Comparative genomics reveals the in planta-secreted *Verticillium dahliae* Av2 effector protein recognized in tomato plants that carry the V2 resistance locus

Edgar A. Chavarro-Carrero, Jasper P. Vermeulen, David E. Torres, Henk J. Schouten, Yuling Bai, Michael F. Seidl, and Bart P. H. J. Thomma

**Summary**

Plant pathogens secrete effector molecules during host invasion to promote colonization. However, some of these effectors become recognized by host receptors to mount a defence response and establish immunity. Recently, a novel resistance was identified in wild tomato, mediated by the single dominant V2 locus, to control strains of the soil-borne vascular wilt fungus *Verticillium dahliae* that belong to race 2. With comparative genomics of race 2 strains and resistance-breaking race 3 strains, we identified the avirulence effector that activates V2 resistance, termed Av2. We identified 277 kb of race 2-specific sequence comprising only two genes encoding predicted secreted proteins that are expressed during tomato colonization. Subsequent functional analysis based on genetic complementation into race 3 isolates and targeted deletion from the race 1 isolate JR2 and race 2 isolate TO22 confirmed that one of the two candidates encodes the avirulence effector Av2 that is recognized in V2 tomato plants. Two Av2 allelic variants were identified that encode Av2 variants that differ by a single acid. Thus far, a role in virulence could not be demonstrated for either of the two variants.

**Introduction**

In nature, plants are continuously threatened by potential plant pathogens. However, most plants are resistant to most potential plant pathogens due to an efficient immune system that becomes activated by any type of molecular pattern that accurately betrays microbial invasion (Dangl and Jones, 2001; Cook et al., 2015). Throughout time, different conceptual frameworks have been put forward to describe the molecular basis of plant–pathogen interactions and the mechanistic underpinning of plant immunity. Initially, Harold Flor introduced the gene-for-gene model in which a single dominant host gene, termed a resistance (R) gene, induces resistance in response to a pathogen expressing a single dominant avirulence (Avr) gene (Fior, 1942). Isolates of the pathogen that do not express the allele of the Avr gene that is recognized escape recognition and are assigned to a resistance-breaking race. In parallel to these race-specific Avrs, non-race-specific effectors were described as conserved microbial molecules that are often recognized by multiple plant species (Darvill and Albersheim, 1984). The recognition by plants of Avrs and of non-race-specific elicitors, presently known as pathogen- or microbe-associated molecular patterns (P/MAMPs), was combined in the ‘zig-zag’ model (Jones and Dangl, 2006). In this model, P/MAMPs are perceived by cell surface-localized pattern recognition receptors (PRRs) to trigger pattern-triggered immunity (PTI), while effectors are recognized by cytoplasmic receptors that are known as resistance (R) proteins to activate effector-triggered immunity (ETI) (Jones and Dangl, 2006). Importantly, the model recognizes that Avrs function to suppress host immune responses in the first place, implying that these molecules, besides being avirulence determinants, act as virulence factors through their function as effector molecules (Jones and Dangl, 2006). A more recent model, termed the invasion model, recognizes that the functional separation of
PTI and ETI is problematic and proposes that the corresponding receptors, collectively termed invasion pattern receptors (IPRs), detect either externally encoded or self-modified ligands that indicate invasion, termed invasion patterns (IPs), to mount an effective immune response (Thomma et al., 2011; Cook et al., 2015). However, it is generally appreciated that microbial pathogens secrete dozens to hundreds of effectors to contribute to disease establishment, only some of which are recognized as Avrs (Rovenich et al., 2014).

IPRs encompass typical R genes, which have been exploited for almost a century to confer resistance against plant pathogens upon introgression from sexually compatible wild relatives into elite cultivars (Dodds and Rathjen, 2010; Dangl et al., 2013). Most R genes encode members of a highly polymorphic superfamily of intracellular nucleotide-binding leucine-rich repeat (NLR) receptors, while others encode cell surface receptors (Dangl et al., 2013). Unfortunately, most R genes used in commercial crops are short-lived because the resistance that they provide is rapidly broken by pathogen populations as their deployment in monoculture-based cropping systems selects for rapidly broken strains that are contained by Ve1 belong to race 1 (Alexander, 1962). Thus, Ve1 is characterized as a race-specific R gene, and resistance-breaking strains have become increasingly problematic over time (Alexander, 1962; Dobinson et al., 1996). With comparative population genomics of race 1 and race 2 strains, the V. dahliae avirulence effector that is recognized by tomato Ve1 was identified as VdAve1, an effector that is secreted during host colonization (de Jonge et al., 2012). As anticipated, it was demonstrated that VdAve1 acts as a virulence factor on tomato plants that lack the Ve1 gene and that, consequently, cannot recognize VdAve1 (de Jonge et al., 2012). Recent evidence demonstrates that VdAve1 exerts selective antimicrobial activity and has the capacity to manipulate local microbiomes inside host plants as well as in the environment (Snelders et al., 2020). Whereas all race 1 strains carry an identical copy of VdAve1, all race 2 strains analysed to date are characterized by complete loss of the VdAve1 locus (de Jonge et al., 2012; Faino et al., 2016). Intriguingly, phylogenetic analysis has revealed that VdAve1 was horizontally acquired by V. dahliae from plants (de Jonge et al., 2012; Shi-Kunne et al., 2018), after which the effector gene was lost multiple times independently, presumably due to selection pressure exerted by the Ve1 locus that has been introgressed into most tomato cultivars (Faino et al., 2016).

