Genome evolution in the plant-pathogenic fungus *Verticillium dahliae* in the absence of apparent sexual reproduction

Doctoral thesis

for

the award of the doctoral degree

of the Faculty of Mathematics and Natural Sciences

of the University of Cologne

submitted by

Chen-Yu Kuan

2026

Examination Committee:

1st Examiner: Prof. Dr. Bart P.H.J. Thomma

2nd Examiner: Prof. Dr. David Cook

Chair of Examination committee:

Prof. Dr. Mirka Uhlirova

Minutes taker:

Dr. Julie Noah

Thesis Advisory Committee:

Prof. Dr. Bart P.H.J. Thomma

Prof. Dr. Ann-Marie Waldvogel

Prof. Dr. Thomas Wieh

Table of Contents

Thesis Summary

Sexual reproduction is a hallmark of eukaryotes and occurs across nearly all branches of the eukaryotic tree of life. By promoting genetic variation through meiotic recombination, it has played a fundamental role in the evolution of life. However, many eukaryotes present an evolutionary paradox because they do not exhibit apparent sexual reproduction, despite having the conserved genetic machinery required for this process. A classic example is the broad host-range vascular wilt pathogen *Verticillium dahliae*, which is considered strictly asexual because no sexual stage has been observed. Nevertheless, its genome contains genes typically involved in sexual reproduction, including mating-type (*MAT*) and meiotic genes. This raises the intriguing question whether these genes remain functional in *V. dahliae*. If so, they may support cryptic sexual reproduction, meaning rare and hidden sexual reproduction, or, alternatively, have been repurposed for biological processes beyond sexual reproduction. Moreover, the genome of *V. dahliae* is characterized by extensive large-scale chromosomal rearrangements, which are thought to be incompatible with proper chromosome pairing during meiosis and may therefore promote the persistence of asexuality in *V. dahliae*. These rearrangements contribute to the generation of highly dynamic adaptive genomic regions (AGRs) that facilitate host adaptation and are therefore considered important drivers of *V. dahliae* adaptation. Yet, it remains unclear whether such rearrangements represent ancient events inherited from ancestral *V. dahliae* lineages or continue to arise during ongoing genome evolution. During my doctoral research, I investigated how *V. dahliae* evolves in the absence of apparent sexual reproduction, with a particular focus on the occurrence of chromosomal rearrangements and the functional roles of *MAT* genes.

To investigate the adaptability of *V. dahliae*, I first established a collection of *V. dahliae* lineages generated under diverse conditions, including abiotic stress and repeated passage through the host plant *Arabidopsis thaliana*. Among these lineages, I identified a *de novo* inter-chromosomal rearrangement that emerged after passage through *A. thaliana*, and demonstrated that this rearrangement confers a competitive advantage to *V. dahliae* during host colonization. These results show that large-scale chromosomal rearrangements can contribute to ongoing genome evolution and host adaptation in *V. dahliae.* Expanding beyond non-sexual mechanisms of genome evolution, we used transcriptomic analyses to show that the sexual machinery of *V. dahliae* is transcriptionally active, including *MAT* genes and 85 sex-related genes. These findings suggest that *V. dahliae* may have the potential for cryptic sexuality. Moreover, I showed that *MAT* genes influence transcription beyond the 85 identified sex-related genes, indicating noncanonical regulatory roles. These findings may help explain the paradox of *MAT* gene presence in *V. dahliae*, as *MAT* genes may contribute not only to sexual reproduction but also to broader biological processes.

Zusammenfassung

Sexuelle Reproduktion ist ein Kennzeichen der Eukaryoten und kommt in nahezu allen Zweigen des Stammbaum der Eukaryoten vor. Indem sie durch meiotische Rekombination genetische Variation fördert, hat sie eine grundlegende Rolle in der Evolution des Lebens gespielt. Viele Eukaryoten stellen jedoch ein evolutionäres Paradoxon dar, da sie keine offensichtliche sexuelle Reproduktion aufweisen, obwohl sie über die konservierte genetische Maschinerie verfügen, die für diesen Prozess erforderlich ist. Ein klassisches Beispiel hierfür ist der vaskuläre Welkeerreger *Verticillium dahliae* mit breitem Wirtsspektrum, der als strikt asexuell gilt, da bislang kein sexuelles Stadium beobachtet wurde. Dennoch enthält sein Genom Gene, die typischerweise an der sexuellen Reproduktion beteiligt sind, darunter Paarungstyp-Gene (*MAT*-Gene) sowie meiotische Gene. Dies wirft die interessante Frage auf, ob diese Gene in *V. dahliae* weiterhin funktional sind. Falls dies zutrifft, könnten sie kryptische sexuelle Reproduktion ermöglichen, also seltene und verborgene sexuelle Reproduktion, oder alternativ für biologische Prozesse jenseits der sexuellen Reproduktion umfunktioniert worden sein. Darüber hinaus ist das Genom von *V. dahliae* durch umfangreiche großskalige chromosomale Umlagerungen gekennzeichnet, von denen angenommen wird, dass sie mit einer korrekten Chromosomenpaarung während der Meiose unvereinbar sind und daher die Persistenz der Asexualität in *V. dahliae* begünstigen könnten. Diese Umlagerungen tragen zur Entstehung hochdynamischer adaptiver genomischer Regionen (AGRs) bei, die die Anpassung an Wirte erleichtern und daher als wichtige Triebkräfte der Anpassung von *V. dahliae* gelten. Dennoch bleibt unklar, ob solche Umlagerungen uralte Ereignisse darstellen, die von ancestralen *V. dahliae*-Linien geerbt wurden, oder ob sie während der fortlaufenden Genomevolution weiterhin entstehen. Im Rahmen meiner Promotionsforschung untersuchte ich, wie sich *V. dahliae* in Abwesenheit offensichtlicher sexueller Reproduktion entwickelt, wobei der Schwerpunkt insbesondere auf dem Auftreten chromosomaler Umlagerungen und den funktionellen Rollen von *MAT*-Genen lag.

Um die Anpassungsfähigkeit von *V. dahliae* zu untersuchen, etablierte ich zunächst eine Sammlung experimentell evolvierter *V. dahliae*-Linien. Diese wurden unter verschiedenen Bedingungen erzeugt, darunter abiotischer Stress und wiederholte Zyklen aus Inokulation und Reisolierung aus der Wirtspflanze *Arabidopsis thaliana*. Unter diesen Linien identifizierte ich eine de novo entstandene interchromosomale Umlagerung, die nach wiederholten Inokulations- und Reisolierungszyklen in *A. thaliana* auftrat, und zeigte, dass diese Umlagerung während der Wirtsbesiedlung einen kompetitiven Vorteil verleiht. Diese Ergebnisse zeigen, dass großskalige chromosomale Umlagerungen zur fortlaufenden Genomevolution und Wirtsanpassung in *V. dahliae* beitragen können. Über nicht-sexuelle Mechanismen der Genomevolution hinausgehend, zeigten wir mithilfe transkriptomischer

Analysen, dass die sexuelle Maschinerie von *V. dahliae* transkriptionell aktiv ist, einschließlich der *MAT*-Gene und 85 geschlechtsbezogener Gene. Diese Befunde deuten darauf hin, dass *V. dahliae* das Potenzial für kryptische Sexualität besitzen könnte. Darüber hinaus zeige ich, dass *MAT*-Gene die Transkription über die 85 identifizierten geschlechtsbezogenen Gene hinaus beeinflussen, was auf nichtkanonische regulatorische Rollen hinweist. Diese Ergebnisse könnten dazu beitragen, das Paradoxon der Präsenz von *MAT*-Genen in *V. dahliae* zu erklären, da *MAT*-Gene möglicherweise nicht nur zur sexuellen Reproduktion, sondern auch zu weiter gefassten biologischen Prozessen beitragen.

Chapter 1: General introduction

Plant health shapes human history and ecosystems

Plants are essential for life on Earth (Robbins 1944). They constitute approximately 80% of the Earth's total biomass and therefore form the foundation of the biosphere (Bar-On et al. 2018). Through photosynthesis, plants produce oxygen that sustains aerobic life while also providing food resources and habitats for numerous other organisms (Angelini et al. 2011; Robbins 1944). Plants have also played a central role in human livelihoods, providing essential resources such as food, fuel, materials, and medicine (Pironon et al. 2024; Angourakis et al. 2022). However, plants are continually exposed to pathogens and pests that can cause plant diseases (Savary et al. 2019). Such plant diseases can have significant societal and ecological impacts. A prominent historical example of a devastating plant disease that significantly affected mankind is provided by potato late blight, caused by the oomycete pathogen *Phytophthora infestans*, which emerged in Western Europe in the mid-nineteenth century (Yoshida et al. 2013). The resulting destruction of potato crops triggered the Irish Great Famine, causing approximately one million deaths and forcing another million people to emigrate from Ireland (Yoshida et al. 2013). As a consequence, Ireland's population in the twenty-first century remains less than three-quarters of its level in the early 1840s (Yoshida et al. 2013). Plant diseases affect not only food production but can also profoundly disrupt natural ecosystems. A striking example of large-scale ecological disruption is provided by chestnut blight, a disease caused by the ascomycete fungus *Cryphonectria parasitica* that emerged in North America in the late nineteenth century (Fernandes et al. 2022). Within a few decades, the pathogen eliminated an estimated four billion American chestnut trees (*Castanea dentata*), a keystone species in eastern North American forests, thereby reshaping ecosystem structure and human land use (Fernandes et al. 2022). The impact of these epidemics was amplified by the lack of effective disease management strategies, including genetic, ecological, and chemical approaches, as well as limited systems for early detection and disease monitoring (Ristaino et al. 2021).

The plant holobiont

Plants continuously interact with diverse microorganisms present in their surrounding environment or colonizing their tissues, and these microorganisms are collectively referred to as the plant microbiota (Sokol et al. 2022). The plant microbiota plays a crucial role in plant health, as microorganisms interact with the host plant across a spectrum, ranging from negative (parasitic) to neutral (commensal) and beneficial (mutualistic) relationships (Osayande et al. 2025). For example, plants can recruit beneficial microbes in response to pathogen attack through a mechanism known as the "cry for help" (Rolfe et al. 2019). In this

process, changes in root exudate composition attract beneficial microbes that suppress invading pathogens either through direct antagonism or by enhancing plant immunity (Rolfe et al. 2019). These dynamic interactions between plants and the plant microbiota have led to the concept of the plant holobiont, in which the plant and its associated microbial communities function as an integrated biological unit (Spooren et al. 2024; Vandenkoornhuyse et al. 2015). Disruptions of the stability of this holobiont can compromise host immunity and genereate conditions that favor pathogen infection, ultimately leading to plant disease (Spooren et al. 2024). Among plant pathogens, fungi are responsible for approximately 70–80% of plant diseases worldwide (Peng et al. 2021). Understanding how fungal plant pathogens evolve and manipulate the plant holobiont is therefore essential for developing sustainable strategies for plant disease management.

The evolutionary arms race between plants and fungal pathogens

The ability of fungal plant pathogens to evade host immunity is shaped by continuous evolutionary arms races with their plant hosts. A key step in the establishment of plant colonization by fungal pathogens is the secretion of effector molecules, including proteins, secondary metabolites, and small RNAs, that manipulate host cellular processes, including the suppression of host immune responses, or modulate host-associated microbiota (Rovenich et al. 2014; Collemare et al. 2019; Snelders et al. 2022). In turn, plants have evolved immune receptors that detect pathogen effectors or cellular changes induced by these effectors, thereby activating defense responses that restrict pathogen colonization (Cook et al. 2015). To overcome recognition, fungal pathogens frequently discard, modify, or evolve novel effectors to escape detection while maintaining virulence (Rovenich et al. 2014). In response, plants diversify their immune receptor repertoires to detect these modified or newly evolved effectors (Chisholm et al. 2006; Jones and Dangl 2006). This ongoing cycle of adaptation and counter-adaptation is described as the molecular arms race between hosts and fungal pathogens (Seidl and Thomma 2017; Strotz et al. 2018). In many pathogens, including many fungal plant pathogens, adaptation is facilitated by a “two-speed genome” (Croll and McDonald 2012; Dong et al. 2015). The “two-speed genome” hypothesis proposes that the genome is organized into a conserved core compartment and a dynamic accessory compartment (Dong et al. 2015). The core compartment encodes housekeeping genes required for basic cellular functions and evolves slowly, whereas the accessory compartment evolves more rapidly, shows greater structural variation, and is enriched in genes involved in host interactions, such as effectors (Dong et al. 2015). This compartmentalization promotes effector diversification during host–pathogen coevolution while maintaining stability of the core genome (Torres et al. 2020).

Sexual reproduction drives evolution

Sexual reproduction has played a central role in the genome evolution of eukaryotes, including fungi (Speijer et al. 2015). The ubiquity of sexual reproduction has led to the hypothesis that sexual reproduction evolved once and was already present in the last eukaryotic common ancestor (LECA) (Speijer et al. 2015). By reshuffling parental alleles through meiotic recombination and uniting two parental genomes during fertilization, sexual reproduction generates novel genetic combinations (Fu et al. 2019). The generation of novel genetic combinations can facilitate fungal pathogen outbreaks by giving rise to new virulent fungal lineages (Drenth et al. 2019). Thus, interrupting fungal sexual reproduction has been employed as a strategy for disease control (Drenth et al. 2019). A classic example of disease control by interrupting sexual reproduction involves the stem rust fungus *Puccinia graminis* (Leonard and Szabo 2005). This pathogen infects cereal crops such as wheat, oats, barley, and rye, where it reproduces asexually, but it requires the alternate host berberis (*Berberis* spp.) to complete its sexual cycle (Leonard and Szabo 2005). In the early 20th century, large-scale barberry eradication was implemented in North America to disrupt the sexual reproduction of *P. graminis*, thereby protecting cereal crops (Leonard and Szabo 2005). Following barberry eradication, the number of *P. graminis* f. sp. *tritici* races recovered from wheat in Minnesota declined sharply, from 21 races in the 1920s to only 5 races in the 1980s (Peterson et al. 2005). No significant wheat stem rust epidemics have occurred in the United States since 1974 (Leonard and Szabo 2005). These results demonstrate that sexual reproduction plays a key role in generating genetic diversity that gives rise to virulent fungal lineages.

Despite the benefits of sexual reproduction in generating genomic diversity, it also imposes substantial costs (Gibson et al. 2017; Lee et al. 2010; Heitman 2015). One well-known cost of sex is the theoretical “two-fold cost”, under which sexual populations are predicted to reproduce less efficiently than asexual populations capable of rapid clonal propagation without mating and meiosis (Gibson et al. 2017; Heitman 2015). Sexual reproduction requires two parents that each contribute half of their genetic material to the offspring. Consequently, asexual populations, in which individuals reproduce independently, can theoretically increase in population size approximately twice as fast under equal growth conditions (Gibson et al. 2017; Heitman 2015). In addition, sexual reproduction requires additional physiological investment because processes such as meiosis, mating signaling, and cellular fusion divert resources from vegetative growth and are absent in asexual reproduction (Lee et al. 2010). Sexual reproduction may also be time-consuming, because in some fungi the completion of sexual reproduction requires days to months (Ni et al. 2011). Furthermore, recombination during sex can disrupt advantageous allelic combinations that are already well suited to the environment (Luijckx et al. 2017). These costs raise a fundamental evolutionary question: why

does sexual reproduction persist across the diverse eukaryotic tree of life?

The widespread occurrence of sexual reproduction despite substantial costs is commonly referred to as the paradox of sex (Otto and Lenormand 2002). This paradox is particularly evident in fungi, where many species reproduce primarily asexually, yet retain the ability to reproduce sexually (Nieuwenhuis and James 2016). Several hypotheses have been proposed to explain the persistence of sexual reproduction (Ashu and Xu 2015). One hypothesis proposes that sexual reproduction is maintained because it facilitates the removal of deleterious mutations that arise spontaneously in genomes (Ashu and Xu 2015). In asexual reproduction, offspring inherit the parental genome without meiotic recombination, limiting the elimination of harmful mutations (Felsenstein 1974). Consequently, strictly asexual species may experience an irreversible accumulation of deleterious mutations, a process known as Muller's ratchet, which can ultimately lead to extinction (Muller 1964). In addition to preventing the accumulation of deleterious mutations, sexual reproduction facilitates adaptation. The Red Queen hypothesis proposes that species must continuously evolve to maintain their fitness relative to other species that are also evolving (Van Valen 1973). The hypothesis takes its name from a metaphor in Lewis Carroll's novel *Through the Looking-Glass*, in which the Red Queen tells Alice: "Now, here, you see, it takes all the running you can do, to keep in the same place" (Van Valen 1973). Consequently, species that fail to generate sufficient genetic variation may be unable to keep pace with the evolution of other species, potentially leading to competitive disadvantage or extinction (Van Valen 1973). Sexual reproduction is therefore advantageous because it generates high genetic diversity among offspring, which reduces the ability of coevolving species, such as pathogens, to adapt to host genotypes that vary between generations (Hamilton and Zuk 1982). Experimental evolution studies also support the adaptive benefits of sexual reproduction in complex environments. For example, research on the rotifer *Brachionus calyciflorus*, which can reproduce both sexually and asexually, showed that populations evolved higher rates of sexual reproduction when adapting to more complex environments (Luijckx et al. 2017). Given the inherent complexity of natural environments, the genetic diversity generated by sexual reproduction may enhance adaptive potential in heterogeneous environments, thereby contributing to the long-term persistence of sex in eukaryotes (Bürger 1999).

Thriving without sex: asexuality in fungi

Asexual reproduction, in contrast to sexual reproduction, enables rapid clonal propagation because offspring are generated through mitotic division of a single parent, bypassing the processes of mating and meiosis that are required for sexual reproduction (Taylor et al. 2015). Fungi with broad host ranges have been reported to rely more strongly on asexual reproduction

than host specialists that infect only one or a few host species, suggesting a potential association between broader host range and increased reliance on asexual reproduction (Newman and Derbyshire 2020). Moreover, although both sexual and asexual reproduction can contribute to the emergence of fungal outbreaks, rapid epidemic spread is often driven by asexual clonal expansion (Ashu and Xu 2015). A classic example in banana cultivation is provided by the Panama disease epidemic of the 1950s, caused by *Fusarium oxysporum* f. sp. *cubense* race 1, a presumed asexual fungal plant pathogen (Pegg et al. 2019). The epidemic emerged under extensive banana monoculture, as export production in Central America depended almost exclusively on the highly susceptible 'Gros Michel' cultivar (Pegg et al. 2019). *F. oxysporum* spread rapidly across banana production regions, devastating the global banana trade and ultimately forcing the replacement of 'Gros Michel' with the resistant 'Cavendish' cultivar (Pegg et al. 2019). However, this apparent solution proved temporary, as the subsequent emergence of *F. oxysporum* f. sp. *cubense* race 4, first recorded in 1967, triggered renewed epidemics across Asia, Australia, and Africa by the early 2000s (Pegg et al. 2019). These outbreaks illustrate how asexual pathogens can spread rapidly across large geographic distances and sustain recurrent epidemics even in the absence of sexual reproduction (Drenth et al. 2019).

Although asexual reproduction can confer advantages, evolutionary theory predicts that strictly asexual lineages should experience long-term disadvantages (Muller 1964). In the absence of meiotic recombination, deleterious mutations are expected to accumulate irreversibly through the process known as Muller's ratchet, potentially reducing fitness and limiting long-term persistence (Muller 1964). Consequently, the loss of sexual reproduction has traditionally been regarded as an evolutionary dead end (Muller 1964). However, genomic evidence increasingly challenges the existence of strictly asexual fungi and suggests that exclusive clonal reproduction may be rare or nonexistent in fungi (Nieuwenhuis and James 2016; Seidl and Thomma 2014). In fungi, mating-type (*MAT*) genes determine sexual identity and regulate sexual reproduction, and homologs of *MAT* genes are present in nearly all sequenced genomes of the Dikarya, regardless of whether a sexual stage has been observed (Nieuwenhuis and James 2016). Similar evidence has also been reported in arbuscular mycorrhizal fungi, which have existed for at least 400 million years and were once regarded as an "ancient asexual scandal" because of their presumed asexuality (Judson and Normark 1996). Nevertheless, arbuscular mycorrhizal fungi are increasingly considered capable of cryptic sexual reproduction, a rare and hidden sexual cycle that has yet to be discovered, as their genomes harbor mating-type (*MAT*) loci and meiotic genes (Taylor et al. 2015). These findings suggest that apparent asexuality in fungi may reflect hidden reproductive strategies rather than genuine asexual reproduction, raising the unresolved question of whether fungi classified as asexual truly lack sexual reproduction (Seidl and Thomma 2014; Drenth et al.

2019; Nieuwenhuis and James 2016). An example of sexual reproduction occurring under specific environmental conditions is provided by the human fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii* (Xue et al. 2007). It has long been an ecological mystery why these species are associated with diverse plant hosts in nature and how they complete their sexual cycles to produce infectious spores despite the absence of observed mating in nature (Xue et al. 2007). This question was later resolved by demonstrating that these fungi can use plants as a natural niche for sexual reproduction, completing their sexual cycle directly on plant surfaces (Xue et al. 2007).

The widespread retention of *MAT* genes and meiotic machinery in fungi lacking an observed sexual cycle raises the question of why these genes are retained (Wallen and Perlin 2018). One explanation is that sexual reproduction still occurs in these predominantly asexual fungi but only rarely or under specific tissue or environmental conditions that are difficult to observe, leaving sexual cycles undetected (Dyer and O'Gorman 2012). In this scenario, the conservation of *MAT* and meiotic genes reflects their roles in a cryptic sexual cycle. For example, *Aspergillus fumigatus* was originally considered to reproduce only asexually, yet its genome contains *MAT* genes (Dyer and Paoletti 2005). Subsequent studies demonstrated that *A. fumigatus* can reproduce sexually *in vitro*, although sexual fruiting bodies form only after approximately six months when compatible mating strains are co-cultured (O'Gorman et al. 2009). Population genetic analyses also indicate that the genetic structure of natural *A. fumigatus* populations reflects the occurrence of sexual recombination (Klaassen et al. 2012). An alternative explanation for the persistence of sex-related genes is that they may facilitate alternative mechanisms of genetic recombination outside canonical sexual reproduction. For example, the conserved meiotic recombinase SPO11, known for generating meiotic double-strand breaks, is repurposed to mediate recombination during the parasexual cycle of the human fungal pathogen *Candida albicans* (Forche et al. 2008). In parasexuality, genetic variation is generated during vegetative growth via mitotic recombination rather than via meiosis (Forche et al. 2008). In addition to parasexual processes, sex-related genes may persist because they have been co-opted for other functions, such as mediating resistance to environmental stress (Wallen and Perlin 2018). For example, deletion of the *MAT* genes in the plant-pathogenic fungus *Cochliobolus lunatus* and *Villosiclava virens* resulted in altered sensitivity to osmotic stress, with either increased or decreased tolerance depending on which *MAT* gene was disrupted, suggesting that *MAT* genes function beyond reproduction (Liu et al. 2022; Yong et al. 2020). Additionally, abiotic stresses such as nutrient starvation and oxidative stress have been shown to activate sex-related pathways that initiate mating and meiosis in several fungi (Wallen and Perlin 2018). These findings suggest that sex-related genes may have been co-opted for roles in stress responses, consistent with evolutionary models proposing that sexual processes evolved from stress-induced DNA recombination and repair

pathways under early environmental pressures on Earth (Wallen and Perlin 2018). These findings indicate that genes traditionally associated with canonical fungal sexual reproduction may also play roles in cryptic sexual reproduction, parasexual recombination, or nonsexual processes such as stress responses.

