ADDING BY SUBTRACTING

Impairing tomato susceptibility genes to obtain resistance to Verticillium wilt

Katharina Hanika

Propositions

1. For the identification of susceptibility genes in crops, impairing orthologues of known susceptibility genes is the most straightforward approach.
   (this thesis)

2. Even under controlled greenhouse conditions, precise monitoring of environmental factors that may influence plant disease susceptibility is crucial.
   (this thesis)

3. In laboratory data management, the transition from hard copy notebooks to electronic lab notebooks is inevitable.

4. Due to the lack of sex-disaggregated data, artificial intelligence only enforces male dominance in urban transportation planning.

5. An artistic interpretation of research results would be more powerful than textual and quantitative statements to arouse interest of the general public in science.

6. When facing a health issue, going to the psychologist should be as normal as going to the dentist.

Propositions belonging to the thesis entitled
Adding by subtracting – Impairing tomato susceptibility genes to obtain resistance to Verticillium wilt
Katharina Hanika
Wageningen, 11 December 2020

Adding by subtracting – Impairing tomato susceptibility genes to obtain resistance to Verticillium wilt

Katharina Hanika

Thesis committee

Promoters
Prof. Dr Yuling Bai
Professor of Plant Breeding
Wageningen University & Research

Prof. Dr Bart P.H.J. Thomma
Professor of Phytopathology
Wageningen University & Research

Co-promoter
Dr Henk Schouten
Senior Researcher, Plant Breeding
Wageningen University & Research

Other members
Prof. Dr Gert Smant, Wageningen University & Research
Prof. Dr Ralph Hückelhoven, Technische Universität München, Germany
Dr Frank L.W. Takken, University of Amsterdam
Dr Kamila Koropacka, Axia Vegetable Seeds B.V., Naaldwijk

This research was conducted under the auspices of the Graduate School of Experimental Plant Science (EPS).

Adding by subtracting – Impairing tomato susceptibility genes to obtain resistance to Verticillium wilt

Katharina Hanika

Thesis
submitted in fulfilment of the requirement for the degree of doctor
at Wageningen University
by the authorship of the Rector Magnificus,
Prof. Dr A.P.J. Mol
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 11 December 2020
at 11 a.m. in the Aula.

Katharina Hanika
Adding by subtracting - Impairing tomato susceptibility genes to obtain resistance to Verticillium wilt, 212 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2020)
With references, with summary in English

DOI: 10.18174/531120
ISBN: 978-94-6395-542-3

TABLE OF CONTENT

Chapter 1

General introduction

In 2002, the concept of plant disease susceptibility genes in plant-microbe interactions was put into the spotlight for the first time (Eckardt, 2002). At that time, only one out of 524 documents identified via a Web of Science search for "plant disease resistance" used the term "plant disease susceptibility", highlighting the focus of research on resistance rather than on susceptibility. Now, 18 years later, a similar search in Web of Science still shows a large number of publications per year with terms such as '"resistance gene" + plant' or "plant disease resistance" (Figure 1) while, at the same time, the number of publications with the phrase "plant disease susceptibility" is low. Nevertheless, if queried for the term '"susceptibility gene" + plant' a steady annual increase can be observed since 2010. This indicates that plant disease susceptibility (*S*) genes gain more attention in research.

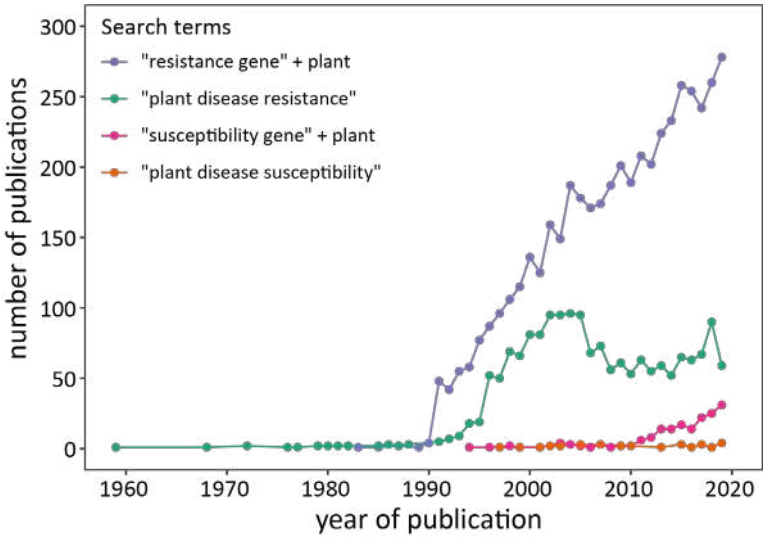


Figure 1 | Number of publications per year covered in Web of Science (data retrieved April 2020, www.webofknowledge.com).

THE CHAIN IS NO STRONGER THAN ITS WEAKEST LINK – SUSCEPTIBILITY GENES AND THEIR ROLE IN DISEASE

S genes encode host proteins that are exploited by the pathogen to establish a compatible interaction with the host, and thus facilitate infection (Pavan *et al.*, 2010; Gawehns *et al.*, 2013; Hückelhoven *et al.*, 2013; van Schie and Takken, 2014; Engelhardt *et al.*, 2018). While from the pathogen perspective this plant gene aids to establish disease, for the host this gene presents a weak link in its defence. Besides the intrinsic function *S* genes have for the host, these genes also have a plethora of functions in plant-pathogen interaction that can be categorized into three classes (van Schie and

Takken, 2014). The first class comprises gene products that are involved in the early interactions between host and pathogen. An example is the well-studied *mildew resistance locus O* (*mlo*) mutant, which provides resistance against *Erysiphe graminis* f. sp. *hordei* in barley (Buschgel *et al.*, 1997; Acevedo-Garcia *et al.*, 2014). This gene encodes a transmembrane protein and *mlo*-mediated resistance is based on the failure of the fungus to penetrate the host cell in early stages of infection. A second example is a *Medicago truncatula* mutant which inhibits germ tube growth of *Puccinia emaculata* due to the loss of epicuticular waxes, namely *inhibitor of rust germ tube differentiation 1* (*irg1*) (Uppalapati *et al.*, 2012). The second class of *S* genes concerns those genes that are involved in negative regulation of immunity. This class contains mutants with a constitutive expression of the defence hormone salicylic acid (SA), such as the *constitutive expresser of PR 5* (*cpr5*) mutant in Arabidopsis, which is resistant to *Pseudomonas syringae* and *Peronospora parasitica* (Bowling *et al.*, 1997). Moreover, other negative regulators of immunity, for example genes involved in the stress-related signalling cascade can act as *S* genes. An example is the *enhanced disease resistance 1* (*edr1*) mutant that provides resistance to *E. cichoracearum* in Arabidopsis and that was found to carry a mutation in a gene encoding a *mitogen-activated protein kinase kinase kinase* (*MAPKKK*) (Frye and Innes, 1998; Christiansen *et al.*, 2011). The third and last class of *S* genes encompasses genes that allow pathogen proliferation at late infection stages. In Arabidopsis, the *downy mildew resistant 1* (*dmr1*) mutant is resistant to *Hyaloperonospora arabidopsidis* and it was found that *DMR1* encodes a homoserine kinase (HSK) (van Damme *et al.*, 2005, 2009). HSKs are involved in the aspartate metabolic pathway and *dmr1* mutants show elevated levels of homoserine in the chloroplasts, which seems to be linked to *dmr1*-mediated resistance. Another well-studied example is the efflux sugar transporter *SWEET11* which underlies the recessive *xa13* resistance in rice against *Xanthomonsas oryzae*. This sugar transporter transports sugars into the apoplastic space, thereby providing nutrients to the pathogen (Chu *et al.*, 2006; Chen *et al.*, 2010). In general, it should be acknowledged that *S* genes have a wide variety of functions in the interaction with pathogens and act at different stages of the infection.

From the above-mentioned examples, it already becomes clear that *S* genes play a role in disease susceptibility to a wide range of pathogens, concerning biotrophs, such as discussed in the examples above, as well as necrotrophs. For example, downregulation or knock-out of the *expansin-like A2* (*EXLA2*) gene that encodes a cell wall-modifying enzyme confers resistance against the necrotrophic fungi *B. cinerea* as well as *Alternaria brassicola* (Abuqamar *et al.*, 2013). Besides playing roles in interactions with fungi, bacteria and oomycetes, *S* genes also play roles in interactions with viruses that rely on host factors for their replication (Garcia-Ruiz, 2018). For example, simultaneous mutations in the two Arabidopsis genes *Tobamovirus Multiplication 1 and 3* (*TOM1* and

TOM3), which encode transmembrane proteins required for tobamovirus replication, leads to undetectable viral titres (Yamanaka *et al.*, 2002). Another essential and well-studied host factor required by different viruses is the eukaryotic translation initiation factors (eIFs) which provided resistance when mutated (Wang and Krishnaswamy, 2012). To a far smaller extent, *S* genes were also characterized in interactions with nematodes. A mutant in the *heavy metal associated isoprenylated plant protein 27* (*HIPP27*) provides resistance against the cyst nemtode *Heterodera schachtii* (Radakovic *et al*., 2018). Lastly, studying *S* genes in the interaction with insects has been proposed, for example for aphids for which omics studies may lay the foundation to identify insect-related *S* genes (Ahman *et al*., 2019).

ADDING BY SUBTRACTING – IMPAIRING *S* GENES TO OBTAIN RESISTANCE

Resistance breeding aims at improving crops in order to withstand infections by pathogens (Bai and Lindhout, 2007; Bharadwaj, 2016). The main strategy in resistance breeding, which is also reflected in the annually increasing numbers of publication on *resistance* (*R*) genes (Figure 1), is the introgression of dominant *R* genes from wild germplasm into elite cultivars. In contrast, in order to utilize *S* genes in resistance breeding, their function in disease susceptibility needs to be impaired (Pavan *et al.*, 2010). Thus, *S* genes can be characterized as dominant genes while the resistance that is based on their impairment inherits recessively. For resistance breeding this, in return, requires the presence of impaired *S* gene alleles in both parental lines in a cross of a diploid crop such as tomato. Taken together, the concept of *S* gene-mediated resistance breeding is based on impairing – or subtracting – a *S* gene from the plant and thereby adding resistance.

Impairment of *S* genes can be achieved in multiple ways. Firstly, non-functional *S* gene alleles present in wild germplasm can be exploited in breeding programmes. An example for a naturally occurring *S* gene allele is a wild tomato accession from Ecuador that was shown to be resistant to powdery mildew which was found to be caused by an impaired *mlo* allele (*SlMlo1*) (Bai *et al.*, 2008). Accordingly, a major QTL conferring resistance to powdery mildew in cucumber was caused by a transposable element insertion in *Mlo8* (Berg *et al.*, 2015). Alternatively, impaired *S* genes might be found in mutant populations that can be generated by radiation or treatment with ethyl methanesulfonate (EMS). Subsequently, mutations in a specific target sequence can be identified using 'Targeting Induced Local Lesions IN Genomes' (TILLING) (Kurowska *et al.*, 2011). A screening of an EMS-mutagenized population of the tomato genotype Micro-Tom for resistance against powdery mildew, for example, identified a resistant mutant which turned out to carry a point mutation in the *SlMlo1* gene (Appiano, 2016). Other examples of using TILLING include the identification of virus resistance in

pepper due to nucleotide changes in the aforementioned eIFs (Ibiza *et al.*, 2010) or the identification of *mlo*-mediated resistance against powdery mildew in polyploid wheat (Acevedo-Garcia *et al.*, 2017).

STRIKE WHILE THE IRON IS HOT – *S* GENES IN THE ERA OF GENOME EDITING

A more targeted approach for impairing *S* genes than identifying naturally occurring *S* genes or screening mutant populations is the use of genome editing. Genome editing entails the alteration of an organism's DNA at a specific location in the genome by deleting, adding or modifying DNA. Such techniques have gained increasing attention over the last years and have revolutionized the field of biology.

The first genome editing technique made use of an artificial fusion between zinc finger DNA-binding domains and a non-specific endonuclease domain (*Fok I*), creating a so called zinc finger nuclease (ZFN) (Kim *et al.*, 1996). To induce a double stranded break (DSB) in a target sequence, ZFNs need to be designed in pairs, one targeting each strand, and binding specificity is determined by DNA triplets in each of up to three zinc finger domains. The large-scale application of ZFNs was hampered by the difficulties in protein engineering and the limited availability of zinc finger motives for target site selection. The first report in plants was on the use of ZFNs to create DSBs that stimulated homologous recombination in tobacco (Wright *et al.*, 2005). The next milestone in the field of genome editing was the discovery of a DNA-binding domain from transcription activator-like effectors (TALEs) found in many *Xanthomonas* spp. (Boch *et al.*, 2009; Bogdanove *et al.*, 2010). These TALEs are injected into host cells by the bacterial type III secretion system and, upon entry into the nucleus, bind to TALE-specific DNA to activate gene expression. For the purpose of genome editing the TALE binding domain was fused with the *Fok I* nuclease, substituting the zinc finger domain, generating a TALE nuclease (TALEN) (Christian *et al.*, 2010). Similar to ZFNs, pairs of TALEN need to be designed on both strands of the target sequence, but target site specificity of TALEN, in contrast to ZFNs, can be engineered more easily. The TALE DNA-binding domains consist of tandem amino acid repeats responsible for binding to only one base pair in the target sequence and a so-called repeat-variable di-residue (RVD) in each repeat determines this specificity. Hence, in contrast to ZFNs, specificity of TALEN is determined by a single nucleotide facilitating its design. The TALEN system was first used in plants in a proof-of-concept study in Arabidopsis to mutate *alcohol dehydrogenase 1* (*ADH1*) (Cermak *et al.*, 2011).

The latest breakthrough that advanced genome editing was the engineering of a RNA-guided nuclease complex that makes use of clustered regularly interspaced short palindromic repeats (CRISPR) and a CRISPR associated (Cas9) nuclease, referred to as CRISPR-Cas9 (Jinek *et al*., 2012). This system is based on the adaptive immune system of

bacteria against viruses (Doudna and Charpentier, 2014). For genome editing purposes, the Cas9 nuclease needs to be introduced into the organism together with a guide RNA (gRNA) that guides the complex to the desired target sequence (Sander and Joung, 2014). As the CRISPR-Cas9 system is based on an only 20 nucleotide sequence for target site recognition it is much easier to design than ZFNs and TALENS. Furthermore, for CRISPR-Cas9 only one monomeric protein is required, while the function of ZFNs and TALEN depends on a pair. Hence, numerous studies were published only shortly after the original report for many plant species (Bortesi and Fischer, 2015). These latest advances in genome editing most likely also reflect the increase in publications with the search term '"susceptibility gene" + plant' after 2011 (Figure 1). Furthermore, recent reviews on the application of CRISPR-Cas9 in plants are dedicated to crop improvement and crop protection (Bortesi and Fischer, 2015; Liu *et al.*, 2016, 2017; Yin *et al.*, 2017; Chen *et al.*, 2019; Metje-Sprink *et al.*, 2019) with increasing attention to the impairment of *S* genes (Andolfo *et al.*, 2016; Borrelli *et al.*, 2018; Langner *et al.*, 2018; Zaidi *et al.*, 2018; Das *et al.*, 2019; Mushtaq *et al.*, 2019). One the one hand, these advances represent an enormous opportunity for biology, including resistance breeding, but on the other hand the legislation of genetically modified organisms (GMOs) in Europe currently restrains its applications in agriculture (Eriksson *et al.*, 2020).

TWO SIDES OF THE SAME COIN – ADVANTAGES AND DISADVANTAGES OF USING IMPAIRED *S* GENES

The increasing popularity of the impairment of *S* genes for crop protection and resistance breeding can be explained by several reasons. Firstly, overcoming resistance mediated by an impaired *S* gene is thought to be more challenging for a pathogen than to overcome *R* gene-mediated resistance. The latter is based on the specific recognition of an invasion pattern (IP), such as an effector, of the pathogen by a host IP receptor (IPR) or a R protein (Cook *et al.*, 2015). This recognition can be abolished by, for example, the loss of the effector (de Jonge *et al.*, 2012) or even by only a single nucleotide polymorphism (SNP) in the recognized effector (Joosten *et al.*, 1994). For overcoming loss of susceptibly by an impaired *S* gene, however, the pathogen needs to gain a new manner to establish disease, which is considered more challenging than a loss-of-function mutation. Hence, resistance mediated by impaired *S* genes is considered more durable, which is exemplified by the long-lasting resistance mediated by the barely *mlo* mutant which has been used since 1979 (Buschgel *et al.*, 1997; Lngkjær *et al.*, 2000; Acevedo-Garcia *et al.*, 2014). Secondly, impaired *S* genes are known to not only provide resistance to one strain or race of a given pathogen, but to many if not all (Pavan *et al.*, 2010). This makes their resistance less specific than resistance mediated by *R* genes. This is again exemplified by the *mlo* mutant in barley, for which resistance has not yet

been overcome by any powdery mildew strain tested so far (Jørgensen, 1992; Acevedo-Garcia *et al.*, 2014). Thirdly, impaired *S* genes were also found to provide resistance to multiple pathogens and therefore can lead to broad-spectrum resistance. An example is the cucumber *STAYGREEN* gene, for which a naturally occurring single nucleotide polymorphism confers resistance to three diseases, namely downy mildew, bacterial angular leaf spot and fungal anthracnose (Wang *et al.*, 2018). Lastly, many *S* genes were found to be conserved between plant species, providing the possibility to impair the *S* gene in different plant species to obtain resistance to one or multiple pathogens. For example, silencing of tomato orthologues of the known *S* genes *dmr1* (van Damme *et al.*, 2005) and *powdery mildew resistance 4* (*pmr4*) (Vogel and Somerville, 2000) from Arabidopsis resulted in enhanced resistance to powdery mildew (Huibers *et al.*, 2013). Similarly, silencing of orthologues of six previously identified *S* genes from Arabidopsis in potato led to enhanced resistance to late blight (Sun *et al.*, 2016b). Many of the above-mentioned examples demonstrate why impairment of *S* genes can provide an alternative strategy for resistance breeding, which certainly also explains the increased attention the strategy received over recent years.

As with many methods, also the use of impaired *S* genes has challenges that need to be overcome. One major drawback concerns especially *S* genes in class two, which are negative regulators of plant defence and which are frequently associated with constitutively elevated SA levels. Even though high SA levels might lead to enhanced disease resistance, they can also result in severe fitness costs or pleiotropy. One example is the *defense no death 1* (*dnd1*) mutant, which was isolated in a mutant screening in Arabidopsis for resistance to *P. syringae* (Clough *et al.*, 2000). The *dnd1* mutant accumulated less bacteria than inoculated wild type plants, but at the same time these plants were also severely dwarfed. Nevertheless, such pleiotropic side effects may vary between plant species. It was found that silencing of *DND1* in tomato leads to dwarfing and autonecrosis, while silencing in potato resulted only in mild autonecrosis (Sun *et al.*, 2016a). Another aspect that needs to be evaluated is that mutants with a disturbed hormonal balance might, on the one hand, gain resistance to one pathogen, but on the other hand, loose resistance to another (Thomma *et al.*, 1998). For example, the *symptoms to multiple avr genotypes 4* (*sma4*) mutant is resistant to the necrotrophic pathogen *B. cinerea*, while it became more susceptible to *P. syringae* than wild type plants (Tang *et al.*, 2007). Impairment of *S* genes can also influence other traits, such as sensitivity to abiotic stresses. The previously mentioned *exla2* mutant that shows enhanced resistance to *B. cinerea*, for example, was also more sensitive to salt and cold stress than wild type plants (Abuqamar *et al.*, 2013). In conclusion, these examples illustrate possible side effects of impairing *S* genes which are dependent on the type of gene that is impaired, as well as differences between plant species.

THE NEEDLE IN THE HAYSTACK – IDENTIFICATION OF (NOVEL) *S* GENES

The core component of using impaired *S* genes for any given plant-pathogen interaction is the identification of these genes in the first place. Generally, two main strategies can be pursued to identify *S* genes: forward and reverse genetics. In an attempt to shed light on how *S* genes are generally identified, 168 *S* genes mentioned in an extensive review by van Schie and Takken (2014) were categorised by their method of identification through either forward or reverse genetics. This search revealed that 60 *S* genes (35.7%) were identified via a forward genetics screening, while 108 genes (64.3%) were identified using reverse genetics (Figure 2).

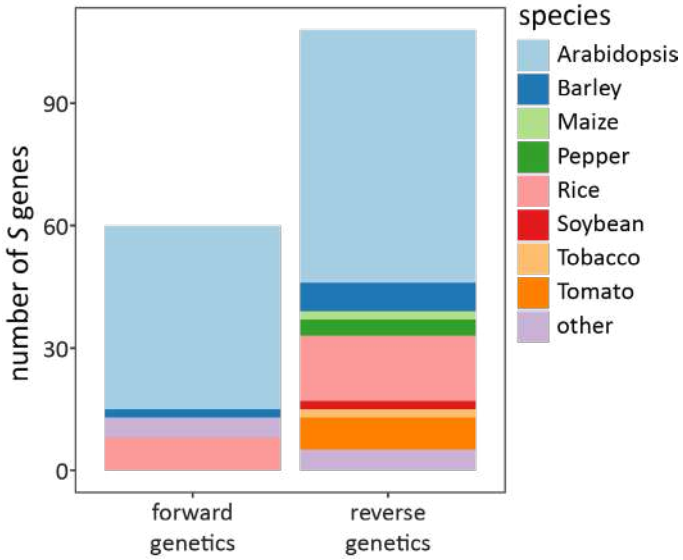


Figure 2 | Number of *S* genes identified via a forward or reverse genetics colour-coded by plant species based on *S* genes listed in the Supplementary Table 1 of van Schie and Takken (2014).

A forward genetics approach entails the screening of a mutant population to identify plants that show loss of susceptibility to a given pathogen. Such assays are only feasible if a large number of mutants can be screened simultaneously and if the phenotyping allows rapid identification of resistant mutants. Hence, the majority of studies which used forward genetics to identify *S* genes were performed in Arabidopsis. Examples are the *pmr* mutants which were isolated from a screening of 26,000 Arabidopsis mutants with *E. cichoracearum* (Vogel and Somerville, 2000). Other studies, especially in crops, made use of forward genetics by mapping naturally occurring resistance. For example, the recessive resistance conferred by *xa5* in rice against *X. oryzae* was cloned and found to be due to a single amino acid change between resistant and susceptible rice plants (Iyer and McCouch, 2004). Moreover, fine mapping of a recessive resistance in an Ecuadorian tomato accession revealed a small deletion in a *mlo* allele associated with powdery mildew resistance (Bai *et al.*, 2008).

The identification of *S* genes via reverse genetics can follow three main strategies. Frequently used are expression analyses followed by functional characterization. Such analyses are based on the fact that pathogens induce transcriptomic changes in the host (van Esse *et al.*, 2009; Su *et al*., 2018), and transcriptomic studies can cover a wide spectrum of research questions. For example, in a study on systemic acquired resistance, a microarray array analysis revealed a negative regulator, *WRKY58*, in Arabidopsis as potential *S* gene (Wang *et al.*, 2006). Another study focused on transcriptional changes induced by fungal trichothecene toxins that revealed the involvement of a homologue of the putative human transcription repressor *NF-X1* (*AtNFXL1*), which was further shown to be a negative regulator in defence to *P. syringae* in Arabidopsis (Masuda *et al.*, 2007; Asano *et al*., 2008). The second reverse genetics strategy is based on the fact that some *S* genes are effector targets or more generally targets of IPs (Gawehns *et al*., 2013). These targets can be identified with a yeast two-hybrid assay as demonstrated for the viral genome-linked protein (VPg) (Huang *et al.*, 2010). The host target of VPg is a DEAD-box RNA helicase from Arabidopsis (AtRH8) and *AtRH8* mutants were found to be resistant to potyviruses. The third reverse genetics strategy focuses on the identification of orthologues of known *S* genes in other plant species. One example is the *suppressor of salicylate insensitivity of npr1-5* (*ssi2*) mutant of Arabidopsis that showed enhanced resistance to *P. parasitica*, presently known as *H. arabidopsidis*, due to an impairment in a stearoyl acyl carrier protein fatty acid desaturase (SACPD) (Shah *et al*., 2001). In soybean, silencing of a *SACPD* orthologue resulted in reduced bacterial titers of *P. syringae* and reduced lesion size in response to *P. sojae* (Kachroo *et al*., 2008). Also in rice, downregulation of a *SACPD* orthologue lowered the number and size of lesions caused by *M. grisea* and *X. oryzae* (Jiang *et al.*, 2009).

FRIEND OR FOE – VASCULAR WILT PATHOGEN *VERTICILLIUM DAHLIAE*

In this thesis, the interaction between the vascular wilt pathogen *Verticillium dahliae* and tomato was explored. *V. dahliae* belongs to the Verticillium genus that comprises ten species, of which also *V. albo-atrum*, *V. alfalfae*, *V. non-alfalfae*, and *V. longisporum* are plant pathogens (Inderbitzin *et al*., 2011). In contrast to the other pathogenic species of the Verticillium genus, *V. dahliae* has a particularly large host range including most, if not all, solanaceous crops such as tomato (Gao *et al*., 2010). Disease incidences with *V. dahliae* have been reported in countries all over the world (Figure 3).

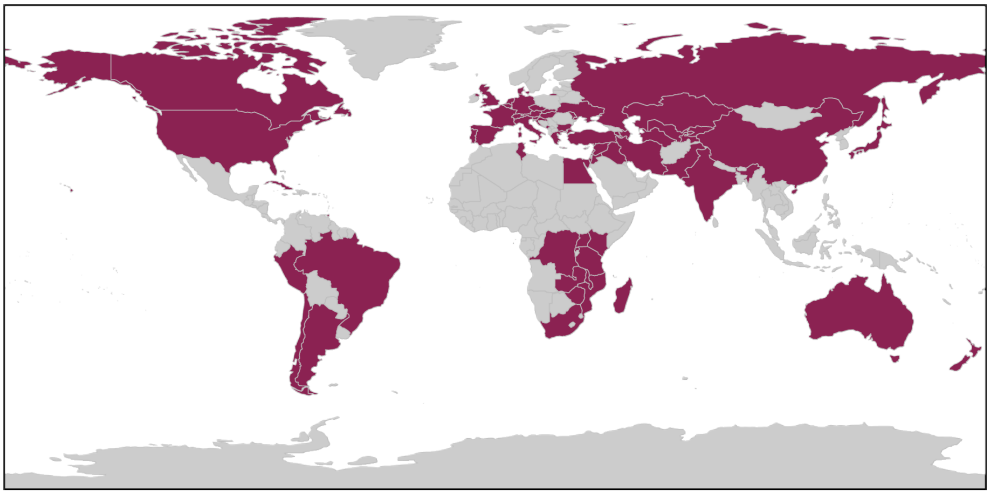


Figure 3 | Global distribution of *Verticillium dahliae*. Data retrieved from EPPO Global Database (April 2020, https://gd.eppo.int/).

As a soil-borne pathogen the life cycle of *V. dahliae* starts and ends in the soil (Figure 4). In the dormant phase, *V. dahliae* can endure in the form of resting structures, so-called microsclerotia, for many years. The parasitic phase is marked by the germination of microsclerotia upon stimuli released by plant roots (Fradin and Thomma, 2006). Emerging hyphae then penetrate root tips or sites of lateral root formation in order to cross the endodermis and subsequently colonize the plant's vascular tissue. *V. dahliae* flourishes inside the xylem where it produces conidia, asexual non-motile spores that can rapidly spread throughout the plants by the xylem sap stream (Klosterman *et al.*, 2011; Yadeta and Thomma, 2013). Finally, the saprophytic phase is characterized by advancing plant senescence, necrosis and tissue decomposition. Through the latter process newly formed microsclerotia are released into the soil.

Owing to its niche colonization and highly sophisticated life cycle, *V. dahliae* is difficult to control in the field. Disease management using crop rotation is not suitable due to the large host range of *V. dahliae*, which also includes many weeds (Fradin and Thomma, 2006). Furthermore, the application of fungicides is generally inefficient as the fungus cannot be reached inside in the plant. Even though *V. dahliae* can be controlled by soil fumigation with methyl bromide, such environmentally hazardous measure is no longer permitted (Inderbitzin *et al*., 2014).

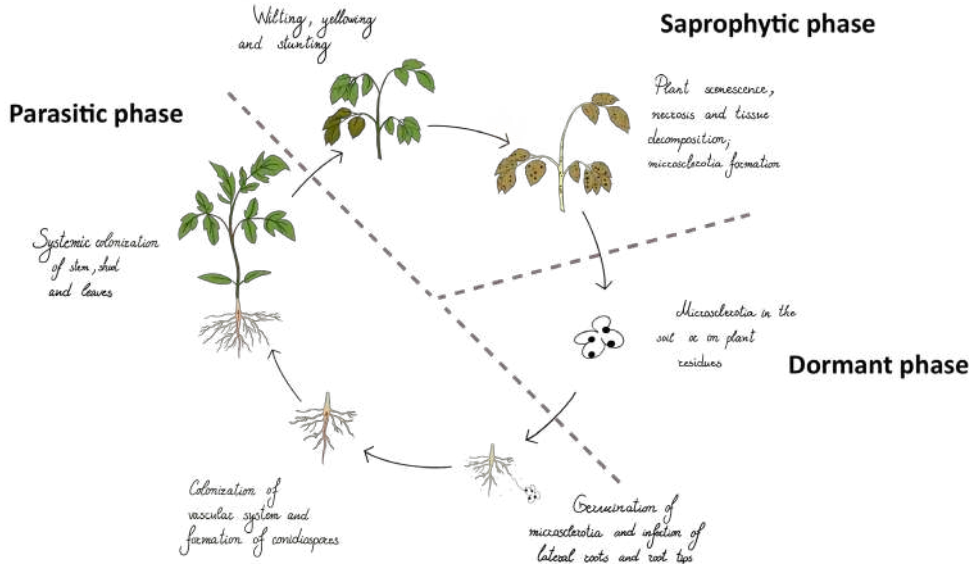


Figure 4 | Disease cycle of *Verticillium dahliae* on tomato (design Tanya Vasileva).

EVERY CLOUD HAS A SILVER LINING – DISEASE RESISTANCE TO *V. DAHLIAE* IN TOMATO

Due to the lack of efficient disease management practises for *V. dahliae* in tomato, the identification of resistance sources has become a prime interest in order to combat Verticillium wilt. So far, the only available monogenic resistance in tomato is mediated by *Ve1* (Kawchuk *et al*., 2001; Fradin *et al*., 2009). Starting in 1952, this resistance has been introgressed into most tomato cultivars (Schaible *et al*., 1951; Labate *et al*., 2007; Fradin *et al*., 2009). Homologs of *Ve1* have been characterized in few other plant species, such as tobacco and eggplant, to provide resistance to *V. dahliae* (Song *et al*., 2017). *Ve1* encodes a cell surface receptor protein that recognizes the *V. dahliae* effector Avirulence on Ve1 tomato (Ave1) (Fradin *et al*., 2009; de Jonge *et al*., 2012). *V. dahliae* strains that carry Ave1, and are therefore recognized by Ve1, are grouped into race 1. The occurrence of *V. dahliae* strains that have purged the *Ave1* gene, collectively named race 2, are posing a reoccurring challenge to crop protection of tomato worldwide (Grogan, 1979; Dobinson *et al*., 1996; de Jonge *et al*., 2012). Another dominant resistance was described more recently in a tomato rootstock, denoted *V2* (Usami *et al*., 2017). Also in this case, *V. dahliae* strains were found that are contained by this resistance, termed race 2, while other strains were able to overcome *V2*-mediated resistance, called race 3. Resistance against *V. dahliae* in other crops such as lettuce or cotton are mostly quantitative in nature, complicating their application in breeding (Atallah *et al*., 2011; Guo *et al*., 2016).

In order to obtain resistance against *V. dahliae* alternative strategies need to be explored, such as the use of impaired *S* genes. So far, only a few genes have been described to act as susceptibility factors in the interaction with *V. dahliae*, most of which were identified in Arabidopsis (Table 1). The role of these genes in susceptibility to *V. dahliae* in other crops such as tomato can be further studied. Moreover, other approaches can be pursued to identify novel *S* genes for *V. dahliae* in tomato.

Table 1 | Previously described/characterized/studied *S* genes for *Verticillium dahliae*.

S gene	Gene product	*S* gene class[1]	Plant species	Pathogen species	Method of identification	Reference
ETR4	Ethylene receptor	I or II	tomato	*V. dahliae*	Reverse genetics (studying the role of ethylene receptors in *V. dahliae* – tomato interaction)	Pantelides *et al.*, 2010
WAT1	Auxin transporter	II	Arabidopsis	*V. dahliae* *V. albo-atrum* *R. solanacearum* *X. campestris*	Forward genetics (screening of cell wall mutants)	Denancé *et al.*, 2013
			cotton	*V. dahliae*	Reverse genetics (orthologue identification)	Tang *et al.*, 2019
PDC1	Pyruvate de-carboxylase	II	Arabidopsis	*V. dahliae* *F. oxysporum*	Reverse genetics (microarray analysis and functional characterization)	Papastolopoulou *et al.*, 2018
ETR1	EF-TU receptor	I or II	Arabidopsis	*V. dahliae*	Reverse genetics (testing different immune signalling mutants)	Gkizi *et al.*, 2016
MPK3	Mitogen-activated protein kinase	II	Arabidopsis			
MPK6						

[1] According to van Schie and Takken (2014)

NECESSITY IS THE MOTHER OF INVENTION – SCOPE OF THIS THESIS

The core of this thesis concerns the identification of *S* genes for *V. dahliae* in tomato by pursing two reverse genetics approaches (Figure 5).

In **Chapter 2**, a collaborative effort was made to set up large scale phenotyping assays for resistance and susceptibility to *V. dahliae* in tomato. Such assays require a reproduceable phenotype to be able to distinguish resistant from susceptible plants. We identified plant canopy area as the most robust phenotyping parameter by calculating stunting of *V. dahliae*-inoculated plants.

In **Chapter 3** transcriptional profiling of a *V. dahliae* – tomato interaction was utilized to identify genes that are specifically induced in a compatible interaction. Subsequent functional validation using virus-induced gene silencing led to the identification of two candidates that could be implicated in Verticillium wilt disease as potential *S* gene for multiple *V. dahliae* strains. Subsequently, these two candidates were further studied by CRISPR-Cas9-mediated genome editing in **Chapter 4**. However, unfortunately, targeted deletion in these two candidate genes could not confirm their involvement in disease susceptibility to *V. dahliae* in tomato.

In **Chapter 5** a reverse genetics approach was pursued to study orthologues of known *S* genes in tomato. Transient silencing of tomato *Walls Are Thin 1* (*WAT1*) resulted in significantly reduced Verticillium wilt development. Hence, we studied the role of *WAT1* further in **Chapter 6** by generating stable knock-down and knock-out lines of tomato through RNAi and CRISPR-Cas9, respectively. Whereas silencing of *WAT1* via RNAi could not confirm loss of *V. dahliae* susceptibility in tomato, targeted deletion in *WAT1* resulted in enhanced resistance to *V. dahliae* as well as to *V. albo-atrum*, albeit this loss of Verticillium susceptibility in *WAT1* mutant lines was accompanied by severe growth defects.

Finally, in **Chapter 7** the major results of this PhD thesis are discussed and implications of this work on further studies on *S* genes for *V. dahliae* are given.

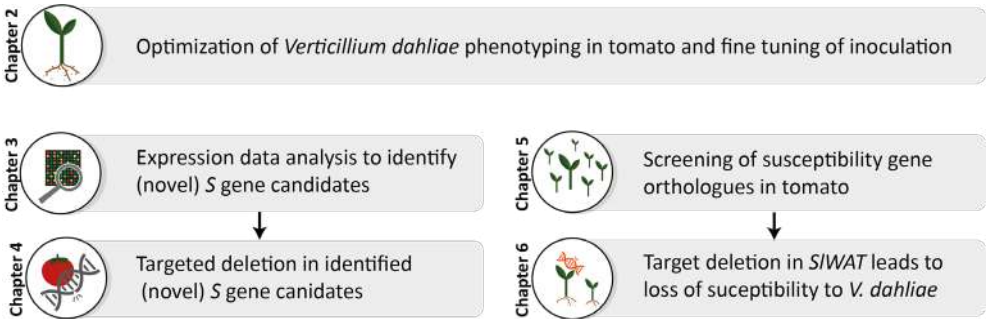


Figure 5 | Schematic outline and strategy pursued in this PhD thesis to identify *S* genes for *Verticillium dahliae* in tomato.

Chapter 2

Evaluation of phenotyping and inoculation procedures for the identification of enhanced resistance against *Verticillium dahliae* in tomato

Jasper P. Vermeulen*, Katharina Hanika*, Bart P.H.J. Thomma, Yuling Bai, Henk J. Schouten

* These authors contributed equally to this work.

ABSTRACT

Plants possess an innate immune system that provides resistance against most pathogens and pests. Still, particular pathogens evolved to overcome this resistance. In agriculture, a common approach to control such pathogens is to breed for genetic resistance. This approach aims at the exploration of plant germplasm for resistance sources, identification of the underlying resistance genes or quantitative trait loci (QTLs), followed by the introduction of these genes or QTLs into commercial cultivars. In order to reliably explore germplasm for resistance sources, an accurate method for phenotyping is essential. In other words, the phenotyping method should provide a good "discriminative power" to be able to detect differences in symptom expression between host genotypes. In the search for resistance against the vascular wilt pathogen *Verticillium dahliae*, various symptoms have been used to score disease development. The most commonly scored symptoms are reduced overall plant stature and size (stunting) and foliar symptoms such as yellowing, chlorosis and necrosis. In this study, we compared the discriminative power of several plant size-related disease symptoms on tomato in *V. dahliae* resistance screens. We obtained the highest discriminative power to detect differences in symptom expression between host genotypes by measuring the canopy area of *V. dahliae*-inoculated plants. The discriminative power was furthermore higher at 21 days after inoculation than at 14 days after inoculation. To assess whether the discriminative power of scoring the canopy area as a phenotyping method could be further optimized, we attempted to streamline the root-dipping inoculation method. We could not find a meaningful effect on the discriminative power by increasing the inoculum concentration, trimming of the roots prior to dipping them in inoculum or applying nutrients to the soil after the inoculation.

INTRODUCTION

Plants are continuously exposed to a wide range of organisms, including a wide diversity of insects and microbes, some of which are potentially harmful. To protect themselves against pests and pathogens, plants possess a sophisticated immune system. Nevertheless, particular pests and pathogens can overcome these immune responses and cause disease. For example, the tomato is susceptible to over 200 different plant pests and pathogens (Jones *et al*., 2014). Although the use of chemical control agents has been a common approach to tackle these organisms, the use of such chemicals has become increasingly restricted over the last decades (Lamichhane *et al*., 2016). Another strategy to combat pests and pathogens is to breed for resistance (Bai and Lindhout, 2007, 2008). Like many other genetic traits, also resistance can be classified as either qualitative or quantitative (Corwin and Kliebenstein, 2017). Qualitative resistance is genetically relatively simple and is based on single resistance (*R*) or susceptibility (*S*) genes, whereas quantitative resistance is based on multiple, often small-effect loci. Because of its simple genetics, qualitative resistance has been studied and used in breeding more frequently than quantitative resistance (St.Clair, 2010). As quantitative resistance is based on many loci that often make a limited contribution on their own, carefully designed disease screens and sensitive, accurate and robust phenotyping methods are crucial to successfully study the genetics of such resistance.

Vascular wilt pathogens typically concern soil-borne organisms that infect plants through the roots and invade the xylem vessels to spread to distal tissues of the plant host, often leading to wilting symptoms (Yadeta and Thomma, 2013). Throughout the majority of the disease cycle, the pathogen is confined to the interior of the plant and pathogen colonization is often difficult to assess. An important vascular pathogen of tomato is *Verticillium dahliae*, an ascomycete fungus that belongs to the Verticillium genus. The Verticillium genus consists of ten species, of which five have a mostly saprophytic lifestyle, and the other five are plant pathogens (Inderbitzin *et al*., 2011). *V. dahliae* infects its hosts through the roots and then attempts to penetrate the root cortex to enter the xylem. Once in the xylem, it produces conidiospores that are carried with the sap stream and spread throughout the plant. Although symptoms depend largely on the host and environmental conditions, they may include stunting, vascular browning, wilting, yellowing and necrosis of the leaves (Fradin and Thomma, 2006). Because *V. dahliae* resides inside the plant throughout most of the disease cycle, most fungicides are ineffective. However, resistance breeding is an alternative approach to control this disease.

Although several resistance sources to *V. dahliae* have been identified in tomato, only one resistance gene, *Ve1*, has been successfully mapped and cloned thus far (Kawchuk *et al*., 2001; Fradin *et al*., 2009). Soon after the deployment of this resistance gene, resistance-overcoming race 2 strains emerged (Robinson, 1957; Alexander, 1962).

Therefore, identification of additional resistance genes or quantitative trait loci (QTLs) is desired to control *V. dahliae* race 2 strains. Although QTLs have been identified for *V. dahliae* resistance in other crops (Bolek *et al*., 2005; Rygulla *et al*., 2008; Wang *et al*., 2008; Zhao *et al*., 2014; Antanaviciute *et al*., 2015; Toppino and Barchi, 2016), these studies often report quantitative resistance based on multiple loci, often with small effects. To detect such small-effect loci, accurate methods are required to quantify *V. dahliae* symptoms or the colonization in plants. In other words, these methods should provide a high "discriminative power" to be able to accurately detect differences in symptom expression or pathogen colonization between host genotypes.

Generally, research on resistance against *V. dahliae* is performed under controlled conditions, to be able to achieve robust inoculation protocols that result in reproducible disease phenotypes. One of the most commonly used *V. dahliae* inoculation methods for tomato is the root-dipping method, which involves dipping tomato roots directly into a *V. dahliae* conidiospore suspension (Fradin *et al*., 2009; Shittu *et al*., 2009; Uribe *et al*., 2014; Parisi *et al*., 2016; Papadaki *et al*., 2017). Alternative inoculation methods include the injection of conidiospores into the stem, watering of the soil with conidial suspensions, or the introduction of microsclerotia into the soil (Bletsos *et al*., 2003; Antoniou *et al*., 2008; Buhtz *et al*., 2015; Jiménez-Díaz *et al*., 2017; Depotter *et al*., 2019). Because *V. dahliae* symptoms vary considerably among host species, methods to phenotype

V. dahliae also differ between host species (Fradin and Thomma, 2006). In tomato, the most common methods for phenotyping are scoring systems that categorize plants based on the severity of foliar symptoms such as wilting or yellowing (Busch and Smith, 1981; Chen *et al*., 2004; Shittu *et al*., 2009; Jiménez-Díaz *et al*., 2017). For example, plants can be scored based on the number of leaves that are affected by yellowing and wilting on a scale from 0 – 5 (Shittu *et al*., 2009). Other methods focus on quantitative determination of the impact of *V. dahliae* infection on elements of plant development, for example by measuring reduced plant height, stem length, fresh weight or canopy diameter, collectively referred to as stunting (Yadeta, 2012; Papadaki *et al*., 2017). Finally, methods exist to quantify the colonization of *V. dahliae* using real-time PCR, or to qualitatively assess resistance by fungal outgrowth assays (Lievens *et al*., 2006; Fradin *et al*., 2009). Since solely the scoring of symptom expression cannot always distinguish tolerant from resistant plants, quantification of the colonization of *V. dahliae* needs to be performed to conclusively determine whether a host genotype is resistant to *V. dahliae*. However, these methods are generally more laborious because they require further processing of samples, in contrast to the direct measurements of symptoms. These methods are therefore too labour-intensive for large-scale resistance screens or QTL mapping experiments and thus we focus on plant growth parameters to phenotype Verticillium wilt in tomato.

Our long-term goal is to screen for novel sources of resistance against *V. dahliae* in tomato. However, to accommodate large-scale screens, we decided to first compare phenotyping methods in order to identify the most robust method to be performed in our plant growth facilities. Next, we optimized inoculation procedures to further optimize the robustness of our disease screens to, eventually, be able determine the most effective method to detect differences in disease symptoms between different host genotypes.

MATERIALS & METHODS

Plant and pathogen material

All tomato accessions (Table 1) were grown in potting soil (Potgrond 4, Horticoop, Katwijk, The Netherlands) in the greenhouse (Unifarm, Wageningen University & Research, the Netherlands) at 25°C/19°C (day/night) with 60% relative humidity and a minimal light intensity of 100 W/m^2. *Verticillium dahliae* race 2 strain DVDS26 was grown on potato dextrose agar (PDA) at room temperature in the dark.

Table 1 | Tomato genotypes used in this study.

Species	Accession
Solanum lycopersicum	Moneymaker
Solanum lycopersicum	Moneymaker 35S:Ve1
Solanum pimpinellifolium	VG-3
Solanum cheesmanii	VG-20
Solanum pimpinellifolium	VG-21
Solanum pimpinellifolium	VG-22
Solanum pimpinellifolium	VG-55
Solanum pimpinellifolium	VG-63
Solanum lycopersicum Moneymaker x *Solanum pimpinellifolium* VG-3	RIL660 (F6)
Solanum lycopersicum Moneymaker x *Solanum pimpinellifolium* VG-3	RIL708 (F6)

V. dahliae inoculation & phenotyping

To screen the tomato accessions, *V. dahliae* inoculations were carried out with the root dipping method as described by Fradin *et al*. (2009). Phenotyping was done at 14- and

21-days post-inoculation (dpi) by measuring the stem diameter (cm) just above the cotyledons with a digital calliper and by taking top- and side-view pictures of the plants. Plant height (cm) was measured from the side-view pictures from the cotyledons upwards, and canopy area (cm^2) and canopy diameter (cm) were measured from the top view pictures using ImageJ (Schneider *et al.*, 2012). Stunting was calculated as follows:

$$stunting\ (\%) = \left(1 - \frac{canopy\ area\ of\ V.\ dahliae{-}inoculated\ plant}{average\ canopy\ area\ of\ mock{-}inoculated\ plants}\right) \times 100.$$

For the comparison of different inoculation protocols, two inoculum concentrations (1 x 10^6 and 1 x 10^7 conidiospores/mL) were used and roots of half of the plants were trimmed to approximately 1 cm. Also, at two and at three weeks after inoculation, a nutrient solution (Supplementary Table 1) was applied twice per week to half of the plants.

Estimating the discriminative power of the resistance test

Ten tomato genotypes (Table 1) were inoculated with the race 2 strain *V. dahliae* DVDS26. At 14- and 21 days post-inoculation, the stem diameter, plant height, canopy diameter, and canopy area were scored on mock-inoculated and *V. dahliae*-inoculated plants as described above. To estimate the discriminative power of the *V. dahliae*-associated symptoms, a one-way ANOVA was performed on these measurements. In this ANOVA, we tested for each symptom, per genotype, for significant differences between mock-inoculated and *V. dahliae*-inoculated plants. The experiment number was included in the analysis as a blocking factor. Where necessary, we performed a square-root or log10-transformation to guarantee the data met the normality and equality of variance assumptions. To estimate the discriminative power of each *V. dahliae*-associated symptom, the F-value from the ANOVA of the interaction between genotype * treatment was used. This F-value reflects the significance of the difference of the effect of the *V. dahliae* inoculation on plant size between genotypes.

RESULTS

Canopy area measurements at 3 weeks after inoculation provide the highest discriminative power to distinguish resistance level differences

In order to explore tomato germplasm for sources of resistance against race 2 *V. dahliae* strains, we queried for a phenotyping method to score Verticillium wilt disease that would provide the highest degree of resolution. In other words, this phenotyping method should provide the best discriminative power to best detect differences in *V. dahliae* symptoms between host genotypes. This discriminative power can be estimated with a one-way ANOVA, by testing which method yields the most significant differences in symptom expression between genotypes. Using a panel of ten tomato genotypes (Table 1), we compared the discriminative power of multiple phenotyping methods. Based on our earlier observations in greenhouse trials with *V. dahliae*-inoculated tomato plants, for instance the trials described by Fradin *et al*. (2009) and Yadeta (2012), we know that symptoms start to develop around 10 dpi, after which they aggravate. Taking the practicalities of large-scale screenings in mind, in which we prefer to terminate experiments 3 weeks after inoculation, we decided to focus the phenotyping efforts on 14 and 21 dpi. Initially, we aimed to assess both the scoring foliar *V. dahliae* symptoms and plant-size related Verticillium wilt symptoms. However, unfortunately, some of the tomato genotypes, especially S. cheesmanii VG-20, developed yellowing of the lower leaves in mock-inoculated plants (Supplementary Figure 1). Simultaneously, several plants of the susceptible Moneymaker did not develop apparent yellowing symptoms upon inoculation with *V. dahliae* strain DVDS26. Therefore, we concluded that yellowing cannot be used as a symptom that is consistently associated with Verticillium wilt disease. Consequently, we discarded yellowing of leaves as a trait to be scored for *V. dahliae* susceptibility. Therefore, we continued with measurements of the reduction in stem diameter, canopy diameter, canopy area, and plant height on mock-inoculated and *V. dahliae*-inoculated plants. Of these parameters, canopy area displayed the highest discriminative power both at 14 days and 21 days post-inoculation. Moreover, the discriminative power of the canopy area at 21 dpi was higher than at 14 dpi (Figure 1).

Given the typically relatively large degree of variation in *V. dahliae* symptoms among plants of the same genotype, we also assessed the effect of the removal of outliers on the discriminative power of each of the symptoms. Removal of these outliers (Supplementary Table 3) yielded a clear effect on the canopy area and canopy diameter, as it increased the discriminative power both at 14 and 21 dpi. At 14 dpi, outlier removal also resulted in a slight increase in the discriminative power of plant height and stem diameter. Overall, our analysis thus demonstrates that canopy diameter measurements at 21 dpi provide the highest discriminative power to distinguish resistant from susceptible genotypes, and that outlier removal may help to further improve the discriminative power of the resistance test.

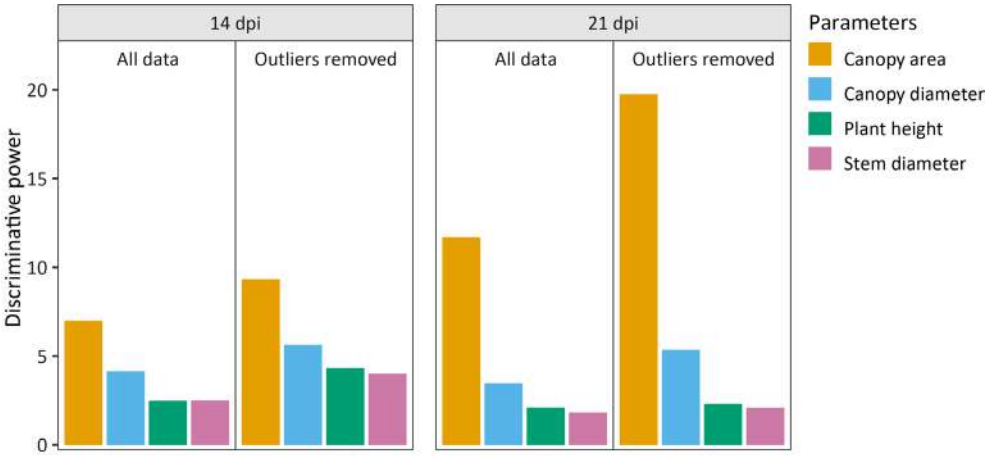


Figure 1 | Estimated discriminative power of canopy area, canopy diameter, plant height and stem diameter at 14 and 21 days after inoculation with *Verticillium dahliae* DVDS26, based on the complete dataset and on the dataset from which outliers were or were not removed based on the studentized residuals (Supplementary Table 3). Discriminative power was estimated with a one-way ANOVA, of which the F-value of the interaction genotype * treatment was used as the value for discriminative power.

Optimization of the inoculation procedure

In order to assess whether the scoring of canopy area at 21 dpi as a phenotyping measure could be optimized further, an attempt was made to optimize the inoculation method. To do this, two wild tomato accessions that were previously found by Yadeta (2012) to display a relatively high degree of resistance against *V. dahliae* race 2 were selected together with the susceptible control Moneymaker. The effect of three modifications of the root-dipping inoculation method on the discriminative power of the disease test was evaluated. First, the effect of the inoculum concentration was tested by increasing the conidiospore concentration from 106 to 107 conidiospores/ml (Fradin *et al*., 2009; Parisi *et al*., 2016; Jiménez-Díaz *et al*., 2017; Tsolakidou *et al*., 2019). Secondly, the addition of nutrients (Supplementary Table 1) twice a week after the second week post sowing was assessed. Finally, trimming of the roots before inoculation was tested as this has been suggested to promote *V. dahliae* infection (Parisi *et al*., 2016; Papadaki *et al*., 2017).

In contrast to the different phenotyping methods, only minor differences in the discriminative power of the different inoculation methods were detected (Figure 2). No significant effect of the increase in conidiospore concentration on stunting of *V. dahliae*-inoculated plants was found for any of the tested genotypes, neither with or without root-trimming or nutrient application (Figure 3). Furthermore, while the addition of nutrients significantly increased the canopy area of mock-inoculated plants of all genotypes (Supplementary Figure 2), we found that the addition of nutrients had

no significant effect on *V. dahliae*-induced stunting for any of the genotypes (Figure 3). Interestingly, the application of nutrients reduced the overall variation in stunting of *V. dahliae*-inoculated VG-20 plants and reduced the number of yellowing leaves of mock-inoculated plants (Supplementary Figure 1). Finally, although no effect of root-trimming on the canopy area of mock-inoculated plants was detected (Supplementary Figure 2), also no effect of root-trimming on *V. dahliae*-induced stunting was observed for any of the genotypes (Figure 3). Collectively, our data thus indicates that increasing the conidiospore concentration, root-trimming and nutrient application do not improve the discriminative power of the resistance test.

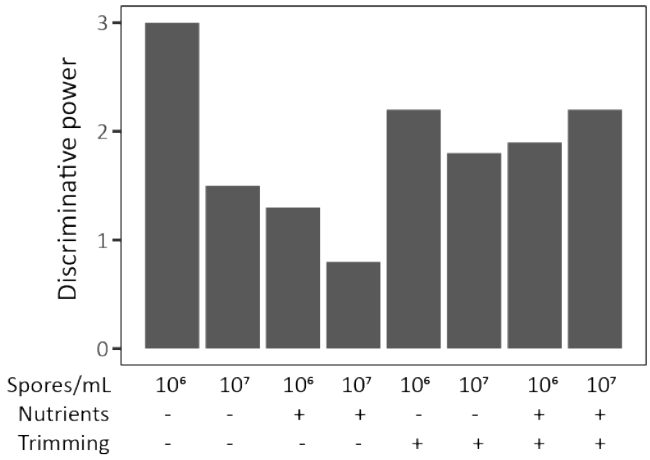


Figure 2 | Estimated discriminative power of canopy area at 21 days after inoculation with *Verticillium dahliae* DVDS26. Outliers were removed based on the studentized residuals (Supplementary Table 4). Discriminative power was estimated with a one-way ANOVA, of which the F-value of the interaction between the genotype and the treatment.

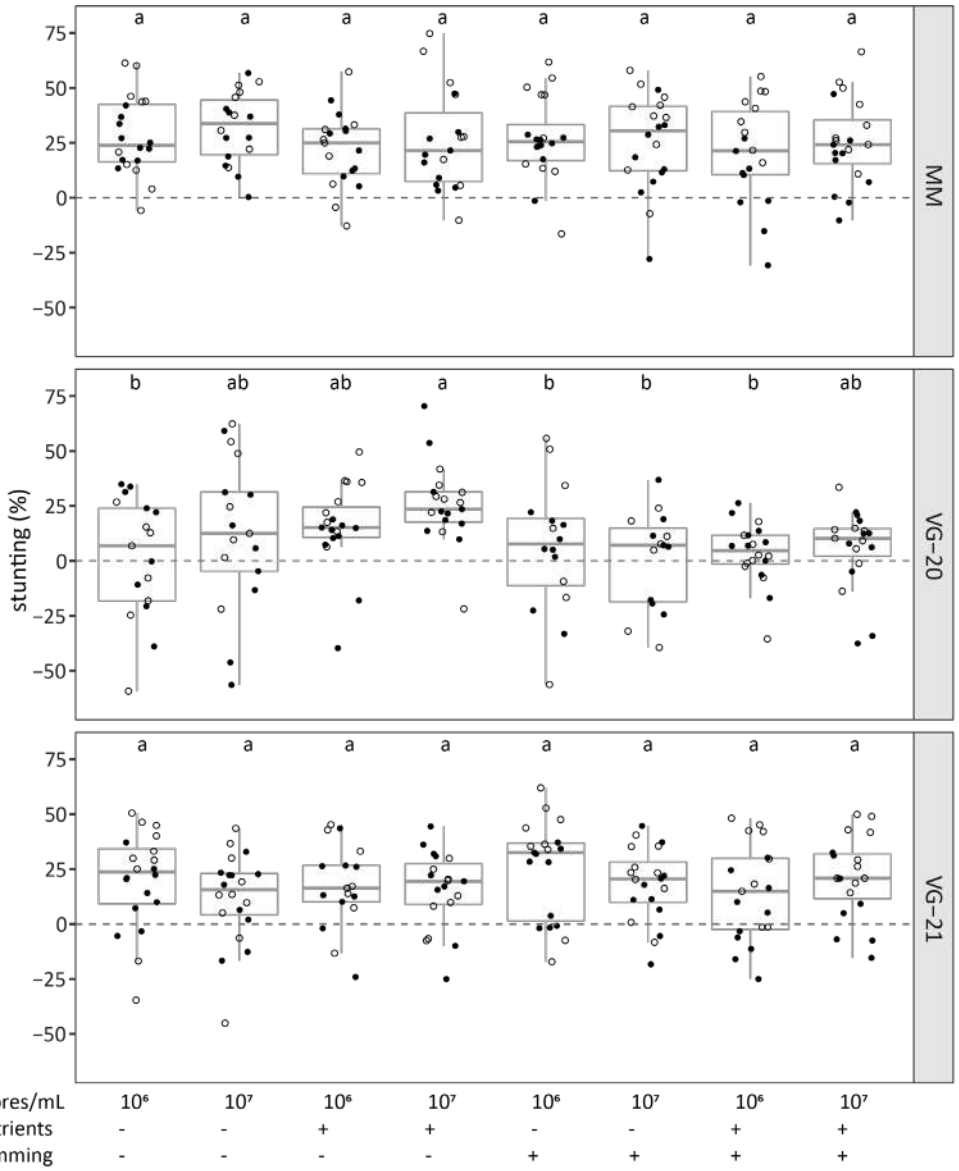


Figure 3 | Stunting (%) based on the canopy area of *Verticillium dahliae*-inoculated plants when compared with mock-inoculated plants at 21 dpi of the tomato genotypes Moneymaker (top), VG-20 (middle) and VG-21 (bottom). Conidiospore concentration, addition of nutrients and trimming of the roots were compared. The depicted data comes from two independent experiments (filled versus non-filled dots) with n ≥ 9 (ANOVA with Fisher's unprotected LSD, α = 0.001).

DISCUSSION

Since the emergence of *V. dahliae* race 2 strains on tomato, several additional resistance sources to Verticillium wilt were reported in tomato and other crops (Okie and Gardner, 1982; Okie and Gardner, 1982; Laterrot, 1984; Baergen *et al*., 1993; Stamova, 2005; Klosterman *et al*., 2009; Yadeta, 2012; Usami *et al*., 2017). Although these studies all had the similar goal to identify resistances to Verticillium wilt and to unravel the underlying genetics, the methods to inoculate plants and subsequently phenotype Verticillium wilt symptoms varied considerably among these studies.

Typically, disease screens aim to compare disease symptoms among different host genotypes or different treatments, to draw conclusions about the effect of the host genotype or treatment on the susceptibility/resistance against the disease. To best be able to draw such conclusions, the phenotyping method must have a high discriminative power to detect differences in symptom expression between host genotypes. In this chapter, we compared several methods to measure *V. dahliae* symptoms on tomato plants. By comparing the effect of *V. dahliae*-inoculation on the plant height, stem diameter, canopy area and canopy diameter, we demonstrated that some parameters better detect differences in symptom expression among tomato genotypes than other methods. We demonstrated that differences in symptom expression were most profound when the canopy area is measured at 21 dpi. The effect of *V. dahliae* -inoculation differed less profoundly between the tomato genotypes when stem diameter or plant height were determined.

The differences in the discriminative power of the parameters that were assessed in our study are not very surprising. First of all, the accuracy of some measurements may be better than that of other measurements. For example, we observed that the thickness of the stems of some wild tomato accessions was irregular, increasing variation in the stem diameter measurements and thereby likely reducing its discriminative power. Secondly, the difference in discriminative power of the canopy area and canopy diameter may be because canopy diameter solely measures the distance between the furthest leaf tips. Canopy area, in contrast, captures the area of the entire canopy and therefore also captures changes in leave size. Besides differences in the accuracy of the measurements, *V. dahliae* infection may also affect specific aspects of plant size to a more considerable extent than it affects other aspects. The higher discriminative power of canopy area and canopy diameter may thus also indicate that Verticillium infection has a stronger effect on canopy size than it has on plant height or stem diameter. In agreement with this, the susceptible control Moneymaker displayed a greater reduction in canopy area and canopy diameter than in plant height or stem diameter upon inoculation with *V. dahliae* (Supplementary Table 2).

Overall, our analysis thus demonstrates that when searching novel resistance sources against *V. dahliae*, determining the right parameter to score disease symptoms is an important success factor for finding such resistance sources, including the subsequent QTL mapping analyses. Based on the appropriate parameter, the stunting of *V. dahliae*-inoculated plants should be calculated relative to mock-inoculated plants of the same genotype instead of directly comparing surface areas. In this way, genotypes which differ in size in absence of *V. dahliae* inoculation can still be compared with respect to their *V. dahliae* susceptibility.

Besides growth-related Verticillium wilt symptoms, many studies also score foliar symptoms such as wilting, yellowing and necrosis of the leaves. Thus, we initially aimed to also include these symptoms in our analysis. However, our Moneymaker plants did not develop apparent yellowing symptoms on all *V. dahliae*-inoculated plants, neither at 14 dpi nor at 21 dpi. Simultaneously, mock-inoculated VG-20 plants also developed yellowing leaves in nutrient-deficient conditions (Supplementary Figure 1), indicating that yellowing of leaves is not always necessarily associated with *V. dahliae* infection. This thus indicates that yellowing symptoms could not be used to accurately identify genotypes which are more resistant than Moneymaker in our set of tomato germplasm. Besides testing different phenotyping parameters to measure disease caused by *V. dahliae*, we also compared different inoculation methods to further optimize our assays. We assessed the effect of conidiospore concentration, nutrient application, and root-trimming on the discriminative power of the disease test. Although the highest discriminative power was found with 106 conidiospores/ml without trimming of roots and nutrient applications (Figure 2), the differences in discriminative power between inoculation methods were much less pronounced than the differences between the different phenotyping methods (Figure 1). No clear differences in stunting were observed between the different inoculation treatments. Firstly, the two most commonly used conidiospore concentrations were tested for their effect on stunting (Fradin *et al*., 2009; Parisi *et al*., 2016; Jiménez-Díaz *et al*., 2017; Tsolakidou *et al*., 2019). As no difference was found, it can be concluded that a concentration of 106 conidiospores/ml is sufficient to result in a robust *V. dahliae* infection. Possibly, the use of a higher conidiospore concentration does not increase stunting, as it does not lead to more penetration sites or higher levels of xylem colonization. Potentially, the use of a lower concentration may reduce the infection efficiency, but this would need to be tested further.

Some studies describe the application of nutrients during disease assays with *V. dahliae* (Shittu *et al*., 2009; Parisi *et al*., 2016; Jiménez-Díaz *et al*., 2017). However, a direct effect on disease development was not evaluated in those studies. In our study, the addition of nutrients did not affect overall stunting of the genotypes, although it mitigated yellowing in VG-20 and reduced the overall variation of this genotype (Figure

3 and Supplementary Figure 1). This suggests that adding nutrients can affect symptom development for particular genotypes.

Finally, we found that root-trimming as described in previous studies (Parisi *et al*., 2016; Papadaki *et al*., 2017) does not aid *V. dahliae* infection, as no effect on stunting was found. It may be speculated that root-trimming creates additional wounds which can be used by *V. dahliae* as an entry point for infection. However, roots have many natural openings, such as sites of lateral root emergence, and furthermore they are unavoidably further damaged during uprooting before inoculation. Consequently, the potential benefit of root-trimming for *V. dahliae* infection may be limited. Furthermore, trimming drastically reduces the size of the roots and therefore reduces the available root surface to which conidiospores could attach. As no beneficial effect of root-trimming on stunting could be found, we demonstrate that it is not necessary to trim the roots before infection under our experimental conditions.

Collectively, our study demonstrated that canopy area at 21 days post-inoculation yields the best discriminative power to detect differences in *V. dahliae*-symptom expression in our set of tomato genotypes and under our experimental conditions. Furthermore, we demonstrated that applying nutrients to the soil post-inoculation, trimming the roots of seedlings prior to inoculation or increasing the inoculum concentration from 106 to 107 conidiospores/ml has no meaningful beneficial effect on the discriminative power of the resistance test.

SUPPLEMENTARY MATERIAL

Supplementary Table 1 | Composition of nutrient solution used in this study.

Macro-elements	mmol/L	Micro-elements	µmol/L
NH_4	1.2	Fe	35.0
K	7.2	Mn	8.0
Ca	4.0	Zn	5.0
Mg	1.82	B	20.0
NO_3	12.4	Cu	0.5
SO_4	3.32	Mo	0.5
P	1.0		

Supplementary Table 2 | Plant size parameters of the susceptible controls.

Treatment group	Canopy area (cm^2)		Canopy diameter (cm)		Height (cm)	
	Moneymaker	Moneymaker 35S:Ve1	Moneymaker	Moneymaker 35S:Ve1	Moneymaker	Moneymaker 35S:Ve1
***V. dahliae*-inoculated plants**	301.89 (102.6)	266.66 (80.96)	30.37 (5.96)	28.27 (5.23)	27.52 (4.74)	21.89 (3.80)
Mock-inoculated plants	527.38 (88.99)	470.68 (114.59)	42.43 (5.07)	39.24 (3.61)	31.69 (2.80)	24.95 (3.80)
Stunting of *V. dahliae*-inoculated plants	42.76 (19.4)	43.35 (17.2)	28.41 (14.1)	27.97 (13.32)	13.17 (14.95)	12.28 (15.22)

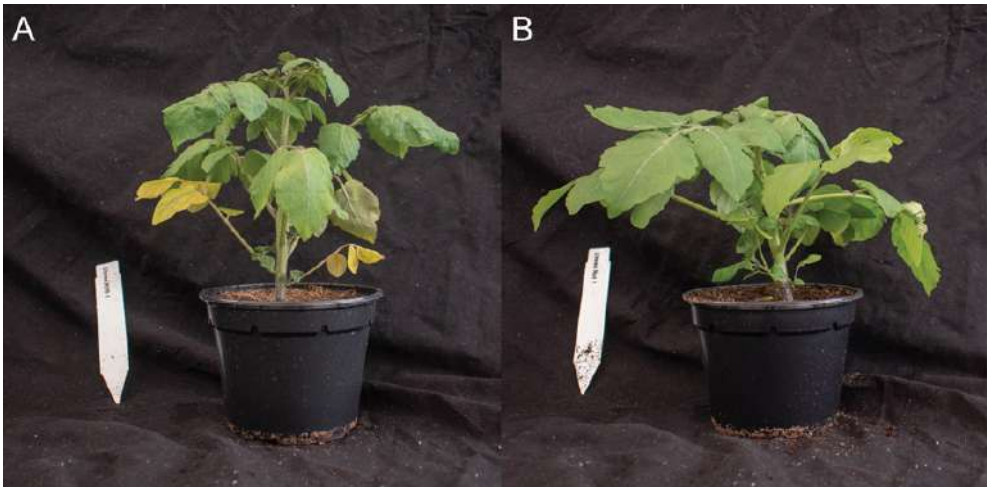
Averages of canopy area, canopy diameter and height of mock-inoculated and *Verticillium dahliae*-inoculated plants of the susceptible control Moneymaker and race 1 resistant control Moneymaker 35S:Ve1. Plants were inoculated with the *V. dahliae* race 2 strain DVDS26. Stunting represents the reduction in size of *V. dahliae*-inoculated plants relative to the average size of the mock-inoculated plants of the same genotype. Numbers in brackets indicate the standard deviation.

Supplementary Table 3 | Number of removed outliers in the analysis of the data presented in Figure 1.

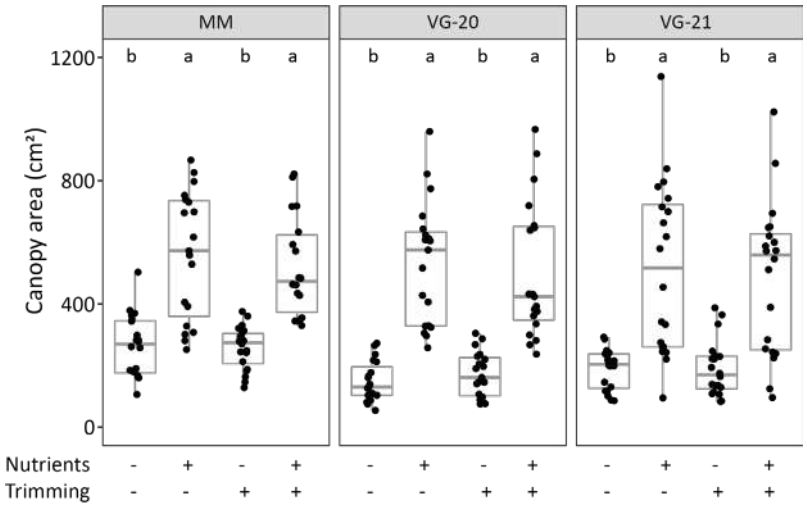
Genotype	14 days post inoculation				21 days post inoculation			
	Canopy area	Stem diameter	Plant height	Canopy diameter	Canopy area	Stem diameter	Plant height	Canopy diameter
Moneymaker	1	0	0	0	0	2	0	2
Moneymaker 35S:Ve1	0	2	0	0	0	0	1	0
VG-3	0	1	0	0	2	1	0	0
VG-20	2	1	0	0	1	0	2	0
VG-21	0	2	0	0	1	0	0	0
VG-22	0	1	1	2	0	0	1	1
VG-55	3	0	0	3	2	1	0	1
VG-63	0	0	2	0	2	1	0	2
RIL660	0	2	1	0	0	2	2	2
RIL708	0	0	0	0	0	0	0	2
Total	6	9	4	5	8	7	6	10

Supplementary Table 4 | Number of removed outliers in the analysis of the data presented in Figure 2.

Trimming	-	-	-	-	+	+	+	+
Nutrients	-	-	+	+	-	-	+	+
Inoculum concentration	10^6	10^7	10^6	10^7	10^6	10^7	10^6	10^7
Moneymaker	0	0	0	0	0	0	0	0
VG-20	3	2	0	0	3	3	0	0
VG-21	0	0	2	1	0	0	1	1
Total	3	2	2	1	3	3	1	1



Supplementary Figure 1 | Appearance of non-inoculated plants of *Solanum cheesmanii* VG-20 at four weeks after sowing without nutrient addition (A) or after the receipt of additional nutrients twice a week after the second week post sowing (B).



Supplementary Figure 2 | Canopy area (cm^2) of mock-inoculated plants (21 dpi) with and without the addition of nutrients and with and without trimming of the roots. Different letter labels indicate significant differences as determined with a one-way ANOVA followed by a fishers LSD test ($p < 0.001$).

Chapter 3

Expression analysis-based selection and functional analysis of candidate susceptibility genes for vascular wilt disease caused by *Verticillium dahliae* in tomato

Katharina Hanika, Dagmar Kuiper*, Timo d'Hont*,
Jillis Grubben*, Marian Oortwijn, Henk J. Schouten,
Bart P.H.J. Thomma, Yuling Bai

* These authors contributed equally to this work.

ABSTRACT

The use of resistant plant varieties represents a core strategy for sustainable agriculture to reduce crop losses due to pests and diseases, which is often based on qualitative resistance. However, depending on the pathogen and crop species, qualitative resistance is not always available. An example is the vascular pathogen *Verticillium dahliae* for which the only available monogenic resistance is broken, posing a recurrent problem for agriculture. The use of impaired disease susceptibility (*S*) genes, which encode host components essential for the pathogen to establish disease, offers a promising alternative approach to obtain disease resistance in crops. As only a few *S* genes are known in Arabidopsis for *V. dahliae*, we combined transcriptional profiling with a reverse genetics approach to identify novel *S* gene candidates for *V. dahliae* in tomato in this study. Since many *S* genes are induced upon pathogen challenge, we filtered publicly available expression data for induced genes in a compatible interaction that were not induced in an incompatible interaction and identified genes that were expressed in foliage or roots, respectively. In total we identified 100 and 262 genes induced in foliage and roots, respectively. To allow the identification of potential *S* genes, the most highly induced genes were selected and functional analysis in tomato was performed by virus-induced gene silencing (VIGS) in combination with *V. dahliae* inoculation. Out of 135 genes tested, two could be implicated in Verticillium wilt disease as potential *S* gene for multiple race 2 strains of *V. dahliae*. Both candidates will be studied further to confirm their role as *S* gene in the interaction of *V. dahliae* with tomato.

INTRODUCTION

Within the core of strategies for sustainable agriculture lays the use of resistant plant varieties to minimize losses due to pests and diseases and to reduce the use of chemicals for crop protection (Bai and Lindhout, 2007; Bharadwaj, 2016). Most commonly exploited in resistance breeding is the introgression of dominant resistance (*R*) genes from a wild species into an elite cultivar. *R* genes belong to a wider group of host genes that encode invasion pattern receptors (IPRs) for recognizing invasion patterns (IPs), microbial- or host-derived molecules of various nature that reliably betray pathogen invasion, to activate IP-triggered resistance (IPTR) (Cook *et al*., 2015). Such qualitative *R* gene-mediated resistance is often quickly overcome by fast evolving pathogen populations that can break the resistance by evolving strains that are no longer recognized. Other resistance mechanisms are more complex and quantitative in nature, and thought to be more difficult to overcome for pathogen populations (Corwin and Kliebenstein, 2017).

For the vascular wilt pathogen *Verticillium dahliae* only one monogenic resistance gene has been described for tomato, known as *Ve1*, of which homologues are distributed in several other plant species (Fradin *et al*., 2009; Song *et al*., 2017). Other resistances in crops such as cotton and lettuce are mostly quantitative in nature (Atallah *et al*., 2011; Guo *et al*., 2016). *Ve1* recognizes the *V. dahliae*-secreted effector protein Avirulence on Ve1 tomato (Ave1) which triggers the immune response (Fradin *et al*., 2009; de Jonge *et al*., 2012). *V. dahliae* strains that are contained by Ve1 are grouped in race 1, whereas resistance-breaking strains are assigned to race 2. Thus far, all race 2 strains that have been characterized to date lack the complete *Ave1* gene and have become increasingly problematic (de Jonge *et al*., 2012; Faino *et al*., 2016). More recently, race 2-resistant tomato root stocks have been developed in Japan. However, several strains were found to escape recognition by these root stocks, that were consequently assigned to race 3 (Usami *et al*., 2017).

An alternative strategy to mediate genetic resistance to plant diseases is the use of impaired disease susceptibility (*S*) genes (Eckardt, 2002; Pavan *et al*., 2010; Gawehns *et al*., 2013). *S* genes encode host genes that are essential for the pathogen to establish colonization. These genes can have various functions, such as in metabolite transport, or act as negative regulator of defence responses (van Schie and Takken, 2014). In order to exploit *S* genes in resistance breeding, their function for the pathogen needs to be impaired, resulting in loss of susceptibility. Such impairments can be found as naturally occurring alleles in wild germplasm, for instance as mutations in promoters that lead to reduced or impaired expression, or as loss-of-function mutations (Chu *et al*., 2006; Bai *et al*., 2008; Gao *et al*., 2015). In contrast to *R* gene-mediated resistance that is typically inherited dominantly, loss of susceptibility by an impaired *S* gene is inherited recessively. Nevertheless, the use of impaired *S* genes represents a major opportunity

for resistance breeding, especially in cases where qualitative resistances are insufficient or not available. For *V. dahliae*, only two *S* genes have been described in literature, namely *Walls are thin 1* (*WAT1*) and *Pyruvate decarboxylase 1* (*PDC1*), but these studies were carried out in *Arabidopsis thaliana* (Denancé *et al*., 2013; Papastolopoulou *et al*., 2018), whereas their role as *S* gene in crop species such as tomato remains unclear.

Several strategies can be used to identify *S* genes in crops. Evidently, orthologues of previously characterized *S* genes, for instance in models such as *A. thaliana*, can be studied in crop species of interest (Huibers *et al*., 2013; Sun, *et al*., 2016) (Chapter 5). Furthermore, novel *S* genes have been identified via forward genetics screenings of mutant populations (Vogel and Somerville, 2000; van Damme *et al*., 2005; Ranocha *et al*., 2010). It is well known that pathogens induce severe transcriptional changes in their hosts (Esse *et al*., 2009; Su *et al*., 2018). In fact, many *S* genes identified so far have been found to be pathogen-inducible, allowing to mine differentially expressed genes upon pathogen inoculation for potential *S* gene candidates (Piffanelli *et al*., 2002; van Damme *et al*., 2008). Here, we combine transcriptional profiling with a reverse genetics approach to identify novel *S* gene candidates for *V. dahliae* in tomato. Subsequently, these candidates are validated via a reverse genetics approach using virus-induced gene silencing (VIGS) in combination with *V. dahliae* inoculation (Liu *et al*., 2002; Fradin *et al*., 2009).

MATERIALS & METHODS

Microarray analysis and filtering

Tomato gene expression data were downloaded from the ArrayExpress Archive of Functional Genomics Data (https://www.ebi.ac.uk/arrayexpress/; ID: E-MEXP-1844; van Esse *et al*., 2009) and a subset of 48 microarrays containing probe sets of 22,721 tomato transcripts with *V. dahliae*-specific data was used for the analysis. The *affy* and *limma* packages were obtained from Bioconductor (https://www.bioconductor.org/) and used to process the data (Gautier *et al*., 2004; Ritchie *et al*., 2015). Data comprised two interactions, compatible and incompatible, with *V. dahliae* race 1 strain St14.01 inoculated on tomato cultivars Moneymaker (lacking *Ve1*) and Motelle (carrying *Ve1*) respectively. Foliage and root samples were harvested at 3, 5 and 7 days post inoculation. Data were normalized using robust multi-array average and compared to mock-inoculated plants at 3 dpi. Subsequently data were filtered with log2fc > 1 and p-value < 0.01 to obtain highly-induced genes.

Generation of silencing constructs and virus-induced gene silencing (VIGS)

VIGS was carried out as described previously using tobacco rattle virus (TRV) (Liu *et al*., 2002; Fradin *et al*., 2009; Verlaan *et al*., 2013). To generate silencing constructs, a gene-specific 150–300 bp region was amplified using Phusion High-Fidelity DNA polymerase (New England Biolabs, Bioké, Leiden, The Netherlands) with primers mentioned in Supplementary Table 2 and Supplementary Figure 2. Exonic regions with little or no homology to other genes were selected preferentially to minimize off-target effects. The obtained fragments were cloned into the pENTR/D-TOPO vector (Invitrogen, Bleiswijk, The Netherlands), transformed into *E. coli* DH5α (Invitrogen, Bleiswijk, The Netherlands), and sequenced to verify correct inserts. Subsequently, the fragments were cloned into the TRV2 vector (Liu *et al*., 2002) using Gateway cloning. Finally, the obtained plasmids were transformed into *Agrobacterium tumefaciens* GV3101 for transient transformation of tomato.

As the empty TRV2 vector was reported to display severe viral symptoms (Wu *et al*., 2011; Senthil-Kumar and Mysore, 2014), instead a TRV2 vector containing a fragment of the *β-Glucuronidase* (*GUS*) gene, which is not endogenous to plants, was used as a negative control. Further, a TRV2 vector carrying a fragment of the tomato *phytoene desaturase* (*PDS*) gene was included as silencing control, leading to bleaching of plant parts in case of effective silencing.

Plant & pathogen material

Tomato cultivar Moneymaker (MM) was grown in potting soil (Potgrond 4, Horticoop, Katwijk, The Netherlands) in a greenhouse (Unifarm, Wageningen University & Research, The Netherlands) at 21°C/19°C (day/night) with 60% relative humidity and a minimal light intensity of 100 W/m^2 at day-time. All *Verticillium dahliae* strains were maintained on potato dextrose agar (PDA) at room temperature in the dark.

***V. dahliae* inoculation and phenotyping**

For *V. dahliae* inoculation, plants were root-dipped at 11-14 days after *A. tumefaciens* treatment as described previously (Fradin *et al*., 2009). Stunting (%) was calculated based on plant canopy area measurements at 14 and 21 days post inoculation (dpi) using Image J (Abramoff *et al*., 2004) as follows:

$$stunting\ (\%) = \left(1 - \frac{canopy\ area\ of\ V.\ dahliae{-}inoculated\ plant}{average\ canopy\ area\ of\ mock{-}inoculated\ plants}\right) \times 100.$$

RESULTS

Different genes are induced in foliage and in roots upon *V. dahliae* challenge

To identify potential *S* gene candidates, a previously generated microarray data set was used in which tomato cultivars Motelle (carrying *Ve1*) and Moneymaker (lacking *Ve1*) were inoculated with a race 1 strain of *V. dahliae*, leading to an incompatible and compatible interaction, respectively, and samples were harvested after 3, 5 and 7 days (van Esse *et al*., 2009) (Figure 1). Firstly, the data set was queried for induced genes in roots as well as in foliage (log2fc > 1, p < 0.01). Subsequently, the retrieved gene sets for the compatible and for the incompatible interaction were overlaid to select only those genes that were induced during the compatible interaction, to exclude the selection of general defence-related genes. In total, we identified 104 and 279 probes that were specifically induced during the compatible interaction in either the foliage or the roots, respectively, which corresponded to 100 and 262 genes (Figure 1 and Supplementary Table1). Interestingly, no overlap among the induced genes that were selected in this manner was observed between these two tissues. Most of the genes, namely 92.0% and 98.1% induced in foliage and roots, respectively, were induced at 7 dpi, and only 4.0% and 1.1% of the genes were induced at 5 dpi in the foliage and roots, respectively.

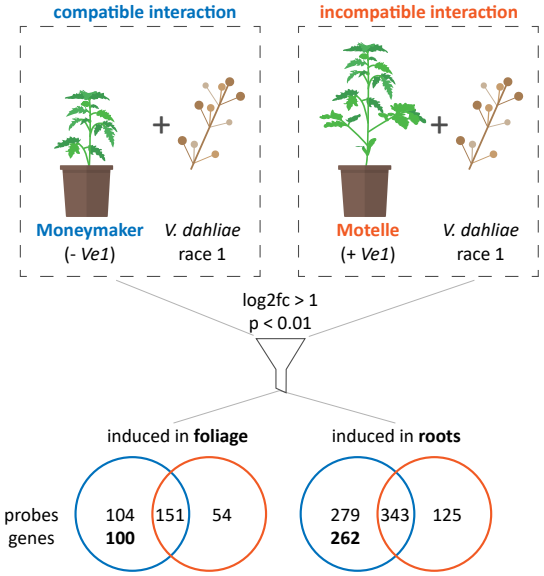


Figure 1 | Strategy to identify potential S gene candidates for the interaction between the vascular wilt fungus Verticillium dahliae and tomato. A previously generated microarray data set of compatible (blue, left) and incompatible (orange, right) interaction between *V. dahliae* and tomato was filtered by log2fc >1 with p < 0.01 for induced genes at 5 and 7 days post inoculation. Subsequently, induced genes in foliage (left) and in roots (right) from the compatible and incompatible interaction were overlaid to select only those genes induced in the compatible interaction. In total we identified 104 and 279 probe IDs corresponding to 100 and 262 genes induced in foliage and roots, respectively.

Virus-induced gene silencing-mediated screening for reduced susceptibility reveals three candidate *S* genes

To allow the identification of potential *S* genes, we ranked the selected tomato genes according to their level of induction (log2fc) and selected the 80 most highly induced genes in the foliage and in the roots for functional validation (160 genes). To this end, constructs for *Agrobacterium tumefaciens*-mediated virus-induced gene silencing (VIGS) were designed. In total, 64 and 71 constructs for genes induced in foliage and roots, respectively, were generated, amounting to a total of 135 constructs (Supplementary Table 2). For the first 40 genes two constructs per gene were designed, whereas for all following genes only one construct was generated, resulting in a total of 175 constructs. Subsequently, tomato seedlings were infiltrated with *A. tumefaciens* carrying the TRV constructs, while TRV::GUS was included as a negative control. Two weeks after *A. tumefaciens* infiltration, plants were inoculated with the *V. dahliae* race 1 strain JR2 and screened for reduced susceptibility by determining levels of stunting between mock-inoculated and inoculated plants (Fradin *et al*., 2009).

To discover the most relevant S gene candidates, two separate approaches were taken. Firstly, stunting was calculated for each of the constructs relative to the TRV::GUS control (Figure 2). Those constructs for which the average stunting was lower than the 95% confidence interval of the TRV::GUS control were marked as “insensitive”. Overall, 66 of 175 constructs (37.7%) caused reduced stunting when compared with TRV::GUS, of which 27 (40.9%) were labelled as “insensitive”, corresponding to 15.4% of all constructs. For 11 of these 27 constructs, a second construct targeting the same gene was used and only for one gene both constructs were labelled as “insensitive”. Construct TRV::224 showed the least stunting of 0.34. In parallel, a second approach was adopted in which the number of plants with less, equal or higher stunting compared to TRV::GUS was determined (Supplementary Figure 1). As the efficiency of VIGS is known to vary between individual plants, we chose a threshold of minimum four plants that displayed reduced stunting when compared with TRV::GUS-treated plants as a criterium to select candidates for further study. In this approach, 26 of all constructs (14.3%) showed four or more plants out of ten that displayed less stunting than plants inoculated with TRV::GUS. For 16 of these 26 constructs, a second construct targeting the same gene was used, for which two were both labelled as “insensitive”.

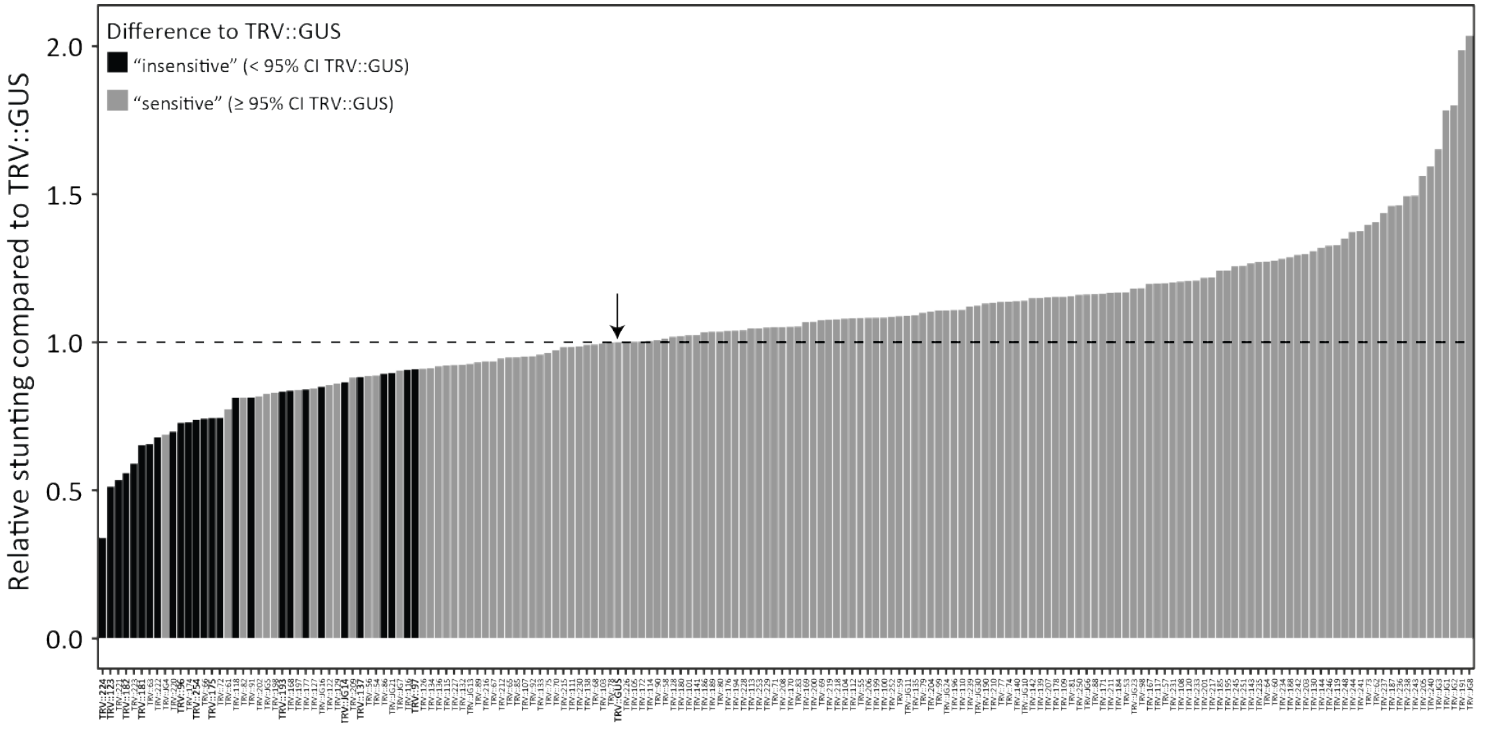


Figure 2 | Relative stunting (compared to TRV::GUS control) of tomato Moneymaker plants that were first treated with 175 different TRV constructs and subsequently challenged with *Verticillium dahliae*. Constructs were grouped as "insensitive" (black bars) if their average stunting was less than the 95% confidence interval (CI) of TRV::GUS or as "sensitive" (grey bars) if their average stunting was equal or more than the 95% CI of TRV::GUS. In total 175 constructs were tested, of which 26 were marked as "insensitive". The presence of "sensitive" constructs in between the "insensitive" ones is caused by the experimental variation in TRV::GUS-treated plants in the different experiments.

To identify the most likely *S* gene candidates, the results from both analyses were combined, which resulted in the identification of 17 constructs with plants that showed significantly reduced stunting according to both analyses (Table 1). Of these candidates, 11 constructs could be re-screened to confirm the effect of reduced susceptibility to *V. dahliae*. Whereas for seven constructs a role in reduced stunting when compared with TRV::GUS could not be confirmed (Supplementary Figure 2), for four candidates (TRV::96, TRV::175, TRV::181 and TRV::224) repeated assays resulted in significantly reduced stunting when compared with the TRV::GUS control (Figure 3). Three of these candidates (TRV::175, TRV::181 and TRV::224) were originally identified to be induced in foliage, while one candidate (TRV::96) was found to be induced in roots.

Table 1 | Overview of 17 constructs for virus-induced gene silencing in tomato that resulted in significantly reduced stunting upon *Verticillium dahliae* inoculation with race 1 strain JR2 when compared with plants that were treated with TRV::GUS.

Construct name	Induced in (microarray)	Tomato genome ID of target gene	Predicted function of target gene
TRV::86	foliage	Solyc10g079200	Mitochondrial carrier protein
TRV::91	foliage	Solyc08g079900	Subtilisin-like protease
TRV::96	roots	Solyc06g067950	Acyl-protein thioesterase 2
TRV::97			
TRV::116	roots	Solyc05g055990	Aquaporin 2
TRV::123	roots	Solyc10g055190	Dirigent-like protein
TRV::137	roots	Solyc09g090210	Serine/threonine protein kinase
TRV::174	foliage	Solyc11g009020	HAD-superfamily hydrolase subfamily IA variant 3
TRV::175	foliage	Solyc12g008650	Inositol oxygenase
TRV::181	foliage	Solyc03g093140	Glycerol-3-phosphate transporter
TRV::182	roots	Solyc08g080660	Osmotin-like protein
TRV::193	roots	Solyc10g051270	RING finger protein 5
TRV::224	foliage	Solyc06g053830	Auxin-regulated IAA7
TRV::254	roots	Solyc10g006755	CONSTANS-like zinc finger protein
TRV::JG14	roots	Solyc08g078220	RING-H2 finger protein ATL22
TRV::JG16	foliage	Solyc05g051200	Ethylene-responsive transcription factor 1A
TRV::JG21	foliage	Solyc11g006250	GDSL esterase/lipase

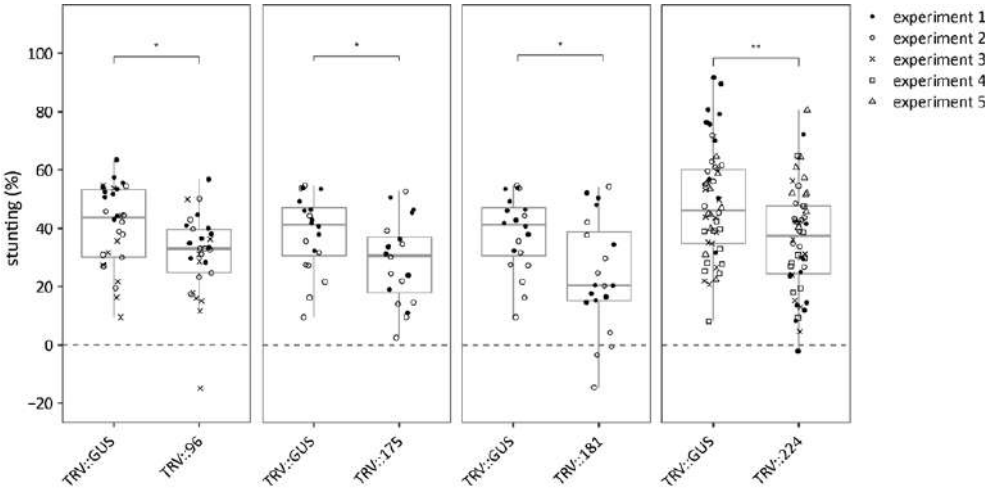


Figure 3 | Stunting (%) of *Verticillium dahliae*-inoculated plants when compared with the average stunting of mock-inoculated plants at 21 dpi after *Agrobacterium tumefaciens* treatment of constructs TRV::96, TRV::175, TRV::181 and TRV::224 (left to right). Box plots represent data with n ≥ 5 per experiment per genotype (t-test when compared with TRV::GUS with * $p < 0.05$, ** and $p < 0.01$).

Three of the candidates were further studied by analysing the induction of the candidates in roots or stems of Moneymaker plants (lacking Ve1) upon *V. dahliae* inoculation with the race 1 strain JR2 and the race 2 strain DVDS26 in a time-course experiment (4, 7, 11, 14 and 18 dpi). This analysis revealed only a slight induction at 18 dpi for the candidate genes targeted with constructs TRV::96 and TRV::181 (Supplementary Figure 3). The target gene of construct TRV::224 was not induced at any time point tested. In addition, silencing levels were evaluated for candidates TRV::96, TRV::181 and TRV::224 in stem samples at 14 days after *A. tumefaciens* treatment (Supplementary Figure 4). Silencing was confirmed in stems at two weeks after *A. tumefaciens* treatment with constructs TRV::96 and TRV::224, in which the target gene expression was significantly reduced when compared with TRV::GUS-treated plants. Silencing levels for construct TRV::181 could not be confirmed, as variable degrees of expression were found for the individual replicates.

Two candidates show non-race specific resistance to multiple *V. dahliae* strains

The three candidates were further characterized by testing for reduced susceptibility to additional *V. dahliae* strains. To this end, four strains were used that belong to different races; the race 1 strain JR2, the additional race 2 strains DVDS26 and DVDS29, and the race 3 strain HoMCF (Usami *et al*., 2017). For constructs TRV::96 and TRV::181, significantly reduced susceptibility when compared with TRV::GUS was confirmed for *V. dahliae* strain JR2, and similarly observed for race 2 strain DVDS29 and race 3 strain

HoMCF (Figure 4A). Only for race 2 strain DVDS26 this pattern was not observed, but it needs to be noted that the overall degree of stunting of TRV::GUS plants was the lowest upon inoculation with strain DVDS26. Thus, the absence of susceptibility phenotypes may be due to too weak virulence of the DVDS26 strain. Overall, these data suggest that constructs TRV::96 and TRV::181 target susceptibility genes for a wide spectrum of *V. dahliae* strains across races.

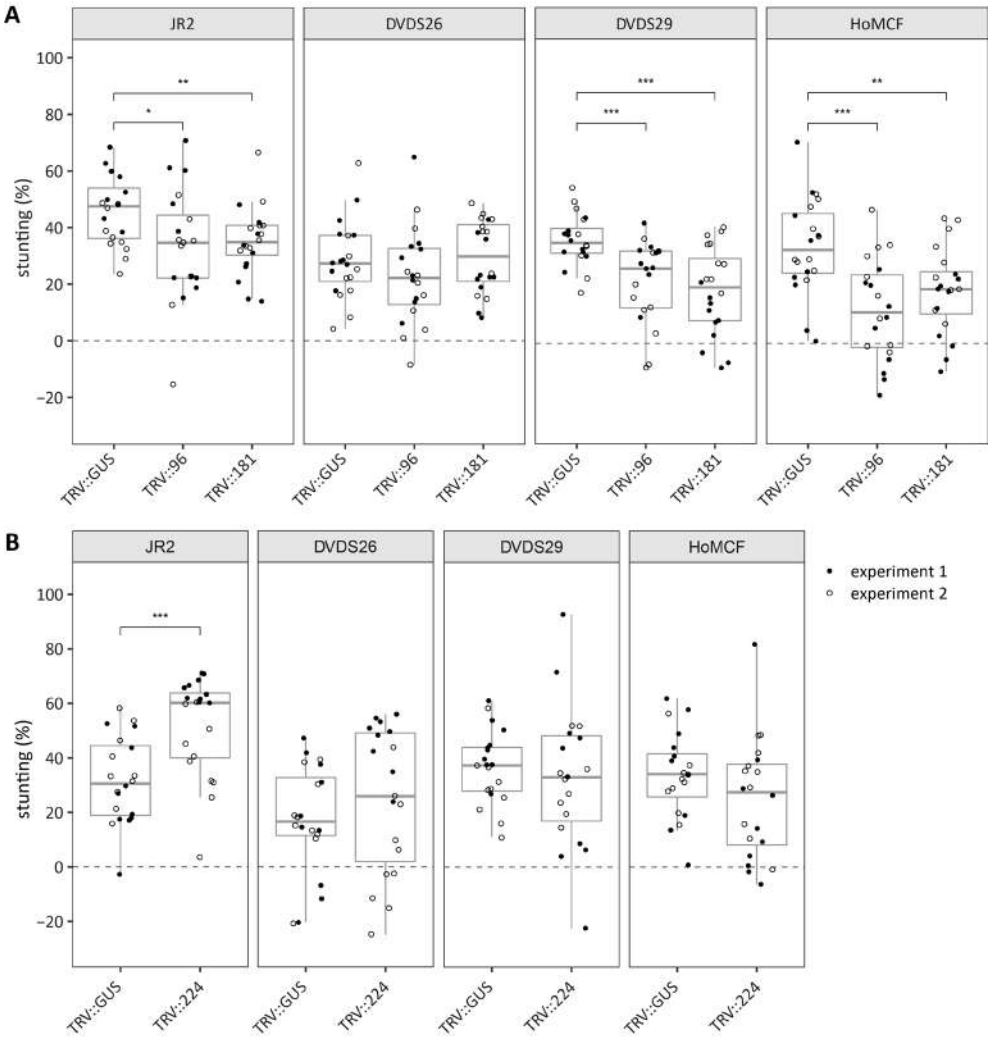


Figure 4 | Stunting (%) of *Verticillium dahliae*-inoculated plants when compared with the average stunting of mock-inoculated plants at 21 dpi after *Agrobacterium tumefaciens* treatment with constructs TRV::96, TRV::181 (A) and TRV::224 (B) with *V. dahliae* strains JR2 (race 1), DVDS26 and DVDS29 (race 2) and HoMCF (race 3) (left to right). Box plots represent data with n ≥ 9 per experiment per genotype (t-test when compared with TRV::GUS with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

In contrast to the observation for constructs TRV::96 and TRV::181, construct TRV::224 increased susceptibility towards the race 1 strain JR2 in these assays (Figure 4B), which is in contrast to previously obtained data with the same strain (Figure 3). Furthermore, the susceptibility towards the strains that were tested in addition remained unaltered (Figure 4B). Overall, these data suggest that the target gene of construct TRV::224 needs to be dismissed as bona fide S gene candidate.

DISCUSSION

Identification of novel *S* genes for pathogens like *V. dahliae*, for which very limited qualitative resistance is available, is a promising approach for resistance breeding. Here we combined transcriptional profiling with a reverse genetics approach to identify novel *S* gene candidates for *V. dahliae* in tomato. Out of 135 tested gene candidates, silencing of two genes repeatedly reduced susceptibility to different strains of *V. dahliae* when compared with TRV::GUS control plants (Figure 3 and 4). The first candidate, Solyc06g067950 (TRV::96), is annotated as acyl-protein thioesterase 2 (APT 2). APTs are de-acylating enzymes which play an important role in S-acetylation for protein interactions with membranes in animals, yeast and toxoplasma (Hemsley, 2015). Knowledge on APTs in plants is limited (Hemsley, 2017) and therefore a link to plant immunity is not yet established. The second candidate, Solyc03g093140 (TRV::181), is a glycerol-3-phosphate (G3P) transporter. The role of G3P as signalling molecule of systemic acquired resistance is well-described (Chanda *et al*., 2011). Interestingly, another G3P-related protein has been described as susceptibility factor for *Phytophthora palmivora* in Arabidopsis. *Required for Arbuscular Mycorrhization 2* (*RAM2*), encodes a G3P acyl transferase and a loss-of-function mutant shows loss of susceptibility to *P. palmivora* (Wang *et al*., 2012). Although we could not confirm effective gene silencing upon TRV::181 treatment (Supplementary Figure 4), a clear treatment effect on reduced susceptibility to *V. dahliae* was found in all assays. Confirming VIGS-mediated silencing levels in tomato is challenging due to the patchiness, but also with respect to expression levels and localized expression patterns of the gene. For both candidates reduced stunting was also found for *V. dahliae* strains that belong to other races (Figure 4). These findings suggest that the two *S* gene candidates contribute to non-race specific susceptibility, which has been reported for other *S* genes as well such as the *mildew locus O* (*mlo*) mutant that provides resistance to all tested powdery mildew isolates (Jørgensen, 1977; Piffanelli *et al*., 2004). Not only was non-race specific resistance based on the targeting of *S* genes described, but also broad-spectrum resistance to multiple pathogen species is reported. Examples are the *walls are thin 1* (*wat1*) mutant in Arabidopsis that shows loss of susceptibility, amongst others, to *V. dahliae* and *Ralstonia solanacearum* or the cucumber *staygreen* (*sgr*)

mutant that is resistant to *Pseudoperonospora cubensis* and *Pseudomonas syringae* pv. *lachrymans* (Denancé *et al*., 2013; Wang *et al*., 2018). Therefore, additional *V. dahliae* strains should be tested, but also other pathogen species. To further confirm the role of these candidates as genuine *S* genes for *V. dahliae* in tomato as well as for other possible diseases, functional analysis by using stable transformation based on RNAi or CRISPR-Cas9 should be used (Zaidi *et al*., 2018; Dong and Ronald, 2019).

Notwithstanding the clear and unambiguous results for constructs TRV::96 and TRV::181, the results for TRV::224 proved not to be consistent and reproducible. Despite earlier findings (Figure 3), repeated assays with the same *V. dahliae* strain, JR2, did not result in consistent observation of reduced stunting in subsequent assays (Figure 4). To some extent, these discrepancies may be attributed to the transient and patchy nature of the VIGS assay, which does not result in complete gene silencing. Perhaps, the obtained level of silencing is just around a threshold level that is required to have a phenotypic effect in the disease assay. Even though VIGS is a frequently used method for functional validation of candidate genes (Liu *et al*., 2002; Senthil-Kumar *et al*., 2007; Fradin *et al*., 2009; Ramegowda *et al*., 2014), it also poses its challenges in tomato as silencing occurs in a patchy fashion throughout the plant (Liu *et al*., 2002; Lu *et al*., 2003; Orzaez *et al*., 2009). Moreover, environmental influences such as temperature were reported to be crucial for VIGS experiments (Lu *et al*., 2003; Senthil-Kumar and Mysore, 2014) and therefore influence silencing efficiency and efficacy that, depending on the target gene, may have a larger or smaller impact. Seasonal variations in temperature, but also the availability of natural light, therefore might have caused discrepancies between assays. These difficulties become even more clear considering that the effect on reduced stunting could only be confirmed for four out of originally 11 selected candidates (Table 1). Evidently this stresses the difficulties to reproduce data using VIGS in tomato and certainly also makes it clear that other candidates may have been missed during the initial screening.

Besides concerns with respect to the use of VIGS for functional validation of candidate genes, also other factors may have influenced the overall success of the strategy that was chosen to identify potential *S* genes in this study. For example, the selection of candidate genes may have been compromised in several steps of the analysis. Firstly, the data were filtered for induced genes as many *S* genes are induced upon infection (Piffanelli *et al*., 2002; van Damme *et al*., 2008). However, it is known that other *S* genes are actually repressed upon pathogen challenge. An example is *Walls are thin 1* (*WAT1*), a functionally characterized *S* gene that is repressed upon inoculation with *R. solanacearum* in Arabidopsis (Denancé *et al*., 2013). Therefore, potential candidates were certainly excluded by filtering for induced genes only. However, considering that most *S* genes are induced, it would therefore not be most evident to also included down-regulated genes in the analysis, as this would also have led to the inclusion of

a wealth of unsuitable candidates in our screening. Secondly, we could further not confirm the induction of three selected candidates (targeted by TRV::96, TRV::181 and TRV::224) in a time course infection experiment. However, while the expression study on which the selection of candidate genes was based was performed with the ST14.01 strain of *V. dahliae*, our time course expression study was performed with the JR2 and DVDS26 strains of that species. As different *V. dahliae* strains carry highly divergent effector catalogues, they may manipulate different host targets (Gibriel *et al*., 2019), leading to divergent host expression profiles. Thus, if a particular expression profile is considered a solid criterium for the selection of candidate genes, confirmation of gene expression based on transcriptomic analysis should be conducted before functional analysis.

Overall, it is certainly difficult to estimate how many *S* gene could be expected in any given plant – pathogen interaction. In this chapter, we pursued an approach combining transcriptional profiling with reverse genetics to identify *S* genes for *V. dahliae* in tomato. As presented in the general introduction (Chapter 1), there are additional approaches to identify *S* genes such as studying orthologues of known *S* genes in other plant species (Chapter 5) or screening a mutant population. For the latter, it is known that many hundreds to thousands of mutants need to be tested to find a loss of susceptibility mutant (Appiano, 2016). Considering that many candidates were discarded based the filtering criteria and the variability in the functional analysis based on our VIGS assays, the selection of two candidates out of 135 tested genes can be considered as encouraging. These candidates will be further studied to confirm their role as *S* gene in the interaction with *V. dahliae* in tomato.

ACKNOWLEDGMENT

This project is financially supported by Topsector Tuinbouw & Uitgangsmaterialen (project: 1409-026). The authors would like to thank Bert Essenstam and Casper Pillen at Unifarm for excellent plant care, Danny Schipper for technical assistance, Nick Snelders for reviewing the manuscript, Dr. Michael Seidl for facilitating expression data analysis and Dr. Toshiyuki Usami for providing the *V. dahliae* race 3 strain.

SUPPLEMENTARY MATERIAL

Supplementary Table 1 | List of induced genes (log2fc > 1, p < 0.01) in foliage (100) and roots (262) specific for the compatible *Verticillium dahliae* – tomato interaction.

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
foliage	Le003154_at1	5 dpi	2.45	0.008	Solyc07g053540.1.12
foliage	Le006075_at1	5 dpi	1.93	0.001	Solyc04g024840.3.12
foliage	Le015984_at1	5 dpi	1.78	0.002	Solyc07g054580.3.12
foliage	Le008995_at	5 dpi	1.75	0.003	Solyc11g072980.1.1
foliage	Le001453_at1	5 dpi	1.35	0.001	Solyc01g087560.3.12
foliage	Le001467_at	5 dpi	1.31	0.005	Solyc09g075180.3.1
foliage	Le009072_at	5 dpi	1.25	0.009	Solyc05g012100.3.1
foliage	Le011073_at	5 dpi	1.21	0.003	Solyc01g110060.3.1
foliage	Le002476_at	7 dpi	3.06	0.000	Solyc10g076240.2.12
foliage	Le015687_s_at	7 dpi	2.56	0.005	Solyc10g076240.2.12
foliage	Le012485_at	7 dpi	2.52	0.001	Solyc08g079900.3.1
foliage	Le002727_at	7 dpi	2.44	0.000	Solyc03g096540.3.1
foliage	Le010130_at	7 dpi	2.15	0.003	Solyc08g007060.3.1
foliage	Le014670_at	7 dpi	1.99	0.001	Solyc04g008210.2.1
foliage	Le003154_at1	7 dpi	1.96	0.000	Solyc07g053540.1.12
foliage	Le008814_at	7 dpi	1.96	0.003	Solyc01g108030.3.1
foliage	Le000874_at	7 dpi	1.92	0.005	Solyc06g065970.1.1
foliage	Le013238_at	7 dpi	1.82	0.003	Solyc10g086580.2.1
foliage	Le015921_at	7 dpi	1.70	0.000	Solyc08g066740.3.1
foliage	Le017369_at	7 dpi	1.69	0.000	Solyc12g013850.2.1
foliage	Le001652_at	7 dpi	1.68	0.003	Solyc10g079200.2.1
foliage	Le012438_at	7 dpi	1.63	0.003	Solyc06g060640.1.1
foliage	Le016603_at	7 dpi	1.63	0.010	Solyc03g082610.1.1
foliage	Le003588_at	7 dpi	1.62	0.004	Solyc03g096550.3.1
foliage	Le018254_at	7 dpi	1.62	0.000	Solyc02g072260.3.1
foliage	Le005250_at	7 dpi	1.60	0.006	Solyc05g014120.1.1
foliage	Le015870_s_at	7 dpi	1.60	0.004	Solyc06g073180.3.1
foliage	Le002355_s_at	7 dpi	1.59	0.001	Solyc03g096290.3.12
foliage	Le017453_at	7 dpi	1.57	0.000	Solyc06g069760.3.1
foliage	Le018165_at	7 dpi	1.55	0.001	Solyc07g009435.1.1
foliage	Le008609_at	7 dpi	1.54	0.002	Solyc11g009020.2.1

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
foliage	Le012611_at	7 dpi	1.50	0.000	Solyc12g094720.2.1
foliage	Le001911_s_at	7 dpi	1.49	0.008	Solyc12g008650.2.12
foliage	Le002377_at	7 dpi	1.48	0.001	Solyc10g007600.3.1
foliage	Le006075_at1	7 dpi	1.48	0.000	Solyc04g024840.3.12
foliage	Le003649_at	7 dpi	1.46	0.002	Solyc10g080600.2.1
foliage	Le012650_at	7 dpi	1.46	0.000	Solyc01g104775.1.1
foliage	Le017529_at	7 dpi	1.46	0.000	Solyc01g068560.3.12
foliage	Le000605_s_at	7 dpi	1.45	0.000	Solyc12g006140.2.1
foliage	Le004805_at	7 dpi	1.45	0.002	Solyc02g063030.3.1
foliage	Le013103_at	7 dpi	1.44	0.010	Solyc03g093140.3.1
foliage	Le013941_at	7 dpi	1.42	0.000	Solyc07g006550.2.1
foliage	Le003171_at	7 dpi	1.41	0.009	Solyc05g015300.3.1
foliage	Le010828_at	7 dpi	1.37	0.002	Solyc03g121180.3.1
foliage	Le018581_at	7 dpi	1.37	0.000	Solyc01g068560.3.12
foliage	Le002347_at	7 dpi	1.36	0.000	Solyc10g052530.1.1
foliage	Le006084_at	7 dpi	1.35	0.002	Solyc06g076630.3.1
foliage	Le016266_at	7 dpi	1.35	0.004	Solyc05g008920.3.1
foliage	Le016686_at	7 dpi	1.35	0.008	Solyc03g113910.3.1
foliage	Le000207_at	7 dpi	1.34	0.001	Solyc07g044860.2.1
foliage	Le007086_at	7 dpi	1.34	0.005	Solyc04g079960.1.1
foliage	Le010895_at	7 dpi	1.34	0.004	Solyc07g053140.3.1
foliage	Le000279_at	7 dpi	1.33	0.001	Solyc12g009650.2.1
foliage	Le001312_at	7 dpi	1.32	0.002	Solyc03g096290.3.12
foliage	Le007160_at	7 dpi	1.30	0.001	Solyc03g117850.3.1
foliage	Le011091_at	7 dpi	1.28	0.008	Solyc03g006410.3.1
foliage	Le016729_at	7 dpi	1.28	0.004	Solyc05g053960.3.1
foliage	Le000291_s_at	7 dpi	1.27	0.002	Solyc03g005780.3.1
foliage	Le001453_at1	7 dpi	1.27	0.000	Solyc01g087560.3.12
foliage	Le017597_at	7 dpi	1.27	0.000	Solyc12g094580.2.1
foliage	Le017584_at	7 dpi	1.26	0.001	Solyc07g066430.2.1
foliage	Le004626_at	7 dpi	1.25	0.001	Solyc02g092700.3.1
foliage	Le000238_at	7 dpi	1.24	0.001	Solyc07g009380.3.1
foliage	Le008782_at	7 dpi	1.24	0.002	Solyc06g005710.3.1
foliage	Le006468_at	7 dpi	1.23	0.001	Solyc01g097770.3.1

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
foliage	Le014773_at	7 dpi	1.23	0.001	Solyc10g039290.2.1
foliage	Le001019_at	7 dpi	1.22	0.001	Solyc04g009050.3.1
foliage	Le003358_at	7 dpi	1.21	0.007	Solyc03g118410.3.1
foliage	Le009541_at	7 dpi	1.21	0.008	Solyc03g121600.3.1
foliage	Le002317_at	7 dpi	1.20	0.000	Solyc09g098160.3.1
foliage	Le009150_at	7 dpi	1.20	0.000	Solyc06g053830.3.1
foliage	Le006387_at	7 dpi	1.19	0.007	Solyc05g051200.1.1
foliage	Le015984_at1	7 dpi	1.19	0.001	Solyc07g054580.3.12
foliage	Le005239_at	7 dpi	1.18	0.006	Solyc11g044910.2.1
foliage	Le008715_at	7 dpi	1.18	0.008	Solyc04g040160.3.1
foliage	Le018943_at	7 dpi	1.18	0.002	Solyc01g105020.3.1
foliage	Le002516_at	7 dpi	1.16	0.001	Solyc11g006250.2.1
foliage	Le013811_s_at	7 dpi	1.16	0.009	Solyc09g011810.3.1
foliage	Le000512_at	7 dpi	1.15	0.000	Solyc08g082400.1.1
foliage	Le003347_at	7 dpi	1.15	0.003	Solyc11g066410.2.1
foliage	Le014333_at	7 dpi	1.15	0.009	Solyc04g080040.3.1
foliage	Le001741_at	7 dpi	1.13	0.000	Solyc09g098170.3.1
foliage	Le008694_at	7 dpi	1.13	0.001	Solyc02g088390.3.1
foliage	Le012902_at	7 dpi	1.13	0.002	Solyc07g063690.1.1
foliage	Le015432_at	7 dpi	1.13	0.002	Solyc02g065170.3.1
foliage	Le016548_at	7 dpi	1.13	0.007	Solyc07g053630.3.1
foliage	Le000759_at	7 dpi	1.12	0.009	Solyc02g069860.3.1
foliage	Le000893_at	7 dpi	1.12	0.003	Solyc09g075460.3.1
foliage	Le004292_at	7 dpi	1.11	0.001	Solyc11g072710.2.1
foliage	Le013337_at	7 dpi	1.11	0.010	Solyc03g031620.3.1
foliage	Le016225_at	7 dpi	1.11	0.003	Solyc06g076790.1.1
foliage	Le000491_at	7 dpi	1.10	0.002	Solyc01g091530.3.1
foliage	Le015418_s_at	7 dpi	1.10	0.010	Solyc12g008650.2.12
foliage	Le009865_at	7 dpi	1.08	0.002	Solyc08g068290.3.1
foliage	Le002650_at	7 dpi	1.07	0.005	Solyc04g071800.3.1
foliage	Le000661_at	7 dpi	1.06	0.002	Solyc01g096660.3.1
foliage	Le004020_at	7 dpi	1.06	0.001	Solyc06g083580.3.1
foliage	Le013198_at	7 dpi	1.06	0.003	Solyc12g089210.2.1
foliage	Le000890_at	7 dpi	1.05	0.003	Solyc08g079180.3.1

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
foliage	Le007662_at	7 dpi	1.05	0.005	Solyc05g013510.3.1
foliage	Le009919_at	7 dpi	1.04	0.005	Solyc10g007670.3.1
foliage	Le015713_at	7 dpi	1.04	0.000	Solyc10g083170.2.1
foliage	Le000507_at	7 dpi	1.03	0.002	Solyc12g096550.2.1
foliage	Le000624_at	7 dpi	1.03	0.000	Solyc12g042780.2.1
foliage	Le013488_at	7 dpi	1.03	0.000	Solyc10g038130.2.1
foliage	Le008617_at	7 dpi	1.02	0.001	Solyc02g090100.1.1
foliage	Le015228_at	7 dpi	1.01	0.001	Solyc12g042920.2.1
foliage	Le018150_at	7 dpi	1.00	0.005	Solyc04g005090.3.1
root	Le017047_at1	5 dpi	2.42	0.008	Solyc09g074570.1.12
root	Le014999_at1	5 dpi	2.14	0.001	Solyc06g067950.3.12
root	Le006688_at	5 dpi	2.10	0.005	Solyc05g024415.1.1
root	Le015962_at	5 dpi	1.97	0.009	Solyc01g101070.3.1
root	Le013316_at	5 dpi	1.62	0.001	Solyc09g090210.3.1
root	Le003269_at	7 dpi	3.97	0.000	Solyc10g075150.2.1
root	Le006092_at	7 dpi	2.69	0.008	Solyc12g096620.1.1
root	Le016891_s_at	7 dpi	2.56	0.000	Solyc01g106610.2.1
root	Le003654_at	7 dpi	2.52	0.003	Solyc04g011480.3.1
root	Le003763_at	7 dpi	2.48	0.007	Solyc00g026160.3.1
root	Le000600_at	7 dpi	2.45	0.001	Solyc07g006560.3.1
root	Le002238_at	7 dpi	2.39	0.003	Solyc03g083990.1.1
root	Le020449_at	7 dpi	2.38	0.005	Solyc01g010040.2.1
root	Le021300_at	7 dpi	2.34	0.003	Solyc08g067960.3.1
root	Le014232_at	7 dpi	2.33	0.000	Solyc07g052370.3.1
root	Le016906_at	7 dpi	2.25	0.000	Solyc03g120470.2.1
root	Le016191_at	7 dpi	2.20	0.000	Solyc05g055990.3.1
root	Le009631_at	7 dpi	2.18	0.006	Solyc03g095780.2.1
root	Le010856_at	7 dpi	2.10	0.004	Solyc03g120320.1.1
root	Le001361_at	7 dpi	2.08	0.002	Solyc01g111075.1.1
root	Le007742_at	7 dpi	2.04	0.006	Solyc10g055190.1.1
root	Le005113_at	7 dpi	1.99	0.002	Solyc01g104110.3.1
root	Le018398_at	7 dpi	1.97	0.001	Solyc01g010180.3.1
root	Le019830_at	7 dpi	1.96	0.009	Solyc07g018400.2.1
root	Le000449_at	7 dpi	1.92	0.000	Solyc09g014280.1.1

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
root	Le006031_at	7 dpi	1.90	0.004	Solyc03g032060.1.1
root	Le006771_at	7 dpi	1.84	0.000	Solyc06g074710.1.1
root	Le002219_at	7 dpi	1.83	0.001	Solyc03g114100.1.1
root	Le001382_at	7 dpi	1.82	0.001	Solyc08g080660.1.1
root	Le003216_at	7 dpi	1.77	0.002	Solyc02g092550.3.1
root	Le003733_at	7 dpi	1.77	0.000	Solyc09g097810.3.1
root	Le001733_at	7 dpi	1.76	0.000	Solyc02g078380.3.1
root	Le004053_at	7 dpi	1.76	0.004	Solyc08g060810.3.1
root	Le012974_at	7 dpi	1.76	0.003	Solyc03g044910.1.12
root	Le004223_at	7 dpi	1.75	0.002	Solyc03g111710.3.1
root	Le005300_at	7 dpi	1.75	0.002	Solyc03g044910.1.12
root	Le005591_at	7 dpi	1.74	0.001	Solyc02g079410.2.1
root	Le014933_at	7 dpi	1.74	0.000	Solyc09g008740.1.1
root	Le014536_at	7 dpi	1.73	0.003	Solyc08g076960.1.1
root	Le015960_at	7 dpi	1.73	0.001	Solyc04g064530.1.1
root	Le002933_at	7 dpi	1.72	0.002	Solyc10g051270.2.1
root	Le008596_at	7 dpi	1.72	0.002	Solyc07g054080.2.1
root	Le008066_at	7 dpi	1.71	0.010	Solyc10g080500.1.1
root	Le001417_at	7 dpi	1.71	0.005	Solyc12g009560.2.1
root	Le019696_at	7 dpi	1.71	0.004	Solyc09g013140.2.1
root	Le021186_at	7 dpi	1.71	0.001	Solyc02g064800.3.1
root	Le004827_at	7 dpi	1.70	0.001	Solyc08g082180.3.1
root	Le011110_at	7 dpi	1.69	0.010	Solyc09g090730.2.12
root	Le009570_at	7 dpi	1.68	0.000	Solyc10g006750.3.1
root	Le009588_at	7 dpi	1.67	0.001	Solyc08g078940.1.1
root	Le003702_at	7 dpi	1.66	0.000	Solyc12g056360.1.1
root	Le006518_at	7 dpi	1.65	0.003	Solyc11g011190.1.1
root	Le012570_at	7 dpi	1.65	0.004	Solyc07g006420.1.1
root	Le012782_at	7 dpi	1.62	0.000	Solyc01g098390.3.1
root	Le005337_at	7 dpi	1.61	0.004	Solyc06g065900.3.1
root	Le009356_at	7 dpi	1.59	0.002	Solyc05g052030.1.1
root	Le009788_at	7 dpi	1.59	0.005	Solyc07g008250.3.12
root	Le010889_at	7 dpi	1.59	0.001	Solyc01g086730.3.1
root	Le011991_at	7 dpi	1.59	0.002	Solyc04g071130.1.1

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
root	Le015699_at	7 dpi	1.58	0.008	Solyc01g007750.3.1
root	Le015786_at	7 dpi	1.58	0.001	Solyc02g085745.1.1
root	Le003389_at	7 dpi	1.56	0.001	Solyc09g011560.2.1
root	Le011285_at	7 dpi	1.56	0.000	Solyc08g061010.3.1
root	Le015880_at	7 dpi	1.56	0.007	Solyc02g070280.3.1
root	Le003285_at	7 dpi	1.55	0.005	Solyc04g007790.3.1
root	Le009087_at	7 dpi	1.55	0.001	Solyc11g012360.2.12
root	Le010851_at	7 dpi	1.55	0.006	Solyc02g077880.3.1
root	Le008118_at	7 dpi	1.53	0.000	Solyc03g005820.3.1
root	Le003798_at	7 dpi	1.52	0.004	Solyc01g005440.3.12
root	Le013564_at	7 dpi	1.52	0.004	Solyc09g090730.2.12
root	Le003910_at	7 dpi	1.51	0.001	Solyc04g079320.3.1
root	Le003086_at	7 dpi	1.50	0.001	Solyc07g054270.3.1
root	Le005876_at	7 dpi	1.50	0.003	Solyc04g082060.3.1
root	Le002783_s_at	7 dpi	1.49	0.000	Solyc09g011470.3.1
root	Le016145_at	7 dpi	1.49	0.001	Solyc02g091430.3.12
root	Le004663_at	7 dpi	1.48	0.006	Solyc04g005530.2.1
root	Le013901_at	7 dpi	1.48	0.000	Solyc08g079440.1.1
root	Le003688_at	7 dpi	1.47	0.003	Solyc05g008100.1.1
root	Le009424_at	7 dpi	1.46	0.000	Solyc06g054270.3.1
root	Le013458_at	7 dpi	1.46	0.000	Solyc03g113570.1.1
root	Le016357_at	7 dpi	1.46	0.001	Solyc03g096840.3.1
root	Le000005_at	7 dpi	1.45	0.003	Solyc02g065400.3.1
root	Le000407_s_at	7 dpi	1.45	0.000	Solyc01g005440.3.12
root	Le005774_at	7 dpi	1.45	0.001	Solyc08g078203.1.1
root	Le013654_at	7 dpi	1.45	0.002	Solyc11g012360.2.12
root	Le014376_at	7 dpi	1.45	0.006	Solyc04g082700.3.1
root	Le008661_at	7 dpi	1.44	0.008	Solyc08g007460.2.1
root	Le013712_at	7 dpi	1.44	0.000	Solyc07g008620.1.1
root	Le021181_at	7 dpi	1.44	0.002	Solyc06g066760.3.1
root	Le008610_at	7 dpi	1.43	0.001	Solyc01g095700.3.1
root	Le010647_at	7 dpi	1.43	0.009	Solyc08g014080.3.1
root	Le010940_at	7 dpi	1.43	0.002	Solyc02g091430.3.12
root	Le021925_at	7 dpi	1.43	0.007	Solyc12g008620.2.1

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
root	Le008438_at	7 dpi	1.42	0.003	Solyc01g098210.3.1
root	Le008618_s_at	7 dpi	1.42	0.002	Solyc10g055800.2.1
root	Le003598_at	7 dpi	1.41	0.007	Solyc09g014480.2.1
root	Le002970_at	7 dpi	1.41	0.000	Solyc02g089870.2.1
root	Le016443_at	7 dpi	1.40	0.003	Solyc02g087970.1.1
root	Le005012_at	7 dpi	1.40	0.003	Solyc04g056340.3.1
root	Le017688_at	7 dpi	1.40	0.007	Solyc12g099175.1.1
root	Le019960_at	7 dpi	1.40	0.005	Solyc08g069120.3.1
root	Le007472_at	7 dpi	1.40	0.001	Solyc04g025940.3.1
root	Le013312_at	7 dpi	1.39	0.008	Solyc02g080070.3.1
root	Le022131_at	7 dpi	1.39	0.000	Solyc09g064750.2.1
root	Le000928_at	7 dpi	1.37	0.005	Solyc03g078160.3.1
root	Le001622_at	7 dpi	1.36	0.004	Solyc06g048820.1.1
root	Le004220_at	7 dpi	1.36	0.000	Solyc09g020190.3.1
root	Le001797_at	7 dpi	1.36	0.005	Solyc10g047210.1.1
root	Le004234_at	7 dpi	1.36	0.002	Solyc07g065010.3.1
root	Le006646_at	7 dpi	1.35	0.004	Solyc06g011350.3.1
root	Le013616_at	7 dpi	1.34	0.006	Solyc07g008250.3.12
root	Le013667_at	7 dpi	1.34	0.000	Solyc04g071160.3.1
root	Le016349_at	7 dpi	1.33	0.005	Solyc03g120690.3.1
root	Le001762_at	7 dpi	1.33	0.000	Solyc08g068700.1.1
root	Le005146_at	7 dpi	1.33	0.003	Solyc04g082770.3.1
root	Le001168_at	7 dpi	1.32	0.003	Solyc09g097770.3.1
root	Le009952_at	7 dpi	1.32	0.000	Solyc07g065270.1.1
root	Le010327_at	7 dpi	1.32	0.000	Solyc05g055550.3.1
root	Le005569_at	7 dpi	1.32	0.001	Solyc02g082060.2.1
root	Le015202_at	7 dpi	1.31	0.000	Solyc01g088550.3.1
root	Le002564_at	7 dpi	1.31	0.002	Solyc01g067660.3.1
root	Le007606_at	7 dpi	1.31	0.004	Solyc05g009870.3.1
root	Le000949_at	7 dpi	1.30	0.002	Solyc09g008610.3.1
root	Le012763_at	7 dpi	1.30	0.004	Solyc08g078670.2.1
root	Le003693_at	7 dpi	1.30	0.002	Solyc06g073170.1.1
root	Le005724_at	7 dpi	1.30	0.002	Solyc06g008740.3.1
root	Le023075_at	7 dpi	1.30	0.002	Solyc06g049030.3.1

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
root	Le014883_at	7 dpi	1.30	0.000	Solyc10g008000.1.1
root	Le014999_at1	7 dpi	1.30	0.000	Solyc06g067950.3.12
root	Le018241_at	7 dpi	1.29	0.000	Solyc12g008510.2.1
root	Le007211_at	7 dpi	1.29	0.006	Solyc00g170200.1.1
root	Le009353_at	7 dpi	1.29	0.000	Solyc06g005500.3.1
root	Le004546_at	7 dpi	1.29	0.002	Solyc12g019740.2.1
root	Le000676_at	7 dpi	1.28	0.003	Solyc07g006570.3.1
root	Le014649_at	7 dpi	1.28	0.007	Solyc11g073200.2.1
root	Le005646_at	7 dpi	1.28	0.001	Solyc02g062780.3.1
root	Le004132_at	7 dpi	1.28	0.009	Solyc06g061240.3.1
root	Le009704_at	7 dpi	1.28	0.002	Solyc06g076440.2.1
root	Le007884_at	7 dpi	1.27	0.004	Solyc05g009670.3.1
root	Le008614_at	7 dpi	1.27	0.002	Solyc05g013750.3.1
root	Le001291_at	7 dpi	1.27	0.000	Solyc06g051270.3.1
root	Le007843_at	7 dpi	1.26	0.000	Solyc05g008815.1.1
root	Le002938_at	7 dpi	1.26	0.000	Solyc01g091520.3.1
root	Le007159_at	7 dpi	1.26	0.000	Solyc01g096630.3.1
root	Le005024_at	7 dpi	1.25	0.004	Solyc06g065330.3.1
root	Le015144_at	7 dpi	1.25	0.000	Solyc01g095860.3.1
root	Le004907_at	7 dpi	1.25	0.004	Solyc05g052620.3.12
root	Le004247_at	7 dpi	1.24	0.009	Solyc04g005100.3.1
root	Le017047_at1	7 dpi	1.24	0.006	Solyc09g074570.1.12
root	Le003895_at	7 dpi	1.24	0.001	Solyc03g118290.3.1
root	Le011519_s_at	7 dpi	1.24	0.005	Solyc06g048520.3.12
root	Le004604_at	7 dpi	1.24	0.005	Solyc01g080080.3.1
root	Le003594_at	7 dpi	1.24	0.001	Solyc06g053670.1.1
root	Le006582_at	7 dpi	1.24	0.008	Solyc01g105290.3.1
root	Le003664_at	7 dpi	1.24	0.003	Solyc10g007990.3.1
root	Le009212_at	7 dpi	1.23	0.001	Solyc02g064940.1.1
root	Le006482_at	7 dpi	1.23	0.003	Solyc06g065320.3.1
root	Le012905_at	7 dpi	1.22	0.000	Solyc08g076180.3.1
root	Le013965_at	7 dpi	1.22	0.001	Solyc09g082530.2.1
root	Le002305_at	7 dpi	1.22	0.007	Solyc11g069750.2.1
root	Le007851_s_at	7 dpi	1.21	0.000	Solyc06g009050.3.1

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
root	Le016560_at	7 dpi	1.21	0.004	Solyc03g026360.1.1
root	Le001749_at	7 dpi	1.20	0.001	Solyc03g096390.3.1
root	Le004839_at	7 dpi	1.20	0.003	Solyc02g014130.2.1
root	Le004086_at	7 dpi	1.20	0.003	Solyc02g036370.3.1
root	Le001903_at	7 dpi	1.20	0.000	Solyc08g076520.3.1
root	Le016613_at	7 dpi	1.20	0.001	Solyc10g078310.2.1
root	Le008539_at	7 dpi	1.19	0.001	Solyc01g008810.3.12
root	Le007415_at	7 dpi	1.19	0.000	Solyc10g012430.3.1
root	Le016310_at	7 dpi	1.19	0.003	Solyc02g063440.3.1
root	Le007291_at	7 dpi	1.19	0.002	Solyc02g082430.3.1
root	Le006555_at	7 dpi	1.19	0.002	Solyc09g066210.3.1
root	Le003335_at	7 dpi	1.19	0.000	Solyc09g063070.3.1
root	Le016449_at	7 dpi	1.18	0.004	Solyc02g078840.3.1
root	Le022072_at	7 dpi	1.17	0.004	Solyc04g049690.3.1
root	Le008885_at	7 dpi	1.17	0.007	Solyc01g111750.3.1
root	Le009783_at	7 dpi	1.17	0.000	Solyc01g110020.3.1
root	Le016433_s_at	7 dpi	1.17	0.001	Solyc02g093130.2.1
root	Le015135_at	7 dpi	1.16	0.003	Solyc04g014790.1.1
root	Le000285_at	7 dpi	1.16	0.006	Solyc01g111660.3.1
root	Le022197_at	7 dpi	1.16	0.004	Solyc11g044470.2.1
root	Le006863_at	7 dpi	1.16	0.005	Solyc09g074240.1.1
root	Le012961_at	7 dpi	1.15	0.001	Solyc04g080720.3.1
root	Le003596_at	7 dpi	1.15	0.003	Solyc02g076830.1.1
root	Le017841_at	7 dpi	1.15	0.009	Solyc01g044240.3.1
root	Le000228_at	7 dpi	1.15	0.000	Solyc01g009170.3.1
root	Le017072_at	7 dpi	1.14	0.000	Solyc08g067430.3.1
root	Le012554_at	7 dpi	1.14	0.006	Solyc08g077380.3.1
root	Le015959_at	7 dpi	1.14	0.000	Solyc07g042680.3.1
root	Le006887_at	7 dpi	1.14	0.008	Solyc05g047640.3.1
root	Le005157_at	7 dpi	1.13	0.002	Solyc07g006350.3.12
root	Le018850_s_at	7 dpi	1.13	0.007	Solyc06g051360.3.1
root	Le013697_at	7 dpi	1.12	0.001	Solyc06g054420.3.1
root	Le014549_at	7 dpi	1.12	0.002	Solyc05g056140.3.1
root	Le006215_s_at	7 dpi	1.11	0.001	Solyc04g050470.3.1

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
root	Le004454_at	7 dpi	1.11	0.007	Solyc01g067000.3.1
root	Le009042_at	7 dpi	1.11	0.002	Solyc01g108620.3.1
root	Le005402_at	7 dpi	1.11	0.001	Solyc01g008230.3.1
root	Le002972_at	7 dpi	1.11	0.007	Solyc07g025510.3.1
root	Le013233_at	7 dpi	1.11	0.006	Solyc01g073810.2.1
root	Le006411_at	7 dpi	1.10	0.000	Solyc02g077560.3.1
root	Le020035_at	7 dpi	1.10	0.003	Solyc03g097670.3.1
root	Le019778_at	7 dpi	1.10	0.003	Solyc12g027760.1.1
root	Le013986_at	7 dpi	1.10	0.001	Solyc06g071370.1.1
root	Le018290_at	7 dpi	1.09	0.001	Solyc01g067280.3.1
root	Le000682_at	7 dpi	1.09	0.001	Solyc07g063190.3.1
root	Le008633_at	7 dpi	1.09	0.000	Solyc12g044820.2.1
root	Le000082_at	7 dpi	1.09	0.000	Solyc03g117770.3.1
root	Le011011_at	7 dpi	1.09	0.000	Solyc04g081980.2.1
root	Le007284_at	7 dpi	1.09	0.000	Solyc06g076820.1.1
root	Le013904_at	7 dpi	1.09	0.004	Solyc05g012850.2.1
root	Le019155_at	7 dpi	1.09	0.003	Solyc02g062800.2.1
root	Le020823_at	7 dpi	1.09	0.004	Solyc01g008810.3.12
root	Le016179_at	7 dpi	1.08	0.001	Solyc05g050380.3.1
root	Le017906_s_at	7 dpi	1.08	0.006	Solyc01g017560.1.1
root	Le017934_at	7 dpi	1.08	0.006	Solyc11g007130.1.1
root	Le009111_at	7 dpi	1.08	0.000	Solyc03g115610.3.1
root	Le015450_s_at	7 dpi	1.08	0.002	Solyc03g025170.1.1
root	Le002569_at	7 dpi	1.08	0.010	Solyc06g063070.3.1
root	Le020619_at	7 dpi	1.08	0.005	Solyc02g078520.3.1
root	Le005669_at	7 dpi	1.07	0.002	Solyc08g069000.3.1
root	AAK63012.1_at	7 dpi	1.07	0.000	Solyc12g009470.2.1
root	Le009537_at	7 dpi	1.06	0.000	Solyc03g117050.3.1
root	Le001561_at	7 dpi	1.06	0.001	Solyc03g098220.3.1
root	Le005725_at	7 dpi	1.06	0.004	Solyc06g048520.3.12
root	Le008067_at	7 dpi	1.06	0.009	Solyc01g111880.3.1
root	Le001332_at	7 dpi	1.05	0.001	Solyc12g096500.2.1
root	Le008134_at	7 dpi	1.05	0.000	Solyc01g098140.3.1
root	Le000843_at	7 dpi	1.05	0.006	Solyc02g062140.2.1

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
root	Le004789_at	7 dpi	1.05	0.004	Solyc04g082210.3.1
root	Le006035_at	7 dpi	1.05	0.002	Solyc03g114120.3.1
root	Le006774_at	7 dpi	1.05	0.002	Solyc07g006350.3.12
root	Le012260_at	7 dpi	1.05	0.008	Solyc03g118425.1.1
root	Le003149_at	7 dpi	1.05	0.001	Solyc08g080920.3.1
root	Le004570_at	7 dpi	1.05	0.007	Solyc01g098500.3.1
root	Le005985_at	7 dpi	1.05	0.000	Solyc04g078390.2.1
root	Le013408_at	7 dpi	1.05	0.001	Solyc05g052620.3.12
root	Le017012_at	7 dpi	1.04	0.000	Solyc02g005200.3.1
root	Le017357_at	7 dpi	1.04	0.002	Solyc01g087995.1.1
root	Le011023_at	7 dpi	1.04	0.004	Solyc03g096950.3.1
root	Le003338_at	7 dpi	1.04	0.002	Solyc01g090180.3.1
root	Le005340_at	7 dpi	1.04	0.007	Solyc06g050760.1.1
root	Le000777_at	7 dpi	1.04	0.001	Solyc03g082710.3.1
root	Le000090_at	7 dpi	1.04	0.003	Solyc08g083330.2.1
root	Le015993_at	7 dpi	1.04	0.003	Solyc01g108840.3.1
root	Le006227_at	7 dpi	1.03	0.009	Solyc06g065190.1.1
root	Le014813_at	7 dpi	1.03	0.000	Solyc02g063270.3.1
root	Le004638_at	7 dpi	1.03	0.008	Solyc02g070630.3.1
root	Le000835_at	7 dpi	1.03	0.004	Solyc08g075470.3.1
root	Le004150_at	7 dpi	1.03	0.000	Solyc02g086830.3.1
root	Le003331_s_at	7 dpi	1.03	0.005	Solyc03g120630.3.1
root	Le015231_at	7 dpi	1.03	0.003	Solyc08g061920.2.1
root	Le005814_at	7 dpi	1.03	0.001	Solyc11g071920.2.1
root	Le012876_at	7 dpi	1.03	0.008	Solyc10g047930.2.1
root	Le006736_at	7 dpi	1.02	0.005	Solyc02g062690.3.1
root	Le014155_at	7 dpi	1.02	0.002	Solyc03g097210.3.1
root	Le005972_at	7 dpi	1.02	0.005	Solyc09g075140.3.1
root	Le000483_at	7 dpi	1.02	0.000	Solyc05g056000.3.1
root	Le004343_at	7 dpi	1.02	0.001	Solyc01g104500.2.1
root	Le009372_at	7 dpi	1.02	0.001	Solyc04g076250.3.1
root	Le018231_at	7 dpi	1.02	0.010	Solyc03g063220.1.1
root	Le017224_at	7 dpi	1.02	0.002	Solyc01g090940.3.1
root	Le006833_at	7 dpi	1.02	0.000	Solyc10g005040.3.1

Tissue	Probe ID	Time point	log2fc	adj. p-value	Tomato genome ID
root	Le018362_at	7 dpi	1.02	0.001	Solyc03g096840.2.1
root	Le005320_at	7 dpi	1.02	0.001	Solyc12g099390.2.1
root	Le015289_at	7 dpi	1.01	0.001	Solyc04g082810.3.1
root	Le002381_at	7 dpi	1.01	0.003	Solyc07g056540.3.1
root	Le017855_s_at	7 dpi	1.01	0.001	Solyc01g087680.3.1
root	Le014214_at	7 dpi	1.01	0.000	Solyc12g008770.2.1
root	Le011064_at	7 dpi	1.01	0.004	Solyc09g063140.3.1
root	Le004532_at	7 dpi	1.01	0.001	Solyc03g031920.3.1
root	Le017730_at	7 dpi	1.01	0.001	Solyc02g083320.3.1
root	Le019970_at	7 dpi	1.00	0.007	Solyc06g076970.3.1
root	Le009903_s_at	7 dpi	1.00	0.009	Solyc09g059270.3.1

[1] Probe IDs found multiple times.
[2] Genes found multiple times.

Supplementary Table 2 | Overview of 135 *Verticillium dahliae*-induced genes in foliage and roots of tomato plants tested in this study.

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
foliage	Le003154_at	5 dpi	2.45	0.008	Solyc07g053540	59 and 60	KH_059_Fw	caccCCACCAAGGATTAGCCCCAA
							KH_059_Rv	TATTGGTCCTGAGGGTGCTG
		7 dpi	1.96	0.000			KH_060_Fw	caccCTGATGAGCCATCACCAGCA
							KH_060_Rv	ACACACAACTCATGTAATTGCAA
foliage	Le006075_at	5 dpi	1.93	0.001	Solyc04g024840	61 and 62	KH_061_Fw	caccTGCCCTCTAATACCAACCGAC
							KH_061_Rv	TCCAGGATCAAACATGGAGTCT
		7 dpi	1.48	0.000			KH_062_Fw	caccATGCTGTGGGTTTGGTCCAT
							KH_062_Rv	AGGACACAAGCGACGAACAA
foliage	Le015984_at	5 dpi	1.78	0.002	Solyc08g068490	63 and 64	KH_063_Fw	caccCCACCCTTGGCCTAACAAAt
							KH_063_Rv	GCATGAGAAGCAAGAGGGACTA
		7 dpi	1.19	0.001			KH_064_Fw	caccTGACAGGATCAATGATGCCAT
							KH_064_Rv	TGACTTGACACAAGGACACAAG
foliage	Le008995_at	5 dpi	1.75	0.003	Solyc11g072980	65 and 66	KH_065_Fw	caccCGAGAGAGGCACCAAAATTGT
							KH_065_Rv	AGGACATGCTTCACGACCAT
							KH_066_Fw	caccATAGTTGTGCACAAAGGGAGGA
							KH_066_Rv	AGGCCCTCCTTTTGTTGAGC
foliage	Le001453_at	5 dpi	1.35	0.001	Solyc01g087560	53 and 54	KH_053_Fw	caccACGTCTCGACTCACGTTCTC
							KH_053_Rv	AGGAAATGCCAAAACGGAGAC
		7 dpi	1.27	0.000			KH_054_Fw	caccTGCATTGCTTGTTTGTCACCA
							KH_054_Rv	CCAATCCGGTCCATATATACCCC

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
foliage	Le001467_at	5 dpi	1.31	0.005	Solyc09g075180	67 and 68	KH_067_Fw	caccTCAGAAGCCCAAACACCTCA
							KH_067_Rv	GCTCAGAAGGACGAAGAGGG
							KH_068_Fw	caccACTACTTGCGTAGGAGCTGC
							KH_068_Rv	GCGACTTGCTAGAAATGGCG
foliage	Le009072_at	5 dpi	1.25	0.009	Solyc05g012100	69 and 70	KH_069_Fw	caccGGAGACAGAGGTGGTTGGTG
							KH_069_Rv	TTTCTTCCGGGTTCTTCGGG
							KH_070_Fw	caccACAGGCGAGGAGATTAGGGT
							KH_070_Rv	CTCCGATACCACGATGTTCCA
foliage	Le011073_at	5 dpi	1.21	0.003	Solyc01g110050	71 and 72	KH_071_Fw	caccGGGATCTTGGTCAGAGTTTCGA
							KH_071_Rv	TATCTCATGATTCCCCGCGG
							KH_072_Fw	caccCATGACGGAGCCACAACCAG
							KH_072_Rv	ATCATGATTTGGCTGCTGTGG
foliage	Le002476_at	7 dpi	3.06	0.000	Solyc10g076240	55 and 56	KH_055_Fw	caccTCGTGCATGAATTGTCCTCT
							KH_055_Rv	GAAAACGCCATGCCTGCTAG
	Le015687_s_at	7 dpi	2.56	0.005			KH_056_Fw	caccCAAGCCATTGACCGGAAAGC
							KH_056_Rv	AGGTATGGTTCGGTCCCTCT
foliage	Le012485_at	7 dpi	2.52	0.001	Solyc08g079900	91 and 92	KH_091_Fw	caccATAATGGTCCTTCGCCTGCC
							KH_091_Rv	CTCCAGCATCCTTCACAGCT
							KH_092_Fw	caccCTCGCGGACCAAGTAAACCT
							KH_092_Rv	TGTGTCAGCGGTTGTCATCA

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
foliage	Le002727_at	7 dpi	2.44	0.000	Solyc03g096540	57 and 58	KH_057_Fw	caccCAGAATGGTGGAGGTAGCCG
							KH_057_Rv	TGGCTGGTTCTTTTGTTCTCCT
							KH_058_Fw	caccTGAGCAACAGTCGCTTTACCT
							KH_058_Rv	GTATCGCTTAGGGGTTCCCC
foliage	Le010130_at	7 dpi	2.15	0.003	Solyc08g007060	73 and 74	KH_073_Fw	caccCTGAACTTCAGCGGATGGGT
							KH_073_Rv	TGCTTGCGCGTTGAAGAATT
							KH_074_Fw	caccAGCAACTGATAACATGCCTGGA
							KH_074_Rv	GAGTTGGAGCACTGGAGTGG
foliage	Le014670_at	7 dpi	1.99	0.001	Solyc04g008210	75 and 134	KH_075_Fw	caccACTGCTGGAGTGGTGGTTG
							KH_075_Rv	TGATGTGGCTGTCAGTCCAA
							KH_134_Fw	caccAAAATGGTGAATTTTCTTCTG-GAAATT
							KH_134_Rv	TGAACTGATTTTCCATCTTGAATGA
foliage	Le008814_at	7 dpi	1.96	0.003	Solyc01g108030	77 and 78	KH_077_Fw	caccGCTTTCCTTCCGAAGCAAGC
							KH_077_Rv	TACAAGAGGAGGTCCAGCCA
							KH_078_Fw	caccATGCTATGATGCCTGAGCCC
							KH_078_Rv	CAGCCACGGAACCACAGTTA
foliage	Le000874_at	7 dpi	1.92	0.005	Solyc06g065970	79 and 80	KH_079_Fw	caccGCCCTCTCCTCCACCATTTT
							KH_079_Rv	GGGTTGTCACTTGGCTACCA
							KH_080_Fw	cacccTTGTTGCTCATTGCTTGAAGG
							KH_080_Rv	CACCCCACCCTTGGCTAATT

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
foliage	Le013238_at	7 dpi	1.82	0.003	Solyc10g086580	81 and 82	KH_081_Fw	caccGCCCCAAGGTTTCAAACAGG
							KH_081_Rv	TTTAAGAGCCTGGCTGACGT
							KH_082_Fw	caccGGGCACGAGTATATGACGATGA
							KH_082_Rv	GTTAGCATCTCCGAGAGCTG
foliage	Le015921_at	7 dpi	1.70	0.000	Solyc08g066740	83 and 135	KH_083_Fw	caccCCTCTGCTTCGTCTTCTGGG
							KH_083_Rv	AGGCAAATTGGGAAGCTCCA
							KH_135_Fw	caccTGGCTACTCTTAGAAATCCAAGAA
							KH_135_Rv	TGTGATTCTTGGACCATTTGAGA
foliage	Le001652_at	7 dpi	1.68	0.003	Solyc10g079200	85 and 86	KH_085_Fw	caccTTCCAGGGCAGCTTCCAAAA
							KH_085_Rv	TAAGCTCAGTTGGGCAAGCA
							KH_086_Fw	caccCGTGAAATCCCCGGAAATGC
							KH_086_Rv	CTCCCTCCGATGCCAAGATC
foliage	Le016603_at	7 dpi	1.63	0.010	Solyc03g082610	136 and 88	KH_088_Fw	caccACATCCGCTGCGAAGATGAT
							KH_088_Rv	CATTACGAACACGCCGCAAA
							KH_136_Fw	caccCATGCCTTCTCCCATCACCA
							KH_136_Rv	GTAGAGGGTAGGGGGACGTT
foliage	Le012438_at	7 dpi	1.63	0.003	Solyc06g060640	89 and 90	KH_089_Fw	caccACCTTCTCTTCTTCACAATGGT
							KH_089_Rv	GCAGCATCAACATCAGCAAGA
							KH_090_Fw	caccTGTTGGAAGCCCATTATTGTCA
							KH_090_Rv	TTTACGCGCATTTGAAACCTT
foliage	Le003588_at	7 dpi	1.62	0.004	Solyc03g096550	167	KH_167_Fw	caccTCGAGAATTGGCTTGGTGCT
							KH_167_Rv	CGAATTGGAAAAAGGGAAAAGGGA

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
foliage	Le018254_at	7 dpi	1.62	0.000	Solyc02g072260	168	KH_168_Fw	caccTCCTCAGCTTAAAGAACTGGCA
							KH_168_Rv	AGCATTGGATGGATGACTTGA
foliage	Le005250_at	7 dpi	1.60	0.006	Solyc05g014120	169	KH_169_Fw	caccAGCACTGAAACGAGTCGGAG
							KH_169_Rv	AAATCCCGCCGGTCTTCAAT
foliage	Le015870_s_at	7 dpi	1.60	0.004	Solyc06g073180	170	KH_170_Fw	caccATTCATTCAGCCAGCAGCCT
							KH_170_Rv	CGACAGGCCAAGATGGTGAT
foliage	Le002355_s_at	7 dpi	1.60	0.001	Solyc03g096290	171	KH_171_Fw	caccTGGAGCATAGAGAAGAGGATGTG
							KH_171_Rv	AGAAACGCCCATGACGGTTA
foliage	Le017453_at	7 dpi	1.57	0.000	Solyc06g069760	172	KH_172_Fw	caccGTTTGGTCCCGACTTACCCC
							KH_172_Rv	TGTGGGGCTCTCCTTTACCT
foliage	Le008609_at	7 dpi	1.54	0.002	Solyc11g009020	174	KH_174_Fw	caccTGGCGTCTTCCATTAACCTACT
							KH_174_Rv	ATCAGGGCATCGCACATTGA
foliage	Le012611_at	7 dpi	1.50	0.000	Solyc12g094720	175	KH_175_Fw	caccTCGAGTACACTCTCCAAAATCCC
							KH_175_Rv	CCAGTGAGAGTATACAGTCTGTTGT
foliage	Le001911_s_at	7 dpi	1.49	0.008	Solyc12g008650	176	KH_176_Fw	caccGGATCACAAGTGGAGGAGAAA
							KH_176_Rv	TGCATTTATGTCTGGCACTTCA
foliage	Le002377_at	7 dpi	1.48	0.001	Solyc10g007600	177	KH_177_Fw	caccGGCTGTCGTTCACTGAAGGA
							KH_177_Rv	CACTAAGCTTTCACTACGCGC
foliage	Le003649_at	7 dpi	1.46	0.002	Solyc10g080600	178	KH_178_Fw	caccTCTCAACTTCCTTTCATGAGACCA
							KH_178_Rv	GCGTTTTGATGTCCTCCTAGG
foliage	Le012650_at	7 dpi	1.46	0.000	Solyc01g068563	248	KH_248_Fw	caccTTTTGGTCCCTGGAGAGCTG
							KH_248_Rv	ACAATCCATCAGAGTTGCCGT

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
foliage	Le004805_at	7 dpi	1.45	0.002	Solyc02g063030	180	KH_180_Fw	caccTGGGAACACAAAGCATTTCAGC
							KH_180_Rv	TGACTGGAGAGTGATCTGAGGA
foliage	Le013103_at	7 dpi	1.44	0.010	Solyc03g093140	181	KH_181_Fw	caccCAGCAAGCCCCCTGGAATTA
							KH_181_Rv	CAAGTTCACCAAGCATCGCC
foliage	Le013941_at	7 dpi	1.42	0.000	Solyc07g006550	200	KH_200_Fw	caccTGTTGAGGCTATACAAAAGTTGG
							KH_200_Rv	AGGGCTTCATTAGGGCAACT
foliage	Le003171_at	7 dpi	1.41	0.009	Solyc05g015300	201	KH_201_Fw	caccAGACGGTGTTGTTCCTGAGC
							KH_201_Rv	TCCCCTTCTCCAAGTCCTTCT
foliage	Le018581_at	7 dpi	1.37	0.000	Solyc01g068560	202	KH_202_Fw	caccTGGTTCACCAAGCTTCCCAA
							KH_202_Rv	TCACGATGTGGCTTCAACCC
foliage	Le010828_at	7 dpi	1.37	0.002	Solyc03g121180	203	KH_203_Fw	caccTGGGTATCTACAGACAGCAAAA
							KH_203_Rv	CATTGTTTCCTACATCCACAGCG
foliage	Le006084_at	7 dpi	1.35	0.002	Solyc06g076630	204	KH_204_Fw	caccAGTCTGAGCAGGCCTTTGAC
							KH_204_Rv	GATACGACAGTTGGCTCGCA
foliage	Le016686_at	7 dpi	1.35	0.008	Solyc03g113910	205	KH_205_Fw	caccGCAACAAGACCAAGTGCCCT
							KH_205_Rv	ACTCCGCCACTTTGAACAAA
foliage	Le016266_at	7 dpi	1.35	0.004	Solyc05g008920	206	KH_206_Fw	caccTTATAGTGCTGCGAAGGCTC
							KH_206_Rv	GCTGTAGATTACAAAGCGGCAAA
foliage	Le010895_at	7 dpi	1.34	0.004	Solyc07g053140	207	KH_207_Fw	caccCGGAAGTTCGTCGTGTCAAC
							KH_207_Rv	TTGAGTCTACAGAGCTCGGC
foliage	Le007086_at	7 dpi	1.34	0.005	Solyc04g079960	208	KH_208_Fw	caccGAGGATCCTCGTTGCTGTCG
							KH_208_Rv	CGAGCTCCGACGATGACTTT

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
foliage	Le007086_at	7 dpi	1.33	0.001	Solyc12g009650	209	KH_209_Fw	caccCTCACAAGCCCAAAAAGCCC
							KH_209_Rv	GACCGGTGGAATCCCAAC
foliage	Le007160_at	7 dpi	1.30	0.001	Solyc03g117850	210	KH_210_Fw	caccAGCTCCTCTCACTCTCCACT
							KH_210_Rv	ATCAATGACGCCAGCCCTAG
foliage	Le011091_at	7 dpi	1.28	0.008	Solyc03g006410	211	KH_211_Fw	caccGTCATCGGATTTTAACGTGCGA
							KH_211_Rv	AGTTGATGTTGTGTTTCCCAAAA
foliage	Le016729_at	7 dpi	1.28	0.004	Solyc05g053960	212	KH_212_Fw	caccTCTCATGTTTTTGATGGCTGCA
							KH_212_Rv	CAATTAGCACCAATTGTCCTCTT
foliage	Le001453_at	7 dpi	1.27	0.000	Solyc10g081300	250	KH_250_Fw	caccTTCGGGTTCGATTCACAGCA
							KH_250_Rv	TCCAGTCAATGTACCATGACCA
foliage	Le017584_at	7 dpi	1.26	0.001	Solyc10g086150	251	KH_251_Fw	caccGAAATCGACGGGAGGGCATT
							KH_251_Rv	CATCGACACCCCATGAGAGG
foliage	Le004626_at	7 dpi	1.25	0.001	Solyc02g092700	215	KH_215_Fw	caccTGGCTCCATAGTTTCTAGTCCG
							KH_215_Rv	GCCTTTGTTCAAATGGCACCT
foliage	Le000238_at	7 dpi	1.25	0.001	Solyc07g009380	216	KH_216_Fw	caccTGTTTCTTCGTAGTGGTGGCT
							KH_216_Rv	CCAGCAGAATTACCAGGGACA
foliage	Le008782_at	7 dpi	1.24	0.002	Solyc06g005710	217	KH_217_Fw	caccACCTGCTAGCACGAACACAT
							KH_217_Rv	TAGCTCTTGTGCAGCACCAA
foliage	Le014773_at	7 dpi	1.23	0.001	Solyc10g039290	218	KH_218_Fw	caccGGTGGTGTAAGGGGAGCATT
							KH_218_Rv	TGGTGGAATTTGAGCAGGAAGT
foliage	Le006468_at	7 dpi	1.23	0.001	Solyc01g097770	219	KH_219_Fw	caccGGCGGAATCTGCAAGTAGGA
							KH_219_Rv	TCGACTCCTCGGATGTCCTT

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
foliage	Le001019_at	7 dpi	1.22	0.001	Solyc01g105120	220	KH_220_Fw	caccTGGGATGAGTTCTGCCACAA
							KH_220_Rv	TCCAGCATTTTGGGGCTTCT
foliage	Le003358_at	7 dpi	1.21	0.007	Solyc03g118410	221	KH_221_Fw	caccAGGCAGAGACAATTGATAAGGT
							KH_221_Rv	AGTGTCCAGAGAGTCGGCTC
foliage	Le009541_at	7 dpi	1.21	0.008	Solyc03g121600	222	KH_222_Fw	caccTTGGGAGCCCTCACATTCTG
							KH_222_Rv	GCTGAAGCGACTCCATGATG
foliage	Le002317_at	7 dpi	1.20	0.000	Solyc09g098160	223	KH_223_Fw	caccTGCTACACCAAGACATACCAAAG
							KH_223_Rv	TCCGAAGAACCAAACGCTCC
foliage	Le009150_at	7 dpi	1.20	0.000	Solyc06g053830	224	KH_224_Fw	caccCTCAAACTGTTGATCTCAAACTCA
							KH_224_Rv	TGGTGGCTTGTTGGAGGAAT
foliage	Le006387_at	7 dpi	1.19	0.007	Solyc05g051200	JG16	JG_16_FW	caccATGAGGGGTCCTTGGTCTCT
							JG_16_RV	TTCAACTCCCAAGTCTTGAAAAA
foliage	Le002516_at	7 dpi	1.16	0.001	Solyc11g006250	JG21	JG_21_FW	caccTGGAGCTCGTCGTGTAATTG
							JG_21_RV	TGCATTTGTTGTGTATTTGCAG
foliage	Le003347_at	7 dpi	1.15	0.003	Solyc11g066410	JG23	JG_23_FW	caccCCATTTTCCGAAACAGTGAAA
							JG_23_RV	TTTGCCCAATGGTAGCAAAT
foliage	Le014333_at	7 dpi	1.15	0.003	Solyc04g080040	JG24	JG_24_FW	caccCCACAGATCTTCCCACGTTT
							JG_24_RV	AATGCTTCCCTCCCTTCAGT
foliage	Le000759_at	7 dpi	1.12	0.009	Solyc02g069860	JG30	JG_30_FW	caccGGAGCATCTTTGCACCGTAT
							JG_30_RV	GATTGTCGACGCCTCAAGAT

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
root	Le014999_at	5 dpi	2.14	0.001	Solyc06g067950	96 and 97	KH_096_Fw	caccGGCTTAGATGCTTCCGTTGC
							KH_096_Rv	CCCATGCTGAAGCCTCCAAT
		7 dpi	1.3	0.000			KH_097_Fw	caccGCCTCGTCTTTGCCCATTCT
							KH_097_Rv	GCAAGGTTCCTGAATCCAGC
root	Le006688_at	5 dpi	2.10	0.005	Solyc05g024410	98 and 99	KH_098_Fw	caccAAGCTCACATGCCATCACCA
							KH_098_Rv	GTCATCGAGTGGTAGCTCCG
							KH_099_Fw	cacctGGACCTGGTTTGACAGTGG
							KH_099_Rv	CCTGCAAATTTCCCAGCACC
root	Le015962_at	5 dpi	1.97	0.009	Solyc01g101070	100 and 101	KH_100_Fw	caccGGCGCCAGACTTCGATTTTG
							KH_100_Rv	GCCTTTCTACAGCTCCAAGC
							KH_101_Fw	caccTGGACGTTGAATGGGATGCT
							KH_101_Rv	TGCAGCATCACCTTCTTTGGA
root	Le013316_at	5 dpi	1.62	0.001	Solyc09g090210	137 and 103	KH_103_Fw	caccCTGCAGCCACTTCTTAACGC
							KH_103_Rv	TAGTCCATGTTTGCGAGGGC
							KH_137_Fw	caccTCCTGGTTTACGAGTCTGCA
							KH_137_Rv	TCGCGATGGATAATTGGTTCA
root	Le003269_at	7 dpi	3.97	0.000	Solyc10g075150	104 and 105	KH_104_Fw	caccTCTACCCAACACTCCTCACAC
							KH_104_Rv	CCCAACCTGAATTTGGCCAC
							KH_105_Fw	caccGAACCGTGGGCCAATAGGAG
							KH_105_Rv	ACTCCAGAAAGACCAGCAGC

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
root	Le006092_at	7 dpi	2.69	0.008	Solyc12g096620	138 and 107	KH_107_Fw	caccCCTGAACCTGAGGTACGTACg
							KH_107_Rv	TCCCAACCAAGACGCGTATC
							KH_138_Fw	caccAACGTGATCCGCATACCTGG
							KH_138_Rv	TCGGGAGAGGAACGTTGAAC
root	Le016891_s_at	7 dpi	2.48	0.007	Solyc00g026160	108 and 109	KH_108_Fw	caccAGCTGGAGAAATAGCGACGT
							KH_108_Rv	TCCCAGGTAAGATCATGCATGT
							KH_109_Fw	caccGGAGTTGTTGTTTGTGGGCC
							KH_109_Rv	TCAGAAACTTGCACACAACCA
root	Le000600_at	7 dpi	2.45	0.001	Solyc07g006560	110 and 111	KH_110_Fw	caccACTCAACGTATTTTGGCCCTCT
							KH_110_Rv	AGCAGAGTCACAAGGGACAA
							KH_111_Fw	caccTGGAATTGTCCCTTGTGACTCT
							KH_111_Rv	GAGGCGAAGGACGAGGAAC
root	Le002238_at	7 dpi	2.39	0.003	Solyc03g083990	112 and 113	KH_112_Fw	caccTGGCTTCCAAAACAATTGCA
							KH_112_Rv	AGCACACACACCAAGTTTCA
							KH_113_Fw	caccTCGGGTCTCCACCAACTTTG
							KH_113_Rv	TCCACATGTGTTTAGTATGAGGCT
root	Le021300_at	7 dpi	2.34	0.003	Solyc08g067960	114 and 115	KH_114_Fw	caccGATTGAACATTCAGGGTCGCC
							KH_114_Rv	GCTTCGGTGCAAGTGGATTT
							KH_115_Fw	caccATTGCTGCAACACCAATGCC
							KH_115_Rv	CTCTTGTTTGGCGGGTGTTG

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
root	Le016191_at	7 dpi	2.20	0.000	Solyc05g055990	116 and 117	KH_116_Fw	caccCAAAGGACTACCACGAGCCA
							KH_116_Rv	CAAGCAACACCAAGAAGCCC
							KH_117_Fw	caccCAGCAGTGACCTTTGGGCTA
							KH_117_Rv	AGTGGCCGATAAAACCGTGT
root	Le009631_at	7 dpi	2.18	0.006	Solyc03g095780	118 and 119	KH_118_Fw	caccGGAAACTTCCACAAACCGCC
							KH_118_Rv	GCTTATACGCTTGCGGGTTG
							KH_119_Fw	caccGCCTCTAGCACGGAGAGATT
							KH_119_Rv	ACGAATACACACGTTTCTTCCT
root	Le010856_at	7 dpi	2.10	0.004	Solyc03g120320	120 and 139	KH_120_Fw	caccGCTACTGGTTCAACCCGTCA
							KH_120_Rv	CAAAAACGGCGTTCGAAGGT
							KH_139_Fw	caccTGAATCTGCCACGTGTCCAA
							KH_139_Rv	GATTGAACGTGGCCGGAAAA
root	Le007742_at	7 dpi	2.04	0.006	Solyc10g055190	122 and 123	KH_122_Fw	caccAGGTCCCAACGCTGTTGAAA
							KH_122_Rv	ATTGTCGAGTTGGGCTCTGG
							KH_123_Fw	caccAGTAGGTCGAGCTCAAGGGA
							KH_123_Rv	ACCAATACGTCTTCGCGGTT
root	Le005113_at	7 dpi	1.99	0.003	Solyc01g104110	140 and 141	KH_140_Fw	caccTGGGAACAGTGTCATGGTGG
							KH_140_Rv	TTGCGCTGTTGGATGCATTT
							KH_141_Fw	caccACGTACAGAGAATTATCGGGTG
							KH_141_Rv	ACCGTCGATGTTATTTTCAAGC

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
root	Le018398_at	7 dpi	1.97	0.001	Solyc01g010180	126 and 127	KH_126_Fw	caccTGGAGACCATCGATTTCAAGTC
							KH_127_Fw	caccTCCGTGGTGCGCTAGAAAAA
							KH_127_Rv	GCGTGAGAGTCCCTTCCAAA
root	Le000449_at	7 dpi	1.92	0.000	Solyc09g014280	128 and 129	KH_128_Fw	caccTGGCGATTCAGGTTACGGAG
							KH_128_Rv	TTCCGGGAACTTAAGCACCG
							KH_129_Fw	caccAGACGGGTTTGAAAAACGGC
							KH_129_Rv	CGCGTTCCCAAAATAGAGCG
root	Le006031_at	7 dpi	1.90	0.004	Solyc03g032060	130 and 142	KH_130_Fw	caccGCTAACTTCGAACGCCTCCT
							KH_130_Rv	GGAGCCAAGTACAACGAGCT
							KH_142_Fw	caccCGAGTGTTGCCGAATTGTGG
							KH_142_Rv	GCCGGAAATTGCAGGAACTT
root	Le006771_at	7 dpi	1.84	0.000	Solyc06g074710	132 and 133	KH_132_Fw	caccTGAAGACAACATCATCCCTCCT
							KH_132_Rv	GCTGATGCCTCAACGAATCG
							KH_133_Fw	caccTGGAGGGCCATCACTAAAGC
							KH_133_Rv	CAACCGCGTCATGAATGAGC
root	Le002219_at	7 dpi	1.83	0.001	Solyc03g114100	143 and 144	KH_143_Fw	caccACGGTTCAATCAAAACACACAC
							KH_143_Rv	ATAGCGCTACTGCTGGAACC
							KH_144_Fw	caccGGCCGGTGAGGGAGATTTC
							KH_144_Rv	ATGTCACCGACCGGAAACAT
root	Le001382_at	7 dpi	1.82	0.001	Solyc08g080660	182	KH_182_Fw	caccGTCAGAGTTGGACTACAGACTACA
							KH_182_Rv	TGTAGGCATCTCCAATGGGA

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
root	Le003733_at	7 dpi	1.77	0.000	Solyc09g097810	252	KH_252_Fw	caccTTCAACAACTGACTAATATGAAGCTCT
							KH_252_Rv	AGACACAACAATAGCCACAAAGG
root	Le003216_at	7 dpi	1.77	0.002	Solyc02g092550	184	KH_184_Fw	caccCAGTCAACGGTGCTGTAGGT
							KH_184_Rv	GATTCTCCGGCAAAGGGTGA
root	Le001733_at	7 dpi	1.76	0.000	Solyc02g078380	185	KH_185_Fw	caccTCTGCATCTTTTCTGGTGGT
							KH_185_Rv	AGCAAGAAAAAGAGTGGAGGCT
root	Le012974_at	7 dpi	1.76	0.003	Solyc03g044910	186	KH_186_Fw	caccGCAACACGTGGGTTTTCCTG
							KH_186_Rv	TTTGTACCTCCGCCAGAACC
root	Le004053_at	7 dpi	1.76	0.004	Solyc08g060810	187	KH_187_Fw	caccGGGGGAAGTCTCGAGTCTCT
							KH_187_Rv	GCTACTGACCCCATTGCAGT
root	Le005300_at	7 dpi	1.75	0.002	Solyc03g111710	188	KH_188_Fw	caccACATAGAAGGGAGCAGAACTTGT
							KH_188_Rv	GCTGCCACATCCTTTTGCAT
root	Le005591_at	7 dpi	1.74	0.001	Solyc02g079410	189	KH_189_Fw	caccAAAGCCACCACCAGAATCCG
							KH_189_Rv	TTTGCACAGCCCATTTGACG
root	Le014933_at	7 dpi	1.74	0.000	Solyc09g008740	190	KH_190_Fw	caccAATGGCGTGAGTTCCTTCGT
							KH_190_Rv	AGAACCGCGTTTGGTGAAGT
root	Le015960_at	7 dpi	1.73	0.001	Solyc04g064530	191	KH_191_Fw	caccGTATGGGCTTTCAGCATGCG
							KH_191_Rv	ATGATGGCTTCCATGCACCA
root	Le014536_at	7 dpi	1.73	0.003	Solyc08g076960	253	KH_253_Fw	caccGTTCCGAAACCGGTTCCTCT
							KH_253_Rv	GGTGGATCCGACGGTCATAC
root	Le002933_at	7 dpi	1.72	0.002	Solyc10g051270	193	KH_193_Fw	caccGCAAGACAACCTCTGACCCA
							KH_193_Rv	GGAACTCCTGTTGTTGCACC

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
root	Le008596_at	7 dpi	1.72	0.002	Solyc07g054080	194	KH_194_Fw	caccACCAACTACCGATTCAAGTCCA
							KH_194_Rv	TTGGGTATGCATACAGAGTTGT
root	Le019696_at	7 dpi	1.71	0.004	Solyc09g013140	195	KH_195_Fw	caccTGGCTTACACCCTGCTGTTT
							KH_195_Rv	GCCTTTTCCCAAGCCTTTTGAT
root	Le001417_at	7 dpi	1.71	0.005	Solyc12g009560	196	KH_196_Fw	caccTGTTGTCTTTGGGTCATGCTG
							KH_196_Rv	CTCAACACTGTAAGCCAGCG
root	Le021186_at	7 dpi	1.71	0.001	Solyc02g064800	197	KH_197_Fw	caccGGCCAGTATGCAGGAGAAAGT
							KH_197_Rv	AGTATTGAATTGACTGCACGCA
root	Le004827_at	7 dpi	1.70	0.001	Solyc08g082180	198	KH_198_Fw	caccTCAATTATCACTGTTCACCCGA
							KH_198_Rv	TGTCGATAGGCTCCATATGGC
root	Le011110_at	7 dpi	1.69	0.010	Solyc09g090730	199	KH_199_Fw	caccTGGATTCATCGGCCGACATT
							KH_199_Rv	ACCCGGCGAAATCGATTACA
root	Le009570_at	7 dpi	1.68	0.000	Solyc10g006755	254	KH_254_Fw	caccTGTGAGTTATGTGGTGAGAACA
							KH_254_Rv	AAAGCTTTGGACCAGAGGCA
root	Le009588_at	7 dpi	1.67	0.001	Solyc08g078940	226	KH_226_Fw	caccGACTGGTTGCAACAATCCACC
							KH_226_Rv	CCGACCGGACCGTTCAATAC
root	Le003702_at	7 dpi	1.66	0.000	Solyc12g056360	227	KH_227_Fw	caccATTTAGTCCTGTGTCCGCGG
							KH_227_Rv	AACCTCGAATAGTTGGTCGGAC
root	Le012570_at	7 dpi	1.65	0.004	Solyc07g006420	228	KH_228_Fw	caccGCCATAGCTAAAGGTGGAAGACT
							KH_228_Rv	TCAACTTCCTCTTCTCCATGGT
root	Le006518_at	7 dpi	1.65	0.003	Solyc11g011190	229	KH_229_Fw	caccCCGGACGTTAGGAGCTGATC
							KH_229_Rv	CGTCTTCCCGTCCCATTTCA

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
root	Le012782_at	7 dpi	1.62	0.000	Solyc01g098390	230	KH_230_Fw	caccTCGTCACTTGGCAGAGTTCC
							KH_230_Rv	TGCCTTGCAAATGCCAACAA
root	Le005337_at	7 dpi	1.61	0.004	Solyc06g065900	231	KH_231_Fw	caccCAGGTGGTTCGGAAGCAGAT
							KH_231_Rv	CGACGTCTCTGATGATGCGA
root	Le011991_at	7 dpi	1.59	0.002	Solyc04g071130	233	KH_233_Fw	caccCTTGGTGCAAACATGGGTCG
							KH_233_Rv	AGCAAGCAATCTAGGGCGTT
root	Le009788_at	7 dpi	1.59	0.005	Solyc07g008250	234	KH_234_Fw	caccTTGCGTTTCTAAGCGTTGGC
							KH_234_Rv	GAGAGCTTTCCAAGACCCCC
root	Le009356_at	7 dpi	1.60	0.002	Solyc05g052030	235	KH_235_Fw	caccCGATCACATCTCCTCCTCCG
							KH_235_Rv	GTCTTCATTAGAACCGGCGGA
root	Le015786_at	7 dpi	1.58	0.001	Solyc02g085745	236	KH_236_Fw	caccCAACAGAGGTGGCAAGGCTA
							KH_236_Rv	AGCCTCCACCATTCACCAAG
root	Le015699_at	7 dpi	1.58	0.008	Solyc01g007750	237	KH_237_Fw	caccACACCCACACCCCAAAACTT
							KH_237_Rv	ACACTTTGTGAGTAGCCGGG
root	Le003389_at	7 dpi	1.56	0.000	Solyc08g061010	238	KH_238_Fw	caccCGTCAGTGACAAAAGTGGAGG
							KH_238_Rv	ACGATCATATGTTGCAAGAGAGA
root	Le015880_at	7 dpi	1.56	0.007	Solyc02g070280	239	KH_239_Fw	caccCATCCTCGGGTCCTCAATCG
							KH_239_Rv	GCATGCAGCCCGAAAAAGAA
root	Le009087_at	7 dpi	1.55	0.001	Solyc11g012360	240	KH_240_Fw	caccAACACCACTCCTTCCAATTCA
							KH_240_Rv	ATGACTGAGCAACCTCGTCG
root	Le010851_at	7 dpi	1.55	0.006	Solyc02g077880	241	KH_241_Fw	caccGGTGGTGAATCAGGAGAAGGA
							KH_241_Rv	ACAGTAGGAGCGTTAGGGTG

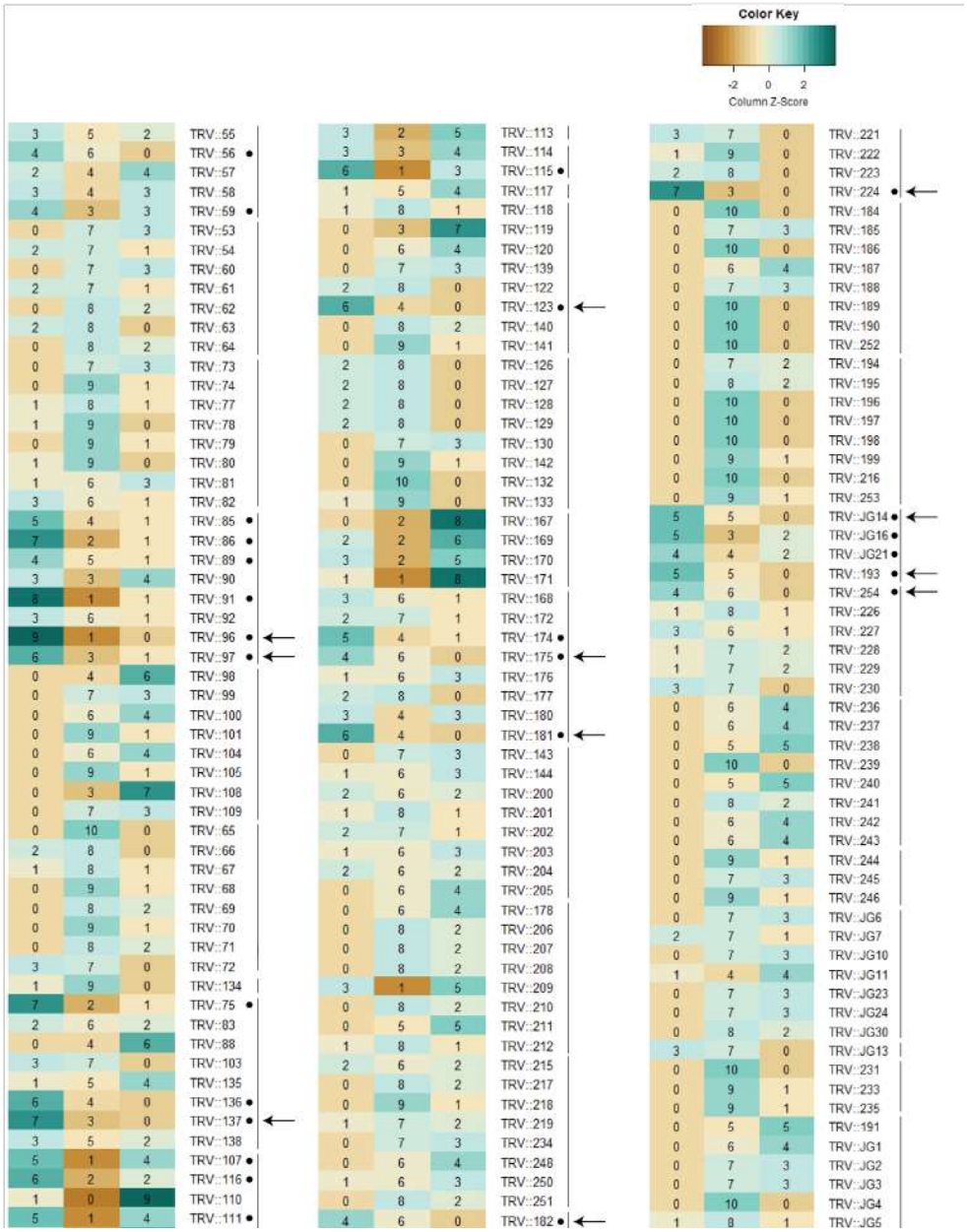
Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
root	Le003285_at	7 dpi	1.55	0.005	Solyc04g007790	242	KH_242_Fw	caccAGAGGGAGATACGTTGGAATTGT
							KH_242_Rv	AATCTTGTGGAGGTGACCCT
root	Le008118_at	7 dpi	1.53	0.000	Solyc03g005820	243	KH_243_Fw	caccGGTGTGTTACTTGGTGGGGT
							KH_243_Rv	GACGTTTCCCCAGACTCGTT
root	Le003798_at	7 dpi	1.52	0.004	Solyc01g005440	244	KH_244_Fw	caccTGTTTTTGGCTGGACATGGC
							KH_244_Rv	TGACATGGGGAGTCACTGAA
root	Le003910_at	7 dpi	1.51	0.001	Solyc04g079320	245	KH_245_Fw	caccGCATCGAGACAGTGGGACAA
							KH_245_Rv	GACCAGGACTTGTTGGATGGT
root	Le003086_at	7 dpi	1.50	0.001	Solyc07g054270	246	KH_246_Fw	caccTGGAGAATGGCAAAAGAAAACGA
							KH_246_Rv	CCACGACGACTACCCATGAAATA
root	Le005876_at	7 dpi	1.50	0.003	Solyc04g082060	JG01	JG_01_FW	caccCGACTCCCAAAAACCTACCA
							JG_01_RV	GAGAAACCGGTGGAAGATCA
root	Le016145_at	7 dpi	1.49	0.001	Solyc02g091430	JG02	JG_02_FW	caccGGTCAGTGCCGACAACTTTT
							JG_02_RV	CGAACGAAGCATATCAACGA
root	Le002783_s_at	7 dpi	1.49	0.000	Solyc09g011470	JG03	JG_03_FW	caccAAGCTGTCGGCCAGCAAG
							JG_03_RV	TTGTGCATGTAGAGCTCTGATTA
root	Le004663_at	7 dpi	1.48	0.006	Solyc04g005530	JG04	JG_04_FW	caccGGATTCCCAGTCCCTTCTTC
							JG_04_RV	AACCCAAGAAACTGCACACC
root	Le013901_at	7 dpi	1.48	0.000	Solyc08g079440	JG05	JG_05_FW	caccTTTGGATTCCATTCCTTCCA
							JG_05_RV	AAGCCCGTTTTAGACCTTGG
root	Le003688_at	7 dpi	1.47	0.003	Solyc05g008100	JG06	JG_06_FW	caccCCTCCTCAACTTTCCAGCAA
							JG_06_RV	AGGGACAATTGGTTCTGGTG

Tissue	Probe ID[1]	Time point	log2fc	adj. p-value	Tomato genome ID	TRV construct[2]	Primers	Sequence (5'→ 3')[3]
root	Le009424_at	7 dpi	1.46	0.000	Solyc06g054270	JG07	JG_07_FW	caccAAGCTTCACGCAAAGGAGAA
							JG_07_RV	TAATAGCCCCAGCGATGAAG
root	Le013458_at	7 dpi	1.46	0.000	Solyc03g113570	JG08	JG_08_FW	caccTGGGGAACGTTCTTTCATTC
							JG_08_RV	ATTTGGAAATGCACCAGTCC
root	Le000407_s_at	7 dpi	1.45	0.000	Solyc01g005440	JG10	JG_10_FW	caccGCAGCCAAGGTTTCAACTTC
							JG_10_RV	TGACATGGGGAGTCACTGAA
root	Le014376_at	7 dpi	1.45	0.006	Solyc04g082690	JG11	JG_11_FW	caccCACCGGGATCGGTAAAGTC
							JG_11_RV	GGAAGCGACTTGAGAACCTT
root	Le000005_at	7 dpi	1.45	0.003	Solyc02g065400	JG13	JG_13_FW	caccCCGGATGGCACTGTTAAGTT
							JG_13_RV	AAGCTCCTCCTCGTCTCCTC
root	Le005774_at	7 dpi	1.45	0.001	Solyc08g078220	JG14	JG_14_FW	caccTCCCCTGTCTAAGTGGCAAT
							JG_14_RV	TCCTTTAAACTGCCCTGTTGTT

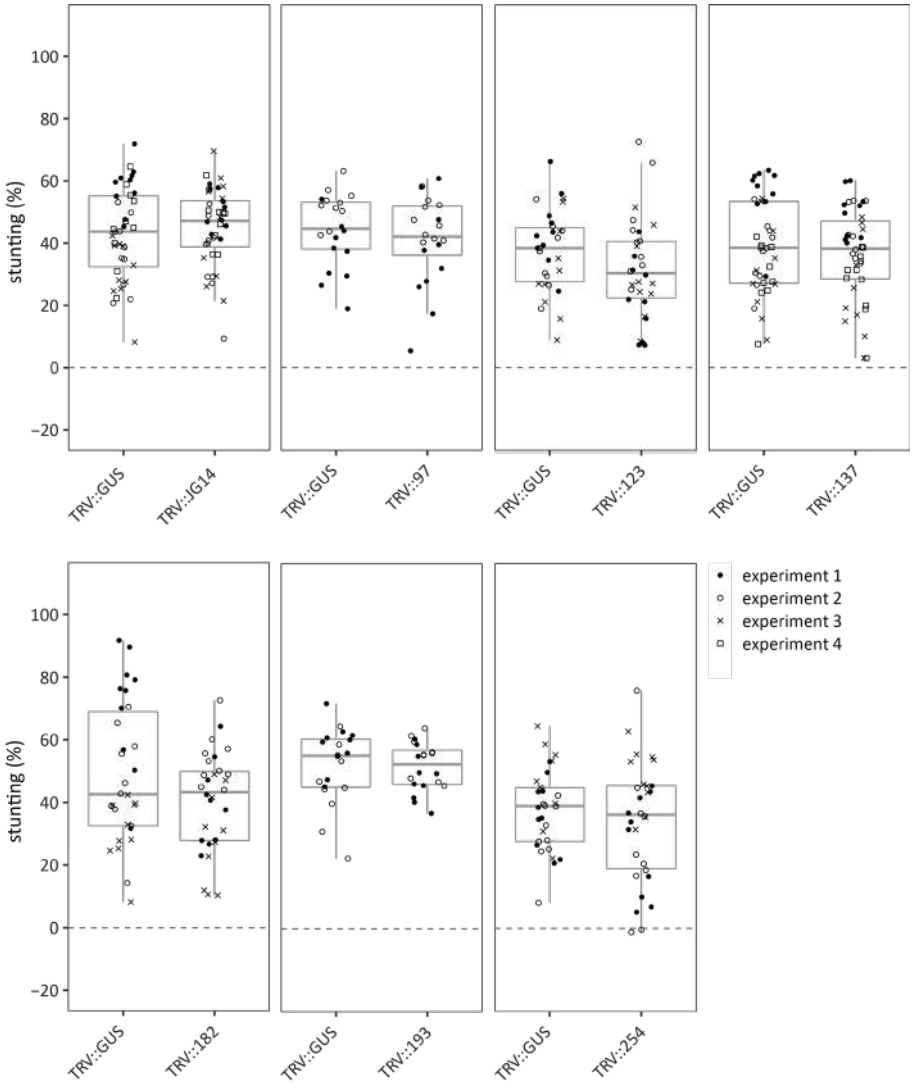
[1] Probe IDs that could not be assigned a Tomato genome ID after balstN search were discarded.
[2] Genes for which constructs could not be cloned were discarded.
[3] Small letters indicate 'cacc' overhang for Gateway cloning.

Supplementary Table 3 | Real-time PCR primers used in this study.

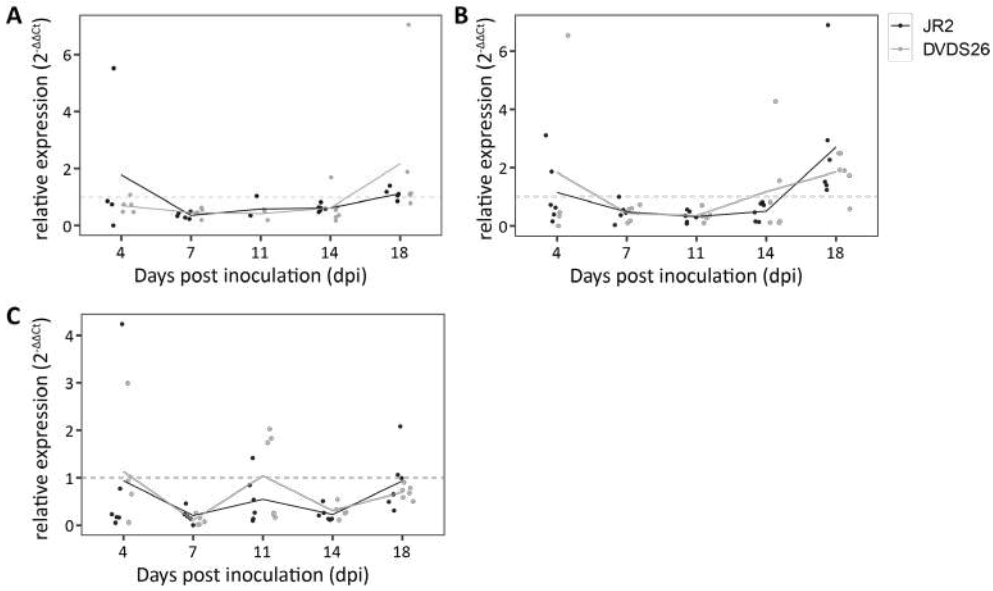
Primer	Sequence (5'→ 3')	ID
SlEF1α_Fw	ATTGGAAACGGATATGCCCCT	Solyc06g005060
SlEF1α_Rv	TCCTTACCTGAACGCCTGTCA	
KH_266_Fw	GCTTTGCACAAGGAAAATACGG	Solyc06g067950
KH_266_Rv	TCCGCTTAGACCTATGATAGCC	
KH_281_Fw	TAACTGCTGCCAGCTTCATG	Solyc03g093140
KH_281_Rv	TCCGGTGATCAACATGAGGATG	
KH_289_Fw	GAGGAAACTGATGGATGTCCTC	Solyc06g053830
KH_289_Rv	ATGGCTCTTGGTGCTAGTCC	



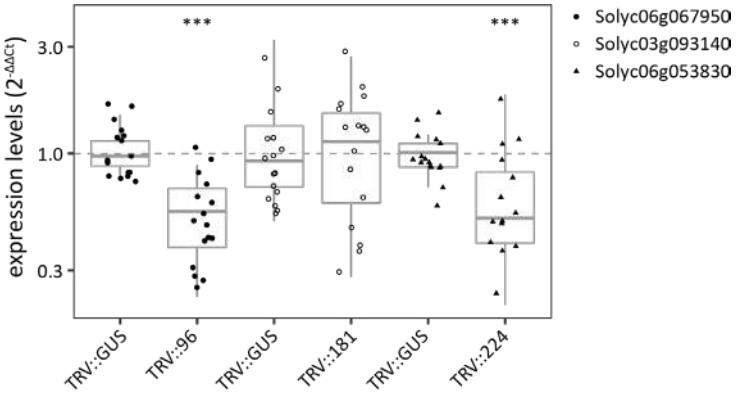
Supplementary Figure 1 | Heatmap of the number of plants with lower, equal or higher stunting when compared with TRV::GUS (considering all data points except outliers based on inter quantile range) and colour-coded per group. Vertical lines indicate constructs tested in the same experiment and dots highlight candidates with ≤ 4 plants with reduced stunting. Arrows indicate candidates also found in the first approach (relative stunting compared to TRV::GUS).



Supplementary Figure 2 | Stunting (%) of plants inoculated with *Verticillium dahliae* strain JR2 when compared with the average stunting of mock-inoculated plants at 21 dpi after *Agrobacterium tumefaciens* treatment of constructs TRV::JG14, TRV::97, TRV::123, TRV::137, TRV::182, TRV::193 and TRV::254 (left to right). Box plots represent data with n ≥ 9 per experiment per genotype (t-test compared to TRV::GUS with no significant difference).



Supplementary Figure 3 | Relative expression of candidates Solyc06g067950 (TRV::96) (A), Solyc03g093140 (TRV::181) (B) and Solyc08g080660 (TRV::224) (C) at five different time points after inoculation with *Verticillium dahliae* strain JR2 (race 2, black line) and DVDS26 (race 2, grey line). Expression was measured in either roots (A and B) or stems (C) depending on the tissue in which induction was found in the microarray analysis. Data were normalized to mock-inoculated samples per time point using the 2-ΔΔCT method. Data of two independent experiments with n ≥ 3 per experiment per genotype with a trendline.



Supplementary Figure 4 | Relative silencing levels ($2^{-\Delta\Delta Ct}$) of target genes for constructs TRV::96 (Solyc06g067950), TRV::181 (Solyc03g093140) and TRV::224 (Solyc06g053830) in stems when normalized to TRV::GUS 14 days after *Agrobacterium tumefaciens* treatment on a log10 scale. Data of two independent experiments with n ≥ 6 per experiment per genotype (t-test on ΔCt compared to TRV::GUS with *** < 0.01).

Chapter 4

Functional analysis of two susceptibility gene candidates for *Verticillium dahliae* in tomato using CRISPR-Cas9

Katharina Hanika, Danny Schipper, Dagmar Kuiper*, Shravya Chinnappa*, Timo d'Hont*, Henk J. Schouten, Bart P.H.J. Thomma, Yuling Bai

* These authors contributed equally to this work.

ABSTRACT

Plant disease susceptibility (*S*) genes play a key role in plant - microbe interactions as they facilitate establishment of disease by pathogens. We previously identified two *S* gene candidates for *Verticillium dahliae* in tomato via reverse genetics. The first candidate, Solyc06g067950, encodes an acyl-protein thioesterase 2 (APT2) which de-acylates proteins required for protein interactions with membranes. The second candidate, Solyc03g093140, encodes a glycerol-3-phosphate (G3P) transporter (GlpT) and G3P was shown to act as signaling molecule in systemic acquired resistance. Here, we used CRISPR-Cas9 to generate knock-outs in these two candidates to confirm their role in susceptibility to *V. dahliae*. For *APT2*, one mutant line with a large deletion was obtained which was predicted to result in a truncated protein lacking 90 of 256 amino acids (aa) at the C-terminus. For *GlpT*, two mutant lines were obtained for which the deletions were predicted to result in truncated proteins, lacking the N-terminal 284 and 176 of 521 aa. A third mutant line for *GlpT* carried a deletion which was predicted to cause a 125 aa deletion in the middle of the protein. Surprisingly, for none of the mutant lines loss of susceptibility upon challenge with *V. dahliae* was found. Hence, these results do not confirm the role of APT2 or GlpT in susceptibility to *V. dahliae* in tomato. However, additional CRISPR mutants are required for *APT2* to support these results.

INTRODUCTION

Recent advances in genome editing have revolutionized the field of biology and have found wide-ranging applications in various disciplines, including plant sciences. At the onset, zinc finger proteins were fused to endonuclease domains to generate DNA-cleaving enzymes (Kim *et al*., 1996). In 2005, this technology was reported in plants for the first time to study homologous recombination in tobacco (Wright *et al*., 2005). Only a few years later the discovery of transcription activator-like (TAL) effectors of *Xanthomonas* bacteria advanced site-directed genome editing when these effectors were fused with nucleases, creating TAL effector nucleases (TALEN) (Christian *et al*., 2010). A proof-of-concept was published shortly afterwards, in a study on the a*lcohol dehydrogenase 1* (*ADH1*) gene in Arabidopsis protoplasts (Cermak *et al*., 2011). The breakthrough in genome editing was reported only one year later, when an element of the adaptive immune system of bacteria against viruses was exploited to generate an RNA-guided nuclease complex (Jinek *et al*., 2012). This system uses clustered regularly interspaced short palindromic repeats (CRISPR) together with the Cas9 nuclease to facilitate cleavage of double-stranded DNA; now commonly referred to as CRISPR-Cas9. Due to its simplicity and ease of use, several publications on the use of CRISPR-Cas9 in various plant species appeared only one year later (Bortesi and Fischer, 2015), highlighting the immense potential of this genome editing technique in plant research. The application of the CRISPR-Cas9 system in plant genome editing has been extensively reviewed and future perspectives are generally dedicated to crop improvement and plant breeding (Bortesi and Fischer, 2015; Liu *et al*., 2017; Yin *et al*., 2017; Chen *et al*., 2019; Metje-Sprink *et al*., 2019).

With respect to crop protection and resistance breeding, the use of genome editing for the impairment of plant disease susceptibility (*S*) genes has gained increasing attention over recent years (Andolfo *et al*., 2016; Borrelli *et al*., 2018; Langner *et al*., 2018; Zaidi *et al*., 2018; Das *et al*., 2019; Mushtaq *et al*., 2019). *S* genes encode host components that are required by the pathogen to mediate a compatible interaction with the host, and thus foster host susceptibility. In order to obtain resistance, or rather loss of susceptibility, *S* genes need to be impaired in their beneficial function for the pathogen (Pavan *et al*., 2010; Gawehns *et al*., 2013; Hückelhoven *et al*., 2013; van Schie and Takken, 2014). This can be achieved via targeted genome editing, although loss-of-function alleles may also be identified in wild germplasm or in mutant populations. The discussion on *S* genes was sparked in 2002 after the discovery of the recessive *powdery mildew resistant 6* (*pmr6*) mutant that provides resistance to powdery mildew in Arabidopsis (Eckardt, 2002; Vogel *et al*., 2002). Ever since, and further boosted due to advances in genome editing, research on *S* genes has been expanded to other plant-pathogen interactions, although most studies on *S* genes are still conducted in Arabidopsis today.

To identify (novel) *S* genes in crops, we focused on the interaction between tomato and *Verticillium dahliae*. This vascular pathogen is posing a reoccurring threat to crop protection as its niche colonization renders fungicide treatment inefficient and also because its persisting resting structures remain viable in the soil for many years (Fradin and Thomma, 2006; Yadeta and Thomma, 2013). Moreover, crop protection is not feasible as *V. dahliae* has an enormous host range, including many weeds. As sources of monogenic resistance to *V. dahliae* are limited (Fradin *et al*., 2009; Song *et al*., 2017; Usami *et al*., 2017), the use of impaired *S* genes to combat *V. dahliae* represents an alternative strategy for resistance breeding. To this end, we previously used transcriptional profiling to identify induced genes in a compatible interaction between *V. dahliae* and tomato and functionally validated selected genes using virus-induced gene silencing (VIGS) in combination with *V. dahliae* inoculation (Chapter 3). Transient silencing of two candidates, Solyc06g067950 and Solyc03g093140, repeatedly resulted in reduced susceptibility to multiple *V. dahliae* strains. The first candidate, Solyc06g067950, is annotated as acyl-protein thioesterase 2 (APT2), which belongs to a group of enzymes that reverse S-acylation in eukaryotes. S-acylation is a lipid modification process in which fatty acids are added to cysteine residues of proteins through thioester bonds (Hemsley, 2015). Such post-translational lipid modifications play a role in anchoring proteins to membranes, but also in the regulation of signalling pathways and are therefore involved in many biological processes (Hurst and Hemsley, 2015; Li and Qi, 2017). Even though S-acylation is a ubiquitous mechanism in eukaryotes, not much is known about de-S-acylation. Besides APTs, also palmitoyl protein thioesterases (PPTs) regulate de-S-acylation, but knowledge on both APTs and PPTs is still lacking in plants (Hemsley, 2017). In mammals, two highly similar APTs are known (Won *et al*., 2018). APT1 plays a major role in de-S-acylation of the proto-oncogene Ras, and inhibition of APT1 disturbs acylation and trafficking of Ras in the cell (Conibear and Davis, 2010). APT2 was shown to de-acylate the neural growth-associated protein 43 (GAP-43) (Tomatis *et al*., 2010). However, a link of *APTs* to a role in plant immunity is not yet established and it is not known whether this candidate indeed acts in reversal of S-acylation in tomato.

The second candidate, Solyc03g093140, is annotated as glycerol-3-phosphate (G3P) transporter (GlpT). In *E. coli*, GlpT is an antiporter in the cytoplasmic membrane which regulates the uptake of G3P and the release of inorganic phosphate (Pi) (Lemieux *et al*., 2005; Law *et al*., 2009). In Arabidopsis, five proteins with high homology to the prokaryotic GlpT were characterized as G3P permeases (G3Pps) and were shown to be involved in Pi transport and homeostasis (Ramaiah *et al*., 2011). One of these proteins, G3Pp4, was further shown to play a role in regulation of seed lipid content (Kawai *et al*., 2014). A pair of Pi efflux transporters with homology to *E. coli* GlpT was also identified in rice (Xu *et al*., 2019). Even though a direct link of GlpTs to plant immunity

is not yet established, G3P was already shown to be a signaling molecule in systemic acquired resistance (Chanda *et al*., 2011). Furthermore, another G3P-related protein was shown to be involved in susceptibility to *Phytophthora palmivora* in Arabidopsis. Loss-of-function of a G3P acyl transferase, *Required for Arbuscular Mycorrhization 2* (*RAM2*), resulted in enhances resistance to *P. palmivora* (Wang *et al*., 2012). However, also for this candidate, its genuine functionality as a G3P transporter in tomato remains undemonstrated.

Here, we generated targeted deletion lines for *APT2* and *GlpT* using CRISPR to confirm the role of these two candidate genes in tomato susceptibility to Verticillium wilt.

MATERIALS & METHODS

Generation of CRISPR-Cas9 lines

To design sgRNAs the 'CCTop - CRISPR/Cas9 target online predictor' (https://crispr.cos.uni-heidelberg.de/; Stemmer *et al*., 2015) was used and for target site evaluation the tomato genome (*Solanum lycopersicum* Solyc2.5) was used as reference. Only sgRNAs with a maximum of one exonic off-target site were selected. All sgRNAs were verified to contain a GC-content (http://www.endmemo.com/bio/gc.php) between 30 and 80% and presence of required secondary structures was evaluated (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form; Zuker, 2003) according to Liang *et al*., 2016. Different scoring tools (https://sgrnascorer.cancer.gov/; Chari *et al*., 2017); https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design; Sanson et al., 2018; http://crispr.wustl.edu/; Wong *et al*., 2015) were used to select the best sgRNAs which met most of the criteria. In total, four sgRNAs were designed within a range of 1,700 bp per gene (Supplementary Table 1).

Golden Gate Cloning (Engler *et al*., 2008) was used to clone the constructs, and plasmids were obtained from Addgene (https://www.addgene.org/): pICH86966 (level 0 plasmid for amplification), pICSL01009 (level 0 plasmid containing AtU6), pICH47751 (level 1 position 1), pICH47761 (level 1 position 2), pICH47772 (level 1 position 3), pICH47781 (level 1 position 4), pICH47732 (level 1 containing NPTII), pICH47742 (level 1 containing Cas9), pICH41822 (linker) and pAGM4723 (level 2 binary vector) (Weber *et al*., 2011). Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Bleiswijk, The Netherlands) was used to amplify sgRNAs, and PCR products were purified with QIAquick PCR Purification Kit (Qiagen Benelux B.V., Venlo, The Netherlands). Level 1 plasmids were digested using *Bsa*I/*Eco31*I and ligated using T4 DNA ligase (Thermo Scientific, Bleiswijk, The Netherlands) and cloned into *Escherichia coli* strain DH5α (Thermo Scientific, Bleiswijk, The Netherlands). Plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen Benelux B.V., Venlo, The Netherlands). Level 2 plasmids were

digested using *Bpi*I/*Bps*I and ligated using T4 DNA ligase (Thermo Scientific, Bleiswijk, The Netherlands), cloned into *E. coli* strain DH5α, purified and sequenced. All plasmids were cloned into *Agrobacterium tumefaciens* strain AGL1+virG. Transformation of tomato cultivar Moneymaker (MM) was carried out as described previously (Huibers *et al*., 2013).

Plant growth conditions

All CRISPR lines and susceptible MM control plants were grown in the greenhouse (Unifarm, Wageningen University & Research, The Netherlands) with 60% relative humidity at 21°C/19°C (day/night) and a minimal light intensity of 100 W/m² in potting soil (Potgrond 4, Horticoop, Katwijk, The Netherlands). Plants for seed production were kept under the same conditions.

Pathogen inoculation & phenotyping

Inoculations with *V. dahliae* (strain JR2, race 1) and *V. albo-atrum* (strain CBS385.91, race 1) were carried out using root dipping in a conidial spore suspension as described previously (Fradin *et al.*, 2009). To phenotype the plants, stunting (%) was calculated between mock-inoculated and *V. dahliae*-inoculated plants based on plant canopy area at 21 days post inoculation (dpi) using Image J (Abramoff et al., 2004):

$$stunting\ (\%) = \left(1 - \frac{canopy\ area\ of\ V.\ dahliae{-}inoculated\ plant}{average\ canopy\ area\ of\ mock{-}inoculated\ plants}\right) \times 100.$$

For fungal biomass quantification, stems sections (~2 cm around the cotyledons) were harvested at 21 dpi, freeze-dried for 48 hours, and ground for DNA isolation using CTAB buffer (200 mM Tris-HCl pH 7.5, 50 mM EDTA pH 8.0, 2 M NaCl, 2% CTAB). Using a CFX96 Real-time System (Bio-Rad, Veenendaal, The Netherlands) and SYBR Green Master Mix (Bio-Rad, Veenendaal, The Netherlands) fungal biomass biomass was determined on genomic DNA targeting the *ITS* region relative to the reference gene *SlRUB* (Supplementary Table 1) and normalized to MM plants with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

DNA isolation & genotyping

To determine the presence of a mutation in the CRISPR lines, DNA was isolated from young leaves using CTAB buffer (1 M Tris-HCl pH 7.5, 0.5 M EDTA pH 8.0, 5 M NaCl, 2% CTAB). A gene-specific PCR was performed using DreamTaq DNA polymerase (Thermo Scientific, Bleiswijk, The Netherlands) with corresponding primers (Supplementary Table 1). PCR products were sent for Sanger sequencing to Marcrogen Europe (Amsterdam, The Netherlands).

RESULTS

Targeted deletion in *APT2* does not affect susceptibility to *V. dahliae*

To confirm the role of the two previously identified *S* gene candidates in susceptibility to *V. dahliae* in tomato (Chapter 3), CRISPR-Cas9-mediated knock-outs were generated. For each candidate gene, we designed four single guide RNAs (sgRNA) targeting different locations in the gene. The use of multiple sgRNAs aimed at creating large deletions due to simultaneous double stranded breaks at multiple target sites (Do *et al*., 2019). Genotyping of primary transformants (T1) was performed with a gene-specific PCR and gel electrophoresis to detect PCR products with aberrant sizes. The wild type PCR products were also sent for sequencing. For *APT2*, 45 T1 plants were genotyped and five were found to carry relatively large deletions (Supplementary Figure 1). From these plants, T2 seeds were obtained of three plants, which could be used for further testing (Table 1).

Table 1 | Overview of T2 CRISPR families obtained for *APT2* (Solyc06g067950).

TV number	T1 plant number[1]	T2 seed production	T2 genotyping
TV191152	4	In vitro plant did not root	-
TV191159	22	Seeds obtained	Deletion (segregating)
TV191160	20	No seeds obtained	-
TV191161	21	Seeds obtained	Not a mutant
TV191175	43	Seeds obtained	Not a mutant

[1] See also Supplementary Figure 1.

To verify the presence of a mutation in the T2 generation, the obtained T2 families were genotyped and sequenced as described before. For only one of the three T2 families, TV191159, the mutation could be confirmed (Figure 1A). This family had an 815 bp deletion between sgRNA 1 and 3, affecting the C-terminus of the gene. Following a Mendelian segregation, in total 25.4% of the plants were wild type, 49.7% heterozygous and 24.9% homozygous for the deletion (Figure 1B). This deletion resulted in a truncated protein lacking 90 out of the 256 amino acids at the C-terminus (Figure 1C), showing that *APT2* was mutated, but the N-terminus of the protein was not affected. To further understand the effect of this deletion, we predicted protein domains using InterPro (https://www.ebi.ac.uk/interpro/). One alpha/beta hydrolase domain was predicted which is found in phospholipases, carboxylesterases and thioesterases and which is in line with the annotated function of this candidate as acyl-protein thioesterase 2 in the Sol genomics database (https://solgenomics.net/). In order to quantify whether the deletion had any effect on plant growth, canopy area was measured in absence

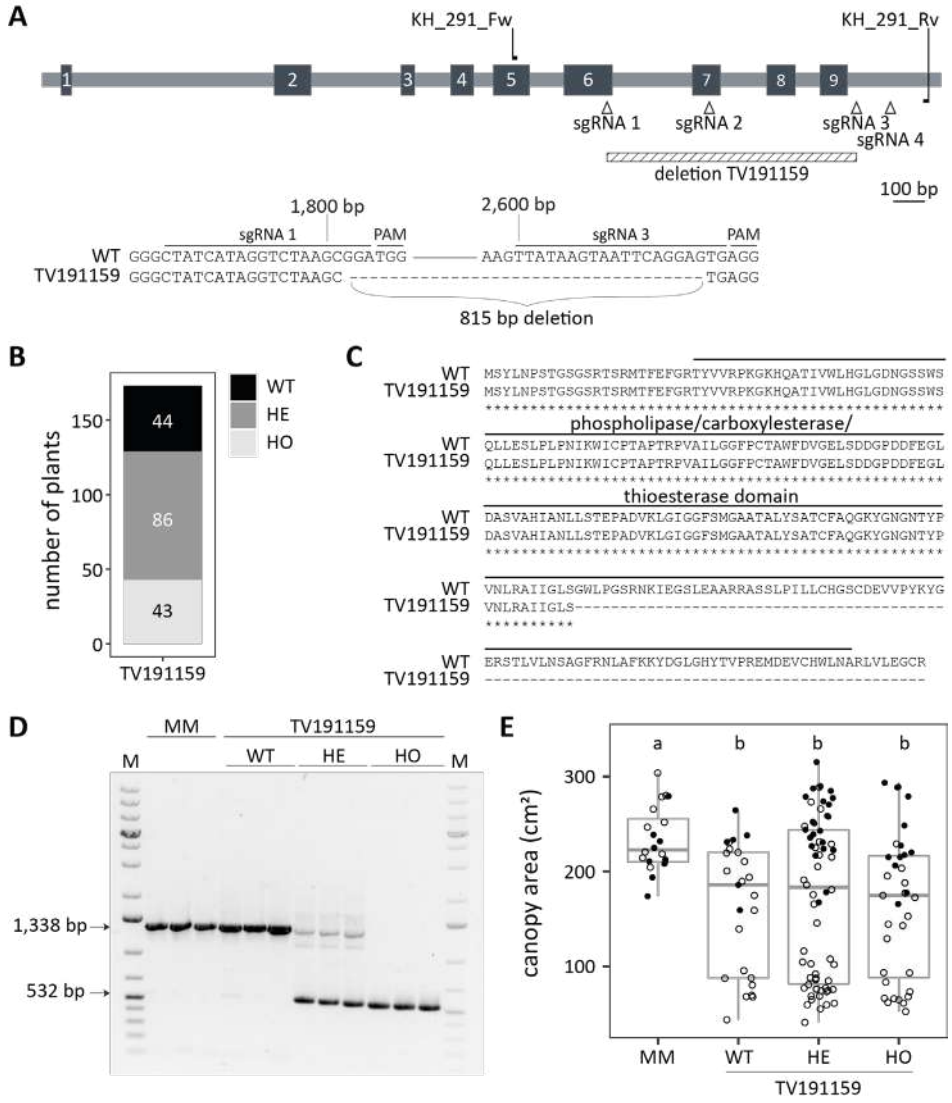


Figure 1 | Targeted deletion in *APT2* (Solyc06g067950) of T2 family TV191159. (A) Schematic overview of Solyc06g067950 indicating the exons (dark grey), the locations of the sgRNAs and the primers used for genotyping. Sanger sequencing of the wild type (WT) and the TV191159 mutant allele revealed a 815 bp deletion between sgRNAs 1 and 3. (B) Total number of TV191159 plants with a WT allele (black), a heterozygous (HE, dark grey) or a homozygous deletion (HO, light grey). (C) Protein alignments of Solyc06g067950 WT with TV191159 showing a 90 amino acid deletion. Alleles were translated into protein using http://www.softberry.com/berry.phtml. CLUSTAL multiple sequence alignment was done using https://www.ebi.ac.uk/Tools/msa/muscle/. Solid bars indicate predicted protein domains annotated as phospholipase/carboxylesterases/thioesterases domain (https://www.ebi.ac.uk/interpro). (D) Gel electrophoresis (1% TAE, ethidium bromide) of gene-specific PCR with primers KH_291 showing the Moneymaker (MM) allele and the TV191159 WT allele with a PCR band at 1,338 bp as well as the deletion allele at 532 bp with a 1 kb ladder (M). (E) Canopy area of mock-inoculated plants at 21 dpi for MM and T2 family TV191159. Data of two independent experiments indicated with shapes (experiment 1 full circle, experiment 2 open circles) with n ≥ 6 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.001).

of *V. dahliae* inoculation for all genotypes. Compared with MM plants, TV191159 plants showed similar (data of experiment 1, full circles in Figure 1E) or significant smaller canopy area (experiment 2, open circles in Figure 1E). The variation between the experiments could be caused by environmental conditions as experiments were conducted in different greenhouse compartments. This indicates that the mutation had no effect on plant growth of any TV191159 genotype even though a large variation was found between experimental repeats. The difference in experiment 2 when compared with MM plants can most likely be attributed to the overall transformation procedure and/or environmental conditions.

To assess whether the mutation in *APT2* affected susceptibility to *V. dahliae*, T2 plants were challenged with *V. dahliae*. Stunting of mock-inoculated and *V. dahliae*-inoculated plants was determined for all genotypes and compared with MM control plants. Surprisingly, no significant difference in stunting between any genotypes of T2 family TV191159 was found, and also not when compared with MM control plants (Figure 2A). Additionally, fungal biomass was quantified in stems of *V. dahliae*-inoculated plants. No difference in fungal biomass was found for any of the genotypes (Figure 2B). Taken together, these data indicate that the deletion found in T2 family TV191159 did not affect susceptibility to *V. dahliae*.

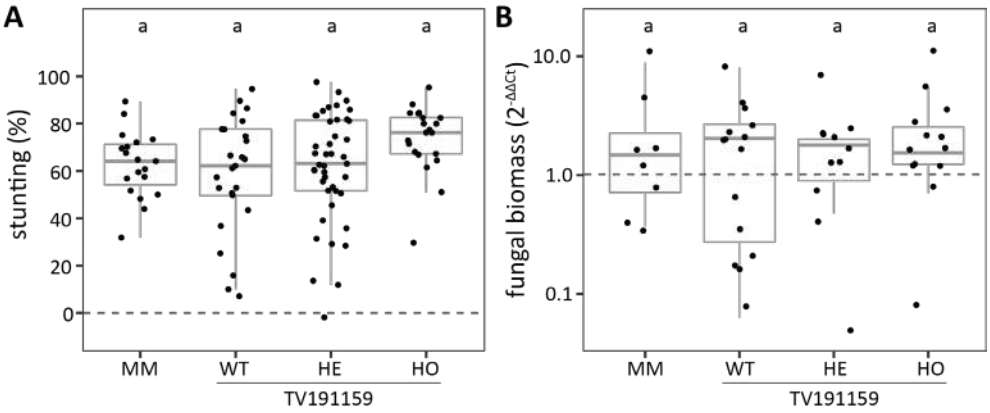


Figure 2 | Targeted deletion in *APT2* (Solyc06g067950) of T2 family TV191159 does not affect susceptibility to *Verticillium dahliae*. (A) Stunting (%) of *V. dahliae*-inoculated Moneymaker (MM) plants and TV191159 T2 plants with wild type (WT) allele, heterozygous (HE) or homozygous (HO) deletion when compared with the average stunting of mock-inoculated plants at 21 dpi. Box plots represent data of two independent experiments with n ≥ 9 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.01). (B) Fungal biomass of *V. dahliae*-inoculated T2 plants relative to *V. dahliae*-inoculated MM plants in stems at 21 dpi and normalized using $2^{-\Delta\Delta Ct}$ on a log10 scale. Data of two independent experiments with n ≥ 3 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.01 on ΔCt).

For subsequent experiments, homozygous T2 plants were kept for seed production and T3 plants were genotyped as before. Two T3 lines, TV191159-4 and -62, were found to be homozygous for the same 815 bp deletion as the corresponding T2 genotype (Figure 3A). In contrast to the T2 generation, the canopy area in absence of *V. dahliae* inoculation of T3 plants was not significantly different when compared with MM control plants (Figure 3B). Plants of the two T3 lines were challenged with *V. dahliae* and no reduced stunting was found when compared with MM plants (Figure 3C). Finally, fungal biomass quantification did also not reveal reduced biomass in plants of the T3 lines when compared with MM control plants.

As *S* genes can provide broad spectrum resistance to multiple pathogens, we also inoculated the T3 plants with *V. albo-atrum* (*Vaa*). Also for this pathogen, no reduced stunting was found for *Vaa*-inoculated plants when compared with MM plants (Figure 3C). Moreover, no reduced fungal biomass was determined for *Vaa*-inoculated plants when compared with MM plants (Figure 3D). Collectively, the data of the T2 and T3 generation did not confirm the role of *APT2* in susceptibility to *V. dahliae* and in addition *APT2* does not seem to be involved in susceptibility to *Vaa* either.

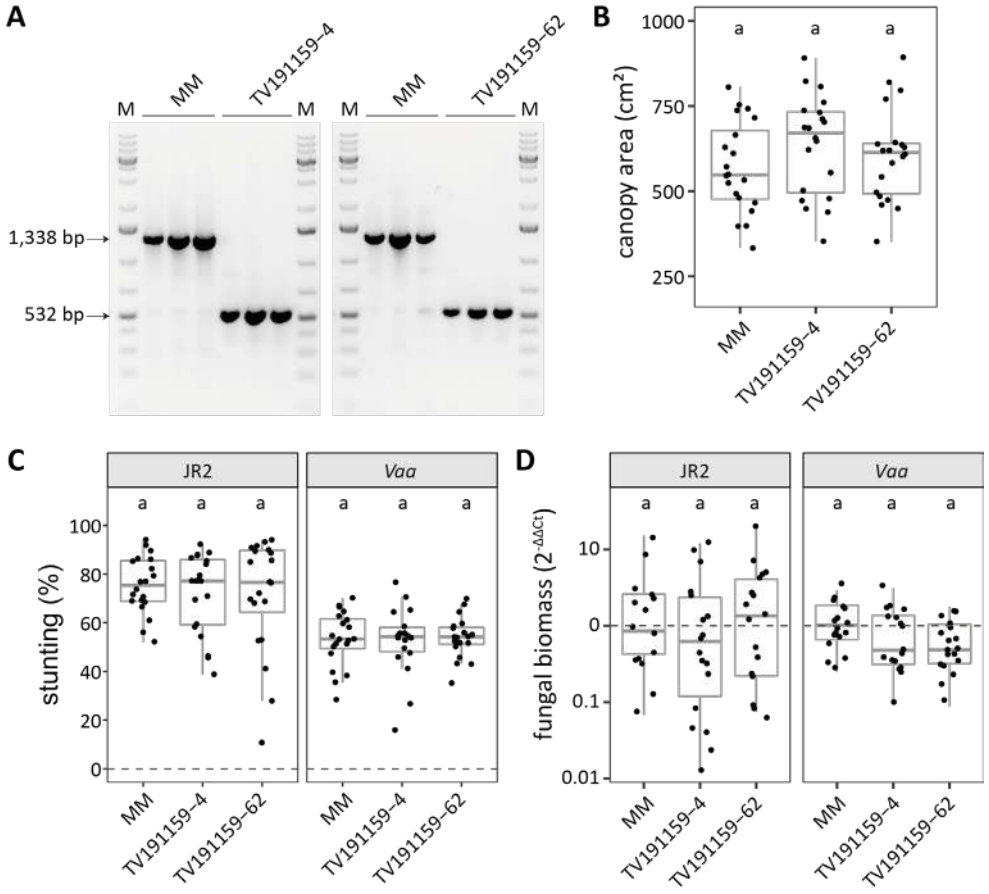


Figure 3 | Targeted deletion in *APT2* (Solyc06g067950) of T3 lines TV191159-4 and -62. (A) Gel electrophoresis (1% TAE, ethidium bromide) of gene-specific PCR with primers KH_291 showing the Moneymaker (MM) and wild type (WT) allele for TV191159-4 and -62 with a PCR band at 1,338 bp as well as the deletion allele at 532 bp with a 1 kb ladder (M). (B) Canopy area of mock-inoculated plants at 21 dpi for MM and T3 lines TV191159-4 and -62. Data of two independent experiments with n ≥ 9 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.001). (C) Stunting (%) of *Verticillium dahlia* (JR2) or *V. albo-atrum* (*Vaa*) -inoculated T3 plants when compared with the average stunting of mock-inoculated plants at 21 dpi. Box plots represent data of two independent experiments with n ≥ 9 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.01). (D) Fungal biomass of *V. dahliae*- or *Vaa*-inoculated T3 plants relative to *V. dahliae*- or *Vaa*-inoculated MM plants in stems at 21 dpi and normalized using $2^{-\Delta\Delta Ct}$ on a log10 scale. Data of two independent experiments with n ≥ 6 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.01 on ΔCt).

Targeted deletion in *GlpT* does not affect susceptibility to *V. dahliae*

For the second candidate, GlpT, the same strategy was pursued as for the first candidate. After transformation with a CRISPR-Cas9 construct, 56 T1 plants were obtained and genotyped. Four transformants showed relatively large deletions and plants were maintained for seed production (Supplementary Figure 2). From all four T1 plants, T2 seeds were obtained which were further analyzed. Genotyping revealed that families TV191153, TV191155 and TV191177 carried different deletions for this candidate, while family TV191154 was not found to carry a mutation (Table 2). Firstly, T2 family TV191153 had a 462 bp deletion between sgRNAs 3 and 4, and 30.7%, 50.0% and 19.3% of the plants were found to carry the wild type allele, a heterozygous or a homozygous deletion, respectively (Figure 4 A and B). Subsequently, the mutant allele was translated into protein (http://www.softberry.com/berry.phtml) and compared with the wild type protein (Figure 4C). For family TV191153 a 125 of 521 amino acid deletion in the middle of the protein was predicted. The second T2 line, TV191155, was found to be homozygous for a large 1,105 bp deletion. In addition, this line was found to carry a random 99 bp insertion for which no hit was found in a regular blast search (Figure 4 A and B). Line TV191155 was predicted to lack the N-terminal 284 amino acids (Figure 4C). The third T2 family, TV191177, carried a small 280 bp deletion at the N-terminus (Figure 4 A and B). This family segregated with 27.4%, 48.8% and 23.8% for the wild type allele, a heterozygous or a homozygous deletion, respectively. All heterozygous plants were found to have an additional band in-between the wild type and deletion allele, however, sequencing of this band was not successful (Figure 4C). Also for this T2 family, protein prediction of the mutant allele showed a 176 amino acid deletion affecting the N-terminus of the protein (Figure 4C).

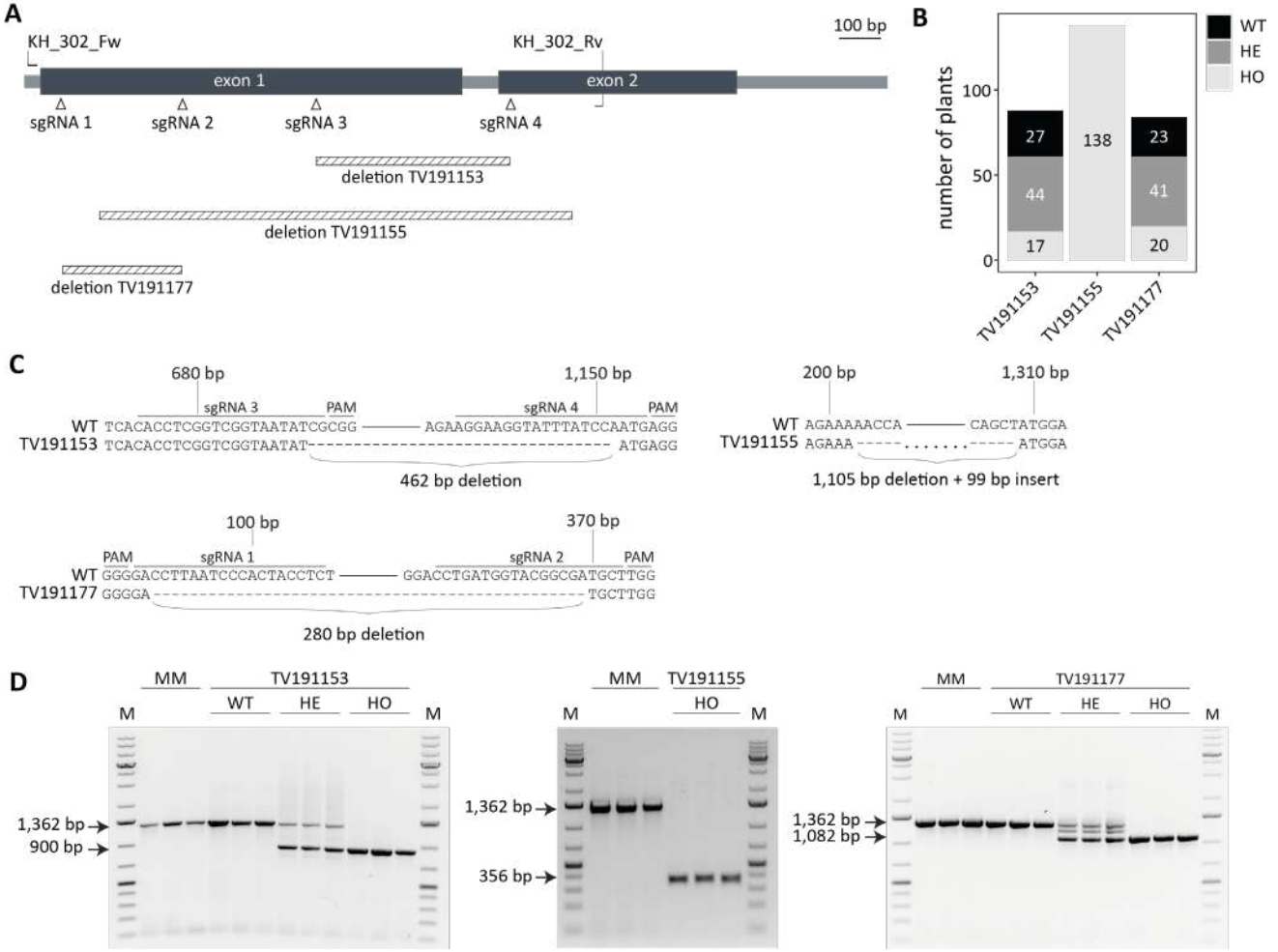
Table 2 | Overview of T2 CRISPR families obtained for GlpT (Solyc03g093140).

TV number	T1 plant number[1]	T2 seed production	T2 genotyping
TV191153	4	Seeds obtained	Segregating
TV191154	8	Seeds obtained	Not a mutant
TV191155	14	Seeds obtained	Homozygous deletion
TV191177	53	Seeds obtained	Segregating

[1] See also Supplementary Figure 2.

To further study the deletions in these three T2 families, we first predicted the domains of the wild type protein using InterPro (https://www.ebi.ac.uk/interpro/) and found one domain annotated as major facilitator superfamily (MFS) domain spanning across the protein. All known organisms have MFS proteins which function as transporters and carry single-polypeptides and small solutes based on ion gradients (Pao *et al.*,

1998). All three mutant proteins were predicted to affect the MFS domain (Figure 4D). As MFS proteins are known to consist of 12 transmembrane domains, we also predicted the transmembrane domains for the wild type protein and the three mutant proteins (http://www.cbs.dtu.dk/services/TMHMM/). As expected, the wild type protein was predicted to contain 12 transmembrane domains. In contrast, the three mutant proteins were predicted to carry only nine, three and eight transmembrane regions for T2 families TV191153, TV191155 and TV191177, respectively (Supplementary Figure 3). Moreover, the effect of the different deletions on plant growth was assessed in absence of *V. dahliae* inoculation. Only T2 family TV191155 was found to have a significantly smaller canopy area compared to MM plants (Figure 4E). Collectively, our data show that the three obtained T2 families carry different deletions in GlpT, which all affect the predicted MFS domain as well as the transmembrane regions.


A
KH_302_Fw
KH_302_Rv
100 bp
exon 1
exon 2
sgRNA 1
sgRNA 2
sgRNA 3
sgRNA 4
deletion TV191153
deletion TV191155
deletion TV191177
B
number of plants
WT
HE
HO
27
44
17
138
23
41
20
TV191153
TV191155
TV191177
C
680 bp
1,150 bp
sgRNA 3
PAM
sgRNA 4
PAM
WT TCACACCTCGGTCGGTAATATCGCGG AGAAGGAAGGTATTTATCCAATGAGG
TV191153 TCACACCTCGGTCGGTAATAT ATGAGG
462 bp deletion
200 bp
1,310 bp
WT AGAAAAACCA CAGCTATGGA
TV191155 AGAAA ATGGA
1,105 bp deletion + 99 bp insert
100 bp
370 bp
PAM
sgRNA 1
sgRNA 2
PAM
WT GGGGACCTTAATCCCACTACCTCT GGACCTGATGGTACGGCGATGCTTGG
TV191177 GGGGA TGCTTGG
280 bp deletion
D
MM
TV191153
WT
HE
HO
M
M
1,362 bp
900 bp
MM
TV191155
HO
M
M
1,362 bp
356 bp
MM
TV191177
WT
HE
HO
M
M
1,362 bp
1,082 bp

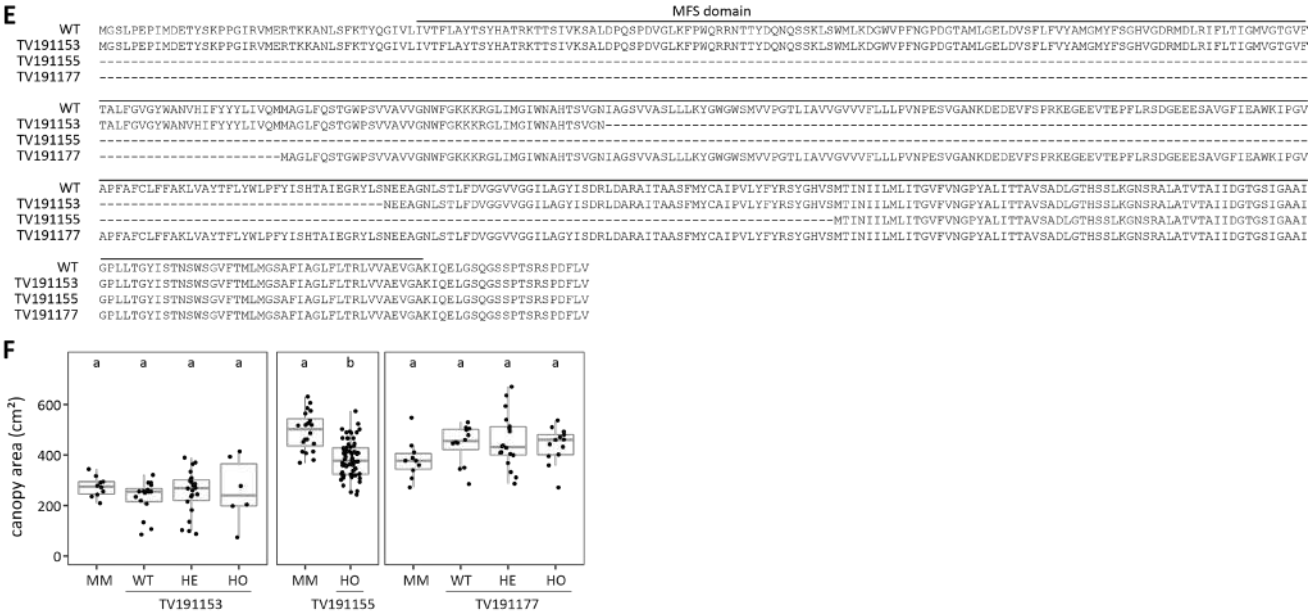


Figure 4 | Targeted deletion in *GlpT* (Solyc03g093140) of T2 families TV191153, TV191155 and TV191177. (A) Schematic overview of Solyc03g093140 indicating the exons, the locations of the sgRNAs, primers used for genotyping and the mutant alleles of TV191153, TV191155 and TV191177. (B) Total number of TV191153, TV191155 and TV191177 plants with a wild type (WT) allele (black), a heterozygous (HE, dark grey) or a homozygous deletion (HO, light grey). (C) Sanger sequencing of the WT and mutant alleles showed a 462 bp deletion between sgRNAs 3 and 4 for TV191153, a 1,105 bp deletion with a random 99 bp insert for TV191155 and a 280 bp deletion between sgRNAs 1 and 2 for TV191177. Gel electrophoresis (1% TAE, ethidium bromide) of gene-specific PCR with primers KH_302 showing the Moneymaker (MM) and WT allele with a PCR band at 1,362 bp as well as the deletion alleles for T2 families TV191153, TV191155 and TV191177 at 900 bp, 356 bp and 1,082 bp, respectively, with a 1 kb ladder (M). (D) Protein alignments of Solyc06g067950 WT with TV191153, TV191155 and TV191177 showing 125, 284 and 176 amino acid deletions, respectively. Alleles were translated into protein using http://www.softberry.com/berry.phtml. CLUSTAL multiple sequence alignment was done using https://www.ebi.ac.uk/Tools/msa/muscle/. Solid bars indicate predicted protein domains annotated as major facilitator superfamily (MFS) domain (https://www.ebi.ac.uk/interpro). (E) Canopy area of mock-inoculated plants at 21 dpi for MM and T2 families TV191153, TV191155 and TV191177. Data of one or two independent experiments with n ≥ 3 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.001).

All T2 families of GlpT were challenged with *V. dahliae*. For *V. dahliae*-inoculated plants of T2 families TV191153 and TV191177 no significant difference in stunting was found compared with *V. dahliae*-inoculated MM control plants or compared with *V. dahliae*-inoculated wild type plants (Figure 5A). Only for the homozygous T2 line TV191155 significantly reduced stunting was found with a large variation between plants. However, fungal biomass quantification for all genotypes revealed no significant difference when compared with MM control plants (Figure 5B). These results do not confirm the role of GlpT in susceptibility to *V. dahliae*.

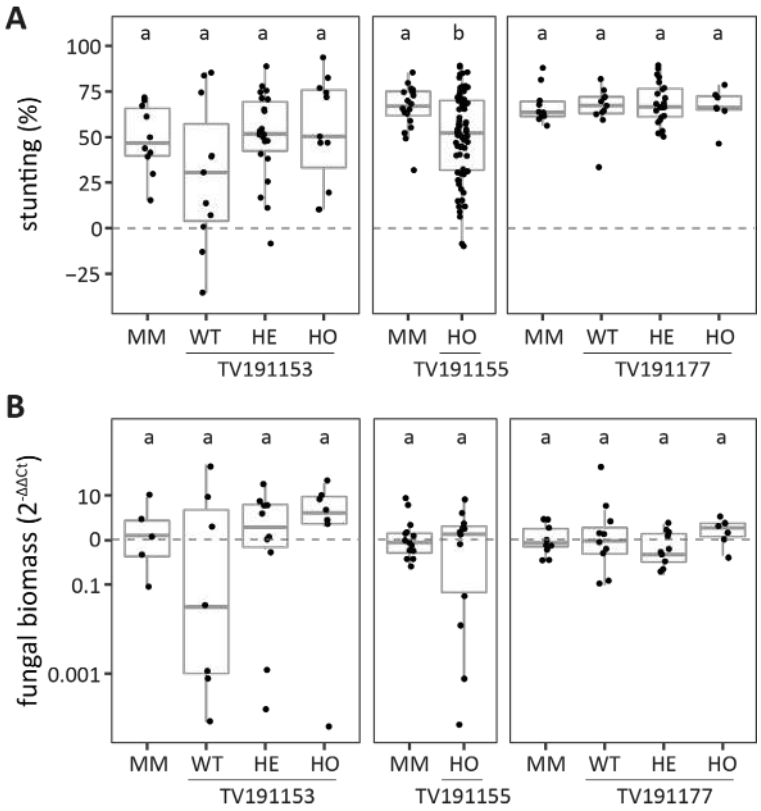


Figure 5 | Targeted deletions in Solyc03g093140 for T2 families TV191153, TV191155 and TV191177 do not affect susceptibility to *Verticillium dahliae*. (A) Stunting (%) of *V. dahliae*-inoculated Moneymaker (MM) and T2 plants with wild type (WT) allele, homozygous (HO) or heterozygous (HE) deletion when compared with the average stunting of mock-inoculated plants at 21 dpi. Box plots represent data of one or two independent experiments with n ≥ 9 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.01). (B) Fungal biomass of *V. dahliae*-inoculated T2 plants relative to *V. dahliae*-inoculated MM plants in stems at 21 dpi and normalized using $2^{-\Delta\Delta Ct}$ on a log10 scale. Data of one or two independent experiments with n ≥ 3 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.01 on ΔCt).

Several plants per T2 family were kept for T3 seed production. For family TV191153, accidentally heterozygous instead of homozygous plants were selected, resulting in segregation in the T3 generation once again. For line TV191155, homozygous T3 were seeds obtained, while seeds for family TV191177 were not available on time for subsequent experiments. The mutations in T3 family TV191153-59 and T3 line TV191155-1 were confirmed with PCR and gel electrophoresis as described previously (Figure 6A). T3 family TV191153-59 was segregating as expected with 25.0%, 51.8% and 23.2% for the wild type allele, the heterozygous and homozygous deletion, respectively (Figure 6B). The effect of the mutation on plant growth in the absence of *V. dahliae* inoculation was assessed, and no significant differences were found for plants of TV191153-59 and TV191155-1 when compared with MM plants (Figure 6C).

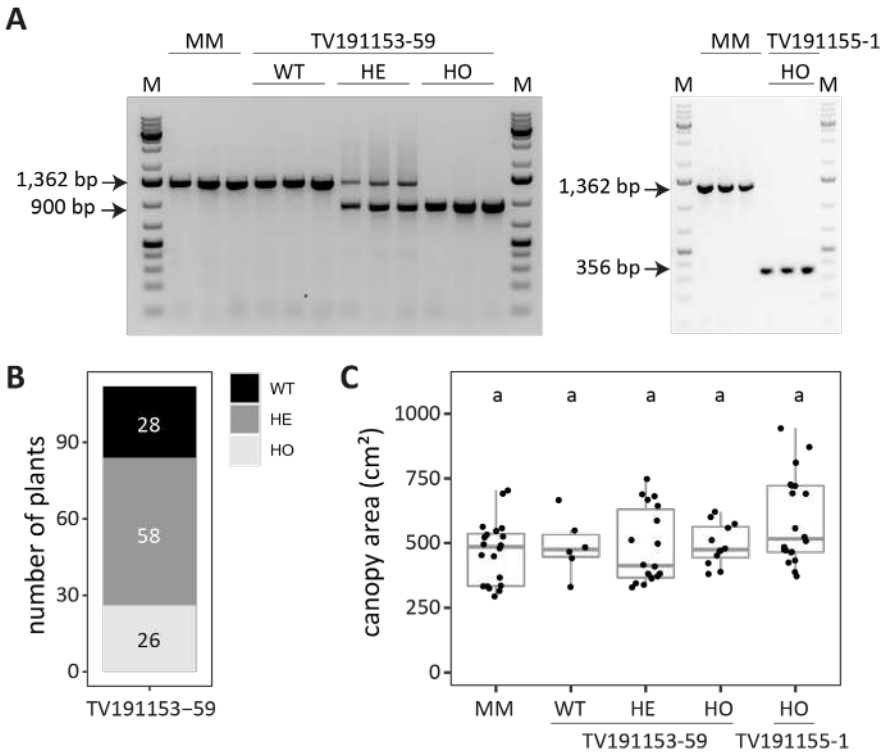


Figure 6 | Targeted deletion in GlpT (Solyc03g093140) of T3 family TV191153-59 and T3 line TV191155-1. (A) Gel electrophoresis (1% TAE, ethidium bromide) of gene-specific PCR with primers KH_302 showing the Moneymaker (MM) and wild type (WT) allele with a PCR band at 1,362 bp as well as the deletion alleles of TV191153-59 (heterozygous (HE) or homozygous (HO) deletion) and TV191155-1 at 900 bp and 356 bp, respectively, with a 1 kb ladder (M). (B) Total number of TV191153-59 plants with WT allele (black), a HE (dark grey) or a HO deletion (light grey). (C) Canopy area of mock-inoculated plants at 21 dpi for T3 family TV191153-59 and T3 line TV191155-1. Data from two independent experiments with n ≥ 3 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.001).

The T3 plants were challenged with *V. dahliae* to test for loss of susceptibility, but all genotypes except TV191155-1 showed no reduced stunting when compared with inoculated MM plants (Figure 7A). Further biomass quantification also did not reveal significant differences between the CRISPR mutants and the MM plants (Figure7B). In addition, the T3 genotypes were also challenged with *Vaa*, but neither reduced stunting nor reduced fungal biomass was found for *Vaa*-inoculated plants when compared with MM plants (Figures 7 A and B). Together with the data of the T2 generation, this data did not confirm the role of the GlpT in susceptibility to *V. dahliae* in tomato and GlpT also is not involved in susceptibility to *Vaa*.

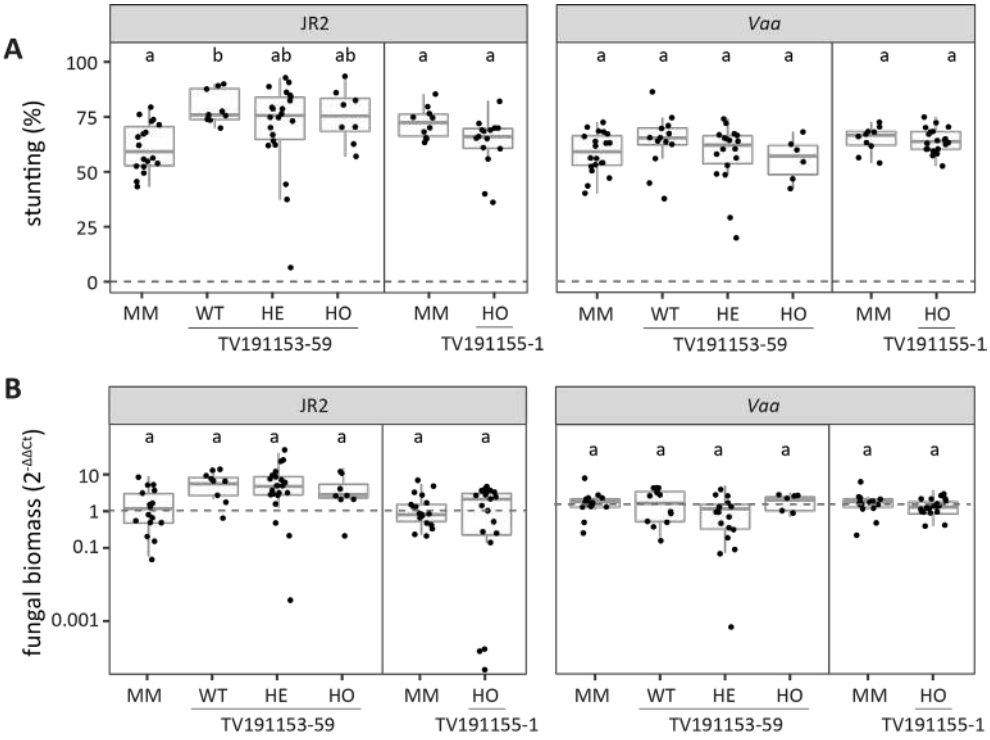


Figure 7 | Targeted deletions in GlpT (Solyc03g093140) of T3 family TV191153-59 and T3 line TV191155-1 do not affect susceptibility to *Verticillium dahliae* (JR2) and *V. albo-atrum* (*Vaa*). (A) Stunting (%) of *V. dahliae*- or *Vaa*-inoculated T3 plants when compared with the average stunting of mock-inoculated plants at 21 dpi. Box plots represent data from two independent experiments with n ≥ 3 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.01). (B) Fungal biomass of *V. dahliae*- or *Vaa*-inoculated T3 plants relative to *V. dahliae*- or *Vaa*-inoculated MM plants in stems at 21 dpi and normalized using $2^{-\Delta\Delta Ct}$ on a log10 scale with n ≥ 6 per experiment per genotype (ANOVA, Fisher's unprotected LSD with p = 0.01 on ΔCt).

DISCUSSION

To obtain resistance to plant pathogens, genome editing can be used to create loss-of-function alleles of disease *S* genes (Andolfo *et al*., 2016; Borrelli *et al*., 2018; Langner *et al*., 2018; Zaidi *et al*., 2018; Das *et al*., 2019; Mushtaq *et al*., 2019). Here we used CRISPR-Cas9 to impair two *S* gene candidates that we previously identified in the interaction between *V. dahliae* and tomato (Chapter 3). These candidates were selected based on transcriptomic profiling and were functionally characterized using VIGS. Silencing of these two genes repeatedly reduced susceptibility to different strains of *V. dahliae*. Surprisingly, the generated CRISPR mutants did not show loss of susceptibility upon challenge with *V. dahliae* or V. albo-atrum. For GlpT, three independent CRISPR lines with different deletions in the target gene were equally stunted as MM plants when inoculated with *V. dahliae* and no reduced fungal biomass was found. The role of this candidate in susceptibility to *V. dahliae* could therefore not be confirmed. As it is known that Arabidopsis contains five putative GlpTs and rice two (Ramaiah *et al*., 2011; L. Xu *et al*., 2019), it could be possible that multiple GlpTs are also present in tomato. Phylogenetic analysis indeed indicated at least two proteins with high homology to our candidate, which were also annotated as GlpTs (Supplementary Figure 4A). In case of a knock-out of our candidate GlpT, one of these potential homologues could be functionally redundant and hence the effect on susceptibility to *V. dahliae* would be masked. In order to circumvent this, RNAi silencing constructs could be generated. In contrast to knock-outs, silencing might not trigger the expression of one of the other homologues and could therefore affect susceptibility. Alternatively, multiple GlpTs could be targeted simultaneously with a single RNAi construct. With respect to potential functional redundancy, it is also possible that even though the VIGS constructs were carefully designed (Chapter 3), these also could have had off-targets. A blastN search of the gene-specific sequences used for silencing against the tomato genome revealed four potential off-targets with sequence homologies between 80 and 100% (Supplementary Figure 4B). In fact, two of these potential off-targets concern potential homologues of our GlpT candidate. It is therefore possible that our previous results in the VIGS assays were caused by silencing multiple genes, or even by silencing one of the homologs that was not our initial target gene (Chapter 3). By targeting only one candidate using CRISPR in this chapter, this would explain why the role of GlpT in susceptibility to *V. dahliae* could not be confirmed. Another explanation could be the differences in tools used to identify and verify this *S* gene candidate. When using VIGS for transient silencing, plants are treated with an Agrobacterium suspension that contains a viral vector (Senthil-Kumar and Mysore, 2014). In addition to these two organisms, also *V. dahliae* is introduced to screen for reduced susceptibility. It could be possible that the presence of *Agrobacterium* and the virus alters certain biological processes in the plant, for example, hormone homeostasis. The role of an *S* gene candidate in this context

might be different than in a knock-out assays that does not make use of *Agrobacterium* and a viral vector.

For the second candidate only one T2 CRISPR line was obtained, and also no loss of susceptibility to *V. dahliae* was found. The predicted domain of this candidate, an alpha/beta hydrolase domain, was predicted to span nearly the entire protein (Figure 1C). The mutant line was predicted to lack the last 82 amino acids of this domain, meaning that 64% of the domain was intact. Nevertheless, the functionality of the mutant protein was not further tested and it currently cannot be excluded that the mutant protein still displays (partial) activity. In order to conclusively confirm the role of *APT2* in susceptibility to *V. dahliae*, additional CRISPR mutants should be generated with sgRNAs also in the first exons of the gene. The design of the sgRNAs in the first place was strictly based on selecting sgRNAs with no predicted off-targets. This was not possible in the first exons of *APT2*, indicating potential homology to other genes. New sgRNAs could be designed with less strict criteria and if off-targets are predicted, this could be tested for. Also for this candidate, phylogenetic analysis revealed three proteins with >67% homology to our candidate (Supplementary Figure 4C). These potential homologues were also annotated as *APT2*. Similar as in the case of GlpT, also for this candidate functional redundancy could have caused the discrepancies between the VIGS and the CRISPR assays. An additional analysis of the VIGS construct also showed potential off-targeting towards two of the identified homologues (Supplementary Figure 4D). Designing a RNAi silencing construct could therefore also circumvent potential functional redundancy for this candidate.

To create large deletions in the two candidates, four sgRNAs were designed per gene. Multiple primary transformants with relatively large deletions were successfully obtained for both candidates (Supplementary Figures 1 and 2). In soybean, the use of two sgRNAs resulted in deletions of more than 1,000 bp in the targeted *fatty acid desaturase 2* (*FAD2*) genes (Do *et al*., 2019). Moreover, four sgRNAs were used to target the *powdery mildew resistant 4* (*pmr4*) gene in tomato and genotyping revealed large deletions as well as an insertion and an inversion in the primary transformants (Santillán Martínez *et al*., 2020). As we focused on those mutants with large deletions that were visible using PCR and gel electrophoresis, the exact mutation efficiency cannot be determined as small indels (insertions or deletions) are not considered. Genotyping of the subsequent generation (T2) revealed a mutant line for *APT2* with a 815 bp deletion in close proximity to the protospacer adjacent motif (PAM) (Figure 1). The PAM is essential for the initial target site recognition and double stranded breaks (DSB) are usually within 3-4 bp downstream of the PAM (Wu *et al*., 2014). Similarly, for GlpT, also two mutant lines were identified with deletions close to the PAM sequence (Figure 4). In contrast, T2 line TV191155 showed a large deletion far away from an sgRNA which could be due to the error-prone nature of DNA repair after a DSB in plants

(Manova and Gruszka, 2015). It is likely that DSBs occurred between sgRNAs 2 and 4 in TV191155 and that nonhomologous end joining occurred after partial degradation of the ends. Moreover, this T2 line also carried a 99 bp random insert which most likely also occurred upon repair of the DSB.

Collectively, this work on *GlpT* and *APT2* in susceptibility to *V. dahliae* in tomato requires further investigation due to potential functional redundancy for both candidates.

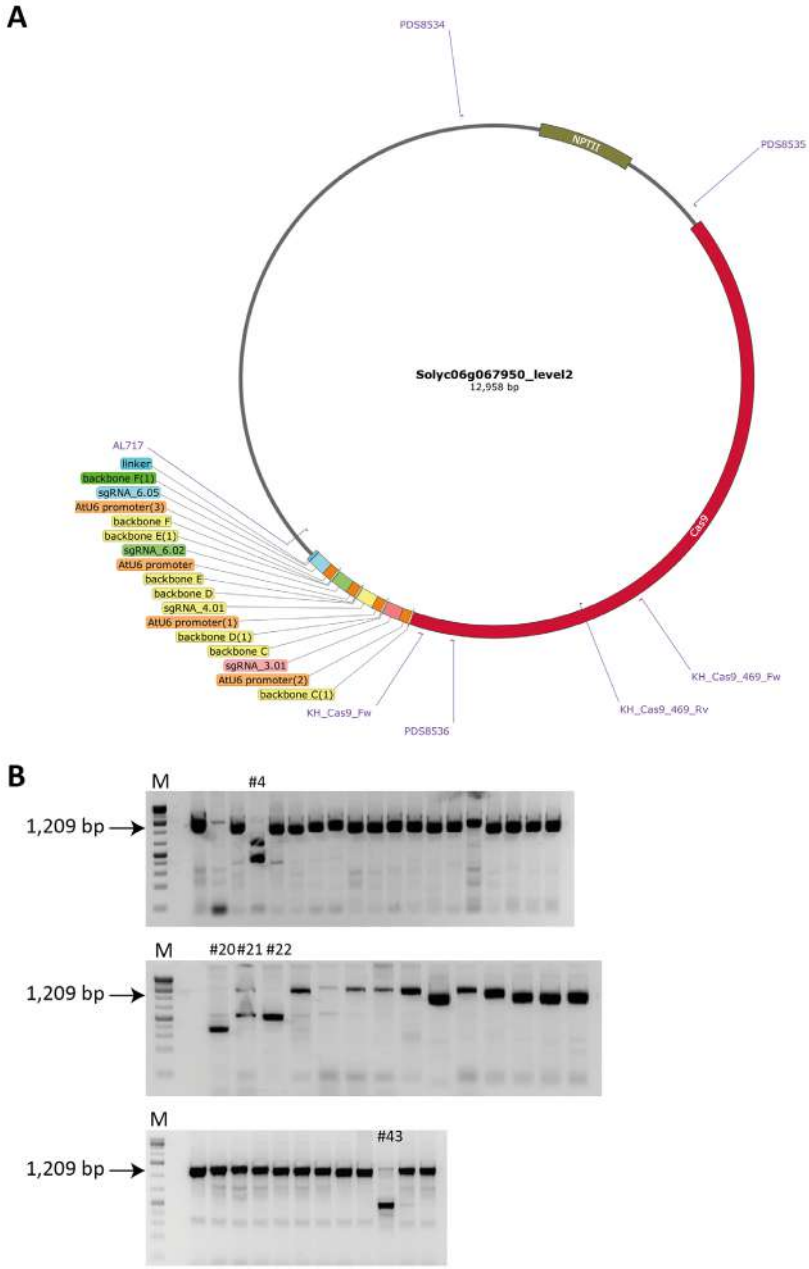
ACKNOWLEDGMENT

This project is financially supported by Topsector Tuinbouw & Uitgangsmaterialen (project: 1409-026). Special thanks go Bert Essenstam and Andre Maassen at Unifarm for excellent plant care and Annelies Loonen for technical support.

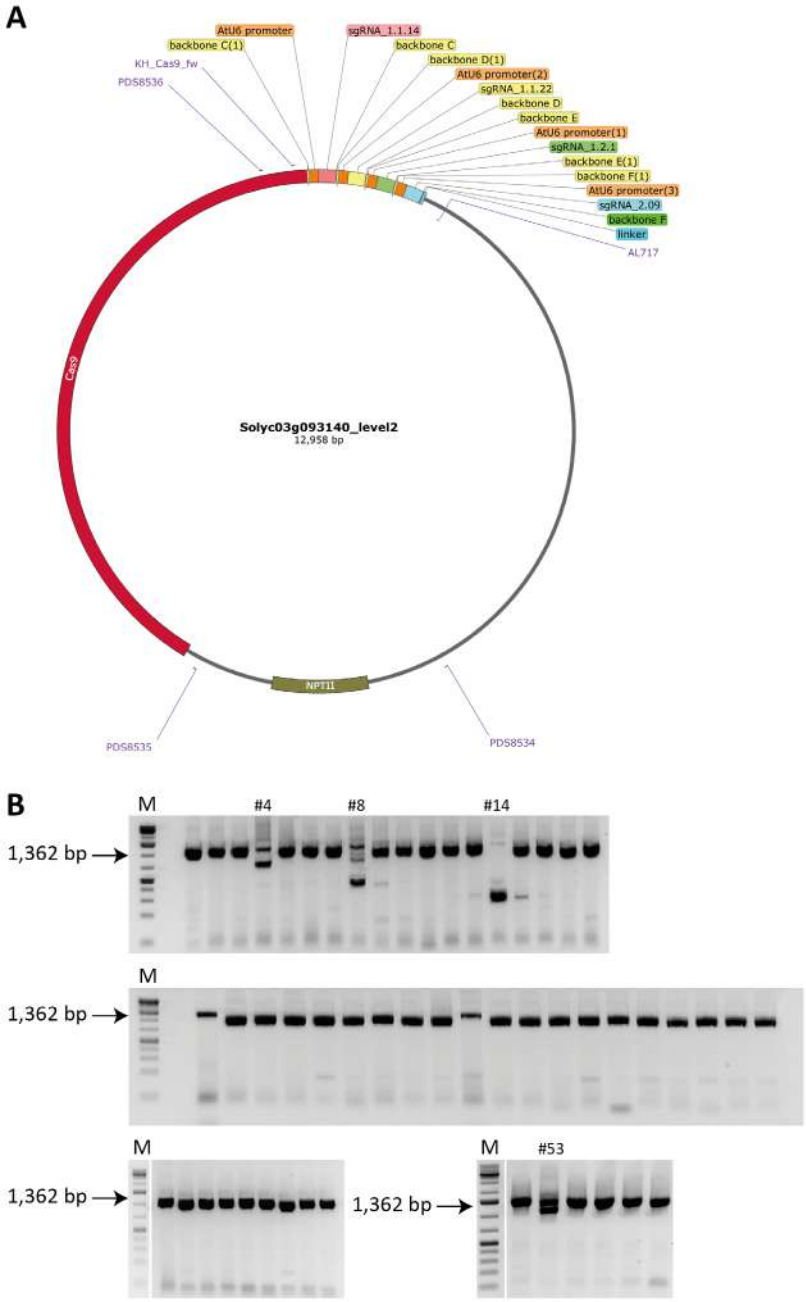
SUPPLEMENTARY MATERIAL

Supplementary Table 1 | Primers and sgRNAs used in this study.

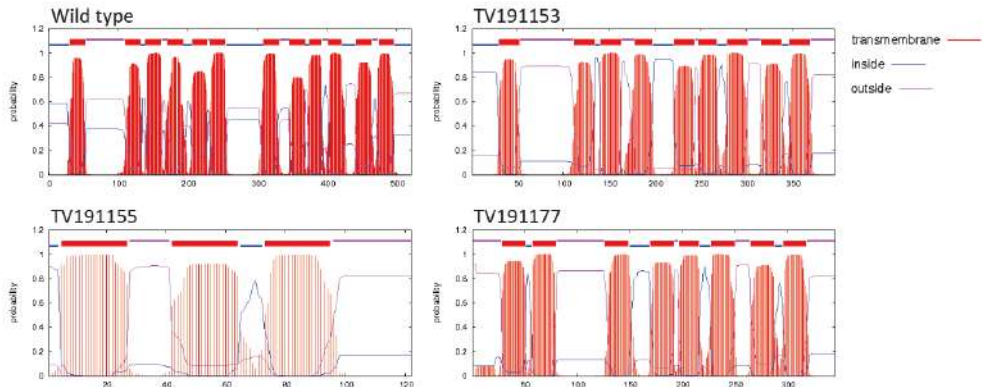
Primer/sgRNA	Sequence (5'→ 3')	Used for
sgRNA_1	CTATCATAGGTCTAAGCGGA	sgRNAs Solyc06g067950
sgRNA_2	GAGTAGAATGGGCAAAGACG	
sgRNA_3	TTATAAGTAATTCAGGAGTG	
sgRNA_4	ATATGCCTTGGGTGAATTCT	
sgRNA_1	CTCCATCACCCTAATTCCAG	sgRNAs Solyc03g093140
sgRNA_2	CCTGATGGTACGGCGATGCT	
sgRNA_3	CACCTCGGTCGGTAATATCG	
sgRNA_4	AGGAAGGTATTTATCCAATG	
SlRub_QPCR_F	GAACAGTTTCTCACTGTTGAC	Tomato rubisco gene
SlRub_QPCR_R	CGTGAGAACCATAAGTCACC	
Vd-ITS-Fw	AAAGTTTTAATGGTTCGCTAAGA	*V. dahliae* biomass
Vd-ITS-Rv	CTTGGTCATTTAGAGGAAGTAA	
KH_291_Fw	GGCTTAGATGCTTCCGTTGC	Genotyping primers Solyc06g067950
KH_291_Rv	ACAACCATGATACAATGACTACCA	
KH_302_Fw	TCCTCTTTATCAGTTTGTTTGGGT	Genotyping primers Solyc03g093140
KH_302_Rv	ACCATTCACGAATACTCCGGT	



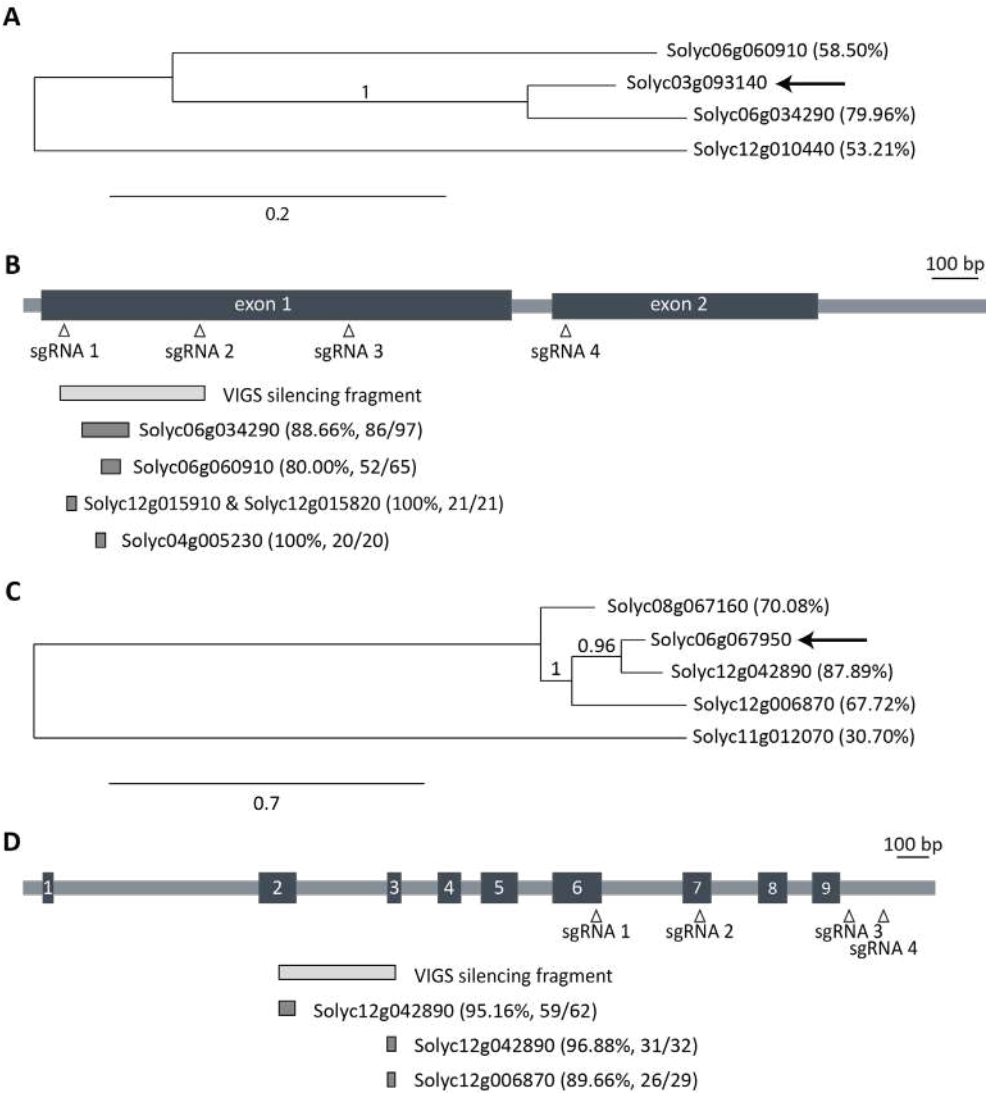
Supplementary Figure 1 | T1 transformants for Solyc06g067950 generated using CRISPR-Cas9. (A) CRISPR-Cas9 level 2 plasmid for knock-out of Solyc06g067950. Plasmid map generated using SnapGene. Gel electrophoresis (1% TBE, Gelred) of gene-specific PCR on primary transformants (T1) of plants transformed with Solyc06g067950 CRISPR-Cas9 construct. Wild type PCR product (1,209 bp) indicated with an arrow. Mutants #4, #20, #21, #22 and #43 were kept for seed production.



Supplementary Figure 2 | T1 transformants for Solyc03g093140 generated using CRISPR-Cas9. (A) CRISPR-Cas9 level 2 plasmid for knock-out of Solyc03g093140. Plasmid map generated using SnapGene. Gel electrophoresis (1% TBE, Gelred) of gene-specific PCR on primary transformants (T1) of plants transformed with Solyc03g093140 CRISPR-Cas9 construct. Wild type PCR product (1,209 bp) indicated with an arrow. Mutants #4, #8, #14 and #53 were kept for seed production.



Supplementary Figure 3 | Predicted transmembrane domains for GlpT (Solyc03g093140) wild type, TV191153, TV191155 and TV191177. Graphs were generated with TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).



Supplementary Figure 4 | (A) Phylogenetic tree based on amino acid sequences for GlpT (Solyc03g093140) including potential homologues in tomato. Percentages indicate sequence similarity to GlpT (arrow). Numbers above nodes indicate branch support values. (B) Schematic overview of Solyc03g093140 indicating the target location of the sgRNAs and the VIGS silencing fragment. A blastN search with the silencing fragment revealed four potential off-targets with sequence identity and aligned nucleotides. (C) Phylogenetic tree based on amino acid sequences for APT2 (Solyc06g067950) including potential homologues in tomato. Percentages indicate sequence similarity to APT2 (arrow). Numbers above nodes indicate branch support values. (B) Schematic overview of Solyc06g067950 indicating the target location of the sgRNAs and the VIGS silencing fragment. A blastN search with the silencing fragment revealed two potential off-targets with sequence identity and aligned nucleotides.

Chapter 5

Screening of susceptibility gene orthologues in tomato identified *WAT1* as a susceptibility factor for *Verticillium dahliae*

Katharina Hanika, Henk J. Schouten, Bart P.H.J. Thomma, Yuling Bai

ABSTRACT

As an alternative strategy to the exploitation of resistance (*R*) genes, genetic resistance against microbial disease may be established through the impairment of disease susceptibility (*S*) genes. *S* genes are host genes that are required by the pathogen to establish disease, and their role in susceptibility might be conserved across plant species. As knowledge transfer from model to crop species can be useful for resistance breeding, homologs of previously identified *S* genes from *Arabidopsis thaliana* may be tested for a role in susceptibility to *Verticillium dahliae* in tomato. Here, homologs of three previously identified *S* genes from *A. thaliana* were identified in tomato and their role in disease susceptibility to *V. dahliae* was tested using virus-induced gene silencing (VIGS) followed by disease phenotyping. Whereas neither targeting of the tomato orthologue of *Pyruvate Decarboxylase 1* (*PDC1*) nor of WRKY27 resulted in reduced symptoms of Verticillium wilt disease when compared with control plants, silencing of the tomato orthologue of *Walls Are Thin 1* (*WAT1*). This finding suggests that the tomato orthologue of Arabidopsis *WAT1* acts as a susceptibility gene for *V. dahliae*.

INTRODUCTION

Verticillium dahliae is a soil-borne fungus that infects hundreds of host species and that causes yield losses on a wide diversity of crops, including tomato, lettuce, olive and cotton (Pegg and Brady, 2002). Disease symptoms include, amongst others, stunting, wilting and necrosis (Fradin and Thomma, 2006). *V. dahliae* is particularly hard to control due to its persistent resting structures in the soil, limited options to clear infested soils, and the inefficacy of fungicides to cure infected plants once the fungus has entered. Furthermore, its enormous host range that also includes many weeds, makes crop rotation ineffective. Due to these constrains, host plant resistance is considered the most suitable strategy for disease control. Breeding for resistance historically aimed at introducing dominant resistance (*R*) genes from wild donor species into elite cultivars. Since 1952, breeders rely on the thus far only identified *R* gene for *V. dahliae*, termed *Ve1*, which has been introgressed into most tomato cultivars (Schaible *et al*., 1951; Labate *et al*., 2007; Fradin *et al*., 2009; Song *et al*., 2017). The Ve1 protein recognizes the *V. dahliae* effector Avirulence on Ve1 tomato (Ave1), leading to dominantly inherited resistance (de Jonge *et al*., 2012). Fungal strains that are contained by the *Ve1* resistance gene product are designated race 1, whereas strains that overcome *Ve1*-mediated resistance have been assigned to race 2. In addition, race 2-resistant rootstocks were developed in Japan (Usami *et al*., 2017). However, several of the tested *V. dahliae* strains caused Verticillium wilt on these plants, and were therefore grouped into race 3 (Usami *et al*., 2017). Taken together, the *R* genes that have been broken by the evolving *V. dahliae* stains, the lack of suitable other resistance sources, and the inefficacy of common containment strategies, fuel the necessity to explore additional approaches to combat Verticillium wilt in tomato.

As an alternative strategy for the exploitation of *R* genes, genetic resistance against microbial disease may be established through the impairment of disease susceptibility (*S*) genes. The latter are host genes which are required by the pathogen to establish disease and can be involved in diverse processes, such as early interaction with the pathogen, suppression of immunity, liberation of nutrients, or pathogen proliferation (Pavan *et al*., 2010; Gawehns *et al*., 2013; van Schie and Takken, 2014). In contrast to dominant *R* genes that need to be functional to establish resistance, *S* genes need to be impaired to achieve resistance, basically through loss of susceptibility, leading to recessively inherited resistance. Using impaired *S* genes is considered advantageous for several reasons (Pavan *et al*., 2010). Firstly, *S* gene-mediated resistance is believed to be durable because overcoming loss of susceptibility due to an impaired *S* gene requires gain-of-function by the pathogen, which is more challenging than loss-of-function which is typically associated with overcoming *R* gene-mediated resistance. The latter can already be achieved by a single nucleotide polymorphism in an effector gene (Joosten *et al*., 1994), or loss of the recognized effector as occurred in *V. dahliae*

to overcome *Ve1*-mediated resistance (de Jonge *et al*., 2012). An example of the durability of impaired *S* genes for resistance is the well-studied *Mildew locus O* (*mlo*) mutant of barley that provides resistance to powdery mildew in the field since 1979 (Buschgel *et al*., 1997; Lngkjær *et al*., 2000; Acevedo-Garcia *et al*., 2014). Secondly, as it is difficult to overcome, *S* genes like *Mlo* generally provide non-race specific resistance that contains all strains of a given pathogen species (Jørgensen, 1977). Thirdly, loss of susceptibility can be even more broad-spectrum to confine multiple pathogens, such as the loss of susceptibility to three diseases, e.g. downy mildew, bacterial angular leaf spot, and fungal anthracnose, that is conferred by a single nucleotide polymorphism in the cucumber *STAYGREEN* gene (Wang *et al*., 2018). Finally, in many cases *S* genes are conserved across plant species. This is also the case for *Mlo*, which was originally identified in barley, but loss-of-function variants in orthologues of pea and tomato similarly provide resistance to powdery mildew (Bai *et al*., 2008; Humphry *et al*., 2011). This offers a great opportunity and value for interspecies knowledge transfer from model plants, such as *Arabidopsis thaliana*, to crops. In tomato, resistance to powdery mildew was obtained by impairing orthologues of *PMR4* (*Powdery Mildew Resistance 4*) and *DMR1* (*Downy Mildew Resistance 1*) from *A. thaliana* (Huibers *et al*., 2013). In potato, loss of susceptibility to late blight was achieved by silencing *S* gene orthologues from *A. thaliana* including *PMR4* and *DMR1* (Sun, *et al*., 2016).

For *V. dahliae* only few *S* genes are described in literature, most of which were identified in *A. thaliana*. The only study on tomato showed that transient silencing of the ethylene receptor *ETR4* reduced the amount of symptoms caused by *V. dahliae* (Pantelides *et al*., 2010). However, a decrease in fungal biomass was not detected in *etr4* mutants, suggesting that impairment of *ETR4* can be utilized to mediate enhanced tolerance, but not enhanced resistance to *V. dahliae*. As such plants are likely to still accumulate significant amounts of pathogen biomass, which ultimately leads to enhanced pathogen biomass accumulation in the soil, exploitation of enhanced tolerance is not desirable. Therefore, the most interesting *S* gene candidate found in Arabidopsis is *Walls Are Thin 1* (*WAT1*). A mutant of *WAT1* provides broad-spectrum resistance against several bacterial and fungal vascular pathogens, including *V. dahliae* and *Ralstonia solanacearum* which is accompanied by reduced fungal and bacterial colonization respectively (Denancé *et al*., 2013). *WAT1* encodes a tonoplast-localized auxin transporter that is involved in secondary cell wall formation, however its exact function in so-called “vascular immunity” is not yet understood (Ranocha *et al*., 2010, 2013). A second interesting candidate from Arabidopsis is *pyruvate decarboxylase 1* (*PDC1*) which is a negative regulator of disease resistance against vascular wilt fungi *V. dahliae* and *Fusarium oxysporum*. A lower percentage of diseased leaves and reduced fungal biomass were found for the *pdc1* mutant for both fungi (Papastolopoulou *et al*., 2018). PDC1 is an enzyme that catalyses the decarboxylation of pyruvic acid to

acetaldehyde and carbon dioxide (Kursteiner *et al*., 2003; Mithran *et al*., 2014), but its role in plant immunity remains to be elucidated. Another candidate that acts as *S* gene in vascular wilt disease caused by the bacterial pathogen *R. solanacearum* is WRKY27 which belongs to the WRKY transcription factor family that is known to have a plethora of functions including transcriptional regulation of (a)biotic defence responses (Rushton *et al*., 2010; Bai *et al*., 2018). Strongly reduced wilting symptoms but no difference in bacterial growth was found for *WRKY27* T-DNA insertion lines upon challenge with *R. solanacearum* (Mukhtar *et al*., 2008).

In this study, a reverse genetics approach was used to study tomato orthologues of known *S* genes from *A. thaliana* for *V. dahliae* and other vascular pathogens. Two previously identified *S* genes for Verticillium wilt in Arabidopsis were selected, namely *PDC1* (At4g33070), and *WAT1* (At1g75500). Additionally, the transcription factor *WRKY27* (At5g52830) that was found in the interaction with *R. solanacearum* was assessed as well. The potential tomato orthologues of these *S* genes were targeted using virus-induced gene silencing (VIGS) and subsequently plants were screened for reduced susceptibility to *V. dahliae*.

MATERIALS & METHODS

Selection of *S* genes and orthologue identification

Amino acid sequences of PDC1 (At4g33070), WRKY27 (AtWRKY27) and WAT1 (At1g75500) were obtained from TAIR (https://www.arabidopsis.org/) and used as query in a blastP search against the Sol genomics database (ITAG release 4.0; https://solgenomics.net/tools/blast/). For each candidate, the closest orthologues were selected and phylogenetic trees were constructed using Phylogeny.fr (Dereeper *et al*., 2008).

Generation of silencing constructs and virus-induced gene silencing (VIGS)

Silencing constructs for VIGS were designed as reported previously (Chapter 3). Briefly, a gene-specific 150-300 bp fragment was cloned into the tobacco rattle virus 2 (TRV2) vector (Liu *et al*., 2002) using Gateway cloning (Supplementary Table 1) and subsequently transformed into *Agrobacterium tumefaciens* strain GV3103. VIGS was performed as described previously (Liu *et al*., 2002; Fradin *et al*., 2009; Verlaan *et al*., 2013). As negative control a TRV2 vector containing a fragment of the *β-Glucuronidase* (*GUS*) gene was used (Wu *et al*., 2011; Senthil-Kumar and Mysore, 2014). Moreover, a TRV2 vector carrying a fragment of the tomato *phytoene desaturase* (*PDS*) gene was used as a positive control as it triggers photobleaching upon effective silencing.

Plant growth conditions, *V. dahliae* inoculation and phenotyping

Plants were grown in potting soil (Potgrond 4, Horticoop, Katwijk, The Netherlands) at Unifarm (Wageningen University & Research, The Netherlands) at 21°C/19°C (day/night) with relative humidity of 60% and minimal light intensity of 150 W/m^2. *V. dahliae* inoculation was done as described previously at 11–14 days after *A. tumefaciens* infiltration (Fradin et al., 2009). The canopy area of the plants was quantified at 21 days after inoculation using ImageJ (Schneider et al., 2012) and stunting was calculated as follows:

$$stunting\ (\%) = \left(1 - \frac{canopy\ area\ of\ V.\ dahliae{-}inoculated\ plant}{average\ canopy\ area\ of\ mock{-}inoculated\ plants}\right) \times 100.$$

RNA isolation and quantitative reverse transcription PCR (qRT-PCR)

To determine silencing levels of the target genes, stems were harvested two weeks after *A. tumefaciens* treatment and snap frozen in liquid nitrogen. Total plant RNA was isolated using MagMAX-96 Total RNA Isolation Kit (Invitrogen, Bleiswijk, The Netherlands) using the KingFisher Flex System (Thermo Scientific, Bleiswijk, The Netherlands). The iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands) was used for cDNA synthesis. Subsequently, real-time PCR was performed using a CFX96 Real-time System (Bio-Rad, Veenendaal, The Netherlands) and SYBR Green Master Mix (Bio-Rad, Veenendaal, The Netherlands). The tomato *elongation factor 1 α* (*SlEF1α*) gene was used as reference to determine relative transcript levels. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Experiments were done twice with a minimum of 4 biological replicates per experiment.

RESULTS

Transient silencing of *PDC*-like genes in tomato

To identify potential orthologues of *AtPDC1* in tomato, the corresponding amino acid sequence was used as query in a blastP search against the Sol genomics database (https://solgenomics.net /tools/blast/). Four potential orthologues were identified, Solyc10g076510, Solyc09g005110, Solyc06g082130 and Solyc02g077240, which correspond to four described PDCs in *A. thaliana* (Mithran et al., 2014). As phylogenetic analysis did not convincingly identify a single *PDC1* orthologue (Figure 1A), all four *SlPDC1*-like genes were included in further analyses.

To functionally test the candidate genes for a role as S gene in tomato, *A. tumefaciens*-mediated VIGS was used for transient silencing of the target genes. Silencing constructs were generated for targeting each of the four genes individually,

as well as for two homologues simultaneously (Solyc10g076510 & Solyc09g005110, Solyc06g082130 & Solyc02g077240) (Supplementary Figure 1). Due to the low sequence similarity, simultaneous silencing of all four candidates with one construct was not possible. First, silencing levels were determined by means of qRT-PCR and expression levels were normalized and compared with TRV::GUS control plants. While silencing could not be demonstrated for Solyc06g082130 (A), for the other three *SlPDC* candidates a significant reduction in target gene expression could be monitored, namely for Solyc02g077240 (B), Solyc09g005110 (C) and Solyc10g076510 (D) (Figure 1B). Overall, gene expression was reduced to 34.7%, 66.1% and 23.2% for Solyc02g077240 (B), Solyc09g005110 (C) and Solyc10g076510 (D), respectively, when compared with expression levels of the corresponding target genes in TRV::GUS-treated plants. For the VIGS constructs that targeted two homologues (A/B and C/D) a significant reduction of expression of each target gene was found for both constructs with 64.0% (A), 50.3% (B), 57.8% (C) and 69.4% (D), respectively.

To screen for reduced susceptibility to *V. dahliae*, *A. tumefaciens*-treated plants were inoculated and stunting based on canopy area was calculated between mock- and *V. dahliae*-inoculated plants and compared with stunting of TRV::GUS control plants. For the four individual silencing constructs of *SlPDC*, no reduced stunting was observed, as stunting levels were comparable to the average of *V. dahliae*-inoculated plants treated with TRV::GUS. For the constructs targeting two genes, TRV::SlPDC_A/B and TRV::SlPDC_C/D, also no reduced stunting was found. Thus, a role of any of the SlPDCs as an *S* gene in the interaction with *V. dahliae* could not be confirmed. Furthermore, considering that residual gene expression was at least 23.2%, but in most cases around 61.5%, and taking functional redundancy into account, a role of *SlPDCs* as *S* genes in the interaction with can neither be confirmed nor ruled out.

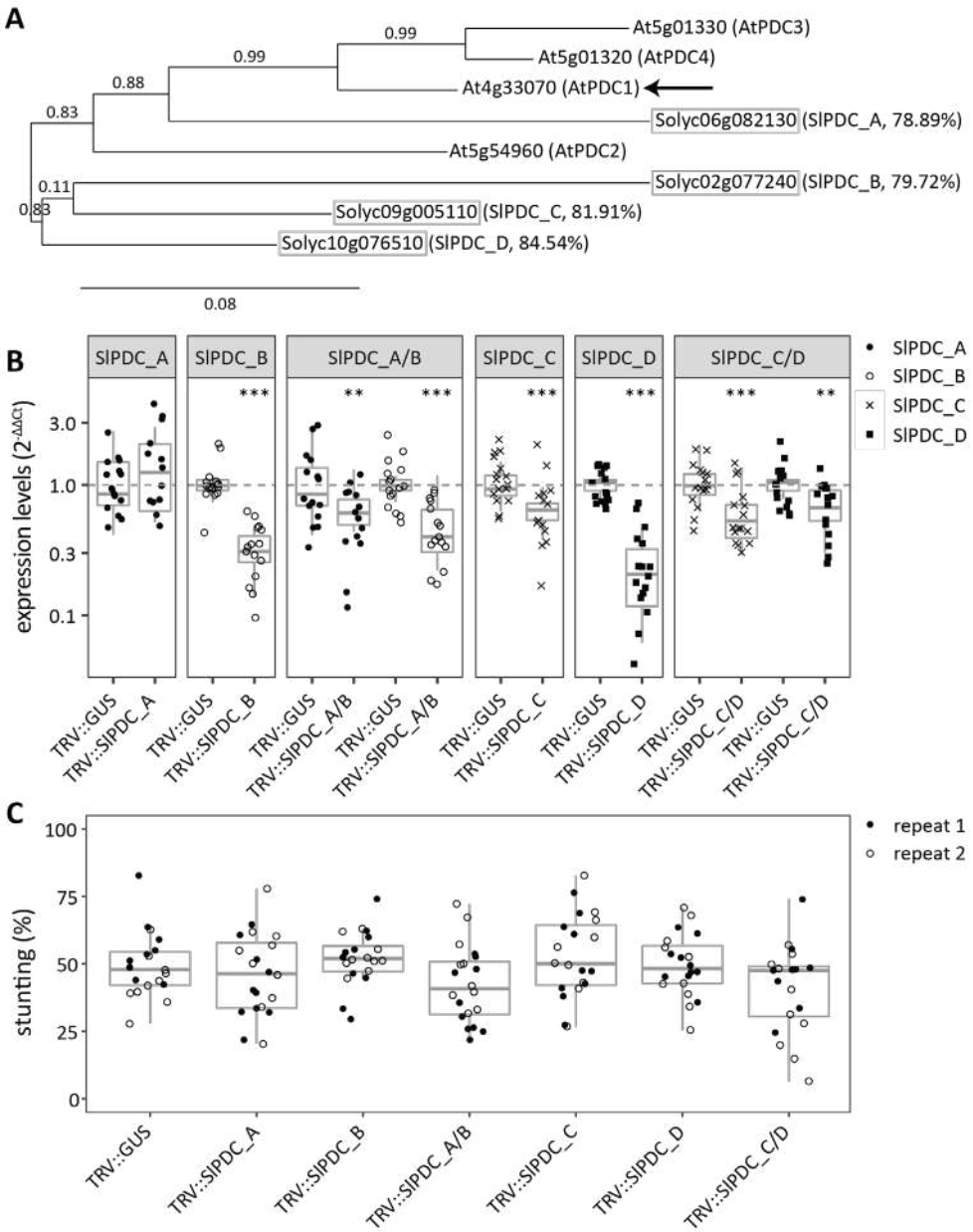


Figure 1 | Transient silencing of four *PDC*-like genes in tomato. (A) Phylogenetic trees based on amino acid sequences for PDC1 including potential orthologues from tomato (grey boxes). Percentages indicate sequence similarity to AtPDC1 (arrow). Numbers above nodes indicate branch support values. (B) Silencing levels ($2^{-\Delta\Delta Ct}$) of plants treated with TRV constructs for *SlPDCs* in stems normalized to TRV::GUS two weeks after *Agrobacterium tumefaciens* treatment on a log10 scale. Data of two independent experiments with n ≥ 6 per experiment per genotype (t-test when compared with TRV::GUS with ** $p < 0.01$ and *** $p < 0.001$). (C) Stunting (%) in *Verticillium dahliae*-inoculated plants when compared with the average of mock-inoculated plants at 21 dpi after transient silencing of *SlPDCs*. Boxplots represent data of two independent experiments with n>5 plants per experiment per genotype (ANOVA, p = 0.05213).

Transient silencing of *WRKY27* orthologues in tomato

A blastP search revealed three potential *WRKY27* orthologues in tomato; Solyc01g095100, Solyc10g011910 and Solyc08g081610 (Figure 2A). The overall sequence similarity was low (44 – 61%) when compared with the similarities found for *SlPDCs* orthologues earlier (78 – 84%). Two silencing constructs were generated to target Solyc01g095100 (A) and two constructs to target Solyc10g011910 (B), as well as a construct to target both genes simultaneously (A/B). Additionally, a silencing construct targeting the third candidate (Solyc08g081610 = C) was included (Supplementary Figure 1). Silencing could not be demonstrated for the constructs targeting Solyc01g095100 (A) as residual expression was on average 41.8% and 15.3% higher compared with expression in TRV::GUS-treated plants (Figure 2B). A significant reduction in gene expression to on average 48.4% was only found for one of the two constructs targeting Solyc10g011910 (B). Finally, the constructs that were designed to simultaneously target the two genes (A/B) could not be demonstrated to silence their target genes. Residual gene expression of the third candidate, Solyc08g081610 (C), was also not significantly reduced.

No significant reduction in stunting was found for *V. dahliae*-inoculated plants treated with any of the constructs when compared with *V. dahliae*-inoculates plants treated with TRV::GUS (Figure 2C). As silencing of most of these *SlWRKY* candidates could not be demonstrated, no conclusion can be drawn on a potential function of these genes as susceptibility factors for *V. dahliae* in tomato. Only for Solyc01g095100 (A) residual gene expression was significantly reduced, but 48.4% may not be sufficient to effectively impair gene function. Furthermore, even if silencing of this candidate was sufficient, potential functional redundancy could prevent potential effects on *V. dahliae* susceptibility.

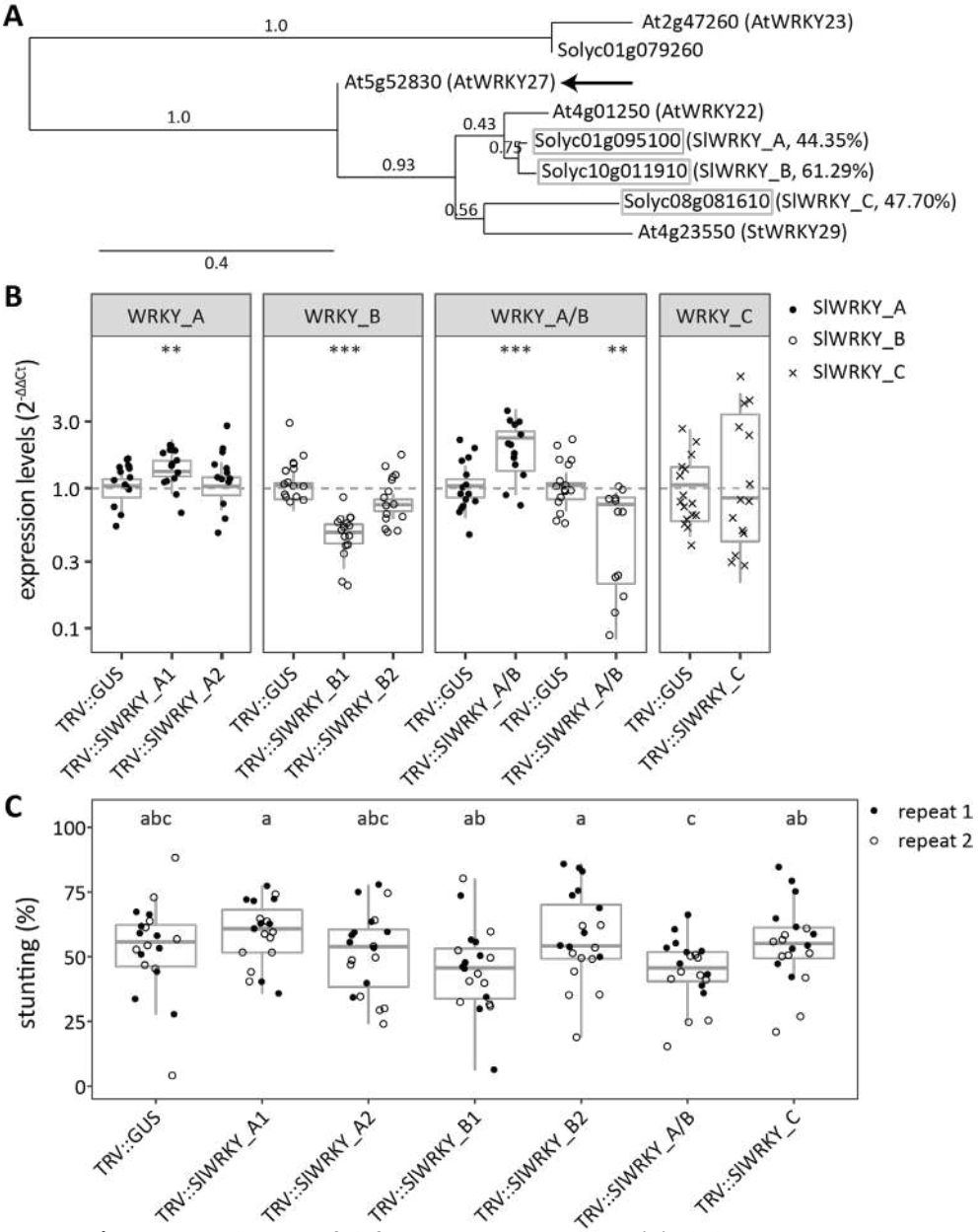


Figure 2 | Transient silencing of different *WRKYs* in tomato. (A) Phylogenetic trees based on amino acid sequences for WRKY27 including potential orthologues from tomato (highlighted in grey square). Percentages indicate sequence similarity to *AtPWRKY27* marked with an arrow. Numbers above nodes indicate branch support values. (B) Silencing levels ($2^{-\Delta\Delta Ct}$) of plants treated with TRV constructs for *SlWRKYs* in stems normalized to TRV::GUS two weeks after *Agrobacterium tumefaciens* treatment on a log10 scale. Data of two independent experiments with n ≥ 6 per experiment per genotype (t-test when compared with TRV::GUS with ** $p < 0.01$ and *** $p < 0.001$). (C) Stunting (%) in *Verticillium dahliae*-inoculated plants when compared with the average of mock-inoculated plants at 21 dpi after transient silencing of *SlWRKYs*. Boxplots represent data with n>5 per experiment per genotype (ANOVA with Fisher's unprotected LSD, $p = 0.05$).

Transient silencing of *WAT1* in tomato

Based on a blastP search and phylogenetic analysis, the tomato gene with the highest homology of 74.7% to *AtWAT1* was identified as Solyc04g080940 (Figure 3A). Silencing was determined in plants treated with TRV::SlWAT1 and a significant reduction in relative expression to approximately 49.6% was found when compared with TRV::GUS-treated plants (Figure 3B). Interestingly, *V. dahliae*-inoculated plants treated with the TRV::SlWAT1 construct showed significantly less stunting when compared with *V. dahliae*-inoculated TRV::GUS plants at 21 dpi (Figure 1C, panels 1, 3 and 8). To confirm these results, the experiment was repeated eight times. Significantly reduced stunting was observed in three of those repeats, whereas this effect could not be observed in the five other repeats. However, this finding implicates that *SlWAT1* may be an *S* gene in the interaction between tomato and *V. dahliae* although further confirmation is needed.

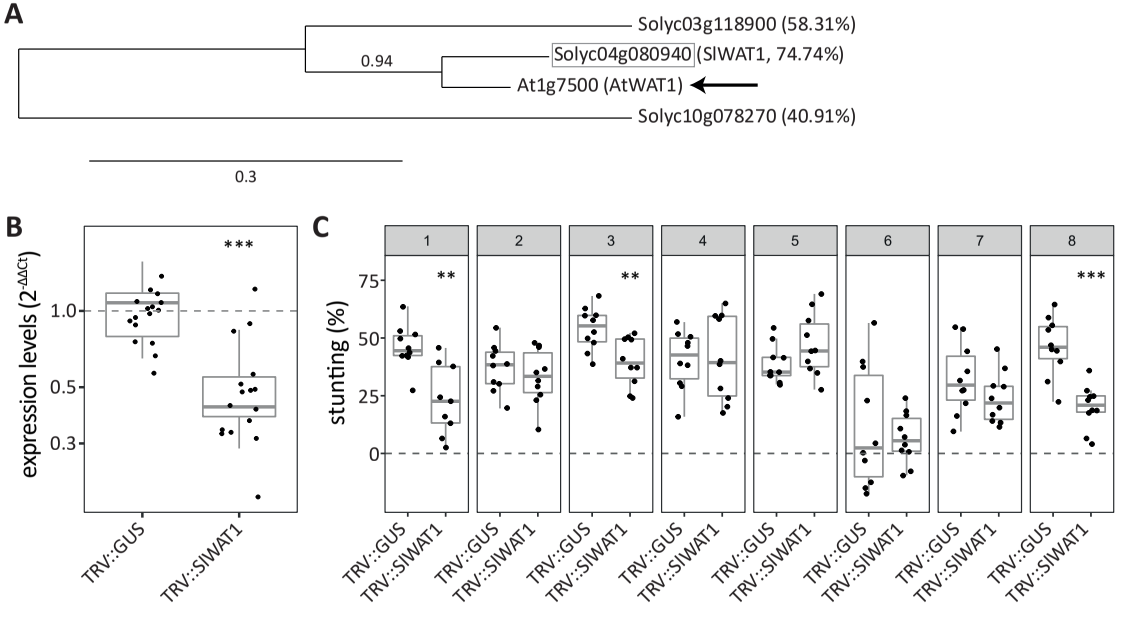


Figure 3 | Transient silencing of *WAT1* in tomato resulted in reduced susceptibility to *Verticillium dahliae*. (A) Phylogenetic tree based on amino acid sequences for *WAT1* including potential orthologue from tomato (highlighted in grey square). Percentages indicate sequence similarity to *AtWAT1* marked with an arrow. Number above node indicate branch support values. (B) Silencing levels ($2^{-\Delta\Delta Ct}$) of plants treated with TRV::SlWAT1 in stems normalized to TRV::GUS two weeks after *Agrobacterium tumefaciens* treatment on a log10 scale. Data of two independent experiments with n ≥ 6 per experiment per genotype (t-test when compared with TRV::GUS with *** $p < 0.001$). (C) Stunting (%) in *V. dahliae*-inoculated plants when compared with the average of mock-inoculated plants at 21 dpi after transient silencing of *SlWAT* in eight independent experiments with n ≥ 8 per experiment per genotype (n ≥8, t-test when compared with TRV::GUS with ** $p < 0.01$ and *** $p < 0.001$).

DISCUSSION

The use of impaired *S* genes provides a strategy for genetic resistance to microbial diseases. So far, only a few *S* genes for *V. dahliae* have been reported, mainly from *A. thaliana*. Here we studied potential tomato orthologues of three *A. thaliana S* genes. Silencing of the tomato orthologue of Arabidopsis *WAT1* resulted in significantly reduced stunting when compared with control plants upon challenge with *V. dahliae* (Figure 3). Notwithstanding, reduced stunting for *SlWAT1* was not observed in all experimental repeats which can be attributed to the method used for functional characterization. We used VIGS as tool for transient gene silencing which is frequently employed for gene functional studies in tomato (Liu *et al*., 2002; Senthil-Kumar *et al*., 2007; Ramegowda *et al*., 2014). It is known, however, that silencing in tomato does not occur throughout the entire plant as silencing is typically observed in a patchy fashion (Liu *et al*., 2002; Lu *et al*., 2003; Orzaez *et al*., 2009). Silencing efficiency is further influenced by temperature and the availability of natural light. Such environmental differences might explain the variation in the WAT1 experiments, as poor silencing efficiency can mask the potential effect on stunting (Figure 3C). To confirm the role of *WAT1* as *S* gene in the interaction with *V. dahliae* and tomato, stable transformants based on RNAi or CRISPR-Cas9 should be analysed.

Another point of consideration is the fact that VIGS is primarily used to study gene function in leaves. However, in the interaction with the soil-borne fungus *V. dahliae* silencing in the roots is certainly essential. VIGS has been successfully used to silence root-specific genes in other studies (Valentine *et al*., 2004; Jablonska *et al*., 2007; Seifi *et al*., 2011). In fact, VIGS has previously been used in combination with *V. dahliae* in tomato to silence *Ve1* and downstream signalling components as well as to study the ethylene receptor *ETR4* (Fradin *et al*., 2009; Pantelides *et al*., 2010). In the case of *Ve1*, VIGS was used to comprise incompatibility, while our aim is to comprise compatibility. Although ultimately the occurrence of a phenotype depends on the extent to which the corresponding protein level is reduced, which arguably differs between targets, it may be argued that modest reduction in protein levels are more likely to compromise incompatibility, which may depend on reaching a threshold level to activate appropriate defences, rather than to compromise compatibility, as lower amounts of target protein may still be sufficient to mediate disease. In the case of *ETR4*, VIGS-mediated silencing of *ETR4* resulted in reduced and delayed symptom development upon challenge with *V. dahliae*, although fungal colonization was not reduced (Pantelides *et al*., 2010).

Recently, it was shown that simultaneous silencing of three *WAT1* orthologs of cotton resulted in reduced susceptibility to *V. dahliae* with significantly reduced fungal biomass (Tang *et al*., 2019). Together with the original identification of *WAT1* in Arabidopsis and our finding that *SlWAT1* impairment leads to enhanced Verticillium wilt resistance, this indicates a conserved role of *WAT1* as *S* gene in the interaction

with *V. dahliae* in *A. thaliana*, cotton and tomato, which may likely be extrapolated to other plant species as well. Certainly, it would also be interesting to test these mutants with other vascular pathogens such as *Xanthomonas campestris*, *V. albo-atrum* and *Plectosphaerella cucumerina* as *wat1* mutants in Arabidopsis also showed enhanced resistance to these pathogens (Denancé *et al*., 2013).

In contrast to *WAT1*, silencing of the different *SlPDCs* did not result in reduced susceptibility to *V. dahliae*. On one hand, this can be attributed to variable silencing efficiency as discussed above. However, besides, residual gene expression also functional redundancy may have played a role in the lack of identification of phenotypes. As described in Arabidopsis, also four *PDC*-like genes were found in tomato and even though we here targeted individual homologues as well as two candidates simultaneously, and as significantly reduced levels of gene expression were obtained in most cases, we did not see an effect on stunting. Theoretically, this still leaves the options that the level of silencing obtained was still not sufficient to compromise functionality, that the proteins do not play a role in mediating Verticillium wilt disease, or that there is functional redundancy in other combinations as tested here. However, simultaneous silencing of all four candidates with one construct was not possible as well as different combinations (e.g. A/C or A/D etc.) due to the low similarity between the two homologous pairs (A/B and C/D). An alternative approach could be the infiltration of multiple single or double constructs into the same plant or the generation of stable transformants using RNAi or CRISPR-Cas9. As we could not silence all four *SlPDCs* simultaneously in this study, we can therefore only conclude that silencing of individual candidates as well as of homologous pairs, did not point to a role of *SlPDCs* in tomato.

Similar to SlPDCs, also for studying *WRKY27* in tomato, functional redundancy complicated this study. At the onset, the identification of the corresponding orthologue for *AtWRKY27* proved difficult as this is a large family of transcription factors. A blastP search showed many potential candidates, all of which had a rather low sequence similarity to *AtWRKY27* (Figure 2C). It is therefore possible that either the wrong candidate was chosen, or that there is no corresponding orthologue in tomato. The latter is further supported by the discrepancies in literature regarding *SlWRKY27*. According to Huang *et al*. (2012) *SlWRKY27* is Solyc03g082750, while Karkute *et al*. (2018) states Solyc05g050300, neither of which were found in our search. Certainly, also functional redundancy plays a major role in such a large gene family. Therefore, and even though impairment of single genes was sufficient in Arabidopsis, we attempted to target similar candidates with one TRV construct. Nevertheless, we could not demonstrate silencing for most constructs, and therefore we cannot rule out that these *SlWRKYs* function as *S* gene for *V. dahliae* in tomato. To avoid further complications due to functional redundancy, these candidates could be further studied using targeting of gene expression in stable RNAi lines. However, also RNAi may lead to complications

due to residual levels of gene expression as we have observed in this study for *SlWAT1*. Therefore, although technically challenging, ultimately targeted mutagenesis by CRISPR-Cas9 through simultaneous knock-out of multiple *SlWRKY* homologues should be pursued. Interestingly, recently transient silencing of another *WRKY* candidate, *WRKY70*, was described to result in reduced susceptibility to *V. dahliae* in cotton (Xiong *et al*., 2019) which supports the assumption that also *WRKY* transcription factors play a role in *V. dahliae* susceptibility.

Taken together we here demonstrate that VIGS can be used to study *S* genes for *V. dahliae* in tomato. Nevertheless, factors such as silencing efficiency and functional redundancy need to be taken into consideration. To confirm the role of *SlWAT1* in *V. dahliae* susceptibility we will further use stable transformation based on CRISPR-Cas9 and RNAi (Chapter 6).

ACKNOWLEDGMENT

This project is financially supported by Topsector Tuinbouw & Uitgangsmaterialen (project: 1409-026). The authors would like to thank Marian Oortwijn, Eleni Koseoglou and Gökhan Gökmen for technical assistance, Nick Snelders for reviewing the manuscript and Bert Essenstam and Casper Pillen at Unifarm for excellent plant care.

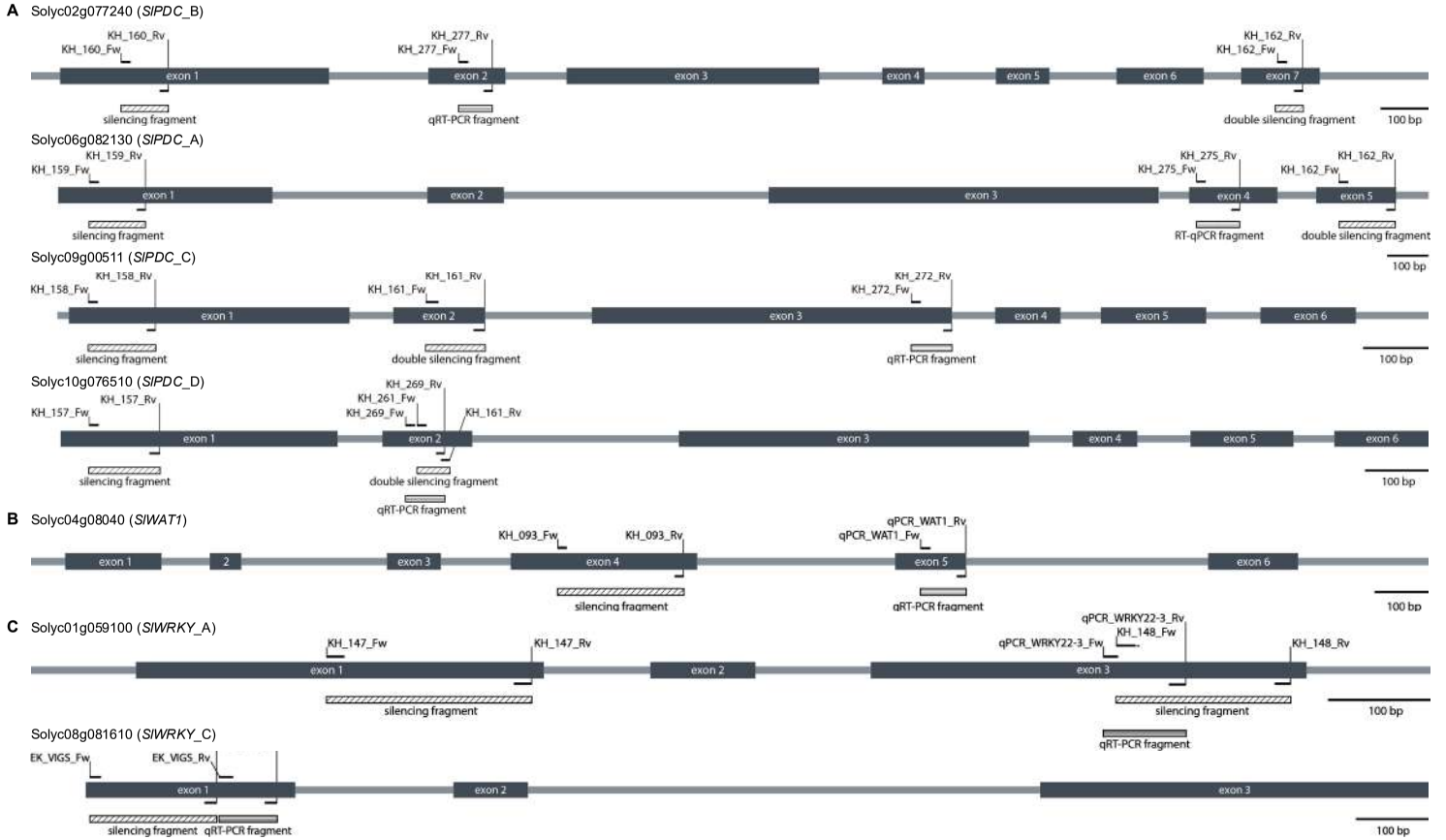
Supplementary Table 1 | Primers used in this study.

Gene	Primer	Sequence (5'→ 3')*	Used for	ID
PDC	KH_157_Fw	caccGGGCTGTTTACCGACGGTTA	VIGS	Solyc10g076510 (SlPDC_D)
	KH_157_Rv	GCCAAAAACATCGGTGACCC		
	KH_158_Fw	caccGGCGACATAGCAAACGTACC	VIGS	Solyc09g005110 (SlPDC_C)
	KH_158_Rv	TCCGATCTGAACTAACCGGC		
	KH_159_Fw	caccGCAAACTTAGGGCGCCATTT	VIGS	Solyc06g082130 (SlPDC_A)
	KH_159_Rv	AAGCCTTGGCTCAGCAATGA		
	KH_160_Fw	caccAACAATGCCATCGGTTCCAC	VIGS	Solyc02g077240 (SlPDC_B)
	KH_160_Rv	TTTGTACTAGCCGACGAGCC		
	KH_161_Fw	caccTCTTTGAAAGAGAGTAAGCCGGT	VIGS	Solyc09g005110 (SlPDC_C, 100%) & Solyc10g076510 (SlPDC_D, 89%)
	KH_161_Rv	GGGAGAGAGGGAAAACGGAA		
	KH_162_Fw	caccATCGCAACTGCAACTGGAGA	VIGS	Solyc06g082130 (SlPDC_A, 100%) & Solyc02g077240 (SlPDC_B, 88%)
	KH_162_Rv	TTGAGGGTTTGGTGGACGTC		
	KH_269_Fw	TGATACCGCGATTTCTACGTC	qRT-PCR	Solyc10g076510 (SlPDC_D)
	KH_269_Rv	CCCGGCAAGTTACAGCTTATAC		
	KH_272_Fw	TGAGAGTGCTGTGATTGCTG	qRT-PCR	Solyc09g005110 (SlPDC_C)
	KH_272_Rv	CGCATCCTTGTGGCAATTTC		
	KH_275_Fw	AGACGTGTCGACAATGATTCG	qRT-PCR	Solyc06g082130 (SlPDC_A)
	KH_275_Rv	GCCCATCATGGATTTCAACCTC		
	KH_277_Fw	AAGGAGAGCAAGCCAGTTTAC	qRT-PCR	Solyc02g077240 (SlPDC_B)
	KH_277_Rv	CTCTAGCAAATGTTGGGTGAGC		
WAT1	KH_093_Fw	caccGGCCCAACAATTTACAGCC	VIGS	Solyc04g080940 (SlWAT1)
	KH_093_Rv	GAACTAGCCAAGCCTGAGGG		

	qRT-PCR_WAT1_Fw	GGGGGTCCAGTTTTTGTTGC	qRT-PCR	
	qRT-PCR_WAT1_Rv	CTCCGATTATCCCGCCCAAG		
WRKY	KH_147_Fw	caccAGCCTTTCTTCCCTAAAGCACA	VIGS	Solyc01g095100 (SlWRKY_A)
	KH_147_Rv	GGGCTTTGAACATGTGAAGCA		
	KH_148_Fw	caccTGAAAGAGAAGACATTTTTGAA-GACGA	VIGS	
	KH_148_Rv	CGGTGGTTGTGGCGTTATTT		
	KH_149_Fw	caccGCCGGCGACATCAGTTTTAC	VIGS	Solyc10g011910 (SlWRKY_B)
	KH_149_Rv	TAAGCTGTTGCGACGGTGAT		
	KH_150_Fw	caccGTTTCCGATGTTGGGTTGGA	VIGS	
	KH_150_Rv	GCCGTAGTTGCTGCGTTATT		
	KH_151_Fw	caccGGTTGTTTGGCCCGAAAACA	VIGS	Solyc10g011910 (SlWRKY_A, 100%) & Solyc01g095100 (SlWRKY_B 89%)
	KH_151_Rv	TTCTTGATTTTCCGGTGCCG		
	EK_Fw	caccGATTGGGGTCTTCAAGCTGT	VIGS	Solyc08g081610 (SlWRKY_C)
	EK_Rv	TTTTGTCCACCATCAACATGA		
	qRT-PCR_WRKY22-3_Fw	GAAAGCAGCAGGGATGAAAG	qRT-PCR	Solyc01g095100 (SlWRKY_A)
	qRT-PCR_WRKY22-3_Rv	TCTGCAGGCTCCATGTTATC		
	qRT-PCR_WRKY25-2_Fw	AGTCAGCGCCTTCTTTTGTC	qRT-PCR	Solyc10g011910 (SlWRKY_B)
	qRT-PCR_WRKY25-2_Rv	ACGGCGTTTAGATCCCAATC		
	KH_153_Fw	TGTTGATGGGATCTTCAATCTCA	qRT-PCR	Solyc08g081610 (SlWRKY_C)
	KH_153_Rv	AGTAGGAGCCACCACTTGTT		
	SlEF1α_Fw	ATTGGAAACGGATATGCCCCT	qRT-PCR	Solyc06g005060
	SlEF1α_Rv	TCCTTACCTGAACGCCTGTCA		

* Small letters indicate ‘cacc’ overhang for Gateway cloning.

Supplementary Figure 1 | Schematic overview of the tomato orthologues for *PDCs* (A), *WAT1* (B) and *WRKYs* (C) including locations of primers to amplify silencing fragments (for single silencing constructs), double silencing fragments (for constructs targeting two genes) and real-time RT-PCR (qRT-PCR) fragments, respectively.




Solyc10g011910(SlWRKY_B)
KH_149-Fw
qPCR_WRKY25-2_Rv
qPCR_WRKY25-2_Fw
qPCR-fragment
exon 1
silencing fragment
KH_149-Rv
exon 2
KH_151_Fw
KH_150_Fw
KH_149_Rv
exon 3
double silencing fragment
KH_150_Rv
silencing fragment
100 bp

Chapter 6

Targeted deletion in tomato *WAT1* leads to enhanced *Verticillium dahliae* resistance at the expense of severe growth defects

Katharina Hanika, Danny Schipper*, Shravya Chinnappa*, Marian Oortwijn*, Henk J. Schouten, Bart P.H.J. Thomma, Yuling Bai

* These authors contributed equally to this work

Manuscript in preparation.

ABSTRACT

Vascular wilt pathogens cause diseases in many annual and perennial crops. *Verticillium dahliae* is a particularly notorious vascular wilt pathogen of tomato and poses a reoccurring challenge to crop protection as limited qualitative resistance is available. Therefore, alternative approaches for crop protection are pursued. One such strategy is the impairment of disease susceptibility (*S*) genes, host genes that are required by the pathogen for disease establishment, leading to loss of susceptibility upon impairment. For example, impairment of *Walls Are Thin 1* (*WAT1*) in *Arabidopsis thaliana* mediated broad-spectrum resistance to various vascular pathogens. The role of *WAT1* as *S* gene against *V. dahliae* is conserved between Arabidopsis and cotton, and we showed previously that transient silencing of tomato *WAT1* resulted in reduced *V. dahliae* susceptibility. In this study, we generated stable tomato knock-down and knock-out lines through RNAi and CRISPR-Cas9 to confirm a role of *WAT1* in Verticillium wilt susceptibility of tomato. Whereas RNAi-mediated *WAT1* silencing could not confirm loss of *V. dahliae* susceptibility in tomato, our data show that targeted deletion in *WAT1* results in enhanced resistance to *V. dahliae* as well as to *V. albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). However, unfortunately the loss of Verticillium susceptibility in *WAT1* mutant lines is accompanied by severe growth defects. Therefore, future efforts should be devoted to identifying *WAT1* alleles that cannot be exploited by *Verticillium* spp. and *Fol* for disease development, yet that do not negatively impact tomato growth and development.

INTRODUCTION

Vascular wilt pathogens cause diseases in many annual and perennial crops (Yadeta and Thomma, 2013). On tomato, vascular pathogens include fungi such as *Fusarium* (Michielse and Rep, 2009) and *Verticillium* (Fradin and Thomma, 2006), as well as bacteria such as *Clavibacter* (Nandi *et al*., 2018), *Ralstonia* (Peeters *et al*., 2013) and *Xanthomonas* (Potnis *et al*., 2015). Due to the particular niche they colonize, namely the xylem vessels, vascular pathogens are hard to combat once they invaded a plant host as only few measures for efficient disease control are available (Yadeta and Thomma, 2013). The soil-borne fungus *V. dahliae* is particularly hard to control due to its enormous host range and its persisting resting structures in the soil (Fradin and Thomma, 2006). Crop protection therefore relies on resistant plant varieties as disease control using chemicals is ineffective and crop rotation is ineffective due to the large host range. For *V. dahliae* only one monogenic resistance conferred by the tomato *Ve1* gene is known so far (Fradin *et al*., 2009). This resistance is based on the recognition of the *V. dahliae* avirulence protein (Ave1) by the resistance (R) protein encoded by the *Ve1* gene (de Jonge *et al*., 2012). However, this resistance has been overcome by *V. dahliae* strains that have purged the *Ave1* gene, posing a reoccurring challenge to crop protection of tomato worldwide (Grogan, 1979; Dobinson *et al*., 1996; de Jonge *et al*., 2012; Usami *et al*., 2017).

To address the recurrent problem of the breakdown of *R* gene-mediated resistance, alternative approaches can be pursued, such as the impairment of disease susceptibility (*S*) genes (Pavan *et al*., 2010; Gawehns *et al*., 2013). *S* genes are host genes that play an important role for disease establishment by the pathogen. *S* genes can function in a multitude of ways, including early recognition of the pathogen, negative regulation of immune responses or pathogen sustenance (van Schie and Takken, 2014). Nevertheless, *S* genes also have functions for the host. *S* gene-mediated resistance, or rather loss of susceptibility, is achieved by circumventing the mis-use of these gene products by the pathogen, preferably whilst keeping the intrinsic function for the host intact. In wild germplasm such impaired *S* gene alleles can occur naturally, for example as loss-of-function mutations or as promoter mutations leading to impaired expression (Chu *et al*., 2006; Bai *et al*., 2008; Gao *et al*., 2015). Alternatively, these impairments can be introduced by random mutagenesis and nowadays targeted genome editing, for example using CRISPR-Cas9 (Zaidi *et al*., 2018; Dong and Ronald, 2019). Impairment of *S* genes can be associated with severe fitness costs as a consequence of not only impairment of its function for the pathogen, but also impairment of its intrinsic role for the host. For instance for the *defense no death 1* (*dnd1*) mutant, loss of susceptibility to *Pseudomonas syringae* is accompanied by dwarfism in Arabidopsis (Clough *et al*., 2000), dwarfism in tomato, and spontaneous lesion formation in potato (Sun *et al*., 2016a). An important benefit, however, is that impairment of *S* genes can lead to non-

race specific resistance to all strains of a given pathogen (Jørgensen, 1992), or even to broad-spectrum resistance to multiple pathogens (Wang *et al*., 2018). This highlights the potential of *S* gene-mediated resistance in crop protection.

An example of broad-spectrum resistance to different vascular pathogens is provided by the *walls are thin 1* (*wat1*) mutant (Denancé *et al*., 2013). This mutant was identified in an Arabidopsis cell wall mutant screening (Ranocha *et al*., 2010), and displays resistance to the bacteria *R. solanacearum* and *X. campestris*, and the fungi *V. dahliae*, *V. albo-atrum* and *Plectoshaerella cucumerina* (Denancé *et al*., 2013). *WAT1* is a tonoplast-localized auxin transporter (Ranocha *et al*., 2013), but its exact role in so-called "vascular immunity" is not yet understood. *WAT1* has orthologues in different plant species (Ranocha *et al*., 2010), and recently its role as susceptibility factor in cotton was investigated, demonstrating that simultaneous transient silencing of three *WAT1* homologues resulted in increased resistance to *V. dahliae* (Tang *et al*., 2019). We previously showed that transient silencing of tomato *WAT1* (*SlWAT1*) similarly resulted in reduced *V. dahliae* susceptibility (Chapter 5), suggesting that the role of *SlWAT1* as an *S* gene for *V. dahliae* infection is conserved across plant families. In this study, we generated stable knock-down and knock-out lines through RNAi and CRISPR-Cas9 to confirm the role of *SlWAT1* in disease susceptibility in tomato.

MATERIALS & METHODS

Plant and fungi growth conditions

All tomato genotypes were grown in the greenhouse (Unifarm, Wageningen University & Research, The Netherlands) at 21°C/19°C (day/night) at 60% relative humidity and a minimal light intensity of 100 W/m^2 in potting soil (Potgrond 4, Horticoop, Katwijk, The Netherlands). *V. dahliae*, *V. albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) strains (Table 1) were maintained on potato dextrose agar (PDA) at room temperature in the dark.

Table 1 | Overview of *V. dahliae*, *V. albo-atrum* and *Fol* strains used in this study.

Species	Strain	Race
V. dahliae	JR2	1
	VdLS17	2
	DVDS26	2
	DVDS29	2
	DVD3	2
	HoMCF	3
V. albo-atrum	CBS385.91	1
Fol	Bt.01	1

Generation of CRISPR-Cas9 and RNAi lines

CRISPR-Cas9 constructs were designed and clones as described before (Chapter 4) (Supplementary Table 1 & Supplementary Figure 1). To generate the *WAT1* silencing construct, the same fragment as used for transient silencing (Chapter 5) (Supplementary Table 1 & Supplementary Figure 1) was cloned into the pHellsgate8 vector (Helliwell and Waterhouse, 2003) and subsequently transformed into *Agrobacterium tumefaciens* strain AGL1+virG. Tomato transformation of cultivar Moneymaker (MM) was carried out as described previously (Huibers et al., 2013).

Pathogen inoculations, phenotyping & fungal biomass quantification

V. dahliae, *V. albo-atrum* and *Fol* inoculations were carried out with root dipping as described previously (Fradin et al., 2009; Boshoven, 2017). For phenotyping, stunting (%) between inoculated and mock-inoculated plants was calculated based on plant canopy area at 21 days post inoculation (dpi) using Image J (Abramoff et al., 2004) as follows:

$$stunting\ (\%) = \left(1 - \frac{canopy\ area\ of\ V.\ dahliae{-}inoculated\ plant}{average\ canopy\ area\ of\ mock{-}inoculated\ plants}\right) \times 100.$$

To quantify fungal biomass, stems sections (~ 2 cm around the cotyledons) were harvested at 21 dpi and freeze-dried for 48 hours. Subsequently, material was ground, and DNA was isolated using CTAB buffer (200 mM Tris-HCl pH 7.5, 50 mM EDTA pH 8.0, 2 M NaCl, 2% CTAB). Fungal biomass was determined on genomic DNA targeting the *ITS* gene (*V. dahliae* and *V. albo-atrum*) relative to the reference gene *SlRUB* (Supplementary Table 1) with the CFX96 Real-time System (Bio-Rad, Veenendaal, The Netherlands) and SYBR Green Master Mix (Bio-Rad, Veenendaal, The Netherlands). Data were normalized to MM with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

DNA isolation and genotyping

To genotype RNAi and CRISPR plants, DNA was isolated from young leaves using CTAB buffer (1 M Tris-HCl pH 7.5, 0.5 M EDTA pH 8.0, 5 M NaCl, 2% CTAB). PCR was performed with DreamTaq DNA polymerase (Thermo Scientific, Bleiswijk, The Netherlands) and corresponding primers (Supplementary Table 1). PCR products were sequenced by Marcrogen Europe (Amsterdam, The Netherlands).

RNA isolation and quantitative reverse transcription PCR (qRT-PCR)

To quantify silencing levels, root material was harvested at 21 dpi and snap-frozen in liquid nitrogen. Total RNA was isolated with the MagMAX-96 Total RNA Isolation Kit (Invitrogen, Bleiswijk, The Netherlands) using a KingFisher Flex System (Thermo

Scientific, Bleiswijk, The Netherlands) and synthesis of cDNA was performed with the iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer's instructions. qRT-PCR was carried out with the CFX96 Real-time System (Bio-Rad, Veenendaal, The Netherlands) and SYBR Green Master Mix (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer's instructions. Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) relative to the *tomato elongation factor 1 α* (*SlEF1α*) (Supplementary Table 2). Data were normalized to transformants devoid of the silencing construct or, when not available, to MM plants.

RESULTS

Knock-down of *SlWAT1* did not confirm loss of susceptibility to *V. dahliae*

Only a single orthologue of *AtWAT1* was previously identified in tomato (Solyc04g080940, hereafter *SlWAT1*) for which transient silencing resulted in reduced susceptibility to *V. dahliae* (Chapter 5). To confirm the role of *SlWAT1* in *V. dahliae* susceptibility, tomato cultivar Moneymaker (MM) was transformed with an RNAi construct to silence *SlWAT1*. The RNAi construct was based on the previously designed transient silencing construct (Chapter 5) (Supplementary Figure 1). Several primary transformants (T1) were evaluated by testing for the presence of the silencing construct and by determining residual *WAT1* expression levels. In the T1 transformants relative *WAT1* expression greatly varied, from 11% to 270%, when compared with the expression levels found in leaves of control plants. Five T1 transformants with reduced *SlWAT1* expression were transferred to the greenhouse for T2 seed production (Supplementary Table 2). However, seeds were only obtained from three transformants, TV181034, TV181036 and TV181037, which were used for further study.

First of all, T2 plants derived from the three transformants, TV181034, TV181036 and TV181037, were tested for presence of the silencing construct with a NPTII- and 35S specific-PCR. This revealed that 15.5%, 19.1% and 10.0% of the plants of the T2 families TV181034, TV181036 and TV181037 respectively, did not carry the silencing construct (Figure 1A). For plants carrying the silencing construct residual SlWAT1 expression levels were determined in roots. *SlWAT1* was most efficiently silenced in plants of family TV181036, as expression was significantly reduced to on average 20.2% in plants that carried the silencing construct (+ NPTII/35S) when compared with plants lacking the construct (- NPTII/35S) (Figure 1B). For plants of family TV181034 expression was also significantly reduced, albeit to on average 54.4%. As a large variation in *SlWAT1* expression was found in plants of family TV181034 that did not carry the silencing construct, an additional analysis was carried out to normalize the expression of

TV181034 plants with the silencing constructs to plants of family TV181036 lacking the silencing construct. A significant reduction in *SlWAT1* expression to on average 20.3 % was determined in this way (Figure 1C). Residual *SlWAT1* expression was the highest in plants of family TV181037, for which expression was significantly reduced to on average 65.5%. For this genotype, several plants showed similar or even higher levels of *SlWAT1* expression when compared with plants lacking the silencing construct. Lastly, we also determined whether the presence of the silencing construct affected plant growth in the absence of *V. dahliae* inoculation. No significant difference in canopy area of mock-inoculated plants was found for any of the T2 families compared with mock-inoculated MM plants (Figure 1D). Taken together, the presence of the silencing construct did not affect canopy area and plants of T2 families TV181034 and TV181036 showed a residual *SlWAT1* expression of approximately only 20%, while plants of family TV181037 were not well silenced.

To test for loss of susceptibility to *V. dahliae*, plants from all three T2 families were challenged with *V. dahliae*. To this end, stunting based on canopy area was calculated between mock- and *V. dahliae*-inoculated plants for each of the genotypes at 21 days post inoculation (dpi) and compared with *V. dahliae*-induced stunting in MM plants (Figure 1E). Plants of family TV181036 and TV181037 showed no significant reduction in stunting of *V. dahliae*-inoculated plants when compared with *V. dahliae*-inoculated MM plants. In contrast, for plants of family TV181034 a significant reduction in stunting of *V. dahliae*-inoculated plants to on average 32.2% was observed compared with an average of 52.6% in *V. dahliae*-inoculated MM plants.

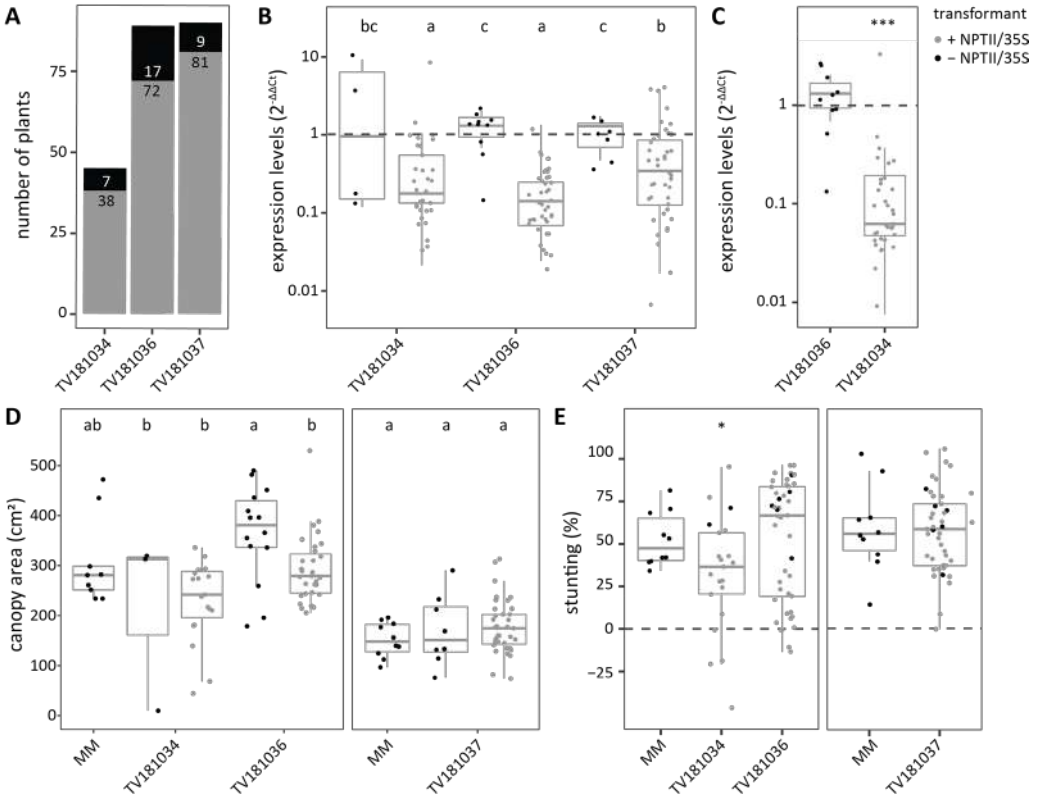


Figure 1 | Reduced susceptibility to *Verticillium dahliae* was found for *SlWAT1* T2 RNAi family TV181034, but not for TV181036 and TV181037. (A) Total number of plants with (grey) and without (black) the silencing construct. (B) Expression levels of *SlWAT1* in roots collected at 21 dpi relative between plants with the silencing construct (+ NPTII/35S, grey) and without the silencing construct (- NPTII/35S, black). Data of two independent experiments were normalized using $2^{-\Delta\Delta Ct}$ with n ≥ 4 per experiment per genotype (ANOVA with Fisher's unprotected LSD with p = 0.05 on ΔCt values). (C) Additional analysis of silencing levels for TV181034 plants with the silencing construct, here normalized to TV181036 plants lacking the silencing construct. Data of two independent experiments with n ≥ 4 per experiment per genotype (t-test with *** p < 0.001). (D) Canopy area (cm^2) of mock-inoculated plants at 21 dpi for T2 RNAi families. Data of two independent experiment with n ≥ 2 per experiment per genotype (ANOVA with Fisher's unprotected LSD, p = 0.05). (E) Stunting of *V. dahliae*-inoculated plants relative to mock-inoculated plants per genotype at 21 dpi. Data of two independent experiments with n ≥ 8 per experiment per genotype (t-test with * p < 0.05).

To confirm the results of the T2 generation, two plants per T2 family were kept for T3 seed production. Notably, all six T3 families except TV181037-74 were still segregating for the presence of the silencing construct and approximately 14% to 26% of the plants did not carry the silencing construct (Figure 2A). Expression levels of the six T3 families confirmed silencing of *SlWAT1* in plants carrying the silencing construct for families TV181034-46 and -53 as well as in TV181036-54 and -59 with a significant reduction of *SlWAT1* expression to 12.0%, 41.4%, 35.2% and 30.6%, respectively (Figure 2A).

Unfortunately, *SlWAT1* was not silenced at all in plants carrying the silencing construct for families TV181037-73 and -74, as *SlWAT1* expression was on average 39.4% and 36.5% higher than in control plants lacking the silencing construct, respectively. This is in accordance with already relatively high residual *SlWAT1* expression determined in the T2 generation (Figure 1B). To assess whether the presence of the silencing constructs induced any pleiotropic effects on plant growth, the canopy area of mock-inoculated plants was compared between all genotypes in the absence of *V. dahliae* inoculation. No statistically significant difference in canopy area was found for plants of any of the T3 RNAi families when compared with the canopy area of MM plants (Figure 2C). This indicates that the presence of the silencing construct did not significantly affect plant growth at this developmental stage.

The plants of the six T3 RNAi families were challenged with *V. dahliae*, and similar levels of stunting of *V. dahliae*-inoculated plants were found for most plants of the T3 families when compared with *V. dahliae*-inoculated MM plants (Figure 2D). However, stunting of *V. dahliae*-inoculated plants of family TV181034-46 was significantly reduced to, on average, 39.5% when compared with *V. dahliae*-inoculated MM plants that displayed an average stunting of 56.6%. For plants of this family residual expression was the lowest, with only 12.0%, suggesting that *SlWAT1* expression needs to be severely reduced in order to see an effect on *V. dahliae* susceptibility. This is further supported by the fact that for families TV181037-73 and -74 that were not well-silenced stunting of *V. dahliae*-inoculated plants was equal or even significantly increased when compared with *V. dahliae*-inoculated MM plants. Strikingly, the overall variation in stunting of families TV181037-73 and -74 was much smaller compared with the large variation observed in families TV181034-46 and -53 as well as TV181036-59 and -62. Considering that silencing levels most likely also greatly vary between individual plants, this large spread in *V. dahliae*-induced stunting may be attributed to varying levels of residual *SlWAT1* expression.

To quantify *V. dahliae* colonization in the T3 RNAi families, fungal biomass was quantified in stems of *V. dahliae*-inoculated plants at 21 dpi and normalized to *V. dahliae*-inoculated MM plants. No significant reduction in fungal biomass in plants of all six T3 RNAi families was found when compared with *V. dahliae*-inoculated MM plants (Figure 2E). Nevertheless, several plants per T3 RNAi family were found to contain relatively little *V. dahliae* biomass, less than 10%, when compared with *V. dahliae*-inoculated MM plants. This might again relate to varying levels of residual *SlWAT1* expression in the individual plants of these RNAi families which might affect stunting and fungal biomass in a dose-dependent manner.

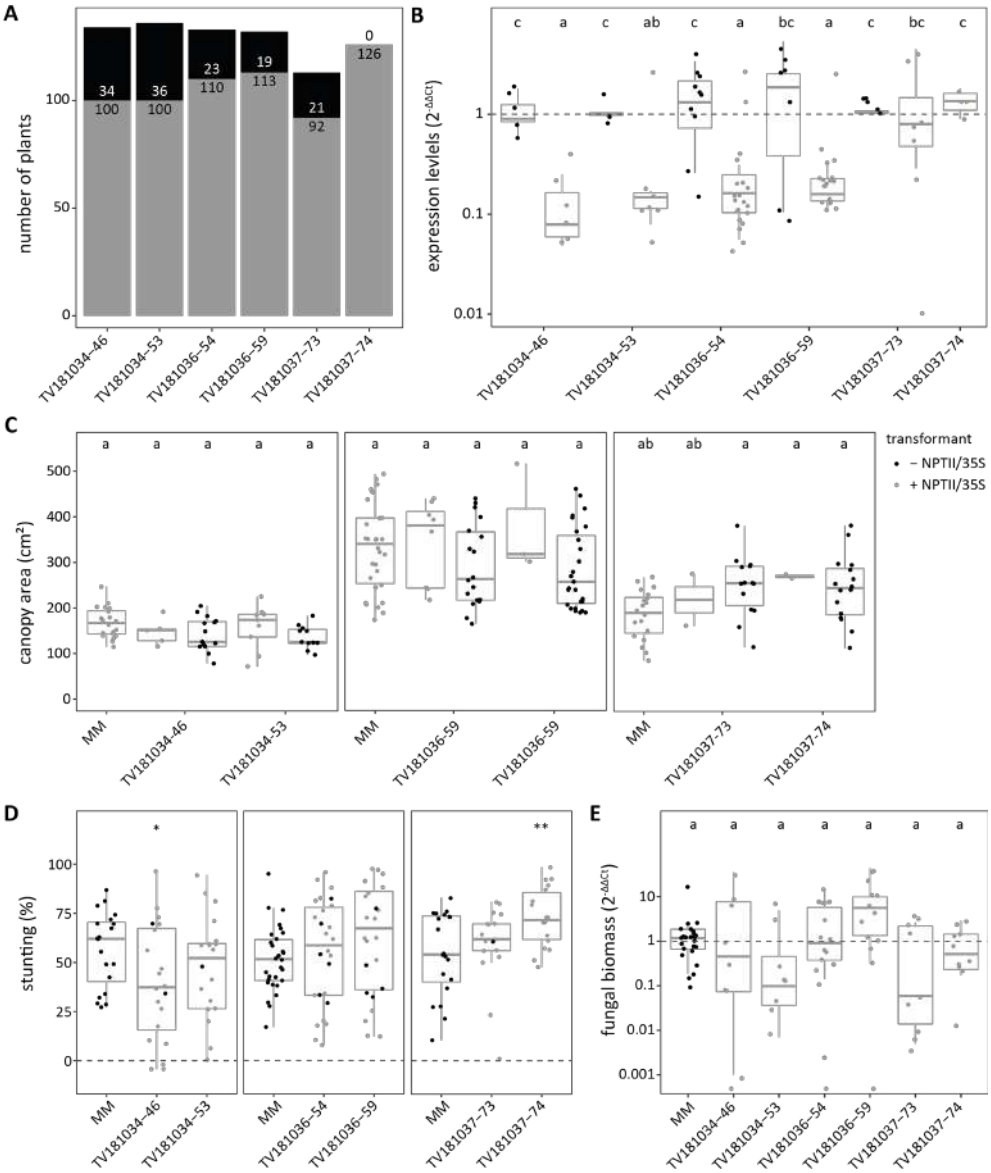


Figure 2 | Loss of susceptibility to *Verticillium dahliae* could not be confirmed in *SlWAT1* T3 RNAi families. (A) Total number of plants with (grey) and without (black) the silencing construct. (B) Expression levels of T3 *SlWAT1* RNAi families relative to plants without the silencing construct (- NPTII/35S, grey) and normalized using $2^{-\Delta\Delta Ct}$. Data of two independent experiments with n ≥ 3 per experiment (ANOVA with Fisher's unprotected LDS with p = 0.05 on ΔCt values). (C) Canopy area (cm^2) of mock-inoculated plants at 21 dpi for T3 SlWAT1 RNAi families. Data from two independent experiments with n ≥ 5 per experiment per genotype (ANOVA with Fisher's unprotected LSD, p = 0.05). (D) Stunting (%) of *V. dahliae*-inoculated plants when compared with the average stunting of mock-inoculated plants at 21 dpi. Box plots represent data with n ≥ 8 plants per experimental repeat (t-test when compared with MM with * $p < 0.05$ and ** $p < 0.01$). (E) Fungal biomass relative to Moneymaker (MM) plants inoculated with *V. dahliae* strain JR2 at 21 dpi and normalized using $2^{-\Delta\Delta Ct}$ on a log10 scale. Data of two independent experiments with n ≥ 5 per experiment per genotype (ANOVA with Fisher's unprotected LSD, p = 0.05 on ΔCt).

The plants of the T3 RNAi families were also challenged with five additional *V. dahliae* strains belonging to different races. Stunting of *V. dahliae*-inoculated plants was equal or even higher in the T3 RNAi plants compared with *V. dahliae*-inoculated MM plants for all tested *V. dahliae* strains (Figure 3). Collectively, these data cannot confirm loss of susceptibility of WAT1 RNAi families to *V. dahliae* in tomato, most likely because residual SlWAT1 expression was too high to have a significant effect on *V. dahliae* susceptibility.

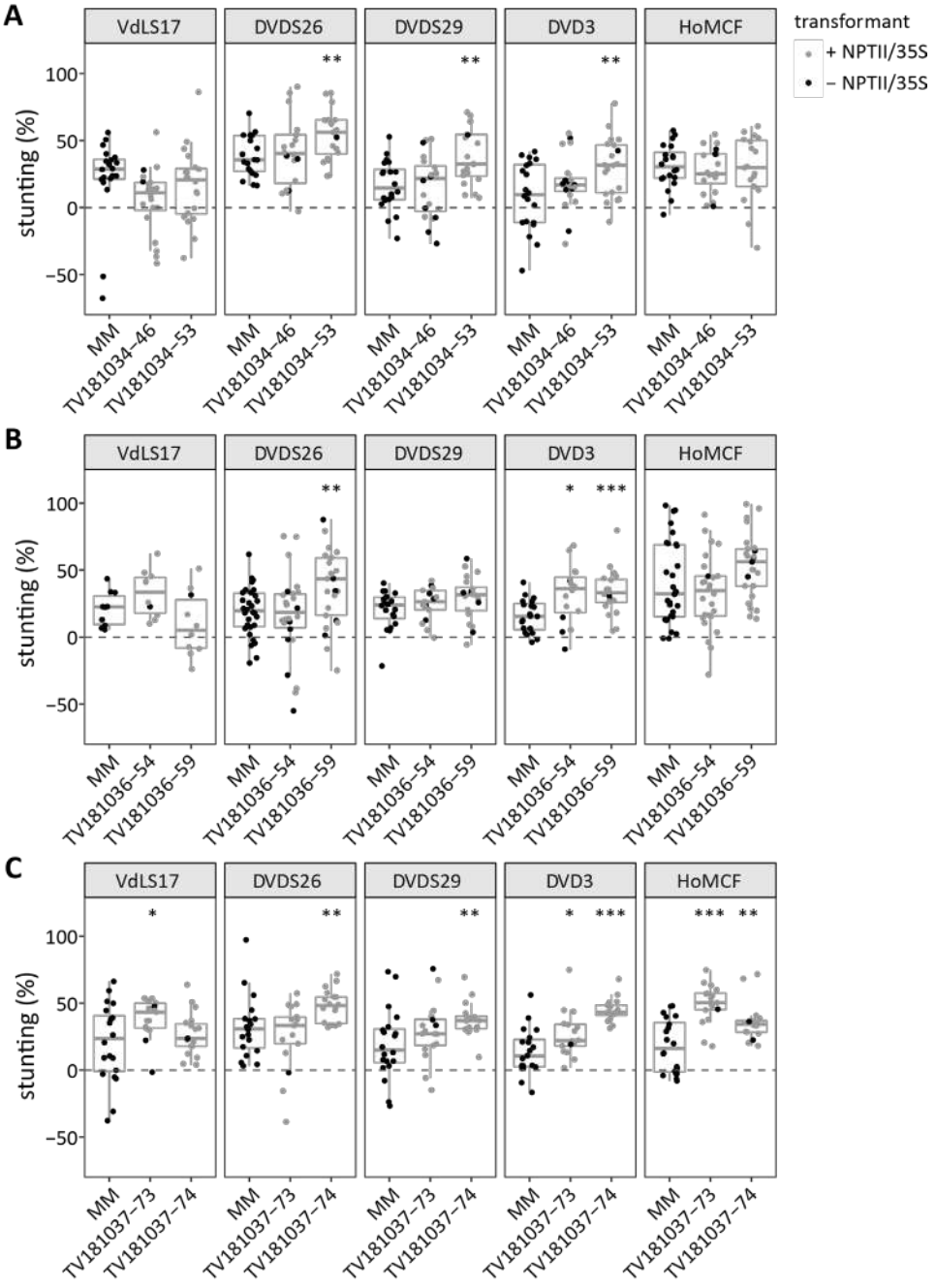


Figure 3 | Stunting (%) of *Verticillium dahliae*-inoculated *SlWAT1* T3 RNAi families TV181034-46 and -53 (A), TV181036-54 and -59 (B) and TV181037-73 and -73 (C) when compared with the average stunting of mock-inoculated Moneymaker (MM) plants at 21 dpi. Box plots represent data of two independent experiments with n ≥ 7 per experiment per genotype (t-test when compared with MM with * $p < 0.05$ and ** $p < 0.01$).

Targeted deletion in *SlWAT1* leads to loss of susceptibility to *V. dahliae* at the expense of severe growth defects

In order to circumvent interference of residual *SlWAT1* expression as shown for the RNAi families, we explored approaches for targeted knock-down. To this end, stable transformants using CRISPR-Cas9 were generated. The CRISPR-Cas9 construct was designed with four single guide RNAs (sgRNAs) that targeted sequences in exons 3, 4 and 5 of the SlWAT1 gene (Supplementary Figure 1). The use of multiple sgRNAs increases the possibility of creating large deletions due to the occurrence of double stranded breaks at multiple sgRNAs locations simultaneously (Do *et al*., 2019). T1 transformants were evaluated for the occurrence of mutations in *SlWAT1* with a gene-specific PCR and gel electrophoresis to detect aberrantly sized PCR products (Supplementary Table 1). By focusing on large deletions, small deletions, small insertions, and single nucleotide polymorphisms might have been missed. Three transformants (#10, #19 and #28) showed a relatively large deletion, while for a fourth transformant (#23) an additional band appeared above the wild-type band (Supplementary Figure 2). However, T2 seeds from only one of these mutants, TV181046 (#19), were obtained as the other transformants were either not successfully transferred from in vitro conditions to soil, did not set fruits, or did not produce seeds.

First, plants from T2 CRISPR family TV181046 were genotyped to confirm the presence of a mutation by sequencing. In fact, seedlings of TV181046 were found to carry bi-allelic mutations with either a smaller deletion (allele 1), or a larger deletion (allele 2), or heterozygous plants that carry both types of deletions (Figure 4 A and B). The smaller 352 bp deletion (allele 1) located in exon 4 led to a 121 amino acid deletion and the larger 1,291 bp deletion (allele 2) spanning exons 3, 4 and partly 5 resulted in a 197 amino acid deletion. As only one T2 CRISPR line was obtained, we propagated plants with the heterozygous deletions as well as homozygous plants for each mutant allele to obtain a larger panel of genotypes (T3) for testing. Seeds from three T3 CRISPR lines were obtained, TV181046-16, -18 and -23, genotyped and found to be heterozygous for the deletions (-16 and -23) and homozygous for allele 1 (-18) (Figure 4B).

As for neither of the two deletions (allele 1 and allele 2) a premature stop codon was predicted (http://www.softberry.com/berry.phtml), we subsequently investigated whether these deletions affected any known domain of SlWAT1. To this end, protein domains were predicted using InterPro (https://www.ebi.ac.uk/interpro/). For wild type SlWAT1 two EamA domains were found. Most EamA domain-containing proteins are classified as metabolite transporters that usually carry two copies of this domain (Jack *et al*., 2001). For the SlWAT1 mutant alleles both predicted EamA domains were affected (Figure 4C). As *AtWAT1* is located in the tonoplast, the membrane of the vacuole (Ranocha *et al*., 2010), and also because many EamA domain-containing proteins carry multiple transmembrane domains, we further predicted the transmembrane domains

for wild type SlWAT1, mutant allele 1 and mutant allele 2. Wild type SlWAT1 was predicted to contain ten transmembrane domains which was described before for WAT1 in Arabidopsis and cotton as well (Ranocha *et al*., 2010; Tang *et al*., 2019). For mutant allele 1 only seven out of ten transmembrane domains were found and for mutant allele 2 only four (Supplementary Figure 3). Collectively, our data suggested that both mutant alleles carried a deletion which affected known domains in *SlWAT1* and thus it allows us to further study these CRISPR lines for loss of susceptibility to *V. dahliae*.

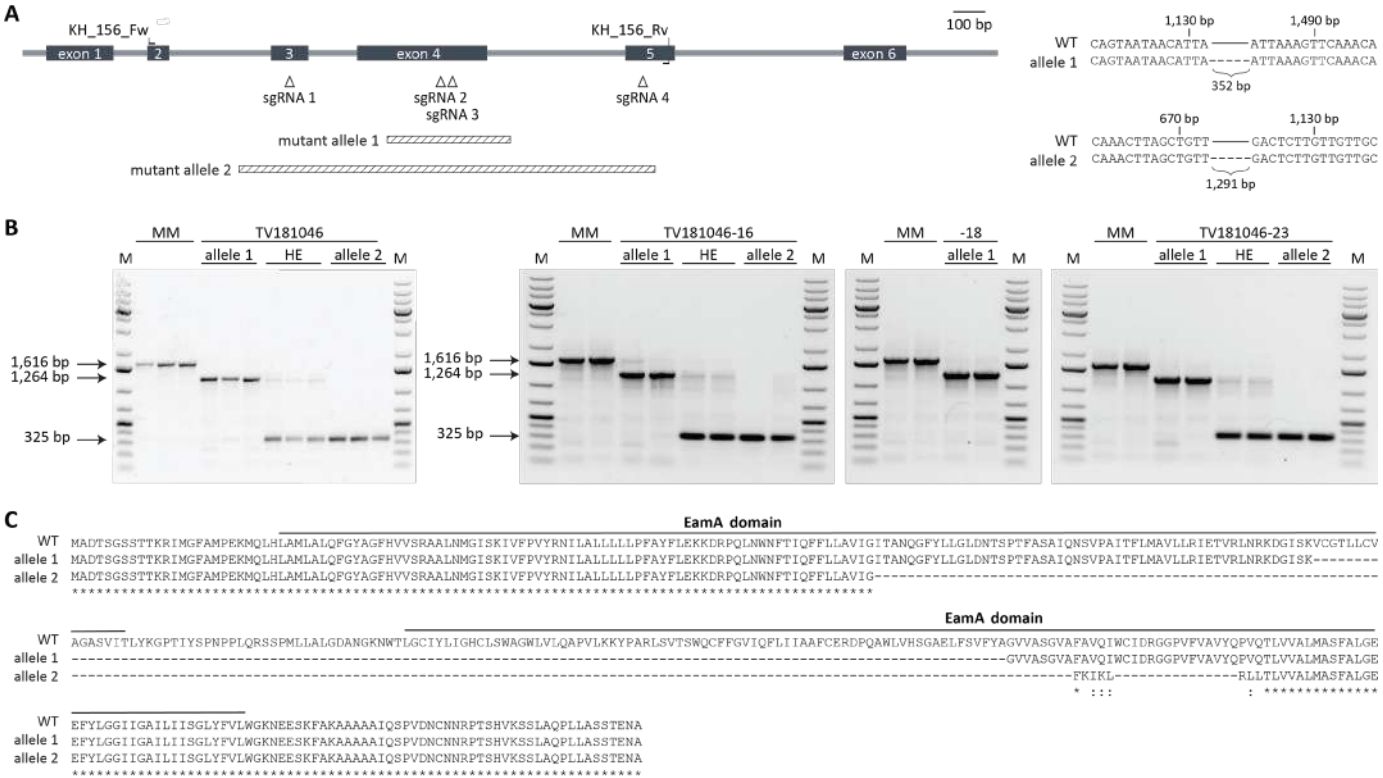


Figure 4 | CRISPR T2 family TV181046 and its T3 progeny TV181046-16, -18 and -23 carry a bi-allelic mutation in *SlWAT1*. (A) Schematic overview of *SlWAT1* (Solyc04g080940) indicating location of the sgRNAs, primers used for genotyping and the two mutant alleles (right). Sequencing revealed a 352 bp and 1,291 bp deletion for mutant alleles 1 and 2 respectively compared with *SlWAT1* wild type (WT) (right). (B) Gel electrophoresis (1% TAE, ethidium bromide) of gene-specific PCR with (WT band at 1,616 bp) showing the two mutant alleles as well as heterozygous (HE) plants for the T2 (left) and the T3 (right) plants with a 1 kb ladder (M). (C) Protein alignments of SlWAT1 WT with mutant alleles 1 and 2 showing a 121 and 197 amino acid deletion respectively. Mutant alleles were translated into protein using http://www.softberry.com/berry.phtml. CLUSTAL multiple sequence alignment was done using https://www.ebi.ac.uk/Tools/msa/muscle/. Solid bars indicate predicted protein domains annotated as EamA domain (https://www.ebi.ac.uk/interpro).

Irrespective of the type of mutation, all plants of the T2 and the T3 generation displayed severe growth and development defects; the germination rate was low, seedlings were small and light in color, and overall plant growth remained severely compromised (Figure 5A). To quantify the size difference, we determined canopy area of mock-inoculated plants for all genotypes in the absence of *V. dahliae* inoculation. Canopy area of mock-inoculated plants was heavily reduced when compared with mock-inoculated MM plants measured at 21 dpi. While the canopy area of MM plants was 300 cm^2 on average, the canopy area of most CRISPR T2 and 3 plants was less than 10 cm^2. However, the observed aberrations alleviated during plant development and even though the CRISPR plants remained smaller than MM plants, they developed flowers and set fruits (Figure 5B and Supplementary Figure 4).

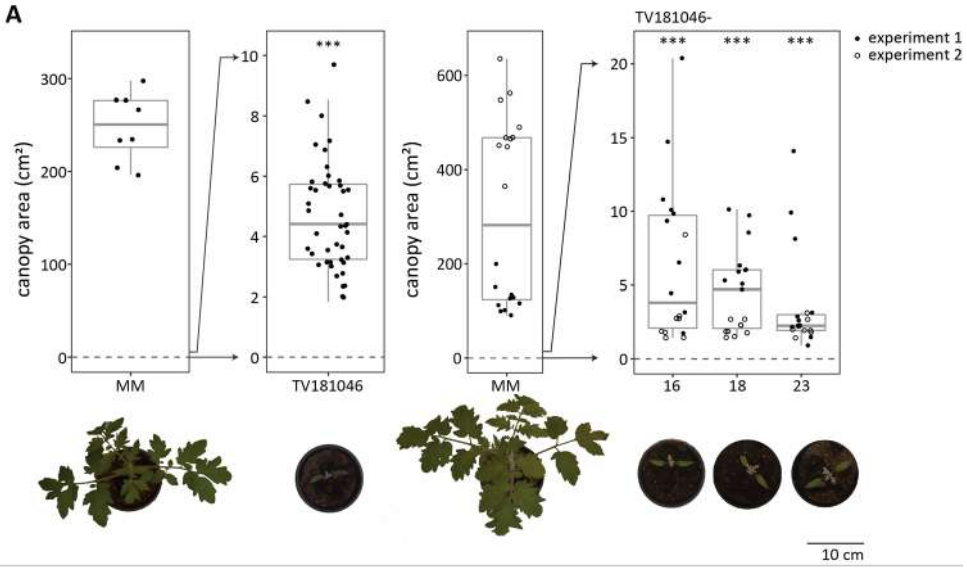


Figure continues on next page

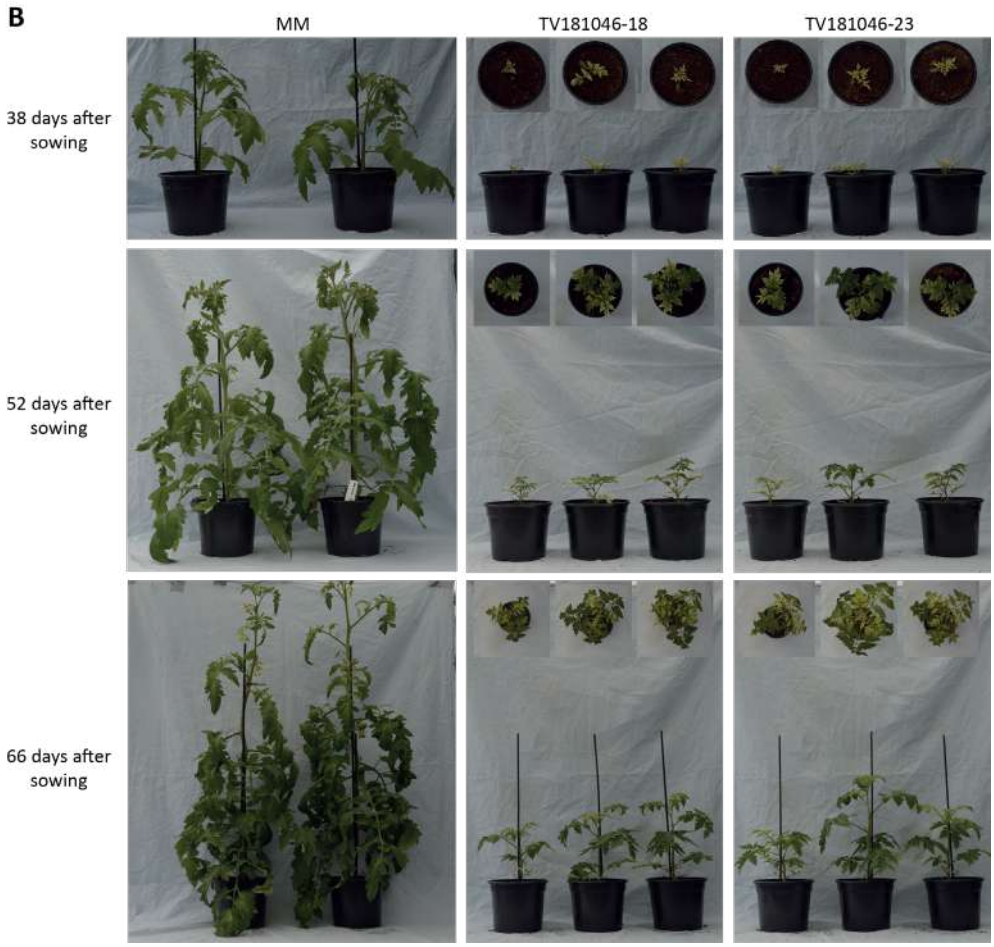


Figure 5 | CRISPR T2 family TV181046 and its T3 progeny TV181046-16, -18 and -23 display severe growth and development defects. Canopy area of mock-inoculated plants at 21 dpi for T2 CRISPR line TV181046 (left) and T3 CRISPR lines TV181046-16, -18 and -23 (right). Data of one or two independent experiments with n ≥ 8 per experiment per genotype (t-test compared with Moneymaker (MM) with *** p = 0.001). (B) Pictures of MM and T3 CRISPR WAT1 plants at different time points.

To test for loss of susceptibility, plants of the T2 and the T3 generation were inoculated with *V. dahliae*. Stunting of *V. dahliae*-inoculated T2 plants was significantly reduced to on average 7.1% for line TV181046 when compared with *V. dahliae*-inoculated MM plants with on average 65.5% stunting (Figure 6A). Similarly, significantly reduced stunting of *V. dahliae*-inoculated T3 plants of all three lines was found compared with *V. dahliae*-inoculated MM plants (Figure 6A). Compared with *V. dahliae*-induced stunting of 60.0% on average in MM plants, stunting in TV181046-16-, -18 and -23 was significantly reduced to 41.7%, 1.4% and 26.2% on average, respectively. Due to the

stunting calculations being based on the average of mock-inoculated plants and due to variation in plant size observed in the mutant lines, the differences in stunting of *V. dahliae*-inoculated plants were pronounced in the mutant lines compared to the MM plants. To quantify the effect *V. dahliae* proliferation, fungal biomass was determined in stems of *V. dahliae*-inoculated T3 plants at 21 dpi. Fungal biomass was significantly reduced to around 1% in *V. dahliae*-inoculated plants of all CRISPR T3 families compared with *V. dahliae*-inoculated MM plants (Figure 6B).

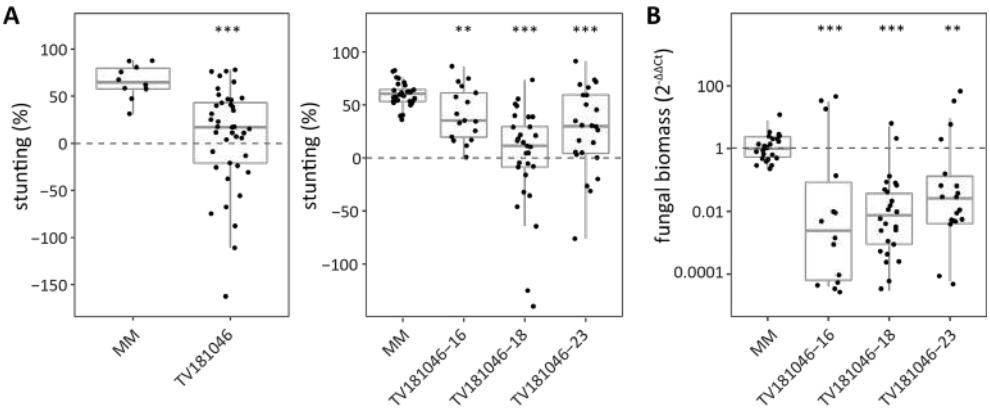


Figure 6 | Targeted knockout of *SlWAT1* lead to loss of susceptibility to *Verticillium dahliae*. (A) Stunting (%) of *V. dahliae*-inoculated T2 (left) and T3 (right) plants when compared with the average stunting of mock-inoculated plants at 21 dpi. Box plots represent data of one or two independent experiments with n ≥ 9 per experiment per genotype (t-test when compared with MM with ** $p < 0.01$ and*** $p < 0.001$). (B) Fungal biomass of *V. dahliae*-inoculated T3 CRISPR plants of all three lines relative to *V. dahliae*-inoculated MM plants in stems at 21 dpi and normalized using $2^{-\Delta\Delta Ct}$ on a log10 scale. Data of two independent experiments with n ≥ 7 per experiment per genotype (t-test on ΔCt when compared with MM with ** $p < 0.01$ and *** $p < 0.001$).

As *S* gene-mediated resistance can lead to broad-spectrum resistance to multiple pathogens (Wang et al., 2018), we also challenged T3 CRISPR plants with *V. albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). For both pathogens, inoculated T3 CRISPR plants showed significantly reduced stunting when compared with inoculated MM plants (Figure 7A). Moreover, fungal biomass was significantly reduced in *V. albo-atrum*- and *Fol*-inoculated T3 CRISPR plants of all three lines when compared with inoculated MM plants (Figure 7B). Collectively, our data show that targeted deletion in *SlWAT1* resulted in enhanced resistance to *V. dahliae, V. albo-atrum* and *Fol* in tomato.

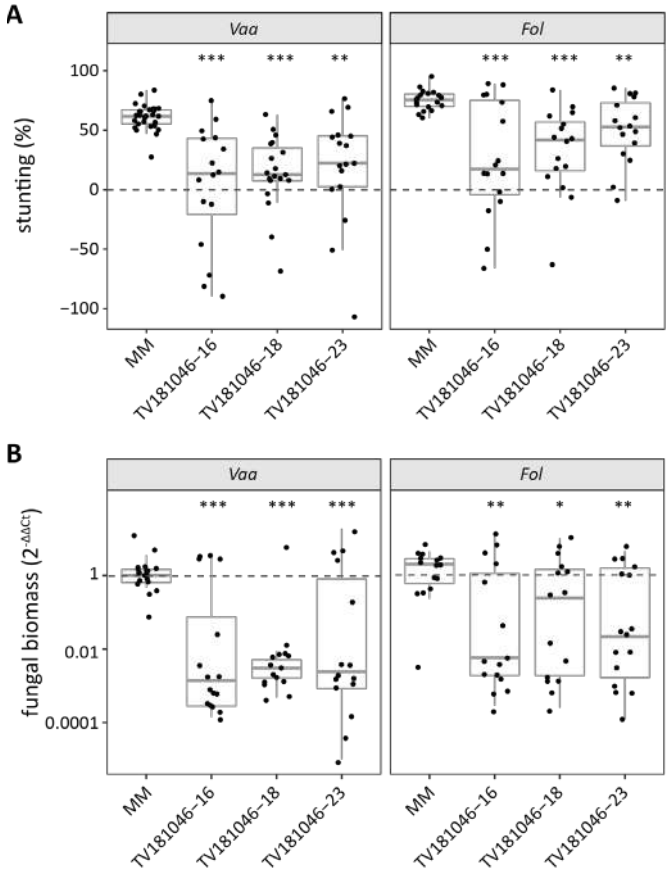


Figure 7 | Targeted knockout of *SlWAT1* also lead to loss of susceptibility to *Verticillium albo-atrum* (*Vaa*) and *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). (A) Stunting (%) of *Vaa* and *Fol* inoculated T3 plants when compared with the average stunting of mock-inoculated plants at 21 dpi. Box plots represent data of two independent experiments with n ≥ 5 per experiment per genotype (t-test when compared with MM with ** $p < 0.01$ and *** $p < 0.001$). (B) Fungal biomass of of *Vaa* and *Fol* inoculated T3 CRISPR plants relative to *Vaa* and *Fol* inoculated MM plants in stems at 21 dpi and normalized using $2^{-\Delta\Delta Ct}$ on a log10 scale. Data of two independent experiments with n ≥ 5 per experiment per genotype (t-test on ΔCt when compared with MM with ** $p < 0.01$ and *** $p < 0.001$).

DISCUSSION

For vascular pathogens such as *V. dahliae*, for which only few sources of monogenic resistance are known, crop protection mainly relies on alternative strategies. The impairment of *S* genes has gained increasing attention in resistance breeding over the last years (Pavan *et al*., 2010; Gawehns *et al*., 2013; van Schie and Takken, 2014), particularly in the light of recent advances in genome editing in plants (Andolfo *et al*., 2016; Langner *et al*., 2018; Zaidi *et al*., 2018; Yin and Qiu, 2019). Here, we show that targeted deletion of *SlWAT1* using CRISPR-Cas9 led to loss of susceptibility to *V. dahliae* in tomato. Plants of T3 CRISPR lines showed reduced disease symptoms upon challenge with *V. dahliae* as well as reduced fungal biomass when compared with susceptible MM plants (Figure 6). These findings are in agreement with our previous experiments concerning transient silencing of *WAT1* in tomato, which similarly resulted in reduced susceptibility (Chapter 5). The loss of susceptibility to *V. dahliae*, as observed in plants of the CRISPR lines, could not be demonstrated in plants carrying the RNAi silencing construct (Figures 1 and 2). This can likely be attributed to the relatively high degree of residual *WAT1* expression in most plants of the T2 and T3 RNAi lines, which likely compromised the efficacy of silencing too much to monitor effects on *V. dahliae* infection. However, in particular plants *V. dahliae*-induced stunting was reduced when compared with MM control plants while in other plants reduced fungal biomass was found, suggesting that in these cases sufficient levels of silencing were likely obtained.

In *WAT* studies in Arabidopsis and cotton, reduced Verticillium wilt symptoms and reduced fungal proliferation were observed in knock out mutants or upon transient silencing of *WAT1*, respectively (Denancé *et al*., 2013; Tang *et al*., 2019). Remarkably, the loss of susceptibility in Arabidopsis *wat1* mutants was further extended to other vascular pathogens including bacteria and fungi (Denancé *et al*., 2013). *S* gene-mediated broad-spectrum resistance to multiple pathogens was described before (Wang *et al*., 2018), and highlights the potential of using impaired *S* genes for the control of multiple pathogens simultaneously. In fact, we also demonstrated loss of susceptibility of *SlWAT1* CRISPR plants to another pathogenic *Verticillium* species, *V. albo-atrum*, as well to another vascular pathogen, *Fol* (Figure 7). Also for these pathogens, disease symptoms and fungal biomass were significantly reduced when compared with susceptible MM plants. Together, this indicates that the function of *WAT1* in susceptibility to different vascular pathogens seems to be conserved across plant species, and therefore impairment of *WAT1* offers a promising approach to combat different vascular pathogens in multiple crops.

To date, the function of *WAT1* in so called “vascular immunity” remains to be elucidated. *WAT1* was originally identified in a cell wall mutant screening in zinnia (*Zinnia elegans*) (Pesquet *et al*., 2005; Ranocha *et al*., 2010) and the homolog of Arabidopsis was shown to be a tonoplast-localized auxin transporter (Ranocha *et*

al., 2013). In Arabidopsis *wat1* mutants, cell wall-related phenotypes in stems were described with altered cell elongation and reduced secondary cell walls of fiber cells, hence its name *Walls Are Thin 1*. Furthermore, *wat1* mutants showed altered contents of auxin (indole-3-acetic acid, IAA), tryptophan and salicylic acid (SA) (Ranocha *et al*., 2010; Denancé *et al*., 2013). The IAA content in roots was reduced in *wat1* mutants while the SA content was found to be elevated when compared with wild type plants (Denancé *et al*., 2013), which is in line with the previously-described antagonism of auxin and SA in plant immunity and development (Wang *et al*., 2007; Robert-Seilaniantz *et al*., 2011). SA does not seem to play a role in basal plant defense against *V. dahliae* as different Arabidopsis mutants with a deficiency in SA signaling, such as *enhanced disease susceptibility* (*eds1-2* and *eds5-1*), *nonexpresser of PR genes* (*npr1-1* and *npr1-3*) and *phytoalexin deficient 4* (*pad4-1*), show similar symptoms and levels of fungal biomass as control plants upon *V. dahliae* infection (Pantelides *et al*., 2010; Fradin *et al*., 2011). In contrast, a role was assigned to auxin in *V. dahliae* susceptibility as two auxin receptor mutants, *auxin signaling F-box 1 and 3* (*afb1* and *afb3*), as well as auxin transporter mutant *auxin resistant 4* (*axr4*) display reduced symptoms and less fungal biomass upon challenge with *V. dahliae* (Fousia *et al*., 2018). For another vascular wilt pathogen, *F. oxysporum*, two transcription factor mutants, *auxin response factor 1* and *2* (*arf1* and *arf2*), showed significantly less disease levels although fungal biomass was not quantified (Lyons *et al*., 2015). Collectively, auxin seems to play a crucial role in *V. dahliae* susceptibility to vascular wilt fungi, and therefore auxin-related genes may be further studied to test their potential as susceptibility factors for *V. dahliae*.

Even though *SlWAT1* CRISPR plants showed loss of susceptibility to *V. dahliae*, the targeted deletion was accompanied by severe growth defects (Figure 5). Impairment of *S* genes is known to cause pleiotropic side effects in some cases (Clough *et al*., 2000; Sun *et al*., 2016b), and also for *WAT1* such effects were described in other plant species. For Arabidopsis *wat1* mutants, no abnormalities were found in early stages of development, but older plants were stunted when compared with wild type plants (Ranocha *et al*., 2010). Transient silencing of *WAT1* in cotton resulted in reduced root length and shorter first internodes (Tang *et al*., 2019). Such growth defects can certainly be attributed to the imbalance between auxin and SA. Firstly, it is well known that auxin plays an essential role in many aspects of plant development (Korver *et al*., 2018) and its downregulation, as shown in Arabidopsis *wat1* mutants, might negatively affect growth. Secondly, Arabidopsis *wat1* mutants also showed higher SA levels, which is known to affect plant growth as observed in the *constitutive expressor of PR genes 5* (*cpr5*) mutant which shows high SA levels accompanied by severe dwarfism (Bowling *et al*., 1997). Evidently, pleiotropic effects of impaired *S* genes are not desirable for breeding purposes, as it might affect yield but also overall development (Hückelhoven *et al*., 2013; Engelhardt *et al*., 2018). Additionally, special attention should also be given

to resistance against other pathogens as an altered hormone balance, as observed in *WAT1*-mediated resistance (Denancé *et al*., 2013), can influence resistance to other pathogens (Thomma *et al*., 1998). Therefore, alternatives for obtaining mutants without such pleiotropic effects needs to be explored. For example, potential natural allelic variants of *WAT1* in wild germplasm that can no longer be exploited by the pathogen, but that do not display pleiotropic effects, could be used for breeding. Alternatively, mutant populations can be used to identify *wat1* mutants omitting the severe growth defects. Certainly, mutants with smaller targeted deletions or even single base pair changes could also be studied, to find essential domains that are only required by *Verticillium* spp. for disease development, but that are not involved in tomato growth and development. Lastly, targeted deletions in the *WAT1* promoter could circumvent pleiotropy in a similar fashion as previously shown for *xa13*-mediated resistance against bacterial blight (Chu *et al*., 2006; Zaka *et al*., 2018). Specific variations in the promoter sequence prevented binding of the effector binding elements (EBEs) of the bacterial effector, which however, kept the host function of this *S* gene intact. In the case of *WAT1*, however, our data already indicated that a reduction in expression is not sufficient to enhance resistance and hence, a mutation in the promoter might not lead to loss of susceptibility. Summarizing, there are many different approaches to identifying *WAT1* alleles or variants that cannot be exploited by *Verticillium* spp. for disease development, yet that do not negatively impact tomato growth and development.

ACKNOWLEDGMENT

This project is financially supported by Topsector Tuinbouw & Uitgangsmaterialen (project: 1409-026). Special thanks to Bert Essenstam and Andre Maassen at Unifarm for excellent plant care and to Eleni Koseoglou for discussions.

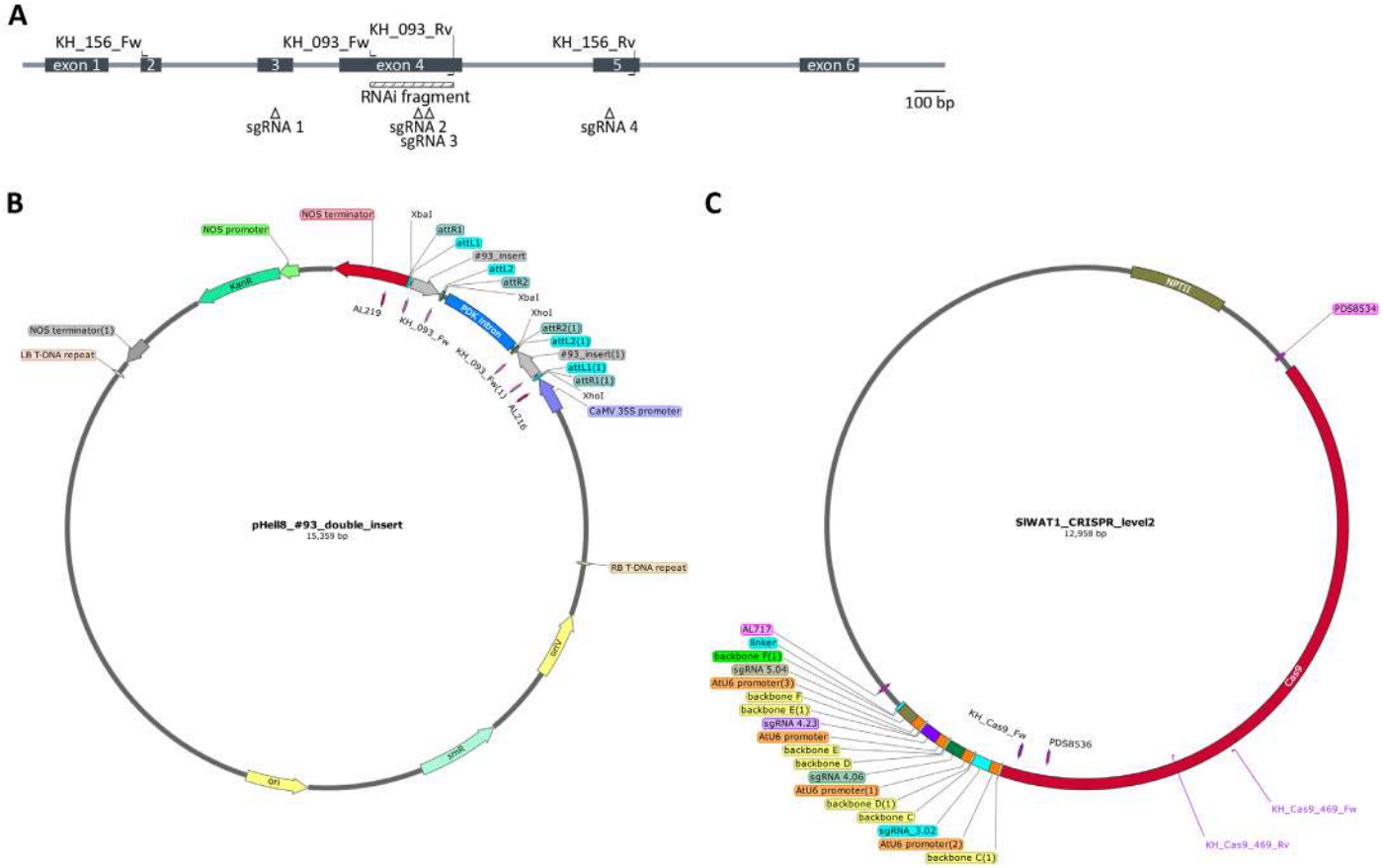
SUPPLEMENTARY MATERIAL

Supplementary Table 1 | Primers used in this study.

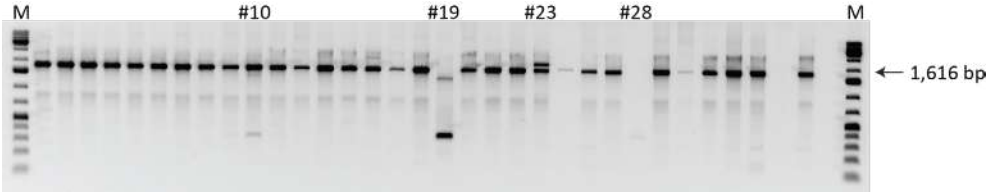
Primer	Sequence (5'→ 3')	Used for
SlEF1α_Fw SlEF1α_Rv	ATTGGAAACGGATATGCCCCT TCCTTACCTGAACGCCTGTCA	RT-qPCR (reference tomato)
WAT1_qPCR_Fw WAT1_qPCR_Rv	GGGGGTCCAGTTTTTGTTGC CTCCGATTATCCCGCCCAAG	RT-qPCR (*SlWAT1* expression)
KH_093_Fw KH_093_Rv	caccCGGCCCAACAATTTACAGCCC GAACTAGCCAAGCCTGAGGG	RNAi construct
MA_NPTII_421_Fw MA_NPTII_421_Rv	GAAGGGACTGGCTGCTATT AATATCACGGGTAGCCAAC	RNAi construct NPTII
MA_35S_597_Fw MA_35S_597_Rv	TACAAAGGCGGCAACAAAC AGCAAGCCTTGAATCGTCC	RNAi construct 35S
SlWat1_1_Fw SlWat1_2_Fw SlWat1_3_Fw SlWat1_4_Fw	GTATGGCAGAAGCAAAAGTA CTAGGCTCTCGGTCACGTCG CGGGTACTTCTTGAGTACGG ATATGGTGCATTGACAGAGG	sgRNAs CRISPR
KH_156_Fw KH_156_Rv	CAGGAAAGACAGGCCACAACT CCTAACGCGAAGGAAGCCAT	genotyping
SlRub_QPCR_F SlRub_QPCR_R	GAACAGTTTCTCACTGTTGAC CGTGAGAACCATAAGTCACC	Tomato *rubisco* gene
Vd-ITS-Fw Vd-ITS-Rv	AAAGTTTTAATGGTTCGCTAAGA CTTGGTCATTTAGAGGAAGTAA	*V. dahliae* biomass

Supplementary Table 2 | Overview of primary transformants (T1) with presence/absence of silencing construct (NPTII/35S), relative *SlWAT1* expression normalized to control plants at 1 ($2^{-\Delta\Delta Ct}$) and plants from which T2 seeds were obtained.

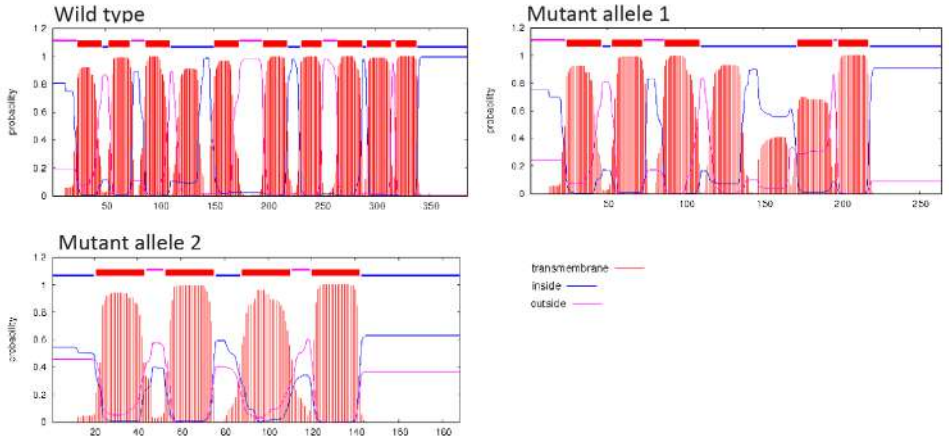
Plant	NPTII/35S	$2^{-\Delta\Delta Ct}$	Seed production
#93-1	-	0.61	NA
#93-2	+	0.11	Yes (no fruits)
#93-3	-	0.54	NA
#93-4	-	0.98	NA
#93-5	+	1.35	NA
#93-6	+	0.59	NA
#93-7	-	2.70	NA
#93-8	+	1.65	NA
#93-9	+	2.04	NA
#93-10	-	0.87	NA
#93-11	+	0.47	Yes (TV181034)
#93-12	-	0.62	NA
#93-13	+	0.29	Yes (no fruits)
#93-14	+	0.46	Yes (TV181036)
#93-15	-	1.01	NA
#93-16	NA	0.23	Yes (TV181037)



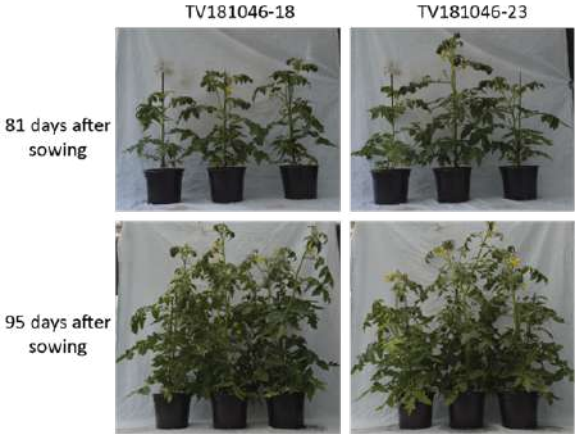
Supplementary Figure 1 | Knock-out and knock-down constructs generated to study *SlWAT1*. (A) Schematic overview of *SlWAT1* (Solyc04g080940) with intron/exon locations as well as primers, RNAi fragment and sgRNAs designed in this study. (B) pHellsgate8 construct for silencing of *SlWAT1*. (C) CRISPR-Cas9 level 2 plasmid for knock-out of *SlWAT1*. Plasmid maps were generated using SnapGene.



Supplementary Figure 2 | Gel electrophoresis (1% TBE, Gelred, image colours inverted) of gene-specific PCR on primary transformants (T1) of plants transformed with *SlWAT1* CRISPR-Cas9 construct. Wild type PCR product (1,616 bp) indicated with an arrow and highlighted are mutants #10, #19, #23 and #28 that were transferred to the greenhouse, and of which seeds were only obtained from #19 (TV181046).



Supplementary Figure 3 | Predicted transmembrane domains for wild type *SlWAT1* (left), mutant allele 1 (right) and mutant allele 2 (bottom). Graphs were generated with TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).



Supplementary Figure 4 | Further development of the CRISPR *WAT1* families until flowering and first fruit set.

Chapter 7

General discussion

Agriculture plays an essential role in human civilization, and one of the greatest global challenges of our time concerns food security. The world population is expected to increase to approximately 11 billion people by 2100 (United Nations, DESA, Population division, 2019) and hence crop production plays a key role in food security. Crop production is influenced by many abiotic and biotic factors and yield losses caused by pests and pathogens are a major threat to food security. In order to minimize crop losses due to pests and pathogens, as well as to reduce the use of environmentally hazardous substances that are used for crop protection, breeding for resistance has become a cornerstone of sustainable agriculture.

The core strategy for resistance breeding is the identification of resistance sources and the subsequent introgression into cultivated crops. Studies on plant immunity focus on the molecular understanding of disease resistance, which contributes to resistance breeding with novel tools and strategies. Plant immunity monitors invasions by pathogens and subsequently mounts appropriate defence responses. It has been described, for instance, in the invasion model (Cook *et al*., 2015) in which a pathogen-derived signal, or a so-called invasion pattern (IP), is detected by an IP receptor (IPR) of the host leading to an IP-triggered response (IPTR). Depending on whether the pathogen can suppress IPTR or not, the interaction between host and pathogen is either continued, leading to plant susceptibility, or stopped, resulting in plant resistance, respectively. A subgroup of IPRs is resistance (R) proteins encoded by dominant R genes. The detection of an IP by an R protein results in the activation of plant immunity leading to resistance. In resistance breeding, introgression of *R* genes from wild germplasm into cultivars have been a core practice. However, in many cases *R* gene-mediated resistance is based on a very specific interaction between the host and the pathogen and therefore fast evolving pathogen populations can compromise *R* gene-mediated resistance, as these can break the resistance by evolving strains that are no longer recognized and contained.

In parallel to the identification of *R* genes, a novel breeding strategy focuses on resistance mediated by impaired susceptibility (*S*) genes. Any host factor that facilitates a compatible interaction between the host and the pathogen can be referred to as susceptibility (*S*) gene (Pavan *et al*., 2010; Gawehns *et al*., 2013; Hückelhoven *et al*., 2013; van Schie and Takken, 2014). Accordingly, *S* genes require impairment in order to achieve loss of host susceptibility. Impaired *S* genes alleles may occur naturally, for example as loss-of-function mutations or as promoter mutations which result in impaired expression (Chu *et al*., 2006; Bai *et al*., 2008; Gao *et al*., 2015). Alternatively, random mutagenesis or genome editing can be used to impair *S* genes (Zaidi *et al*., 2018; Dong and Ronald, 2019).

DIFFERENT ROADS LEAD TO ROME – THE IDENTIFICATION OF *S* GENES IN CROPS

Research on *S* genes has mostly been conducted in the model plant Arabidopsis (Chapter 1 of this thesis). However, in order to be able to use impaired *S* genes in breeding, either the knowledge needs to be translated from Arabidopsis to crops, or studies need to be directly conducted in crop species to identify (novel) *S* genes. Hence, it is essential to identify and functionally characterize *S* genes both in Arabidopsis and in crop species. There are several approaches to identify *S* genes (Figure 1), and it is worthwhile pursuing multiple of these in parallel. These approaches can be grouped into forward and reverse genetics approaches and are discussed in the following sections.

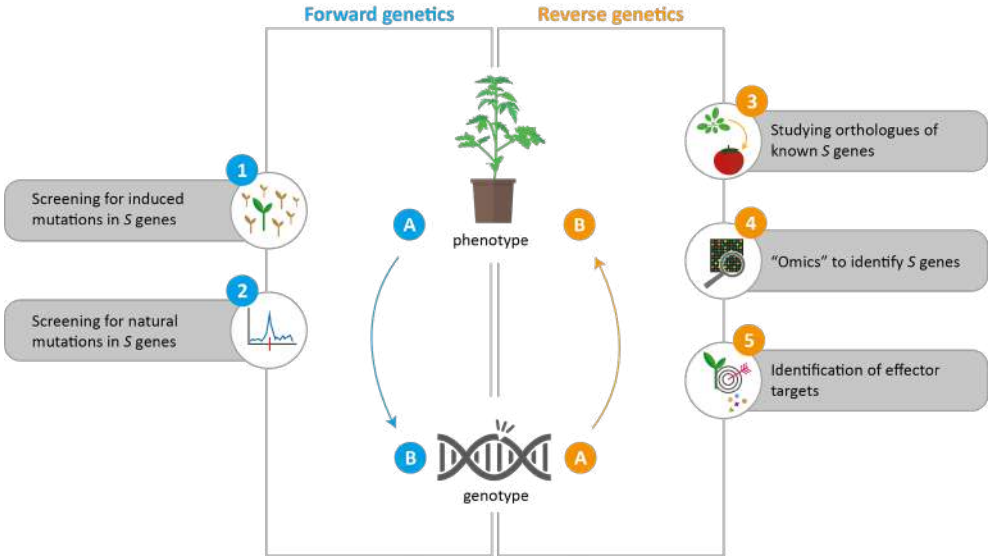


Figure 1 | Overview of five strategies to identify susceptibility (*S*) genes. Forward genetics (blue) is based on finding the desired phenotype (A) and identifying the underlying genetics (B) such as a mutation in an *S* gene. This can be done by screening for induced mutations in *S* genes (strategy 1) and by screening for natural mutations is *S* genes (strategy 2). In reverse genetics (orange), a certain genotype is investigated (A) to find out the corresponding phenotype (B) such as loss of susceptibility to a certain pathogen. This can be done by studying orthologues of known *S* genes in other plant species (strategy 3), by using "Omics" (strategy 4) and by identifying effector targets (strategy 5). All strategies are discussed in the following sections.

Much of a muchness – Studying *S* gene orthologues

Due to the large number of studies conducted on *S* genes in Arabidopsis, one can make use of this knowledge to study *S* genes in crop species. Such model-to-crop translations have proven successful in other areas. For example, the knowledge obtained on fruit opening in Arabidopsis was used to study seed dispersal in oilseed rape (Stephenson *et al*., 2019). Oilseed rape mutants in the orthologue of the Arabidopsis *INDEHISCENT* (*IND*) gene involved in valve margin formation, were found to be resistant to pod

shattering which ultimately reduces yield loss. Model-to-crop translations can also be used for *S* genes and several cases are known in which the role of *S* genes in disease susceptibility was conserved across plant species (van Schie and Takken, 2014). For example, a mutation in the *powdery mildew resistant 4* (*pmr4*) gene in Arabidopsis was found to enhance resistance to powdery mildew (Vogel and Somerville, 2000). In tomato, the corresponding orthologue was identified and both silencing and targeted deletion of this orthologue resulted in resistance to powdery mildew (Huibers *et al*., 2013; Santillán Martínez *et al*., 2020). Similarly, impairment of *Walls Are Thin 1* (*WAT1*) enhanced resistance to *Verticillium dahliae* in Arabidopsis (Denancé *et al*., 2013), cotton (Tang *et al*., 2019) and tomato (Chapter 6 of this thesis). As *WAT1* was identified to play a role in susceptibility to multiple fungal and bacterial vascular pathogens in Arabidopsis (Denancé *et al*., 2013), the generated tomato *WAT1* mutants were also challenged with *V. albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) (Chapter 6 of this thesis). The *WAT1* mutants were indeed also resistant to these two pathogens indicating that *S* genes can provide resistance to multiple pathogens and that the *WAT1* mutants should be tested with additional pathogens. Moreover, it was also shown that some *S* genes play a role in susceptibility even to unrelated pathogens. In a study on *S* genes from tomato and potato it was found that silencing of *defence no death 1* (*dnd1*) resulted in enhanced resistance to powdery mildew and late blight (Sun *et al*., 2016a), while the Arabidopsis mutant was originally found to be resistant to bacterial speck *Pseudomonas syringae* (Clough *et al*., 2000).

Seek and you shall find – Using "Omics" for candidate selection

The identification of *S* genes can also be facilitated by using "omics", such as transcriptomics, proteomics and metabolomics (Cabrera *et al*., 2015). For example, transcriptomic profiling can give insights into transcriptional changes in the host upon pathogen challenge (Wise *et al*., 2007). It is known that many *S* genes are induced upon pathogen infection, for example, the Arabidopsis *Downy Mildew Resistant 6* (*DMR6*) gene is induced upon infection with *Hyaloperonospora parasitica* (van Damme *et al*., 2008) and also the *mildew locus O* (*mlo*) gene is induced in barley upon infection with *Blumeria graminis* f. sp. *hordei* (Piffanelli *et al*., 2002). Hence the selection of induced genes is frequently used to identify *S* genes. By this means, a WRKY transcription factor was identified that was induced in the interaction between pepper and *Xanthomonas axonopodis* (Oh *et al*., 2008). This gene, *CaWRKY1*, was further found to be a negative regulator of immunity and transient silencing resulted in reduced bacterial titres. A transcriptomics approach was also used to select *mlo* candidates in cucumber after a genome-wide identification of *Mlo* genes (Schouten *et al*., 2014).

The mechanism by which the induction of *S* genes upon pathogen infection leads to loss of susceptibility can be linked to their role in plant immunity or pathogen

sustenance. For example, *S* genes can be negative regulators of plant immunity and hence their induction increases susceptibility by suppressing immune responses. Plant hormones such as salicylic acid (SA) play a key role in immune responses to biotic stress and the expression of SA is associated with activation of plant immunity (Zhang and Li, 2019). Therefore, genes involved in negative regulation of SA are potential *S* genes as the induction of these genes suppresses immune responses. The impairment of the Arabidopsis *S* genes *dmr6* and *pmr4* is accompanied with increased levels of the SA marker gene *pathogenesis-related 1* (*PR-1*) and with enhanced resistance (Vogel and Somerville, 2000; van Damme *et al*., 2008). Another example of a negative regulator of plant immunity is the potato NPH3/RPT2-LIKE1 (NRL1) protein, which is a direct target of the *Phytophthora infestans* effector Pi02860 (Yang *et al*., 2016). Pi02860 was shown to suppress cell death mediated by INF1, an IP from *P. infestans*. Silencing of *NRL1* in *Nicotiana benthamiana* reduced *P. infestans* colonization and accelerated cell death mediated by INF1. Besides a role in regulation of plant immunity, the induction of *S* genes upon pathogen challenge can also be directly beneficial for pathogen sustenance. The *Sugars Will Eventually be Exported Transporter* (*SWEET*) genes in rice are well-studied *S* genes in the interaction with *X. oryzae*. These sugar transporters are thought to supply carbohydrates to *X. oryzae* facilitating pathogen sustenance and susceptibility (Chen, 2014).

Apart from the induction of *S* genes in host – microbe interaction, the expression of *S* genes can also be down regulated upon pathogen infection. For example, the expression of *WAT1* is reduced after infection with *Ralstonia solanacearum* (Denancé *et al*., 2013). Such a down regulation of *S* genes upon pathogen challenge could have two reasons. Firstly, considering that *S* genes aid the infection by a pathogen, the downregulation by the host could limit the beneficial effect for the pathogen. As *S* genes also have a function for the host itself, downregulation provides an alternative to a complete loss of the *S* gene as this could be determinantal for the host. Secondly, the down regulation could be actively induced by the pathogen if the *S* gene acts as positive regulators of immunity (Pavan *et al*., 2010). In this scenario the down regulation by the pathogen is required to suppress activation of plant immunity. One example, is the HopAI1 effector from *P. syringae* which interferes with mitogen-activated protein kinases (MAPKs) to suppress activation of immunity in Arabidopsis (Zhang *et al*., 2007). Interestingly the same MAPKs, *MAPK3* and *MAPK6*, were also found to be involved in susceptibility to *V. dahliae* in Arabidopsis (Gkizi *et al*., 2016). Fungal biomass quantification revealed reduced *V. dahliae* biomass in *mapk3* and *mapk6* mutants. Nevertheless, the impairment of positive immune regulators is difficult as these are involved in many immune responses and impairment could influence a plethora of other processes. One possibility could be the use of genome editing to modify the gene in such a way that only the interaction by the pathogen is abolished.

In chapter 3 of this thesis, transcriptional profiling was used to select potential *S* gene candidates in the interaction between *V. dahliae* and tomato. Specifically, only those genes were selected that were induced in the compatible interaction. This was done to exclude genes involved in general defence responses. Alternatively, those genes that are induced in both interactions could also be investigated. Even though the pathogen cannot proliferate in the incompatible interaction, *S* genes might be induced independently at the onset of pathogen invasion. In fact, the aforementioned *S* genes *AtDMR6* and *CaWRKY1* are induced in both a compatible and incompatible interaction (Oh *et al*., 2008; van Damme *et al*., 2008).

Aside from transcriptome analysis, pathogen-specific changes in the host can also be explored using proteomics (Jayaraman *et al*., 2012). Proteomics studies might be conducted in a particular tissue related to the pathogen's niche colonization. For example, the soil-borne pathogen *V. dahliae* infects via the roots and colonizes the host's vasculature and hence the proteome of these tissues can give insights into the infection process. A study conducted on the root proteome of *V. dahliae*-inoculated tomato roots identified different host proteins that are abundant at early or late stages of infection (Witzel *et al*., 2017). Potentially, some of these proteins could play a role in disease susceptibility to *V. dahliae*. Similar as mentioned for the transcriptomics approach, differences or similarities in the proteome between a compatible and an incompatible interaction could be used to select *S* gene candidates. Moreover, host proteins involved in susceptibility do also not necessarily need to be more abundant upon pathogen challenge. Amongst many highly abundant proteins in the xylem sap of tomato stems inoculated with *Fol*, a protein was found with a strong decrease in abundance (Rep *et al*., 2002, 2003). This candidate, *XSP10*, encodes a lipid transfer protein and is constitutively expressed in roots of mock-and *Fol*-inoculated plants. A decrease in XSP10 protein levels occurs upon *Fol* inoculation. Silencing of *XSP10* resulted in reduced *Fol* symptom development and therefore highlights the importance of *XSP10* in susceptibility to *Fol* (Krasikov *et al*., 2011). Therefore, proteomic changes in a given plant – pathogen interaction can be used to search for *S* genes.

Know the enemy's strategy to win – Exploring effector targets

Pathogens secrete effectors to influence a wide variety of interactions, including with the host, but also with other microbes (Rovenich *et al*., 2014). Pathogen effectors may target and manipulate host genes to establish disease and hence effector targets can also represent *S* genes (Gawehns *et al*., 2013). As *S* genes are not exclusively effector targets, this strategy focuses on cases in which the effector targets a host component. This, however, also implies that other aforementioned *S* genes could also be effector targets for which the effector is not yet described. The identification of effector targets

can, for example, be done with yeast two-hybrid assays and may also be facilitated by "omics" data. One example of an effector target from the interaction between wheat and *Puccinia striiformis* f. sp. *tritici* (*Pst*) is a putative component of the cytochrome b6-f complex TaISP (Xu *et al*., 2019). This host protein was found to interact with the *Pst* effector Pst_12806 in a yeast two-hybrid assay and silencing of *TaISP* enhanced resistance to *Pst*. Another example is the nuclear-localized auxiliary spliceosome protein AtSMU2, which was found to interact with the cyst nematode effector 30D08 (Verma *et al*., 2018). A T-DNA insertion line of *AtSMU2* showed enhanced resistance to cyst nematodes.

Exploring effector targets as potential *S* genes depends on the function of the effector in plant susceptibility, but also on its role for the pathogen. Firstly, if the effector is a pathogenicity factor or a major virulence gene for the pathogen, the impairment of its target can severely influence its ability to infect the plant. In the above-mentioned example, the effector Pst_12806 was silenced in *Pst* using host-induced gene silencing to further characterize its role in the interaction between *Pst* and wheat. As the results showed significantly reduced fungal growth, Pst_12806 was concluded to be required for virulence of *Pst* on wheat (Xu *et al*., 2019). This highlights that impairing those host genes which are targeted by effectors with a major role in pathogenicity, can lead to enhanced resistance. Secondly, besides being involved in direct host manipulation, not all pathogen effectors target the host directly. The selection of an effector to identify the corresponding effector target requires insights into the process a given effector is involved in. For example, effectors containing lysin motifs (LysMs), referred to as LysM effectors, were shown to perturb chitin-induced immunity by binding chitin through intermolecular LysM dimerization which protects fungal cell walls against host chitinases (Kombrink and Thomma, 2013; Sánchez-Vallet *et al*., 2020; Tian *et al*., 2020). Another example is the *V. dahliae* effector Avirulence on Ve1 tomato (Ave1). This effector was shown to manipulate the microbiome by targeting antagonistic bacteria in order to promote host susceptibility (Snelders *et al*., 2020). Hence, even though these effectors are involved in plant susceptibility, they do not target host components and are therefore not suitable cases to identify *S* genes. In contrast, recently identified effectors of *V. dahliae* could be used to identify effector targets. Comparative genomics revealed effectors that contribute to the establishment of *V. dahliae* infections on cotton, tomato and sunflower (Li, 2019). Moreover, all these effectors were shown to play a role in pathogenicity or virulence of *V. dahliae*. One example is an effector which causes severe defoliation (D) on cotton and olive, namely the D effector. It was further shown that the D effector mediates pathogenicity also on Arabidopsis and *Nicotiana benthamiana*, which indicates a potentially conserved target in different plant species. Even though neither the host target(s) nor the function of the D effector is yet unrevealed, it was speculated that it involves abscisic acid and ethylene homeostasis (Wiese and Devay,

1970; Li, 2019). This indicates the involvement of a possible host target, which in turn highlights that the D effector represents an interesting candidate to search for the host target. Impairment of this target could lead to loss of susceptibility to *V. dahliae*.

Depending on the reverse genetics approach chosen for the selection of *S* gene candidates, different strategies for functional characterization can be adopted. For example, a transient assay such as virus-induced gene silencing (VIGS) can be used to rapidly screen candidate genes (Senthil-Kumar and Mysore, 2014). However, in tomato VIGS is known to be patchy throughout the plant and to be prone to environmental influences (Liu *et al*., 2002; Lu *et al*., 2003; Orzaez *et al*., 2009). To circumvent these issues, stable transformation with CRISPR-Cas9 or RNA interference (RNAi) can be used as an alternative. Genome editing can be utilized to introduce deletions or single nucleotide polymorphisms in the coding sequence or the promoter region of the S gene candidates. For high throughput assays to screen for *S* gene candidates, it is possible to design CRISPR-Cas9 constructs which target multiple genes simultaneously. Moreover, *S* gene candidates can also be functionally characterized using RNAi. Silencing might circumvent potential pleiotropy accompanied by targeted knock-outs as the residual expression of the target could still be sufficient to fulfil the function for the host. Alternatively, it is also possible to explore natural variation in different genotypes or cultivars to find genotypes with a natural mutation in an *S* gene. This approach was used to find various resistance alleles in rice against *X. oryzae* (Zaka *et al*., 2018). Specifically, the promoters of the effector targets *SWEET13* and *SWEET14* were mined to find mutations that prevent activation by the effector. Several resistance alleles were identified with, for example, a small deletion or a substitution and these variants can be used to breed for resistance to *X. oryzae* in rice.

In this thesis, two of the three above mentioned reverse genetics strategies were followed to identify *S* genes for *V. dahliae* in tomato. The first strategy focused on a model-to-crop translation by studying tomato orthologues of known *S* genes from Arabidopsis and one *S* gene was successfully identified (Figure 1, strategy 3) (Chapter 6 of this thesis). The second reverse genetics strategy used in this thesis was transcriptional profiling and functional characterization using VIGS (Figure 1, strategy 4). Several limiting factors complicated the identification of *S* genes in this approach. Firstly, only one group of genes was selected from the transcriptomic analysis. As discussed above, other groups of genes such as down-regulated genes or genes induced in both a compatible and incompatible interaction are equally valuable groups of genes that can be used for functional characterization. By selecting genes from multiple groups, the chance of finding an *S* gene could be increased. Secondly, the time points and sampled tissues in this transcriptomics approach could be adjusted

to include additional conditions in which *S* genes are relevant. In the transcriptomic data set used in this project, genes in roots and foliage were analysed up to one week post inoculation. A time-course experiment monitoring the colonization of *V. dahliae* in tomato showed the presence of *V. dahliae* in stems by four days post inoculation (Chen *et al*., 2004). As *V. dahliae* infects via the roots and spreads upwards into the stem, host genes differentially expressed in the roots and stems present interesting candidates for *V. dahliae* susceptibility. Genes differentially expressed in the foliage might play a minor role in early stages of infection. To find *S* genes not only involved in early pathogen recognition or negative regulation of immunity, but also in pathogen sustenance, a later time point after inoculation could be included in the analysis. To further optimize this approach, the expression of the selected candidates should be verified by quantitative reverse transcription PCR. To this end, the number of false-positive candidates selected in the transcriptomic analysis can be reduced. A third limiting factor was the combination of VIGS for functional characterization with phenotyping of *V. dahliae*-induced stunting. As discussed above, VIGS is prone to environmental influences and silencing efficiency can vary within and between experiments (Liu *et al*., 2002; Lu *et al*., 2003; Orzaez *et al*., 2009; Senthil-Kumar and Mysore, 2014). Even though VIGS was used in the interaction between *V. dahliae* and tomato before to functionally characterize the *R* gene *Ve1* (Fradin *et al*., 2009), the VIGS results for silencing *S* gene candidates were not as unambiguous as the results of silencing of *Ve1*. The reproducibility between VIGS assays was challenging, especially as screenings were carried out throughout the year with varying seasonal effects. On top of the variation caused by VIGS, inoculations with *V. dahliae* were also seen to be variable and prone to environmental influences. Even though phenotyping of *V. dahliae* symptoms is often based on foliar symptoms such as wilting or yellowing, in our experimental conditions stunting was determined as most reliable phenotyping parameter (Chapter 2 of this thesis). Stunting was based on canopy area differences between mock- and *V. dahliae*-inoculated plants, which is also prone to environmental influences and therefore also caused variations within and between experiments. Collectively, the variation by the VIGS treatment and by *V. dahliae* inoculation certainly complicated the screenings for reduced susceptibility in tomato. A rather labour-intensive alternative is a high-throughput CRISPR-Cas9 knock-out assay. Multiple single guide RNAs targeting different genes could be combined in one construct. In such a way, multiple genes can be targeted in one approach, reducing the number of transformations needed. To target multiple homologues simultaneously it is further possible to use RNAi. Stable transformation with either CRISPR-Cas9 or RNAi could be combined with quantifying fungal biomass instead of phenotyping based on stunting. However, for a first screening to identify candidates, this approach is very time-consuming and labour-intensive.

The needle in the haystack – Forward genetics

A parallel strategy to reverse genetics is the search for *S* genes in forward genetics studies. One such approach is the screening of mutant populations (Figure 1, strategy 1). Mutation breeding is a commonly used strategy to generate genetic variation and typically makes use of X-rays, gamma irradiation or treatment with a mutagen such as ethyl methane sulfonate (EMS) (Holme *et al*., 2019). To identify *S* genes such mutant populations are screened for resistant individuals. Many *S* genes have been identified in this way in Arabidopsis, for example the *pmr* mutants (Vogel and Somerville, 2000) (Chapter 1 of this thesis). Once a resistant individual is found, the underlying mutation needs to be identified which is typically done by crossing and mapping of the resistance. Compared to Arabidopsis, such mutant screenings are challenging when it comes to crops. As thousands of mutants need to be tested for loss of susceptibility, mutant population screenings require large amounts of space as well as straightforward inoculation and phenotyping assays. In tomato, this can be circumvented by using the compact cultivar Microtom, which has been used to identify *S* genes before (Appiano, 2016). Nevertheless, for *V. dahliae* such assays could still be challenging as phenotyping relies on growth differences between mock- and *V. dahliae*-inoculated plants (Chapter 2 of this thesis) and because these differences would be less clear in a genotype that is already stunted. Furthermore, to calculate stunting mock-inoculated plants are needed, however, a mutation population screening is carried out using the M2 generation, in which every plant is genetically unique. Therefore, it is not suitable to screen loss of susceptibility via stunting by using M2 families of an EMS population since an individual M2 plant can only be used once for either mock- or *V. dahliae*-inoculation.

Another forward genetics approach focuses on naturally occurring recessive resistance or natural variation of susceptibility. One such strategy makes use of quantitative trait loci (QTL) mapping which is based on genetic differences between a resistant and susceptible parental line. Several QTLs for resistance against *V. dahliae* have been found in different crops (Bolek *et al*., 2005; Rygulla *et al*., 2008; Wang *et al*., 2008; Zhao *et al*., 2014; Antanaviciute *et al*., 2015; Toppino and Barchi, 2016). However, most of these QTLs were found to be quantitative based on multiple loci. Alternatively, a QTL mapping approach can be combined with a candidate gene approach. The presence of an *S* gene candidate in a known QTL region could be used to further investigate whether the resistance is caused by an impaired *S* gene. This approach was used in a study on powdery mildew resistance in cucumber. A cucumber *Mlo* gene, *CsaMlo8*, was found to co-localize with a known QTL for resistance to powdery mildew. By comparing the *CsaMlo8* alleles from a resistant and susceptible cultivar, it was found that the resistant genotype contained an insertion of a retrotransposable element (Berg *et al*., 2015). This insertion was further shown result in aberrant *CsaMLO8* splicing. For this thesis, the presence of the two candidate *S* genes selected from the transcriptomic analysis in

Chapter 3 was checked in QTL regions identified in a study on resistance to *V. dahliae* in two wild tomato accessions (Vermeulen, 2020). One candidate gene, the glycerol-3-phosphate transporter (GlpT, Solyc03g093140) was found to co-localize with a QTL on chromosome 3. This QTL from the susceptible parent was found to contribute to reduced *V. dahliae* symptom expressions in a cross with *Solanum pimpinellifolium* based on yellowing and in a cross with *Solanum cheesmanii* based on stunting. Even though the resistance in this QTL inherited dominantly, the resistance was further found to be quantitative and most likely based on multiple genes. Therefore, the involvement of an impaired *S* gene in this resistance is possible. In total, this QTL region contained 279 candidate genes and to further investigate the involvement of this candidate, several recombined inbred lines with and without this QTL could be screened with the designed VIGS constructs. As *GlpT* was shown to have potential homologues (Chapter 4 of this thesis), it was also analyzed whether any of the homologues co-localized with this or other identified QTLs on different chromosomes. No overlap was found, indicating that only the *GlpT* gene on chromosome 3 might play a minor role in this resistance.

As QTL mapping is only based on the genetic differences between two parental lines, genome wide association (GWA) mapping might offer an alternative strategy to find variations in susceptibility. To this end wild germplasm can the screened to identify genomic regions involved in susceptibility. In Arabidopsis, such a GWA study found several loci associated with susceptibility to root-knot nematode *Meloidogyne incognita* (Warmerdam *et al*., 2018). The underlying candidate genes can be used to search for loss of susceptibility alleles in other plant species.

DON'T PUT ALL YOUR EGGS IN ONE BASKET – APPLICATION OF IMPAIRED *S* GENES IN BREEDING FOR RESISTANCE

Due to the recessive nature of impaired *S* genes, their integration in a breeding programme is more cumbersome than the introgression of dominant resistance (Pavan *et al*., 2010). For example, in F1 hybrid breeding, dominant resistance needs to be present in one of the two parental lines. In the case of recessive resistance, both parental lines are required to carry the resistance. The use of genome editing could certainly facilitate this process, particularly in polyploid crops. However, current regulations in Europe are restricting its application (Eriksson *et al*., 2020). In order to circumvent genome editing, it is possible to search for naturally occurring impaired *S* genes alleles in wild germplasm. Alternatively, mutations in the candidate *S* genes can also be found using 'Targeting Induced Local Lesions IN Genomes' (TILLING) (Kurowska *et al*., 2011). A study on the role of the *eukaryotic translation initiation factors* (*eIFs*) in susceptibility to potyviruses demonstrated a difference in resistance spectrum between a natural resistance allele and an induced null mutation (Gauffier *et al*., 2016). The natural allele from a wild

tomato species, displayed broad spectrum resistance to potyviruses and was further found to encode a functional *eIF* allele. In contrast, the null mutation isolated from a TILLING population had a premature stop codon leading to a nonfunction protein and was shown to display narrow spectrum resistance. Based on the differences seen in the case of *eIF*, it is therefore suggested to use naturally occurring variants of *S* gene alleles rather than induced mutants (Gauffier *et al*., 2016). It might be possible to mimic the natural mutation using genome editing, but in the present case this would require the editing of up to eight amino acids. Such precise editing could be carried out using base editors which allow the modification of specific nucleotides (Marx, 2018).

The impairment of *S* genes might influence other processes in the host and therefore several other factors need to be evaluated (van Schie and Takken, 2014). One of the most frequent drawbacks of impaired *S* genes is a potential pleiotropic effect. Such pleiotropy can have influences on plant growth or fertility and hence are undesired for breeding. This thesis has shown the severe growth defects of targeted deletion in *SlWAT1*, which was very pronounced in the early stages of development (Chapter 6, this thesis). In contrast, the Arabidopsis *wat1* mutant showed pleiotropic effect at a later stage in development (Ranocha *et al*., 2010). Therefore, pleiotropy needs to be evaluated depending on the candidate gene and the plant species. In other cases, the impairment of *S* genes might affect interactions with other pathogens. For example, silencing of a lipoxygenase gene in rice, *OsHI-LOX*, enhanced resistance to a phloem feeder, while silencing of the same gene increased susceptibility to a chewing herbivore (Zhou *et al*., 2009). These observations were associated with changes in plant hormone levels, which is frequently associated with impairment of *S* genes involved in negative regulation of plant immunity. Such changes in the hormone balance are known to affect interactions between the host and pathogens with different lifestyles (Thomma *et al*., 1998). One example is the *BOTRYTIS-INDUCED KINASE1* (*BIK1*) mutant, which displayed enhanced resistance to the biotrophic fungus *P. syringae*, but at the same time also showed enhanced susceptibility to necrotrophic pathogens (Veronese *et al*., 2006). In the *bik1* mutant it was found that SA was upregulated, which is known to be associated with resistance to biotrophic pathogens. Moreover, due to the antagonistic interaction between SA and the hormones jasmonic acid (JA) and ethylene (ET), it was further shown that the responses mediated by JA and ET were attenuated in the *bik1* mutant. JA and ET are linked to resistance to necrotrophic pathogens.

Finally, the application of impaired *S* genes should also be integrated with for example, *R* gene-mediated resistance. Current developments in plant breeding are moving towards using different forms of resistance simultaneously (Pilet-Nayel *et al*., 2017). Therefore, impaired *S* genes can be used together with *R* genes or quantitative resistance in order to combat diseases.

WHEELS WITHIN WHEELS – ENVIRONMENTAL INFLUENCES ON PLANT SUSEPTIBILITY

Research on plant susceptibility also needs to be explored in a wider context such as the environment. The role of the environment on plants has been acknowledged already many years ago (Populer, 1978), and more recently research is focused on the role of climate extremes and climate change on agriculture (Chappelka and Grulke, 2016; Vogel *et al*., 2019). Research has shown that rising temperatures have an effect on the abundance of soil-borne pathogens on a global scale (Delgado-Baquerizo *et al*., 2020). In the context of climate change, the interaction between abiotic and biotic stress has also become central and evidence on crosstalk between these stress responses accumulates (Bai *et al*., 2018; Bergès *et al*., 2018; Saijo and Loo, 2020). Therefore, studies on plant susceptibility also need to be considered in an environmental context and in relation with multiple stresses.

Major advances are also made in the field of microbiomes and it is known that plant health, and therefore also plant susceptibility, are shaped by the microbiome (Compant *et al*., 2019). A genome-wide association study in Arabidopsis has found several host loci that are involved in microbiome composition (Horton *et al*., 2014). Furthermore, research on the Arabidopsis root microbiome identified a host candidate gene associated with bacterial and fungal richness in the microbiome of different Arabidopsis accessions (Bergelson *et al*., 2019). Interestingly, this gene, a subunit of the SEC 61 protein channel, was also characterized as *S* gene to powdery mildew in barley (Zhang *et al*., 2013). This indicate that impairment of *S* gene could also influence microbiome composition.

References

Abramoff, M. D., Magalhães, P. J. and Ram, S. J. (2004) 'Image Processing with ImageJ', *Biophotonics international*, 11(7), pp. 36–42.

Abuqamar, S., Ajeb, S., Sham, A., Enan, M. R. and Iratni, R. (2013) 'A mutation in the *expansin-like A2* gene enhances resistance to necrotrophic fungi and hypersensitivity to abiotic stress in Arabidopsis thaliana', *Molecular Plant Pathology*, 14(8), pp. 813–827. doi: 10.1111/mpp.12049.

Acevedo-Garcia, J., Spencer, D., Thieron, H., Reinstädler, A., Hammond-Kosack, K., Phillips, A. L. and Panstruga, R. (2017) '*mlo*-based powdery mildew resistance in hexaploid bread wheat generated by a non-transgenic TILLING approach', *Plant Biotechnology Journal*, 15(3), pp. 367–378. doi: 10.1111/pbi.12631.

Acevedo-Garcia, J., Kusch, S. and Panstruga, R. (2014) 'Magical mystery tour: MLO proteins in plant immunity and beyond', *New Phytologist*, 204, pp. 273–281. doi: 10.1111/nph.12889.

Ahman, I., Kim, S. Y. and Zhu, L. H. (2019) 'Plant genes benefitting aphids - Potential for exploitation in resistance breeding', *Frontiers in Plant Science*, 10, pp. 1–14. doi: 10.3389/fpls.2019.01452.

Alexander, L. J. (1962) 'Susceptibility of certain *Verticillium*-resistant tomato varieties to an Ohio isolate of the pathogen', *Phytopathology*, 53, pp. 998–1000.

Andolfo, G., Iovieno, P., Frusciante, L. and Ercolano, M. R. (2016) 'Genome-editing technologies for enhancing plant disease resistance', *Frontiers in Plant Science*, 7, pp. 1–8. doi: 10.3389/fpls.2016.01813.

Antanaviciute, L., Surbanovski, N., Harrison, N., McLeary, K. J., Simpson, D. W., Wilson, F., Sargent, D. J. and Harrison, R. J. (2015) 'Mapping QTL associated with *Verticillium dahliae* resistance in the cultivated strawberry (*Fragaria* X *ananassa*)', *Horticulture Research*, 2, pp. 1–8. doi: 10.1038/hortres.2015.9.

Antoniou, P. P., Markakis, E. A., Tjamos, S. E., Paplomatas, E. J. and Tjamos, E. C. (2008) 'Novel methodologies in screening and selecting olive varieties and root-stocks for resistance to *Verticillium dahliae*', *European Journal of Plant Pathology*, 122(4), pp. 549–560. doi: 10.1007/s10658-008-9323-0.

Appiano, M. (2016) 'Susceptibility pays off: Insights into the *mlo*-based powdery mildew resistance'. Wageningen University. doi: 10.18174/391598.

Asano, T., Masuda, D., Yasuda, M., Nakashita, H., Kudo, T., Kimura, M., Yamaguchi, K. and Nishiuchi, T. (2008) '*AtNFXL1*, an Arabidopsis homologue of the human transcription factor *NF-X1*, functions as a negative regulator of the trichothecene phytotoxin-induced defense response', *Plant Journal*, 53(3), pp. 450–464. doi: 10.1111/j.1365-313X.2007.03353.x.

Atallah, Z. K., Hayes, R. J. and Subbarao, K. V. (2011) 'Fifteen years of Verticillium wilt of lettuce in America's salad bowl: A tale of immigration, subjugation, and abatement', *Plant Disease*, 95(7), pp. 784–792. doi: 10.1094 /PDIS-01-11-0075.

Baergen, K. D., Hewitt, J. D. and Clair, D. A. S. (1993) 'Resistance of tomato genotypes to four isolates of *Verticillium dahliae* race 2', *HortScience*, 28(8), pp. 833–836. doi: 10.21273/HORTSCI.28.8.833 .

Bai, Y., Pavan, S., Zheng, Z., Zappel, N. F., Reinstädler, A., Lotti, C., De Giovanni, C., Ricciardi, L., Lindhout, P., Visser, R., Theres, K., and Panstruga P. (2008) 'Naturally occurring broad-spectrum powdery mildew resistance in a Central American tomato accession is caused by loss of *Mlo* function', *Molecular Plant-Microbe*

Interactions, 21(1), pp. 30–39. doi: 10.1094/MPMI-21-1-0030.

Bai, Y., Sunarti, S., Kissoudis, C., Visser, R. G. F. and van der Linden, C. G. (2018) 'The role of tomato *WRKY* genes in plant responses to combined abiotic and biotic stresses', *Frontiers in Plant Science*, 9, pp. 1–7. doi: 10.3389/fpls.2018.00801.

Bai, Y. and Lindhout, P. (2007) 'Domestication and breeding of tomatoes: What have we gained and what can we gain in the future?', *Annals of Botany*, 100(5), pp. 1085–1094. doi: 10.1093/aob/mcm150.

Bai, Y. and Lindhout, P. (2008) 'New challenges for durable resistance breeding in tomato', *Acta Horticulturae*, 789, pp. 61–73. doi: 10.17660/actahortic.2008.789.8.

Berg, J. a, Appiano, M., Santillán Martínez, M., Hermans, F. W., Vriezen, W. H., Visser, R. G., Bai, Y. and Schouten, H. J. (2015) 'A transposable element insertion in the susceptibility gene *CsaMLO8* results in hypocotyl resistance to powdery mildew in cucumber', *BMC Plant Biology*, 15(1), p. 243. doi: 10.1186/s12870-015-0635-x.

Bergelson, J., Mittelstrass, J. and Horton, M. W. (2019) 'Characterizing both bacteria and fungi improves understanding of the Arabidopsis root microbiome', *Scientific Reports*, 9(1), pp. 1–11. doi: 10.1038/s41598-018-37208-z.

Bergès, S. E., Vile, D., Vazquez-Rovere, C., Blanc, S., Yvon, M., Bédiée, A., Rolland, G., Dauzat, M. and van Munster, M. (2018) 'Interactions between drought and plant genotype change epidemiological traits of Cauliflower mosaic virus', *Frontiers in Plant Science*, 9, pp. 1–11. doi: 10.3389/fpls.2018.00703.

Bharadwaj, D. N. (2016) 'Sustainable agriculture and plant breeding', in Molecular Breeding to Improve Plant Resistance to Abiotic Stresses, pp. 3–34. doi: 10.1007/978-3-319-22518-0_1.

Bletsos, F., Thanassoulopoulos, C. and Roupakias, D. (2003) 'Effect of grafting on growth, yield, and Verticillium wilt of eggplant', *HortScience*, 38(2), pp. 183–186. doi: 10.21273/hortsci.38.2.183.

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A. and Bonas, U. (2009) 'Breaking the code of DNA binding specificity of TAL-type III effectors', *Science*, 11(5959), pp. 1509–1512. doi: 10.1126/science.1178811.

Bogdanove, A. J., Schornack, S. and Lahaye, T. (2010) 'TAL effectors: Finding plant genes for disease and defense', *Current Opinion in Plant Biology*, 13(4), pp. 394–401. doi: 10.1016/j.pbi.2010.04.010.

Bolek, Y., El-Zik, K. M., Pepper, A. E., Bell, A. A., Magill, C. W., Thaxton, P. M. and Reddy, O. U. K. (2005) 'Mapping of Verticillium wilt resistance genes in cotton', *Plant Science*, 168(6), pp. 1581–1590. doi: 10.1016/j.plantsci.2005.02.008.

Borrelli, V. M. G., Brambilla, V., Rogowsky, P., Marocco, A. and Lanubile, A. (2018) 'The enhancement of plant disease resistance using CRISPR/Cas9 technology', *Frontiers in Plant Science*, 9. doi: 10.3389/fpls.2018.01245.

Bortesi, L. and Fischer, R. (2015) 'The CRISPR/Cas9 system for plant genome editing and beyond', *Biotechnology Advances*, 33(1), pp. 41–52. doi: 10.1016/j.biotechadv.2014.12.006.

Boshoven, J. C. (2017) 'Virulence contribution and recognition of homologs of the *Verticillium dahliae* effector Ave1'. Wageningen University. doi: 10.18174/420169.

Bowling, S. A., Clarke, J. D., Liu, Y., Klessig, D. F. and Dong, X. (1997) 'The *cpr5* mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance', *The Plant Cell*, 9, pp. 1573–1584. doi: 10.1105/tpc.9.9.1573.

Buhtz, A., Witzel, K., Strehmel, N., Ziegler, J., Abel, S. and Grosch, R. (2015) 'Perturbations in the primary metabolism of tomato and *Arabidopsis thaliana* plants infected with the soil-borne fungus *Verticillium dahliae*', *PLoS ONE*, 10(9), pp. 1–14. doi: 10.1371/journal.pone.0138242.

Busch, L. V. and Smith, E. (1981) 'Susceptibility of Ontario-grown alfalfa cultivars and certain *Medicago* species to *Verticillium albo-atrum*', *Canadian Journal of Plant Pathology*, 3(3), pp. 169–172. doi: 10.1080/07060668109501938.

Buschgel, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Dalen, R., van der Lee, T., Diergaarde, P., Groenendijk, J., Topsch, S., Vos, P., Salamini, F. and Schulze-Lefert, P. (1997) 'The barley *Mlo* gene: A novel control element of plant pathogen resistance', *Cell*, 88, pp. 695–705. doi: 10.1016/s0092-8674(00)81912-1.

Cabrera, J., Barcala, M., Fenoll, C. and Escobar, C. (2015) 'The power of omics to identify plant susceptibility factors a*nd to study resistance to root-knot nematodes',* Current Issues in Molecular Biology, 19, pp. 53–72.

Cermak, T., Doyle, E. L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J. A., Somia, N. V., Bogdanove, A. J. and Voytas, D. F. (2011) 'Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting', *Nucleic Acids Research*, 39(12), pp. 1–11. doi: 10.1093/nar/gkr218.

Chanda, B., Xia, Y., Mandal, M. K., Yu, K., Sekine, K. T., Gao, Q. M., Selote, D., Hu, Y., Stromberg, A., Navarre, D., Kachroo, A., and Kachroo, P. (2011) 'Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants', *Nature Genetics*, 43(5), pp. 421–429. doi: 10.1038/ng.798.

Chappelka, A. H. and Grulke, N. E. (2016) 'Disruption of the "disease triangle" by chemical and physical environmental change', *Plant Biology*, 18, pp. 5–12. doi: 10.1111/plb.12353.

Chari, R., Yeo, N. C., Chavez, A. and Church, G. M. (2017) 'sgRNA Scorer 2.0 - a species independent model to predict CRISPR/Cas9 activity', *ACS Synthetic Biology*, 19, pp. 902–904. doi: 10.1021/acssynbio.6b00343.

Chen, K., Wang, Y., Zhang, R., Zhang, H. and Gao, C. (2019) 'CRISPR/Cas genome editing and precision plant breeding in agriculture', *Annual Review of Plant Biology*, 70(1), pp. 667–697. doi: 10.1146/annurev-arplant-050718-100049.

Chen, L. Q., Hou, B. H., Lalonde, S., Takanaga, H., Hartung, M. L., Qu, X. Q., Guo, W. J., Kim, J. G., Underwood, W., Chaudhuri, B., Chermak, D., Antony, G., White, F. F., Somerville, S., Mudgett, M. B. and Frommer, W. B. (2010) 'Sugar transporters for intercellular exchange and nutrition of pathogens', *Nature*, 468(7323), pp. 527–532. doi: 10.1038/nature09606.

Chen, L. Q. (2014) 'SWEET sugar transporters for phloem transport and pathogen nutrition', *New Phytologist*, 201(4), pp. 1150–1155. doi: 10.1111/nph.12445.

Chen, P., Lee, B. and Robb, J. (2004) 'Tolerance to a non-host isolate of *Verticillium dahliae* in tomato', *Physiological and Molecular Plant Pathology*, 64(6), pp. 283–291. doi: 10.1016/j.pmpp.2004.10.002.

Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A. J. and Voytas, D. F. (2010) 'Targeting DNA double-strand breaks with TAL effector nucleases', *Genetics*, 186(2), pp. 756–761. doi: 10.1534/genetics.110.120717.

Christiansen, K. M., Gu, Y., Rodibaugh, N. and Innes, R. W. (2011) 'Negative regulation

of defence signalling pathways by the EDR1 protein kinase', *Molecular Plant Pathology*, 12(8), pp. 746–758. doi: 10.1111/j.1364-3703.2011.00708.x.

Chu, Z., Yuan, M., Yao, J., Ge, X., Yuan, B., Xu, C., Li, X., Fu, B., Li, Z., Bennetzen, J. L., Zhang, Q. and Wang, S. (2006) 'Promoter mutations of an essential gene for pollen development result in disease resistance in rice', *Genes and Development*, 20(10), pp. 1250–1255. doi: 10.1101/gad.1416306.

Clough, S. J., Fengler, K. A., Yu, I. C., Lippok, B., Smith, R. K. and Bent, A. F. (2000) 'The Arabidopsis *dnd1* "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel', *Proceedings of the National Academy of Sciences of the United States of America*, 97(16), pp. 9323–9328. doi: 10.1073/pnas.150005697.

Compant, S., Samad, A., Faist, H. and Sessitsch, A. (2019) 'A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application', *Journal of Advanced Research*, 19, pp. 29–37. doi: 10.1016/j.jare.2019.03.004.

Conibear, E. and Davis, N. G. (2010) 'Palmitoylation and depalmitoylation dynamics at a glance', *Journal of Cell Science*, 123(23), pp. 4007–4010. doi: 10.1242/jcs.059287.

Cook, D. E., Mesarich, C. H. and Thomma, B. P. H. J. (2015) 'Understanding plant immunity as a surveillance system to detect invasion', *Annual Review of Phytopathology*, 53(1), pp. 541–563. doi: 10.1146/annurev-phyto-080614-120114.

Corwin, J. A. and Kliebenstein, D. J. (2017) 'Quantitative resistance: More than just perception of a pathogen', *The Plant Cell*, 29(4), pp. 655–665. doi: 10.1105/tpc.16.00915.

van Damme, M., Andel, A., Huibers, R. P., Panstruga, R., Weisbeek, P. J. and van den Ackerveken, G. (2005) 'Identification of Arabidopsis loci required for susceptibility to the downy mildew pathogen *Hyaloperonospora parasitica*', *Molecular Plant-Microbe Interactions*, 18(6), pp. 583–592. doi: 10.1094/MPMI-18-0583.

van Damme, M., Huibers, R. P., Elberse, J. and van den Ackerveken, G. (2008) 'Arabidopsis *DMR6* encodes a putative 2OG-Fe(II) oxygenase that is defense-associated but required for susceptibility to downy mildew', *The Plant Journal*, 54(5), pp. 785–793. doi: 10.1111/j.1365-313X.2008.03427.x.

van Damme, M., Zeilmaker, T., Elberse, J., Andel, A., de Sain-van der Velden, M. and van den Ackerveken, G. (2009) 'Downy mildew resistance in Arabidopsis by mutation of HOMOSERINE KINASE', *The Plant Cell*, 21(7), pp. 2179–2189. doi: 10.1105/tpc.109.066811.

Das, A., Sharma, N. and Prasad, M. (2019) 'CRISPR/Cas9: A novel weapon in the arsenal to combat plant diseases', *Frontiers in Plant Science*, 9, pp. 1–8. doi: 10.3389/fpls.2018.02008.

Delgado-Baquerizo, M., Guerra, C. A., Cano-Díaz, C., Egidi, E., Wang, J. T., Eisenhauer, N., Singh, B. K. and Maestre, F. T. (2020) 'The proportion of soil-borne pathogens increases with warming at the global scale', *Nature Climate Change*, 10. doi: 10.1038/s41558-020-0759-3.

Denancé, N., Ranocha, P., Oria, N., Barlet, X., Rivière, M. P., Yadeta, K. A., Hoffmann, L., Perreau, F., Clément, G., Maia-Grondard, A., van den Berg, G. C. M., Savelli, B., Fournier, S., Aubert, Y., Pelletier, S., Thomma, B. P. H. J., Molina, A., Jouanin, L., Marco, Y. and Goffner, D. (2013) 'Arabidopsis *wat1* (*walls are thin1*)-mediated resistance to the bacterial vascular pathogen, *Ralstonia solanacearum*, is accompanied by cross-regulation of salicylic acid and tryptophan metabolism', *The*

Plant Journal, 73(2), pp. 225–239. doi: 10.1111/tpj.12027.

Depotter, J. R. L., Thomma, B. P. H. J. and Wood, T. A. (2019) 'Measuring the impact of *Verticillium longisporum* on oilseed rape (*Brassica napus*) yield in field trials in the United Kingdom', *European Journal of Plant Pathology*, 153(1), pp. 321–326. doi: 10.1007/s10658-018-1537-1.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.-M. and Gascuel, O. (2008) 'Phylogeny.fr: robust phylogenetic analysis for the non-specialist', *Nucleic Acids Research*, 36, pp. 465–9. doi: 10.1093/nar/gkn180.

Do, P. T., Nguyen, C. X., Bui, H. T., Tran, L. T. N., Stacey, G., Gillman, J. D., Zhang, Z. J. and Stacey, M. G. (2019) 'Demonstration of highly efficient dual gRNA CRISPR/Cas9 editing of the homeologous *GmFAD2-1A* and *GmFAD2-1B* genes to yield a high oleic, low linoleic and α-linolenic acid phenotype in soybean', *BMC Plant Biology*, 19(1), pp. 1–14. doi: 10.1186/s12870-019-1906-8.

Dobinson, K. F., Tenuta, G. K. and Lazarovits, G. (1996) 'Occurrence of race 2 of *Verticillium dahliae* in processing tomato fields in southwestern Ontario', *Canadian Journal of Plant Pathology*, 18(1), pp. 55–58. doi: 10.1080/07060669609500655.

Dong, O. X. and Ronald, P. C. (2019) 'Genetic engineering for disease resistance in plants: Recent progress and future perspectives', *Plant Physiology*, 180(1), pp. 26–38. doi: 10.1104/pp.18.01224.

Doudna, J. A. and Charpentier, E. (2014) 'The new frontier of genome engineering with CRISPR-Cas9', *Science*, 346(6213). doi: 10.1126/science.1258096.

Eckardt, N. A. (2002) 'Plant disease susceptibility genes?', *The Plant Cell*, 14(9), pp. 1983–1986. doi: 10.1105/tpc.140910.

Engelhardt, S., Stam, R. and Hückelhoven, R. (2018) 'Good riddance? Breaking disease susceptibility in the era of new breeding technologies', *Agronomy*, 8(114), pp. 1–16. doi: 10.3390/agronomy8070114.

Engler, C., Kandzia, R. and Marillonnet, S. (2008) 'A one pot, one step, precision cloning method with high throughput capability', *PLoS ONE*, 3(11), pp. 1–7. doi: 10.1371/journal.pone.0003647.

Eriksson, D., Custers, R., Edvardsson Björnberg, K., Hansson, S. O., Purnhagen, K., Qaim, M., Romeis, J., Schiemann, J., Schleissing, S., Tosun, J. and Visser, R. G. F. (2020) 'Options to reform the European Union legislation on GMOs: Scope and definitions', *Trends in Biotechnology*, 38(3), pp. 231–234. doi: 10.1016/j.tibtech.2019.12.002.

van Esse, H. P., Fradin, E. F., de Groot, P. J., de Wit, P. J. G. M. and Thomma, B. P. H. J. (2009) 'Tomato transcriptional responses to a foliar and a vascular fungal pathogen are distinct', *Molecular Plant-Microbe Interactions*, 22(3), pp. 245–258. doi: 10.1094/MPMI-22-3-0245.

van Esse, H., Fradin, E. F., de Groot, P. J., de Wit, P. and Thomma, B. (2009) 'Tomato transcriptional responses to a foliar and a vascular fungal pathogen are distinct', *Molecular Plant-Microbe Interactions*, 22(3), pp. 245–258. doi: 10.1094/mpmi-22-3-0245.

Faino, L., Seidl, M. F., Shi-Kunne, X., Pauper, M., van den Berg, G. C. M., Wittenberg, A. H. and Thomma, B. P. H. J. (2016) 'Transposons passively and actively contribute to evolution of the two-speed genome of a fungal pathogen', *Genome Research*, pp.

1091–1100. doi: 10.1101/gr.204974.116.

Fousia, S., Tsafouros, A., Roussos, P. A. and Tjamos, S. E. (2018) 'Increased resistance to *Verticillium dahliae* in Arabidopsis plants defective in auxin signalling', *Plant Pathology*, 67, pp. 1749–1757. doi: 10.1111/ppa.12881.

Fradin, E. F., Zhang, Z., Juarez Ayala, J. C., Castroverde, C. D. M., Nazar, R. N., Robb, J., Liu, C.-M. and Thomma, B. P. H. J. (2009) 'Genetic dissection of Verticillium wilt resistance mediated by tomato Ve1', *Plant Physiology*, 150(1), pp. 320–332. doi: 10.1104/pp.109.136762.

Fradin, E. F., Abd-El-Haliem, A., Masini, L., van den Berg, G. C. M., Joosten, M. H. A. J. and Thomma, B. P. H. J. (2011) 'Interfamily transfer of tomato *Ve1* mediates Verticillium resistance in Arabidopsis', *Plant Physiology*, 156(4), pp. 2255–2265. doi: 10.1104/pp.111.180067.

Fradin, E. F. and Thomma, B. P. H. J. (2006) 'Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*', Molecular *Plant Pathology*, 7(2), pp. 71–86. doi: 10.1111/j.1364-3703.2006.00323.

Frye, C. A. and Innes, R. W. (1998) 'An Arabidopsis mutant with enhanced resistance to powdery mildew', *The Plant Cell*, 10(6), pp. 947–956. doi: 10.1105/tpc.10.6.947.

Gao, D., Appiano, M., Huibers, R. P., Loonen, A. E. H. M., Visser, R. G. F., Wolters, A. M. A. and Bai, Y. (2015) 'Natural loss-of-function mutation of *EDR1* conferring resistance to tomato powdery mildew in *Arabidopsis thaliana* accession C24', *Molecular Plant Pathology*, 16(1), pp. 71–82. doi: 10.1111/mpp.12165.

Gao, Y., Zitter, T. and Veilleux, R. (2010) 'Verticillium wilt in solanaceous crops', in Plant Breeding Reviews. Hoboken, NJ, USA, pp. 115–144.

Garcia-Ruiz, H. (2018) 'Susceptibility genes to plant viruses', *Viruses*, 10(9), p. 484. doi: 10.3390/v10090484.

Gauffier, C., Lebaron, C., Moretti, A., Constant, C., Moquet, F., Bonnet, G., Caranta, C. and Gallois, J. L. (2016) 'A TILLING approach to generate broad-spectrum resistance to potyviruses in tomato is hampered by *eIF4E* gene redundancy', *The Plant Journal*, 85(6), pp. 717–729. doi: 10.1111/tpj.13136.

Gautier, L., Cope, L., Bolstad, B. M. and Irizarry, R. A. (2004) 'Affy - Analysis of Affymetrix GeneChip data at the probe level', *Bioinformatics*, 20(3), pp. 307–315. doi: 10.1093/bioinformatics/btg405.

Gawehns, F., Cornelissen, B. J. C. and Takken, F. L. W. (2013) 'The potential of effector-target genes in breeding for plant innate immunity', *Microbial Biotechnology*, 6(3), pp. 223–229. doi: 10.1111/1751-7915.12023.

Gibriel, H. A. ., Li, J., Zhu, L., Seidl, M. F. and Thomma, B. P. H. J. (2019) '*Verticillium dahliae* strains that infect the same host plant display highly divergent effector catalogs', *bioRxiv*. doi: 10.4165/kapps1958.25.0_45.

Gkizi, D., Lehmann, S., Haridon, F. L., Serrano, M. and Paplomatas, E. J. (2016) 'The innate immune signalling system as a regulator of disease resistance and induced systemic resistance activity against *Verticillium dahliae*', *Molecular Plant-Microbe Interactions*, 29(4), pp. 313–323. doi: 10.1094/MPMI-11-15-0261-R.

Grogan, R. G. (1979) 'Verticillium wilt on resistant tomato cultivars in California: Virulence of isolates from plants and soil and relationship of inoculum density to disease incidence', *Phytopathology*, 69(11), pp. 1176–1180. doi: 10.1094/phyto-69-1176.

Guo, X. H., Cai, C. P., Yuan, D. D., Zhang, R. S., Xi, J. L. and Guo, W. Z. (2016) 'Development and identification of Verticillium wilt-resistant upland cotton accessions by pyramiding QTL related to resistance', *Journal of Integrative Agriculture*, 15(3), pp. 512–520. doi: 10.1016/S2095-3119(15)61083-8.

Helliwell, C. and Waterhouse, P. (2003) 'Constructs and methods for high-throughput gene silencing in plants', *Methods*, 30(4), pp. 289–295. doi: 10.1016/S1046-2023(03)00036-7.

Hemsley, P. A. (2015) 'The importance of lipid modified proteins in plants', *New Phytologist*, 205(2), pp. 476–489. doi: 10.1111/nph.13085.

Hemsley, P. A. (2017) 'An outlook on protein S-acylation in plants: What are the next steps?', *Journal of Experimental Botany*, 68(12), pp. 3155–3164. doi: 10.1093/jxb/erw497.

Holme, I. B., Gregersen, P. L. and Brinch-Pedersen, H. (2019) 'Induced genetic variation in crop plants by random or targeted mutagenesis: Convergence and differences', *Frontiers in Plant Science*, 10, pp. 1–9. doi: 10.3389/fpls.2019.01468.

Horton, M. W., Bodenhausen, N., Beilsmith, K., Meng, D., Muegge, B. D., Subramanian, S., Vetter, M. M., Vilhjálmsson, B. J., Nordborg, M., Gordon, J. I. and Bergelson, J. (2014) 'Genome-wide association study of *Arabidopsis thaliana* leaf microbial community', *Nature Communications*, 5, pp. 1–7. doi: 10.1038/ncomms6320.

Huang, S., Gao, Y., Liu, J., Peng, X., Niu, X., Fei, Z., Cao, S. and Liu, Y. (2012) 'Genome-wide analysis of WRKY transcription factors in *Solanum lycopersicum*', *Molecular Genetics and Genomics*, 287(6), pp. 495–513. doi: 10.1007/s00438-012-0696-6.

Huang, T. S., Wei, T., Laliberté, J. F. and Wang, A. (2010) 'A host RNA helicase-like protein, AtRH8, interacts with the potyviral genome-linked protein, VPg, associates with the virus accumulation complex, and is essential for infection', *Plant Physiology*, 152(1), pp. 255–266. doi: 10.1104/pp.109.147983.

Hückelhoven, R., Eichmann, R., Weis, C., Hoefle, C. and Proels, R. K. (2013) 'Genetic loss of susceptibility: A costly route to disease resistance?', *Plant Pathology*, 62, pp. 56–62. doi: 10.1111/ppa.12103.

Huibers, R. P., Loonen, A. E. H. M., Gao, D., van den Ackerveken, G., Visser, R. G. F. and Bai, Y. (2013) 'Powdery mildew resistance in tomato by impairment of *SlPMR4* and *SlDMR1*', *PLoS ONE*, 8(6), pp. 2–9. doi: 10.1371/journal.pone.0067467.

Humphry, M., Reinstädler, A., Ivanov, S., Bisseling, T. and Panstruga, R. (2011) 'Durable broad-spectrum powdery mildew resistance in pea *er1* plants is conferred by natural loss-of-function mutations in *PsMLO1*', Molecular *Plant Pathology*, 12(9), pp. 866–878. doi: 10.1111/j.1364-3703.2011.00718.

Hurst, C. H. and Hemsley, P. A. (2015) 'Current perspective on protein S-acylation in plants: More than just a fatty anchor?', *Journal of Experimental Botany*, 66(6), pp. 1599–1606. doi: 10.1093/jxb/erv053.

Ibiza, V. P., Cañizares, J. and Nuez, F. (2010) 'EcoTILLING in *Capsicum* species: Searching for new virus resistances', *BMC Genomics*, 11(1), pp. 1–15. doi: 10.1186/1471-2164-11-631.

Inderbitzin, P., Bostock, R. M., Davis, R. M., Usami, T., Platt, H. W. and Subbarao, K. V. (2011) 'Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species', *PLoS ONE*, 6(12). doi: 10.1371/journal.pone.0028341.

Inderbitzin, P., Thomma, B. P. H. J., Klosterman, S. J. and Subbarao, K. V. (2014) '*Verticillium alfalfae* and *V. dahliae*, Agents of Verticillium Wilt Diseases', in Genomics of Plant-Associated Fungi and Oomycetes: Dicot Pathogens. Springer, Berlin, Heidelberg, pp. 65–97.

Iyer, A. S. and McCouch, S. R. (2004) 'The rice bacterial blight resistance gene *xa5* encodes a novel form of disease resistance', *Molecular Plant-Microbe Interactions*, 17(12), pp. 1348–1354. doi: 10.1094/MPMI.2004.17.12.1348.

Jablonska, B., Ammiraju, J. S. S., Bhattarai, K. K., Mantelin, S., De Ilarduya, O. M., Roberts, P. A. and Kaloshian, I. (2007) 'The *Mi-9* gene from *Solanum arcanum* conferring heat-stable resistance to root-knot nematodes is a homolog of *Mi-1*', *Plant Physiology*, 143(2), pp. 1044–1054. doi: 10.1104/pp.106.089615.

Jack, D. L., Yang, N. M. and Saier, M. H. (2001) 'The drug/metabolite transporter superfamily', *European Journal of Biochemistry*, 268(13), pp. 3620–3639. doi: 10.1046/j.1432-1327.2001.02265.x.

Jayaraman, D., Forshey, K. L., Grimsrud, P. A. and Ané, J. M. (2012) 'Leveraging proteomics to understand plant-microbe interactions', *Frontiers in Plant Science*, 3, pp. 1–6. doi: 10.3389/fpls.2012.00044.

Jiang, C. J., Shimono, M., Maeda, S., Inoue, H., Mori, M., Hasegawa, M., Sugano, S. and Takatsuji, H. (2009) 'Suppression of the rice fatty-acid desaturase gene *OsSSI2* enhances resistance to blast and leaf blight diseases in rice', *Molecular Plant-Microbe Interactions*, 22(7), pp. 820–829. doi: 10.1094/MPMI-22-7-0820.

Jiménez-Díaz, R. M., Olivares-García, C., Trapero-Casas, J. L., Jiménez-Gasco, M. M., Navas-Cortés, J. A., Landa, B. B. and Milgroom, M. G. (2017) 'Variation of pathotypes and races and their correlations with clonal lineages in *Verticillium dahliae*', *Plant Pathology*, 66(4), pp. 651–666. doi: 10.1111/ppa.12611.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A. and Charpentier, E. (2012) 'A programmable dual-RNA– guided DNA endonuclease in adaptive bacterial immunity', *Science*, 337, pp. 816–822. doi: 10.1126/science.1225829.

Jones, J. B., Zitte, T. A., Momol, T. M. and Miller, S. A. (2014) 'Compendium of tomato diseases', *American Phytopathological Society*. doi: 10.1094/9780890544341

de Jonge, R., van Esse, P. H., Maruthachalam, K., Bolton, M. D., Santhanam, P., Saber, M. K., Zhang, Z., Usami, T., Lievens, B., Subbarao, K. V. and Thomma, B. P. H. J. (2012) 'Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing', *Proceedings of the National Academy of Sciences of the United States of America*, 109(13), pp. 5110–5115. doi: 10.1073/pnas.1119623109.

Joosten, M. H. A. J., Cozijnsen, T. J. and de Wit (1994) 'Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene', *Nature*, 367, pp. 384–386. doi: 10.1016/0168-9525(94)90208-9.

Jørgensen, J. H. (1992) 'Discovery, characterization and exploitation of *Mlo* powdery mildew resistance in barley', *Euphytica*, 63(1–2), pp. 141–152.

Jørgensen, J. H. (1977) 'Spectrum of resistance conferred by *ML-O* powdery mildew resistance genes in barley', *Euphytica*, 26(1), pp. 55–62. doi: 10.1007/BF00032068.

Kachroo, A., Fu, D. Q., Havens, W., Navarre, D. R., Kachroo, P. and Ghabrial, S. A. (2008) 'An oleic acid-mediated pathway induces constitutive defense signalling and enhanced resistance to multiple pathogens in soybean', *Molecular Plant-Microbe*

Interactions, 21(5), pp. 564–575. doi: 10.1094/MPMI-21-5-0564.

Karkute, S. G., Gujjar, R. S., Rai, A., Akhtar, M., Singh, M. and Singh, B. (2018) 'Genome wide expression analysis of *WRKY* genes in tomato (*Solanum lycopersicum*) under drought stress', *Plant Gene*, 13, pp. 8–17. doi: 10.1016/j.plgene.2017.11.002.

Kawai, H., Ishikawa, T., Mitsui, T., Kore-Eda, S., Yamada-Kawai, M. and Ohnishi, J. I. (2014) 'Arabidopsis glycerol-3-phosphate permease 4 is localized in the plastids and involved in the accumulation of seed oil', *Plant Biotechnology*, 31(2), pp. 159–165. doi: 10.5511/plantbiotechnology.14.0222a.

Kawchuk, L. M., Hachey, J., Lynch, D. R., Kulcsar, F., van Rooijen, G., Waterer, D. R., Robertson, A., Kokko, E., Byers, R., Howard, R. J., Fischer, R. and Prüfer, D. (2001) 'Tomato *Ve* disease resistance genes encode cell surface-like receptors', *Proceedings of the National Academy of Sciences of the United States of America*, 98(11), pp. 6511–5. doi: 10.1073/pnas.091114198.

Kim, Y. G., Cha, J. and Chandrasegaran, S. (1996) 'Hybrid restriction enzymes: Zinc finger fusions to *Fok* I cleavage domain', *Proceedings of the National Academy of Sciences of the United States of America*, 93(3), pp. 1156–1160. doi: 10.1073/pnas.93.3.1156.

Klosterman, S. J., Atallah, Z. K., Vallad, G. E. and Subbarao, K. V. (2009) 'Diversity, pathogenicity, and management of Verticillium species', *Annual Review of Phytopathology*, 47(1), pp. 39–62. doi: 10.1146/annurev-phyto-080508-081748.

Klosterman, Steven J., Subbarao, K. V., Kang, S., Veronese, P., Gold, S. E., Thomma, B. P. H. J., Chen, Z., Henrissat, B., Lee, Y. H., Park, J., Garcia-Pedrajas, M. D., Barbara, D. J., Anchieta, A., de Jonge, R., Santhanam, P., Maruthachalam, K., Atallah, Z., Amyotte, S. G., Paz, Z., Inderbitzin, P., Hayes, R. J., Heiman, D. I., Young, S., Zeng, Q., Engels, R., Galagan, J., Cuomo, C. A., Dobinson, K. F. and Ma, L.-J. (2011) 'Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens', *PLoS Pathogens*, 7(7). doi: 10.1371/journal.ppat.1002137.

Kombrink, A. and Thomma, B. P. H. J. (2013) 'LysM Effectors: Secreted proteins supporting fungal life', *PLoS Pathogens*, 9(12), pp. 1–4. doi: 10.1371/journal.ppat.1003769.

Korver, R. A., Koevoets, I. T. and Testerink, C. (2018) 'Out of shape during stress: A key role for auxin', *Trends in Plant Science*, 23(9), pp. 783–793. doi: 10.1016/j.tplants.2018.05.011.

Krasikov, V., Dekker, H. L., Rep, M. and Takken, F. L. W. (2011) 'The tomato xylem sap protein XSP10 is required for full susceptibility to Fusarium wilt disease', *Journal of Experimental Botany*, 62(3), pp. 963–973. doi: 10.1093/jxb/erq327.

Kurowska, M., Daszkowska-Golec, A., Gruszka, D., Marzec, M., Szurman, M., Szarejko, I. and Maluszynski, M. (2011) 'TILLING - a shortcut in functional genomics', *Journal of Applied Genetics*, 52(4), pp. 371–390. doi: 10.1007/s13353-011-0061-1.

Kursteiner, O., Dupuis, I. and Kuhlemeier, C. (2003) 'The *pyruvate decarboxylase 1* gene of Arabidopsis is required during anoxia but not other environmental stresses', *Plant Physiology*, 132(2), pp. 968–978. doi: 10.1104/pp.102.016907.

Labate, J. A. et al. (2007) 'Chapter 1 - Tomato', in Genome Mapping and Molecular Breeding in Plants. doi: 10.1201/9780203743539.

L**amichhane, J. R., Dachbrodt-Saaydeh, S., Kudsk, P. and Messéan, A.** (2016) 'Conventional pesticides in agriculture: Benefits versus risks', *Plant Disease*, 100(1),

pp. 10–24. doi: 10.1094/PDIS-05-15-0574-FE.

Langner, T., Kamoun, S. and Belhaj, K. (2018) 'CRISPR crops: Plant genome editing toward disease resistance', *Annual Review of Phytopathology*, 56(1). doi: 10.1146/annurev-phyto-080417-050158.

Laterrot, H. (1984) 'Specific resistance of *Verticillium dahliae* race 2 in tomato', *Tomato Genetics Cooperative*, Rpt. 34, pp. 10–11.

Law, C. J., Enkavi, G., Wang, D. N. and Tajkhorshid, E. (2009) 'Structural basis of substrate selectivity in the glycerol-3-phosphate: Phosphate antiporter GlpT', *Biophysical Journal*, 97(5), pp. 1346–1353. doi: 10.1016/j.bpj.2009.06.026.

Lemieux, M. J., Huang, Y. and Wang, D. N. (2005) 'Crystal structure and mechanism of GlpT, the glycerol-3-phosphate transporter from *E. coli*', *Journal of Electron Microscopy*, 54, pp. 43–46. doi: 10.1093/jmicro/dfi017.

Li, J. (2019) 'Identification of host-specific effectors mediating pathogenicity of the vascular wilt pathogen *Verticillium dahliae*'. Wageningen University. doi: 10.18174/464935.

Li, Y. and Qi, B. (2017) 'Progress toward understanding protein S-acylation: Prospective in plants', *Frontiers in Plant Science*, 8, pp. 1–20. doi: 10.3389/fpls.2017.00346.

Liang, G., Zhang, H., Lou, D. and Yu, D. (2016) 'Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing', *Scientific Reports*, 6(1), pp. 1–8. doi: 10.1038/srep21451.

Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Cammue, B. P. A. and Thomma, B. P. H. J. (2006) 'Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples', *Plant Science*, 171(1), pp. 155–165. doi: 10.1016/j.plantsci.2006.03.009.

Liu, D., Hu, R., Palla, K. J., Tuskan, G. A. and Yang, X. (2016) 'Advances and perspectives on the use of CRISPR/Cas9 systems in plant genomics research', *Current Opinion in Plant Biology*, 30, pp. 70–77. doi: 10.1016/j.pbi.2016.01.007.

Liu, X., Wu, S., Xu, J., Sui, C. and Wei, J. (2017) 'Application of CRISPR/Cas9 in Plant Biology', *Acta Pharmaceutica Sinica B*, 7(3), pp. 292–302. doi: 10.1016/j.apsb.2017.01.002.

L**iu, Y., Schiff, M. and Dinesh-Kumar, S. P.** (2002) 'Virus-induced gene silencing in tomato', *The Plant Journal*, 31(6), pp. 777–786. doi: 10.1046/j.1365-313X.2002.01394.

Livak, K. J. and Schmittgen, T. D. (2001) 'Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method', *Methods*, 25(4), pp. 402–408. doi: 10.1006/meth.2001.1262.

Lngkjær, M. F. L., Ewton, A. C. N., Tzema, J. L. A. and Aker, S. J. B. (2000) 'The Barley *mlo*-gene: An important powdery mildew resistance source', *Agronomie*, 20, pp. 745–756. doi: 10.1051/agro:2000173.

Lu, R., Martin-Hernandez, A. M., Peart, J. R., Malcuit, I. and Baulcombe, D. C. (2003) 'Virus-induced gene silencing in plants', *Methods*, 30(4), pp. 296–303. doi: 10.1016/S1046-2023(03)00037-9.

Lyons, R., Stiller, J., Powell, J., Rusu, A. and Manners, J. M. (2015) '*Fusarium oxysporum* triggers tissue-specific transcriptional reprogramming in *Arabidopsis thaliana*', *PLoS ONE*, pp. 1–23. doi: 10.1371/journal.pone.0121902.

Manova, V. and Gruszka, D. (2015) 'DNA damage and repair in plants – From models to crops', *Frontiers in Plant Science*, 6, pp. 1–26. doi: 10.3389/fpls.2015.00885.

Marx, V. (2018) 'Base editing a CRISPR way', *Nature Methods*, 15(10), pp. 767–770. doi: 10.1038/s41592-018-0146-4.

Masuda, D., Ishida, M., Yamaguchi, K., Yamaguchi, I., Kimura, M. and Nishiuchi, T. (2007) 'Phytotoxic effects of trichothecenes on the growth and morphology of *Arabidopsis thaliana*', *Journal of Experimental Botany*, 58(7), pp. 1617–1626. doi: 10.1093/jxb/erl298.

Metje-Sprink, J., Menz, J., Modrzejewski, D. and Sprink, T. (2019) 'DNA-Free genome editing: Past, present and future', *Frontiers in Plant Science*, 9, pp. 1–9. doi: 10.3389/fpls.2018.01957.

Michielse, C. B. and Rep, M. (2009) 'Pathogen profile update: *Fusarium oxysporum*', *Molecular Plant Pathology*, 10(3), pp. 311–324. doi: 10.1111/j.1364-3703.2009.00538.x.

Mithran, M., Paparelli, E., Novi, G., Perata, P. and Loreti, E. (2014) 'Analysis of the role of the pyruvate decarboxylase gene family in *Arabidopsis thaliana* under low-oxygen conditions', *Plant Biology*, 16(1), pp. 28–34. doi: 10.1111/plb.12005.

Mukhtar, M. S., Deslandes, L., Auriac, M. C., Marco, Y. and Somssich, I. E. (2008) 'The Arabidopsis transcription factor WRKY27 influences wilt disease symptom development caused by *Ralstonia solanacearum*', *The Plant Journal*, 56(6), pp. 935–947. doi: 10.1111/j.1365-313X.2008.03651.

Mushtaq, M., Sakina, A., Wani, S. H., Shikari, A. B., Tripathi, P., Zaid, A., Galla, A., Abdelrahman, M., Sharma, M., Singh, A. K. and Salgotra, R. K. (2019) 'Harnessing genome editing techniques to engineer disease resistance in plants', *Frontiers in Plant Science*, 10, pp. 1–16. doi: 10.3389/fpls.2019.00550.

Nandi, M., Macdonald, J., Liu, P., Weselowski, B. and Yuan, Z. C. (2018) '*Clavibacter michiganensis* ssp. *michiganensis*: Bacterial canker of tomato, molecular interactions and disease management', *Molecular Plant Pathology*, 19(8), pp. 2036–2050. doi: 10.1111/mpp.12678.

Oh, S. K., Baek, K. H., Park, J. M., Yi, S. Y., Yu, S. H., Kamoun, S. and Choi, D. (2008) '*Capsicum annuum* WRKY protein CaWRKY1 is a negative regulator of pathogen defense', *New Phytologist*, 177(4), pp. 977–989. doi: 10.1111/j.1469-8137.2007.02310.x.

Okie, W.R. and Gardner, R. G. (1982) 'Breeding for resistance to *Verticillium dahliae* race 2 of tomato in North Carolina', *Journal of the American Society for Horticultural Science*, 107, pp. 552–555.

Okie, W. R. and Gardner, R. G. (1982) 'Screening tomato seedlings for resistance to *Verticillium dahliae* races 1 and 2', *Plant Disease,* 66(1), pp. 34–37. doi: 10.1094/pd-66-34.

Orzaez, D., Medina, A., Torre, S., Fernández-Moreno, J. P., Rambla, J. L., Fernández-del-Carmen, A., Butelli, E., Martin, C. and Granell, A. (2009) 'A visual reporter system for virus-induced gene silencing in tomato fruit based on anthocyanin accumulation', *Plant Physiology*, 150(3), pp. 1122–1134. doi: 10.1104/pp.109.139006.

Pantelides, I. S., Tjamos, S. E. and Paplomatas, E. J. (2010) 'Ethylene perception via *ETR1* is required in Arabidopsis infection by *Verticillium dahliae*', *Molecular Plant Pathology*, 11(2), pp. 191–202. doi: 10.1111/j.1364-3703.2009.00592.

Pantelides, I. S., Tjamos, S. E. and Paplomatas, E. J. (2010) 'Insights into the role of ethylene perception in tomato resistance to vascular infection by *Verticillium dahliae*',

Plant Pathology, 59(1), pp. 130–138. doi: 10.1111/j.1365-3059.2009.02206.

Pao, S. S., Paulsen, I. T. and Saier, M. H. (1998) 'Major Facilitator Superfamily (MFS)', *Microbiology and Molecular Biology Review*s, 62(1), pp. 1–34. doi: 10.1007/978-1-4020-6754-9_9778.

Papadaki, A. M., Bletsos, F. A., Eleftherohorinos, I. G., Menexes, G. and Lagopodi, A. L. (2017) 'Effectiveness of seven commercial rootstocks against Verticillium wilt and their effects on growth, yield, and fruit quality of tomato', *Crop Protection*, 102, pp. 25–31. doi: 10.1016/j.cropro.2017.08.006.

Papastolopoulou, C., Diakou, G., Gkizi, D., Dimitrakas, V., Paplomatas, E. J. and Tjamos, S. E. (2018) 'The *pyruvate decarboxylase 1* (*PDC1*) gene: Negative regulator of disease resistance for *Fusarium oxysporum* and *Verticillium dahliae*', *European Journal of Plant Pathology*, 1, pp. 1–9. doi: 10.1007/s10658-018-1448-1.

Parisi, M., Aversano, R., Graziani, G., Ruggieri, V., Senape, V., Sigillo, L. and Barone, A. (2016) 'Phenotypic and molecular diversity in a collection of "Pomodoro di Sorrento" Italian tomato landrace', *Scientia Horticulturae*, 203, pp. 143–151. doi: 10.1016/j.scienta.2016.02.038.

Pavan, S., Jacobsen, E., Visser, R. G. F. and Bai, Y. (2010) 'Loss of susceptibility as a novel breeding strategy for durable and broad-spectrum resistance', *Molecular Breeding*, 25(1), pp. 1–12. doi: 10.1007/s11032-009-9323-6.

Peeters, N., Guidot, A., Vailleau, F. and Valls, M. (2013) '*Ralstonia solanacearum*, a widespread bacterial plant pathogen in the post-genomic era', *Molecular Plant Pathology*, 14(7), pp. 651–662. doi: 10.1111/mpp.12038.

Pegg, G. F. and Brady, B. L. (2002) 'Verticillium wilts', CABI Publishing.

Pesquet, E., Ranocha, P., Legay, S., Digonnet, C., Barbier, O., Pichon, M. and Goffner, D. (2005) 'Novel markers of xylogenesis in zinnia are differentially regulated by auxin and cytokinin', *Plant Physiology*, 139(4), pp. 1821–1839. doi: 10.1104/pp.105.064337.

Piffanelli, P., Zhou, F., Casais, C., Orme, J., Jarosch, B., Schaffrath, U., Collins, N. C., Panstruga, R. and Schulze-Lefert, P. (2002) 'The barley MLO modulator of defense and cell death is responsive to biotic and abiotic stress stimuli', *Plant Physiology*, 129, pp. 1076–1085. doi: 10.1104/pp.010954.ror2.

Piffanelli, P., Ramsay, L., Waugh, R., Benabdelmouna, A., D'Hont, A., Hollricher, K., Jørgensen, J. H., Schulze-Lefert, P. and Panstruga, R. (2004) 'A barley cultivation-associated polymorphism conveys resistance to powdery mildew', *Nature*, 430(7002), pp. 887–891. doi: 10.1038/nature02781.

Pilet-Nayel, M. L., Moury, B., Caffier, V., Montarry, J., Kerlan, M. C., Fournet, S., Durel, C. E. and Delourme, R. (2017) 'Quantitative resistance to plant pathogens in pyramiding strategies for durable crop protection', *Frontiers in Plant Science*, 8, pp. 1–9. doi: 10.3389/fpls.2017.01838.

Populer, C. (1978) 'Changes in host susceptibility with time', in Plant Disease: An Advanced Treatise Volume II: How Disease Develops in Populations. Academic Press, pp. 239–262. doi: 10.1016/b978-0-12-356402-3.50020-7.

Potnis, N., Timilsina, S., Strayer, A., Shantharaj, D., Barak, J. D., Paret, M. L., Vallad, G. E. and Jones, J. B. (2015) 'Bacterial spot of tomato and pepper: Diverse *Xanthomonas* species with a wide variety of virulence factors posing a worldwide challenge', *Molecular Plant Pathology*, 16(9), pp. 907–920. doi: 10.1111/mpp.12244.

Radakovic, Z. S., Anjam, M. S., Escobar, E., Chopra, D., Cabrera, J., Silva, A. C., Escobar, C., Sobczak, M., Grundler, F. M. W. and Siddique, S. (2018) 'Arabidopsis *HIPP27* is a host susceptibility gene for the beet cyst nematode *Heterodera schachtii*', *Molecular Plant Pathology*, 19(8), pp. 1917–1928. doi: 10.1111/mpp.12668.

Ramaiah, M., Jain, A., Baldwin, J. C., Karthikeyan, A. S. and Raghothama, K. G. (2011) 'Characterization of the phosphate starvation-induced *Glycerol-3-phosphate permease* gene family in Arabidopsis', *Plant Physiology*, 157(1), pp. 279–291. doi: 10.1104/pp.111.178541.

Ramegowda, V., Mysore, K. S. and Senthil-Kumar, M. (2014) 'Virus-induced gene silencing is a versatile tool for unravelling the functional relevance of multiple abiotic-stress-responsive genes in crop plants', *Frontiers in Plant Science*, 5, pp. 1–12. doi: 10.3389/fpls.2014.00323.

Ranocha, P., Denancé, N., Vanholme, R., Freydier, A., Martinez, Y., Hoffmann, L., Köhler, L., Pouzet, C., Renou, J.-P., Sundberg, B., Boerjan, W. and Goffner, D. (2010) '*Walls are thin 1* (*WAT1*), an Arabidopsis homolog of *Medicago truncatula NODULIN21*, is a tonoplast-localized protein required for secondary wall formation in fibers', *The Plant Journal*, 63(3), pp. 469–483. doi: 10.1111/j.1365-313X.2010.04256.x.

Ranocha, P., Dima, O., Nagy, R., Felten, J., Corratgé-Faillie, C., Novák, O., Morreel, K., Lacombe, B., Martinez, Y., Pfrunder, S., Jin, X., Renou, J.-P., Thibaud, J.-B., Ljung, K., Fischer, U., Martinoia, E., Boerjan, W. and Goffner, D. (2013) 'Arabidopsis WAT1 is a vacuolar auxin transport facilitator required for auxin homoeostasis', *Nature Communications*, 4, pp. 1–9. doi: 10.1038/ncomms3625.

Rep, M., Dekker, H. L., Vossen, J. H., de Boer, A. D., Houterman, P. M., Speijer, D., Back, J. W., de Koster, C. G. and Cornelissen, B. J. C. (2002) 'Mass spectrometric identification of isoforms of PR proteins in xylem sap of fungus-infected tomato', *Plant Physiology*, 130(2), pp. 904–917. doi: 10.1104/pp.007427.

Rep, M., Dekker, H. L., Vossen, J. H., de Boer, A. D., Houterman, P. M., de Koster, C. G. and Cornelissen, B. J. C. (2003) 'A tomato xylem sap protein represents a new family of small cysteine-rich proteins with structural similarity to lipid transfer proteins', *FEBS Letters*, 534(1–3), pp. 82–86. doi: 10.1016/S0014-5793(02)03788-2.

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W. and Smyth, G. K. (2015) 'limma powers differential expression analyses for RNA-sequencing and microarray studies', *Nucleic Acids Research*, 43(7), p. e47. doi: 10.1093/nar/gkv007.

Robert-Seilaniantz, A., Grant, M. and Jones, J. D. G. (2011) 'Hormone crosstalk in plant disease and defense: More than just JASMONATE-SALICYLATE antagonism', *Annual Review of Phytopathology*, 49(1), pp. 317–343. doi: 10.1146/annurev-phyto-073009-114447.

Robinson, D. B. (1957) 'Verticillium wilt of potato in relation to symptoms, epidemiology and variability of the pathogen', in University of Wisconsin Agricultural Experiment Station Results Bulletin, p. 49.

Rovenich, H., Boshoven, J. C. and Thomma, B. P. H. J. (2014) 'Filamentous pathogen effector functions: Of pathogens, hosts and microbiomes', *Current Opinion in Plant Biology*, 20, pp. 96–103. doi: 10.1016/j.pbi.2014.05.001.

Rushton, P. J., Somssich, I. E., Ringler, P. and Shen, Q. J. (2010) 'WRKY transcription factors', *Trends in Plant Science*, 15(5), pp. 247–258. doi: 10.1016/j.tplants.2010.02.006.

Rygulla, W., Snowdon, R. J., Friedt, W., Happstadius, I., Cheung, W. Y. and Chen, D.

(2008) 'Identification of quantitative trait loci for resistance against *Verticillium longisporum* in oilseed rape (*Brassica napus*)', *Phytopathology*, 98(2), pp. 215–221. doi: 10.1094/PHYTO-98-2-0215.

Saijo, Y. and Loo, E. P. (2020) 'Plant immunity in signal integration between biotic and abiotic stress responses', *New Phytologist*, 225(1), pp. 87–104. doi: 10.1111/nph.15989.

Sánchez-Vallet, A., Tian, H., Rodriguez-Moreno, L., Valkenburg, D.-J.,Saleem-Batcha, R., Wawra, S., Kombrink, A., Verhage, L., de Jonge, R., van Esse, H. P., Zuccaro, A., Croll, D., Mesters, J. R. and Thomma, B. P. H. J. (2020) 'A secreted LysM effector protects fungal hyphae through chitin-dependent homodimer polymerization', *PLoS Pathogens*, 16, pp. 1–21. doi: 10.1371/journal.ppat.1008652.

Sander, J. D. and Joung, J. K. (2014) 'CRISPR-Cas systems for editing, regulating and targeting genomes', *Nature Biotechnology*, 32(4), pp. 347–355. doi: 10.1038/nbt.2842.

Sanson, K. R., Hanna, R. E., Hegde, M., Donovan, K. F., Strand, C., Sullender, M. E., Vaimberg, E. W., Goodale, A., Root, D. E., Piccioni, F. and Doench, J. G. (2018) 'Optimized libraries for CRISPR-Cas9 genetic screens with multiple modalities', *Nature Communications*, 9(1), pp. 1–15. doi: 10.1038/s41467-018-07901-8.

Santillán Martínez, M. I., Bracuto, V., Koseoglou, E., Appiano, M., Jacobsen, E., Visser, R. G. F., Wolters, A. M. A. and Bai, Y. (2020) 'CRISPR/Cas9-targeted mutagenesis of the tomato susceptibility gene *PMR4* for resistance against powdery mildew', *BMC Plant Biology*, 20(1), pp. 1–13. doi: 10.1186/s12870-020-02497-y.

Schaible, L., Cannon, O. and Waddoups, V. (1951) 'Inheritance of resistance to Verticillium wilt in a tomato cross', *Phytopathology*, 41, pp. 986–990.

van Schie, C. C. N. and Takken, F. L. W. (2014) 'Susceptibility genes 101: How to be a good host', *Annual Review of Phytopathology*, pp. 1–31. doi: 10.1146/annurev-phyto-102313-045854.

Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012) 'NIH Image to ImageJ: 25 years of image analysis', *Nature Methods*, 9(7), pp. 671–675. doi: 10.1038/nmeth.2089.

Schouten, H. J., Krauskopf, J., Visser, R. G. F. and Bai, Y. (2014) 'Identification of candidate genes required for susceptibility to powdery or downy mildew in cucumber', *Euphytica*, 200(3), pp. 475–486. doi: 10.1007/s10681-014-1216-z.

Seifi, A., Kaloshian, I., Vossen, J., Che, D., Bhattarai, K. K., Fan, J., Naher, Z., Goverse, A., Tjallingii, W. F., Lindhout, P., Visser, R. G. F. and Bai, Y. (2011) 'Linked, if not the same, *Mi-1* homologues confer resistance to tomato powdery mildew and root-knot nematodes', *Molecular Plant-Microbe Interactions*, 24(4), pp. 441–450. doi: 10.1094/MPMI-06-10-0145.

Senthil-Kumar, M., Hema, R., Anand, A., Kang, L., Udayakumar, M. and Mysore, K. S. (2007) 'A systematic study to determine the extent of gene silencing in *Nicotiana benthamiana* and other Solanaceae species when heterologous gene sequences are used for virus-induced gene silencing', *New Phytologist*, 176(4), pp. 782–791. doi: 10.1111/j.1469-8137.2007.02225.x.

Senthil-Kumar, M. and Mysore, K. S. (2014) 'Tobacco rattle virus-based virus-induced gene silencing in *Nicotiana benthamiana'*, *Nature protocols*, 9(7), pp. 1549–62. doi: 10.1038/nprot.2014.092.

Shah, J., Kachroo, P., Nandi, A. and Klessig, D. F. (2001) 'A recessive mutation in the

Arabidopsis *SSI2* gene confers SA- and *NPR1*-independent expression of *PR* genes and resistance against bacterial and oomycete pathogens', *The Plant Journal*, 25(5), pp. 563–574. doi: 10.1046/j.1365-313x.2001.00992.x.

Shittu, H. O., Castroverde, D. C. M., Nazar, R. N. and Robb, J. (2009) 'Plant-endophyte interplay protects tomato against a virulent Verticillium', *Planta*, 229(2), pp. 415–426. doi: 10.1007/s00425-008-0840-z.

Snelders, N. C., Rovenich, H., Petti, G., Rocafort, M., Vorholt, J. A., Mesters, J. R., Seidl, M. F., Nijland, R. and Thomma, B. P. H. J. (2020) 'A plant pathogen utilizes effector proteins for microbiome manipulation', *bioRxiv*. doi: 10.1101/2020.01.30.926725.

Song, Y., Zhang, Z., Seidl, M. F., Majer, A., Jakse, J., Javornik, B. and Thomma, B. P. H. J. (2017) 'Broad taxonomic characterization of Verticillium wilt resistance genes reveals an ancient origin of the tomato Ve1 immune receptor', *Molecular Plant Pathology*, 18(2), pp. 195–209. doi: 10.1111/mpp.12390.

St.Clair, D. A. (2010) 'Quantitative disease resistance and quantitative resistance loci in breeding', *Annual Review of Phytopathology*, 48(1), pp. 247–268. doi: 10.1146/annurev-phyto-080508-081904.

Stamova, L. (2005) 'Resistance to *Verticillium dahliae* race 2 and its introgression into processing tomato cultivars', *Acta Horticulturae*, 695, pp. 257–261. doi: 10.17660/actahortic.2005.695.28.

Stemmer, M., Thumberger, T., del Sol Keyer, M., Wittbrodt, J. and Mateo, J. L. (2015) 'CCTop: An intuitive, flexible and reliable CRISPR/Cas9 target prediction tool', *PLoS ONE*, 10(4), pp. 1–11. doi: 10.1371/journal.pone.0124633.

S**tephenson, P., Stacey, N., Brüser, M., Pullen, N., Ilyas, M., O'Neill, C., Wells, R. and Østergaard, L.** (2019) 'The power of model-to-crop translation illustrated by reducing seed loss from pod shatter in oilseed rape', *Plant Reproduction*, 32(4), pp. 331–340. doi: 10.1007/s00497-019-00374-9.

Su, X., Lu, G., Guo, H., Zhang, K., Li, X. and Cheng, H. (2018) 'The dynamic transcriptome and metabolomics profiling in *Verticillium dahliae* inoculated *Arabidopsis thaliana*', *Scientific Reports*, 8(1), pp. 1–11. doi: 10.1038/s41598-018-33743-x.

Sun, K., Wolters, A. A., Loonen, A. E. H. M., Huibers, R. P., van der Vlugt, R., Goverse, A., Jacobsen, E., Visser, R. G. F. and Bai, Y. (2016a) 'Down-regulation of Arabidopsis *DND1* orthologs in potato and tomato leads to broad-spectrum resistance to late blight and powdery mildew', *Transgenic Research*, 25(2), pp. 123–138. doi: 10.1007/s11248-015-9921-5.

Sun, K., Wolters, A. A., Vossen, J. H., Rouwet, M. E., Loonen, A. E. H. M., Jacobsen, E., Visser, R. G. F. and Bai, Y. (2016b) 'Silencing of six susceptibility genes results in potato late blight resistance', *Transgenic Research*, 25(5), pp. 731–742. doi: 10.1007/s11248-016-9964-2.

Tang, D., Simonich, M. T. and Innes, R. W. (2007) 'Mutations in *LACS2*, a long-chain acyl-coenzyme A synthetase, enhance susceptibility to avirulent *Pseudomonas syringae* but confer resistance to *Botrytis cinerea* in Arabidopsis', *Plant Physiology*, 144(2), pp. 1093–1103. doi: 10.1104/pp.106.094318.

Tang, Y., Zhang, Z., Lei, Y., Hu, G., Liu, J., Hao, M., Chen, A., Peng, Q. and Wu, J. (2019) 'Cotton *WATs* modulate SA biosynthesis and local lignin deposition participating in plant resistance against *Verticillium dahliae*', *Frontiers in Plant Science*, 10, pp. 1–15. doi: 10.3389/fpls.2019.00526.

Thomma, B. P. H. J., Eggermont, K., Penninckx, I. A. M. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. A. and Broekaert, W. F. (1998) 'Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens', *Proceedings of the National Academy of Sciences of the United States of America*, 95(25), pp. 15107–15111. doi: 10.1073/pnas.95.25.15107.

Tian, H., Fiorin, G. L., Kombrink, A., Mesters, J. R. and Bart, P. H. J. (2020) 'Fungal LysM effectors that comprise two LysM domains bind chitin through intermolecular dimerization', *bioRxiv*. doi: 10.1101/2020.06.11.146639.

Tomatis, V. M., Trenchi, A., Gomez, G. A. and Daniotti, J. L. (2010) 'Acyl-protein thioesterase 2 catalizes the deacylation of peripheral membrane-associated GAP-43', *PLoS ONE*, 5(11). doi: 10.1371/journal.pone.0015045.

Toppino, L. and Barchi, L. (2016) 'QTLs mapping for *Fusarium oxysporum* and *Verticillium dahliae* resistance in eggplant (*Solanum melongena* L)', in Proceedings of XVIth EUCARPIA Capsicum and Eggplant Working Group Meeting, pp. 248–255.

Tsolakidou, M. D., Pantelides, I., Tzima, A. K., Kang, S., Paplomatas, E. J. and Tsaltas, D. (2019) 'Disruption and overexpression of the gene encoding ACC (1-Aminocyclopropane-1-Carboxylic Acid) deaminase in soil-borne fungal pathogen *Verticillium dahliae* revealed the role of ACC as a potential regulator of virulence and plant defense', *Molecular Plant-Microbe Interactions*, 32(6), pp. 639–653. doi: 10.1094/MPMI-07-18-0203-R.

Uppalapati, S. R., Ishiga, Y., Doraiswamy, V., Bedair, M., Mittal, S., Chen, J., Nakashima, J., Tang, Y., Tadege, M., Ratet, P., Chen, R., Schultheiss, H. and Mysore, K. S. (2012) 'Loss of abaxial leaf epicuticular wax in *Medicago truncatula irg1/palm* mutants results in reduced spore differentiation of anthracnose and nonhost rust pathogens', *The Plant Cell*, 24(1), pp. 353–370. doi: 10.1105/tpc.111.093104.

Uribe, P., Jansky, S. and Halterman, D. (2014) 'Two CAPS markers predict Verticillium wilt resistance in wild Solanum species', *Molecular Breeding*, 33(2), pp. 465–476. doi: 10.1007/s11032-013-9965-2.

Usami, T., Momma, N., Kikuchi, S., Watanabe, H., Hayashi, A., Mizukawa, M., Yoshino, K. and Ohmori, Y. (2017) 'Race 2 of *Verticillium dahliae* infecting tomato in Japan can be split into two races with differential pathogenicity on resistant rootstocks', *Plant Pathology*, 66(2), pp. 230–238. doi: 10.1111/ppa.12576.

Valentine, T., Shaw, J., Blok, V. C., Phillips, M. S., Oparka, K. J. and Lacomme, C. (2004) 'Efficient virus-induced gene silencing in roots using a modified tobacco rattle virus vector', *Plant Physiology*, 136, pp. 3999–4009. doi: 10.1104/pp.104.051466.1.

Verlaan, M. G., Hutton, S. F., Ibrahem, R. M., Kormelink, R., Visser, R. G. F., Scott, J. W., Edwards, J. D. and Bai, Y. (2013) 'The tomato yellow leaf curl virus resistance genes *Ty-1* and *Ty-3* are allelic and code for DFDGD-class RNA-dependent RNA polymerases', *PLoS Genetics*, 9(3). doi: 10.1371/journal.pgen.1003399.

Verma, A., Lee, C., Morriss, S., Odu, F., Kenning, C., Rizzo, N., Spollen, W. G., Lin, M., McRae, A. G., Givan, S. A., Hewezi, T., Hussey, R., Davis, E. L., Baum, T. J. and Mitchum, M. G. (2018) 'The novel cyst nematode effector protein 30D08 targets host nuclear functions to alter gene expression in feeding sites', *New Phytologist*, 219(2), pp. 697–713. doi: 10.1111/nph.15179.

Vermeulen, J. P. (2020) 'Identification and inheritance of quantitative resistance against

Verticillium dahliae in tomato'. Wageningen University.

Veronese, P., Nakagami, H., Bluhm, B., AbuQamar, S., Chen, X., Salmeron, J., Dietrich, R. A., Hirt, H. and Mengiste, T. (2006) 'The membrane-anchored *BOTRYTIS-INDUCED KINASE1* plays distinct roles in Arabidopsis resistance to necrotrophic and biotrophic pathogens', *The Plant Cell*, 18, pp. 257–273. doi: 10.1105/tpc.105.035576.1.

Vogel, E., Donat, M. G., Alexander, L. V., Meinshausen, M., Ray, D. K., Karoly, D., Meinshausen, N. and Frieler, K. (2019) 'The effects of climate extremes on global agricultural yields', *Environmental Research Letters*, 14(5). doi: 10.1088/1748-9326/ab154b.

Vogel, J. P., Raab, T. K., Schiff, C. and Somerville, S. C. (2002) '*PMR6*, a pectate lyase-like gene required for powdery mildew susceptibility in Arabidopsis', *The Plant Cell*, 14(9), pp. 2095–2106. doi: 10.1105/tpc.003509.

Vogel, J. and Somerville, S. (2000) 'Isolation and characterization of powdery mildew-resistant Arabidopsis mutants', *Proceedings of the National Academy of Sciences of the United States of America*, 97(4), pp. 1897–1902. doi: 10.1073/pnas.030531997.

Wang, A. and Krishnaswamy, S. (2012) 'Eukaryotic translation initiation factor 4E-mediated recessive resistance to plant viruses and its utility in crop improvement', *Molecular Plant Pathology*, 13(7), pp. 795–803. doi: 10.1111/j.1364-3703.2012.00791.x.

Wang, D., Pajerowska-Mukhtar, K., Culler, A. H. and Dong, X. (2007) 'Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway', *Current Biology*, 17(20), pp. 1784–1790. doi: 10.1016/j.cub.2007.09.025.

Wang, D., Amornsiripanitch, N. and Dong, X. (2006) 'A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants', *PLoS Pathogens*, 2(11), pp. 1042–1050. doi: 10.1371/journal.ppat.0020123.

Wang, E., Schornack, S., Marsh, J. F., Gobbato, E., Schwessinger, B., Eastmond, P., Schultze, M., Kamoun, S. and Oldroyd, G. E. D. (2012) 'A common signaling process that promotes mycorrhizal and oomycete colonization of plants', *Current Biology*, 22(23), pp. 2242–2246. doi: 10.1016/j.cub.2012.09.043.

Wang, H. M., Lin, Z. X., Zhang, X. L., Chen, W., Guo, X. P., Nie, Y. C. and Li, Y. H. (2008) 'Mapping and quantitative trait loci analysis of Verticillium wilt resistance genes in cotton', *Journal of Integrative Plant Biology*, 50(2), pp. 174–182. doi: 10.1111/j.1744-7909.2007.00612.x.

Wang, Y., Tan, J., Wu, Z., VandenLangenberg, K., Wehner, T. C., Wen, C., Zheng, X., Owens, K., Thornton, A., Bang, H. H., Hoeft, E., Kraan, P. A. G., Suelmann, J., Pan, J. And Weng, Y. (2018) 'STAYGREEN, STAY HEALTHY: A loss-of-susceptibility mutation in the *STAYGREEN* gene provides durable, broad-spectrum disease resistances for over 50 years of US cucumber production', *New Phytologist*, pp. 1–16. doi: 10.1111/nph.15353.

Warmerdam, S., Sterken, M. G., van Schaik, C., Oortwijn, M. E. P., Sukarta, O. C. A., Lozano-Torres, J. L., Dicke, M., Helder, J., Kammenga, J. E., Goverse, A., Bakker, J. and Smant, G. (2018) 'Genome-wide association mapping of the architecture of susceptibility to the root-knot nematode *Meloidogyne incognita* in *Arabidopsis thaliana*', *New Phytologist*, 218(2), pp. 724–737. doi: 10.1111/nph.15034.

Weber, E., Engler, C., Gruetzner, R., Werner, S. and Marillonnet, S. (2011) 'A modular cloning system for standardized assembly of multigene constructs', *PLoS ONE*, 6(2),

pp. 1–11. doi: 10.1371/journal.pone.0016765.

Wiese, M. V. and Devay, J. E. (1970) 'Growth regulator changes in cotton associated with defoliation caused by *Verticillium albo-atrum*', *Plant Physiology*, 45(3), pp. 304–309. doi: 10.1104/pp.45.3.304.

Wise, R. P., Moscou, M. J., Bogdanove, A. J. and Whitham, S. A. (2007) 'Transcript profiling in host-pathogen interactions', *Annual Review of Phytopathology*, 45, pp. 329–369. doi: 10.1146/annurev.phyto.45.011107.143944.

Witzel, K., Buhtz, A. and Grosch, R. (2017) 'Temporal impact of the vascular wilt pathogen *Verticillium dahliae* on tomato root proteome', *Journal of Proteomics*, 169, pp. 215–224. doi: 10.1016/j.jprot.2017.04.008.

Won, S. J., Cheung See Kit, M. and Martin, B. R. (2018) 'Protein depalmitoylases', *Critical Reviews in Biochemistry and Molecular Biology*, 53(1), pp. 83–98. doi: 10.1080/10409238.2017.1409191.

Wong, N., Liu, W. and Wang, X. (2015) 'WU-CRISPR: Characteristics of functional guide RNAs for the CRISPR/Cas9 system', **Genome Biology**, 16(1), pp. 1–8. doi: 10.1186/s13059-015-0784-0.

Wright, D. A., Townsend, J. A., Winfrey, R. J., Irwin, P. A., Rajagopal, J., Lonosky, P. M., Hall, B. D., Jondle, M. D. and Voytas, D. F. (2005) 'High-frequency homologous recombination in plants mediated by zinc-finger nucleases', *The Plant Journal*, 44(4), pp. 693–705. doi: 10.1111/j.1365-313X.2005.02551.x.

Wu, C., Jia, L. and Goggin, F. (2011) 'The reliability of virus-induced gene silencing experiments using tobacco rattle virus in tomato is influenced by the size of the vector control', *Molecular Plant Pathology*, 12(3), pp. 299–305. doi: 10.1111/j.1364-3703.2010.00669.x.

Wu, X., Kriz, A. J. and Sharp, P. A. (2014) 'Target specificity of the CRISPR-Cas9 system', *Quantitative Biology,* 2, pp. 1–24. doi: 10.1007/s40484-014-0030-x.Wu.

Xiong, X., Sun, S., Li, Y., Zhang, X., Sun, J. and Xue, F. (2019) 'The cotton WRKY transcription factor *GhWRKY70* negatively regulates the defense response against *Verticillium dahliae*', *The Crop Journal*, 7(3), pp. 393–402. doi: 10.1016/j.cj.2018.10.005.

Xu, L., Zhao, H., Wan, R., Liu, Y., Xu, Z., Tian, W., Ruan, W., Wang, F., Deng, M., Wang, J., Dolan, L., Luan, S., Xue, S. and Yi, K. (2019) 'Identification of vacuolar phosphate efflux transporters in land plants', *Nature Plants*, 5(1), pp. 84–94. doi: 10.1038/s41477-018-0334-3.

Xu, Q., Tang, C., Wang, Xiaodong, Sun, S., Zhao, J., Kang, Z. and Wang, X. (2019) 'An effector protein of the wheat stripe rust fungus targets chloroplasts and suppresses chloroplast function', *Nature Communications*, 10(1). doi: 10.1038/s41467-019-13487-6.

Yadeta, K. A. (2012) 'Verticillium wilt resistance in Arabidopsis and tomato: Identification and functional characterization'. Wageningen University. ISBN: 978-94-6173-384-9.

Yadeta, K. A. and Thomma, B. P. H. J. (2013) 'The xylem as battleground for plant hosts and vascular wilt pathogens', *Frontiers in Plant Science*, 4, pp. 1–18. doi: 10.3389/fpls.2013.00097.

Yamanaka, T., Imai, T., Satoh, R., Kawashima, A., Takahashi, M., Tomita, K., Kubota, K., Meshi, T., Naito, S. and Ishikawa, M. (2002) 'Complete inhibition of tobamovirus multiplication by simultaneous mutations in two homologous host genes', *Journal*

of Virology, 76(5), pp. 2491–2497. doi: 10.1128/jvi.76.5.2491-2497.2002.

Yang, L., McLellan, H., Naqvi, S., He, Q., Boevink, P. C., Armstrong, M., Giuliani, L. M., Zhang, W., Tian, Z., Zhan, J., Gilroy, E. M. and Birch, P. R. J. (2016) 'Potato NPH3/RPT2-like protein StNRL1, targeted by a *Phytophthora infestans* RXLR effector, is a susceptibility factor', *Plant Physiology*, 171(1), pp. 645–657. doi: 10.1104/pp.16.00178.

Yin, K., Gao, C. and Qiu, J.-L. (2017) 'Progress and prospects in plant genome editing', *Nature Plants*, 3(8), pp. 1–6. doi: 10.1038/nplants.2017.107.

Yin, K. and Qiu, J.-L. (2019) 'Genome editing for plant disease resistance: Applications and perspectives', *Philosophical Transactions B*, 374(1767), doi: 10.1098/rstb.2018.0322.

Zaidi, S. S., Mukhtar, M. S. and Mansoor, S. (2018) 'Genome editing: Targeting susceptibility genes for plant disease resistance', *Trends in Biotechnology*, 36(9), pp. 898–906. doi: 10.1016/j.tibtech.2018.04.005.

Zaka, A., Grande, G., Coronejo, T., Quibod, I. L., Chen, C. W., Chang, S. J., Szurek, B., Arif, M., Cruz, C. V. and Oliva, R. (2018) 'Natural variations in the promoter of *OsSWEET13* and *OsSWEET14* expand the range of resistance against *Xanthomonas oryzae* pv. *oryzae*', *PLoS ONE*, 13(9), pp. 1–15. doi: 10.1371/journal.pone.0203711.

Zhang, J., Shao, F., Li, Y., Cui, H., Chen, L., Li, H., Zou, Y., Long, C., Lan, L., Chai, J., Chen, S., Tang, X., Zhou, J.-M. (2007) 'A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants', *Cell Host and Microbe*, 1(3), pp. 175–185. doi: 10.1016/j.chom.2007.03.006.

Zhang, W. J., Hanisch, S., Kwaaitaal, M., Pedersen, C. and Thordal-Christensen, H. (2013) 'A component of the Sec61 ER protein transporting pore is required for plant susceptibility to powdery mildew', *Frontiers in Plant Science*, 4, pp. 1–8. doi: 10.3389/fpls.2013.00127.

Zhang, Y. and Li, X. (2019) 'Salicylic acid: Biosynthesis, perception, and contributions to plant immunity', *Current Opinion in Plant Biology*, 50, pp. 29–36. doi: 10.1016/j.pbi.2019.02.004.

Zhao, Y., Wang, H., Chen, W. and Li, Y. (2014) 'Genetic structure, linkage disequilibrium and association mapping of Verticillium wilt resistance in elite cotton (*Gossypium hirsutum* L.) germplasm population', *PLoS ONE*, 9(1). doi: 10.1371/journal.pone.0086308.

Zhou, G., Qi, J., Ren, N., Cheng, J., Erb, M., Mao, B. and Lou, Y. (2009) 'Silencing *OsHI-LOX* makes rice more susceptible to chewing herbivores, but enhances resistance to a phloem feeder', *The Plant Journal*, 60(4), pp. 638–648. doi: 10.1111/j.1365-313X.2009.03988.x.

Zuker, M. (2003) 'Mfold web server for nucleic acid folding and hybridization prediction', *Nucleic Acids Research*, 31(13), pp. 3406–3415. doi: 10.1093/nar/gkg595.

Summary
Acknowledgments
About the author
Education statement

SUMMARY

In the field of plant-microbe interactions the concept of plant disease susceptibility (*S*) genes is relatively new as research has largely focused on plant resistance in the past. However, advances in genome editing and the finding that pathogen effectors target host components to establish disease have contributed to the increasing interest in *S* genes over recent years. *S* genes are host factors that are required by the pathogen to establish disease. They fulfil a plethora of functions in disease, for example, in early interaction, negative regulation of immunity, or pathogen sustenance. Unlike the introgression of dominant resistance (*R*) genes from wild germplasm into elite cultivars, the use of *S* genes in breeding requires their impairment in order to establish resistance. Hence resistance, or rather loss of susceptibility, mediated by an impaired *S* gene inherits recessively. Despite the herewith associated increased complexity for breeding, the use of impaired *S* genes provides a parallel strategy to breed for resistance, especially in those cases in which dominant resistance is not available or quickly overcome. One example of a pathogen for which only one dominant *R* gene is described so far is the notorious soil-borne vascular wilt fungus *Verticillium dahliae* which affects a wide range of crops, including tomato. Due to the colonization in the vascular system of the host, *V. dahliae* is particularly hard to control as fungicide applications are generally ineffective once the fungus entered the plant. Furthermore, *V. dahliae* produces persisting resting structures in the soil, and only limited options are available to clear infested soils. For *V. dahliae*, also only a few *S* genes have been described in literature so far. Generally, many S genes have been identified in the model plant Arabidopsis. However, in order to be able to use impaired *S* genes in resistance breeding, it is essential to identify and functionally characterize *S* genes in crop species as well as in model species. In this thesis, strategies to identify plant *S* genes are reviewed (**Chapter 1**) and two reverse genetics strategies were pursued to identify *S* genes for *V. dahliae* in tomato.

Firstly, in order to be able to screen for loss of susceptibility against *V. dahliae* in tomato, a phenotyping assay was set up in **Chapter 2**. Several resistant and susceptible genotypes were tested to find the most reliable and reproducible phenotype caused by *V. dahliae*. To this end, several plant growth-related parameters were evaluated and plant canopy area was found to provide the highest discriminative power between mock- and *V. dahliae*-inoculated plants. The relative difference in canopy area between mock- and *V. dahliae*-inoculated plants, hereafter referred to as stunting, was used to asses *V. dahliae* susceptibility in subsequent inoculation experiments. To determine whether the discriminative power based on canopy area measurements could be improved, the inoculation procedure was further evaluated. Neither an increased inoculum concentration, nor trimming of the roots at the time of inoculation, nor the application of nutrients to the soil after inoculation significantly improved the discriminative power.

The first strategy to identify *S* genes for *V. dahliae* in tomato builds on the observation that the expression of many *S* genes is induced upon pathogen challenge. Therefore, available expression data were mined for candidate genes that were specifically induced in a compatible *V. dahliae* – tomato interaction. In total, 100 and 262 genes induced in foliage and roots, respectively, were identified in **Chapter 3** and the most highly induced genes were selected for transient silencing using virus-induced gene silencing (VIGS). Subsequently, VIGS-treated plants were challenged with *V. dahliae* to screen for reduced susceptibility. Out of 135 genes tested, two candidates could be implicated in Verticillium wilt disease as potential *S* gene. As *S* gene-mediated resistance is known to be non-race specific, the role of these candidates in *V. dahliae* susceptibility was further tested with additional *V. dahliae* strains. The results indicate these candidates are indeed *S* genes to multiple *V. dahliae* strains. The first candidate, Solyc06g067950, encodes an acyl-protein thioesterase 2 (APT 2) which catalyses the de-acylation of proteins required for protein interactions with membranes. As knowledge on APTs is limited in plants, a link to plant immunity is not yet established. The second candidate, Solyc03g093140, encodes a glycerol-3-phosphate (G3P) transporter and G3P is known to function as signalling molecule in systemic acquired resistance. In order to study these candidates further, CRISPR-Cas9-mediated genome editing was used in **Chapter 4** to generate knock-outs. CRISPR mutants were obtained for both candidates. For *APT 2*, one mutant line with a 815 bp deletion at the rear part of the gene was obtained. This deletion was predicted to result in a truncated protein lacking lacking 90 of 256 amino acids at the C-terminus. For the G3P transporter, three different mutant lines were obtained carrying deletions in the front or middle part of the gene. In two cases, these deletions were predicted to result in truncated proteins, lacking the first 284 and 176 of 521 amino acids, and for the third mutant line the deletion was predicted to cause a 125 amino acid deletion in the middle of the protein. In all cases, several of the predicted transmembrane domains of this transporter were affected. Overall, targeted deletions in both candidates did not affect plant growth in early stages of development when compared with control plants. Surprisingly, however, none of the mutant lines showed loss of susceptibility upon challenge with *V. dahliae*. Collectively, these findings do not confirm the role of the G3P transporter in susceptibility to *V. dahliae* in tomato as three independent mutant lines showed no loss of susceptibility to *V. dahliae*. However, the role of *APT 2* as *S* gene for *V. dahliae* requires further study because the generated mutant line only affected the rear part of the gene.

The second reverse genetics strategy employed in this thesis research is based on the fact that many *S* genes have a role in plant susceptibility which is conserved in different plant species. As extensive knowledge on *S* genes is available in the model plant Arabidopsis, a literature search was conducted in **Chapter 5** to select candidates in this model species. For three previously identified *S* genes in Arabidopsis their

tomato homologues were identified, and their role in *V. dahliae* susceptibility was determined in tomato using VIGS followed by disease phenotyping. Targeting of the tomato orthologue of *Pyruvate Decarboxylase 1* (*PDC1*) and of *WRKY27* did not result in reduced susceptibility to *V. dahliae*. However, transient silencing of the tomato orthologue of *Walls Are Thin 1* (*WAT1*) indicated an involvement in susceptibility to *V. dahliae*. *WAT1* encodes a tonoplast-localized auxin transporter and was previously found to be involved in *V. dahliae* susceptibility in both Arabidopsis and cotton. To further study the role of *WAT1* in susceptibility to *V. dahliae* in tomato, CRISPR-Cas9-mediated knock-outs as well as *WAT1*-silenced lines using RNA interference (RNAi) were generated in **Chapter 6**. Silencing of *WAT1* did not confirm the role of *WAT1* in *V. dahliae* susceptibility in the RNAi lines. This can be attributed to the relatively high levels of residual *WAT1* expression in these RNAi lines which likely compromised the silencing efficacy too much to monitor effects on *V. dahliae* infection. By means of CRISPR-Cas9, one *WAT1* mutant line was obtained, which carried a biallelic mutation of a 121 and a 197 deletion out of 385 amino acids. Both mutant alleles affected the middle part of the gene and the deletions were predicted to affect the number of transmembrane domains of *WAT1*. Plants which were hetero- or homozygous for these deletions in *WAT1* displayed severe growth defects in early plant development, such as severe discoloration of the leaves and strongly reduced overall growth. However, the targeted deletions in *WAT1* also enhanced resistance to *V. dahliae*, as reduced stunting as well as reduced fungal biomass was monitored when compared with control plants. Furthermore, in line with previous findings that *WAT1* is involved in susceptibility to multiple vascular pathogens, *WAT1* CRISPR lines also showed loss of susceptibility to *V. albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici* in tomato.

In the Chapter 7, a general discussion on the identification of *S* genes in crops using different approaches is provided.

ACKNOWLEDGMENTS

One thing is for sure: I did not do this by myself! Without the help and support of many of you, I would not have completed my PhD. So many people were involved from the beginning until the end. Now it is my turn to thank you all and show my appreciation for what you have done for me. Thank you!

I would like to express my special thanks of gratitude to my supervisors. **Yuling**, thank you for sparkling my interest in *S* genes in one of your lectures during my MSc. You truly fascinated me and certainly paved my way in plant breeding. Thank you for shaping me as a scientist and for believing in me through my MSc thesis and of course during my PhD. You always encouraged me to do my best and to work on myself. **Bart**, thank you for always having and eye on me and thank you for making me realize things that I sometimes tried to deny. You are one of the most inspiring people in my life and I admire your sense of leadership. I also would like to thank you for your support during the writing phase; I learned so much from you! **Henk**, you were the first person who told me about the opportunity to do a PhD. Thank you for your support during my PhD journey, in both difficult and easy moments. Thank you for giving me the lovely nick name "VIGS machine" and thank you for your appreciation of my organizational talent.

Nora, thank you for being my external advisor. We met during the research master cluster course and once I had to propose an external advisor you were the first person I thought of. I enjoyed our talks especially in the beginning of my PhD. Thanks to you I still remind myself of "Don't sweat the small stuff".

I was fortunate to work on a TKI project together with several breeding companies. My thanks go to **Bejo Zaden, East West Seeds, Hazera, Nunhems, Rijk Zwaan, Syngenta and Takii Seeds** for the nice discussions during our half-yearly meetings. Your input was very inspiring and much appreciated.

A big thank you goes of course to you, **Jasper**! It was great to have a buddy like you and I appreciate everything you have done for me. Our PhD project certainly had crazy moments (e.g. inoculating thousands of your plants), looooong moments (e.g. way too many meetings all week long), awesome moments (e.g. day trip to Loch Lomond after MPMI), sad moments (e.g. Bart leaving) and funny moments (e.g. when the compartment and I got flooded accidently). I wish you all the best for the future and I know we will stay in touch!

I spend most of my PhD time in the lab at Plant Breeding and working in the lab would not have been so much fun without all the lovely people around. Special thanks go to **Fien, Jos** and **Johan** from my lab in the corner. Thanks for letting me put up self-made Christmas decoration each year. P.S.: If you haven't done so this year, I am happy to pass by! **Danny, Doret, Gert and Linda** from the middle lab: Thank you for your radio tunes and the positive vibes, I enjoyed those so much. **Danny**, you certainly are the one reason why I managed to finish my PhD! You did an awesome job with the continuous

amount of stems I gave you for isolation. Thank you for always keeping the spirit up, you are awesome! **Doret**, hallöchen popöchen, thank you for making the early morning very entertaining. Thank you also for always listening to me, you are great! Hallo leuke meneer, **Gert**, thanks for all the help I got from you in the lab, you are a true genius! Also thank you for greeting me with "Hallo lekker ding" in the hallway, which causes some raised eyebrows once in a while. **Linda**, thank you for always helping me out whenever I needed something. **Marian, Annelies** and **Marion** from the third lab: Thank you for all your support. Special thanks go to you, **Annelies**, you taught me so much about CRISPR and transformation! My dear **Marian**, thank you for always being there for me! I learned so much from you and you became more than just a colleague to me! You are a very special person to me and I am grateful for our friendship! **Bernadette, Isolde, Iris** and **Marion**, from the tissue culture lab, thank you so much for every chat we had and all the advice that I got from you throughout the years. Liebe **Isolde**, danke, dass Du immer für mich da warst! Du bist wirklich toll, danke!

Besides being in the lab, many of you might remember me going to Unifarm with my "picknick" basket. My thanks go to all the lovely people in the greenhouse who took care of my plants and who helped me out whenever I had a problem! Special thanks go to **Bertus, Andre, Bert, Sean, Erik, Henk, Casper, Teus** and **Maarten**. Without you I would not have had so many happy tomatoes, despite the fact that I murdered more than 15 thousand plants in my PhD. My sincere and deep gratitude goes to you, **Bert**, you made so much possible and you always had a trick or two to teach me, thank you!

I was fortunate to had great mentors during my education – thank you **Mr Bramstedt**, **Hassan Ghareeb** and **Michela Appiano** – which certainly inspired me to supervise students myself. It all started with you, **Lisa**, thank you for being an awesome first student and thank you for being patient with me! My thanks also go to you, **Gökhan**. Thanks for all you help with the potatoes. **Jillis**, how awesome that you are back as PhD student yourself now. You certainly contributed to my PhD thesis and I remember a funny Christmas sweater under the greenhouse lab coat. **Timo**, I will always remember you, your ice tea label and the moment we climbed into the GMO bin! Thank you for all your help on my project! **Dagmar**, you did excellent work on my project, and I enjoyed to see you grow throughout our discussions. Thank you for your help! **Shravya**, we did not only work hand-in-hand together in the lab, but also shared a passion for belly dancing. Thank you for your contributions to my thesis! All of you were absolutely fantastic and I wish you the best for your future!

I would also like to thank all the PhD students (past and present) at Plant Breeding. Amongst other my thanks go to **Alejandro, Carolina, Charlotte, Cynara, Daniel, Eleni, Jillis, Jarst, Jasper, Jeroen, Kaile, Lampros, Mas, Michela, Miguel S., Miguel R., Pauline, Sri, Yan** and **Xuexue**. Thank you for all the chats, support and advice!

A big thank you also goes to other colleagues at Plant Breeding. **Anne-marie**, thank you for always helping me out when I had a question! Even though you were not involved in my PhD project, you were always there to help! **Lei**, I enjoyed working with you on the pepper project. Thank you for everything! Irma, thank you so much for being so lovely, I always enjoyed coming into your office to introduce a new student. **Patrick**, thank you for your support with eLabjournal throughout the years. And thank you for helping me fix things when they were broken! **Jaap**, thank you for all the nice chats, of course especially during the Colombian dinners! **Danielle**, thank you for always giving me a big smile and for being so supportive, you are awesome! **Nicole**, thank you so much for being my Dutch mum! You helped me through so many rough moments, and I enjoyed your warm hugs (when that was still possible) and the dinners with **Louis, Luke** and **Madelief**.

Even though I spent most of my time at Plant Breeding, I was fortunate to be part of the Laboratory of Phytopathology as well. My thanks go amongst others to **Ali, Aranka, Ciska, Carolina, Einar, Francine, Gert, Giuliana, Harold, Jan, Jochem, Kiki, Laurens, Lorena, Maikel, Matthieu, Michele, Sander, Si, Sergio, Wen and Yaohua**.

My deep gratitude and special thanks go to all past and present Verticillium group members: **Bart, Edgar, David, Gabriel, Grardy, Hannah, Hui, Jasper D., Jasper V., Jinling, Luis, Malaika, Martin, Michael, Mireille, Nelia, Nick, Yin and Xiaoqian** (Bart, you managed to put quite a bunch of people together!). Thank you everyone for the weekly work discussions and all the support throughout all ups and down. I wish everyone all the best!

A project very close to my heart was my involvement with eLabjournal which all started with you, **Patrick** and **Eliana**. Thank you for letting me be involved and thank you for driving this project with me. Of course, I would also like to take this opportunity and thank the Bio-ITech team in Groningen and in particular **Wouter, Ivelina, Jasper, Florian** and **Annette**. It has been fantastic and very inspiring to work with you and I have learned so much from you. Through my eLabjournal activities I also got to know and to work with you, **Jacques** and **René**. Thank you for supporting all my activities and thank you for the appreciation I have received from you throughout my PhD!

None of this of course would have been possible without two very special people: my paranymphs. **Eliana**, we met at the beginning of our PhD journeys and ever since you have become a very special person to me and my best friend. I am so thankful for having you in my life and that you always have my back. You inspire me and you widen my horizon. Thank you for supporting my crazy eLabjournal endeavour and thank you for being you! **Nick**, we did it: MSc and PhD together! Thank you for your support throughout the last 6 years! You are a truly awesome person and I have always (and will probably always) looked up to you! I known our friendship sometimes suffered, but at the end of the day we were always there for each other. Thank you so much!

A definite highlight of my PhD was the participation in the “Dance your PhD” competition. Special thanks go to my ladies **Belén, Inge, Johanna, Lara, Pam, Sabine** and **Tanya**. You were fantastic and your creativity has made this video unforgettable. Of course, this would not have been possible without a charming fungus, **Edgar**, and the hard work of **David** and **Thijs** behind the scenes. Certainly, teaching belly dance throughout the years was the best time of the week! A big thank you also goes to lovely **Sabine** for painting my cover and to lovely **Tanya** for drawing the disease cycle. Both artworks make this thesis very special to me, thank you!

Besides of a lot of highlights, I also had very bad moments in my PhD. I cried my eyes out many times (especially on Sunday nights) and had to deal with anxiety and depression. In these moments I was fortunate to get help from professionals. Thank you, **Fenny**, for always being available for a talk. You helped me with a lot of tips and tricks, some of which I am still using today. Also a big thank you to you, **Wytske**. Thank you for giving me valuable insights into myself and for teaching me to prevent and to deal with difficult moments. With all my heart I encourage those of you who struggle to seek help; you are not alone!

Lieber **Herr Bramstedt**, Sie wissen es vielleicht nicht, aber Sie sind der Grund warum ich meinen Bachelor in Biologie begonnen habe. Sie haben wirklich einen sehr grossen Einfluss gehabt auch meinen Werdegang und ich war immer mächtig stolz von Ihnen zu hören. Danke für alles!

Edgar, gracias por todo! Tu eres el mejor novio del mundo (and that is where my Spanish ends, but I am working on it!). Thank you so much for your unconditional love and support no matter what. Thank you for never giving up on me! I cannot wait to build a future with you! Te amo mucho!

Liebe Herde, ihr seid die Besten und ich liebe euch über alles! Liebe **Mama**, lieber **Papa**, liebe **Franziska** und lieber **Sebastian**. Danke, dass ihr immer für mich da wart! Ich weiß, dass es nicht immer einfach war mit mir, wenn ich mal wieder “abgetaucht” bin und in meiner Blase hier in Wageningen verschwunden bin. Ich weiß auch, dass es schwierig war mich manchmal so leiden zu sehen, aber ich danke euch sehr, dass ihr immer da wart für mich. Ich bin so stolz eine so tolle Familie zu haben und ich lieb euch über alles! Ein großes Dankeschön an dich, Mama, für die Inspiration meines Titels und den ‘About the author’ Text.

Thank you!

ABOUT THE AUTHOR

Katharina Hanika was born in Coesfeld, North-Rhine Westphalia, Germany, on 22 August 1992 – five weeks ahead of time, characterising the pronounced trait of curiosity throughout her life: After having watched TV for the first time at the age of 18 months, she ran behind the TV set in order to find out where all the animals in the programme had gone …

She spent two years in Hamburg from 1993 to 1995. Since Katharina's father serves in the German Armed Forces, the family subsequently moved right through the Republic to the southern part of Germany near Lake Constance for another two years before ending up in Great Britain from 1997 to 1998. Now, the Hanika number was five. After six years of secondary education at the Konrad-Adenauer-Gymnasium in Meckenheim, two important decisions were taken: First, she moved to Great Britain again for 9 months – this time alone – to attend the Royal Hospital School in Ipswich. After the GB experience, Katharina was now curious for new things at home, hence no longer willing to return to her old school. Her parents succeeded in finding an alternative with the Friedrich-Ebert-Gymasium in Bonn. Here, Katharina passed her German Abitur and the International Baccalaureate in English in 2011. Her teacher Uwe Bramstedt helped her discover the passion for biology. We took it almost for granted that Katharina – Little Miss Helpful, the eldest of three, no comments – might become a primary school teacher. But at the end of her school time, she changed plans, turning to biology thanks to Mr Bramstedt. As a result, she made her Bachelor's Degree in Göttingen at the Georg-August-Universität in 2014 with a BSc thesis supervised by Hassan Ghareeb in the laboratory of Volker Lipka – after having forced her entire family to climb trees, walk through the woods, and pick any sorts for plants for her herbarium.

In autumn 2014, she started her new academic life in Wageningen. After two years, she successfully passed her Master of Science in Plant Biotechnology with a MSc thesis supervised by Michaela Appiano in the group of Yuling Bai at Wageningen University (WUR). As of September 2016, Katharina was a PhD candidate at the Laboratory of Plant Breeding and the Laboratory of Phytopathology, working on the TKI project "Identification of genes in tomato and other crops for resistance or susceptibility to Verticillium wilt" together with her colleague Jasper Vermeulen. With this project, Katharina stayed on the suseptiblity gene track which she got hooked on during one of Yuling's lectures.

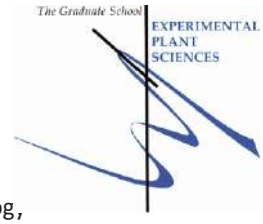
Besides being actively involved in department activities, Katharina found her passion for using eLabjournal on a daily basis in 2016. Together with her close colleague Eliana Papoutsoglou, she developed and gave workshops at WUR; she was also invited to national and international meetings. The objective was to train university staff and students to get started with eLabjournal. Since December 2018, Katharina has been a reference customer for eLabjournal, helping others to get familiar with the system.

The second important decision during her teenage time was to ensure her participation in international belly dance classes with teachers from France, Turkey, and England. This provided her stage experience and performance, finally making her "the" ISOW belly dance teacher for more than 6 years in Wageningen. The ladies helped her dancing her PhD (and dancing her through her PhD): The group won the biology category of Dance Your PhD devised by the Science Magazine. Despite Covid-19, she has found ways and means to keep teaching her belly dance ladies, either online or in a park.

In August – you remember, Katharina does not like to be late (e.g., seeing the light of day) and loves meeting deadlines –, she handed in her PhD thesis within her contract period. So, Katharina was able to move house to Utrecht where she started working as a researcher in the start-up company Jongerius Research.

Written by Hanna Hanika, Katharina's mum

EDUCATION STATEMENT OF THE GRADUATE SCHOOL EXPERIMENTAL PLANT SCIENCES



Issued to: Katharina Hanika
Date: 11 December 2020
Group: Laboratory of Plant Breeding & Laboratory of Phytopatholog,
University: Wageningen University & Research

1) Start-Up Phase	date	cp
► **First presenation of your project**		
Impairing tomato susceptibility genes to obtain resistance to Verticillium wilt	28 Jun 2016	1.5
► **Writing or rewriting a project proposal**		
Impairing tomato susceptibility genes to obtain resistance to Verticillium wilt	Sep 2016	3.0
► **MSc courses**		
Subtotal Start-up Phase		4.5

2) Scientific Exposure	date	cp
► **EPS PhD student days**		
EPS PhD student days 'Get2Gether', Soest (NL)	09 - 10 Feb 2017	0.6
EPS PhD student days 'Get2Gether', Soest (NL)	15 - 16 Feb 2018	0.6
► **EPS theme symposia**		
EPS theme 2: Interactions between plants and biotic agents, Wageningen University (NL)	23 Jan 2017	0.3
EPS theme 2: Interactions between plants and biotic agents, Wageningen University (NL)	01 Feb 2019	0.3
► **Lunteren Days and other national platforms**		
Annual Meeting 'Experimental Plant Sciences', Lunteren (NL)	10 - 11 Apr 2017	0.6
Annual Meeting 'Experimental Plant Sciences', Lunteren (NL)	08 - 09 Apr 2019	0.6
► **Seminars (series), workshops and symposia**		
Seminars		
EPS Seminar Dr. Sotirios Fragkostefanakis "Alternative splicing of a heat stress transcription factor mediates thermotolerance in tomato"	02 Nov 2016	0.1
EPS Seminar Mark Varrelmann "The rhizomania complex in sugar beet - virus variation and resistance breaking"	18 April 2017	0.1
KNAW "CRISPR-Cas – from evolution to revolution"	08 Mar 2018	0.2
PHP Seminar Sylvia Brugman "Intestinal immunity in fish"	13 Apr 2018	0.1
PBR Seminar Mary C. Wildermuth "Salycilic acid and cell cycle manipulation take center stage"	25 Jun 2018	0.1
Wageningen Molecular Life Science Seminar Prof. Wolf Frommer "Logistics: allocation of carbon and energy for yield and pathogen resistance"	17 Jan 2019	0.1
PHP Seminar Luigi Faino "Insights into Nanopore MinION sequencing"	22 Feb 2019	0.1

PHP Seminar Guido v.d. Ackerveken "Why are my leafy greens having these downy mildews?"	08 Mar 2019	0.1
Ritzema Bos Lecture Nick Talbot "Investigating the biology of plant infection by rice blast fungus *Magnaporthe oryzae*"	02 Apr 2019	0.1
Seminar of two invited speakers (inaugural address Yuling Bai): Ralph Panstruga "Phenotypic and molecular characterization of partially *mlo*-virulent isolates of the barley powdery mildew pathogen (*Blumeria graminis* f.sp. *hordei*)" and Jaime Prohens "Introgression breeding from wild species for crops adaptation to climate change"	23 May 2019	0.2
PHP Seminar Dr. Jijie Chai "Structure, mechanism and biochemical insight of plant NLR protein"	05 Jun 2019	0.1
Ritzema Bos Lecture Malcolm Bennett "Uncovering the hidden half of plants: revealing how roots adapt to water availability"	14 Oct 2019	0.1
Open Plant Pathology Seminar Luigi Faino "Evolution of mini-chromosomes in Fusarium"	25 May 2020	0.1
Workshops		
Workshop "Plants & Patents"	21 Oct 2019	0.2
Symposia		
WURomics Symposium 2016	15 Dec 2016	0.3
PHP Mini Symposium: Applied Phytopathology	01 Mar 2017	0.2
PHP Mini Symposium: Ronnie de Jonge & Andrea Sanchez-Vallet	20 Feb 2019	0.2
► **Seminar plus**		
Phytopathology PhD master class Nick Talbot (Ritzema Bos Lecture), Wageningen University (NL)	02 April 2019	0.1
► **International symposia and congresses**		
Host-Microbe Genetics Meeting 2018, University Medical Center Groningen (NL)	26 Oct 2018	0.3
XVIII Congress on Molecular Plant-Microbe Interactions, Glasgow (UK)	14 - 18 Jul 2019	1.2
► **Presentations**		
Presentation at TKI-U project kick-off meeting (KV1409 026): "Identification of genes in tomato and other crops for resistance or susceptibility to Verticillium wilt", Wageningen University (NL)	30 May 2016	1.0
Poster at Annual meeting XVIII Congress on Molecular Plant-Microbe Interactions: "Expression analysis-based selection of candidates to screen for reduced susceptibility to Verticillium dahliae in tomato", Glasgow (UK)	14 - 18 Jul 2019	1.0
Presentation at 10th PhD Summer School Environmental Signalling in Plants: "Expression analysis-based selection of candidates to screen for reduced susceptibility to Verticillium dahliae in tomato", Utrecht (NL)	27 Aug 2019	1.0
► **3rd year interview**		
► **Excursions**		
EPS excursion to TomatoWorld, Honselersdijk (NL)	14 Oct 2016	0.2
EPS excursion Averis Seeds, Veendam (NL)	07 Jul 2019	0.2
	Subtotal Scientific Exposure	10.4

3) In-Depth Studies	*date*	*cp*
► **Advanced scientific courses & workshops**		
Data analyses and visualization in R, Wageningen University (NL)	12 - 13 Dec 2016	0.6
Introduction to R for Statistical Analysis (PE&RC & WIMEK), Wageningen University (NL)	13 - 14 May 2019	0.6
SLU-WUR Symposium Plant Breeding and Biotechnology, Wageningen (NL)	11 - 13 Jun 2019	1.6
10th PhD Summer school – Environmental Signalling in Plants, Utrecht (NL)	26 - 28 Aug 2019	0.9
► **Journal club**		
► **Individual research training**		
	Subtotal In-Depth Studies	3.7

4) Personal Development	*date*	*cp*
► **General skill training courses**		
EPS Introduction course, Wageningen University (NL)	16 Feb 2017	0.3
WGS PhD workshop carousel	07 April 2017	0.3
PhD Peer Consultation (WGS), Wageningen University (NL)	Sep 2017 - Jan 2018	0.3
Brain training (WGS), Wageningen University (NL)	08 Nov 2017	0.3
Scientific publishing (WGS), Wageningen University (NL)	05 Apr 2018	0.3
Participation PBR PhD discussion club (peer consultation), Wageningen University (NL)	Apr 2018 - Aug 2020	1.3
Brain friendly working and writing (WGS), Wageningen University (NL)	06 Mar 2019	0.3
Efficient Writing Strategies (Wageningen Into Languages), Wageningen University (NL)	Apr - Jun 2019	1.3
Career Orientation (WGS), Wageningen University (NL)	Oct 2019	1.5
Adobe InDesign (WGS), Wageningen University (NL)	04 - 05 Nov 2019	0.6
Infographics and Iconography (WGS), Wageningen University (NL)	26 Nov 2019	0.3
EPS Postdoc Career Day, Wageningen University (NL)	07 Feb 2020	0.3
edX online course "Analytics storytelling for impact" (DAT248X)	Apr 2020	0.3
edX online course "Creative thinking: Techniques and tools for success" (CTT102)	Apr 2020	0.3
Young WUR online workshop Ben Hartwig "Resilience through applied improvisation"	07 May 2020	0.1
edX online course "Framing your communication to inspire and convince" (LEfE3x)	May - Jun 2020	0.3
edX online course "Open Science: Sharing your research with the world" (OS101X)	May - Jun 2020	0.3
► **Organisation of meetings, PhD courses or outreach activities**		
Organization PBR PhD discussion club, Wageningen University (NL)	Apr 2017 - Apr 2018	0.5
Organization and administration of eLabjournal workshops, Wageningen University (NL)	Apr 2017 - Feb 2019	1.0

Presentation at Digital notebooks event (organizer: Marta Teperek): "From paper to screen - What users really think of electronic lab notebooks", TU Delft (NL)	15 Mar 2018	0.5
Presentation at Research Notebooks Software Demo Day (organizer Valerie McCutcheon): "Behind the scenes: What users really think of electronic lab notebooks - eLabjournal case study", University of Glasgow (UK)	18 Mar 2019	0.5
Participation in 12th annual "Dance your Ph.D." contest organized by Science and AAAS	21 Jan 2020	0.5
► **Membership of EPS PhD Council**		
Subtotal Personal Development		11.4

5) Teaching & Supervision Duties	<u>date</u>	<u>cp</u>
► **Courses**		
PHP-30306 Plant-Microbe Interactions, Wageningen University (NL)	2017 - 2019	1.5
PBR-30306 Breeding for Quality & Resistance, Wageningen University (NL)	2018	0.1
► **Supervision of BSc/MSc students**		
MSc major thesis student Lisa Mulder "Discovery and functional analysis of candidate *S* genes in pepper (*Capsicum annuum*) against *Phytophthora capsici*"	Sep 2017 - Mar 2018	1.0
HBO Internship student Gökhan Gökmen "The search for susceptibility genes"	Feb - Jul 2018	0.0
MSc major thesis student Jillis Grubben "Screening for loss-of-susceptibility to *Verticillium dahliae* in tomato using virus induced gene silencing"	Sep 2018 - Mar 2019	1.0
MSc major thesis student Timo d'Hont "Design and analysis of VIGS and CRISPR-Cas9 constructs for loss-of-susceptibility to *Verticillium dahliae* in tomato (*Solanum lycopersicum*)"	Jan - Jul 2019	1.0
MSc major thesis student Dagmar Kuiper "Studying potential *S* gene candidates for *Verticillium dahliae* in tomato (*Solanum lycopersicum*) using VIGS and CRISPR-Cas9"	Jul 2019 - Jan 2020	0.0
MSc major thesis student Shravya Chinnappa "Analysing CRISPR-Cas9 and RNAi lines targeting *S* gene candidates for loss-of-susceptibility to *Verticillium dahliae* in *Solanum lycopersicum*"	Jul 2019 - Jan 2020	0.0
Subtotal Teaching & Supervision Duties		3.0

TOTAL NUMBER OF CREDIT POINTS*	34.6

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

The research described in this thesis was funded through the Topconsortium voor Kennis en Innovatie (TKI) Tuinbouw & Uitgangsmaterialen, with project number 1409-026, which is (partly) financed by the breeding companies Bejo Zaden, BASF, EastWest Seed, Hazera, Rijk Zwaan, Syngenta and Takii Seed.

Financial support from Wageningen University for the printing of this thesis is gratefully acknowledged.

Cover design: Sabine Hoek
Layout design: Katharina Hanika
Printing: ProefschriftMaken | www.proefschriftmaken.nl