Despite significant efforts, attempts to identify genetic sources for race 2 resistance in tomato have remained unsuccessful for a long time (Baergen et al., 1993). Recently, however, a source of race 2 resistance was identified in the wild tomato species Solanum neoricikii (Usami et al., 2017). This genetic material was used to develop the tomato rootstock cultivars Aibou, Ganbarune-Karis and Back Attack by Japanese breeding companies, in which resistance is controlled by a single dominant locus, denoted V2 (Usami et al., 2017). However, experimental trials using race 2-resistant rootstocks revealed resistance-breaking V. dahliae strains that, consequently, are assigned to race 3 (Usami et al., 2017). In this study, we performed comparative genomics combined with functional assays to identify the avirulence effector Av2 that activates race-specific resistance in tomato genotypes that carry V2.
Results

Identification of Verticillium dahliae strains that escape V2 resistance

To identify Av2 as the V. dahliae gene that mediates avirulence on tomato V2 plants, we pursued a comparative genomics strategy by searching for genomic regions that are absent from all race 3 strains. To this end, we performed pathogenicity assays with a collection of V. dahliae strains on a differential set of tomato genotypes, comprising (I) Moneymaker plants that lack V. dahliae resistance genes, (II) Ve1-transgenic Moneymaker plants that are resistant against race 1 and not against race 2 strains (Fradin et al., 2009), and (III) Aibou plants that carry Ve1 and V2 and are therefore resistant against race 1 as well as race 2 strains (Usami et al., 2017) (Fig. 1A).

First, we aimed to confirm the race assignment of eight V. dahliae strains that were previously tested by Usami et al. (2017) (Table 1). Additionally, three strains that were previously assigned to race 2 were included (de Jonge et al., 2012) as well as V. dahliae strain JR2 (race 1) because of its gapless telomere-to-telomere assembly (Faino et al., 2015).

At 3 weeks post inoculation, all strains caused significant stunting on the universally susceptible Moneymaker control (Fig. 1A and B), while all strains except for the race 1 strain JR2 caused significant stunting on Ve1-transgenic Moneymaker plants (Fig. 1A and C), corroborating that, except for strain JR2, none of the strains belongs to race 1 and that a potential containment on Aibou plants cannot be caused by Ve1 recognition of the VdAve1 effector. Importantly, all of the strains that were used by Usami et al. (2017) and that were previously assigned to race 2 did not cause significant stunting on Aibou, whereas all of the strains that were assigned to race 3 caused clear symptoms of Verticillium wilt disease (Fig. 1, Table 1; Usami et al., 2017). The previously assigned race 2 strain DVDS26 (de Jonge et al., 2012) caused no significant stunting on Aibou plants, confirming that this remains a race 2 strain, while strains DVD161 and DVD3 caused significant stunting, implying that these strains should actually be assigned to race 3.

As expected, the race 1 strain JR2 did not cause stunting on Aibou plants, which can at least partially be attributed to VdAve1 effector recognition by the Ve1 gene product in these plants. However, the finding that a transgenic VdAve1 deletion line (JR2ΔAve1; de Jonge et al., 2012) caused significant stunting on Ve1-transgenic Moneymaker and not on Aibou plants, indicates that the JR2 strain might also encode Av2. Currently, it is not known whether this is the case, or whether it is simply that basal defence is enhanced in the absence of Ave1. After all, we previously showed that the virulence of the VdAve1 deletion strain on tomato is severely compromised (de Jonge et al., 2012), which can also be observed on Moneymaker plants in our assays (Fig. 1B). This observation, combined with the observation that stunting on Aibou plants by any race 3 strain is generally less than stunting on Moneymaker plants (Fig. 1B and D), could indicate that basal defence against Verticillium wilt is enhanced in Aibou plants, and thus that incompatibility of the VdAve1 deletion strain may be due to enhanced basal defence rather than due to V2-mediated recognition of the JR2 strain.

Comparative genomics identifies Verticillium dahliae Av2 candidates

Besides the gapless genome assembly of strain JR2 (Faino et al., 2015), genome assemblies were also available for strains DVDS26, DVD161 and DVD3, albeit that these assemblies were highly fragmented as these were based on Illumina short-read sequencing data (de Jonge et al., 2012) (Table 1). In this study, we determined the genomic sequences of the race 2 strains TO22, UD1-4-1, GF1207 and GFCA2, and the race 3 strains GF-CB5, GF1192, VT2A and HOMCF with Oxford Nanopore sequencing Technology (ONT) using a MinION device (Table 1). For each strain, ~2–4 Gb of sequence data was produced, representing 50–100x genome coverage based on the ~35 Mb gapless reference genome of V. dahliae strain JR2 (Faino et al., 2015). Subsequently, we performed self-correction of the reads, read trimming and genome assembly, leading to genome assemblies ranging from 18 contigs for strain UD1-4-1 to 69 for strain GF1207 (Table 1).

Based on the genome sequences, we pursued comparative genomics analyses by exploring two scenarios. The first scenario is that Av2 is race 2-specific and thus present in race 2 lineage sequences while absent from race 3. The second scenario is that Av2 is present in isolates that belong to race 1 and race 2, but that the resistance phenotype against race 2 is masked by Ve1 resistance directed against Ave1. In scenario I, comparative genomics was performed making use of race 2 strain TO22 (Usami et al., 2017) as a reference, while in scenario II race 1 strain JR2 (Faino et al., 2015) was used (Table 2).

To this end, self-corrected reads from the V. dahliae race 3 strains were mapped against the assembly of V. dahliae strain TO22 (scenario I) or strain JR2 (scenario II) and regions that were not covered by race 3 reads were retained (Table 2). Next, self-corrected reads from the race 2 strains were mapped against the retained reference genome-specific regions that are absent from the race 3 strains, and sequences that were found in every race 2 strain were retained as candidate regions to encode the Avr molecule. Sequences that are shared by the V. dahliae strain TO22 reference assembly and all race 2 strains, and that are absent from all race 3 strains, were
mapped against the *V. dahliae* strain JR2 genome assembly, and common genes were extracted. Sequences that did not map to the *V. dahliae* strain JR2 genome assembly were de novo annotated and signal peptides for secretion at the N-termini of the encoded proteins were predicted to identify potential effector genes.

Our strategy identified 563 kb of race 2-specific regions, containing 110 genes of which six encode putative secreted proteins, for scenario I (Table 2). For approach II, 222 kb of sequence that lacks in race 3 strains was identified with 40 genes of which only two are predicted to encode secreted proteins; XLOC_00170 (VDAG_JR2_Chr4g03680a) and evm.model.contig1569.344 (VDAG_JR2_Chr4g03650a), further referred to as Evm_344. Intriguingly, both these genes were previously recognized as being among the most highly expressed effector genes during colonization of *Nicotiana benthamiana* plants (de Jonge et al. 2013; Faino et al., 2015).
Only two of the Av2 candidates are expressed in planta

We anticipate that the genuine Av2 gene may not necessarily be expressed in N. benthamiana (de Jonge et al., 2013) but should be expressed particularly in tomato. Real-time PCR analysis on a time course of tomato cultivar Moneymaker plants inoculated with the V. dahliae JR2 strain revealed that the two candidate genes are expressed during tomato colonization, with a peak in expression around 7 days post inoculation, whereas little to no expression could be recorded upon growth in vitro (Fig. 2A). Both genes are similarly expressed in V. dahliae strain TO22, albeit that the expression peaks slightly later, at 11 dpi (Fig. 2B). However, whereas the expression level of both genes is similar in V. dahliae strain JR2, Evm_344 is higher expressed than XLOC_00170 in V. dahliae strain TO22. Importantly, none of the four additional avirulence effector gene candidates that were identified in comparative genomics scenario I is expressed in planta in V. dahliae strain TO22 (Fig. 2B). Thus, based on the transcriptional profiling, these four avirulence effector genes can be disqualified as Av2 candidates, and only two genes that display an expression profile that can be expected for a potential avirulence effector gene remain; XLOC_00170 and Evm_344.
XLOC_00170 encodes Av2

To identify which of the two candidates encodes Av2, a genetic complementation approach was pursued in which the two candidate genes were introduced individually into the V. dahliae race 3 strains GF-CBS and HOMCF. Subsequently, inoculations were performed on a differential

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*Fig. 2.* Expression of *V. dahliae* candidate avirulence effector genes *in vitro* and during colonization of tomato plants. To assess *in planta* expression, 12-day-old tomato cv. Moneymaker seedlings were root-inoculated with *V. dahliae* strain JR2 (A) or strain TO22 (B), and plants were harvested from 4 to 14 days post inoculation (dpi), while conidiospores were harvested from 5-day-old cultures of *V. dahliae* on potato dextrose agar (PDA) to monitor *in vitro* expression. Real-time PCR was performed to determine the relative expression of *XLOC_00170, Evm_344* and the race 1-specific effector gene *VdAve1* as a positive control (de Jonge *et al.*, 2012) for strain JR2, using *V. dahliae* GAPDH as reference (A). Similarly, the relative expression of *XLOC_00170, Evm_344* and six additional avirulence effector genes for strain TO22, using *V. dahliae* GAPDH as reference (B).
set of tomato genotypes, comprising Moneymaker plants, Ve1-transgenic Moneymaker plants (Fradin et al., 2009), and Aibou plants (Usami et al., 2017). As expected, the non-transformed race 3 strains GF-CB5 and HOMCF as well as the complementation lines containing XLOC_00170 or Evm_344 caused clear stunting of the universally susceptible Moneymaker as well as of the Ve1-transgenic Moneymaker plants (Fig. 3A and B). Interestingly, non-transformed race 3 strains GF-CB5 and HOMCF and the Evm_344 complementation lines caused clear stunting on Aibou plants, whereas the XLOC_00170 complementation lines did not induce disease symptoms and stunting on these plants (Fig. 3A and B). As such, these complementation transformants of the race 3 strains GF-CB5 and HOMCF behaved essentially as the race 2 strain TO22 (Fig. 3A and B). Thus, these findings suggest that XLOC_00170 encodes Av2. All visual observations of stunting were supported by quantifications of fungal biomass by real-time PCR (Fig. 3C). These measurements revealed that fungal biomass levels were only reduced on Aibou plants when inoculated with the race 2 strain TO22, and with the race 3 strains GF-CB5 and HOMCF that were complemented with XLOC_00170. Thus, our data confirm that reduced symptomatology is accompanied by significantly reduced fungal colonization and indicate that XLOC_00170 encodes the race 2-specific avirulence effector Av2.

To further confirm that XLOC_00170 encodes Av2, targeted gene deletions were pursued in race 2 strain TO22 as well as in the JR2ΔAvr1 strain and inoculations were performed on Moneymaker plants, Ve1-transgenic Moneymaker plants (Fradin et al., 2009), and Aibou plants (Usami et al., 2017). All V. dahliae genotypes caused clear stunting on wild-type and Ve1-transgenic Moneymaker plants, except for wild-type JR2 on Ve1-transgenic Moneymaker plants (Fig. 4A and B). Interestingly, whereas V. dahliae strains TO22 and JR2ΔAvr1 were contained on Aibou plants, the XLOC_00170 deletion strain caused stunting of these plants in a similar fashion as the race 3 strains GF-CB5 and HOMCF (Fig. 4C). All visual observations were supported by quantification of biomass by real-time PCR (Fig. 4C). Collectively, our data unambiguously demonstrate that XLOC_00170 encodes the Av2 effector that is recognized on V2 tomato plants.

Av2 does not seem to contribute to virulence

It has been widely recognized that the intrinsic function of Avrs is to support host colonization by acting as virulence determinants (Jones and Dangl, 2006; Rovenich et al., 2014; Cook et al., 2015). Thus, we assessed the virulence of the complementation lines alongside their wild-type progenitor genotypes on wild-type and Ve1-transgenic Moneymaker plants (Fig. 3). However, no significant increase in symptomatology nor in fungal colonization could be recorded upon Av2 introduction. Similarly, no

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significant decrease in symptomatology, nor a decrease in fungal colonization could be recorded upon inoculation with V. dahliae. A top pictures of Moneymaker plants that lack known resistance genes (MM), a. Top pictures of Moneymaker plants that lack known resistance genes (MM), Ve1-transgenic Moneymaker plants that are resistant against race 1 and not against race 2 strains of V. dahliae (SSS:Ve1), and Abou plants that carry Ve1 and V2 and are therefore resistant against race 1 as well as race 2 strains of the pathogen (Usami et al., 2017) inoculated with the race 3 strains GF-CB5 and HOMCF, the race 1 WT strain JR2, the deletion line JR2ΔAv2, one independent knock-out lines of XLOC_00170 in JR2ΔAv2, the race 2 WT strain TO22 and two independent knock-out lines of XLOC_00170 in TO22. B. Quantification of fungal biomass with real-time PCR caused by the various V. dahliae genotypes on the various tomato genotypes as detailed for panel (A). Different symbols (empty circles, filled circles and triangles) refer to five plants from three different experiments. Asterisks indicate significant differences between V. dahliae- and mock-inoculated plants as determined with an ANOVA followed by a Fisher’s LSD test ($P < 0.01$). [Color figure can be viewed at wileyonlinelibrary.com]

Av2 distribution and allelic variation

Av2 encodes a 91 amino acid protein that, after removal of a predicted signal peptide, leaves a mature protein of 73 amino acids that includes four cysteine residues and that lacks known protein domains. Intriguingly, an Av2 homologue is found in V. nonalfalfae (78% identity), V. longisporum (68% identity) and V. alfalfae (49%) that, like V. dahliae, belong to the Flavononexudans clade of Verticillium spp. (Fig. 5; Shi-Kunne et al., 2018). Furthermore, BLAST searches revealed homologues in Fusarium phyllophilum (79% identity), Fusarium mundagurra (78%), F. oxysporum f. sp. narcissi (77%), Fusarium oxysporum NRRL32931 (75%), F. oxysporum f. sp. pisi (73%) and Fusarium sp. NRRL66182 (41%) (Fig. 5). No homologues are found in any of the other Fusarium spp nor in any other species.

To assess Av2 distribution in V. dahliae, presence–absence variations (PAV) were assessed in a collection of 52 previously sequenced V. dahliae strains (Fig. 6; de Jonge et al., 2012; Faino et al., 2015; Fan et al., 2018; Gibriel et al., 2019), revealing that Av2 occurred in 17 of the isolates including the four race 2 isolates that were sequenced in this study (Fig. 6). To assess the phylogenetic relationships between strains that carry Av2, a phylogenetic tree was generated, showing that the strains can be grouped into three major clades, two of which comprising strains that contain Av2. However, within these clades closely related strains occur that lost Av2, suggesting the occurrence of multiple independent losses (Fig. 6). Overall, no obvious phylogenetic structure is apparent with respect to effector presence within the V. dahliae population.

Next, we investigated the genomic organization surrounding Av2 based on the gapless genome assembly of V. dahliae strain JR2 (Faino et al., 2015). Interestingly, Av2 resides in close proximity to Evm_344, separated by only two additional genes, in a lineage-specific (LS) region on chromosome 4 (Fig. 7). Furthermore, as typically observed in LS regions that are enriched in repetitive elements (de Jonge et al., 2013; Faino et al., 2016), Av2 is surrounded by repetitive elements such as transposons that mostly belong to the class II long terminal repeat (LTR) retrotransposons (Fig. 7). Typically, LS regions are characterized by the high abundance of PAV. As expected, the flanking genomic regions (100 kb) are highly variable between V. dahliae strains (Fig. 7).

As many Avr effectors are under strong selection pressure and thus often display enhanced allelic variation (Stergiopoulos et al., 2007), we assessed allelic variation among the 17 Av2 alleles identified in this study. We
identified only two allelic variants within the 17 Av2 alleles that differed by a single nucleotide polymorphism (SNP) in exon 3 leading to a polymorphic amino acid at position 73. Whereas 10 isolates carry a glutamic acid at this position (E73), seven other carry a valine (V73) (Fig. 8). Interestingly, strains carrying V73 are clustered in the same branch, suggesting that a single event caused this polymorphism (Fig. 6). We noticed that all isolates carrying E73 carry an extra transposable element of the DNA/TC-1 Mariner class in the upstream region of the Av2 gene (Fig. 8). Intriguingly, as strains GF-Ca2, TO22, UD-1-4-1 DVDS26 and GF1207, that encode the Av2 variant with E73, as well as JR2ΔAve1, that encodes the variant with E73, are contained on Aibou plants, we conclude that both allelic variants are recognized by V2. Moreover, the Av2 deletion strain of TO22 (with V73) as well as of JR2ΔAve1 (with E73) is not compromised in aggressiveness on wild-type Moneymaker plants when compared with the TO22 or JR2ΔAve1 progenitor strain, indicating that both alleles make no noticeable contribution to V. dahliae virulence.

Discussion

Historically, the identification of avirulence genes has been challenging for fungi that reproduce asexually, as genetic mapping cannot be utilized. However, since the advent of affordable genome sequencing, cumbersome and laborious methods to identify avirulence genes, that include functional screenings of fungal cDNAs or protein fractions for the induction of immune responses in plants (Takken et al., 2000; Luderer et al., 2002), have been supplemented with comparative genomics and transcriptomics strategies (Gibriel et al., 2016). Less than a decade ago, we identified the first avirulence gene of V. dahliae, known as VdAve1 for mediating avirulence on Ve1 plants, through a comparative population genomics strategy combined with transcriptomics by utilizing race 1 strains that were contained by the Ve1 resistance gene of tomato, and resistance-breaking race 2 strains (de Jonge et al., 2013). In this study, we used a similar approach based on comparative population genomics of race 1 and 2 strains with race 3 strains to successfully identify XLOC_00170 as the Av2 effector that mediates avirulence on V2 plants. Intriguingly, besides VdAve1, XLOC_00170 has been identified previously as one of the most highly induced genes of V. dahliae during host colonization (de Jonge et al., 2013).

Ve1 and the V2 locus are the only two major resistance sources that have been described in tomato against V. dahliae thus far (Fradin et al., 2009; Usami et al., 2017). Since its initial introduction from a wild Peruvian tomato accession into cultivars in the 1950s (Deseret News and Telegram, 1955), Ve1 has been widely exploited as it is incorporated in virtually every tomato cultivar today. Even though soon after the introduction of these cultivars resistance-breaking race 2 strains emerged, first in the United States (Robinson, 1957; Alexander, 1962), and soon thereafter also in Europe (Cirilli, 1969; Pegg and Dixon, 1969), Ve1 is still considered useful for Verticillium wilt control today. An important factor that contributes to the durability of resistance is the fitness penalty for the pathogen upon losing the corresponding avirulence factor (Brown, 2015). The VdAve1 effector contributes considerably to V. dahliae virulence on tomato, which explains why race 2 strains that lack VdAve1 are generally less aggressive (de Jonge et al., 2012). Based on our current observations that differences in aggressiveness between race 2 and race 3 strains on Moneymaker plants are not obvious (Fig. 1), that genetic complementation of race 3 strains with Av2 did not lead to a striking increase in aggressiveness on Moneymaker plants (Fig. 3), and that targeted deletion of Av2 from race 2 strains did not lead to a striking decrease in aggressiveness on Moneymaker plants (Fig. 4), we conclude that the contribution of Av2 to V. dahliae virulence under the conditions tested in this study is modest at most.

Thus far, V2 resistance has been exploited scarcely when compared with Ve1, as it has only been introduced in a number of Japanese rootstock cultivars since 2006 (Usami et al., 2017). Previously, V2 resistance-breaking race 3 strains have been found in several Japanese prefectures on two separate islands (Usami et al., 2017).
Intriguingly, our genome analyses demonstrate that race 3 strains that lack \( \text{Av2} \) are ubiquitous and found worldwide, as our collection of sequenced strains comprises specimens that were originally isolated in Europe, China, Canada, and the United States. Arguably, most of these race 3 strains arose in the absence of \( \text{V2} \) selection by tomato cultivation. It is conceivable that, similar to \( \text{Ve1} \) homologues that are found in other plant species besides tomato (Song et al., 2017), functional homologues of \( \text{V2} \) occur in other plant species as well, which may have selected against the presence of \( \text{Av2} \) in many \( \text{V. dahliae} \) strains. However, as long as \( \text{V2} \) is not cloned this hypothesis cannot be tested.

Like \( \text{VdAve1} \), \( \text{Av2} \) also resides in an LS region of the \( \text{V. dahliae} \) genome, albeit in another region on another chromosome. Typically, these LS regions are gene-sparse and enriched in repetitive elements, such as transposons, causing these regions to be highly plastic which is thought to mediate accelerated evolution of effector catalogues (de Jonge et al., 2013; Faino et al., 2016; Cook, et al., 2020). We previously demonstrated that \( \text{VdAve1} \) has been lost from the \( \text{V. dahliae} \) population multiple times, and to date only \( \text{PAV} \) has been identified as mechanism to escape \( \text{Ve1} \)-mediated immunity (de Jonge et al., 2012, 2013; Faino et al., 2016). Similarly, our phylogenetic analysis reveals that \( \text{Av2} \) has been lost multiple times independently, and although we identified two allelic variants, both variants are recognized by \( \text{V2} \). Consequently, \( \text{PAV} \) remains the only mechanism to overcome \( \text{V2} \)-mediated immunity thus far. Despite the observation that \( \text{PAV} \) is the only observed mechanism for \( \text{V. dahliae} \) to overcome host immunity, pathogens typically exploit a wide variety of mechanisms, ranging from SNPs (Joosten et al., 1994) to altered expression of the avirulence gene (Na and Gijzen, 2016). Nevertheless, avirulence gene deletion to overcome host immunity is common and has been reported for various fungi, including \( \text{C. fulvum} \) (Stergiopoulos et al., 2007), \( \text{Fusarium oxysporum} \) (Niu et al., 2016; Schmidt et al., 2016), \( \text{Leptosphaeria maculans} \) (Gout et al., 2007; Petit-Houdenot et al., 2019), \( \text{Blumeria graminis} \) (Praz et al., 2016) and \( \text{Magnaporthe oryzae} \) (Pallaghy et al., 1994; Zhou et al., 2007).

It was previously demonstrated that frequencies of SNPs are significantly reduced in the area surrounding the \( \text{VdAve1} \) locus when compared with the surrounding genomic regions (Faino et al., 2016), which was thought to point toward recent acquisition through horizontal transfer (de Jonge et al., 2012). However, we recently noted that enhanced sequence conservation through reduced nucleotide substitution is a general feature of LS regions in \( \text{V. dahliae} \) (Depotter et al., 2019). Although a mechanistic underpinning is still lacking, we hypothesized that differences in chromatin organization may perhaps explain this phenomenon. Interestingly, while DNA methylation is generally low and only present at TEs, only TEs in the core genome are methylated while LS TEs are largely devoid of methylation (Cook et al., 2020). Furthermore, TEs within LS regions are more transcriptionally active and display increased DNA accessibility, representing a unique chromatin profile that could contribute to the plasticity of these regions (Faino et al., 2016; Cook et al., 2020). Possibly, the increased DNA accessibility...
contributes to the high \textit{in planta} expression of genes residing in these regions, and \textit{VdAv1} as well as \textit{Av2} belong to the most highly expressed genes during host colonization (de Jonge et al., 2013).

Our identification of \textit{Av2} concerns the cloning of only the second avirulence gene of \textit{V. dahliae}. This identification may permit its use as a functional tool for genetic mapping of the \textit{V2} gene. Typically, \textit{V. dahliae} symptoms on tomato display considerable variability, and disease phenotyping is laborious. Possibly, injections of heterologously produced \textit{Av2} protein can be used to screen tomato plants in genetic mapping analyses, provided that such injections result in a visible phenotype such as a hypersensitive response. Similar effector-assisted resistance breeding has previously been used successfully to identify resistance sources in tomato against the leaf
mould pathogen *Cladosporium fulvum* (Lauge et al., 1998; Takken et al., 1999) and potato against the late blight pathogen *Phytophthora infestans* (Vleeshouwers and Oliver, 2014; Du et al., 2015). The identification of *Av2* can furthermore be exploited for race diagnostics of *V. dahliae* to determine whether cultivation of resistant tomato genotypes is useful, but also to monitor *V. dahliae* population dynamics and race structures. Based on the

**Fig. 8. Allelic variation of Av2 in Verticillium dahliae.**
A. Gene model for Av2. The asterisk indicates the approximate position of the single (A to T) nucleotide substitution in 7 of the 17 isolates that carry the gene, leading to a single amino acid substitution (E73V).
B. Genomic sequence of Av2. The arrow shows the position of the single nucleotide substitution found in particular strains.
C. Alignment of exon 3 of Av2 in the 17 strains containing the avirulence effector gene. The arrow shows the single nucleotide substitution that occurs in seven of the strains when compared with strain JR2.
D. Av2 amino acid sequence as encoded by *V. dahliae* strain JR2 with E73 that is substituted by V in seven isolates indicated by an arrow. [Color figure can be viewed at wileyonlinelibrary.com]
identification of avirulence genes, rapid in-field diagnostics can be developed to aid growers to cultivate disease-free crops.

Materials and methods

V. dahliae inoculation and phenotyping

Plants were grown in potting soil (Potgrond 4, Horticoop, Katwijk, the Netherlands) under controlled greenhouse conditions (Unifarm, Wageningen, the Netherlands) with day/night temperature of 24/18°C for 16-h/8-h periods, respectively, and relative humidity between 50% and 85%. For V. dahliae inoculation, 10-day-old seedlings were root-dipped for 10 min as previously described (Fradin et al., 2009). Disease symptoms were scored at 21 days post inoculation (dpi) by measuring the canopy area to calculate stunting as follows:

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

To test for significant stunting, an ANOVA was performed which tests for significant differences in canopy area between mock-inoculated and V. dahliae inoculated plants. Outliers were detected based on the studentized residuals from the ANOVA analysis. All datapoints with studentized residuals below −2.5 or above 2.5 were classified as outliers and removed. In total, approximately 1.8% of the datapoints were classified as outlier.

High-molecular weight DNA isolation and nanopore sequencing

Conidiospores were harvested from potato dextrose agar (PDA) plates, transferred to Czapek dox medium and grown for 10 days. Subsequently, fungal material was collected on Miracloth, freeze-dried overnight and ground to powder with mortar and pestle of which 300 mg was collected on Miracloth, freeze-dried overnight and ground grown for 10 days. Subsequently, fungal material was transferred to Czapek dox medium and Conidiospores were harvested from potato dextrose agar plates, transferred to Czapek dox medium and

Conidiospores were harvested from potato dextrose agar (PDA) plates, transferred to Czapek dox medium and grown for 10 days. Subsequently, fungal material was collected on Miracloth, freeze-dried overnight and ground to powder with mortar and pestle of which 300 mg was incubated for 1 h at 65°C with 350 μl DNA extraction buffer (0.35 M Sorbitol, 0.1 M Tris-base, 5 mM EDTA pH 7.5), 350 μl nuclease lysis buffer (0.2 M Tris, 0.05 M EDTA, 2 M NaCl, 2% CTAB) and 162.5 μl Sarkosyl (10% w/v) with 1% β-mercaptoethanol. Next, 400 μl of phenol/chloroform/isoamyl alcohol (25:24:1) was added, shaken and incubated at room temperature (RT) for 5 min before centrifugation at 16,000 g for 15 min. After transfer of the aqueous phase to a new tube, 10 μl of RNase (10 mg ml−1) was added and incubated at 37°C for 1 h. Subsequently, half a volume of chloroform was added, shaken and centrifuged at 16,000 g for 5 min at RT, after which the chloroform extraction was repeated. Next, the aqueous phase was mixed with 10 volumes of 100% ice-cold ethanol, incubated for 30 min at RT, and the DNA was fished out using a glass hook, transferred to a new tube, and washed twice with 500 μl 70% ethanol. Finally, the DNA was air-dried, resuspended in nuclease-free water and incubated at 4°C for 2 days. The DNA quality, size and quantity were assessed by Nanodrop, gel electrophoresis and Qubit analyses.

Library preparation with the Rapid Sequencing Kit (SOK-RAD004) was performed according to the manufacturer’s instructions (Oxford Nanopore Technologies, Oxford, UK) with 400 ng HMW DNA. An R9.4.1 flow cell (Oxford Nanopore Technologies) was loaded and run for 24 h. Base calling was performed using Guppy (version 3.1.5; Oxford Nanopore Technologies) with the high-accuracy base-calling algorithm. Adapter sequences were removed using Porechop (version 0.2.4 with default settings; Wick, 2018). Finally, the reads were self-corrected, trimmed and assembled using Canu (Version 1.8; Koren et al., 2017). Sequencing data are available at the NCBI SRA database under accession number PRJNA639910.

Comparative genomics and candidate identification

Self-corrected reads from V. dahliae race 3 strains were mapped against the reference genome using BWA-MEM (version 0.7.17; default settings; Li, 2013). Reads with low mapping quality (score < 10) were removed using Samtools view (version 1.9; setting: -q 10) (Li et al., 2009), and reads mapping in regions with low coverage (<10x) were discarded using Bedtools coverage (version 2.25.0; setting: -d) (Quinlan and Hall, 2010). Self-corrected race 2 strain reads were mapped against the retained reference genome-specific regions that are absent from the race 3 strains. Retained sequences shared by the reference and every race 2 strain, while absent from every race 3 strain, were retained as Av2 candidate regions.

The previously determined annotation of V. dahliae strain JR2 (Faino et al., 2015) was used to extract genes when JR2 or TO22 were used as alignment references. To this end, retained sequences shared by the TO22 reference assembly and race 2 strains, absent from race 3 strains, were mapped against the JR2 genome assembly, and genes in the shared sequences were extracted. The remaining sequences that did not map to the V. dahliae strain JR2 genome assembly were annotated using Augustus (version 2.1.5; default settings; Stanke et al., 2006). SignalP software (version 4.0; Petersen et al., 2011) was used to identify N-terminal signal peptides in predicted proteins.

Real-time PCR

To determine expression profiles of Av2 candidate genes during V. dahliae infection of tomato, 2-week-old tomato
cv. Moneymaker) seedlings were inoculated with V. dahliae strain JR2 or TO22, and stems were harvested up to 14 dpi. Furthermore, conidiospores were harvested from 5-day-old PDA plates. Total RNA extraction and cDNA synthesis were performed as previously described (Santhanam et al., 2013).

Real time-PCR was performed with primers listed in Table 3, using the V. dahliae glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) as endogenous control. The PCR cycling conditions were as follows: an initial 95°C/14°C denaturation step for 10 min followed by denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension at 72°C for 40 cycles.

Genome mining

In total, 44 previously sequenced V. dahliae strains and eight strains sequenced in this study were mined for Av2 gene candidates using BLASTn. Gene sequences were extracted using Bedtools (setting: getfasta) (Quinlan and Hall, 2010) and aligned to determine allelic variation using Espript (version 3.0; default settings) (Robert and Gouet, 2014). Similarly, amino acid sequences were aligned using Espript (Robert and Gouet, 2014).

To determine the genomic localization of XLOC_00170 and Evm_344, the V. dahliae strain JR2 assembly and annotation were used (Faino et al., 2015) together with coverage plots from reads of race 3 and race 2 strains as described in comparative genomics approach IV (Table 2) using R scripts, with the package karyoploteR for R (version 3.6) using kpPlotBAMCoverage function. The schematic representation of the genomic region on chromosome 4 with XLOC_00170 and Evm_344 was generated using Integrative Genomics Viewer (IGV) software v2.6.3 (Robinson et al., 2011) and R package (version 3.6) Gviz (Hahne and Ivanek, 2016).

Phylogenetic tree construction

The phylogenetic tree of 52 V. dahliae strains was generated with Realphy (version 1.12) (Bertels et al., 2014) using Bowtie2 (version 2.2.6) (Langmead and Salzberg, 2012) to map genomic reads against the V. dahliae strain JR2 assembly. A maximum likelihood phylogenetic tree was inferred using RAxML (version 8.2.8) (Stamatakis, 2014).

Presence–absence variation analysis

Presence–absence variation (PAV) was identified by using whole-genome alignments for 17 V. dahliae strains.

Table 3. Primers used in this study.

| Primer name         | Oligonucleotide sequence (5′→3′) | Usage       |
|---------------------|----------------------------------|-------------|
| XLOC00170-F         | CAGCCCTCAATACACCATGAGATG         | qPCR        |
| XLOC00170-R         | TTCCGTGATGCTTCTACAGAGG           | qPCR        |
| evm1569.344-F       | CACTTCTGGTTGCAATG               | qPCR        |
| evm1569.344-R       | TTCTTACTGTCGTTGAGT              | qPCR        |
| tig00000058-ctd1-F  | GAGTGTAGCTGTTGTTG               | qPCR        |
| tig00000058-ctd1-R  | CACACCAAAACACATCAC              | qPCR        |
| tig00000058-ctd2-F  | CCTGATCTGATGCTGTAATG            | qPCR        |
| tig00000151-F       | GTTCCGCCTCTCTGACTCT             | qPCR        |
| tig00000151-R       | GTTGGGCGGTTGGTTCTC              | qPCR        |
| tig00017428-F       | CCATCCAGACCGAACAAGT             | qPCR        |
| tig00017428-R       | CTGGAAAGCCGAGTTGACT             | qPCR        |
| VdAve1-F            | ACCTTCTACGCTCTGGA               | qPCR        |
| VdAve1-R            | TTGACCCTGAGGATTGCT              | qPCR        |
| VdGAPDH-F           | CGAGTCCTTCGTGCTTCA              | qPCR        |
| VdGAPDH-R           | CCCGAAAGACCTGGTAAA              | qPCR        |
| CO-XLOC00170-F     | cgtctattaattaaATGAGATGCAACCTGACTCTTTTGGC | Complementation |
| CO-XLOC00170-R     | cgtcttaggccccTTTACGTTAAGACTACAAAGTGAACATCC | Complementation |
| CO-Evm344-F         | cgtctattaattaaATGAAACTCTCTCTCCATTACAGCC | Complementation |
| CO-Evm344-R         | cgtcttaggccccTTAAGACCTTCTTCTGCTGCTGCTGCTGCTG | Complementation |
| SiRUB-Fw            | GAACATGTTTCCTGTGAC             | qPCR        |
| SiRUB-Rv            | CTGGAGAAGATAATGAC             | qPCR        |
| ITS1-F              | AAAGTTTTAAATGGTGGCTTAAG        | qPCR        |
| STVe1-R             | CTGGTGCTTATGGAAGATGA          | qPCR        |
| JR2-XLOC170-LB-F   | GGTCTTAAUGAGAAAGTGGTGAGGAGGA | Deletion    |
| JR2-XLOC170-LB-R   | GGCATTAATUGAGGAAAGTGGTGTTG    | Deletion    |
| JR2-XLOC170-RB-F   | GGATCATAUCGAAAAGTACGACGGTGGTTG | Deletion    |
| JR2-XLOC170-RB-R   | GGTCTTAAUGAGAGGACCAAGCCTTTATCAA | Deletion    |
| TO22-XLOC170-RB-F  | GGTCTTAAUGAGGCTTTAAATGGCTGTAATG | Deletion    |
| TO22-XLOC170-RB-R  | GGTCTTAAUGAGGCTTTAATGGCTGTAATG | Deletion    |
| TO22-XLOC170-LB-F  | GGTCTTAAUTGCCAAAGGGAGGAGCATGTAACAA | Deletion    |
| TO22-XLOC170-LB-R  | GGTCTTAAUTGGGAGGAGGGCTGTTGTAATG | Deletion    |

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Paired-end short reads were mapped to *V. dahliae* strain JR2 (Faino *et al*., 2015) using BWA-mem with default settings (Li and Durbin, 2009). Long-reads were mapped using minimap2 with default settings (Li, 2018). Using the Picard toolkit (http://broadinstitute.github.io/picard/), library artefacts were marked and removed with -MarkDuplicates followed by -SortSam to sort the reads. Raw read coverage was averaged per 100 bp non-overlapping windows using the BEDtools -multicov function (Quinlan and Hall, 2010). Next, we transformed the raw read coverage values to a binary matrix by applying a cut-off of 10 reads for short-read data; > = 10 reads indicate presence (1) and < 10 reads indicate absence (0) of the respective genomic region. For long-read data a cut-off of 1 read was used; > = 1 read indicates presence (1) and < 1 read indicates absence (0). The total number of PAV counts for each of the 100 bp genomic windows within 100 kb upstream and downstream of the candidate effectors was summarized.

**Genetic complementation, deletion and functional analysis**

For genomic complementation of race 3 strains GF-CB5 and HOMCF, a genomic construct was generated comprising the coding sequence of *XLOC_00170* or *Evm_344* in vector pFBT005 behind the VdAve1 promoter, using primers CO-XLOC00170-F and CO-XLOC00170-R for *XLOC_00170* or CO-Evm344-F and CO-Evm344-R for *Evm_344* (Table 3).

For genomic deletion of *XLOC_00170* from JR2ΔAve1 and race 2 strain TO22, a genomic construct was generated comprising the flanking regions of *XLOC_00170* in vector pRF-NU2 (for JR2ΔAve1) or pRF-HU2 (for strain TO22), using primers JR2-XLOC170-LB-F, JR2-XLOC170-LB-R, JR2-XLOC170-RB-F and JR2-XLOC170-RB-R for strain JR2, and primers TO22-XLOC170-LB-F, TO22-XLOC170-LB-R, TO22-XLOC170-RB-F and TO22-XLOC170-RB-R for strain TO22 (Table 3).

*Agrobacterium tumefaciens*-mediated transformation (ATMT) was performed as described previously (Ökmen *et al*., 2013) with a few modifications. *A. tumefaciens* was grown in 5 ml minimal medium (MM) supplemented with 50 μg m⁻¹ kanamycin at 28°C for 24 h. Subsequent centrifugation at 3000 g (5 min), cells were resuspended in 5 ml induction medium (IM) supplemented with 50 μg m⁻¹ kanamycin, adjusted to OD₆₀₀ 0.15 and grown at 28°C for minimum 6 h until OD₆₀₀ 0.5. Simultaneously, conidiaospores of *V. dahliae* race 3 strains GF-CB5 and HOMCF were harvested after 1 week of cultivation on PDA plates with water, rinsed, and adjusted to a final concentration of 10⁶ conidiaospores mL⁻¹. The *A. tumefaciens* suspension was mixed with *V. dahliae* conidiaospores in a 1:1 volume ratio and 200 μl of the mixture was spread onto PVDF membranes in the centre of IM agar plates. After 2 days at 22°C, membranes were transferred to fresh PDA plates supplemented with 20 μg ml⁻¹ nourseothricin and 200 μM cefotaxime and incubated at 22°C for two weeks until *V. dahliae* colonies emerged. Transformants that appeared were transferred to fresh PDA supplemented with 20 μg ml⁻¹ nourseothricin and 200 μM cefotaxime. Successful transformation was verified by PCR and DNA sequencing.

*V. dahliae* inoculations were performed as described previously (Fradin *et al*., 2009). Disease symptoms were scored 14 days after inoculation by measuring the canopy area to calculate stunting when compared with mock-inoculated plants. Outgrowth of *V. dahliae* from stem slices was assessed as described previously (de Jonge *et al*., 2012). For biomass quantification, stems were freeze-dried and ground to powder, of which ~100 mg was used for DNA isolation. Real-time PCR was conducted with primers SIRUB-Fw and SIRUB-Rv for tomato RuBisCo and primers ITS1-F and STVe1-R for *V. dahliae* ITS (Table 3). Real-time PCR conditions were as follows: an initial 95°C denaturation step for 10 min followed by denaturation for 15 s at 95°C and annealing for 30 s at 60°C, and extension at 72°C for 40 cycles.

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**Author contributions**

J.P.V., T.U., M.F.S. and B.P.H.J.T. conceived the study; J.P.V., D.T., M.F.S. and B.P.H.J.T. designed the experiments; E.A.C.C., J.P.V., D.T. performed experiments; E.A.C.C., J.P.V., D.T., H.J.S., Y.B., M.F.S. and B.P.H.J.T. analysed data, E.A.C.C., J.P.V. and B.P.H.J.T. T wrote the manuscript; MFS and BPHJT supervised the project, all authors discussed the results and contributed to the final manuscript.

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