Fungal genome evolution in the absence of sexual reproduction

In addition to meiotic recombination, fungi can generate genetic variation through multiple non-meiotic mechanisms, allowing predominantly asexual fungi to maintain highly dynamic genomes (Seidl and Thomma 2014; Mondo and Grigoriev 2025). One source of genetic variation is single nucleotide mutations, which can have substantial consequences in the evolutionary arms race between plants and fungi (Seidl and Thomma 2014). For example, in the tomato leaf mould fungus *Cladosporium fulvum*, a naturally occurring single nucleotide change in the effector gene *Avr4* can prevent the AVR4 protein from being recognized by the tomato immune receptor Cf-4, thereby restoring infectivity and converting an avirulent strain into a virulent pathogen (Joosten et al. 1994). This example illustrates how minimal genetic changes can alter pathogenicity. Beyond point mutations, fungi can acquire adaptive traits through horizontal gene transfer (HGT), defined as the movement of genetic material independent of vertical inheritance, enabling gene exchange within and across species boundaries (Richards et al. 2011). A classic example is provided by the plant-pathogenic fungus *Fusarium oxysporum*, in which entire accessory chromosomes enriched in pathogenicity genes can be horizontally transferred between genetically distinct isolates, resulting in the conversion of non-pathogenic isolates into virulent tomato pathogens (Ma et al. 2010). In addition to accessory chromosomes, transposable elements (TEs) contribute substantially to fungal genome evolution (Seidl and Thomma 2017). TEs are mobile genetic elements that can relocate within the genome and, in some cases, can also be horizontally transferred between species, producing structural variation that may disrupt or alter gene regulation (Seidl and Thomma 2017). Recent studies have identified a distinct class of giant transposable elements in fungi, termed Starships (Bucknell and McDonald 2023; Urquhart et al. 2024). Unlike conventional TEs, which are typically less than 10 kilobases (kb) in length, Starships are characterized by their exceptional size, reaching up to approximately 700 kb, and by carrying hundreds of cargo genes, including pathogenicity-related genes (Urquhart et al. 2025; Sato et al. 2025). Evidence further indicates that Starships undergo horizontal transfer both within and between fungal species, facilitating the redistribution of genes across fungal genomes and contributing to *de novo* virulence gene formation (Sato et al. 2025; Urquhart et al. 2025). Asexual genome evolution in fungi also involves large-scale chromosomal rearrangements that can reshape genome architecture and create evolutionary novelty (Seidl and Thomma 2014; Mondo and Grigoriev 2025). These rearrangements can generate genome

structural variants (SVs), including inversions, translocations, duplications, and deletions, thereby generating genomic innovation (Seidl and Thomma 2014). In the plant pathogenic fungus *Verticillium dahliae*, chromosomal rearrangements preferentially localize to repeat-rich, accessory genomic compartments that are enriched for lineage-specific genes, including effector genes that contribute to virulence (de Jonge et al. 2013; Faino et al. 2016). These findings underscore the ability of fungi to exploit non-meiotic mechanisms to restructure their genomes and generate evolutionary innovation.

The plant pathogenic fungus *Verticillium dahliae*

V. dahliae is a soilborne ascomycete fungus that causes vascular wilt diseases in hundreds of dicotyledonous plants, including numerous crops such as bell pepper, tomato, cotton, and olive (Bhat and Subbarao 1999). Although individual *V. dahliae* strains may exhibit host specificity, most strains infect a broad range of plant species, reflecting considerable ecological flexibility (Bhat and Subbarao 1999; Klosterman et al. 2009). *V. dahliae* forms long-lived melanized resting structures, known as microsclerotia, which enable survival in soil for more than a decade and contribute to its long-term persistence (Fradin and Thomma 2006; Inderbitzin et al. 2011). Germination of microsclerotia is triggered by root exudates, after which emerging hyphae grow toward host roots and penetrate them to initiate infection (Fradin and Thomma 2006). Following root penetration, *V. dahliae* colonizes xylem vessels, where it produces conidiospores and proliferates within the vascular system, ultimately causing Verticillium wilt symptoms, including stunting, wilting, chlorosis, necrosis, vascular discoloration, and early senescence (Fradin and Thomma 2006). As infected plants begin to die, *V. dahliae* transitions from a parasitic to a saprophytic phase, emerging from the xylem to colonize decomposing tissues and produce microsclerotia while feeding on the decaying plant tissues (Fradin and Thomma 2006). Upon tissue decomposition, these microsclerotia are released into the soil, allowing *V. dahliae* to persist in a dormant state for extended periods in the absence of a host (Fradin and Thomma 2006). Given its long-term persistence in soil and broad host range, *V. dahliae* poses a significant challenge for disease management. From a plant breeding perspective, breeding crops resistant to *V. dahliae* remains difficult because only a limited number of resistance loci have been identified in host plants (Chavarro-Carrero et al. 2021; de Jonge et al. 2012; Fradin et al. 2009; Fradin and Thomma 2006).

The genome evolution of *V. dahliae* is intriguing, as it has achieved remarkable evolutionary success, infecting a wide range of plant hosts despite the absence of observed sexual reproduction to facilitate its arms race with plant hosts (Sayari et al. 2024). *V. dahliae* is presumed to be strictly asexual and to rely predominantly on nonsexual mechanisms for genomic diversification (Sayari et al. 2024). However, *MAT* genes, which normally determine

sexual identity and regulate sexual reproduction in fungi, have been identified in *V. dahliae* (Klosterman et al. 2011; Zhang et al. 2024). Two alternative mating-type idiomorphs, *MAT1-1* and *MAT1-2*, are present in the *V. dahliae* population, with individual strains carrying either idiomorph (Klosterman et al. 2011). This genomic organization is consistent with the mating system of heterothallic ascomycetes, in which mating occurs between individuals carrying opposite *MAT* idiomorphs (Klosterman et al. 2011). However, a highly skewed mating-type distribution has been observed among 1,120 *V. dahliae* isolates sampled globally, with the *MAT1-1* idiomorph detected in approximately 1% of isolates and *MAT1-2* present in the remaining 99% (Short et al. 2014). The skewed mating-type distribution is expected to substantially reduce the likelihood of encounters between strains carrying opposite mating types in natural environments, thereby constraining opportunities for sexual reproduction. The biological function of these *MAT* idiomorphs in *V. dahliae* remains unclear (Zhang et al. 2024). Furthermore, even when opposite mating types encounter one another in nature, conventional sexual reproduction is likely constrained by the genomic organization of *V. dahliae*. Comparative genomic analyses have revealed extensive chromosomal rearrangements in *V. dahliae*, both within and between chromosomes, resulting in structural variation (de Jonge et al. 2013; Faino et al. 2016). Analysis of 42 genomes derived from distinct *V. dahliae* strains further demonstrated that chromosomal rearrangements are widespread among *V. dahliae* strains (Torres et al. 2021). The extensive chromosomal rearrangements suggest that conventional sexual reproduction is unlikely to occur in *V. dahliae*, because such rearrangements are considered incompatible with proper chromosome pairing during meiosis (Flot et al. 2013; de Jonge et al. 2013). These rearrangements are spatially co-localized with dynamic genomic compartments, collectively referred to as adaptive genomic regions (AGRs). In contrast to the conserved core genome, AGRs exhibit a high degree of presence/absence variation (PAV) across diverse *V. dahliae* strains (Faino et al. 2016; de Jonge et al. 2013). AGRs also display a distinct chromatin profile characterized by enrichment of histone H3 lysine 27 trimethylation (H3K27me3), depletion of histone H3 lysine 9 methylation (H3K9me3), and increased chromatin accessibility (Cook et al. 2020). Functionally, AGRs are enriched for genes induced during plant infection, including effector genes that facilitate host colonization (de Jonge et al. 2013). The co-localization between chromosomal rearrangements and AGRs suggests that chromosomal rearrangements play a key role in host adaptation in *V. dahliae* (de Jonge et al. 2013).

Chromosomal rearrangements in *V. dahliae* are thought to arise from double-strand DNA breaks followed by unfaithful repair between homologous or repetitive sequences, particularly transposable elements (TEs) that can act as ectopic repair templates (Faino et al. 2016). Notably, segmental duplications and the Starship transposable elements in the *V. dahliae* genome serve as hotspots for chromosomal rearrangements, likely because their repetitive

sequences provide substrates for ectopic recombination (Faino et al. 2016; Mehrabi et al. 2017; Sato et al. 2025). In addition, the spatial organization of the genome within the nucleus may contribute to the formation of chromosomal rearrangements. AGRs are physically clustered in the nucleus through long-range chromatin interactions, which could bring distant homologous sequences into close physical contact (Torres et al. 2024). The clustering of AGRs in the nucleus may create an environment that promotes ectopic recombination, thereby resulting in chromosomal rearrangements (Torres et al. 2024). Nevertheless, the molecular mechanisms underlying chromosomal rearrangement formation remain unclear. Moreover, the widespread chromosomal rearrangements identified among *V. dahliae* strains in comparative genomic analyses are based on collections of naturally evolved field isolates (Torres et al. 2021). While these analyses demonstrate that chromosomal rearrangements have occurred during evolution, it remains unclear whether they represent ancient events inherited from ancestral *V. dahliae* lineages, or whether they continue to arise *de novo* to contribute to the ongoing evolution of the *V. dahliae* genome.

Research objective and thesis outline

Sexual reproduction is traditionally viewed as the primary mechanism driving genome diversification in eukaryotes. However, many fungal species lack an observed sexual cycle yet remain evolutionarily successful. This paradox raises fundamental questions regarding how genomes evolve in the absence of sexual reproduction, as well as the functional roles of conserved sexual machineries in organisms that predominantly reproduce asexually. During my doctoral research, I aimed to deepen our understanding of how the presumed strictly asexual plant fungal pathogen *Verticillium dahliae* evolves in the absence of apparent sexual reproduction, and to study the functional role of its *MAT* genes.

In **Chapter 2** (Figure 1A), we investigated the adaptive potential of *V. dahliae* using experimental evolution. We established *V. dahliae* lineages under diverse conditions, including ultraviolet-C (UV-C) exposure, prolonged growth at elevated temperature, sustained co-cultivation with a bacterial antagonist, and serial passage through the plant host *Arabidopsis thaliana*. Phenotypic characterization revealed potential adaptive traits in a subset of these lineages. This chapter provides a collection of *V. dahliae* lineages as a resource for future studies on *V. dahliae* responses to diverse abiotic pressures and plant hosts.

In **Chapter 3** (Figure 1B), we focus on determining whether chromosomal rearrangements contribute to the ongoing evolution of the *V. dahliae* genome. Using Oxford Nanopore sequencing of the lineages established in Chapter 2, we identified an inter-chromosomal rearrangement in a lineage after a single passage through *A. thaliana.* Furthermore, this rearrangement was shown to confer a competitive advantage to *V. dahliae* during colonization

of *A. thaliana*. This chapter demonstrates that chromosomal rearrangements can contribute to the ongoing genome evolution and host adaptation in *V. dahliae*.

In **Chapter 4** (Figure 1C), we present a transcriptomic analysis to investigate the function of *MAT* genes in the presumed strictly asexual fungus *V. dahliae*. Chromosomal rearrangements are considered incompatible with proper chromosome pairing during meiosis and may therefore reinforce an asexual lifestyle in *V. dahliae*. This raises the question of whether *MAT* genes in *V. dahliae* retain their classical regulatory roles in sexual reproduction. To address this question, we generated *V. dahliae* transformants with identical karyotypes that differ solely in their mating-type idiomorph and analyzed their transcriptomes after *in vitro* growth. Transcriptomic analyses demonstrate that *MAT* genes are transcriptionally active in *V. dahliae*. However, *MAT* genes appear to play noncanonical regulatory roles by influencing the transcription of genes not directly associated with sexual reproduction. These findings suggest that *MAT* genes have diverged from their canonical role in controlling sexual reproduction in *V. dahliae* under the tested *in vitro* conditions.

In **Chapter 5**, I discuss the findings of this thesis in the broader context of genome evolution in plant pathogens.

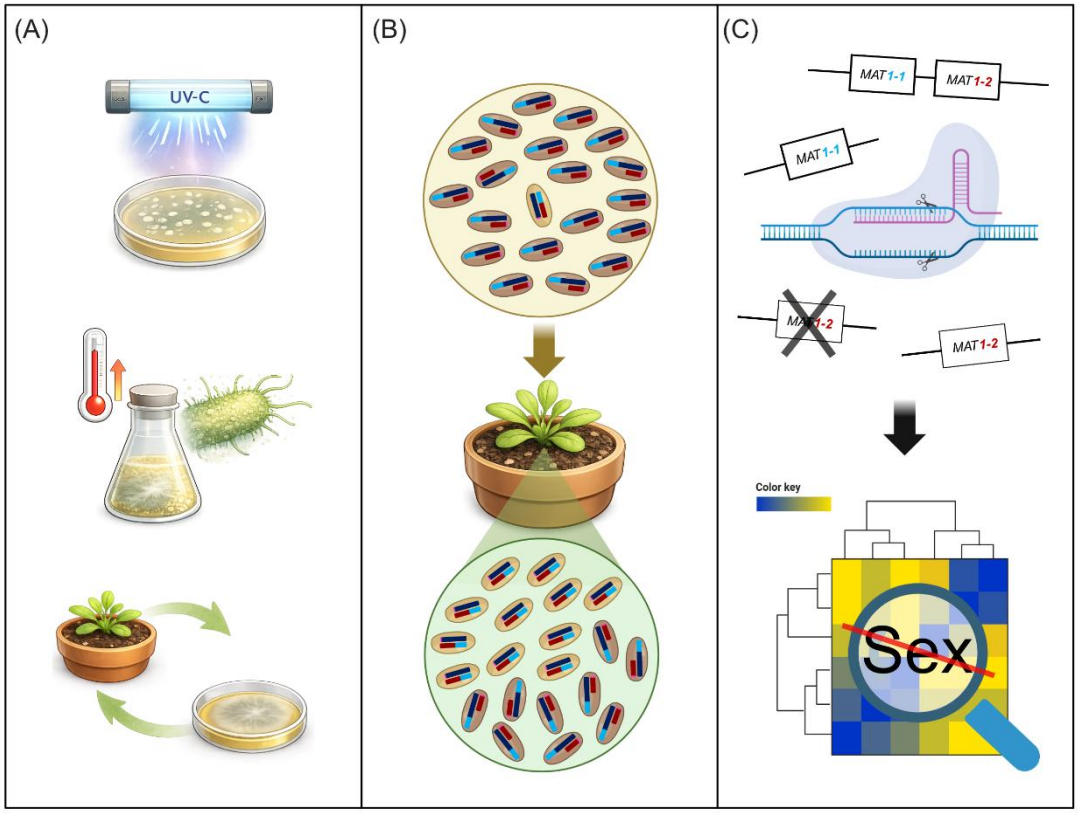


Figure 1. Schematic representation the experimental chapters that compose this thesis. (A) Chapter 2 describes the establishment of *Verticillium dahliae* lineages through experimental evolution under diverse conditions, including ultraviolet-C (UV-C) exposure, prolonged growth at elevated temperatures, sustained co-cultivation with a bacterial antagonist, and serial passage through the plant host *Arabidopsis thaliana.* (B) Chapter 3 presents the

identification of an inter-chromosomal rearrangement in an evolved *V. dahliae* lineage passaged through *A. thaliana*. Furthermore, we demonstrate that this rearrangement confers a competitive advantage to the evolved lineage over its parental lineage during colonization of *A. thaliana*, highlighting that chromosomal rearrangements can contribute to ongoing genome evolution and host adaptation in *V. dahliae*. (C) Chapter 4 explores the function of mating-type (*MAT*) genes in the presumed asexual fungus *V. dahliae* through transcriptomic analysis of transformants differing in their mating-type idiomorph. Although *MAT* genes are transcriptionally active, they do not exhibit detectable regulatory effects on genes directly associated with sexual reproduction, suggesting a divergence from their canonical role in mating and sexual development under the tested *in vitro* conditions.

Chapter 2

Experimental evolution to select adapted lineages of *Verticillium dahliae*

Chen-Yu Kuan, Julie Noah, Hui Tian, Bart P.H.J. Thomma*

University of Cologne, Institute for Plant Sciences, Cluster of Excellence on Plant Sciences (CEPLAS), 50674 Cologne, Germany;

*Corresponding author: bthomma@uni-koeln.de

ABSTRACT

Verticillium dahliae is a globally distributed plant-pathogenic fungus that causes vascular wilt disease in more than 200 dicotyledonous plant species, leading to substantial agricultural losses worldwide. Although host specificity has been reported among *V. dahliae* strains, most strains are capable of infecting a wide range of plant hosts, highlighting the remarkable ecological flexibility of *V. dahliae*. Here, we employed experimental evolution to explore the adaptability of *V. dahliae* to diverse environments. We generated independent *V. dahliae* lineages upon ultraviolet-C (UV-C) exposure, prolonged exposure to elevated temperature, sustained interaction with antagonistic bacteria, and serial passage through the plant host *Arabidopsis thaliana*. Pronounced pigmentation changes were observed from *in vitro* phenotyping of lineages derived from UV-C irradiation and elevated temperature. This study establishes a collection of *V. dahliae* lineages that serves as a resource for future investigations into the phenotypic and genetic adaptive responses of *V. dahliae*.

INTRODUCTION

Earth harbors diverse environments that have sustained life for billions of years, yet these environments are not constant and change over time (Lyons et al. 2024; Hoffmann and Sgrò 2011). Environmental changes, such as shifts in temperature, water availability, nutrient levels, or biotic interactions, can affect an organism's growth, survival, or reproduction, and thereby influence the direction of evolution (Hoffmann and Sgrò 2011). A classic example of evolution driven by environmental change is Darwin's finches on the Galápagos Islands (Grant and Grant 2024). During prolonged droughts, the scarcity of soft seeds favors finches with larger beaks capable of cracking hard seeds, leading to evolutionary changes in beak morphology (Grant and Grant 2024). The changes in beak morphology represent a clear case of adaptation, in which heritable trait modifications enhance survival and reproduction under changing environmental conditions (Bateson 2017). Adaptation occurs continuously in nature and represents one of the central mechanisms enabling life to persist under environmental changes (Bateson 2017). However, in natural ecosystems, multiple environmental changes create complex dynamics and are often poorly documented, making it difficult to determine which specific change is responsible for a given adaptive response (Stroud and Ratcliff 2025).

Experimental evolution is a powerful approach to study adaptation, as it imposes defined selective pressures that favor certain traits in organisms under controlled conditions (Kawecki et al. 2012; Fisher and Lang 2016; Stroud and Ratcliff 2025). This allows researchers to systematically study how organisms evolve adaptative traits and adapt to individual environmental changes. Described by some as "replaying life's tape," experimental evolution allows researchers to observe adaptation in real time (Blount et al. 2018; Fisher and Lang 2016). A widely used framework in experimental evolution involves propagating replicate populations from a single ancestor under defined selective pressures, with two alternative propagation strategies that differ in whether they impose population bottlenecks (Kawecki et al. 2012; Jacobs et al. 2024; Blount et al. 2018). When population bottlenecks are imposed, only a subset of the population is transferred at each passage to the next culture (Jacobs et al. 2024). Conversely, strategies that avoid bottlenecks employ continuous culture systems, in which fresh medium is continuously supplied and excess culture is removed to keep the volume constant, thereby preventing the sharp reductions in population size seen in serial

transfer method (Jacobs et al. 2024). It has been shown that weak population bottlenecks are associated with more parallel evolution, whereas strong population bottlenecks tend to promote more variable evolutionary outcomes among replicate populations (Mahrt et al. 2021). In both approaches, portions of the populations are cryopreserved at regular intervals to create a permanent record of their evolutionary trajectories (Blount et al. 2018; Kawecki et al. 2012). Reviving these cryopreserved populations and comparing them with the ancestor reveals progressive fitness gains and provides insight into how adaptation unfolds over time (Blount et al. 2018; Kawecki et al. 2012). Experimental evolution therefore provides a robust framework for dissecting how defined selective pressures drive the tempo and mode of adaptation over time.

An iconic illustration of the power of experimental evolution is the Long-Term Evolution Experiment (LTEE) with *Escherichia coli*, which at the time of its publication by Good et al. (2017) had been ongoing for more than 25 years and had exceeded 60,000 generations (Lenski et al. 1991; Blount et al. 2008; Wiser et al. 2013; Barrick et al. 2009; Tenaillon et al. 2016). Twelve replicate bacterial populations, all derived from a single ancestor, were propagated in glucose-limited media containing citrate, which *E. coli* typically cannot utilize when oxygen is present (Blount et al. 2008; Lenski et al. 1991). Within just 1,000 generations (~five months), fitness increased by ~20%, highlighting rapid adaptation even in a constant environment (Lenski et al. 1991). Over tens of thousands of generations, replicate populations showed predictable genetic adaptations, acquiring mutations repeatedly in a small set of genes with core metabolic or regulatory functions, including sugar metabolism, which has been shown to be beneficial in the glucose-limited environment of the LTEE (Tenaillon et al. 2016; Cooper et al. 2001). Still, notable differences emerged. In some populations, multiple lineages coexisted for thousands of generations, and in one population, a lineage evolved the novel ability to metabolize citrate in the presence of oxygen, opening a new ecological niche (Blount et al. 2008; Good et al. 2017). The LTEE has provided many valuable insights into evolutionary theory, showing that experimental evolution can uncover predictable trajectories of fitness improvement as well as rare, chance-driven innovations that give rise to evolutionary novelty. Similarly, a long-term experimental evolution study in the yeast *Saccharomyces cerevisiae* tracked over 200 populations for 10,000 generations, making it the first such effort in a eukaryotic system and further demonstrating the power of experimental evolution (Johnson et al. 2021). While many

outcomes mirrored those observed in the LTEE with *E. coli*, such as repeated mutations in key genes, other patterns diverged. For example, no long-term coexistence among lineages was observed in the yeast populations (Johnson et al. 2021). This research highlights that some evolutionary patterns may not apply broadly and underscores the need for diverse experimental models to uncover both general rules and organism-specific features of evolutionary dynamics.

Fungi, as eukaryotic microorganisms, possess relatively small genomes compared to other eukaryotes but still maintain cellular complexity absent in prokaryotes (Fisher and Lang 2016). Many fungal species have short generation times and are easy to propagate, making them an ideal system for experimental evolution, enabling insights into how eukaryotic traits evolve (Fisher and Lang 2016). Experimental evolution in fungi has become a powerful tool for exploring evolutionary processes and addressing challenges in both agriculture and medicine (Jacobs et al. 2024). For example, *Aspergillus fumigatus*, known as a human pathogen but commonly found in soil and decaying plant material where it thrives as a saprophyte, was subjected to experimental evolution through serial passaging in media containing one of five agricultural triazole fungicides (Zhang et al. 2017). Prolonged exposure to any of the five fungicides ultimately led to cross-resistance against clinically used triazoles (Zhang et al. 2017). This study demonstrates that experimental evolution can reveal that agricultural fungicide use may drive the emergence of drug resistance in human pathogens. More studies have used experimental evolution to investigate diverse fungal adaptations. Sustained exposure to heat stress can promote adaptive responses that enhance thermotolerance (Crecy et al. 2009). Repeated exposure to bacteria that inhibit fungal growth can select for delayed spore germination, an adaptive strategy proposed to mitigate bacterial antagonism (Mosquera et al. 2021). Serial passage through a host can select for fungal pathogens with increased virulence (Smith et al. 2022). These studies highlight experimental evolution as a powerful approach to investigate the adaptive strategies of fungi.

Verticillium dahliae is a soil-borne fungal pathogen that causes vascular wilt disease in numerous economically important crops, including vegetable crops such as tomato and lettuce, aromatic herbs like mint, fiber crops such as cotton, and tree crops like olive (Bhat and Subbarao 1999; Sayari et al. 2024). Agricultural losses attributed to *V. dahliae* infection are estimated to exceed several billion dollars annually (Klosterman

et al. 2009). At the species level, *V. dahliae* can infect more than 200 dicotyledonous plant hosts (Klosterman et al. 2011). Although host specificity occurs among *V. dahliae* strains, most strains are able to infect a wide range of plant hosts (Bhat and Subbarao 1999; Klosterman et al. 2009). This broad host range highlights the ecological flexibility of *V. dahliae* and raises questions about the adaptability that enables its persistence across hosts in varying environments. In this study, we use experimental evolution to investigate the adaptability of *V. dahliae* under diverse selective pressures.

RESULTS

Establishment of *V. dahliae* lineages through UV-C exposure

Ultraviolet (UV) radiation has been shown to act as a selective pressure that promotes the evolution of adaptive traits in fungi (Braga et al. 2015). To investigate whether UV exposure can induce an adaptive response in *V. dahliae*, we subjected *V. dahliae* to UV-C radiation under controlled laboratory conditions. To determine a UV-C exposure duration that imposes stress while maintaining fungal viability, 1,000 *V. dahliae* conidiospores were spread onto each of three potato dextrose agar (PDA) plates and exposed to UV-C for 1, 2 or 2.5 minutes, respectively, followed by incubation for 5 days at room temperature in darkness. Based on visual assessment, the number of colonies that formed decreased progressively with longer UV-C exposure times, with a notable reduction starting at 2 minutes of UV-C exposure (Figure 1A). Additionally, some colonies exposed to 2 or 2.5 minutes of UV-C displayed increased size relative to surrounding colonies, suggesting variation in growth among the colonies (Figure 1A). Based on these observations, a 2-minute UV-C exposure was selected for subsequent experiments, as it imposed sufficient stress to allow phenotypic variation without compromising fungal viability too drastically.

To further investigate the effects of UV-C exposure on *V. dahliae*, 4,000 *V. dahliae* conidiospores were first plated onto four PDA plates (1,000 per plate), and subsequently exposed to the selected 2-minute UV-C exposure. As observed previously, colonies displayed size variation, with some appearing larger than average (Figure 1B). Most colonies retained the typical white appearance characteristic of the parental *V. dahliae*. However, we furthermore observed a colony with distinctly reddish pigmentation (Figure 1B). This colony was designated as UV-C-derived lineage U1 and retained for further analysis, as pigmentation changes in fungi have been linked to enhanced UV resistance, suggesting an adaptive response to UV-imposed stress (Avalos and Carmen Limón 2015; Braga et al. 2006). To assess whether the changes in pigmentation are maintained upon subculturing, conidiospore suspensions of lineage U1 and the parental *V. dahliae* lineage were each spotted as a single central spot on a PDA plate. After 14 days of incubation, lineage U1 exhibited a stable reddish pigmentation (Figure 1C), indicating that the altered pigmentation colour is maintained through subculturing.

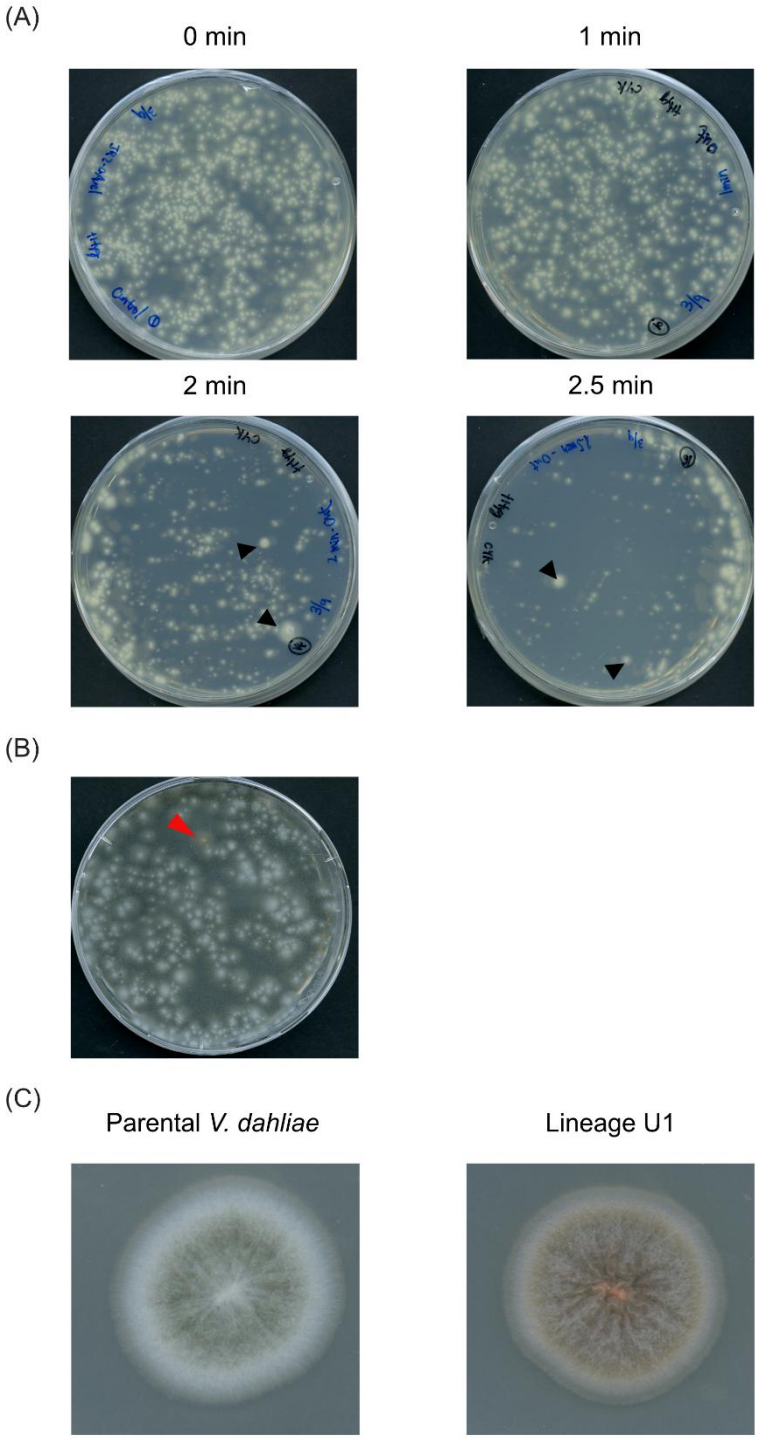


Figure 1. UV-C exposure affects colony formation and pigmentation in *Verticillium dahliae*. (A) Colony formation by *V. dahliae* after UV-C exposure for 0, 1, 2, or 2.5 minutes. Approximately 1,000 conidiospores were spread onto each potato dextrose agar (PDA) plate, exposed to UV-C, and incubated for 5 days at room temperature in darkness. The black arrowheads highlight representative colonies that exhibit increased size relative to surrounding colonies. (B) Colony formation of the *V. dahliae* after 2 minutes of UV-C exposure. Approximately 1,000 conidiospores were spread onto a PDA plate, exposed to UV-C, and incubated for 11 days at room temperature in darkness. The red arrowhead highlights a colony with altered pigmentation. (C) Colony phenotypes of the parental *V. dahliae* lineage

and UV-C–derived lineage U1. Conidiospore suspensions were deposited at the center of PDA plates and incubated for 14 days at room temperature in darkness.

Establishment of *V. dahliae* lineages under prolonged exposure to elevated temperature

Heat stress resulting from climate change is a growing selective pressure on fungi in natural and agricultural environments (Laine and Leino 2025; Konkel Neabore 2024). To explore whether *V. dahliae* adapts under such selective pressure, we first identified a temperature that imposes heat stress on the fungus. Given that the optimal *in vitro* growth temperature for *V. dahliae* ranges from 20°C to 25°C (Sbeiti et al. 2023), we tested its growth on PDA plates at 20°C and 30°C. Based on visual assessment, *V. dahliae* exhibited markedly reduced radial growth at 30°C when compared with 20°C (Figure 2), indicating that 30°C imposes heat stress on *V. dahliae*. Based on this result, we selected 30°C as the temperature for subsequent experimental evolution.

To investigate the effect of prolonged exposure of *V. dahliae* to 30°C*,* four independent cultures were prepared, each containing 400 mL of PDB with *V. dahliae* conidiospores (10^7 conidiospores/mL) and incubated at 30°C while shaking at 130 rpm. Every 3–4 weeks, the biomass from each culture was collected by centrifugation and transferred to 400 mL of fresh PDB. Each cycle of growth followed by biomass transfer was defined as one passage. At the final passage of each culture, 300 µL of culture was plated onto PDA plates, and conidiospores from the resulting colonies were harvested and stored as four heat-derived *V. dahliae* lineages: H1, H2, H3, and H4. Lineages H1 and H2 were harvested after six passages, lineage H3 after seven passages, and lineage H4 after eight passages.

To determine whether the heat-derived *V. dahliae* lineages (H1–H4) adapted to 30°C, we assessed if lineages H1–H4 exhibited improved growth on PDA plates at 30°C relative to the parental *V. dahliae* lineage. To this end, 10 µL of suspension containing 10^3 *V. dahliae* conidiospores were deposited in the middle of PDA plates for each heat-derived lineage and the parental lineage. After 10 days of incubation at 30°C, the parental lineage and lineage H2 displayed greyish pigmentation resulting from melanin accumulation (Figure 3A). In contrast, lineages H1 and H3 produced white, non-melanized colonies with a fluffy appearance resulting from the development of aerial mycelium (Figure 3A). Lineage H4 exhibited an intermediate phenotype, with individual

colonies displaying both melanized regions and areas of non-melanized white aerial mycelium (Figure 3A). Despite these morphological differences, assessment of colony diameters revealed no significant differences in radial growth between lineages H1–H4 and the parental lineage after 10 days of incubation at 30°C on PDA plates (Figure 3B). Thus, all heat-derived *V. dahliae* lineages exhibited radial growth comparable to the parental lineage at 30°C on PDA plates, while colony morphology was altered in all lineages except for lineage H2.

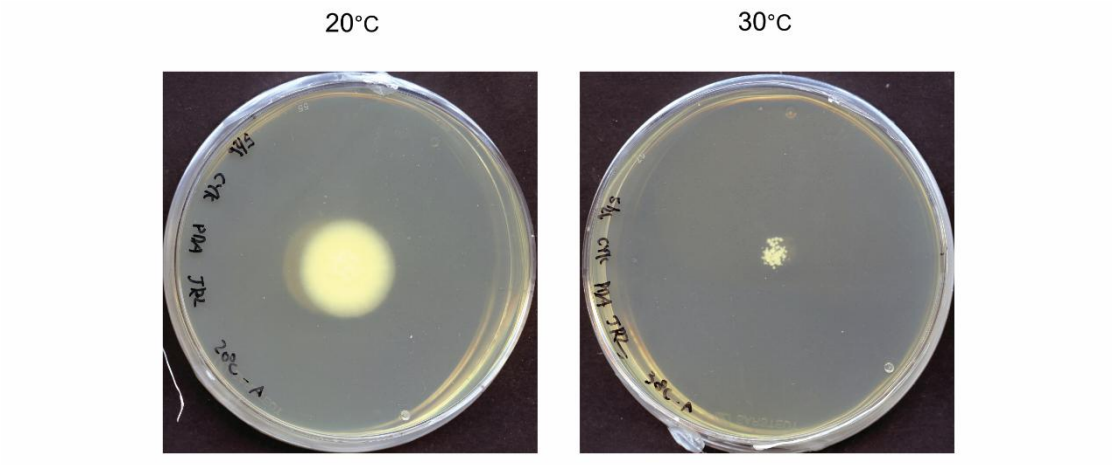


Figure 2. Reduced *Verticillium dahliae* growth at 30°C. Conidiospore suspensions of *V. dahliae* were deposited at the center of potato dextrose agar (PDA) plates. Plates were incubated for 7 days at either 20°C or 30°C in the dark.

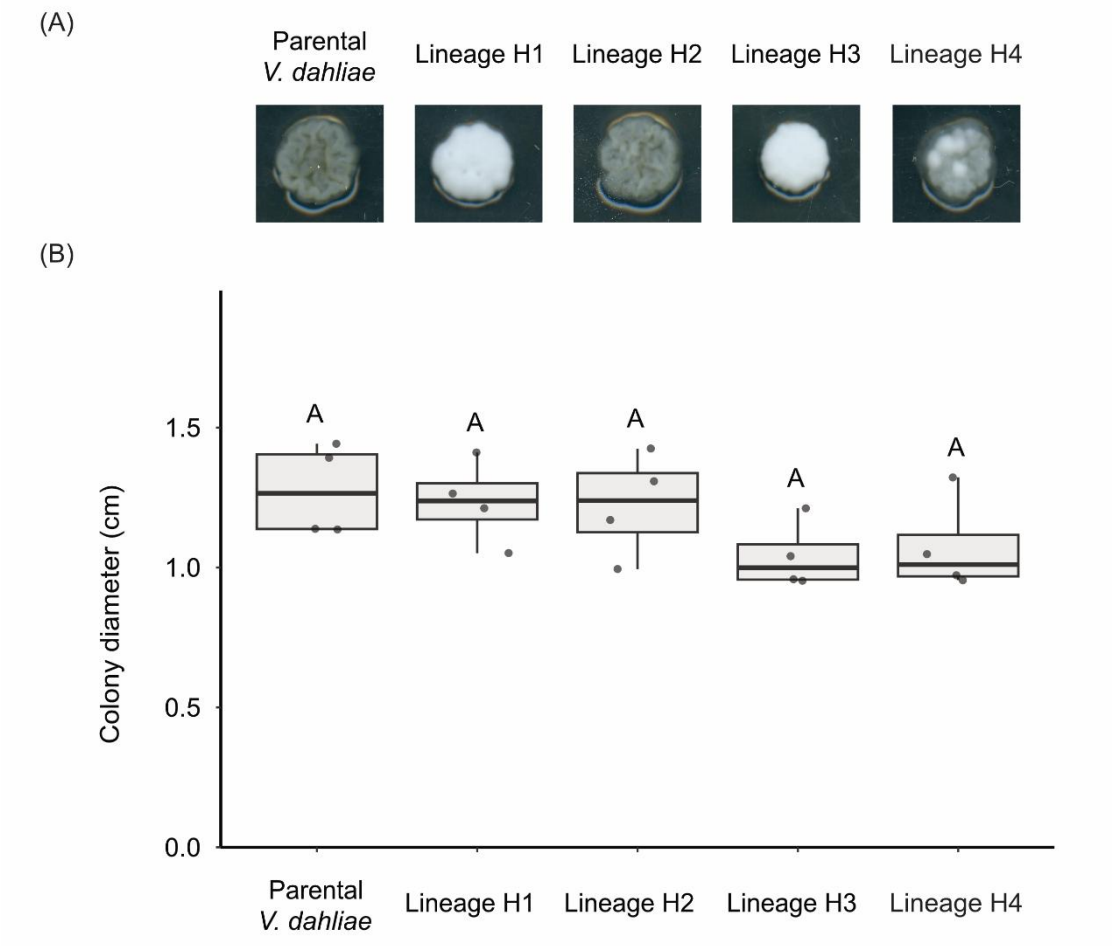


Figure 3. *Verticillium dahliae* lineages exhibit differential colony morphology but comparable radial growth after 10 days of incubation at 30°C. *In vitro* growth of parental *V. dahliae* and the heat-derived *V. dahliae* lineages (H1–H4) was assessed on potato dextrose agar (PDA). (A) Representative colony morphology after 10 days of growth on PDA plates at 30°C. (B) Colony diameter after 10 days of growth on PDA plates at 30°C (one-way ANOVA and Tukey's post-hoc test; $p<0.05$; N=4).

Establishment of *V. dahliae* lineages under prolonged exposure to bacterial antagonism

Fungi in natural environments continuously encounter microbial competitors, and these encounters can result in antagonistic interactions that impose selective pressures, which in turn drive adaptive changes in fungi (Tarkka et al. 2009). We hypothesized that prolonged exposure to such microbial antagonism could drive adaptive evolution in *V. dahliae*. To explore this possibility, we first sought a bacterial strain with antagonistic activity against *V. dahliae*. We selected *Pseudomonas corrugata*, a biocontrol bacterium widely used in crop protection because of its ability to inhibit diverse plant-pathogenic fungi, including *V. dahliae* (Catara 2007). To verify the antagonistic activities of *P. corrugata* against *V. dahliae*, we conducted *in vitro*

confrontation assays on PDA plates, where we grew *V. dahliae* next to *P. corrugata*. On the control plate, in absence of *P. corrugata*, *V. dahliae* formed a circular colony with uniform radial growth (Figure 4A). In contrast, in the presence of *P. corrugata*, *V. dahliae* exhibited restricted and asymmetrical growth, indicating inhibitory effects of *P. corrugata* on *V. dahliae* (Figure 4A). Having confirmed the antagonist activity of *P. corrugata,* we then aimed to establish a co-culture condition in which *P. corrugata* imposes stress on *V. dahliae* without completely inhibiting its growth. To determine an appropriate amount of *P. corrugata* for the co-culture*,* 10 mL of potato dextrose broth (PDB) was cultured with *V. dahliae* conidiospores (10^7 conidiospores/mL) and *P. corrugata* at varying initial OD_{600} values (0.00, 0.01, 0.02, 0.04, and 0.06) while shaking at 80 rpm. The culture of *V. dahliae* lacking *P. corrugata* (OD_{600} = 0) served as control. After three days of incubation, visual assessment showed that all co-cultures exhibited turbidity (Figure 4B). Interestingly, the turbidity decreased with increasing initial OD_{600} values in the co-cultures (Figure 4B), suggesting that higher initial bacterial density leads to greater reduction in *V. dahliae* growth*.* Based on these observations, we selected the lowest tested initial OD_{600} value (0.01) for subsequent co-culture experiments, as this bacterial density affects *V. dahliae* growth without completely inhibiting it.

To investigate the effect of prolonged exposure of *V. dahliae* to *P. corrugata,* three independent co-cultures were prepared, each containing 400 mL of PDB with *V. dahliae* conidiospores (10^7 conidiospores/mL) and *P. corrugata* (OD_{600} = 0.01) and incubated while shaking at 80 rpm. Every 3–4 weeks, the biomass from each co-culture was collected by centrifugation and transferred to 400 mL of fresh PDB containing newly prepared *P. corrugata* (OD_{600} = 0.01). Each cycle of growth followed by biomass transfer was defined as one passage. After 10 passages, we assessed whether *V. dahliae* had undergone adaptive changes in response to prolonged exposure to *P. corrugata*. To isolate *V. dahliae* for further evaluations, 300 µL from each of the three co-cultures was plated onto PDA plates supplemented with tetracycline and chloramphenicol (50 µg/mL each) to suppress *P. corrugata* growth. After seven days of incubation, conidiospores were harvested from plates and stored as three co-culture-derived *V. dahliae* lineages: B1, B2, and B3

Next, we tested whether lineages B1–B3 displayed improved growth in the presence of *P. corrugata.* To this end, 10^7 *V. dahliae* conidiospores from each co-culture-derived

lineage (B1–B3), along with the parental *V. dahliae* lineage, were co-cultured with *P. corrugata* (OD_{600} 0.01) in 10 mL PDB while shaking at 80 rpm. *V. dahliae* biomass accumulation was quantified by real-time PCR after seven days of incubation. However, no significant biomass differences were observed (Figure 5). Thus, the co-culture-derived lineages showed no improvement in biomass accumulation over the parental lineage when co-cultured with *P. corrugata*.

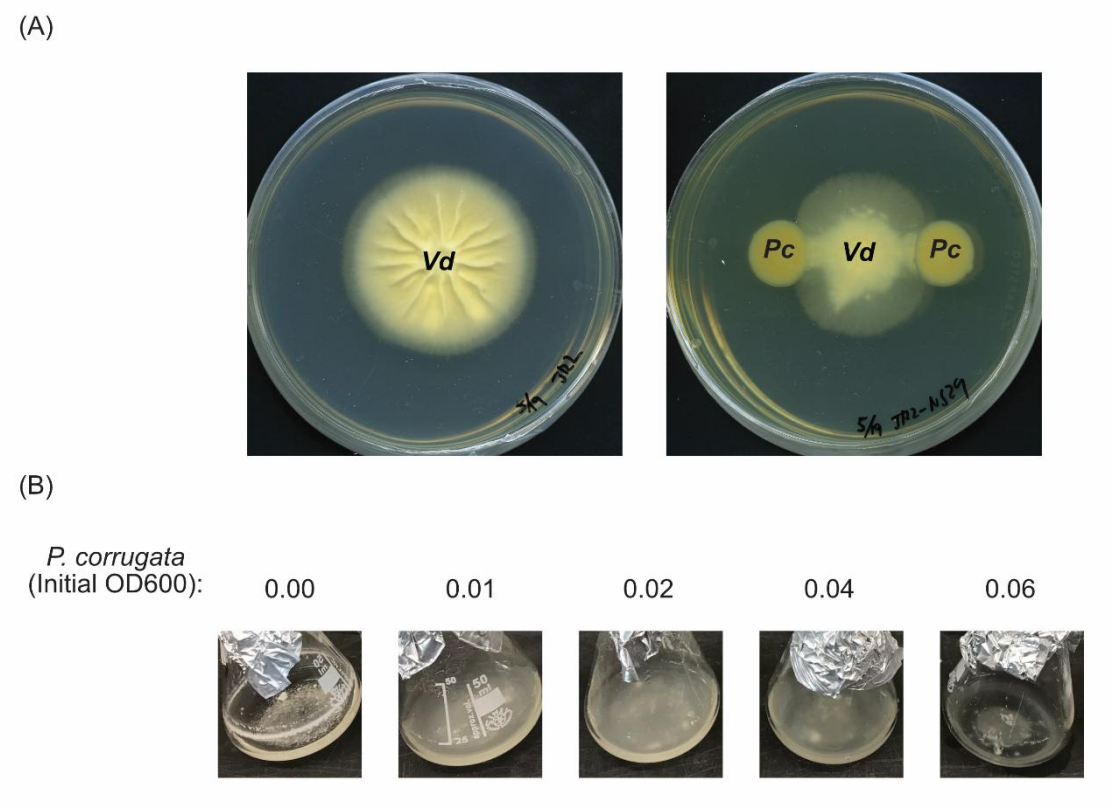


Figure 4. Reduced *Verticillium dahliae* growth in the presence of the bacterial antagonist *Pseudomonas corrugata*. (A) Confrontation assay of *P. corrugata* (*Pc*) and *V. dahliae* (*Vd*). Conidiospore suspensions of *V. dahliae* were deposited at the center of potato dextrose agar (PDA) plates. On the plate with *P. corrugata*, aliquots of a bacterial culture were placed on both sides of *V. dahliae*. Plates were incubated for 14 days at room temperature in the dark. (B) Increasing the initial density of *P. corrugata* resulted in reduced turbidity of the co-cultures with *V. dahliae*. *V. dahliae* was co-cultured with *P. corrugata* in potato dextrose broth for 3 days at increasing initial OD_{600} values (0.00, 0.01, 0.02, 0.04, and 0.06) while shaking at 80 rpm. The control culture (OD_{600} = 0) shows typical *V. dahliae* mycelium in the absence of *P. corrugata*.

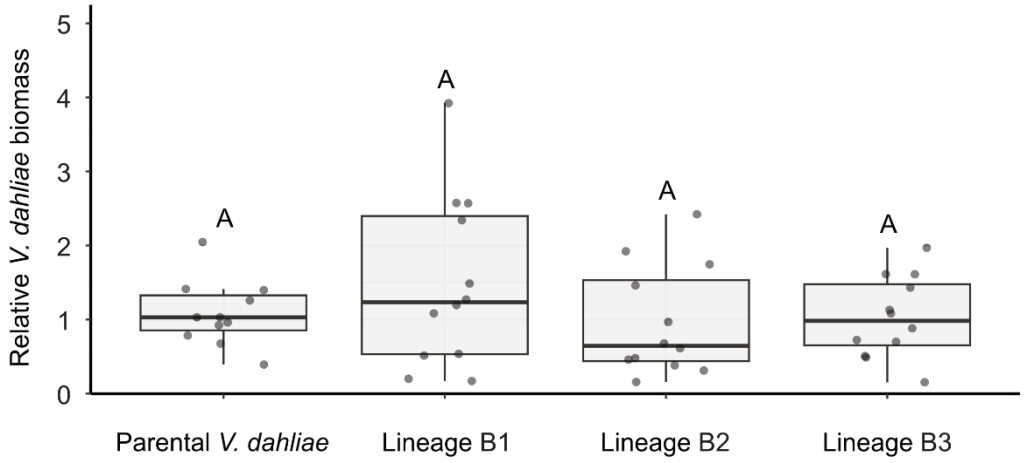


Figure 5. Comparable biomass accumulation between *Verticillium dahliae* lineages after seven days of co-cultivation with the bacterial antagonist *Pseudomonas corrugata*. Relative *V. dahliae* biomass measured by real-time PCR for the parental lineage and co-culture derived lineages (B1–B3), each cultured with *P. corrugata* for seven days in potato dextrose broth. Different letters indicate statistically significant differences according to one-way ANOVA followed by Tukey's post-hoc test ($p<0.05$; $N\geq11$).

Establishment of *V. dahliae* lineages through serial passage in a plant host

Plants represent a complex ecological niche that can impose selective pressures on fungi and influence their evolution (Naranjo-Ortiz and Gabaldón 2019). To investigate how *V. dahliae* can adapt to a plant host, we conducted a serial passage experiment in which *V. dahliae* was repeatedly inoculated onto *Arabidopsis thaliana*. For this, we used a *V. dahliae* strain in which the *Ave1* gene, previously shown to act as a virulence factor on *A. thaliana*, has been deleted (Δ*Ave1*; de Jonge et al. 2012). The absence of this virulence factor reduces the ability to colonize *A. thaliana*, which may put pressure on the strain to become more aggressive in order to overcome the host immune system. To establish each passage, approximately 770 *A. thaliana* were inoculated with *V. dahliae* via root dipping. In the first passage, *V. dahliae* was re-isolated from approximately 150 plants that displayed the most pronounced Verticillium wilt symptoms, including stunting and wilting (Ellendorff *et al.*, 2009), at 4 weeks post inoculation (Figure 6A, C, and E). The re-isolated *V. dahliae* was used to start the next serial passage, following the same procedure described above. However, from the second passage onward, the selection criteria were made more stringent by selecting only the 10 to 15 plants that exhibited the most pronounced Verticillium wilt symptoms at 2 weeks post inoculation, when most plants had not yet developed any disease

symptoms (Figure 6B, D, and F). Thus, <2% of the plants, namely those that developed the earliest and strongest symptoms, were chosen. After completing four consecutive passages, conidiospores from the re-isolated *V. dahliae* were plated onto PDA plates for single-colony isolation. Eventually, three colonies were selected and designated as Arabidopsis-derived lineages P1, P2, and P3. This selection strategy, focused on early symptomatic plants across successive passages, was designed to enrich for *V. dahliae* genotypes potentially adapted to the *A. thaliana*.

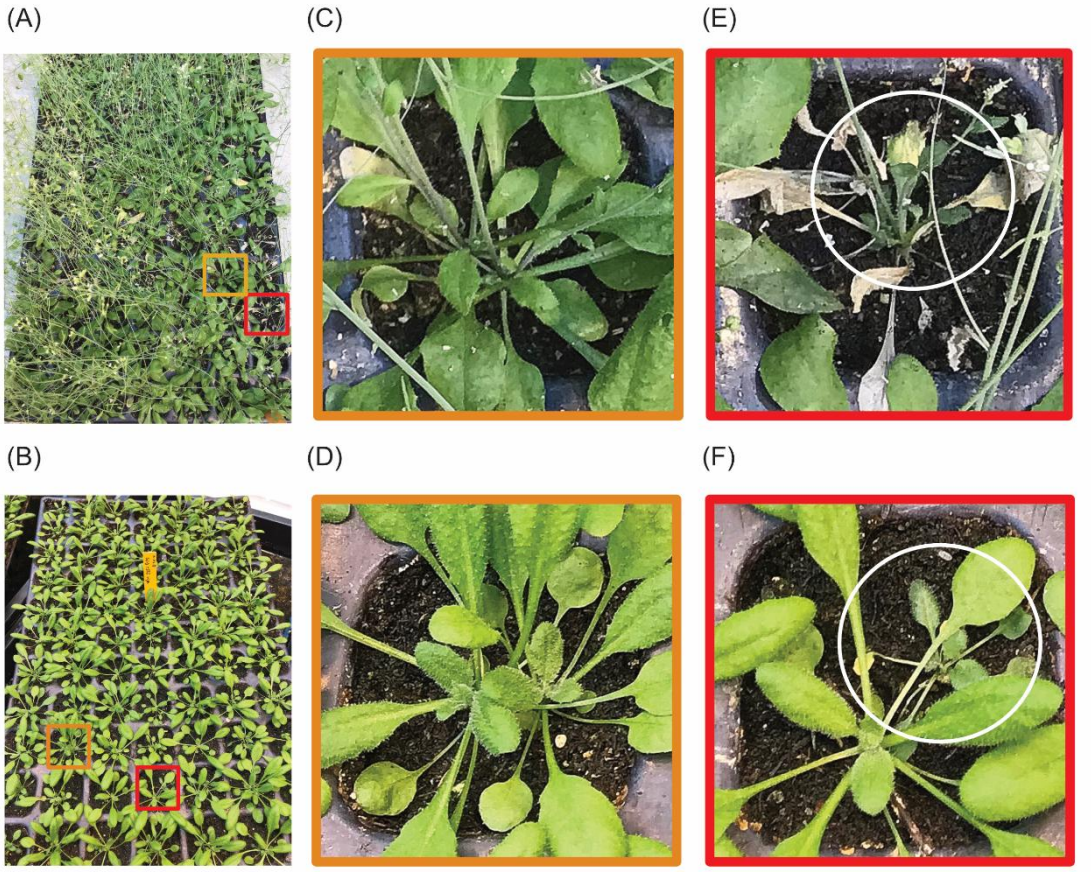


Figure 6. *Arabidopsis thaliana* plants showing varying degrees of disease severity resulting from *Verticillium dahliae* infection. Plants were inoculated with *V. dahliae* and grown in trays containing 77 pots, with two plants per pot. Representative non-symptomatic and symptomatic plants are marked with yellow and red boxes, respectively. A tray of *A. thaliana* is shown at (A) 4 weeks (first passage) and (B) 2 weeks post inoculation (second passage). Higher magnification views of the non-symptomatic (C, D) and symptomatic (E, F) plants marked in A and B, respectively. White circles indicate plants selected for re-isolation based on visible stunting and partial leaf wilting.

DISCUSSION

Experimental evolution has significantly advanced our understanding of adaptive processes, primarily through studies on model microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* (Fisher and Lang 2016). However, its application to plant pathogenic fungi remains limited. *Verticillium dahliae* is a globally distributed fungal plant pathogen that infects a wide range of hosts across diverse environments (Acharya et al. 2020; Sayari et al. 2024). In this study, we used experimental evolution to generate distinct *V. dahliae* lineages that serve as a resource for investigating the adaptability of *V. dahliae*.

Among the tested experimental conditions, a single 2-minute UV-C exposure resulted in a rapid shift to reddish pigmentation in one colony that persisted through subsequent subculturing. This response is likely caused by UV-induced mutagenesis, as UV-C irradiation is known to generate DNA lesions, which are structural alterations in the DNA that, if not accurately repaired, result in random mutations (Rastogi et al. 2010). Notably, pigmentation shifts have been linked to UV protection in fungi (Avalos and Carmen Limón 2015; Braga et al. 2006), raising the question of whether the reddish UV-C-derived lineage might exhibit increased UV-C resistance compared to the parental lineage. This hypothesis can be addressed by comparing survival and growth of the two lineages following UV-C treatment. In addition to potential effects on UV-C resistance, the shift from black to red pigmentation may reflect alterations in melanin biosynthesis, the pathway that normally produces black pigmentation in wild-type *V. dahliae* (Bell et al. 1976). Previous work on UV-derived *V. dahliae* mutants detected accumulation of scytalone, a red-colored intermediate of the DHN-melanin pathway that precedes melanin formation (Fan et al. 2017; Bell et al. 1976) Excessive scytalone accumulation has been shown to cause red pigmentation in both *V. dahliae* and the blue cheese fungus *Penicillium roqueforti* (Bell et al. 1976; Cleere et al. 2024). Thus, the reddish pigmentation observed in our UV-C-derived *V. dahliae* may reflect elevated scytalone accumulation, a hypothesis that can be tested through biochemical analysis.

Sustained exposure to 30°C was hypothesized to select for *V. dahliae* lineages with enhanced growth at 30°C. Following serial passages at 30°C, the heat-derived lineages exhibited pronounced changes in colony morphology, most notably reduced melanized pigmentation. We hypothesized that this reduction in pigmentation may represent an adaptive response to elevated temperature. Consistent with this

hypothesis, lightly pigmented yeasts are more frequently observed in warmer geographic regions, suggesting that reduced pigmentation may limit excessive heat absorption (Cordero et al. 2018). This interpretation is supported by evidence showing that darkly pigmented fungi absorb heat more rapidly than lightly pigmented or non-pigmented fungi, as demonstrated in the yeast *Cryptococcus neoformans* (Cordero et al. 2018). However, despite morphological changes, none of the four heat-derived *V. dahliae* lineages exhibited increased radial growth relative to the parental lineage when assessed on PDA plates at 30°C for 10 days. Nonetheless, because the sustained exposure of *V. dahliae* to 30°C was performed in liquid culture, potential growth advantage at 30°C may be restricted to liquid conditions and therefore not detectable in growth assays on PDA plates. Further studies assessing the growth of heat-derived *V. dahliae* lineages in liquid cultures at 30°C will clarify whether they display enhanced growth at this temperature

We hypothesized that prolonged exposure to *P. corrugata* antagonism would select for *V. dahliae* lineages with increased tolerance to growth inhibition by *P. corrugata*. However, following ten serial co-culture passages with *P. corrugata*, the three co-culture-derived *V. dahliae* lineages (B1–B3) did not exhibit increased biomass accumulation relative to the parental lineage upon re-exposure to *P. corrugata*. Nevertheless, adaptive responses may have occurred through traits not demonstrated by the biomass assay, such as enhanced competitive performance of co-culture-derived lineages relative to the parental lineage in the presence of *P. corrugata*. For example, Mosquera et al. (2021) conducted an experimental evolution study examining prolonged interactions between the filamentous fungus *Aspergillus niger* and the antifungal bacterium *Collimonas fungivorans*. Adaptation of *A. niger* to *C. fungivorans* was evaluated using direct competition assays, in which spores of evolved and parental *A. niger* lineages were co-cultured at equal proportions in the presence of *C. fungivorans* (Mosquera et al. 2021). The evolved *A. niger* produced more spores than the parental *A. niger*, indicating that continuous interaction with *C. fungivorans* selected for a competitive advantage in *A. niger* (Mosquera et al. 2021). Further studies using direct competition assays between co-culture-derived and parental *V. dahliae* lineages in the presence of *P. corrugata* will help determine whether competitive performance differs between lineages.

Experimental evolution involving the serial passage of fungal pathogens through plant

hosts has been applied to *V. dahliae* and other fungal species. For example, Alkher et al. (2009) subjected *V. dahliae* to four successive passages through potato, using 30 host plants per passage and re-isolating *V. dahliae* from plants exhibiting the most severe symptoms at later disease stages. This approach resulted in increased aggressiveness in a subset of the evolved *V. dahliae* lineages (Alkher et al. 2009). Similarly, the plant pathogenic fungus *Stemphylium solani* was serially passed through clover for four rounds, with two ancestral lineages each passed across 11 clover taxa, resulting in 22 evolved *S. solani* lineages (Gilbert and Parker 2010). At each round, each lineage was passed through only 3 clover plants (Gilbert and Parker 2010). Despite this limited number of hosts per passage, the evolved *S. solani* lineages showed a consistently increase in infectivity, as measured by a higher proportion of inoculated leaves from which the *S. solani* could be successfully re-isolated (Gilbert and Parker 2010). These studies demonstrate that *in planta* serial passage can be used successfully to investigate pathogen adaptation. Compared with these studies, our *in planta* experimental evolution involved a larger number of host plants, with approximately 700 *A. thaliana* plants per passage. By preferentially re-isolating from *A. thaliana* exhibiting the earliest and most severe disease symptoms, we imposed a strong selection pressure on *V. dahliae* lineages to favor rapid and aggressive disease progression.

Experimental evolution produces lineages that serve as powerful resources for exploring species adaptability through both phenotypic and genotypic analyses (Kawecki et al. 2012). These lineages can be examined using methods such as whole-genome sequencing to detect genotypic changes and targeted genome engineering to introduce or reverse those changes, thereby allowing researchers to determine how specific modifications drive adaptive outcomes (Stroud and Ratcliff 2025). In this study, we generated multiple *V. dahliae* lineages under a diverse array of experimental conditions, including abiotic treatments such as UV-C irradiation and elevated temperature, as well as biotic interactions with antagonistic bacteria and a plant host. While some lineages exhibited immediate phenotypic changes in the form of altered pigmentation, additional phenotypic variation may exist in other lineages but has yet to be identified. Furthermore, it remains unclear whether the observed phenotypic changes contribute directly to adaptation. Future phenotypic and genotypic analyses of our derived *V. dahliae* lineages will help to elucidate adaptive evolution in *V. dahliae*.

MATERIALS AND METHODS

UV-C exposure

Conidiospores of a *V. dahliae Ave1* deletion strain (Δ*Ave1,* de Jonge et al. 2012) were harvested from three-day-old potato dextrose agar (PDA; Carl Roth, Karlsruhe, Germany). Conidiospores were washed three times with sterile Milli-Q water by centrifugation at 10,000 rpm for 5 minutes, and resuspended in sterile Milli-Q water. Conidial suspension concentration was determined using a haemocytometer and adjusted to a concentration of 10^4 conidiospores/mL in sterile Milli-Q water. Approximately 1,000 conidiospores were evenly spread onto a fresh PDA plate that was placed uncovered inside the front edge of a laminar flow cabinet (BioWizard Silver SL-130, Kojair Tech Oy, Finland) equipped with a germicidal UV-C lamp (254 nm; PURITEC HNS S 11 W G23, Osram GmbH, Germany). To assess the optimal duration of the UV-C exposure, PDA plates with conidiospores were exposed to UV-C irradiation for 1, 2 and 2.5 minutes. After exposure, plates were covered and incubated at room temperature in darkness for 5 days. Fungal growth was documented using a flatbed scanner (Epson Perfection V700, Long Beach, USA).

For the generation of UV-C–derived lineages, four PDA plates with Δ*Ave1* conidiospores were exposed to UV-C irradiation for 2 minutes following the same procedure described above. Plates were incubated at room temperature in darkness for 11 days and documented using a flatbed scanner. Selected colonies were subjected to two rounds of single-colony purification by plating serially diluted conidial suspensions onto PDA plates to ensure clonal purity.

Pigmentation analysis

Conidiospores of the *V. dahliae* Δ*Ave1* strain and the UV-C–derived lineage were prepared as described above. Conidial suspensions were adjusted to 10^5 conidiospores/mL, and a 10 µL aliquot of each suspension was spotted at the center of a PDA plate. Plates were incubated at room temperature in darkness for 14 days. Pigmentation was documented using a flatbed scanner.

Confrontation assay with *P. corrugata*

Conidiospores of *V. dahliae* strain JR2 were prepared as described above (Faino et al. 2015). Conidial suspensions were adjusted to 10^5 conidiospores/mL, and 10 µL of the

suspension was spotted at the center of a PDA plate. Then, 10 µL of *P. corrugata* culture (OD_{600} = 0.05), grown overnight in potato dextrose broth (PDB; VWR, Radnor, PA, USA) at 28°C with shaking at 200 rpm, was spotted on both sides of the *V. dahliae* suspension on the plate. Plates were incubated at room temperature in darkness for 14 days, and documented using a flatbed scanner.

Co-cultivation with *P. corrugata*

Conidiospores of *V. dahliae* strain JR2 were prepared as described above (Faino et al. 2015). The conidial suspension was adjusted to a final concentration of 10^7 conidiospores/mL in five 50-mL flasks, each containing 10 mL of PDB. *P. corrugata* was cultured overnight in PDB at 28°C while shaking at 200 rpm, and then added to each of the five *V. dahliae* cultures to obtain final OD_{600} values of 0.00 (control), 0.01, 0.02, 0.04, and 0.06. The co-cultures were incubated at 20°C while shaking at 80 rpm. After 3 days, pictures were taken.

For the generation of co-culture-derived lineages, the conidial suspension of *V. dahliae* strain JR2 was adjusted to 10^7 conidiospores/mL in three 2-L flasks, each containing 400 mL of PDB. Each flask was then supplemented with *P. corrugata* at an OD_{600} of 0.01, following the procedure described above. Co-cultures were incubated at 20°C while shaking at 80 rpm. Every 3–4 weeks, the biomass from each co-culture was harvested by centrifugation at 4,600 rpm for 5 minutes and transferred into 400 mL of fresh PDB containing freshly prepared *P. corrugata* (OD_{600} = 0.01). Each transfer of biomass into fresh medium was considered one passage. This procedure was repeated independently for each co-culture across 10 sequential passages. Four weeks after the final transfer, 300 µL aliquots were taken from each co-culture and plated onto separate PDA plates supplemented with tetracycline (50 µg/mL) and chloramphenicol (50 µg/mL) to suppress bacterial growth. Plates were incubated in the dark at room temperature for seven days. Conidiospores were harvested and stored as separate lineages.

Quantification of *V. dahliae* biomass after co-cultivation with *P. corrugata*

Conidiospores of *V. dahliae* strain JR2 and the co-culture-derived lineages were prepared and co-cultured with *P. corrugata* as described above, using 10 mL PDB with *V. dahliae* at 10^6 conidiospores/mL and *P. corrugata* at OD_{600} 0.01. Cultures were incubated for 7 days at 20°C while shaking at 80 rpm. Fungal biomass was harvested

by centrifugation at 4,600 rpm for 5 minutes, and pellets were snap-frozen in liquid nitrogen and homogenized with 2.4 mm metal beads (Omni International, Kennesaw, GA, USA) using a Tissue-Lyser (Retsch, Haan, Germany). Genomic DNA was extracted with the RSC Plant DNA Kit (Promega, Madison, WI, USA) using the Maxwell RSC device (Promega, Madison, WI, USA). A spike-in DNA sequence was added during DNA extraction for sample calibration (Guo et al. 2020). The *V. dahliae* biomass was quantified by real-time PCR using *V. dahliae*-specific primers targeting the internal transcribed spacer (ITS) region of the ribosomal DNA (Snelders et al. 2020).

Heat exposure

Conidiospores of *V. dahliae* strain JR2 were prepared as described above (Faino et al. 2015). Conidial suspensions were adjusted to 10^5 conidiospores/mL, and a 10 µL aliquot of each suspension was deposited at the center of a PDA plate. Plates were incubated at 20°C or 30°C in darkness for 7 days and were documented using a flatbed scanner.

For the generation of heat-derived lineages, the conidial suspension of *V. dahliae* strain JR2 was adjusted to 10^7 conidiospores/mL in four 2-L flasks, each containing 400 mL of PDB. Cultures were incubated at 30°C while shaking at 130 rpm. Every 3–4 weeks, the biomass from each culture was harvested by centrifugation at 4,600 rpm for 5 minutes and transferred into 400 mL of fresh PDB. Each transfer of biomass into fresh PDB was considered one passage. This procedure was independently repeated for each culture over 6–8 passages. Four weeks after the final transfer, 300 µL aliquots were taken from each culture and plated onto separate PDA plates. Plates were incubated in the dark at room temperature for 7–10 days. Conidiospores were harvested and stored as separate lineages.

For *In vitro* growth assays of heat-derived *V. dahliae* lineages, conidiospores of the *V. dahliae* strain JR2 and the heat-derived lineages were prepared as described above. Conidial suspensions were adjusted to 10^5 conidiospores/mL, and a 10 µL aliquot of each suspension was deposited at the center of a PDA plate. Plates were incubated at 30°C in darkness. Colony diameters were measured after 10 days, and colony morphology was documented using a flatbed scanner.

***In planta* passage**

Each round of *in planta* passage was performed by inoculating Arabidopsis with a *V.*

dahliae Ave1 deletion strain (Δ*Ave1,* de Jonge et al. 2012) as previously described (Ellendorff et al. 2009), with modifications to facilitate larger-scale inoculation as detailed below. Arabidopsis Col-0 plants were grown in potting soil under controlled greenhouse conditions (17 hours of light at 23°C followed by 7 hours of darkness at 22°C). Conidiospores of the Δ*Ave1* strain were prepared as described above, and the conidial suspension was adjusted to 5 × 10^6 conidiospores/mL. Approximately 770 Arabidopsis seedlings (10–12 days old) were gently uprooted and inoculated by immersing roots in the conidiospore suspension for 6 minutes. Inoculated seedlings were transferred to fresh potting soil and maintained under the above-described greenhouse conditions. The 770 plants were cultivated in five trays, each containing 77 pots, with two plants grown per pot. For the first passage, approximately 150 plants displaying the most pronounced Verticillium wilt symptoms (Ellendorff *et al.*, 2009) were collected at 4 weeks post-inoculation. For subsequent passages, 10–15 plants displaying the most pronounced Verticillium wilt symptoms were collected at 2 weeks post-inoculation. For each collection, aboveground plant tissue was cut into segments and surface-sterilized by immersion in 70% ethanol for 2 minutes, followed by three 30-second washes in 50% commercial bleach (DanKlorix, Colgate-Palmolive, Hamburg, Germany), followed by three rinses with sterile Milli-Q water. Sterilized plant tissue was placed onto PDA plates supplemented with hygromycin B (25 µg/mL) to selectively isolate Δ*Ave1,* in which the *Ave1* gene was replaced by hygromycin B resistance cassette at its original genomic locus. Plates were incubated at room temperature for 5–7 days, after which *V. dahliae* hyphae emerging from internal plant tissues were transferred to fresh PDA plates by streaking with a sterile toothpick. Conidiospores from the resulting colonies were harvested and used to prepare conidial suspensions for the next inoculation cycle, following the procedure described above. This process was carried out for a total of four consecutive *in planta* passages. After the final passage, conidiospores from *V. dahliae* re-isolated from infected plant tissue were suspended in sterile Milli-Q water, serially diluted, and plated onto PDA plates for two rounds of single-colony purification to ensure clonal purity. Selected colonies were stored as separate lineages.

Chapter 3

A *de novo* large-scale chromosomal rearrangement mediates *Verticillium dahliae* adaptation

Chen-Yu Kuan[1], Andrea Doddi [1,2#], Julie Noah [1#], Yukiyo Sato, Gabin Armand[1], Alexander Mandel[1], Edgar A. Chavarro-Carrero[1], Michael F. Seidl[3], Ole Nielsen, Hui Tian, Bart P.H.J. Thomma[1*]

[1] University of Cologne, Institute for Plant Sciences, Cluster of Excellence on Plant Sciences (CEPLAS), 50674 Cologne, Germany

[2] Sapienza University of Rome, Department of Environmental Biology, 00185 Rome, Italy;

[3] Theoretical Biology & Bioinformatics Group, Department of Biology, Utrecht University, Utrecht, the Netherlands.

[#] These authors contributed equally

* Corresponding author: bthomma@uni-koeln.de

ABSTRACT

Chromosomal rearrangements can play an important role in generating genomic variation that facilitates fungal adaptation. Comparative genomic analyses of isolates from the fungal plant pathogen *Verticillium dahliae* have revealed widespread large-scale chromosomal rearrangements, both within and between chromosomes. These rearrangements have been linked to the formation of highly dynamic adaptive genomic regions (AGRs), which harbor genetic variation that supports *V. dahliae* colonization of plant hosts. However, it remains unclear whether such rearrangements represent ancient events inherited from ancestral *V. dahliae* lineages or continue to arise during ongoing genome evolution. Here, we identified a *de novo* inter-chromosomal rearrangement between chromosomes 1 and 5, designated ICR1.5, in an evolved *V. dahliae* lineage after passage through the plant host *Arabidopsis thaliana.* The *V. dahliae* lineage carrying ICR1.5 became increasingly abundant over successive passages through *A. thaliana*, suggesting that the rearrangement provided a selective advantage. Using co-inoculation assays on *A. thaliana*, we further showed that the evolved *V. dahliae* lineage increased in proportion *in planta* when co-inoculated with either the parental lineage, or transformants in which ICR1.5 was reverted to the parental chromosomal configuration. These results indicate that ICR1.5 confers a competitive advantage to *V. dahliae*. However, this competitive advantage was not observed during co-cultivation on potato dextrose agar, suggesting that ICR1.5 confers an advantage to *V. dahliae* specifically during colonization of *A. thaliana*. These results provide direct evidence that a large-scale chromosomal rearrangement can contribute to ongoing genome evolution and host adaptation in *V. dahliae.*

INTRODUCTION

Genome evolution is a fundamental process that drives genetic diversity, allowing organisms to adapt to changing environments (Seidl and Thomma 2014). In eukaryotes, sexual reproduction through meiosis is a key driver of genome evolution (Lee et al. 2010; Lenormand et al. 2016). By reshuffling parental alleles, meiotic recombination creates novel genetic combinations and limits the accumulation of deleterious mutations (Lenormand et al. 2016). Consequently, strict asexuality has typically been regarded as at an evolutionary disadvantage (Felsenstein 1974). The absence of meiotic recombination is thought to result in Muller's ratchet, the accumulation of deleterious mutations that can ultimately drive asexual species toward an evolutionary dead end (Hojsgaard and Hörandl 2015; Felsenstein 1974). However, the evolutionary successes of numerous asexual species challenge this view (Seidl and Thomma 2014). Bdelloid rotifers, a class of microscopic invertebrates, provide a classic example of long-term asexuality, as they are thought to have persisted and diversified for over 60 million years through predominant asexual reproduction (Birky 2010; Terwagne et al. 2022). Genomic analysis of one of the bdelloid rotifer species, *Adineta vaga*, reveals extensive chromosomal rearrangements that generate structural variations (SVs) such as inversions, translocations, deletions, and duplications (Seidl and Thomma 2014; Flot et al. 2013). The resulting chromosome structure is incompatible with the conventional homologous pairing required for meiosis (Flot et al. 2013). Recent genome analyses of the ancient asexual oribatid mite *Platynothrus peltifer* further reveal that genome evolution can proceed in the absence of meiosis through independent haplotype evolution sustained for over millions of years (Öztoprak et al. 2025). The genome of *P. peltifer* is characterized by the long-term maintenance of two independently evolving haplotypes within a single diploid individual, with one remaining largely conserved while the other accumulates genomic variation, thereby generating genetic novelty without sexual recombination (Öztoprak et al. 2025). These observations raise the question of whether other asexual species utilize similar mechanisms to generate genome diversity and facilitate genome evolution.

Verticillium dahliae is a globally distributed, soil-borne fungal plant pathogen that causes vascular wilt disease in hundreds of dicotyledonous plant species (Acharya et al. 2020; Sayari et al. 2024; Bhat and Subbarao 1999). The ecological success of *V. dahliae* is remarkable because it is a strictly asexual fungus (Zhang et al. 2024; Milgroom et al. 2014). Consistent with *V. dahliae*'s asexuality, and thus clonal reproduction, genome comparisons reveal extremely low nucleotide diversity of 0.02% to 0.5% among sequenced strains that have been collected world-wide (de Jonge et al. 2013). However, extensive karyotype variation has been observed among *V. dahliae* strains (de Jonge et al. 2013; Faino et al. 2016). This finding is unexpected, as karyotypic variation observed is typically associated with dispensable

chromosomes, a feature absent in *V. dahliae* (Ma et al. 2010; Langner et al. 2021; Möller et al. 2018; Jones et al. 2008; Johnson Pokorná and Reifová 2021; Seidl et al. 2020) Ancestral chromosome reconstruction based on the genomes of nine haploid *Verticillium* species revealed that their common ancestor possessed a core set of eight chromosomes with no evidence of dispensable chromosomes, indicating that eight represents the fundamental chromosome number of the *Verticillium* genus (Seidl et al. 2020).

A detailed comparison of the gapless *V. dahliae* telomere-to-telomere genome assemblies of strains JR2 and VdLs17 revealed their core genome, which comprise genes shared across all *V. dahliae* strains, showing 99.98% sequence identity (de Jonge et al. 2013). Despite this high conservation, extensive chromosomal rearrangements were observed, including 6 chromosomal rearrangements within the same chromosomes (referred to as intra-chromosomal inversions, ICIs) and 13 chromosomal rearrangements between chromosomes (referred to as inter-chromosomal rearrangements, ICRs)(de Jonge et al. 2013; Faino et al. 2016). The syntenic breakpoints of these rearrangements appeared non-random. A significant correlation ($P < 0.01$) was found between the syntenic breakpoints and genomic regions showing presence-absence variation, where DNA sequences were present in either strain JR2 or Vdls17, but not in both (de Jonge et al. 2013). These genomic regions display a distinct chromatin profile, characterized by enrichment of histone H3 lysine 27 tri-methylation (H3K27me3), depletion of histone H3 lysine 9 methylation (H3K9me), and increased chromatin accessibility, and are collectively referred to as adaptive genomic regions (AGRs) (Cook et al. 2020). AGRs are genome compartments that evolve rapidly and are enriched in genes that are induced *in planta* (de Jonge et al. 2013). Many of these genes encode small, secreted proteins called effectors that manipulate plant host physiology to facilitate successful infections by *V. dahliae* (de Jonge et al. 2013; Cook et al. 2020). For example, of the seven LysM effectors in the VdLs17 genome, only the one located within an AGR is significantly expressed *in planta* and contributes to virulence (de Jonge et al. 2013). The spatial association between chromosomal rearrangements and AGRs suggests that chromosomal rearrangements play a key role in *V. dahliae* adaptation (de Jonge et al. 2013). A comparative genomic analysis of 42 sequenced *V. dahliae* strains identified 919 SVs (Torres et al. 2021). The presence of multiple SVs in every strain indicates that chromosomal rearrangements are a widespread phenomenon within the *V. dahliae* species (Torres et al. 2021).

The formation of chromosomal rearrangements in *V. dahliae* is attributed to double strand DNA breaks followed by unfaithful DNA repair (Faino et al. 2016). Chromosomal rearrangements often occur between homologous sequences and repeated elements, particularly transposable elements (TEs), which are thought to act as ectopic substrates for unfaithful DNA repair (Faino et al. 2016). Of the 13 inter-chromosomal rearrangements identified between *V. dahliae* strains

JR2 and VdLs17, 12 were reconstructed in detail (Faino et al. 2016). These analyses revealed that eight occurred over highly similar TEs in both genomes, two over TEs present only in strain VdLs17, and two over regions showing sequence similarity in the absence of TEs (Faino et al. 2016). Notably, a class of recently characterized giant transposons termed 'Starships' serves as a major hotspot for chromosomal rearrangements in *V. dahliae* (Sato et al. 2025). Approximately 60% of the identified rearrangement breakpoints between *V. dahliae* strains JR2 and VdLs17 are located within syntenic Starship regions, likely due to the repetitive nature of these regions providing abundant substrates for ectopic recombination (Sato et al. 2025). Segmental duplications, defined as large segments of DNA (>1 kb) that exhibit nucleotide sequence identity >80%, represent another genomic feature associated with chromosomal rearrangements (Faino et al. 2016; Mehrabi et al. 2017). These duplications are abundant within the AGRs of *V. dahliae* (Faino et al. 2016). They can arise as a consequence of chromosomal rearrangements while also providing the homologous sequences that serve as substrates for the occurrence of chromosomal rearrangements (Mehrabi et al. 2017; Faino et al. 2016). Finally, it is also hypothesized that the physical colocalization of the genome in the cell nucleus facilitates the formation of chromosomal rearrangements (Torres et al. 2024). DNA proximity labeling has shown that AGRs, which serve as hotspots for chromosomal rearrangements, physically cluster within the nucleus through long-range chromatin interactions in *V. dahliae*, potentially creating a localized environment that promotes rearrangements (Torres et al. 2024; Huang and Cook 2022).

Comparative genomic analyses have revealed widespread chromosomal rearrangements among *V. dahliae* isolates from diverse hosts and environments (Torres et al. 2021). However, whether these rearrangements reflect ancient events inherited from ancestral *V. dahliae* lineages or continue to arise *de novo* during genome evolution remains unresolved. To address this, we analyzed the genomes of 11 *V. dahliae* lineages that resulted from experimental evolution for the occurrence of chromosomal rearrangements.

RESULTS

Verticillium dahliae harbors an inter-chromosomal rearrangement after passage through *Arabidopsis thaliana*

To investigate whether ICRs occurred in *Verticillium dahliae* lineages generated through experimental evolution (Chapter 2), we performed whole-genome sequencing of all lineages using Oxford Nanopore Technology (ONT). First, Nanopore reads exceeding 10 kb were mapped to the *V. dahliae* JR2 reference genome (Faino et al. 2015). Next, SVs were identified in each lineage relative to the JR2 genome using Sniffles (Smolka et al. 2024). Among the detected SVs, candidate ICRs were identified in four *V. dahliae* lineages. The heat-derived lineage H3 exhibited an ICR involving a breakpoint that fused chromosomes 2 and 6 (hereafter referred to as ICR2.6). ICR2.6 was supported by two ~11 kb reads, both spanning Chr2: ~422–423 kb and Chr6: ~2.83–2.84 Mb in the JR2 genome (Table 1). Similarly, the *A. thaliana*-derived lineages P1, P2, and P3 carried an identical ICR involving breakpoints on chromosomes 1 and 5 (hereafter referred to as ICR1.5), with both breakpoints collectively supported by 194 reads in P1, 73 reads in P2, and 36 reads in P3 (Table 1).

To validate the candidate ICRs, PCR amplification was performed using primer pairs flanking the breakpoints of ICR1.5 or ICR2.6. These breakpoint-specific primers (Supplementary Table 1) yielded amplicons either in lineages carrying the corresponding ICRs or in the wild-type JR2. PCR screening of 100 single-colony isolates from the heat-derived lineage H3 detected only the wild-type configuration of chromosome 2, with no evidence of ICR2.6 (Supplementary Figure 1). Thus, ICR2.6 could not be validated and was excluded from further analyses. For validation of the *A. thaliana*-derived lineages, we focused on lineage P1, as all three lineages (P1, P2, and P3) carried ICR1.5 with identical breakpoints. In lineage P1, PCR using primers flanking the breakpoint of ICR1.5 produced the expected amplicons, confirming the presence of the rearrangement (Supplementary Figure 2). Lineage P1 was therefore selected for subsequent analyses.

Table 1. Summary of whole-genome sequencing and inter-chromosomal rearrangements (ICRs) detected in *Verticillium dahliae* lineages generated by experimental evolution.

Experimental Condition	Duration	Lineage	Average Genome Coverage (x-fold)	Sniffles-detected ICRs (Chromosome: position)	Supporting Reads for ICRs	PCR-validated ICRs
UV-C Radiation	2 min	U1	39.2	--	--	--
Elevated Temperature (30℃)	6 passages	H1	38.5	--	--	--
	6 passages	H2	62.9	--	--	--
	7 passages	H3	25.2	Chr2:~422k ↔ Chr6:~2.84Mb	2	No
	8 passages	H4	141.9	--	--	--
Co-Culture with Antagonistic Bacterium	10 passages	B1	21.4	--	--	--
		B2	29.5	--	--	--
		B3	19.6	--	--	--
In planta passage (*A. thaliana*)	4 passages	P1	117.1	Chr1:~6.3Mb ↔ Chr5:~304kb	194	Yes
		P2	41.1		73	--
		P3	26.5		36	--

Whole-genome assembly of the evolved *V. dahliae* lineage P1 carrying inter-chromosomal rearrangement ICR1.5

To determine the chromosomal configuration generated by the inter-chromosomal rearrangement between chromosomes 1 and 5 (ICR1.5), we performed *de novo* whole-genome assembly of the evolved *V. dahliae* lineage P1. The assembly yielded a telomere-to-telomere genome comprising eight chromosomes (Supplementary Figure 3). Subsequently, alignment of the evolved *V. dahliae* genome to the JR2 reference using MUMmer4 (Marçais et al. 2018) showed that the first ~304 kb segment of chromosome 5 is inverted and fused to chromosome 1 at ~6.3 Mb, with the downstream segment of chromosome 1 relocated (Figure 1). The relocated segment of chromosome 1 is inverted and fused to the remaining portion of chromosome 5 beyond the initial ~304 kb segment (Figure 1). As a result, in the evolved *V. dahliae* lineage, chromosome 1, which was originally much longer than chromosome 5, decreased from ~9.2 Mb to ~6.65 Mb, while chromosome 5 increased from ~4.1 Mb to ~6.8 Mb, so that both chromosomes are nearly equal in length (Figure 1). Examination of the ICR1.5 breakpoints revealed that the genes closest to the breakpoints, Chr1g19990a and Chr5g01070a, both encodes an ATP-binding cassette (ABC) transporter family protein. The chromosome 1 breakpoint is located in an intergenic region, with the 5′ UTR of the Chr1g19990a gene positioned ~300 bp downstream (Supplementary Figure 4). The chromosome 5 breakpoint disrupts the first 5 bp of the 5′ UTR of the Chr5g01070a gene, while the coding region of this gene remains intact (Supplementary Figure 4). Thus, the whole-genome assembly revealed the complete chromosomal configuration of lineage P1 and confirmed that ICR1.5 did not directly disrupt coding regions of any genes.

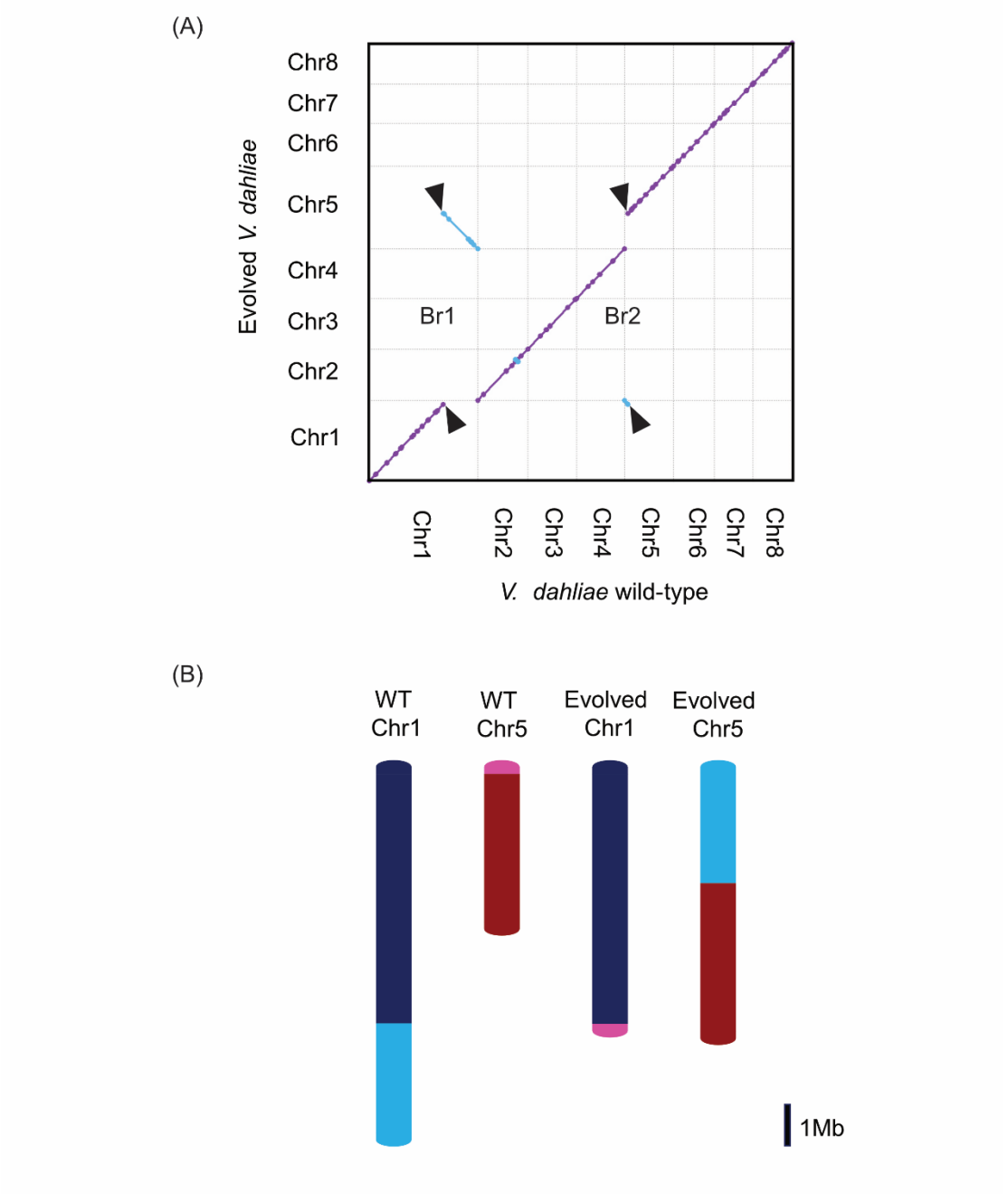


Figure 1. Inter-chromosomal rearrangement between chromosomes 1 and 5 (ICR1.5) in the evolved *Verticillium dahliae* lineage P1. (A) Whole-genome alignment of the evolved *V. dahliae* genome against the reference genome of the wild-type strain JR2. Purple dots indicate syntenic regions, while blue dots represent inverted alignments. Black arrowheads mark the positions of the rearrangement breakpoints (Br1 and Br2). (B) Schematic representation of chromosome configuration in the wild-type (WT) strain JR2 and evolved *V. dahliae*. WT chromosome 1 is represented by dark and light blue segments, and WT chromosome 5 by dark red and pink segments. Each evolved chromosome comprises one segment from each WT chromosome 1 and 5.

The *V. dahliae* that harbors the inter-chromosomal rearrangement ICR1.5 becomes predominant over serial *in planta* passages

Since an inter-chromosomal rearrangement between chromosome 1 and 5 (ICR1.5) was detected in an evolved *V. dahliae* lineage obtained through serial *in planta* passage, we next aimed to determine at which passage this rearrangement emerged. To this end, we quantified the proportion of *V. dahliae* carrying ICR1.5 across successive passages. *V. dahliae* conidiospores collected from each passage were subjected to DNA isolation and real-time PCR using primer pairs flanking the breakpoint of ICR1.5 (Supplementary Table 1). These breakpoint-specific primers (Supplementary Table 1) yielded amplicons either in *V. dahliae* carrying the corresponding ICRs or in the parental lineage. Surprisingly, *V. dahliae* harboring ICR1.5 was already present after the first passage, accounting for ~18% of *V. dahliae*, and gradually rose across successive passages. By the fourth passage, the parental lineage was no longer detected, and only *V. dahliae* harboring ICR1.5 was detected (Figure 2).

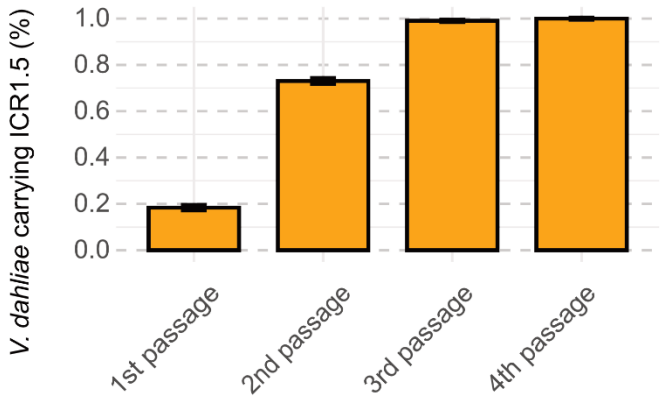


Figure 2. Progressive increase of the *Verticillium dahliae* carrying the inter-chromosomal rearrangement ICR1.5 over four *in planta* passages. Percentages of *V. dahliae* carrying the ICR1.5 were determined by real-time PCR on DNA extracted from *V. dahliae* conidiospores harvested at the end of each passage. Bars represent mean values of two independent real-time PCR assays ± SD.

The inter-chromosomal rearrangement ICR1.5 promotes competitiveness in *Arabidopsis thaliana*

Fixation of evolved *V. dahliae* carrying the inter-chromosomal rearrangement ICR1.5 after passage through *A. thaliana* raised the question whether ICR1.5 enhances *V. dahliae* performance in *A. thaliana.* To address this, we generated two independent *V. dahliae* lineages, R1 and R2, both established by genome editing that restored ICR1.5 to the parental chromosome configuration. Subsequently, *A. thaliana* plants were inoculated with conidial suspensions that were composed of 5% of the evolved *V. dahliae* lineage P1 (harboring ICR1.5) and 95% of either the parental lineage, lineage R1 or lineage R2. Two weeks post-inoculation, the proportion of the lineage P1 *in planta* was quantified by real-time PCR using the same breakpoint-specific primers as described above (Supplementary Table 1). Across all co-inoculation combinations, the proportion of evolved *V. dahliae* lineage P1 *in planta* was significantly higher than the initial 5% input (Figure 3). Notably, in several inoculated plants, lineage P1 accounted for more than 50% of the *V. dahliae* biomass (Figure 3). Overall, our findings indicate that ICR1.5 provides a competitive advantage during colonization of *A. thaliana*.

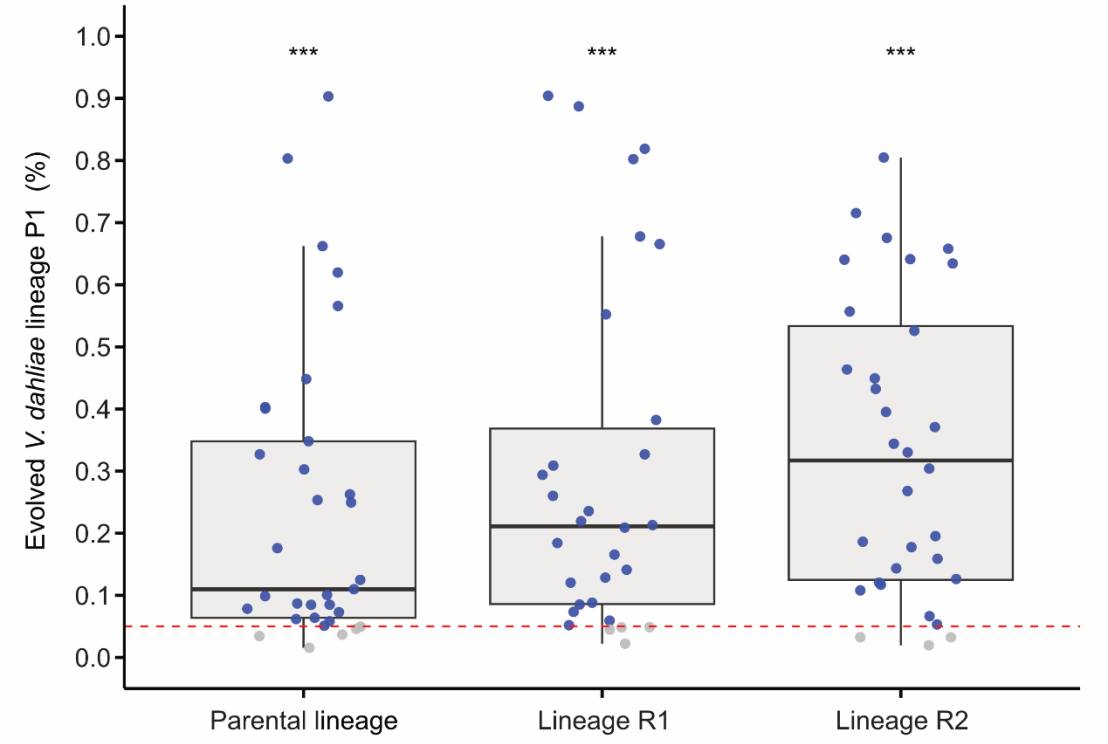


Figure 3. The inter-chromosomal rearrangement ICR1.5 promotes *Verticillium dahliae* lineage P1 competitiveness in *Arabidopsis thaliana*. Lineage P1 carries the inter-chromosomal rearrangement ICR1.5, whereas lineages R1 and R2 retain the parental chromosome configuration after ICR1.5 was reverted to the parental configuration by genome editing. *A. thaliana* was inoculated with *V. dahliae* conidial suspensions containing 5% of the lineage P1 and 95% of either the parental lineage, lineage R1 or lineage R2. Percentages of lineage P1

in *A. thaliana* were quantified by real-time PCR at two weeks post-inoculation. Each data point represents the value obtained from an individual plant. The red dashed line marks the initial 5% input level. Blue dots indicate data points >5%, grey dots indicate data points ≤5%. Data derive from three independent experiments (Wilcoxon signed-rank test; ***p<0.001, **p<0.01, *p<0.05; N≥30).

To determine whether ICR1.5 also promotes the proliferation of *V. dahliae* lineage P1 *in vitro*, pairwise co-cultivation assays were performed on potato dextrose agar (PDA) medium. Conidial suspensions containing 5% of lineage P1 and 95% of either the parental lineage, lineage R1, or lineage R2 were prepared and plated onto multiple independent PDA plates. After two weeks of *in vitro* co-cultivation, conidiospores were harvested from each plate, and the proportion of lineage P1 was determined by real-time PCR with breakpoint-specific primers (Supplementary Table 1), as in the *in planta* co-inoculation assay. In contrast to the *in planta* assay, lineage P1 did not exhibit a strong increase in proliferation *in vitro* (Figure 4). When co-cultivated with either the parental lineage or lineage R1, the proportion of lineage P1 remained close to the initial 5% input, with no significant increase detected (Figure 4). A modest increase was observed during co-cultivation with lineage R2 (Wilcoxon signed-rank test, p = 0.024), with lineage P1 rising from the initial 5% input to a median of ~9% of the total *V. dahliae* biomass (Figure 4). This modest increase contrasts with the *in planta* assay, where co-inoculation of lineage P1 (5%) with lineage R2 (95%) led to a substantial rise, with P1 reaching a median proportion of ~32% of the total *V. dahliae* biomass two weeks after inoculation (Figure 3). These results demonstrate that ICR1.5 promotes *V. dahliae* proliferation more strongly in *A. thaliana* than *in vitro*.

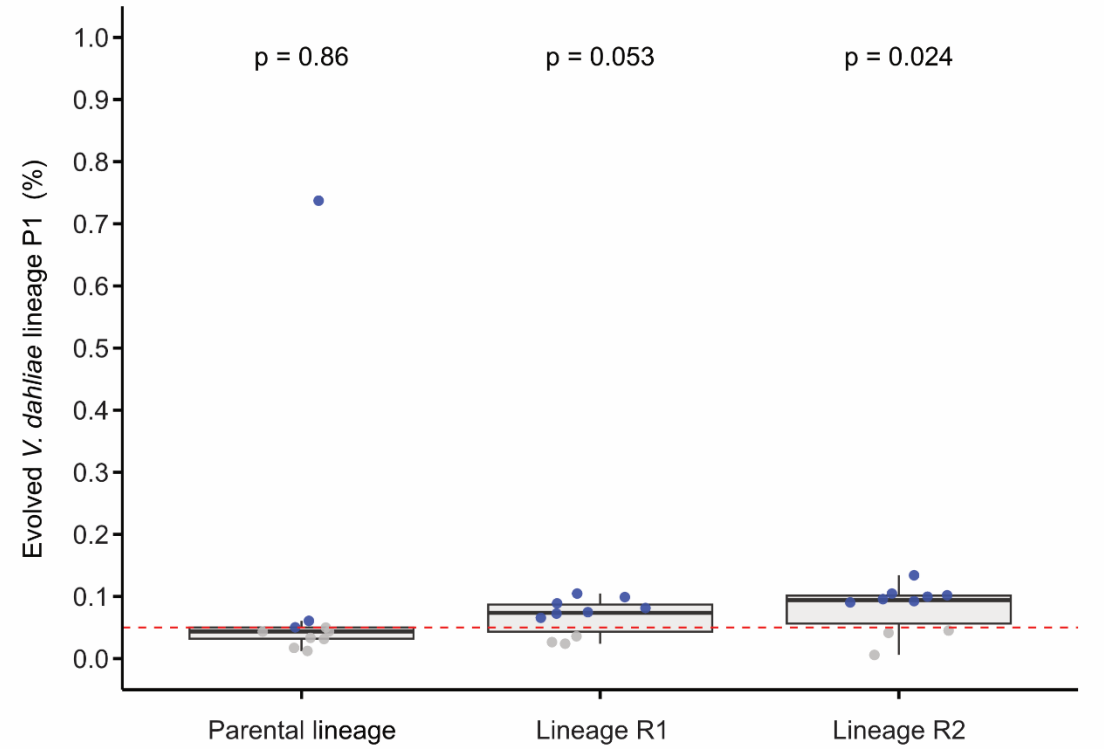


Figure 4. The inter-chromosomal rearrangement ICR1.5 does not promote proliferation of *Verticillium dahliae* lineage P1 *in vitro*. Lineage P1 carries the inter-chromosomal rearrangement ICR1.5, whereas lineages R1 and R2 retain the parental chromosome configuration after ICR1.5 was reverted to the parental configuration by genome editing. Conidial suspensions containing 5% of lineage P1 and 95% of either the parental lineage, lineage R1, or lineage R2 were plated on potato dextrose agar (PDA) plates. Percentages of lineage P1 after two weeks of growth on PDA were quantified by real-time PCR. Each data point represents the value obtained from an individual PDA plate. The red dashed line marks the initial 5% input level. Blue dots indicate data points >5%, grey dots indicate data points ≤5%. P-values were calculated using the Wilcoxon signed-rank test (N=10).

Genomic variation may arise not only through structural variants but also through point mutations. To determine whether additional genomic alterations contribute to the enhanced *V. dahliae* lineage P1 competitiveness in *A. thaliana*, we investigated whether single-nucleotide polymorphisms (SNPs) might also play a role. To this end, we conducted a genome-wide SNP analysis comparing lineage P1 to the wild-type JR2 reference genome. Illumina short-read sequencing of lineage P1 yielded ~ 70× genome coverage, enabling the identification of 29 transcripts harboring non-synonymous SNPs predicted to cause amino acid substitutions (Supplementary Table 2). To assess the potential impact of these SNPs on *V. dahliae* competitiveness in *A. thaliana*, transcript abundance was analyzed using previously published RNA-sequencing data from *V. dahliae*–infected *A. thaliana* (Shi-Kunne et al. 2019). In this dataset, all 29 transcripts harboring SNPs exhibited minimal expression during *V. dahliae* colonization of *A. thaliana*, with average read counts per transcript not exceeding two across biological replicates (Supplementary Table 2). These SNPs are therefore unlikely to contribute significantly to the enhanced competitiveness of lineage P1 in *A. thaliana*. These findings further support that the inter-chromosomal rearrangement ICR1.5 is the primary genomic alteration associated with the enhanced competitiveness of *V. dahliae* lineage P1 in *A. thaliana*.

Evolved *V. dahliae* lineage P1 and the parental lineage cause comparable Verticillium wilt symptoms in *A. thaliana*

To test whether inter-chromosomal rearrangement ICR1.5 contributes to Verticillium wilt disease development, *A. thaliana* were inoculated with either the evolved *V. dahliae* lineage P1, which harbors ICR1.5, or the parental lineage. At 14 days post-inoculation (dpi), *A. thaliana* inoculated with either lineage exhibited mild Verticillium wilt symptoms (Ellendorff *et al.*, 2009), including moderate stunting and initial wilting (Figure 5). Consistent with these symptoms, infected plants displayed reduced canopy areas compared with mock-inoculated plants (Figure 5). However, no significant difference in canopy area was detected between *A. thaliana* inoculated with the parental or the evolved lineage (Figure 5). Verticillium wilt symptoms were more pronounced at 21 dpi, with increased stunting and wilting, yet canopy area remained comparable between plants inoculated with the evolved lineage and the parental lineage

(Figure 6). These results indicate that ICR1.5 does not enhance Verticillium wilt symptom development in *A. thaliana.*

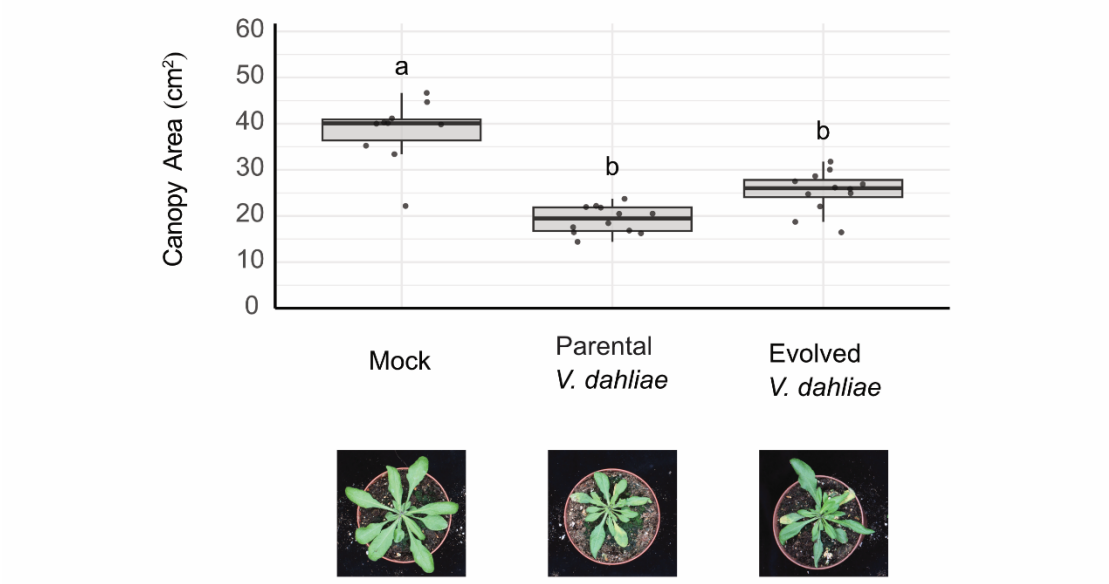


Figure 5. Evolved *Verticillium dahliae* lineage P1 and the parental *V. dahliae* induce comparable Verticillium wilt symptoms in *Arabidopsis thaliana* at 14 dpi. Boxplots display the canopy area (cm²) of *A. thaliana* at 14 days post-inoculation with mock treatment (water), or the parental *V. dahliae*, or the evolved *V. dahliae* lineage P1 harboring Inter-chromosomal rearrangement ICR1.5. Each data point represents the value obtained from an individual plant. Different letters above the boxes indicate statistically significant differences based on one-way ANOVA and Tukey's post-hoc test ($p<0.01$; $N\geq10$). Photos display phenotypes of representative *A. thaliana* for each treatment at 14 days post-inoculation. Plants inoculated with *V. dahliae* displayed early Verticillium wilt symptoms, including stunting and initial leaf wilting.

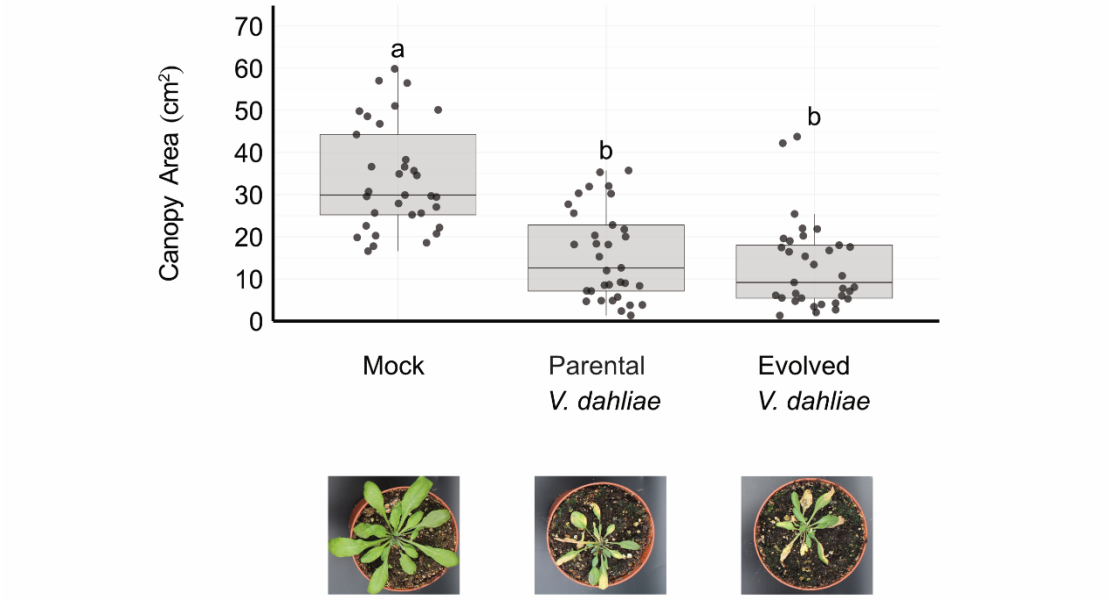


Figure 6. Evolved *Verticillium dahliae* lineage P1 and the parental *V. dahliae* induce comparable Verticillium wilt symptoms in *Arabidopsis thaliana* at 21 dpi. Boxplots display the canopy area (cm²) of *A. thaliana* at 21

days post-inoculation with mock treatment (water), or the parental *V. dahliae*, or the evolved *V. dahliae* lineage P1 harboring Inter-chromosomal rearrangement ICR1.5. Each data point represents the value obtained from an individual plant. Different letters above the boxes indicate statistically significant differences based on one-way ANOVA and Tukey's post-hoc test ($p<0.01$; $N\geq32$). Data derive from three independent experiments. Photos display phenotypes of representative *A. thaliana* for each treatment at 21 days post-inoculation. Plants inoculated with *V. dahliae* displayed typical Verticillium wilt symptoms, including stunting and partial leaf wilting.

In vitro growth reduction of the evolved *V. dahliae* lineage P1 is not caused by inter-chromosomal rearrangement ICR1.5

Given that inter-chromosomal rearrangement ICR1.5 contributes to enhanced *V. dahliae* lineage P1 competitiveness in *A. thaliana* (Figure 3), we assessed whether ICR1.5 also improve *V. dahliae* growth under *in vitro* conditions. To address this, we compared the *in vitro* growth of lineage P1, which harbors ICR1.5, with the parental lineage and the lineages R1 and R2, in which ICR1.5 was edited back to the parental chromosome configuration. Growth impacts were assessed for each lineage based on colony diameter, conidiospore production, and conidiospore germination. Surprisingly, lineage P1 exhibited reduced colony diameter and conidiospore germination efficiency relative to the parental lineage, and similar reductions were observed in the lineages R1 and R2 (Figure 7A–B, D), indicating that these traits are independent of ICR1.5. In contrast, conidiospore production was similar across all lineages (Figure 7C). In summary, lineage P1 exhibits reduced *in vitro* growth, characterized by decreased colony diameter and conidiospore germination efficiency, but this reduction occurs independently of ICR1.5.

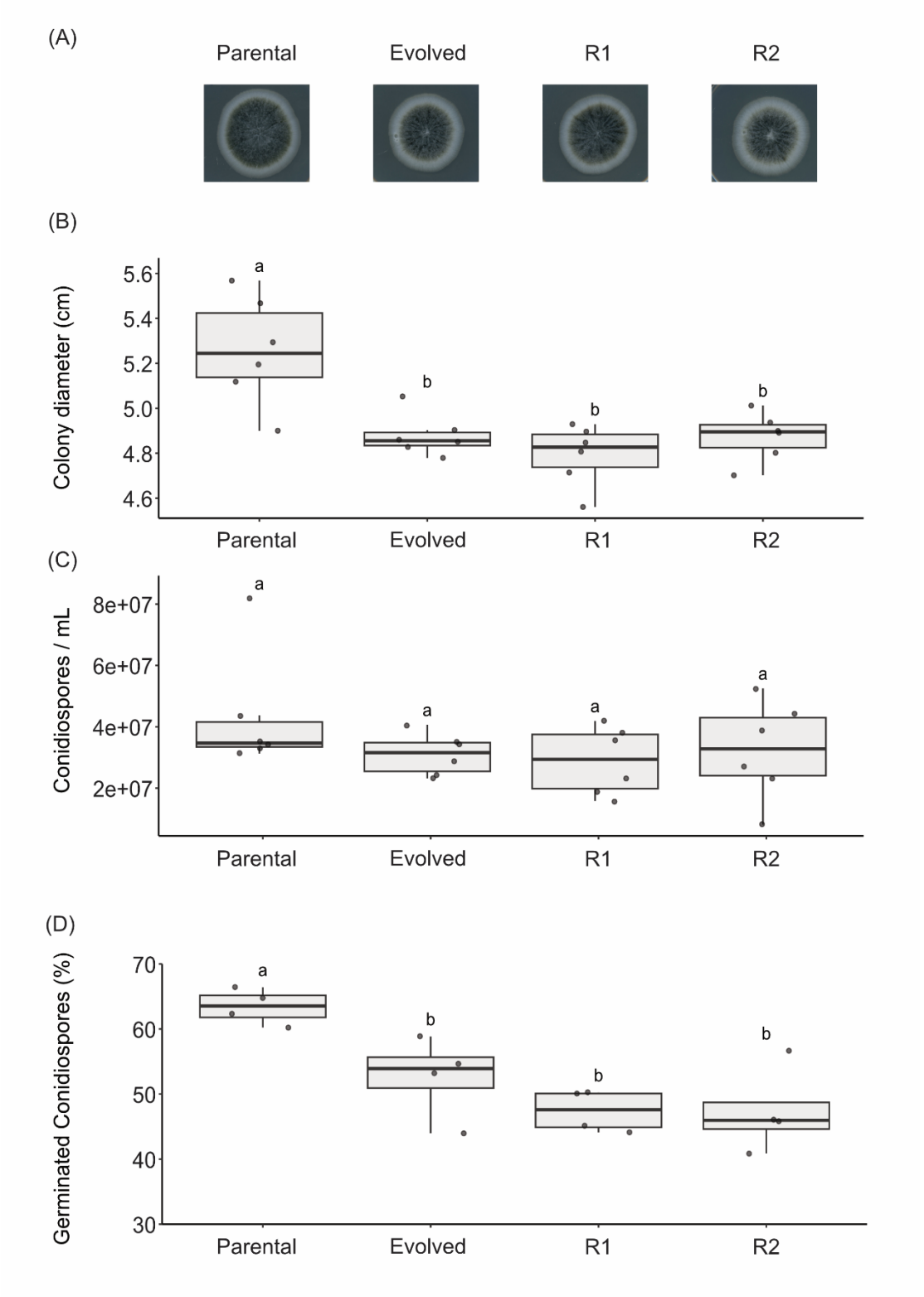


Figure 7. Reduced *in vitro* growth of the evolved *Verticillium dahliae* lineage P1 is independent of inter-chromosomal rearrangement ICR1.5. The parental lineage, evolved *V dahliae* lineage P1 harboring ICR1.5, and lineages R1 and R2 in which ICR1.5 was edited back to the parental chromosome configuration were compared. Different letters above the boxplots indicate statistically significant differences among lineages based on one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$; $N \geq 4$). (A) Representative colony morphology after 14 days of growth on potato dextrose agar (PDA) plates. (B) Colony diameter after 14 days of growth on PDA. (C) Conidiospore production per colony, determined from colonies grown on PDA for 14 days. (D) Germination efficiency of

conidiospores after 11 h incubation in 1% potato dextrose broth (PDB). Each data point is based on scoring germination of at least 100 conidiospores.

DISCUSSION

Comparative genomic analyses have uncovered extensive chromosomal rearrangements among *V. dahliae* isolates and indicated that these rearrangements contribute to *V. dahliae* adaptation (de Jonge et al. 2013; Faino et al. 2016; Torres et al. 2021). However, direct experimental evidence for the *de novo* formation of chromosomal rearrangements in *V. dahliae* has been lacking. To assess the occurrence of chromosomal rearrangements in *V. dahliae*, we subjected *V. dahliae* to experimental evolution. After a single passage through *A. thaliana*, we detected an inter-chromosomal rearrangement between chromosomes 1 and 5 (ICR1.5). This finding provides direct evidence that chromosomal rearrangements contribute to ongoing genome evolution in *V. dahliae*. We subsequently investigated the role of this rearrangement in the adaptation of *V. dahliae* to *A. thaliana*.

The proportion of *V. dahliae* lineages carrying ICR1.5 increased over successive passages through *A. thaliana*. By the fourth passage, the parental lineage lacking ICR1.5 was no longer detectable, suggesting that ICR1.5 was selectively favored during *in planta* passage. We further demonstrated that, during colonization of *A. thaliana*, the evolved *V. dahliae* lineage carrying ICR1.5 exhibited a competitive advantage over both the parental lineage and transformants in which ICR1.5 had been reverted to the parental chromosomal configuration. However, this competitive advantage was not observed *in vitro*. This context-dependent competitive advantage suggests that the advantage associated with ICR1.5 is specifically linked to colonization of the plant host. Future investigations using additional plant hosts will reveal whether the observed competitive advantage extends to other species, such as *Solanum lycopersicum* (tomato) and *Gossypium hirsutum* (cotton). Overall, our results indicate that an inter-chromosomal rearrangement contributes to ongoing host adaptation in *V. dahliae*.

Our serial passage experiment was aimed at selecting *V. dahliae* lineages that cause earlier and more severe disease development on *A. thaliana*. Given the observed increase in the competitive advantage of the evolved *V. dahliae* lineage in *A. thaliana*, we hypothesized that evolved *V. dahliae* lineage would induce more severe Verticillium wilt symptoms than the parental lineage. However, contrary to this hypothesis, the disease assay on *A. thaliana* showed that the evolved *V. dahliae* lineage and the parental lineage caused comparable Verticillium wilt symptoms. Nevertheless, we cannot rule out that the lack of measurable symptom increase results from limitations in the sensitivity of the disease assay, potentially missing transient or subtle effects on Verticillium wilt disease progression.

The contribution of ICR1.5 to the competitive advantage of the evolved *V. dahliae* lineage in *A. thaliana* raises the question of how ICR1.5 provided such an advantage. ICRs are known to alter gene regulation through various mechanisms and are particularly well documented in

cancer research (Harewood and Fraser 2014). First, an ICR can disrupt a gene's coding region, or it can fuse the coding regions of two different genes, resulting in a novel fusion gene. A classic example of the latter is the Philadelphia chromosome in humans, in which an ICR fuses the coding regions of two different genes, resulting in a novel fusion gene called BCR-ABL that promotes tumorigenesis (El-Tanani et al. 2024). However, since ICR1.5 does not intersect coding regions, this mechanism is unlikely to explain the increased competitive advantage of the evolved *V. dahliae* lineage in *A. thaliana*. Alternatively, an ICR can reposition genes relative to regulatory elements. For example, Burkitt's lymphoma is a human cancer in which an ICR relocates the c-MYC gene, a key regulator of cell proliferation, to a genomic region containing a highly active enhancer, resulting in its constitutive activation and uncontrolled cell division (Hayday et al. 1984). Finally, an ICR may induce large-scale alterations in the three-dimensional (3D) genome architecture, which refers to the physical folding of chromatin within the nucleus (Showpnil et al. 2022; Federico et al. 2021). Alterations in 3D genome architecture have been shown to modulate gene expression (Xiao et al. 2021; Showpnil et al. 2022). Thus, further investigation of the transcriptome and 3D genome architecture of *V. dahliae* carrying ICR1.5 could clarify whether ICR1.5 promotes the competitive advantage of *V. dahliae* in *A. thaliana* through any of the above-mentioned mechanisms. Comparative transcriptome profiling of *V. dahliae* lineages with and without ICR1.5 under *in vitro* and *in planta* conditions can identify differentially expressed genes that may contribute to enhanced competitiveness *in planta*. In parallel, analysis of the 3D genome architecture of *V. dahliae* carrying ICR1.5 could determine whether ICR1.5 introduces novel chromatin interactions that change the physical proximity between genes and regulatory elements. We hypothesize that ICR1.5 promotes the competitive advantage of *V. dahliae* in *A. thaliana* by altering the expression of genes associated with host colonization, potentially through repositioning these genes relative to regulatory elements. This could explain why ICR1.5 confers a competitive advantage during colonization of *A. thaliana* but not under *in vitro* conditions.

We also detected a potential inter-chromosomal rearrangement, ICR2.6, which was initially identified in a *V. dahliae* lineage derived from experimental evolution under prolonged exposure to 30°C. Although this rearrangement was supported by two 11 kb Oxford Nanopore reads, we were unable to validate it by PCR, which suggests that it may represent a technical artifact of Oxford Nanopore sequencing, wherein separate DNA fragments may be artificially ligated during library preparation to form a single chimeric read (Yu et al. 2023). Alternatively, if ICR2.6 is a genuine biological event, the inability to validate it by PCR underscores a general challenge in studying genome evolution. Lineages carrying genomic variants that confer a selective advantage are more likely to increase in abundance and become detectable, whereas lineages carrying neutral or deleterious variants may remain at low abundance below the detection limit (Nielsen 2005). The lack of PCR validation for ICR2.6 may indicate that it

does not provide an adaptive advantage under 30°C and therefore remains at an abundance below the limit of PCR detection.

Chromosomal rearrangements were historically regarded as deleterious, largely because of their association with human diseases (Gorkovskiy and Verstrepen 2021). However, growing evidence from diverse organisms, including humans, insects, and microorganisms, indicates that chromosomal rearrangements can be important drivers of adaptation (Gompert et al. 2025; Gorkovskiy and Verstrepen 2021; de Jonge et al. 2013; Faino et al. 2016). In line with this notion, ancestral genome reconstruction in the *Verticillium* genus has revealed that large-scale chromosomal rearrangements occurred repeatedly during its evolution, suggesting a role in *Verticillium* speciation (Shi-Kunne et al. 2018). The identification of ICR1.5 extends current understanding of chromosomal rearrangements in *V. dahliae*. Our findings provide experimental evidence that a large-scale chromosomal rearrangement can contribute to ongoing genome evolution and adaptation of *V. dahliae*, rather than representing merely an ancient event inherited from ancestral lineages.

MATERIALS AND METHODS

High-molecular weight DNA extraction, Oxford Nanopore sequencing, and inter-chromosomal rearrangement (ICR) identification

High-molecular-weight (HMW) DNA was extracted from 11 *V. dahliae* lineages as described previously (Chavarro-Carrero et al. 2021). DNA quality and quantity were assessed using the Nanodrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Qubit fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). DNA integrity was verified by agarose gel electrophoresis as described by (Kraege et al. 2025). HMW DNA was stored at 4°C until further use. DNA sequencing libraries were prepared using the SQK-LSK114 kit (Oxford Nanopore Technologies, Oxford, UK). Libraries were loaded onto R10.4.1 flow cells (Oxford Nanopore Technologies, Oxford, UK) and sequenced on a MinION Mk1B device (Oxford Nanopore Technologies, Oxford, UK). Base calling was performed with Guppy basecaller (version 6.5.7; Oxford Nanopore Technologies, Oxford, UK), and adapter sequences were trimmed with Porechop (version 0.2.4; Wick 2018). Reads shorter than 10 kb were removed using NanoFilt (version 2.8.0; Coster et al. 2018). The remaining reads were mapped to the *V. dahliae* JR2 reference genome (VDAG_JR2v4.0; Faino et al. 2015) using NGMLR (version 0.2.7; Sedlazeck et al. 2018). The resulting mapping files were converted to BAM-format using samtools (version 1.10; Danecek et al. 2021). The BAM files were then used as input for detection of inter-chromosomal rearrangements (ICRs) with Sniffles (version 2.0.7; Smolka et al. 2024). Detected ICRs were validated by PCR using breakpoint-specific primer pairs (Supplementary Table 1) flanking the rearrangement breakpoints.

Chromosome-level genome assembly, pairwise genome alignments

Oxford Nanopore Technology (ONT) long-read sequences of the evolved *V. dahliae* lineage P1 were corrected, trimmed, and assembled using Canu (version 2.0; Koren et al. 2017). The assembled genome was aligned to the *V. dahliae* JR2 reference genome (VDAG_JR2v4.0; Faino et al. 2015) with MUMmer4 (version 4.0.0rc1; Marçais et al. 2018) for whole-genome comparisons. Chromosome assemblies and telomeric regions were assessed with Tapestry (version 1.0.0; Davey et al. 2020) using the conserved [TTAGGG]n repeat. To resolve rearrangement breakpoints, sequences spanning 20 kb upstream and downstream of predicted rearrangement breakpoints in the lineage P1 assembly were aligned against the JR2 reference genome using MUMmer4 (version 4.0.0rc1; Marçais et al. 2018) and visualized with gggenomes (version 1.0.1; Hackl et al. 2024).

Quantification of *V. dahliae* harboring inter-chromosomal rearrangement ICR1.5 after each *in planta* passage

The *V. dahliae Ave1* deletion strain (Δ*Ave1*; de Jonge et al. 2012), in which the *Ave1* gene was replaced by a hygromycin B resistance cassette, was used for inoculation. After each passage, *V. dahliae* was re-isolated by plating surface-sterilized aboveground tissue of symptomatic *A. thaliana* on potato dextrose agar (PDA; Carl Roth, Karlsruhe, Germany) supplemented with hygromycin B (25 µg/mL). Plates were incubated at room temperature for 5–7 days, and hyphae emerging from internal plant tissue were transferred to fresh PDA plates. Conidiospores from all resulting colonies on each plate were harvested and pooled for DNA extraction using the SmartExtract DNA Kit (Eurogentec, Maastricht, The Netherlands). The abundance of the *V. dahliae* harboring inter-chromosomal rearrangement ICR1.5 in conidiospores harvested from each passage was assessed by real-time PCR using primer pairs flanking the breakpoint of ICR1.5 (Supplementary Table 1). Total *V. dahliae* biomass was quantified with primers VdGAPH-F and VdGAPH-R targeting the *V. dahliae* glyceraldehyde 3-phosphate dehydrogenase (VdGAPDH) gene (Supplementary Table 1).

CRISPR-Cas9-mediated genome editing of *Verticillium dahliae*

Lineages R1 and R2 were obtained by CRISPR-Cas9-mediated genome editing in the *V. dahliae* lineage P1 background, following Leisen et al. (2020) with modifications. Single-guide RNAs (sgRNAs) were designed to target the rearrangement breakpoint (Supplementary Table 1). The sgRNA transcription templates were generated by annealing an oligonucleotide encoding the trans-activating CRISPR RNA (tracrRNA) sequence with each protospacer-specific oligonucleotide (Supplementary Table 1). The annealed products were subjected to overhang extension with T4 DNA Polymerase (New England Biolabs, Ipswich, USA) and purified using the Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, USA). sgRNAs were synthesized *in vitro* using the HiScribe™ T7 High Yield RNA Synthesis Kit (New England Biolabs, Ipswich, USA) and purified with RNA Clean & Concentrator 25 (Zymo Research, Irvine, CA, USA). Ribonucleoprotein (RNP) complexes were assembled by combining EnGen® Spy Cas9 HF1 (New England Biolabs, Ipswich, USA) with sgRNA at 2:5 ratio. Donor DNA fragments for homology-directed repair were generated by PCR amplification of regions flanking the rearrangement breakpoint in the *V. dahliae Ave1* deletion strain (Δ*Ave1*; de Jonge et al. 2012), using primers listed in Supplementary Table 1.

Protoplast transformation was performed following Rehman et al. (2016) with minor modifications. In short, conidiospores of the *V. dahliae* lineage P1 were harvested from PDA plates, washed with sterile Milli-Q water, and 3×10^7 conidiospores were cultured in 100 mL Complete Medium (CM; yeast extract 6 g, casein acid hydrolysate 6 g and 10 g sucrose in 1L H_20) for 20 h at 28°C while shaking at 150 rpm. Mycelium was washed with 0.7 M NaCl,

transferred to 10 mL of 2% Driselase solution (Sigma-Aldrich, St. Louis, MO, USA), and incubated for 2.5 h at 33°C while shaking at 60 rpm. Released protoplasts were filtered through a 40 µm cell strainer (Corning Inc., Corning, NY, USA), washed and suspended in STC buffer (20% sucrose, 10 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$) to a final concentration of 5 × 10^6/mL. Transformation was performed by co-incubating protoplasts with assembled Cas9 ribonucleoprotein (RNP) complexes (20 pmol), donor DNA (~13-17 pmol), and pTEL-Hyg (0.1 pmol). Following 30 min incubation on ice, protoplasts were treated with PEG-STC (60 g PEG 4000, 200 g sucrose, 10 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$ per L water) and recovered in TB3 broth (30 g yeast extract, 30 g casein acid hydrolysate, 200 g sucrose per L water) for 18 h at room temperature. Regenerated cells were plated on PDA supplemented with hygromycin B (50 µg/mL) and incubated at room temperature until single colonies appeared. Transformants were selected on PDA plates supplemented with hygromycin B (50 µg/mL), and validated by PCR and sequencing. Marker-free mutants were identified by loss of hygromycin resistance.

In planta co-inoculation and disease assays

Arabidopsis thaliana Col-0 plants were cultivated on potting soil under controlled greenhouse conditions with 17 h of light at 23°C and 7 h of dark at 22°C. Inoculation was performed as previously described in Ellendorff et al. (2009), with minor modifications. Conidiospores of *V. dahliae* were harvested from PDA plates after up to 14 days of growth. Conidiospores were washed three times with sterile Milli-Q water and resuspended in sterile Milli-Q water. Suspensions were adjusted to 5 × 10^6 conidiospores/mL. Two-week-old *A. thaliana* were gently uprooted, roots were rinsed with Milli-Q water, and inoculated by immersing roots for 6 min in the conidiospore suspension before transfer to fresh potting soil.

For co-inoculation assays, suspensions consisted of 5% conidiospores from lineage P1 and 95% conidiospores from either the parental lineage (JR2Δ*Ave1;* de Jonge et al. 2012), lineage R1, or lineage R2. Aboveground *A. thaliana* tissue was harvested at 2 weeks post inoculation, genomic DNA was extracted, and real-time PCR was performed using primer pairs listed in Supplementary Table 1. Primer pair JR2_Chr1_F2/JR2_Chr5_R2 was used to detect the inter-chromosomal rearrangement ICR1.5, while JR2_Chr1_F3/JR2_Chr1_R3 was used to amplify the parental chromosome configuration (Supplementary Table 1). Amplifications were normalized to *V. dahliae* biomass using *V. dahliae*-specific internal transcribed spacer (ITS) primers (Supplementary Table 1).

For disease assays, *A. thaliana* were inoculated with conidiospores of the parental lineage (Δ*Ave1*; de Jonge et al. 2012) and lineage P1 prepared as described above. Disease progression was documented at 14 and 21 dpi by canopy area measurements from overhead photographs using ImageJ (Schneider et al. 2012).

In vitro co-cultivation assay

Conidial suspensions consisting of 5% lineage P1, and 95% of either the parental lineage (JR2Δ*Ave1;* de Jonge et al. 2012), or lineage R1, or lineage R2 were prepared as described above. For each mixture, 80 µL of suspension was spread onto ten PDA plates and incubated at 22°C for 14 days. Conidiospores were subsequently harvested, and genomic DNA was extracted with the RSC Plant DNA Kit (Promega, Madison, WI, USA) using the Maxwell RSC device (Promega, Madison, WI, USA). Lineage abundance was quantified by real-time PCR with primer pairs (Supplementary Table 1) detecting inter-chromosomal rearrangement ICR1.5 (JR2_Chr1_F2/JR2_Chr5_R2), and the parental chromosome configuration (JR2_Chr1_F3/JR2_Chr1_R3). Amplifications were normalized to *V. dahliae* biomass using *V. dahliae*-specific internal transcribed spacer (ITS) primers (Supplementary Table 1).

Single-nucleotide variant and transcriptome analysis

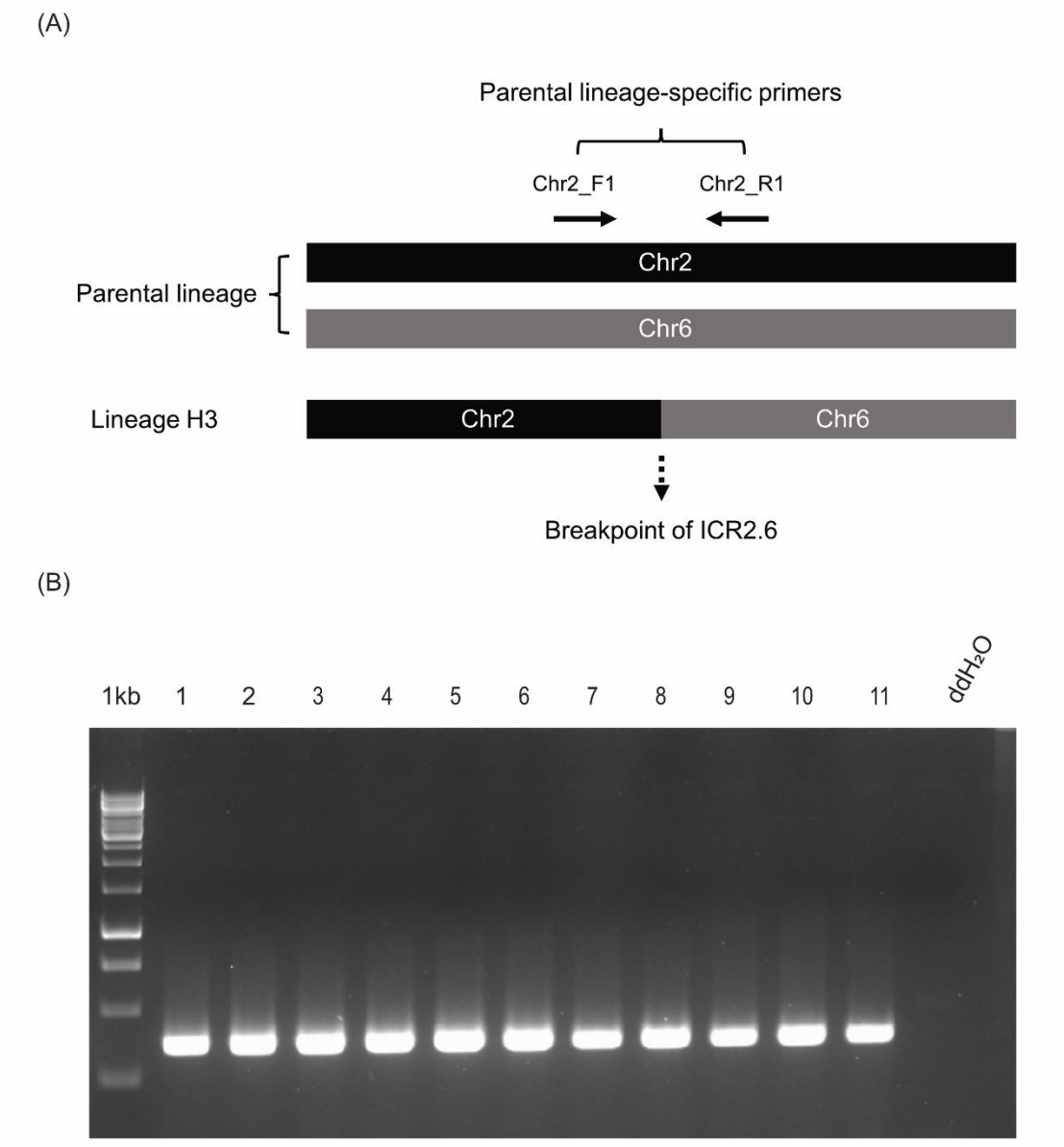
High-molecular-weight (HMW) DNA of lineage P1 was extracted as described above and sequenced on an Illumina NovaSeq X Plus platform (Novogene, Planegg, Germany). Paired-end Illumina reads were processed with Trimmomatic (version 0.39; Bolger et al. 2014) to remove adapters, and aligned to the *V. dahliae* JR2 reference genome (VDAG_JR2v4.0; Faino et al. 2015) using BWA-MEM (version 0.7.17; Li 2013). Read groups were assigned with (version 3.1.1; Broad Institute 2019). SNPs were identified, filtered, and normalized in haploid mode using GATK (version 4.5.0.0; Broad Institute. 2023). Functional annotation was performed using SnpEff (version 5.2; Cingolani et al. 2012), retaining only SNPs predicted to cause high-impact changes in protein-coding sequences. Expression of genes harboring SNPs was assessed using published *in planta* transcriptomes (Shi-Kunne et al. 2019) following the workflow described by Sato et al.(2025).

In vitro growth assays

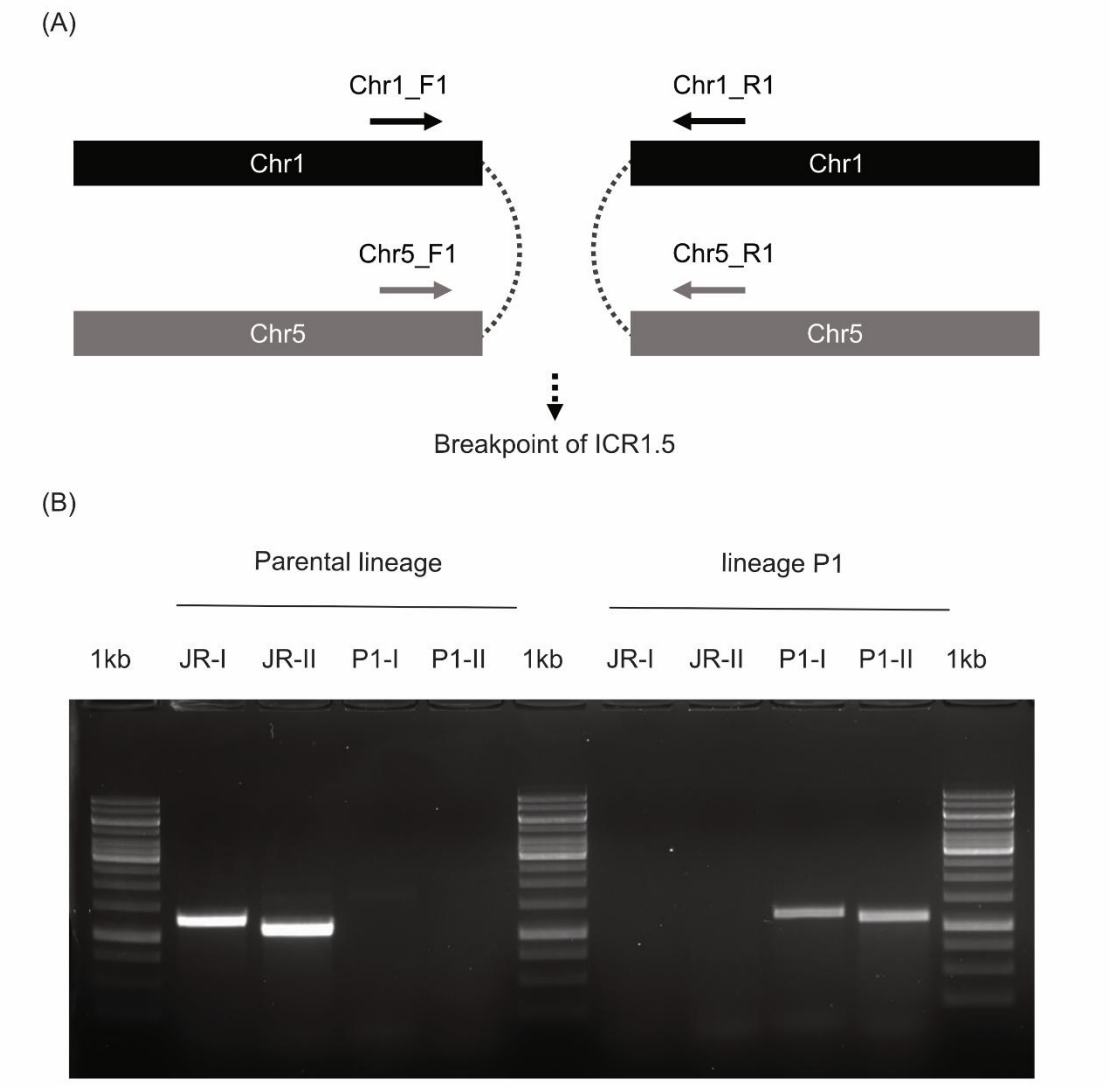
In vitro growth assays were performed with the parental lineage (Δ*Ave1*; de Jonge et al. 2012), the lineage P1, and lineages R1 and R2. Conidiospores were prepared as described above and adjusted to 10^5 conidiospores/mL using a haemocytometer. For radial growth, 10 µL drops containing 10^3 conidiospores were spotted centrally on 90 mm Petri dishes with 25 mL PDA and incubated at 22 °C in darkness. Colony diameters were measured after 14 days, and colony morphology was documented using a flatbed scanner (Epson Perfection V700, Long Beach, USA). For conidiospore production, spores from 14-day-old colonies were harvested in 2 mL sterile Milli-Q water, filtered through a 40 µm cell strainer (Corning Inc., Corning, NY, USA), and quantified using a haemocytometer. For germination assays, 5×10^4 conidiospores were suspended in 500 µL 1% potato dextrose broth (PDB; VWR, Radnor, PA, USA) in 24-well

plates and incubated at 22 °C for 11 h. Germination was scored microscopically by assessing germ tube emergence of ≥100 conidiospores per data point.

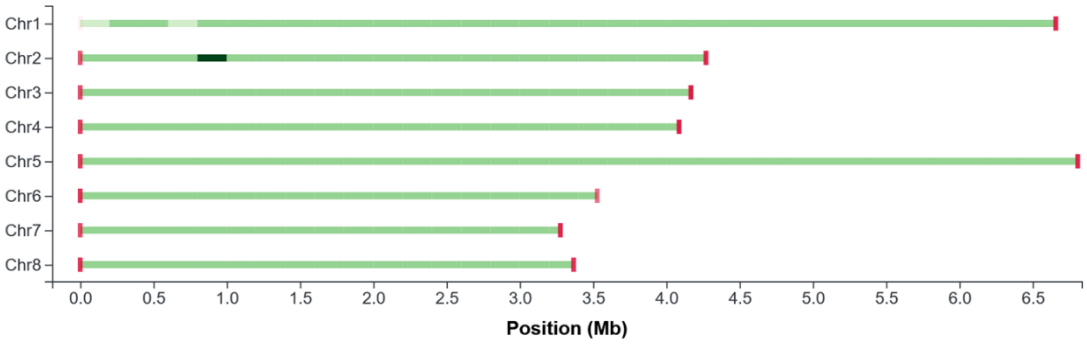
SUPPLEMENTARY DATA



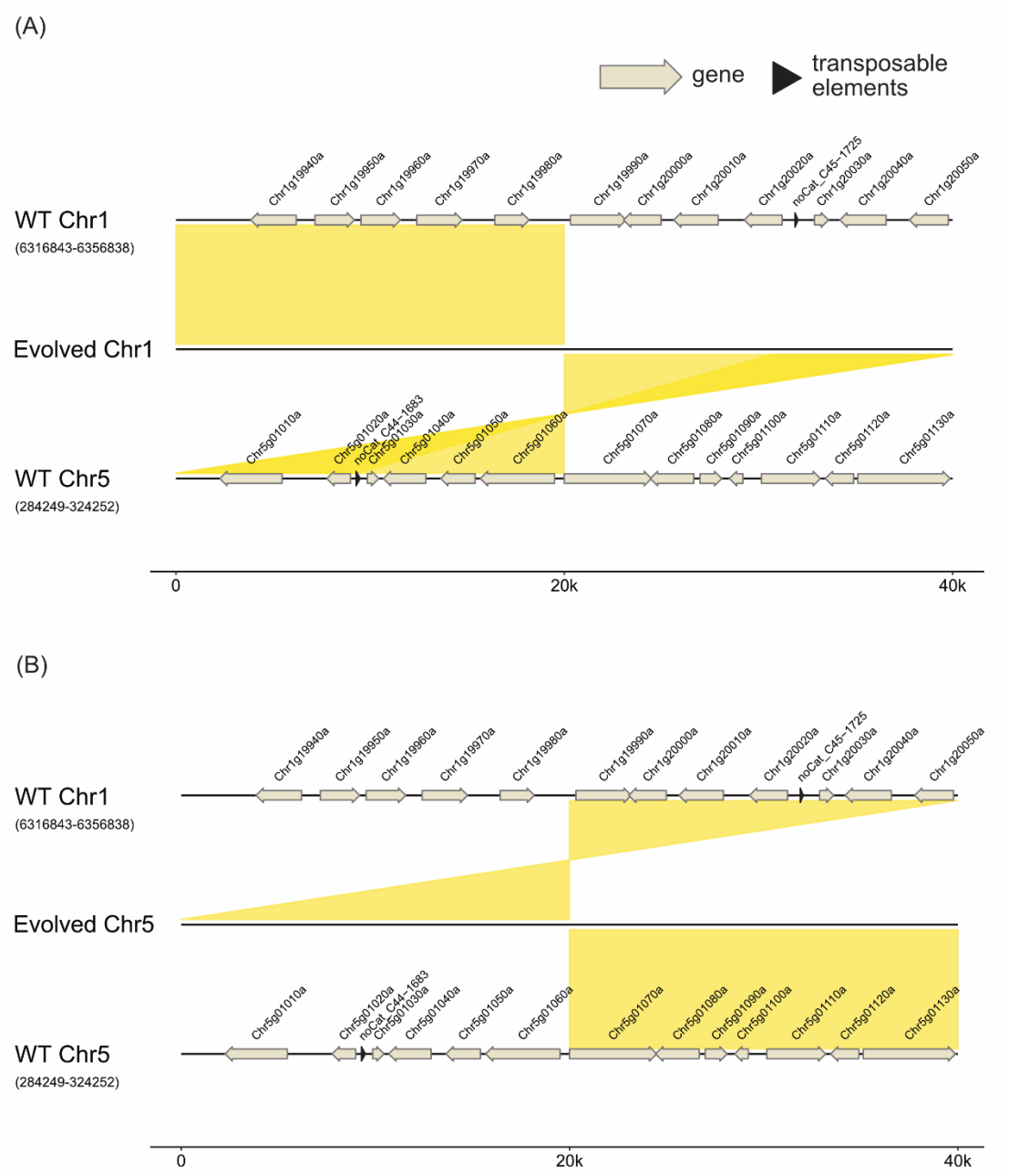
Supplementary Figure 1. PCR validation of inter-chromosomal rearrangement ICR2.6 in heat-derived lineage H3. (A) Black and grey blocks represent chromosome segments in the parental lineage, and dashed line indicates the predicated breakpoint of ICR2.6 in lineage H3. Primer pair Chr2_F1/Chr2_R1 amplifies the parental chromosome 2 configuration around the breakpoint of ICR2.6. (B) Representative colony PCR results from lineage H3 using Chr2_F1/Chr2_R1. Screening of 100 colonies yielded only the parental chromosome 2 amplicon, of which 11 examples are shown.



Supplementary Figure 2. PCR validation of inter-chromosomal rearrangement ICR1.5 in *Arabidopsis thaliana*-derived lineage P1. (A) Black and grey blocks represent chromosome segments in the parental lineage, and dashed lines illustrate how these segments are reconnected in lineage P1 carrying ICR1.5. Primer sets JR-I (Chr1_F1/Chr1_R1) and JR-II (Chr5_F1/Chr5_R1) amplified the parental chromosome configuration, whereas P1-I (Chr1_F1/Chr5_F1) and P1-II (Chr1_R1/Chr5_R1), amplified across the ICR1.5 breakpoints. (B) PCR with the primer sets as detailed for panel A yielded amplicons from JR-I/JR-II only in the parental lineage and from P1-I/P1-II only in lineage P1, respectively.



Supplementary Figure 3. Telomere-to-telomere genome assembly of the evolved *Verticillium dahliae* lineage P1. Green bars represent chromosome sizes, while red bars indicate telomeres. The color intensity reflects differences in sequencing read coverage, which were used to construct the assembly.



Supplementary Figure 4. Pairwise alignment between the *Verticillium dahliae* genomes of evolved lineage P1 and JR2 wild-type (WT) across regions 20 kb upstream and downstream of the rearrangement breakpoints. (A) Breakpoint region on evolved chromosome 1 aligned with the corresponding regions on wild-type chromosomes 1 and 5. (B) Breakpoint region on evolved chromosome 5 aligned with the corresponding regions on wild-type chromosomes 1 and 5.

Supplementary Table 1. Primers used in this study.

Primer ID	Primer sequence	Application
Chr2_F1	CCCAGACTGACAGGCGGACTAT	Breakpoint-specific PCR
Chr2_R1	GCACCTCCTTGGCATTGTTGA	Breakpoint-specific PCR
Chr1_F1	CTTCGACGGATATGAGGGGC	Breakpoint-specific PCR+ PCR genotyping (CRISPR)
Chr1_R1	ACGGATTCGGATGCGATTGA	Breakpoint-specific PCR
Chr5_F1	CTCGTTTCCTTGGGAGGCAT	Breakpoint-specific PCR + PCR genotyping (CRISPR)
Chr5_R1	GTCAGTTAGCCAGGGGCAAG	Breakpoint-specific PCR
VdGAPDH_F	AAGGGTGAGATCACCGAGGA	Real-time PCR
VdGAPDH_R	ATCACGTACATGGGGGCATC	Real-time PCR
VdITS1-F	AAAGTTTTAATGGTTCGCTAAGA	Real-time PCR
VdITS1-R	CTTGGTCATTTAGAGGAAGTAA	Real-time PCR
JR2_Chr1_F2	CGATCTCCTTCCCCAGAGGT	Real-time PCR
JR2_Chr5_R2	TCGTTGCCTCTGTACATGCT	Real-time PCR
JR2_Chr1_F3	AGCAAGCCAGGTAGGAGGAA	Real-time PCR
JR2_Chr1_R3	TGGGCCGTTGGCATATTCAT	Real-time PCR
SgRNA_ constant oligo	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACG GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	sgRNA constant oligo
sgRNA_JR2_1 .4u-5.6u_1	AAGCTAATACGACTCACTATAGGGCGACCCAGGGACCTC TGGTTTTAGAGCTAGAAATAGCAAG	sgRNA protospacer oligo targeting evolved Chr1
sgRNA_JR2_5 .6d-1.4d_2	AAGCTAATACGACTCACTATAGGGTTCCGCGCGCAAGCC CTGTTTTAGAGCTAGAAATAGCAAG	sgRNA protospacer oligo targeting evolved Chr5
Donor_JR2_C hr1_F4	CTGGTCTCTGCTGGGTCTAT	To create donor DNA

Donor_JR2_Chr1_R4	TGGGCCGTTGGCATATTCAT	To create donor DNA
Donor_JR2_Chr5_F3	TCGTTGCCTCTGTACATGCT	To create donor DNA
Donor_JR2_Chr5_R3	CGGACTTGGGGTTGTGGAAT	To create donor DNA
JR2_Chr1_F5	CCTTCAGTCTGAGCTTGGCA	PCR genotyping (CRISPR)
JR2_Chr5_R4	CTGGAGACAACCTACGCCAG	PCR genotyping (CRISPR)

Supplementary Table 2. Single-nucleotide polymorphisms detected in evolved *V. dahliae* lineage P1 relative to the JR2 reference genome.

Annotation	Transcript ID	SNPs	Mean Read Count[a]
Reference transcript with high *in planta* expression included as control	VDAG_JR2_Chr4g03680 (Encodes the Av2 avirulence effector; Chavarro-Carrero et al. 2021)	No	380
Transcripts in lineage P1 carrying non-synonymous SNPs	VDAG_JR2_Chr1g17870	Yes	2
	VDAG_JR2_Chr1g10230	Yes	1.6
	VDAG_JR2_Chr4g09470	Yes	1.6
	VDAG_JR2_Chr6g03850	Yes	1
	VDAG_JR2_Chr1g06140	Yes	0.6
	VDAG_JR2_Chr1g16850	Yes	0.6
	VDAG_JR2_Chr1g17110	Yes	0.6
	VDAG_JR2_Chr7g01570	Yes	0.6
	VDAG_JR2_Chr1g00840	Yes	0
	VDAG_JR2_Chr1g11050	Yes	0
	VDAG_JR2_Chr1g11730	Yes	0
	VDAG_JR2_Chr1g12310	Yes	0
	VDAG_JR2_Chr1g17550	Yes	0
	VDAG_JR2_Chr1g26880	Yes	0
	VDAG_JR2_Chr3g07350	Yes	0

	VDAG_JR2_Chr3g13140	Yes	0
	VDAG_JR2_Chr4g07190	Yes	0
	VDAG_JR2_Chr5g02010	Yes	0
	VDAG_JR2_Chr5g09380	Yes	0
	VDAG_JR2_Chr5g11720	Yes	0
	VDAG_JR2_Chr6g04790	Yes	0
	VDAG_JR2_Chr6g04800	Yes	0
	VDAG_JR2_Chr6g05640	Yes	0
	VDAG_JR2_Chr7g01140	Yes	0
	VDAG_JR2_Chr7g03290	Yes	0
	VDAG_JR2_Chr7g05800	Yes	0
	VDAG_JR2_Chr8g01360	Yes	0
	VDAG_JR2_Chr8g05940	Yes	0
	VDAG_JR2_Chr8g09290	Yes	0

[a] The mean read count refers to the average number of raw sequencing reads that mapped to a given transcript across three biological replicates.

Chapter 4

Transcriptional activity and noncanonical regulatory roles of mating-type genes in the asexual fungus *Verticillium dahliae*

Chen-Yu Kuan[#], Jiyeun Park[#], Julie Noah, Gabin Armand, Anton Kraege, Edgar A. Chavarro-Carrero, Hui Tian, Bart P.H.J. Thomma[*]

University of Cologne, Institute for Plant Sciences, Cluster of Excellence on Plant Sciences (CEPLAS), 50674 Cologne, Germany

[#] These authors contributed equally

[*] Corresponding author: bthomma@uni-koeln.de

ABSTRACT

Sexual reproduction is a major driver of genome evolution in fungi and many other eukaryotes, as it generates genetic diversity and facilitates the removal of deleterious mutations. In sexually reproducing ascomycete fungi, sexual compatibility is determined by a single mating-type (*MAT*) locus with two idiomorphs, *MAT1-1* and *MAT1-2*. Heterothallic ascomycetes carry a single mating-type idiomorph per strain and require a partner with the opposite mating type to mate, whereas homothallic ascomycetes harbor both idiomorphs within a single genome, enabling self-fertility. The soil-borne plant pathogen *Verticillium dahliae* is a presumed asexual ascomycete fungus that exhibits extensive large-scale chromosomal rearrangements that are considered incompatible with chromosome pairing during meiosis. Nonetheless, *V. dahliae* strains retain either the *MAT1-1* or *MAT1-2* mating-type idiomorph, resembling the genomic organization of heterothallic ascomycetes. This raises questions about the functional role of *MAT* genes in *V. dahliae*. Here, we analyzed 52 *V. dahliae* genomes from strains previously collected worldwide and observed a strongly skewed mating-type distribution, with *MAT1-2* predominating and only 7 strains carrying the *MAT1-1* idiomorph. We then generated *V. dahliae* strains with identical karyotypes that differed solely in their mating-type idiomorph in the background of the wild-type strain JR2, including a *MAT* gene deletion strain, strains carrying either *MAT* idiomorph, and a strain carrying both idiomorphs. RNA sequencing of these strains grown in potato dextrose broth or Czapek–Dox broth showed that *MAT* genes, along with 85 sex-related genes, are transcriptionally active in *V. dahliae*. Differentially expressed genes (DEGs) were identified for each *MAT* mutant relative to the wild-type *V. dahliae* under each growth condition. However, DEGs shared among *MAT* mutants were not enriched for the 85 sex-related genes, and were primarily associated with genes involved in general metabolic and cellular processes. Moreover, co-cultures of strains with opposite mating-type idiomorphs also showed no enrichment for sex–related genes among the DEGs. These findings show that *MAT* genes in *V. dahliae* are expressed and associated with transcriptional changes in metabolic and cellular processes, whereas sex-related genes are transcriptionally active but their overall expression is not dependent on *MAT* genotype under the tested conditions.

INTRODUCTION

Sexual reproduction is one of the primary mechanisms by which eukaryotic organisms generate genetic diversity (Speijer et al. 2015). