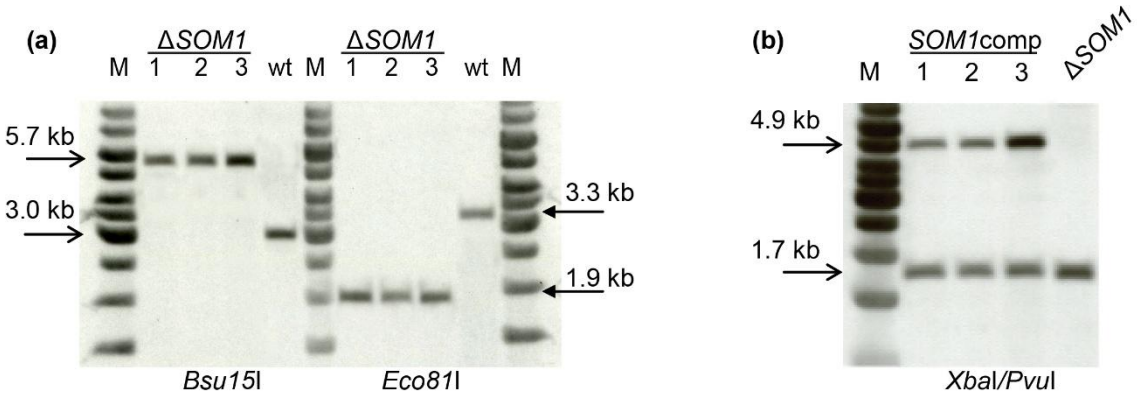


Figure 22. Confirmation of deletion and complementation strains of *V. dahliae SOM1*. (**a**) Confirmation of deletion strains by Southern hybridization with two different restriction enzymes. The probe is the 3' flanking region. Genomic DNA of the wild-type was used as a control. (**b**) Confirmation of complementation strains by Southern hybridization with *Xba*I and *Pvu*I. Genomic DNA of the *SOM1* deletion strain was used as a control. The probe is the terminator of *SOM1* of 0.8 kb. Sizes of expected fragments are indicated. Restriction enzymes and predicted sizes are shown.

Next, deletion and complementation strains of *VTA3* were constructed and confirmed by Southern hybridization (Figure 23). The *VTA3* deletion strain was confirmed by Southern hybridization with two different enzymes *BspE*I and *Xho*I. Genomic DNA of the wild-type was used as a control. The expected bands of 1.8 kb and 4.0 kb were observed in the genome of the *VTA3* deletion strain, and 5 kb and 2.3 kb bands for the genome of the wild-type. The *VTA3* complementation strain was confirmed with the restriction enzyme *Ava*II, using the genomic DNA of the *VTA3* deletion strain as a control. The expected bands for the complementation are 1.8 kb and 3.8 kb, respectively, whereas that for the control strain is 1.8 kb (Figure 23).

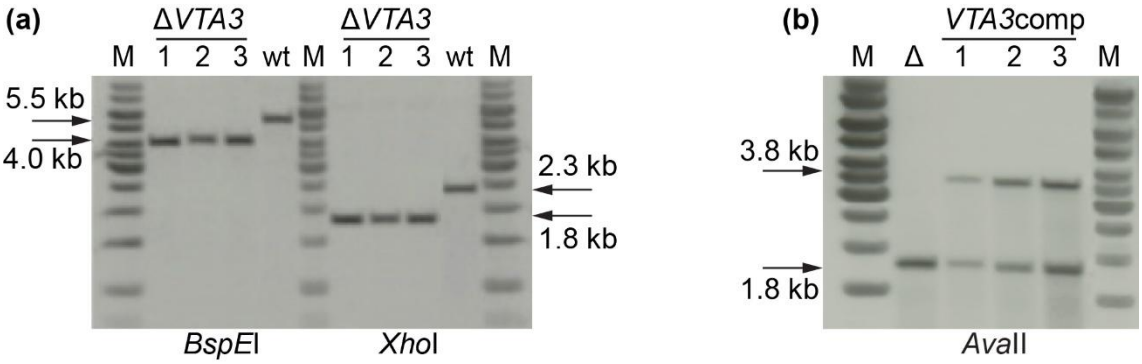


Figure 23. Confirmation of deletion and complementation strain of *VTA3* in *V. dahliae*. (**a**) Confirmation of deletion strains by Southern hybridization with two different restriction enzymes. The probe is the 3' flanking region. Genomic DNA of the wild-type was used as a control. (**b**) Confirmation of complementation strains by Southern hybridization with two different restriction enzymes. Genomic DNA of the *VTA3* deletion strain was used as a control. The probe and sizes of expected fragments are indicated. The name of strains, restriction enzymes, and putative sizes are shown.

3.2.2 Som1 promotes adhesion in *V. dahliae*

The functions of Som1 and Vta3 in *V. dahliae* was studied by comparing the phenotype of deletion strains with that of wild-type and complementation strains. Specifically, the function of Som1 and Vta3 in hyphal clumping, biomass formation, adhesion, virulence, root penetration, conidia and microsclerotia formation, hyphal development, and oxidative stress response was investigated.

3.2.2.1 Som1 is necessary for hyphal clumping and suppresses biomass formation

The biomass of filamentous fungi in liquid medium is antagonistic to flocculation or hyphal clumping (Guebel & Nudel, 1994). Our results show that Som1 is required for adhesion in *V. dahliae* (Figure 24). Therefore, the hyphal clumping and biomass of the *SOM1* deletion strain might be affected. To test this hypothesis, the same number of spores of the wild-type, *SOM1* deletion, and complementation strains was grown in 50 ml liquid PDM on a shaker at 120 rpm at 25^{o}C for seven days. Hyphal ball formation and biomass production were observed. As seen in Figure 24, the *SOM1* deletion strain produced smaller hyphal balls compared to JR2-wt and complementation strains. The size of hyphal balls of the *SOM1* deletion strain was 80% smaller compared to the wild-type and complementation (Figure 24). The size of hyphal balls in deletion strain of *SOM1*

did not increase during growth, whereas that of wild-type and complementation strain was continuously enlarged. Additionally, more hyphal balls were observed in the *SOM1* deletion strain compared to the wild-type and complementation strains (Figure 24).

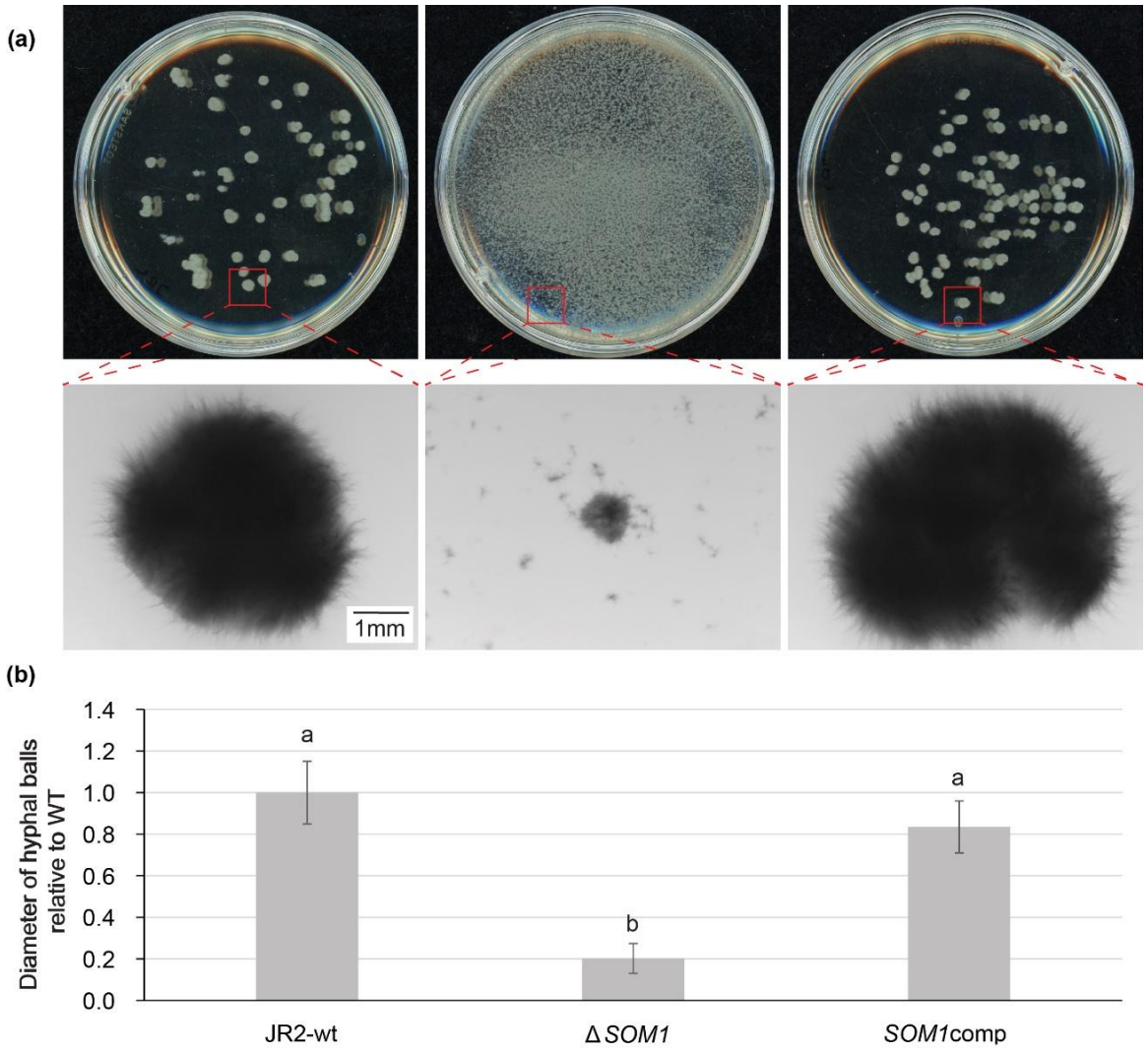


Figure 24. Som1 is required for *V. dahliae* hyphal clumping. (**a**) The same number of spores of JR2-wt, *SOM1* deletion and complementation strains was grown in liquid PDM on a shaker at 25ºC for seven days. The diameter of hyphal balls was observed. (**b**) Diagram of hyphal ball diameter. Experiments were performed in triplicate. Twenty hyphal balls were observed for each repetition. Mean values and standard deviation are presented. The letters a and b indicate groups which are significantly different as calculated by Tukey-Kramer multiple comparison procedures, α= 0.01. The scale bar is shown.

Next, the biomass of the *SOM1* deletion strain was measured (Figure 25). As shown in Figure 25, the biomass of the *SOM1* deletion strain was dramatically increased by 50% when compared to the wild-type and complementation strains

after 7 dpi (Figure 25). The biomass of the deletion strain of *SOM1* is continuously increased whereas that of the wild-type and complementation strains is unchanged after seven days. Taken together, the data suggest that Som1 is required for hyphal clumping and suppression of biomass production in *V. dahliae*.

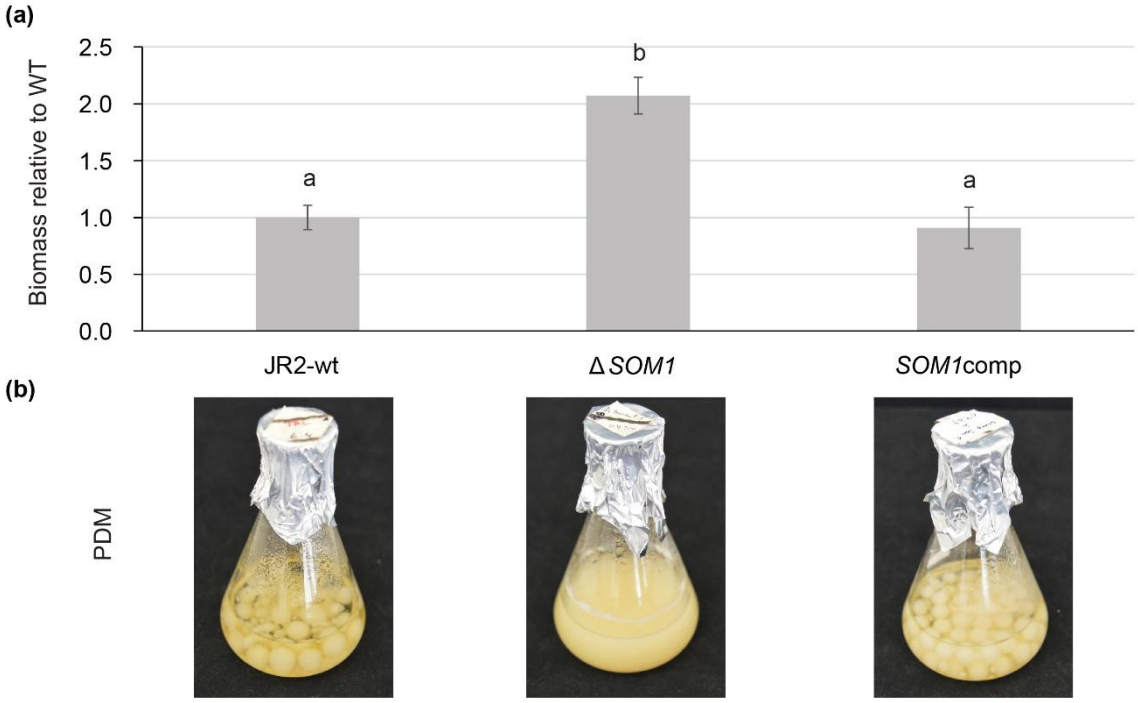


Figure 25. Som1 suppresses *V. dahliae* biomass formation. The same number of spores of JR2-wt, *SOM1* deletion and complementation strains was grown in liquid PDM on a shaker at 25ºC for seven days. (**a**) The diagram of biomasses relative to the wild-type. (**b**) Picture of the culture after seven days of growth. Experiments were performed in triplicate. Mean values and standard deviation are presented. The letter a and b indicate groups which are significant different as calculated by Tukey-Kramer multiple comparison procedures, α= 0.01.

3.2.2.2 Som1 is needed for adherence on abiotic surfaces

As Som1 rescued the non-adhesive phenotype of yeast. It might also play an important role in adhesion of *Verticillium*. Adhesion was tested using two different surfaces polystyrene plates and GelBond films.

To test adhesion to polystyrene plates, the wild-type, *SOM1* deletion and complementation strains were incubated in liquid SXM in a polystyrene plate for seven days at 25ºC and then the plate was washed with water. As seen in Figure 26, the *SOM1* deletion strain was easily washed off the polystyrene, whereas the wild-type and complementation strains remained bound in the plates (Figure 26).

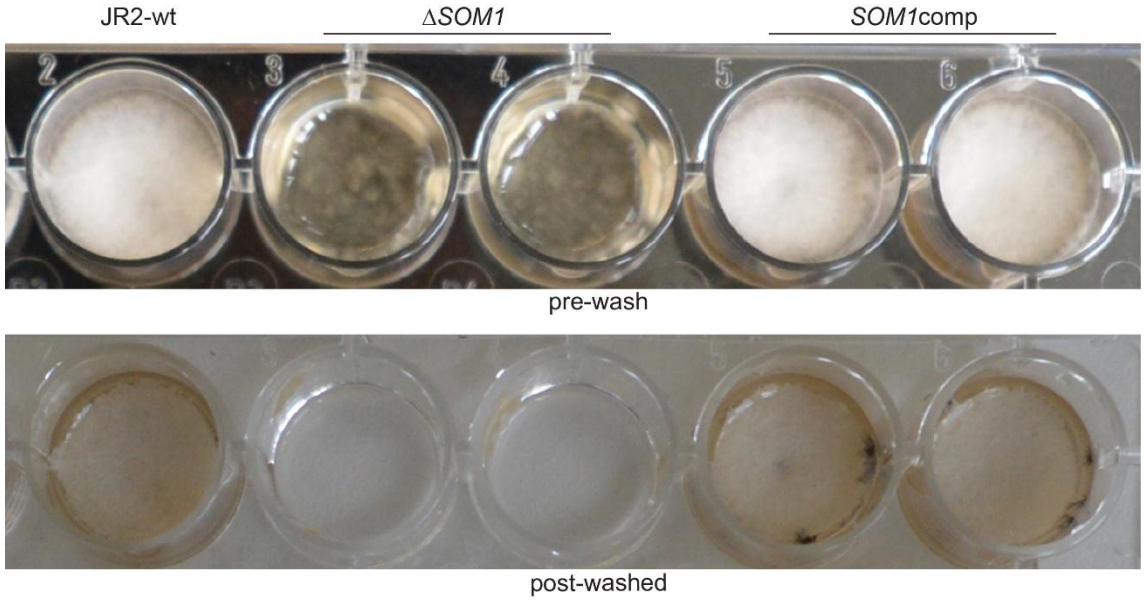


Figure 26. Som1 is necessary for adhesion of *V. dahliae* on polystyrene plates. The JR2-wt, *SOM1* deletion and complementation strains were grown on polystyrene plates containing liquid SXM for seven days at 25ºC. The wells were washed with water. All experiments were performed in triplicates.

The adhesion of *Verticillium* to GelBond films was tested. The same number of spores was inoculated on Gelbond films and then incubated the membrane in a humid chamber. After two days, the GelBond was washed with water and adhering strains were observed by microscopy. The results in Figure 27 show that the *SOM1* deletion strain can grow like the wild-type, but it is unable to adhere to the GelBond film. It was easily washed off when adding some drops of water. Many hyphae were detected in the wild-type and complementation strains but rarely any hyphae were seen in the *SOM1* deletion strain (Figure 27).

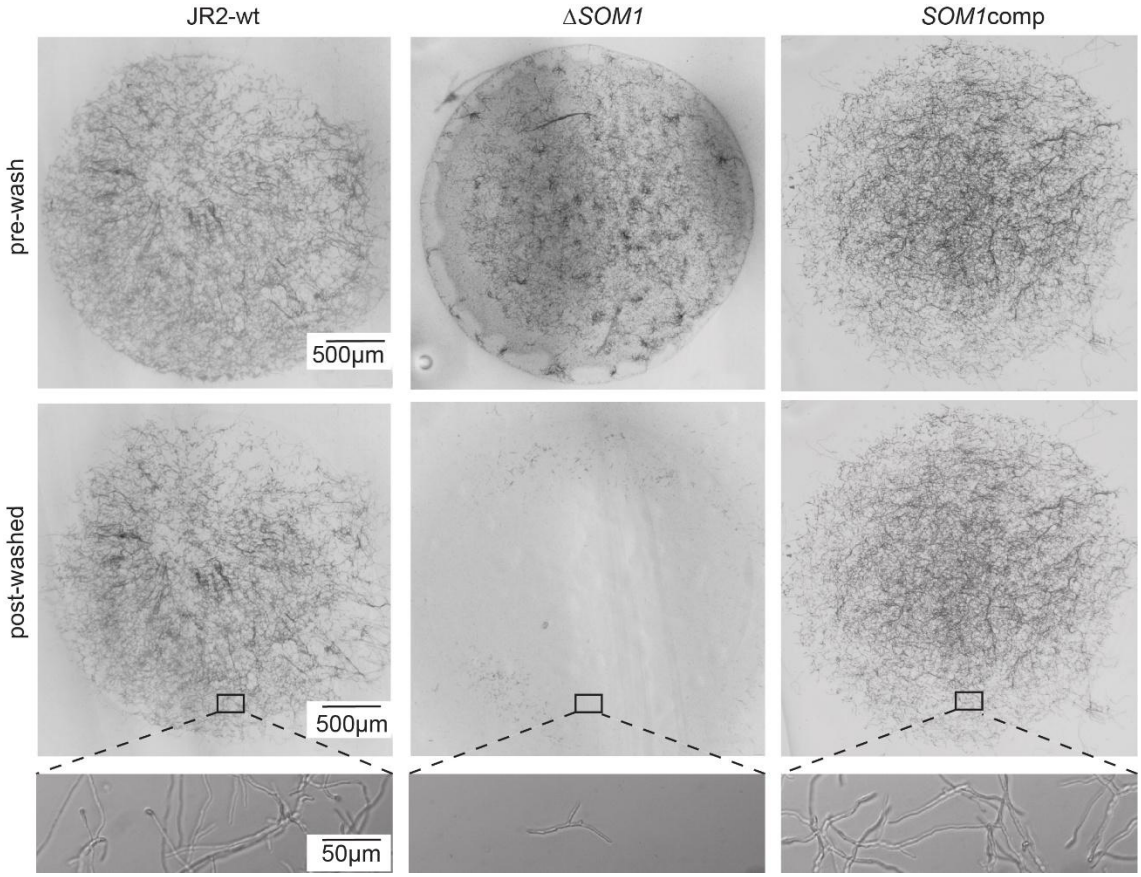


Figure 27. Som1 is necessary for *V. dahliae* adhesion to GelBond film. The same number of spores of the JR2-wt, *SOM1* deletion and complementation strains in liquid MM (1/10 of concentration) were placed on hydrophobic surfaces of the GelBond film and incubated at 25ºC in the dark for two days. Hydrophobic surfaces of the GelBond film were washed with water and further processed under the microscope. All experiments were performed in triplicates. Scale bars are shown.

3.2.3 Som1 and Vta3 are required for pathogenicity

Verticillium dahliae is known to cause wilting disease in more than 200 plant species including tomato plants (Pegg & Brady, 2002; Berlanger & Powelson, 2005). It was reported to cause also wilt disease in *Arabidopsis thaliana* (Wilhelm, 1955; Pegg & Brady, 2002). *V. dahliae* infects plants via the roots, which requires root penetration and colonisation as initial steps of infection. Therefore, the effect of Som1 and Vta3 on virulence was examined in tomato plants and on *A. thaliana* roots.

3.2.3.1 Som1 and Vta3 are involved in fungal pathogenicity

Ten days old tomato plants were used to test the virulence of Som1 and Vta3. The disease symptoms of plants infected by the deletion strains of *SOM1* and *VTA3* were compared to the wild-type and their complementation strains up to 35 dpi (Figure 28).

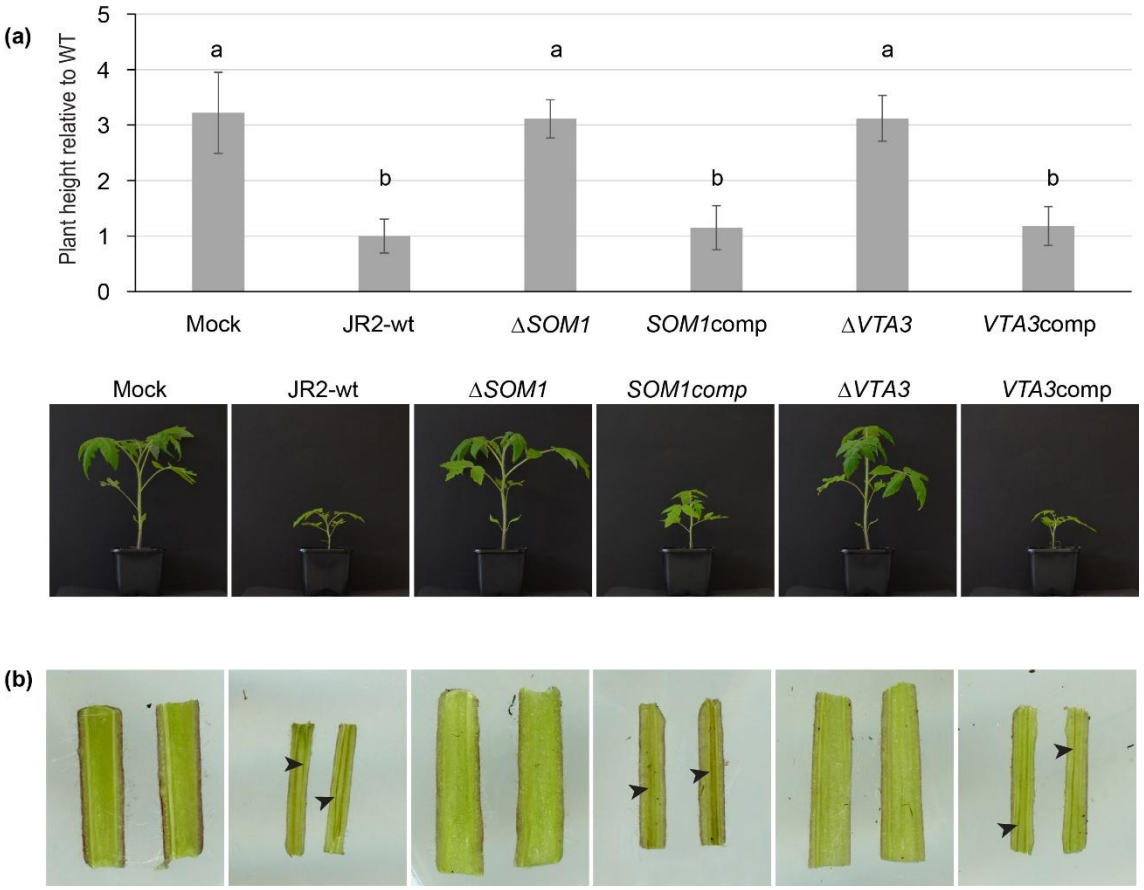


Figure 28. Som1 and Vta3 are required for introduction of disease symptoms in tomatoes. Ten days old tomatoes were infected with the same number of spores of indicated strains or remained uninfected (mock). The plants were incubated in the climate chamber under 16 h light: 8 h dark at 22-25°C. The disease symptoms were assessed at 35 days post infection (dpi). Infection experiments were performed with 16 single plants for each fungal strain and independently repeated twice. (**a**) The plant height was measured. The mean values and standard deviations are indicated. The letters a and b show groups which are significantly different as calculated by Tukey-Kramer multiple comparison procedure α= 0.01. Representative plants are shown. (**b**) Black threads in infected stems were observed. The arrowhead indicates black veins.

The wild-type and the complementation strains initiated severe stunting and leaf chlorosis in infected tomato plants which were accompanied by discoloration of the vascular system (Figure 28). In contrast, plants infected with the *SOM1* or *VTA3*

deletion strains showed no disease symptoms and they were indistinguishable from uninfected plants (Figure 28). The height of plants infected by the deletion strains of *SOM1* and *VTA3* is significantly higher than that of plants infected by the wild-type and complementation strains (Figure 28a). No black veins in stems were found in the plants infected by deletion strains (Figure 28b).

The presence of the pathogenic fungus in the plant was examined by its re-isolation from hypocotyls and quantification of the fungal DNA from the total plant DNA by real-time PCR with *OLG* primer pairs (Eynck *et al.*, 2007; Timpner *et al.*, 2013). The fungal pathogen was successfully re-isolated from hypocotyls of the plants infected with the wild-type and complementation strains, but not from the plants infected by *SOM1* and *VTA3* deletion strains (Figure 29a). The concentration of fungal DNA in the total plant DNA was also examined (Figure 29b). As demonstrated in Figure 29b, fungal DNA was not detected in plants infected with the *SOM1* deletion strain, whereas fungal DNA was reduced by 82% in plants infected with the *VTA3* deletion strain when compared to plants infected with wild-type. Similar results were obtained for fungal DNA extracted either from the root or the hypocotyls.

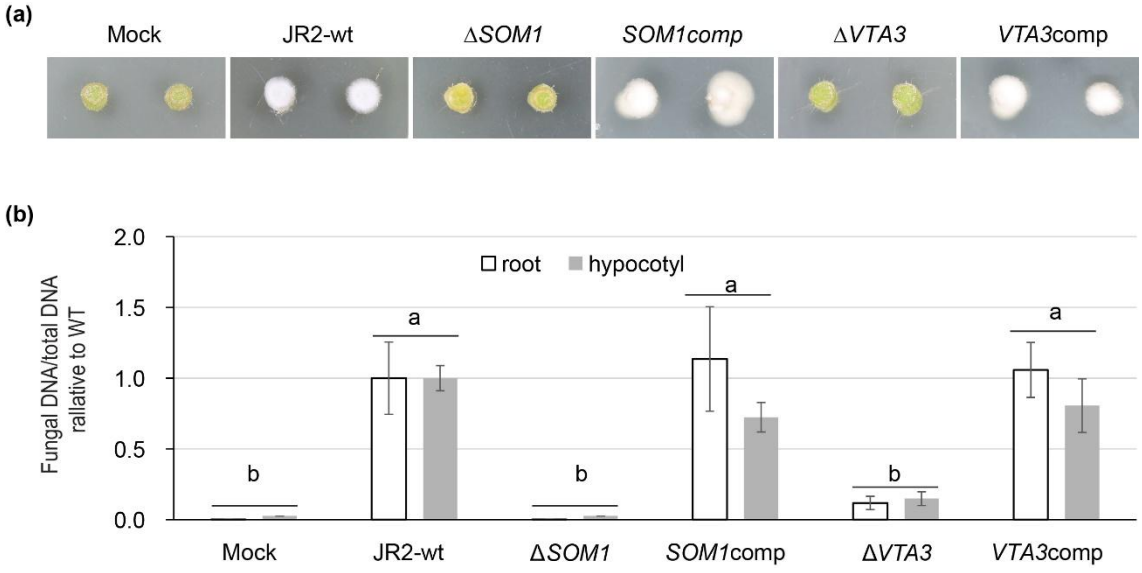


Figure 29. *V. dahliae* Som1 and Vta3 are essential for plant infection. (**a**) Fungal re-isolation from hypocotyl was examined. The surface-sterilized hypocotyls were placed on PDM plates containing cefotaxime and chloramphenicol and incubated for seven days. (**b**) The total DNA from roots and stems was purified. The relative amount of fungal DNA from the total plant DNA was quantified by RT-PCR using the *OLG* primer pairs (Eynck *et al.*, 2007). This experiment was performed with four repetitions. The mean values and standard deviations are indicated. The letters a and b show groups which are significantly different as calculated by Tukey-Kramer multiple comparison procedure α= 0.01.

3.2.3.2 Fungal Som1 and Vta3 are required for sequential steps of plant root penetration and colonisation

Verticillium species belong to soil-borne ascomycete fungi which enter the host plant via the roots. Both, the *SOM1* and *VTA3,* deletion strains are avirulent strains, therefore, *Arabidopsis* root infection was examined to find out why the deletion strains of *SOM1* and *VTA3* are avirulent. Three weeks old *A. thaliana* roots were infected with the same number of spores of the wild-type, *SOM1*, or *VTA3* deletion strains expressing free *GFP* under the control of the *gdpA* promoter. The results shown in Figure 30, indicate that 24 h post inoculation (hpi), the germination of indicated strains is indistinguishable. The *VTA3* deletion strain shows the same potential as wild-type for the initial invasion of plant roots until 48 hpi, whereas the *SOM1* deletion strain is unable to form hyphopodia on the root surface. The plant root infection at 120 hpi revealed that the *SOM1* deletion strain is unable to penetrate the host and proliferate on the root surface. Hyphae of the *SOM1* deletion strain grow between root hairs but not on the root surface, whereas the *VTA3* deletion strain penetrates but is unable to proliferate to the same extend as the wild-type strain (Figure 30).

Hyphal-root interactions were further investigated by scanning electron microscopy at 72 hpi. The results shown in Figure 31, indicate that hyphae of the JR2-wt well colonise and can tightly bind to the root surface. Hyphae of the JR2-wt either enter plant roots directly or form hyphopodia, whereas *SOM1* and *VTA3* deletion strains are impaired in colonisation of root surfaces. The strains, carrying the deletion of *VTA3*, can still develop on root surfaces and forms hyphopodia, but its colonisation is reduced when compared to the wild-type strain (Figure 31).

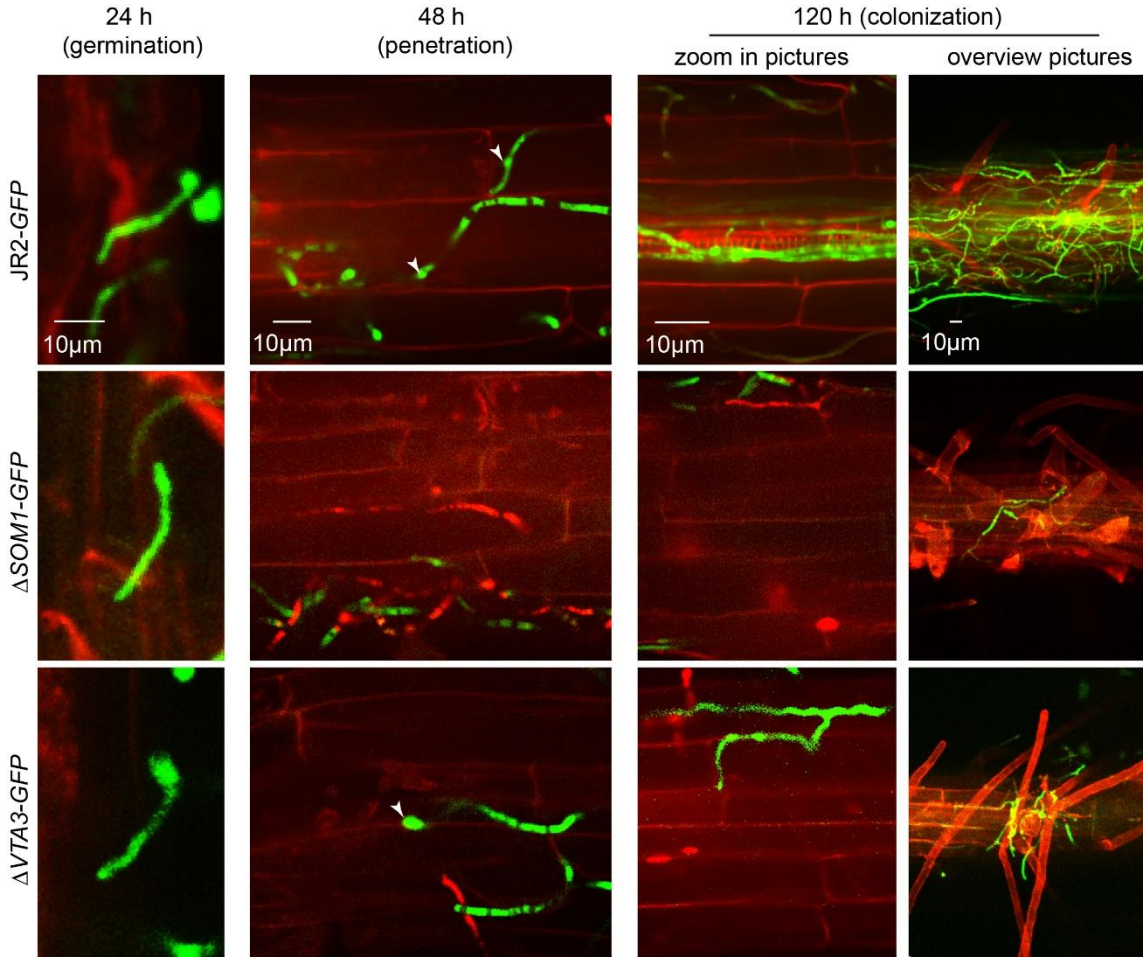


Figure 30. Fungal Som1 and Vta3 are sequentially required for plant root penetration and root colonisation. Three weeks old *Arabidopsis thaliana* plants were infected with the same number of spores of JR2-wt (JR2-*GFP*), *SOM1* deletion (Δ*SOM1-GFP*), and *VTA3* deletion (Δ*VTA3-GFP*) strains expressing free *GFP*. The germination, penetration, and colonisation of fungal hyphae on the root surface were observed after 24 h, 48 h, and 120 h. The experiment was performed with five plants for each fungal strain. The arrowheads indicate the penetration points. Scale bars are represented. The *SOM1* deletion strain is hardly detectable on plant roots. Only short fragments of hyphae could be detected on plant roots which were infected with the *SOM1* deletion strain (Figure 31). In summary, Som1 and Vta3 represent control factors for sequential steps of fungal penetration and colonisation on plant root surfaces.

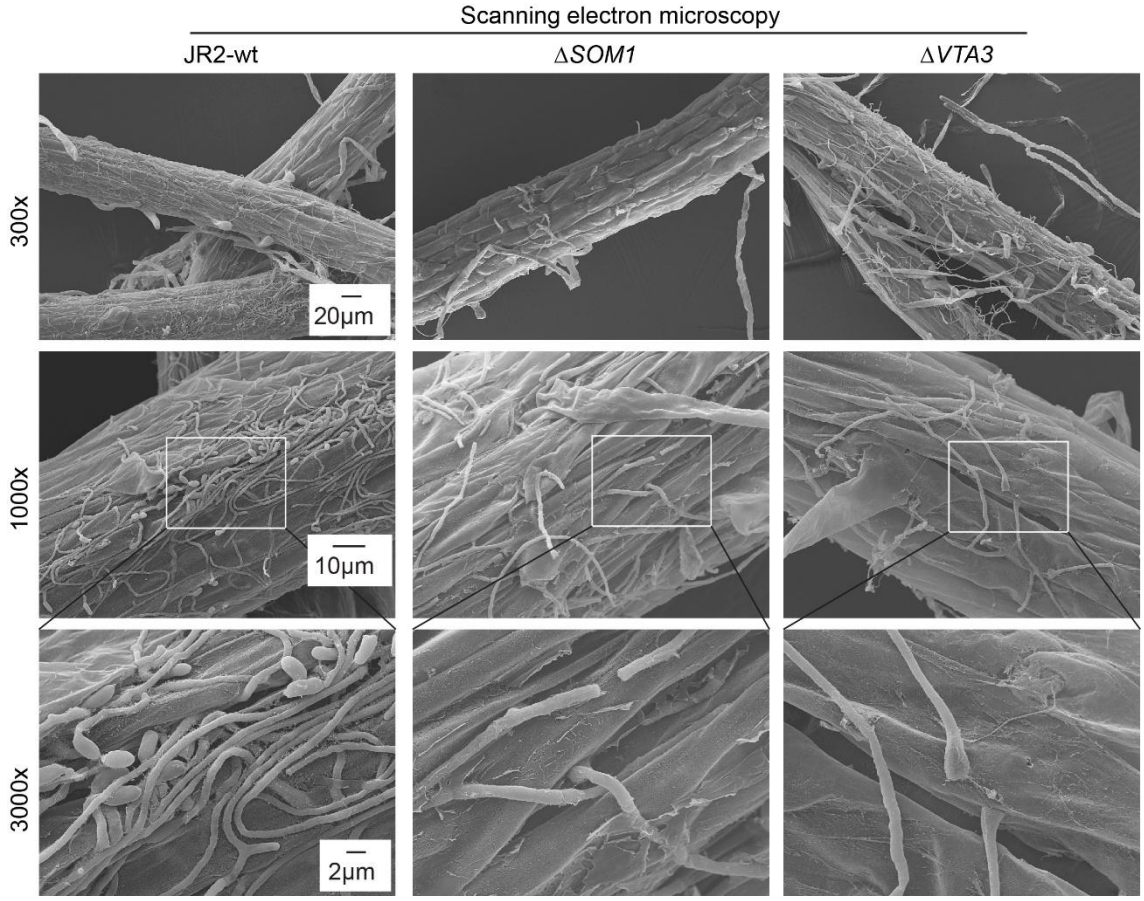


Figure 31. Som1 and Vta3 are required for successful root colonisation. Three weeks old *Arabidopsis thaliana* plants were infected with the same number of spores of JR2-wt, *SOM1* deletion, and *VTA3* deletion strains. The root infection was analysed by scanning electron microscopy at 72 hpi. Scale bars are represented.

3.2.4 Som1 and Vta3 support conidia and microsclerotia formation

Conidia and microsclerotia are important structures for the plant pathogen *V. dahliae*. Conidia are developed on forming phialides (Wilhelm, 1955; Schnathorst, 1982; Xiao *et al.*, 1998; Pegg & Brady, 2002). Each phialide has a mass of spores which is called a conidiospore cluster in the following. Conidia play a major role in the distribution of the pathogen in all plant cells and spreading fungus through water, whereas microsclerotia are important for the distribution of *V. dahliae* worldwide and for survival of fungi in the soil for up to 15 years. The effect of Som1 and Vta3 on conidia and microsclerotia formation was therefore further analysed.

3.2.4.1 Som1 and Vta3 promote conidia formation

The formation of spores in liquid medium and conidiospore clusters on agar plates was examined. Liquid medium was inoculated with the same number of spores and grown for seven days. The spores of each strain were separately counted and plated in Figure 32. The number of spores in the *SOM1* and *VTA3* deletion strains was significantly decreased by 92% and 59%, respectively (Figure 32a).

Conidia are formed at a specialised structure, called the conidiophore. Each conidiophore produces several phialides in which a number of conidia develop. This unit of conidia on a phialide, we called conidiospore cluster. The formation of these structures was tested on CDM plates (Figure 32b). The same number of spores of indicated strains was dropped on CDM plates and grown in the dark for three days. The formation of conidiospore cluster was observed with a binocular microscope. As seen in Figure 32b, the *SOM1* deletion strain has fewer conidiospore clusters than JR2-wt and complementation strains, whereas the number of conidiospore clusters was indistinguishable between the *VTA3* deletion strain and the wild-type. However, 60% of the conidiospore clusters in *VTA3* deletion strain had oval shapes (data not shown), whereas they were round in the wild-type and complementation strains (Figure 32b).

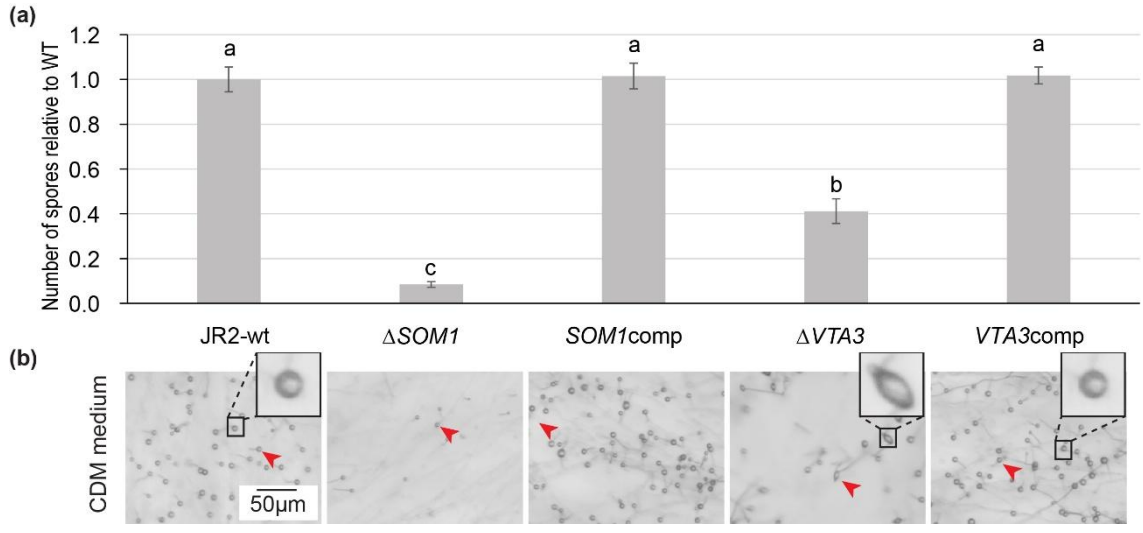


Figure 32. Som1 and Vta3 promote conidia formation. (**a**) The same number of spores of JR2-wt, *SOM1* deletion and complementation, and *VTA3* deletion and complementation strains were cultivated in 50 ml liquid SXM on a shaker at 25ºC for seven days. The spore numbers were counted. The mean values and standard deviations of triplicates are shown. (**b**) The formation of conidiospore clusters on agar plates was examined. The same spore numbers of indicated strains were dropped on CDM plates and grown at 25ºC for seven days. The conidiospore cluster density was investigated. The arrowhead indicates a conidiophore. Scale bars are shown.

3.2.4.2 Som1 and Vta3 control microsclerotia formation

Verticillium pathogens develop resting structures as melanized microsclerotia which help pathogens to persist in the soil for a decade even in the absence of plant hosts (Wilhelm, 1955; Tran *et al.*, 2013). The effects of Som1 and Vta3 on microsclerotia formation were examined on CDM plates containing 3% cellulose (Figure 33). The number of microsclerotia on the surface and in the agar was counted. The data presented in Figure 33a suggest that the *VTA3* deletion strain produces 60% less microsclerotia than the wild-type and the complementation strains, whereas the *SOM1* deletion strain is unable to form microsclerotia after seven days (Figure 33a).

Microsclerotia formation of JR2-wt and *SOM1* deletion strains was further investigated by scanning electron microscopy (Figure 33b). No microsclerotia structures were detected in the *SOM1* deletion strain, whereas they are easily found in the JR2-wt strain.

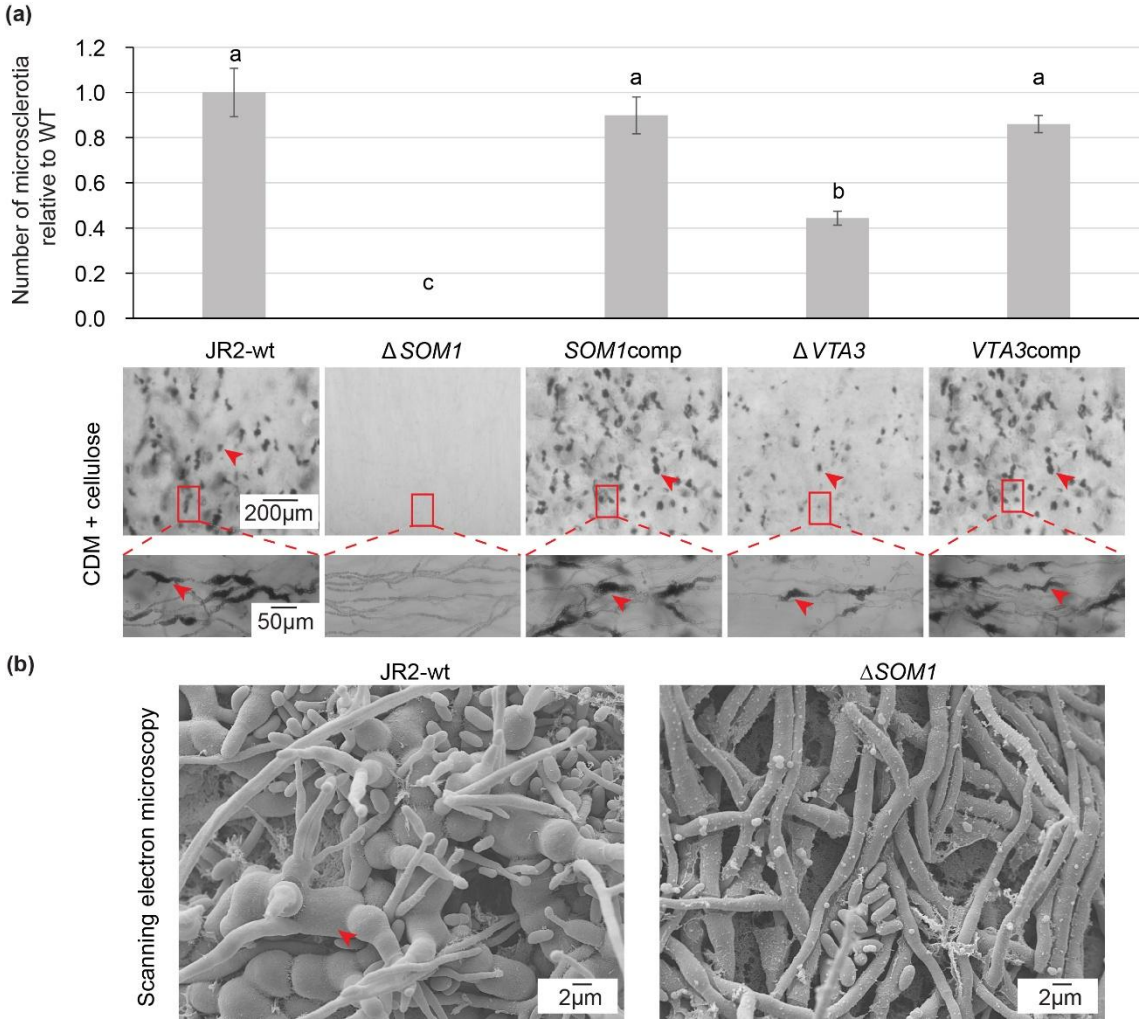


Figure 33. Som1 and Vta3 control microsclerotia formation. (**a**) Spores of the indicated strains were dropped on CDM plates containing 3% cellulose and incubated in the dark. The microsclerotia formation was observed by binocular microscopy. The arrowhead shows a microsclerotium. The number of microsclerotia was counted. Experiments were performed in triplicate. The mean values and standard deviation are shown. The letters a-c indicate the groups which are significant different as calculated by Tukey-Kramer multiple comparison procedures, α= 0.01. (**b**) The formation of microsclerotia of JR2-wt and the *SOM1* deletion strain at five days on SXM plates was examined by scanning electron microscopy. Scale bars are shown.

The formation of microsclerotia in the overexpression strain of *SOM1* was examined, as seen in Figure 34. The data indicate that the *V. dahliae* overexpression strain of *SOM1* produces microsclerotia earlier than the wild-type strain. The overexpression strain of *SOM1* produced microsclerotia and melanin, seen black on PDA plates after seven days while the colony of the wild-type strain was white (Figure 34a). The number of microsclerotia formed on CDM plate by the *SOM1* overexpression strain was also quantified. As seen in Figure 34b, the number of microsclerotia in this strain was significantly higher compared to the wild-type strain by 1.5 fold (Figure 34b).

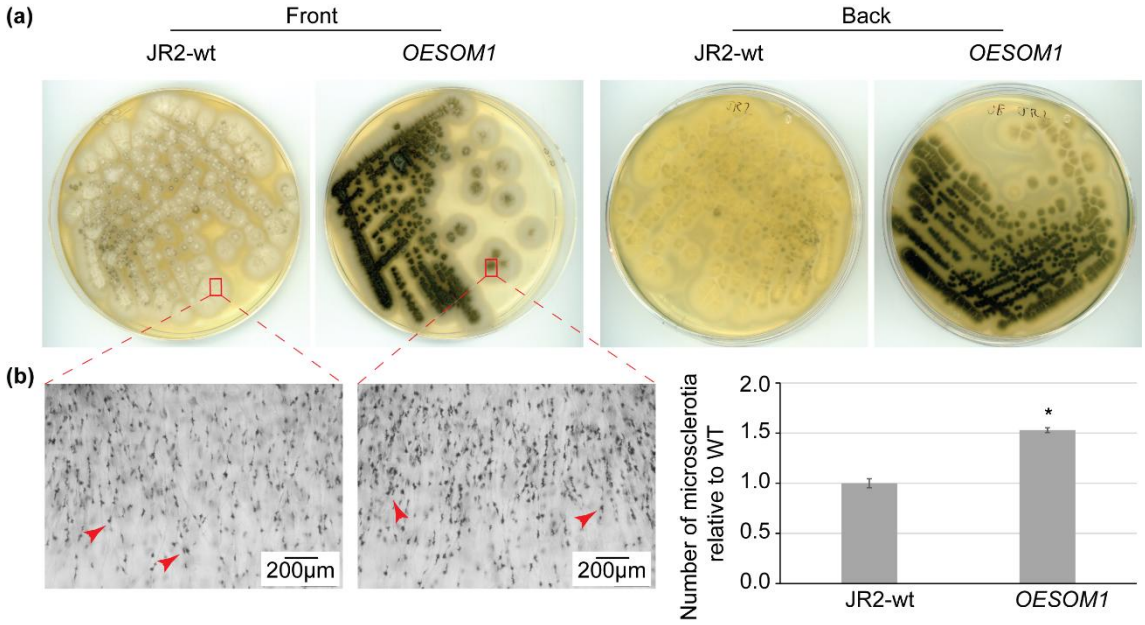


Figure 34. The overexpression of *SOM1* enhances the number of microsclerotia. (**a**) The overexpression of *SOM1* in *V. dahliae* JR2 shows earlier melanin production on PDA plates. The spores of the JR2-wt and the overexpression of *SOM1* strains were spread on PDA plates and grown in the dark at 25°C for seven days. The formation of microsclerotia of the *SOM1* overexpression was examined. (**b**) The same spore number of indicated strains was dropped on CDM plates containing 3% cellulose and grown in the dark at 25°C for seven days. The experiment was performed in triplicate. The microsclerotia number was counted after seven days. The mean values and standard deviations are shown. The asterisk (*) indicates a significant increase of microsclerotia in the *SOM1* overexpression when compared to wild-type strains (Student's t-test, $p<0.05$). The arrowhead indicates a microsclerotium. Scale bars are displayed.

3.2.5 Som1 and Vta3 antagonise in oxidative stress response

Plants can generate reactive oxygen species (ROS) as hydrogen peroxide (H_2O_2) to ward off invading microbial pathogens. Pathogenic fungi need to develop defense systems against the plant immune system. The ability of the *SOM1* and *VTA3* deletion strains to grow in the presence of exogenous H_2O_2 and menadione-induced stress was analysed by measuring the diameter of the inhibition zone around filter papers containing oxidative stress inducing agents (Figure 35). The inhibition zone of the *SOM1* deletion strain was significantly increased, whereas that of the *VTA3* deletion strain decreased when compared to wild-type in both stress tests (Figure 35). This result suggests that Som1 is required for an appropriate stress response, whereas Vta3 inhibits this reaction.

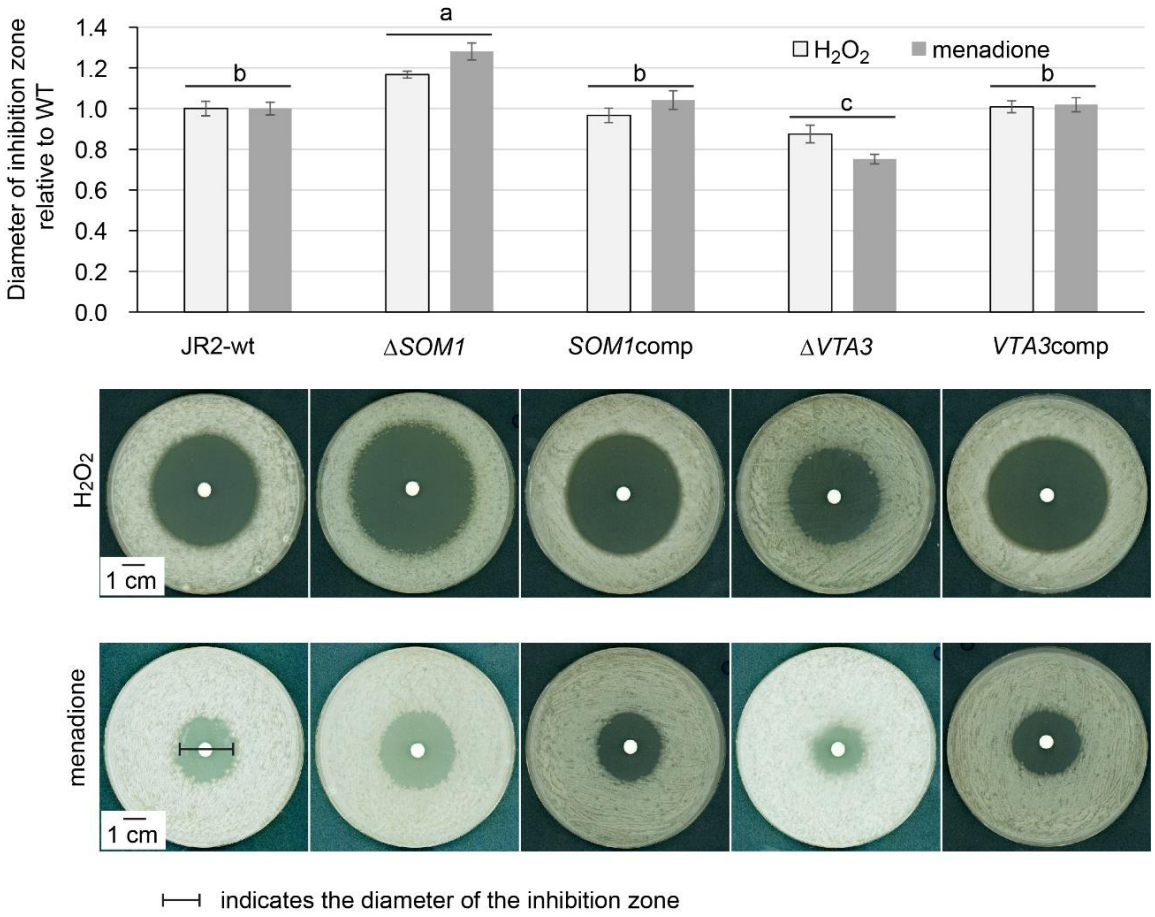


Figure 35. Som1 and Vta3 antagonise in oxidative stress response. The same number of spores of JR2-wt, *SOM1* deletion and complementation, and *VTA3* deletion and complementation strains was spread on PDM plates and a filter paper disc containing 10 µl H_2O_2 10% or menadione 100 µg/µl were placed in the center of plates. Plates were incubated in the dark for four days at 25°C. The diameter of the inhibition zone was measured. The experiment was performed in triplicate. Mean values and standard deviations are shown. The letters a-c indicate groups which are significant different as calculated by Tukey-Kramer multiple comparison procedures, α= 0.01. Scale bars are represented.

3.2.6 Som1 and Vta3 are needed for hyphal growth of *V. dahliae* on agar plates

The growth of *SOM1* and *VTA3* deletion strains was examined in various media including different carbon source media (Figure 36). In general, both deletion strains of *SOM1* and *VTA3* grow significantly slower than the wild-type and complementation strains in all tested media (Figure 36). However, the growth of *SOM1* and *VTA3* deletion strain was similarly slower on glucose-containing medium, whereas significant differences in the growth of *SOM1* deletion strain compared to *VTA3* deletion strain was observed in all other carbon sources. Additionally,

the growth of the *SOM1* deletion strain depends on the carbon source. The deletion of *SOM1* grows faster in glucose-containing medium, whereas it develops very slowly in the medium containing galactose (Figure 36). In summary, Som1 and Vta3 are essential for hyphal growth on agar plates and Som1 is required for growth in galactose-containing medium.

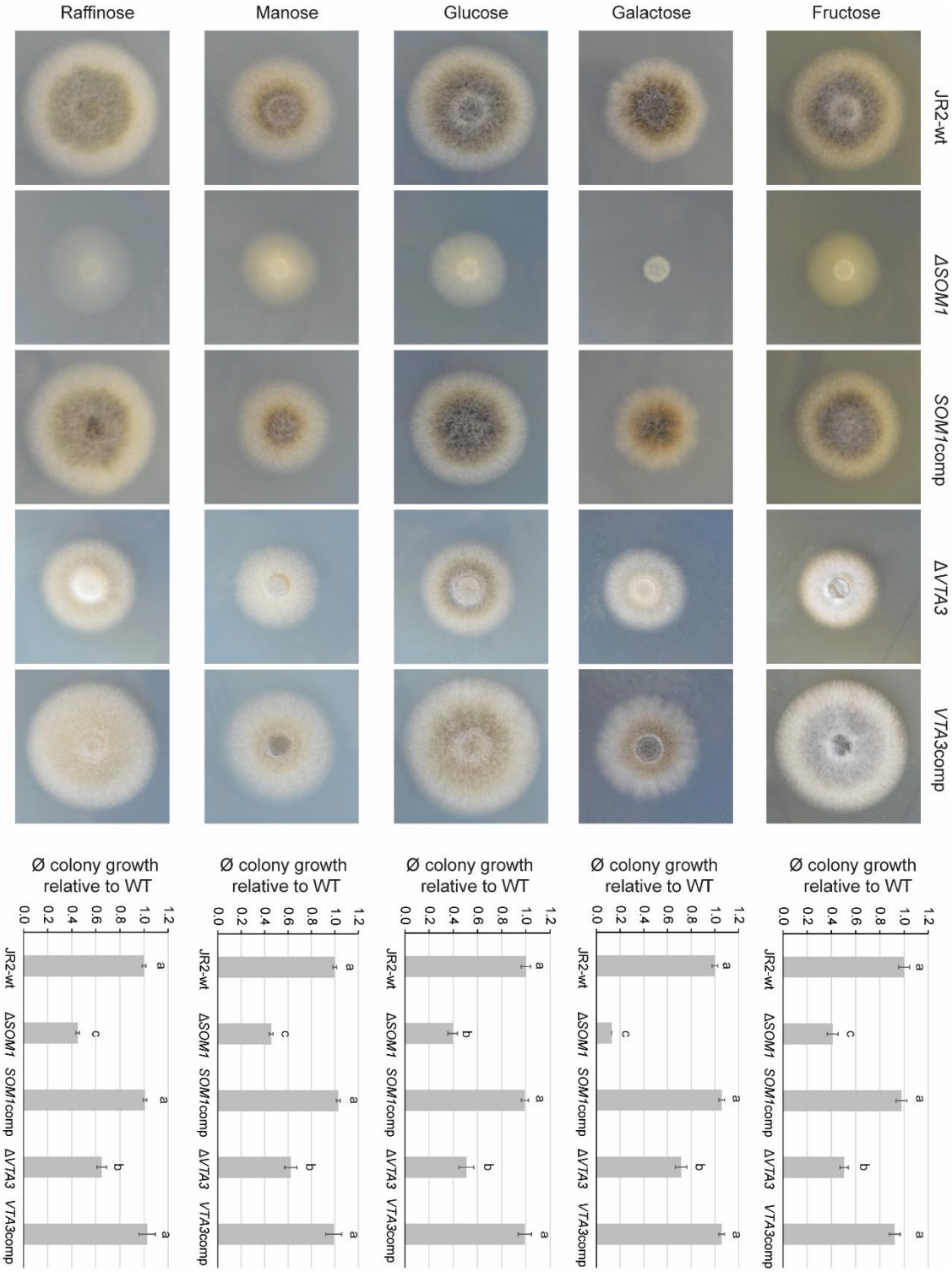


Figure 36. Som1 and Vta3 affect growth on different carbon sources. Czapek Dox agar was used for growth tests with different carbon sources as indicated (3%). The same number of spores of indicated strains was dropped on agar plates and incubated in the dark at 25°C. The diameter of the colony was measured after 11 days post inoculation. The mean values and standard deviations from three repetitions are displayed. The letters a-c are groups which are significantly different as calculated by Tukey-Kramer multiple comparison procedures, α= 0.01.

3.2.7 Som1 is essential for hyphal development in *V. dahliae*

Adhesion of cells to a surface and the clumping of hyphae in liquid medium might represent two aspects of hyphal development. For a more detailed analysis, the JR2-wt and *SOM1* deletion and complementation strains were grown on agar plates and in liquid medium, and the hyphal development was further investigated.

The wild-type, deletion of *SOM1*, and complementation strains were grown on agar plates and in liquid SXM without shaking. The formation of aerial hyphae was investigated, as shown in Figure 37. Aerial hyphae are easily observed in the wild-type and complementation strains either on agar plates or in standing liquid SXM, whereas they are hardly detected in the *SOM1* deletion strain (Figure 37).

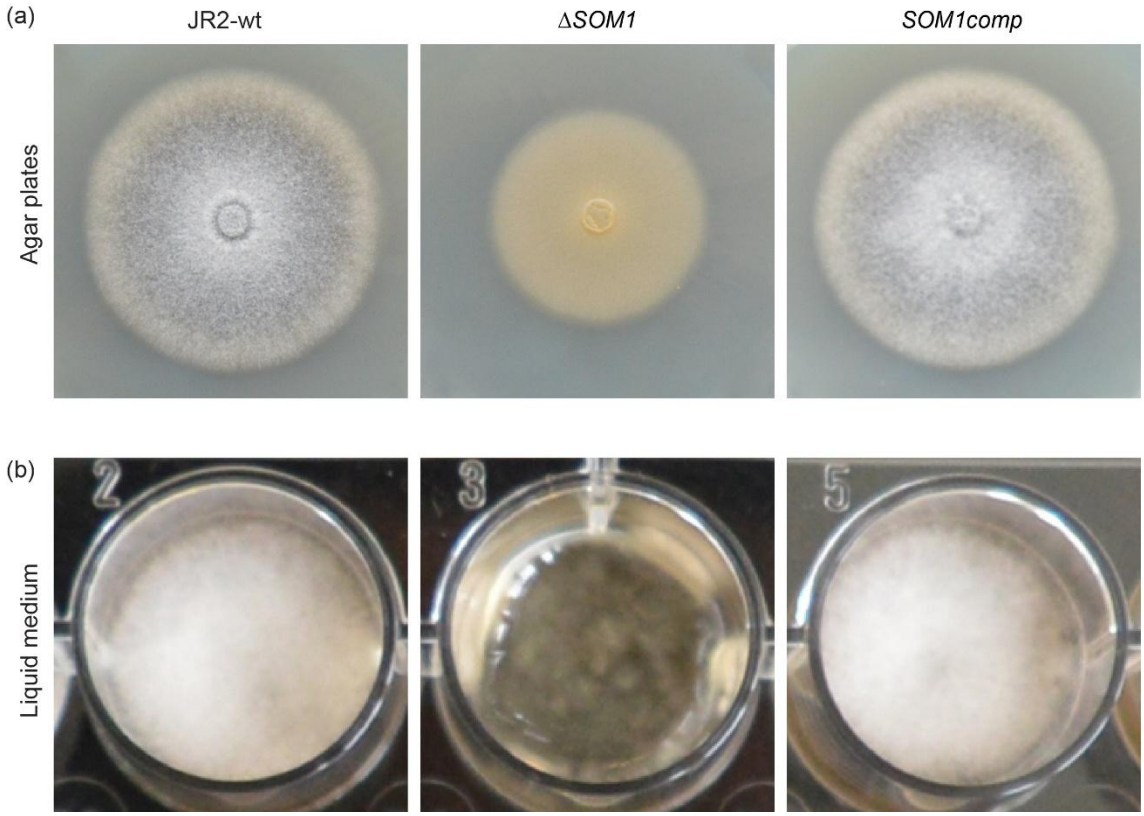


Figure 37. Som1 is necessary for aerial hyphae formation. (**a**) The same number of spores of the wild-type, *SOM1* deletion, and complementation strains was dropped on agar plates and incubated in the dark at 25°C for seven days. (**b**) The indicated strains were grown in standing liquid SXM for seven days.

As mentioned in chapter 3.2.2.1, the biomass of the *SOM1* deletion strain is higher than that of the wild-type and it grows slower on agar plates. Hyphal branching might be affected in the deletion of *SOM1*. Therefore, the branching of hyphae was further examined (Figure 38). As shown in Figure 38a, hyphal branches were observed more often in the *SOM1* deletion strain than in the wild-type on agar

plates. The formation of hyphal branches was further quantified by growing hyphae in devices with multiple channels (Stanley *et al.*, 2014). The formation of hyphal branches was observed with a microscope. As seen in Figure 38b, the *SOM1* deletion strain has on average eight branches whereas wild-type and complementation strains have only two branches in 750 µm hyphae measured from the hyphal tips (Figure 38b). Taken together, Som1 is required for hyphal branching.

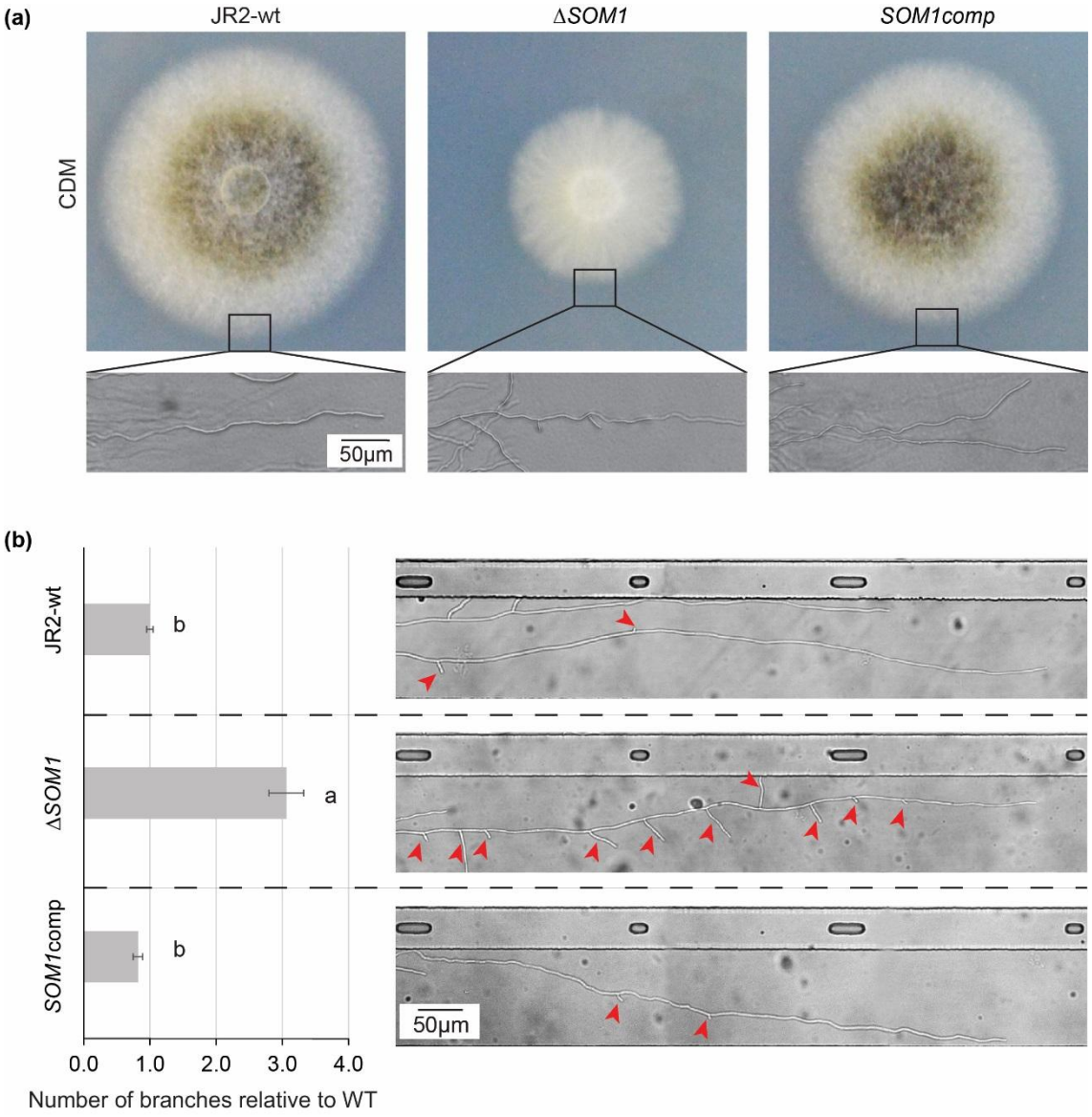


Figure 38. Som1 is required for aerial hyphae and hyphal branching of *V. dahliae*. (**a**) The same number of spores of JR2-wt, the *SOM1* deletion, and complementation strains was dropped on CDM plates and grown in the dark at 25°C for ten days. The development of aerial hyphae and hyphal branching was observed on the surface of plates. (**b**) Quantification of hyphal branches. Single hyphae of indicated strains were grown in microchannels. Arrowheads indicates hyphal branches. The number of branches in 750 µm hyphae was counted. The experiment was performed in triplicate. Average values and standard deviation are indicated. The letters a and b indicate the groups which are significantly different calculated by Tukey-Kramer multiple comparison procedures, α= 0.01. The arrowhead shows a hyphal branch. Scale bars are displayed.

Cell morphology was also examined of the *SOM1* deletion strain grown in liquid SXM for 24 h. Cell walls and septa were stained with congo red and observed by a fluorescence microscope. The data in Figure 39a suggests that the *SOM1* deletion strain has more septa than the wild-type in elongating hyphae. The distance between septa of the *SOM1* deletion strain is approximately 50% shorter than that of the wild-type and complementation strains (Figure 39b).

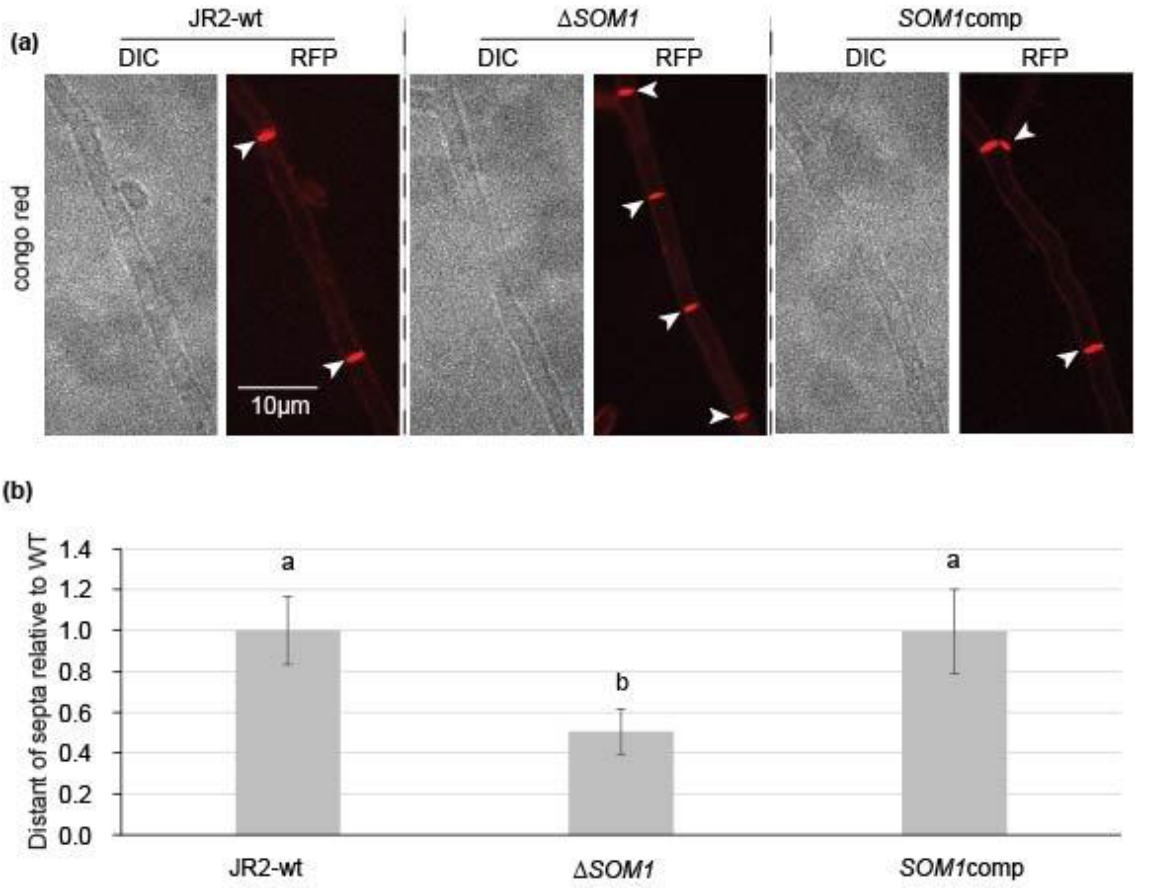


Figure 39. Som1 is required for septa positioning in *V. dahliae*. Spores of JR2-wt and *SOM1* deletion and complementation strains were grown in SXM containing cefotaxime at 25ºC for 24 h. The pictures were taken by fluorescent microscopy with DIC and RFP filters. Septa and cell walls were stained with congo red. The distance between septa was measured (**a**) Septa positioning. White arrowheads indicate septa. (**b**) The diagram of septa distance in elongating hyphae. The experiments were performed in triplicate. One hundred cells were observed for each repetition. Mean values and standard deviation are represented. The letters a and b indicate groups which are significantly different as calculated by Tukey-Kramer multiple comparison procedures, α= 0.01. Scale bars are shown.

Vacuoles in hyphae were dyed with FM4-64 and observed with a fluorescence microscope (Figure 40a). The diameter of vacuoles was also quantified as shown in Figure 40b. The vacuoles of *SOM1* deletion strain were smaller than that of the wild-type strain. The diameter of vacuoles in the deletion strain was reduced by 60% compared to that of the wild-type strain. The vacuoles in wild-type are round and massive, whereas they are small and as dot-like structures in the *SOM1* deletion strain (Figure 40b). In summary, Som1 is necessary for hyphal development including aerial hyphae formation, hyphal branching, septa positioning, and vacuole size.

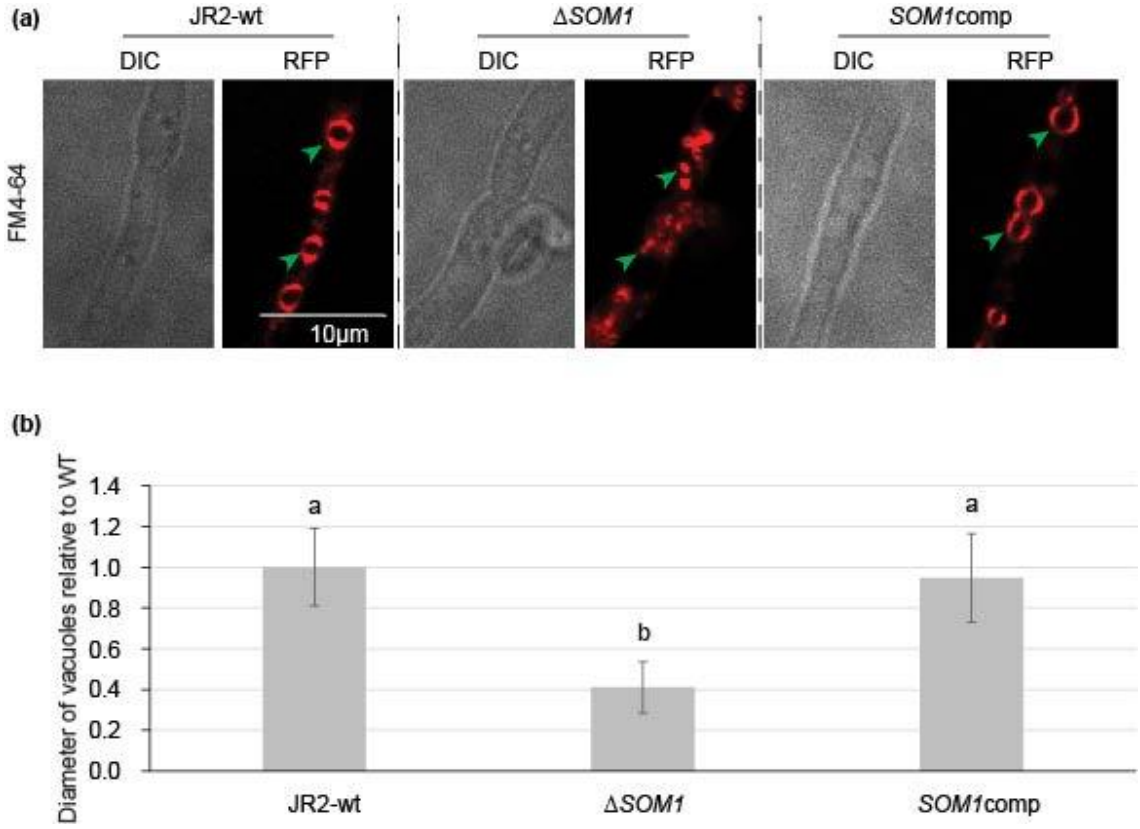


Figure 40. *V. dahliae* Som1 is essential for normal vacuole size. Spores of JR2-wt and *SOM1* deletion and complementation strains were grown in SXM containing cefotaxime at 25ºC for 24 h. The pictures were taken by fluorescence microscopy with DIC and RFP filters. The vacuoles were dyed by FM4-64. The diameter of vacuoles was determined. (**a**) Stained vacuole in *V. dahliae*. The green arrowhead shows a vacuole. (**b**) Diagram of vacuole diameter. The experiments were performed in triplicates. One hundred cells were observed for each repetition. Mean values and standard deviation are shown. The letters a and b indicate groups which are significantly different as calculated by Tukey-Kramer multiple comparison procedures, α= 0.01. Scale bars are displayed.

3.2.8 Som1 and *VTA3* regulate the expression of *VTA* genes and related adhesion, conidia and microsclerotia formation, and virulence genes

Transcription factors for adhesion can reprogram adhesion in non-adhesive *FLO8*-defective *S. cerevisiae* strains. Therefore, Som1 might directly or indirectly regulate *VTA* genes. Additionally, the phenotypes are usually regulated by several genes. The expression of transcription factors for adhesion and genes which are involved in adhesion, conidiation, microsclerotia formation, oxidative stress response, and virulence was examined by real-time PCR in wild-type and the deletion strain.

3.2.8.1 Som1 and Vta3 regulate the expression of *VTA* genes

Verticillium transcription activator of adhesion (*VTA*) genes can rescue *FLO8*-defected yeast strains as shown before (Tran *et al.*, 2014). Som1 is a ortholog of Flo8 and therefore might regulate the expression of *VTA* genes in *Verticillium*. To test this hypothesis, the expression of six *VTA* genes was examined in *SOM1* and *VTA3* deletion strains (Figure 41). The expression of *VTA1*, *VTA2*, *VTA3*, *VTA4*, and *VTA6* was significantly reduced in the deletion strain of *SOM1.* Interestingly, the expression of *VTA2* and *VTA3* is reduced by 90% and 94% in the *SOM1* deletion strain. In the *VTA3* deletion strain, the expression of *VTA1*, *VTA2*, and *VTA6* was significantly decreased, whereas the expression of *VTA4* and *VTA5* was unchanged (Figure 41). In summary, Som1 controls the expression of *VTA3*, and both, Som1 and Vta3, regulate the expression of *VTA1*, *VTA2*, and *VTA6* genes.

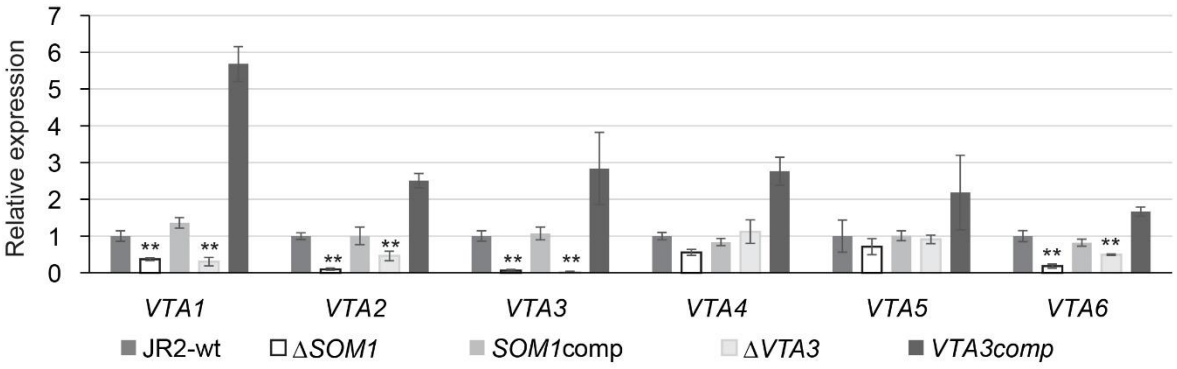


Figure 41. Som1 and Vta3 control the expression of *VTA* genes. The RNAs were purified from three days old mycelia grown in 50 ml liquid SXM. The same amount of RNA was used for cDNA synthesis. The expression of putative target genes was normalized to a housekeeping gene (GAPDH) and the wild-type strain. The mean values and standard deviations of four repetitions are represented. The asterisk (**) indicates a significant decrease when compared to the JR2-wt as calculated by Student's t-test, $p<0.01$.

3.2.8.2 Som1 control expression of genes involved in adhesion

Putative adhesion proteins are usually localised on the cell surface (Sundstrom, 2002). The deletion strain of *SOM1* grown in liquid MM defects in adhesion on abiotic surfaces (Figure 27). Som1 might control the expression of some adhesive factors. Therefore, cell lysate protein of JR2-wt and *SOM1* deletion and complementation strains, which were grown in liquid MM, was isolated and further analysed in 12% SDS gel. Each band was separately digested with trypsin in gels. Peptides from each sample were independently evaluated by mass spectrometry and examined by the MaxQuant program. Proteins, which is significantly different in abundance, are shown in Table 4. The data show that twenty proteins were not found or rarely detected in the *SOM1* deletion strain, whereas they are abundant in the wild-type and complementation strains (Table 4).

Table 4. List of abundant proteins significantly changed in the *SOM1* deletion strain

1	2	3	4	5	6	7	8	9
G2WW49	Fatty acid synthase S-acetyltransferase	24.7	24.5	1.0	26.3	86.3	0.3	303.7
G2XHA0	Serine 3-dehydrogenase	24.2	25.9	1.0	24.9	120.7	0.5	63.5
G2XG83	YcaC	22.7	22.7	1.0	24.1	20.7	0.0	29.5
G2XK31	Sulfite oxidase	22.2	22.6	1.0	23.5	31.0	0.2	47.7
G2XH97	Putative uncharacterized protein	20.8	22.9	1.0	21.3	35.0	0.0	12.8
G2X049	NADPH dehydrogenase	20.5	21.3	1.0	21.6	8.3	0.0	10.5
G2XH96	Putative uncharacterized protein	20.4	22.4	1.0	20.8	28.7	0.0	8.0
G2XJ01	Reticulon-4-interacting protein	20.2	20.5	1.0	21.5	7.7	0.0	13.8
G2WS45	Nitrate reductase	19.2	20.4	1.0	20.0	9.3	0.2	4.7
G2WZ45	Putative uncharacterized protein	18.9	19.9	1.0	19.9	6.3	0.0	5.5
G2XAR8	HpcH/HpaI aldolase/citrate lyase family protein	18.0	22.0	4.0	21.9	15.7	0.5	12.3
G2X086	Tetrahydroxynaphthalene reductase	17.7	13.5	1.0	21.4	6.3	0.0	19.2
G2X5M1	Short-chain dehydrogenase/reductase SDR	16.0	19.6	3.9	20.1	4.0	1.0	6.3
G2XFZ6	Acyl-CoA desaturase	14.4	19.7	3.9	17.5	3.3	0.3	9.7
G2X1C0	Cap20 protein	13.3	19.7	7.1	20.7	4.7	1.8	8.0
G2X9D9	Ribitol kinase	13.3	19.5	6.8	20.3	3.7	0.8	5.5
G2X8Q8	SnodProt1	11.5	20.5	9.7	21.5	4.0	1.3	6.0
G2XGN9	ThiJ/PfpI family protein	11.4	20.5	6.9	17.3	8.7	1.7	5.5
G2X061	Alpha/beta hydrolase	10.5	19.9	7.1	16.5	6.0	2.5	5.5
G2X1B6	Ribonucleoside-diphosphate reductase	10.4	20.1	9.9	20.3	7.3	1.3	9.5

The same amount of cell lysate was run in 12% SDS-PAGE and digested with trypsin. The protein list was identified by LC-MS/MS and MaxQuant analysis and further processed with Perseus. Contaminants, and log2 (fold change) lower than 10 were removed from the final analysis. Columns: 1-accession number, 2-cellular function, 3-log2 (fold change), 4-mean of log2 (LFQ intensity of JR2-wt), 5-mean of log2 (LFQ intensity of Δ*SOM1*), 6-mean of log2 (LFQ intensity of *SOM1*comp), 7-mean of MS/MS count of JR2-wt, 8-mean MS/MS count of Δ*SOM1*, 9-mean of MS/MS count of *SOM1*comp. The table summarized the results from three independent biological repetitions and three technical replicates.

The twenty proteins identified were grouped into three clusters according to their function: cell rescue, defence and virulence (10%), metabolism (70%), and unknown function (20%). The clusters are shown in Figure 42. The cluster cell rescue, defence and virulence included the proteins Cap20 and Snod1.

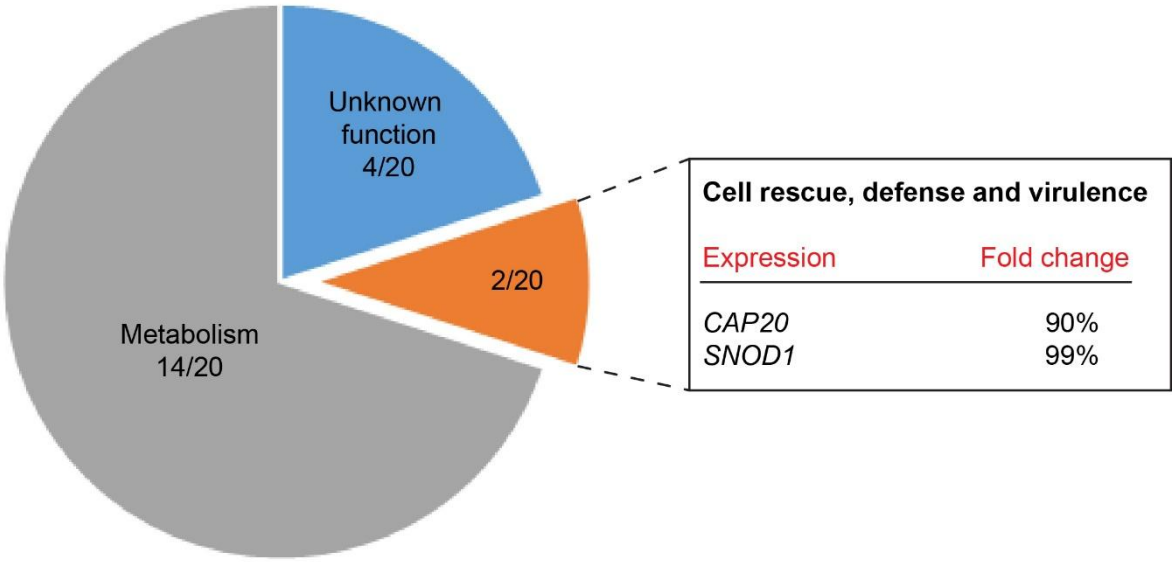


Figure 42. Functional categorisation analyses of twenty proteins significantly down-regulated in the *SOM1* deletion strain. The functional categorisation of proteins was done by using the Funcat version 2.0 (Ruepp *et al.*, 2004). Main groups of protein functions are indicated. The expression of genes was confirmed by real-time PCR. The expression was normalized to a housekeeping gene (GAPDH) and the wild-type. The fold changes are shown.

The expression of genes required for adhesion was investigated by real-time PCR as seen in Figure 43. The expression of *FAS1*, *MAD1*, *MAD2*, *CAP20*, *SNOD1*, and *PLS1* was significantly reduced in the *SOM1* deletion strain by 80%, 94%, 90%, 84%, 87%, and 98%, respectively (Figure 43). Taken together, Som1 promotes adherence via controlling the expression of putative target genes for adhesion.

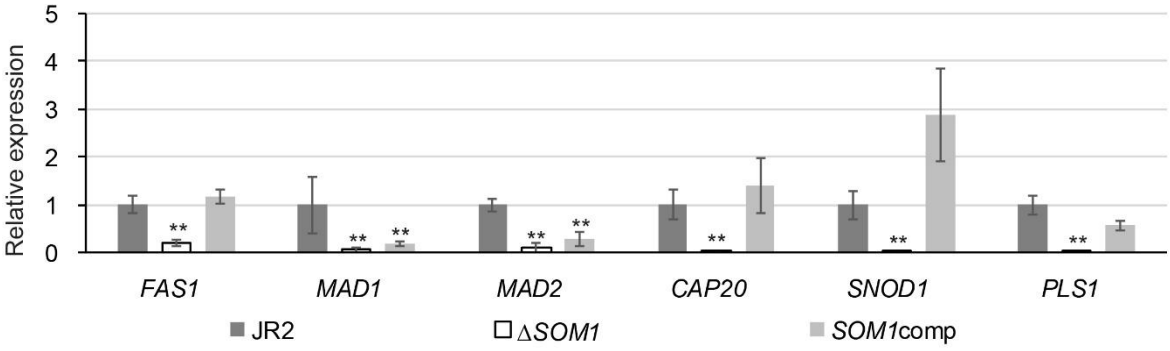


Figure 43. Som1 controls the expression of putative adhesion target genes. The RNAs have been purified from three days old mycelia grown in 50 ml SXM. The same amount of RNA was used for cDNA synthesis. The expression of putative target genes was normalized to a housekeeping gene (GAPDH) and the wild-type strain. The mean values and standard deviations of four repetitions are represented. The asterisk (**) indicates a significant decrease when compared to the JR2-wt as calculated by Student's t-test, p<0.01.

3.2.8.3 Som1 and Vta3 control expression of genes involved in conidia and microsclerotia formation, oxidative stress response and virulence

Som1 and Vta3 are required for conidia and microsclerotia formation (Figure 32, 33, 34), and virulence (Figure 28, 29, 30, 31), antagonised the oxidative stress response (Figure 35) and adhesion (Figure 26, 27), therefore, the expression of genes involved in these processes was investigated. The results are represented in Figure 44.

Five genes involved in conidia formation were investigated, namely *VTA2*, *CON-6*, *CON-8*, *CON-10*, and *ABA1.* The expression of genes involved in conidia formation was significantly reduced in both deletion strains. The transcript levels of *VTA2*, *CON-6*, *CON-8*, *CON-10*, and *ABA1* in the *SOM1* deletion strain were reduced by 90%, 64%, 70%, 92%, and 98% compared to the wild-type, respectively (Figure 41, 44a). Reduced transcript levels were also observed for *CON-8* and *ABA1* in the *VTA3* deletion strain corresponding to 70% and 57% decrease, respectively (Figure 44b).

Significant decrease in the expression of *VDH1*, required for microsclerotia formation, was also observed in *SOM1* and *VTA3* deletion strains when compared to the wild-type corresponding to 95% decrease. The expression of genes involved in oxidative stress response was also examined. While the expression of genes *VEL1*, *CPX2* and *SOD3* was significantly decreased in the *SOM1* deletion strain by 93%, 96% and 94%, respectively, the expression of *INO1* in the *VTA3* deletion strain was significantly enhanced.

Furthermore, the expression of genes which are putatively involved in virulence was significantly lowered in the deletion strains of *SOM1* and *VTA3*. The expression of *VTA2*, *CAP20*, *SNOD1*, *NLP2*, and *PRY1* was significantly reduced in the *SOM1* deletion strain by 90%, 90%, 99%, 71%, and 99%, respectively (Figure 41, 43, 44a). The expression of *VTA2*, *CAP20*, *NLP2,* and *PRY1* was significantly decreased in *VTA3* deletion strain by 54%, 60%, 67%, and 83% (Figure 41, 44b), whereas expression of *SNOD1* is unchanged. Taken together, Som1 and Vta3 control the expression of genes which might be involved in the control of conidia and microsclerotia formation, oxidative stress response, and virulence.

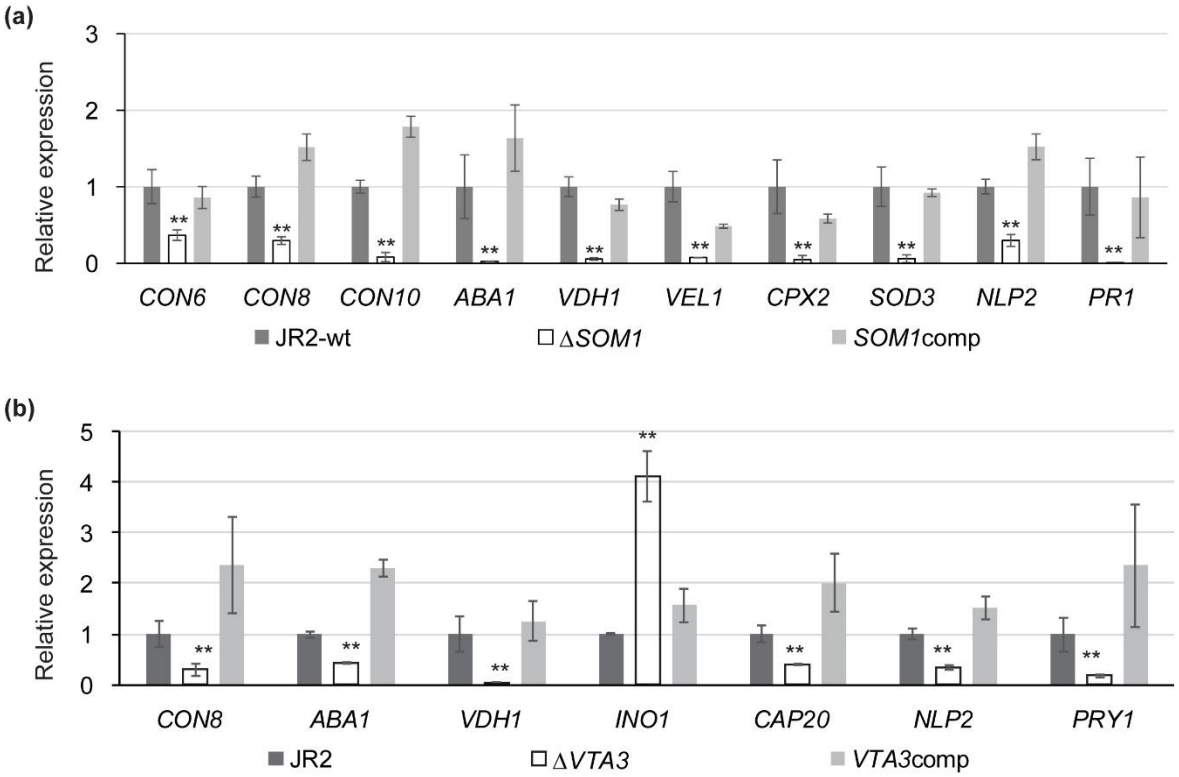


Figure 44. Som1 and Vta3 control the expression of putative target genes of conidia and microsclerotia formation, oxidative stress response, and virulence. The RNAs were purified from three days old mycelia grown in 50 ml SXM. The same amount of RNA was used for cDNA synthesis. The expression of putative target genes was normalized to a housekeeping gene (GAPDH) and the wild-type strain. The mean values and standard deviations of 4 repetitions are represented. (**a**) Som1 regulates the expression of putative target genes for conidiation, microsclerotia formation, oxidative stress response, and virulence genes. (**b**) Vta3 controls the expression of assumed target genes for conidia and microsclerotia formation, stress response, and virulence. The asterisk (**) indicates a significant decrease when compared to the JR2-wt as calculated by Student's t-test, $p<0.01$.

3.2.8.4 Som1 interacts with protein Ptab while Vta3 interacts with the transcriptional co-repressor Cyc8

Often proteins interact with other protein to fulfil their functions (Phizicky & Fields, 1995). The interaction partner of Som1 and Vta3 were investigated by pull- down assays and the results are displayed in Table 5 and Table 6. The Table 5 shows seven proteins which are only found in the Som1:GFP sample (Table 5). In which, Ptab was shown with high log2 (LFQ intensity) and high MS/MS count and appeared in all replication. Ptab is a homolog of Mfg1 in *S. cerevisiae* which is reported to be an interaction partner of Flo8.

Table 5. Som1 putative interaction partners identified by GFP-trap enrichment

1	2	3	4	5	6
G2X5W3	Som1 protein	17.2	18.2	6.4	6/6
G2X127	Ptab protein	12.1	7.6	2.8	6/6
G2X461	Tyrosinase	7.2	1.9	1.4	4/6
G2WW49	Fatty acid synthase S-acetyltransferase	7.2	20.9	13.9	3/6
G2XK31	Sulfite oxidase	7.0	5.0	3.2	4/6
G2WS88	Glutathione S-transferase Gst3	6.1	3.1	2.4	3/6
G2X411	26S protease regulatory subunit 4	4.7	1.1	0.9	3/6

Proteins, identified by LC-MS/MS after enrichment of *SOM1:GFP* by GFP-trap, were examined. Contaminants and proteins shown to interact with free GFP were filtered out. Proteins in this list were only found in the *SOM1*:*GFP* at least five times of log2 (intensity) higher than 11. Columns: 1-protein IDs, 2-cellular function, 3-mean of log2 (LFQ intensity), 4-mean of MS/MS count, 5-min unique peptides, 6-appearance times. The table summarized the results from three independent biological pull downs and two technical replicates.

Table 6 displays five proteins which are only detected in the Vta3:GFP sample (Table 6). The Cyc8 protein was identified in the list of protein enriched by Vta3::GFP. Transcriptional co-repressor Cyc8 is a ortholog of Ssn6 in *S. cerevisiae* which was shown to be a interaction partner of Rfx1 and of SsnF which had been described in *A. fumigatus* (Huang *et al.*, 1998; Johnk *et al*., 2016). Taken together, Som1 might interact with six proteins including Ptab while Vta3 might physically bind to four proteins including Ssn6.

Table 6. Vta3 putative interaction partners identified by GFP-trap enrichment

1	2	3	4	5	6
G2WUC4	Sak1	15.4	26.5	16.0	9/9
G2WR58	Inositol-3-phosphate synthase	6.6	5.0	8.0	3/9
G2XH18	H/ACA ribonucleoprotein complex subunit 1	5.1	2.4	2.0	3/9
G2WXX5	Sulfate adenylyltransferase	5.1	1.8	4.0	4/9
G2XAE9	Transcriptional co-repressor Cyc8	4.1	1.3	5.0	3/9

Proteins, identified by LC-MS/MS analyses after enrichment of *VTA3:GFP* by GFP-trap, were investigated. Contaminants and proteins shown to interact with free GFP were filtered out. Proteins in this list consist of only proteins found in the *VTA3*:*GFP* at least three times of log2 (intensity) higher than 11. Columns: 1-protein IDs, 2-cellular function, 3-mean of log2 (LFQ intensity), 4-mean of MS/MS count, 5-mean unique peptides, 6-appearance times. The table summarized the results from three independent biological pull downs and three technical replicates.

3.3 *A. fumigatus* SomA can rescue the deletion of *SOM1* in *V. dahliae*

Alignment of the plant pathogen *V. dahliae SOM1* sequence and the opportunistic pathogen *A. fumigatus AfSOMA* shows 31% similarity. These proteins fulfill similar functions in different pathogenic fungi such as in adhesion, conidiation, aerial hyphae formation, and virulence (Lin *et al.*, 2015). GFP trap results indicate that Som1 physically interacts with Ptab (Table 5) as AfSomA in the human pathogenic fungus (Lin *et al.*, 2015). It was examined whether Som1 fulfills similar functions as AfSomA in *A. fumigatus* and/or *vice versa*. The ORF of *AfSOMA* under the control of the *gdpA* promoter was transformed into the *SOM1* deletion strain (Table 3). The results of the phenotype analysis indicate that AfSom1 can rescue growth defects of the *SOM1* deletion strain. The strain expressing *AfSOMA* grows faster than the deletion strain, but it still grows slower than the wild-type strain. Its growth can reach approximately 77% of the wild-type strain (Figure 45). Additionally, AfSom1 can rescue the formation of aerial hyphae of deletion strain. The colony of the strain expressing of *AfSOMA* appears with the black ring and has aerial hyphae. Therefore, it might restore conidia and microsclerotia formation.

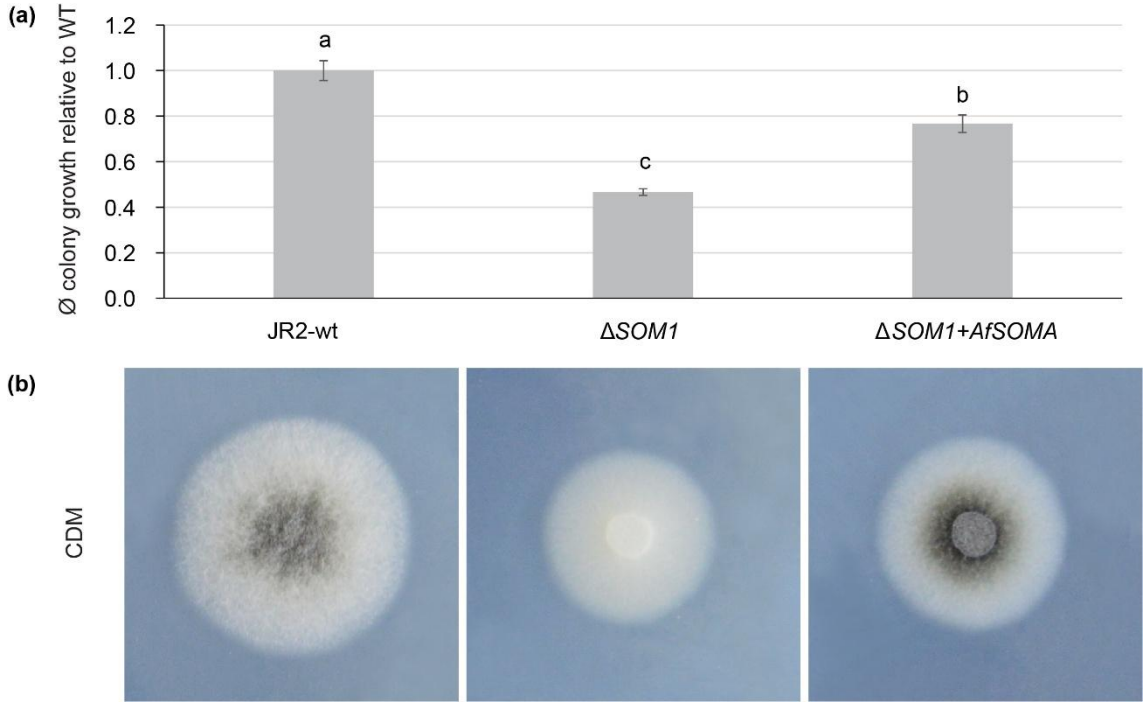


Figure 45. AfSomA can partly rescue the growth of the *SOM1* deletion strain. The same spore numbers of the wild-type, *SOM1* deletion, *AfSOMA* complementation strains were dropped on agar plates and incubated in the dark at 25°C for 11 days. (**a**) The diagram of the diameter of the colony at 11 dpi. (**b**) The colony growth on CDM plate at 11 dpi. The experiments were performed in triplicate. The mean values and standard deviations are shown. The letters a-c indicate groups which are significantly different as calculated by Tukey-Kramer multiple comparison procedures, α= 0.01.

The data of conidia and microsclerotia formation test indicate that AfSomA can rescue both, conidia and microsclerotia formation. Conidia formation of the wild-type and strain expression of *AfSOMA* are indistinguishable, whereas that of deletion strain is significantly reduced by 92% (Figure 46a). The formation of microsclerotia of a strain expressing of *AfSOM1* is not different than that of the wild-type strain. Microsclerotia are easy detected in strain expression of AfSomA and the wild-type, whereas they are unable to find in deletion of *SOM1* (Figure 46b). In summary, Som1 and AfSomA fulfill similar functions in the human pathogen *A. fumigatus* as in the plant pathogen *V. dahliae*.

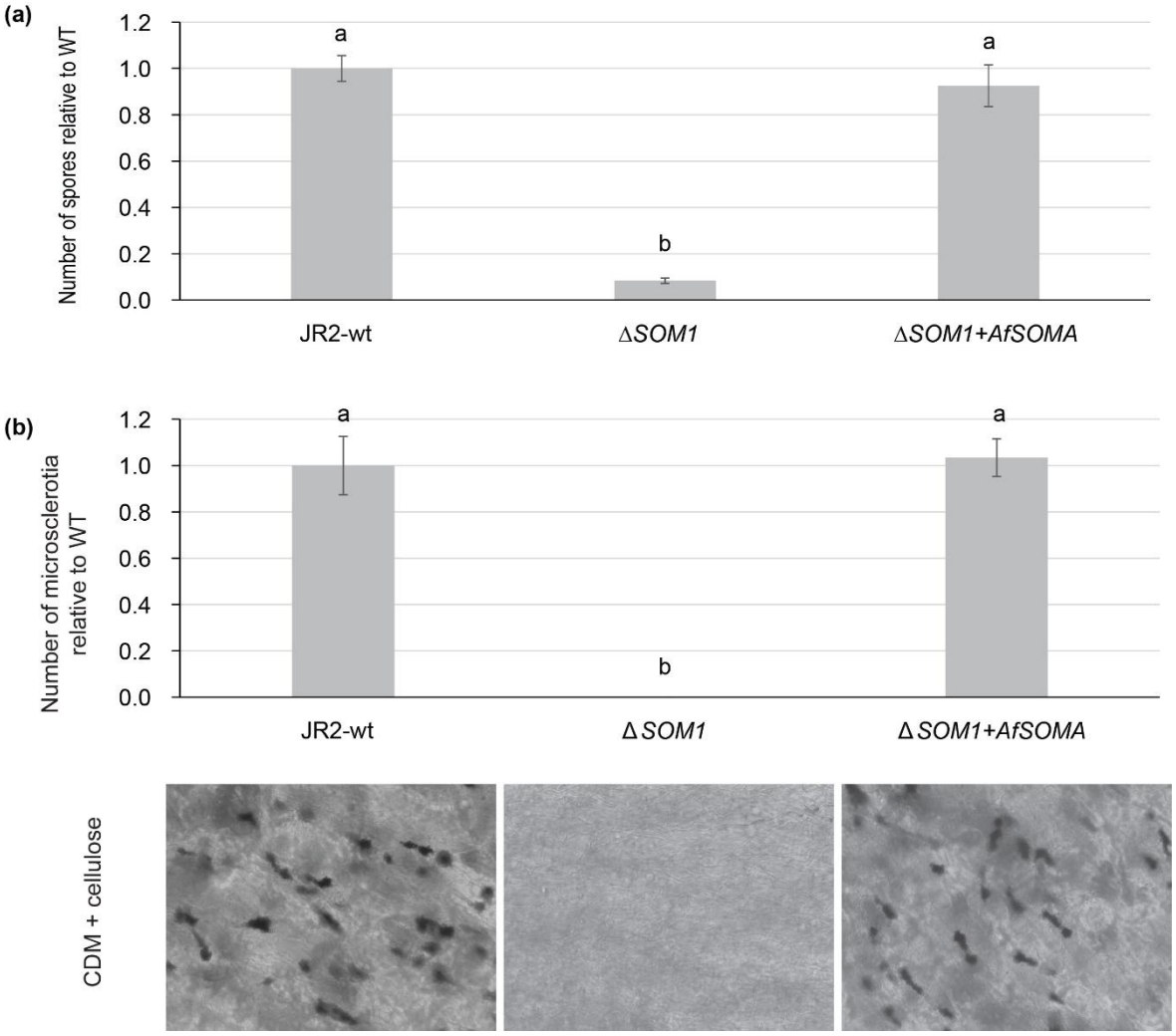


Figure 46. AfSomA can restore the conidia and microsclerotia formation in the *SOM1* deletion strain of *V. dahliae*. (**a**) Conidia formation was examined in 50 ml SXM on a shaker at 25°C. The number of spores was counted at seven days post inoculation. (**b**) The same number of spores of indicated strains was dropped on CDM plates and incubated in the dark at 25°C. Microsclerotia formation was tested on agar plates and incubated in the dark. The number of microsclerotia was counted after seven days. The experiments were performed in triplicates. The mean values and standard deviations are shown. The letters a and b indicate groups which are significantly different as calculated by Tukey-Kramer multiple comparison procedures, α= 0.01.

4. Discussion

4.1 The transcription factors Som1 and Vta3 support in adhesion of *S. cerevisiae*

Adhesion is essential for fungal plant and human infections (Yan *et al.*, 2011; Lin *et al.*, 2015). This is also the case for the plant pathogenic fungus *Verticillium* for which it was shown that *Verticillium* transcriptional regulator of adhesion 2 (Vta2) is important for both, the infection and fungal development (Tran *et al.*, 2014). In this study, we investigated the two proteins Som1 and Vta3, which are involved in fungal differentiation processes and infection and can restore the adhesion process in non-adherent *S. cerevisiae*. Both proteins can activate the expression of flocculation genes in *S. cerevisiae* using two distinct mechanisms.

4.1.1 Som1 presumably binds to promoter regions of flocculation genes in *S. cerevisiae* for activation

Som1, a homolog of Flo8, contains the LisH domain which is highly conserved. Low expression of Som1 can rescue adhesion of non-adhesive *FLO8* defective *S. cerevisiae*. Som1 might directly bind to promoter regions of flocculation genes for activation and interruption of the function of the inhibitor Sfl1 (Figure 47). It might act as its homolog in *A. fumigatus* and *S. cerevisiae* (Rupp *et al.*, 1999; Lin *et al.*, 2015). In *S. cerevisiae*, Flo8 regulates the expression of flocculation gene including *FLO1* and *FLO11* (Kobayashi *et al.*, 1996; Fichtner *et al.*, 2007). Flo1 is required for both, cell-cell and cell-substrate adhesion, whereas Flo11 is essential for adhesion of several layers of cells on agar plates (Fichtner *et al.*, 2007). Flo11 is regulated by the cAMP/PKA and MAPK pathways. The MAPK pathway controls the expression of *FLO11* via transcription factors Ste12 and Tec1, whereas the cAMP/PKA pathway regulates *FLO11* through the activator Flo11 and the inhibitor Sfl1. Flo8 and Sfl1 directly bind and have an overlapping binding side on the promoter region of *FLO11*. Flo8, Ste12, and Tec1 directly bind to the promoter region of *FLO11* for controlling the expression (Octavio *et al.*, 2009). Disruption of *FLO8*, *STE12*, or *TEC1* causes a block of *FLO11* expression and leads to defects in adhesion (Rupp, Steffen *et al.*, 1999; Fichtner *et al.*, 2007). Expression of *AfSOMA*, a homolog of *FLO8* in *A. fumigatus*, can activate *FLO1* and *FLO11* genes in *FLO8*-defective yeast.

AfSomA of *A. fumigatus* can act similarly to yeast Flo8 in the promoter region of *FLO11* (Lin *et al.*, 2015).

Som1, under the control of the *GAL* promoter, was not identified in yeast screen experiments (Tran *et al.*, 2014). In our study, we found the reason why Som1 was not distinguished in adhesive screen experiments. The high expression of *SOM1* cannot reprogram adhesion and activate the expression of *FLO1* and *FLO11*. Additionally, the data show that the expression of *STE12* is significantly down-regulated in the yeast strain with strong expression of *SOM1*. The overexpression of *SOM1* inhibits the expression of *STE12* and might cause an inactivation of *FLO11* expression. Som1 might bind to the promoter region of flocculation genes but lacks the function of *STE12* and therefore it cannot activate the expression of *FLO11*. Flo8 and Ste12 are essential for expression of *FLO11* (Rupp *et al.*, 1999). Lacking either Flo8 or Ste12 leads to defective adhesion in *S. cerevisiae* (Rupp *et al.*, 1999; Kim *et al.*, 2014). Taken together, the low expression of *SOM1* can rescue adhesion in non-adhesion yeast. It might directly bind to the promoter of *FLO11* for activating. The binding sites of Flo8 and Sfl1 in the promoter of *FLO11* are overlapping (Octavio *et al.*, 2009). Sfl1 is essential for the repression of flocculation genes (Conlan & Tzamarias, 2001; Ansanay Galeote *et al.*, 2007).

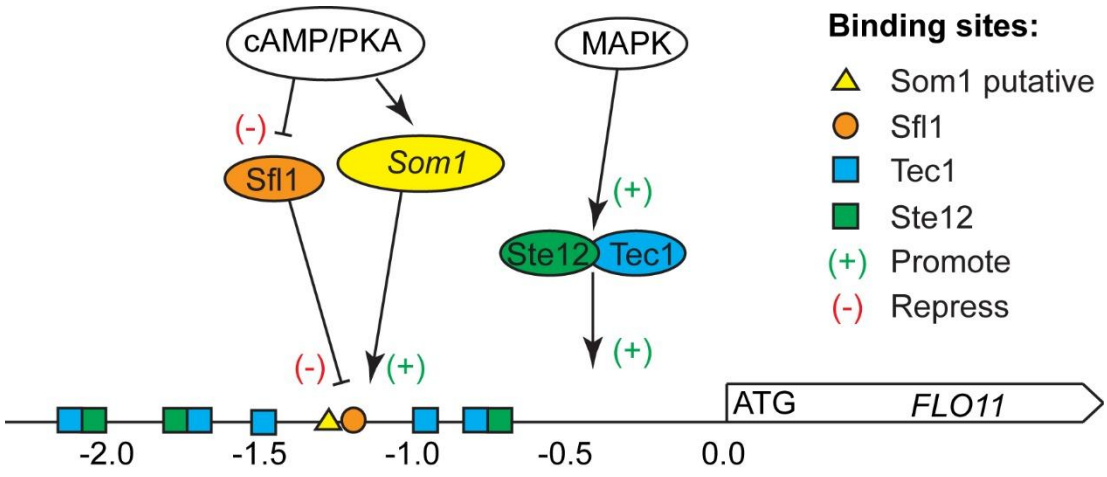


Figure 47. Som1 might directly bind to the promoter of *FLO11* in *S. cerevisiae*. Adhesion of *S. cerevisiae* requires the expression of *FLO11*. The expression of *FLO11* is controlled by the cAMP/PKA and MAPK pathways. The complex of Ste12 and Tec1 promotes the expression of *FLO11*, whereas Sfl1 represses the expression of it. In yeast, Som1 might directly bind to the promoter of *FLO11* for activation. This figure is modified from (Octavio *et al.*, 2009).

4.1.2 Vta3 activate adhesion through repressing the negatively acting *SFL1* in *S. cerevisiae*

The transcription factor Vta3 harbours the winged helix-turn-helix domain which is highly conserved between filamentous fungi. Vta3 together with Ssn6 and Tup1 might indirectly activate *FLO1* and *FLO11* genes via repressing the expression of *SFL1*. Therefore, the expression of *FLO1* and *FLO11* is rescued in the yeast strain expressing *VTA3*. In *S. cerevisiae*, *FLO11* is directly controlled by Flo8, Ste12, Tec1, and Sfl1. Flo8, Ste12, and Tec1 play a role in activation, whereas Sfl1 is involved in inhibiting the expression of *FLO11*. Ste12 together with Tec1 regulated by the MAPK pathway cannot activate *FLO11* when lacking *FLO8*. However, this complex could stimulate the expression of *FLO11* when both, *FLO8* and *SFL1*, are absent. Sfl1 is involved in the repression of related flocculation genes and binds to the promoter region of *FLO11* (Conlan & Tzamarias, 2001; Ansanay *et al.*, 2007). Rfx1 physically interacts with the composite of Ssn6 and Tup1. This complex inhibits the expression of *SFL1*. (Huang *et al.*, 1998; Conlan & Tzamarias, 2001). Additionally, Flo8 and the complex of Ste12 and Tec1 bind to the promoter of *FLO11* for activating, whereas Sfl1 binds to that promoter for suppressing the expression (Rupp *et al.*, 1999; Conlan & Tzamarias, 2001; Octavio *et al.*, 2009). Defects in either Flo8 or Ste12/Tec1 prevent the expression of *FLO11* because of the function of Sfl1 (Rupp *et al.*, 1999). Lacking Sfl1 in the deletion of *FLO8* might explain why the yeast strain expressing *VTA3* can reprogram adhesion (Figure 48).

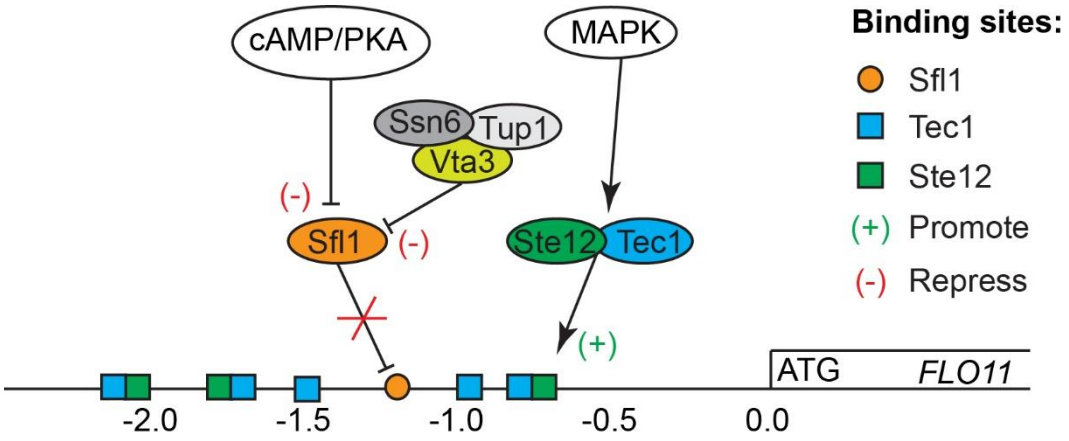


Figure 48. Vta3 activates *FLO11* via repressing the expression of *SFL1*. Adhesion of *S. cerevisiae* requires the expression of *FLO11*. The expression of *FLO11* is controlled by cAMP/PKA and MAPK pathways. Flo8, Sfl1, and Ste12 directly bind to the promoter of *FLO11*. The complex of Vta3, Ssn6, and Tup1 might repress the expression of *SFL1*. The expression of *FLO1* can be activated by the complex of Ste12 and Tec1. This figure is modified from (Octavio *et al.*, 2009).

4.2 The Transcription factors Som1 and Vta3 promote fungal development and virulence

Plant infection requires adhesive proteins in several stages of the host-fungus interaction (Braun & Howard, 1994; Hostetter, 2000). In this study, we show that the transcription factors Som1 and Vta3 are important for morphologies and plant infection at different stages and control the expression of putative transcription factors for adhesion.

4.2.1 Som1 and Vta3 control transcription factors for adhesion

Som1 controls adhesion and root penetration. Both, Som1 and Vta3, promote conidiation, microsclerotia formation, and virulence. They regulate transcription factors which are involved in fungal development, virulence and adhesion in *V. dahliae*. These factors were also shown to be able to restore adhesion in the *FLO8*-deficient *S. cerevisiae* (Tran *et al.*, 2014). Phenotypes of Som1 and Vta3 could be explained by the functions of *VTA* gene. Som1 and Vta3 might control microsclerotia and pigmentation via the regulation of *VTA1*. The transcription factor Vta1 encodes a putative Zn(II)2Cys6 domain. Its homolog in *Fusarium fujikoroi*, Fsr6, is essential for pigmentation of perithecia (Studt *et al.*, 2012). In *Verticillium*, Vta1 is located in melanin biosynthetic cluster of genes. It is upregulated in microsclerotia activation medium (Duressa *et al.*, 2013). Som1 and

Vta3 might control conidiation, virulence via promoting *VTA2*. Vta2 is highly conserved in filamentous fungi (Tran *et al.*, 2014). Its homolog in *M. grisea* plays important roles in conidiation, appressorium formation, and pathogenicity (Odenbach *et al.*, 2007). In *Verticillium*, the transcription factor for adherence Vta2 is involved in conidia formation, stress response, virulence. It also controls the expression of 270 genes including putative target genes for adhesion, conidiation, oxidative stress response, and virulence. Especially, Vta2 controls the expression of *ABA1* and *CON8* which are essential for conidia formation (Tran *et al.*, 2014). It also promotes the expression of virulence factor *NLP2* which is reported to play a role in growth, sporulation, and virulence (Santhanam *et al.*, 2013; Tran *et al.*, 2014). These conidial and virulence factors are down-regulated in deletions of *VTA3* and *SOM1*. Som1 might regulate oxidative stress response via controlling Vta2. Moreover, Vta2 is needed for oxidative stress response and regulate the expression of genes which are related to oxidative stress response such as *CPX2* and *SOD3* (Tran *et al.,* 2014), which are also regulated by Som1. Som1 perhaps promotes growth in medium with galactose as carbon-source though controlling the expression of *VTA6,* a putative CreA regulator, which is the negative regulator mediating carbon catabolism repression in *A. nidulans* (Strauss *et al.*, 1999). CreA is supposed to act in a similar way to the general repressor Tup1 and Ssn6 to suppress the synthesis of enzymes which are involved in the catabolism of carbon sources (Panozzo *et al.*, 1998). Taken together, Som1 and Vta3 might promote fungal development and virulence by regulating *VTA* genes (Figure 49).

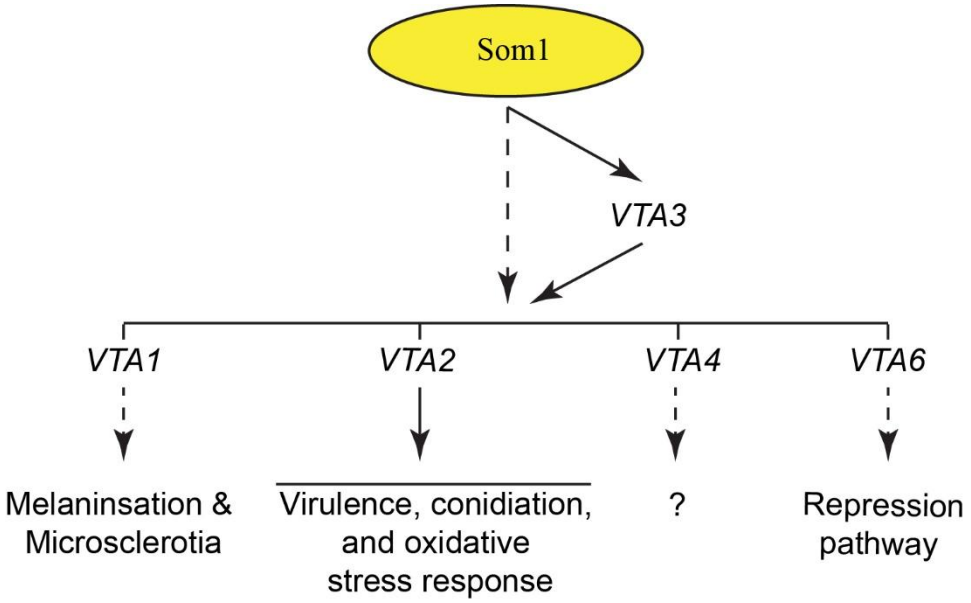


Figure 49. Som1 and Vta3 control fungal development and virulence by regulation of *VTA* genes. Som1 regulates the expression of *VTA3*. Both, Som1 and Vta3, control the expression of *VTA1, VTA2*, VTA4, and *VTA6* genes. Som1 might regulate the *VTA* genes directly or indirectly via *VTA3*. Vta1 might be involved in microsclerotia formation and melanisation. Vta2 is essential for conidiation and virulence. Vta6 might join the repression pathway to control genes which are involved in the catabolism of carbon sources. The solid arrows show the presented data and the result of previous studies. The dashed lines indicate putative function.

4.2.2 Som1 controls adhesion and penetration in *V. dahliae*

In *V. dahliae,* Som1 is essential for adhesion and penetration on plant roots and regulates the expression of adhesion factors such as *FAS1*, *MAD1*, *MAD2*, *SNOD1*, and *PLS1*. A lack of these proteins might explain why the deletion of *SOM1* is defective in adhesion and penetration on plant roots. Homologs of *FAS1* in *Drosophila*, *M. oryzae,* and *Rhodobacter sphaeroides* encode fasciclin domain proteins containing a GPI signal. They are required for conidial adhesion and appressorium turgor formation (Clout *et al.*, 2003; Liu *et al.*, 2009; Moody & Williamson, 2013). Mad1 and Mad2 in *Metarhizium anisopliae* are involved in adhesion. The disruption of *MAD1* blocks adhesion of *M. anisopliae* to insects, whereas *MAD2* mutant impedes the adherence to plant surfaces (Wang & St Leger, 2007). The counterpart of Snod1 in *M. grisea* is essential for the formation of adhesion structures such as appressoria. The *SNODPROT1* deletion strain is impaired in the formation of appressoria which are necessary for adhesion in *Magnaporthe* (Jeong *et al.*, 2007). In *V. dahliae*, Pls1 was reported to play a role in formation of penetration pegs. It is required for the initial colonisation of cotton roots. The deletion

strain of *PLS1* is unable to produce hyphopodia and penetration pegs and loses virulence (Zhao *et al.*, 2016). Studies of Pls1 homologs in other fungi also show that homologs of Pls1 are required for appressoria formation and penetration of plant surfaces (Clergeot *et al.*, 2001; Gourgues *et al.*, 2004). It is not clear if the inability of the Som1 deletion strain to adhere to plants is caused by the down-regulation of one of these factors or if it is a combination of different factors involved in fungal adhesion. To answer this question, further studies on the different proteins and their function in *Verticillium* would be required.

4.2.3 Som1 and Vta3 promote pathogenicity

Som1 and Vta3 are required for virulence and sequential steps of root penetration and colonisation. Som1 promotes the expression of adhesion factors which might be involved in the penetration of plant roots. Additionally, Som1 controls the expression of *VTA3* and together with Vta3 regulates the expression of *VTA2* and several virulence factors as *NLP2*, *CAP20*, and *PRY1*. Therefore, the lack of Vta2 and these virulence factors might explain why the deletion strains of *SOM1* and *VTA3* are defective in plant infection. Vta2 is needed for root colonisation and tomato infection. The absence of *VTA2* causes a block in root colonisation and pathogenicity in *V. dahliae* (Tran *et al.*, 2014). Even though Vta2 and Vta3 have a different function in microsclerotia formation, they play a similar function in root colonisation. Both deletion strains of *SOM1* and *VTA3* show defects in tomato root colonisation as observed in the deletion of Vta2.

NLP2 encodes a cytotoxic necrosis and ethylene-like protein which is expressed during the colonisation of plant roots by the fungus. It was reported to contribute to the pathogenicity and disease symptoms of *V. dahliae* on tomatoes (Santhanam *et al.*, 2013). Plants infected by deletion strains of *SOM1* and *VTA3* have no disease symptom. The homolog of Cap20 in *Colletotrichum gloeosporioides* is essential for appressoria formation and virulence. *CAP20* is expressed during appressoria formation. Deletion of *CAP20* causes defects in the colonisation of tomato fruits (Hwang *et al.*, 1995). Both Som1 and Vta3 are required for root infection. Deletion strains of *SOM1* and *VTA3* were not isolated from plant hypocotyl. The deletion strains might not be able to enter plant root or spread in the plant.

The expression of *PRY1* is significantly down-regulated in the deletions of *SOM1*, *VTA3*, and *VTA2*. The Pry1 counterpart in *F. oxysporum* is required for virulence in mammalian hosts (Prados-Rosales *et al.*, 2012). Som1 and Vta3 may possibly regulate pathogenicity via controlling genes which are related to virulence.

Moreover, our study show that Cyc8 was identified by LC-MS/MS analyses after enrichment of *VTA3*:*GFP* by GFP-trap. Vta3 might physically interact with Cyc8 to control virulence in *V. dahliae*. The homolog of Vta3 in *S. cerevisiae* physically interacts with Ssn6 which is a counterpart of Cyc8 (Huang *et al.*, 1998). SsnF, a homolog of Cyc8 in *A. fumigatus*, was reported to interact with Fbx15 which is essential for virulence (Johnk *et al*., 2016). In *V. dahliae*, Cyc8 is reported to play a major role in virulence. The deletion strain of *CYC8* is impaired in virulence in cotton (Li *et al.*, 2015b). It is not clear whether the complex of Vta3 and Cyc8 controls directly or indirectly the virulence by regulating growth and microsclerotia formation. The function of virulence factors which were found in protein assays has not been studied yet. To understand more about the virulence mechanism of *V. dahliae*, the roles of putative virulence factors should be investigated.

4.2.4 Som1 and Vta3 are essential for conidia and microsclerotia formation

After plant root infection, pathogenic fungi can distribute within the plant by the formation of conidia which are also required to spread the pathogen on the field (Pegg & Brady, 2002; Berlanger & Powelson, 2005). Conidia are formed in a specialised structure, called the conidiophore. Each conidiophore produces several phialides on which a number of conidia develop. This unit of conidia on a phialide, we called conidiospore clusters. We found that Som1 and Vta3 are necessary for conidiation in general and also for conidiospore cluster formation. Conidia formation is reduced in both deletions, whereas the number of conidiospore clusters is only decreased in the deletion strain of *SOM1*.

The counterpart of Vta3 in *F. graminearum* is essential for spore shape (Min *et al.*, 2014). However, in this study, the shape of conidia of the *VTA3* deletion strain was not changed, whereas conidiospore clusters had an abnormal shape. Both, Som1 and Vta3, regulate the expression of conidiation factors such as *VTA2, CON8*, and *ABA1*. Vta2, a homolog of Con7, is essential for conidia formation and promotes the expression of *CON8* and *ABA1* (Tran *et al.*, 2014). The homolog of Con8

in *Neurospora crassa* was shown to be involved in sporulation and is expressed early during this process (Roberts & Yanofsky, 1989). The counterparts of Aba1 in *A. nidulans* and *A. fumigatus* are needed for differentiation of conidia from vegetative hyphae (Tao & Yu, 2011; Son *et al.*, 2013). The absence of Con8 and Aba1 might be the cause of reduction of spore amount in deletion strains of *SOM1* and *VTA3*. Additionally, the expression of *CON6* and *CON10* is significantly declined in the deletion of *SOM1*, whereas it does not change if *VTA3* is deleted. The homolog of Con6 in *N. crassa* is required for early conidia differentiation, whereas the counterpart of Con10 is essential for later stages (Roberts & Yanofsky, 1989). Lacking Con6 and Con10 might explain why the deletion strain of *SOM1* have fewer conidiospore clusters than the wild-type.

Som1 and Vta3 do not regulate the same genes. So there seem to be overlapping regulatory functions but also divergent. Som1 controls *VTA3* and regulated genes in the *VTA3* deletion strain. In contrast, Som1 might have additional functions which are independent of Vta3 as the function encoded by other genes only regulated by Som1 (Figure 50).

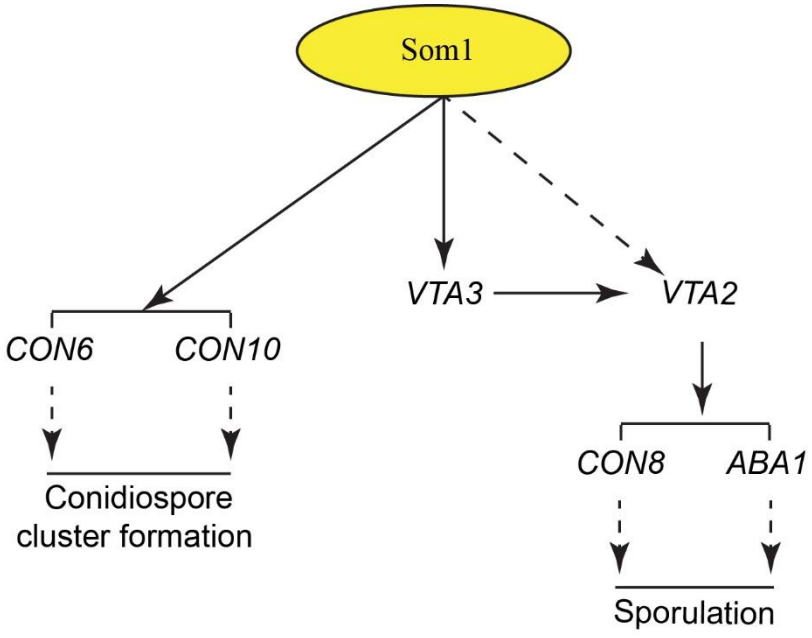


Figure 50. Model illustrating how Som1 and Vta3 can control conidia formation. Som1 regulates the expression of *VTA3*. Both, Som1 and Vta3, control the expression of *VTA2*, *CON8*, and *ABA1* genes which are essential for conidia formation. Som1 might regulate the conidia formation directly or indirectly via *VTA3*. *CON8* and *ABA1* are regulated by transcription factor Vta2. Additionally, Som1 might regulate conidiospore clusters formation via controlling the expression of *CON6* and *CON10*.

Microsclerotia are resting structures to overwinter and also to survive several years without appropriate hosts, which are usually formed in dead plant materials and necessary for the reinfection after the winter (Pegg & Brady, 2002). In this study, we show that both, Som1 and Vta3, support melanised cell formation. Som1 and Vta3 might be a dual control system of microsclerotia development. The deletion strain of *SOM1* was not able to form resting structures, whereas the number of microsclerotia was reduced in the *VTA3* deletion strain. The lacking Vdh1 might explain why no or fewer microsclerotia were developed in the deletion strains of *SOM1* or *VTA3* compared to the wild-type. Vdh1 was described as a key factor which is involved in the formation of melanised cells, whereas it is not required for hyphal growth. Resting structures were not detected in the deletion strain of *VDH1* (Klimes & Dobinson, 2006). Additionally, Vta3 might function together with Cyc8 in microsclerotia formation. They might control the expression of genes which are required for microsclerotia formation and pigmentation. Cyc8, an interaction partner of Vta3, was shown to play a role in the formation of microsclerotia. The deletion strain of *CYC8* shows lack of melanin and fewer microsclerotia (Li *et al.*, 2015b). Cyc8 controls the expression of genes which are involved in melanin synthesis or microsclerotia formation such as encoding laccase (VDAG_00189), conidial yellow pigment biosynthesis polyketide synthase (VDAG_00190), tetrahydroxy-naphthalene reductase (VDAG_03665), and scytalone dehydratase (VDAG_03393) (Li *et al.*, 2015b). Lack of these proteins might explain why the deletion strains of *VTA3* and *CYC8* are defective in microsclerotia formation. Functions of these genes have not been studied yet. To know which of them are specific for microsclerotia formation, further experiments would have to be performed.

4.2.5 Som1 and Vta3 antagonise the oxidative stress response

When pathogens infect host plants, they are confronted with oxidative stress and a proper response to reactive oxygen species is important to successfully colonise the plant (Tanabe *et al.*, 2011; Youseff *et al.*, 2012). We found that Som1 and Vta3 play antagonising roles in oxidative stress response. Som1 positively regulates oxidative stress response, whereas Vta3 negatively influences it. Oxidative stress response of fungi is regulated by several factors. It requires not only the expression of encoding genes but also to stabilize of oxidative stress tolerance proteins (Nahlik *et al.*, 2010; Tanabe *et al.*, 2011;

Youseff *et al.*, 2012). Additionally, oxidative stress tolerance needs a transcription factor which is involved in secondary metabolism (Baidya *et al.*, 2014). Som1 probably controls oxidative stress tolerance by two different mechanisms. On the one hand, Som1 might control the expression of *VEL1*, which encodes a key factor of secondary metabolism (Figure 51). The counterpart of Vel1 in *A. flavus*, a member of velvet complex, was shown to be involved in secondary metabolism and it was essential for oxidative stress response (Baidya *et al.*, 2014). On the other hand, Som1 could promote the expression of genes encoding enzymes which are required for stress response (Figure 51). For example, the homolog of *CPX2* in *M. oryzae* encodes a secreted catalase-peroxidase, whereas the counterpart of *SOD3* in *Histoplasma capsulatum* encrypts a superoxide dismutase. Both, Cpx2 and Sod3, are controlled by Vta2 (Tran *et al.*, 2014) and have a function in fungal defense again the oxidative stress (Tanabe *et al.*, 2011; Youseff *et al.*, 2012). Furthermore, the stability of transcription factors is often controlled by the COP9 signalosome (CSN) including the Ubiquitin-Proteasome system (Chamovitz, 2009; Braus *et al.*, 2010; Nahlik *et al.*, 2010). COP9, a multi-subunit complex, can regulate the stability of VeA protein and other proteins including transcription factors for oxidative stress response (Chamovitz, 2009; Braus *et al.*, 2010; Nahlik *et al.*, 2010). Fbx15 is induced by oxidative stress. In stress condition, Fbx15 protein is elevated 3 times when compared to normal condition (Johnk *et al.*, 2016). It is reported to interact with three subunits of the CSN deneddylase (Nahlik *et al.*, 2010; Johnk *et al.*, 2016). The effect of Som1 on the stability of proteins which are essential for oxidative stress response have not been studied yet. To understand better the oxidative stress response processes, the effect of Som1 on the stability of transcription factor and other functions of candidates for stress response have to be done.

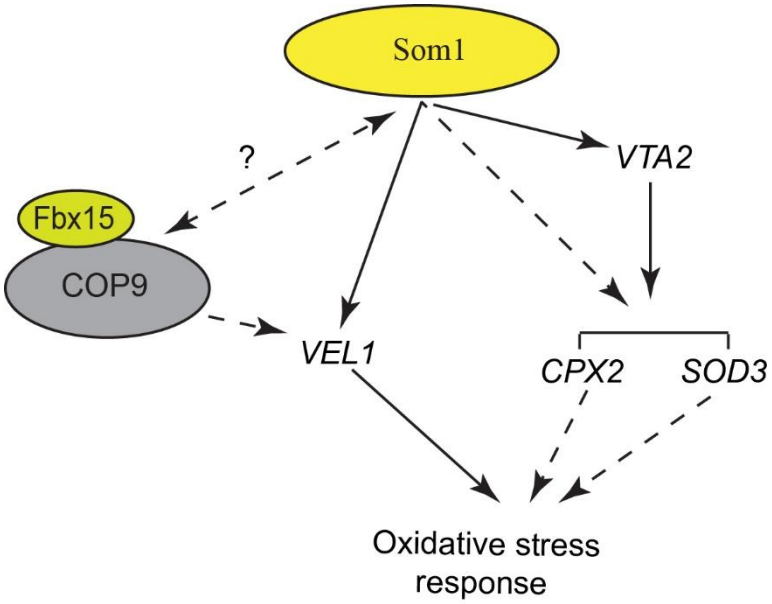


Figure 51. Model of Som1 reregulates the oxidative stress response. Som1 regulates directly or indirectly through Vta2 the expression of *CPX2* and *SOD3*, required for oxidative stress response. On the other hand, Som1 might also regulate stress response and secondary metabolism pathway via synchronising *VEL1*.

Vta3 does not promote the expression of enzymes which are involved in oxidative stress response, however, it represses the expression of *INO1*. The homolog of Vta3 in *S. cerevisiae,* Rfx1*,* was reported to interact with Itc1 and Isw2 which form a multiplex to repress *INO1* expression (Sugiyama & Nikawa, 2001; Gavin *et al.*, 2002). The disruption of either *ITC1* or *ISW2* results in de-repression of the *INO1* expression (Sugiyama & Nikawa, 2001). *INO1* encodes an inositol-3-phosphate synthase which catalyses inositol synthase (Wang *et al.*, 2015). The overexpression of *INO1* enhances the tolerance to inhibitors (Wang *et al.*, 2015). Vta3 might interact with Itc1 and Isw2. This compound might repress the expression of *INO1* to antagonise oxidative stress response (Figure 52). Lacking of Vta3 and the function of the repressor complex of *INO1* might explain why the expression of *INO1* and oxidative stress response are significantly increase in the deletion of *VTA3*. Homologs of Itc1 and Isw2 were found in *V. dahliae*. However, Itc1 and Isw2 were not detected in our pull-down assays neither in the list of proteins interacted with free GFP nor with Vta3:GFP. Probably the interaction of the proteins with Vta3 is week and transient to get them pulled out. It is also possible that the expression of *ITC1* and *ISW2* requires certain conditions such as stress inducing media to be identified in the pull-downs of Vta3:GFP. Therefore, condition in which *ITC1* and *ISW2* are expressed need to be examined to check the possible interaction of Vta3

with both, Itc1 and Isw2. When these conditions are determined chromatin immunoprecipitation can be also used to possibly identify interactions between Vta3, Itc1, and Isw2.

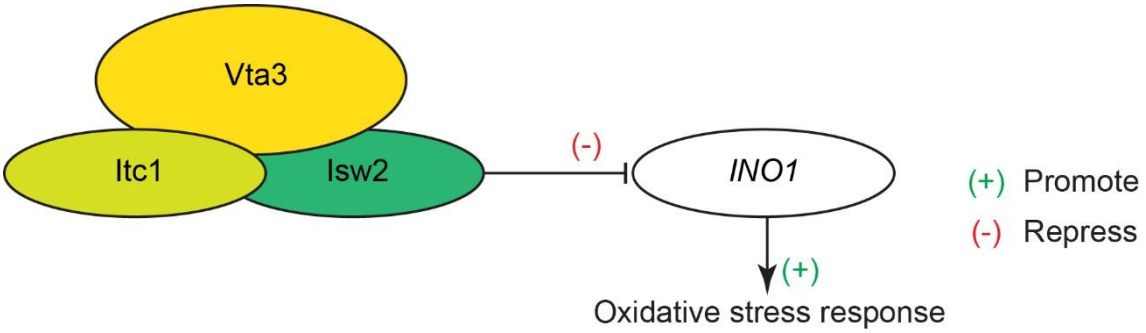


Figure 52. Vta3 can repress oxidative stress response via suppressing *INO1*. Vta3 might interact with Itc1 and Isw2. This complex usually suppresses the expression of *INO1* which is required for oxidative stress response.

4.2.6 Som1 and Vta3 are required for hyphal development

Alterations in the colony phenotype might be directly connected to hyphal development and intracellular processes. We found that Som1 also functions in these processes. Som1 is required for normal hyphal branching, septum positioning, and vacuole size. The genetic mechanisms underlying hyphal branching are not well-studied in filamentous fungi. Septum formation is well studied in *A. nidulans*. Septa are produced from three major rings including actins, septin and formin (Harris, 2001). Formation of septa is a key step in hyphal differentiation (Harris, 1997; Harris, 2001). The formation of septa requires several factors such as SepH, actin, the septin AspB, and the formin SepA (Harris, 2001). Recently, DipA of *A. nidulans* was reported to be required for septa positioning as well (Schinke *et al.*, 2016). The deletion strain of *DIPA* showed an increased number of septa compared to the wild-type (Schinke *et al.*, 2016). This phenotype is similar to what was observed in the *SOM1* deletion strain. The deletion strain had more septa than the wild-type strain. A homolog of DipA is present in *V. dahliae* but its functions have not been studied so far. It is possible that Som1 and Dip1 directly or indirectly interact and/or act as a dual control system for septum positioning.

Vacuoles are essential for cellular differentiation. They were reported to play a major role in cell differentiation during the formation of appressoria which are specialised penetration structures in *C. albicans* and *U. maydis* (Weber, 2002).

Vacuoles help to expand the surface of fungal tips in order to support appressorium formation via degrading lipid stores which generate osmotically active metabolites (Veses *et al.*, 2008). This process supports the forced entry of the fungal penetration peg into the plant epidermis (Weber *et al.*, 2001). Additionally, vacuoles are essential for stress response (Weber, 2002; Palmer *et al.*, 2003; Veses *et al.*, 2008). In this study, we found that Som1 is required for microsclerotia formation, hyphopodia formation, root penetration, and virulence in *V. dahliae*. These phenotypes might be explained by the defect of vacuole size. The small vacuole size might be related to the inability of the *SOM1* deletion strain to form hyphopodia and microsclerotia and to respond to oxidative stress and virulence. Fatty acids are major components of phospholipid and glycolipid compounds which play a key role in the structure and functioning of membranes such as vacuoles and plasma membranes (Michaillat & Mayer, 2013; van Zutphen *et al.*, 2014). Lack of fatty acid might cause the defect in vacuole size. The expression of genes encoding fatty acid synthase S-acetyltransferase which catalyses fatty acid synthesis is significantly down-regulated in the deletion of *SOM1* (Table 4). The hyphal development in *Verticillium* has not yet been studied in detail. To understand more about that process further studies on hyphal development and protein regulated hyphal development would have to be performed.

4.3 AfSom1 and VdSom1 fulfil similar functions in plant and human pathogens

Plant and human pathogenic fungi have massively attracted attention because they have some common pathogenicity factors. Studies of the function of these factors in both, plant and human, pathogens help to understand aspects of infection and virulence (Yu *et al.*, 2017). There are several factors which are reported to play a major role in virulence in plant pathogens and counterparts are involved in pathogenicity in human pathogenic fungi (Yu *et al.*, 2017). They are useful for antifungal strategies and development of new fungicides to control pathogens (Fones *et al.*, 2017; Yu *et al.*, 2017). We recently described that the transcription factor AfSomA in *A. fumigatus* plays important roles in adhesion, conidia formation and virulence in the human pathogenic fungus (Lin *et al.*, 2015).

In this study, we show that AfSomA and VdSom1 fulfil similar functions in the human pathogenic *A. fumigatus* as in the plant pathogen *V. dahliae*. AfSom1 does not only rescues growth, adhesion, conidia formation, and virulence but also reprograms microsclerotia formation in *V. dahliae*, a process which does not exist in *A. fumigatus*. This pathogenicity factor of a plant invader and its counterpart of human pathogenic fungi is highly conserved. The result of this study could help to understand penetration and virulence pathways not only in plant pathogenic fungi but also in human pathogens. Nowadays, antifungal azole is widely used in agriculture and also the clinic to control pathogenic fungi (Fones *et al.*, 2017). However, some new pathogens became resistant against this fungicide. *A. fumigatus,* which causes lung disease, does not respond to azole treatment (Fones *et al.*, 2017). Furthermore, infection of this fungus is often underdiagnosed and treated rather late. Therefore, the effect of fungicides is often limited, and up to 80% of patients infected by *A. fumigatus* die (Fones *et al.*, 2017). It is necessary to develop new fungicides to control this pathogenic fungus. In *V. dahliae*, controlling the expression of *SOM1* might stop the fungus from penetrating plant roots and block virulence, whereas manipulation of the expression of *VTA3* could prevent the development of the pathogen *in planta* to reduce disease. Som1 and Vta3 might be putative targets for antifungal intervention. A new effective fungicide which can treat new pathogenic fungi and *A. fumigatus* can help us save million euro yearly.

In summary, Som1 is required for adhesion on abiotic surfaces, penetration, and colonisation of the root surface, whereas Vta3 is needed for colonisation only. Both, Som1 and Vta3, are essential for conidia and microsclerotia formation in *V. dahliae*. Especially, the *SOM1* deletion strain was unable to produce microsclerotia. Som1 promotes oxidative stress response, whereas Vta3 inhibits this reaction. Additionally, Som1 is required for hyphal development including aerial hyphae, hyphal growth on agar plates, hyphal branching, septum position, and normal vacuole size where Vta3 is essential for hyphal growth on agar plates. (Figure 53).

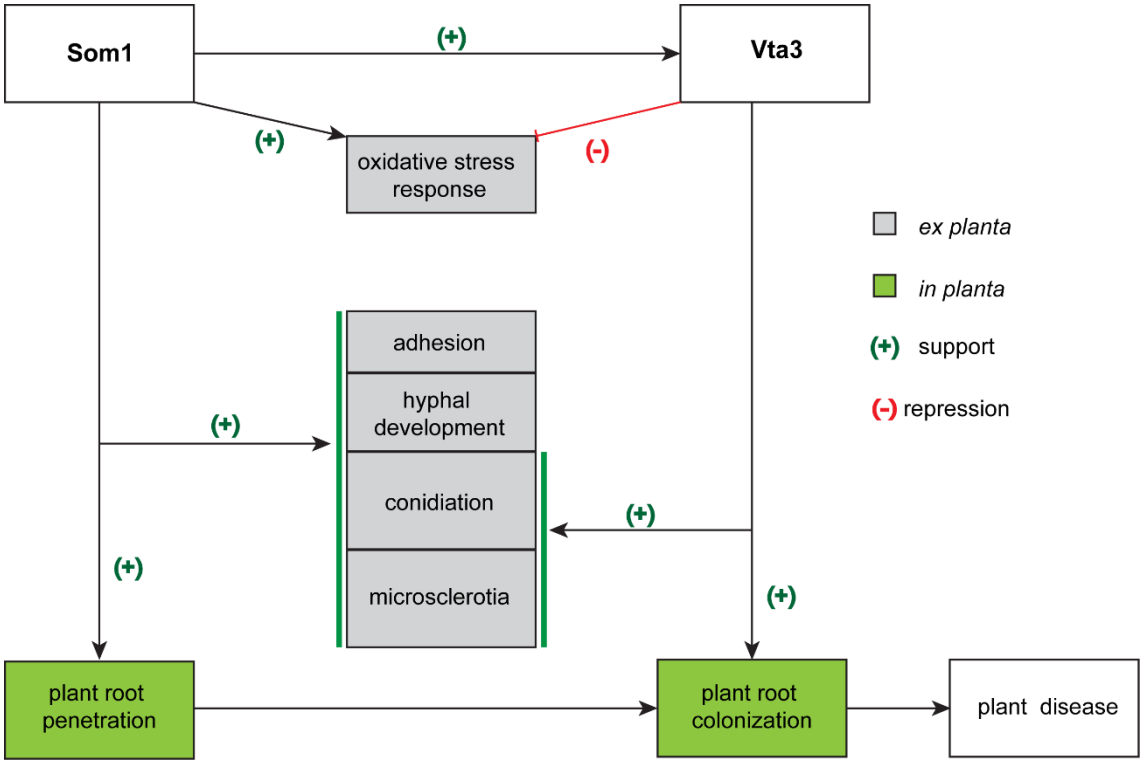


Figure 53. The functions of Som1 and Vta3 in *V. dahliae*. Som1 is a transcription factor which supports adhesion, conidiation, microsclerotia formation, oxidative stress response, and plant root penetration. It controls the expression of *VTA3* which encodes a protein that promotes conidiation, microsclerotia formation, and plant root colonization and represses oxidative stress response.

4.4 Outlook

In this study, we found that the nuclear transcription factors of *Verticillium dahliae* Som1 and Vta3, which can rescue adhesion in a *flo8*-deficient *Saccharomyces cerevisiae* strain, are controlling sequential steps in the infection of a plant host. Som1 might directly bind to the promoter of adhesive genes for activation, whereas Vta3 indirectly induces *FLO1* and *FLO11* genes by repressing the expression of *SFL1*. However, the mechanisms of other adhesion candidates of the yeast screen which can reprogram adherence in non-adhesion yeast and their functions in *V. dahliae* are unclear (Tran *et al.*, 2014). Especially, the transcription factors of adhesion such as *VTA1*, *VTA4*, and *VTA6* which were down-regulated in deletion strains of *SOM1* and *VTA3* have not been studied yet.

Both, Som1 and Vta3, control several similar phenotypes and virulence. Som1 regulates the expression of *VTA3*. Therefore, Som1 might indirectly promote cellular development and virulence via Vta3. To better understand the relation of

Vta3 to Som1, the expression of *VTA3* under the control of a constitutive promoter in the deletion strain of *SOM1* could be examined. In such an experiment Vta3-independent functions of Som1 could be discriminated from effects resulting from a reduced expression of *VTA3* in the *SOM1* deletion strain.

Our proteomic data indicate that twenty abundant proteins were significantly reduced in the deletion of *SOM1* including Cap20 and Snodprot1. They have not been studied yet and might play important roles in *V. dahliae*. Additionally, three putative uncharacterized proteins were also abundant in the wild-type, but they are not found in deletion of *SOM1*. They might fulfil critical functions in *V. dahliae*. The function of Cap20, Snod1, and unknown proteins which were significantly reduced in the *SOM1* deletion strain need to be investigated further.

Therefore, future work will focus on adhesion factors, cellular connections and target genes which are controlled by Som1 and Vta3 and are needed to better understand and control plant and human pathogens more efficiently.

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Abbreviations

^{o}C	degree Celsius
Δ	deletion
Amp^{R}	ampicillin resistance
BLAST	basic local alignment search tool
bp	base pair
cAMP	cyclic adenosine monophosphate
CDM	Czapek-Dox medium
cDNA	complementary DNA
cm	centimeter
C-terminus	carboxyl terminus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole, dilactate
EDTA	ethylenediaminetetraacetic acid
Flo	flocculin
GFP	green fluorescent protein
GPI	glycosyl phosphatidylinositol
g	gram
h	hour
H_2O_2	hydrogen peroxide
HPH	hygromycin phosphotransferase
Kan^{R}	kanamycin resistance
kb	kilobase
kDa	kilo Dalton
l	liter
LB	left border/ Luria Bertani medium
LiAc	lithium acetate
LCMS	liquid chromatography mass spectrometry
LisH	Lis homology
M	molar
MAPK	mitogen-activated protein kinases
mg	milli-gram

min	minute
ml	milliliter
µg	micro-gram
µl	micro-liter
mM	millimolar
µm	micro-meter
MM	minimal medium
NAT	nourseothricin acetyltransferease
NLS	nuclear localisation signal
N-terminus	NH_2 terminus
OD	optical density
ORF	open reading frame
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDM	potato dextrose broth medium
PEG	polyethylene glycol
RB	right border
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SC-Ura	synthetic complete minus uracil medium
SDS	sodium dodecyl sulphate
SXM	simulated xylem medium
TE	Tris-HCl and EDTA
UV	ultraviolet
Va	*Verticillium albo-atrum*
Vd	*Verticillium dahliae*
WT	wild-type
YPD	yeast extract peptone dextrose

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Curriculum vitae

Personal information

Tri-Thuc Bui

Born on May 29th, 1984

In Thai Nguyen, Vietnam

Education

1990 – 1999 Elementary and junior education in Thai Nguyen, Vietnam

2000 – 2002 High school education in Thai Nguyen, Vietnam

Scientific background

2003 – 2007 Bachelor of Science at Thai Nguyen University of Agriculture and Forestry, Thai Nguyen, Vietnam

2007 – 2008 Assistant researcher at Agricultural Genetics Institute

2008 – 2013 Assistant researcher and assistant lecturer at Thai Nguyen University of Agriculture and Forestry, Thai Nguyen, Vietnam

2009 – 2012 Master of Science in Experimental Biotechnology at Thai Nguyen University, Thai Nguyen, Vietnam

2013 – 2017 Scientific assistant and PhD student in the lab of Prof. Dr. Gerhard Braus at Department of Molecular Microbiology and Genetics, Institute of Microbiology and Genetics, Georg-August University Göttingen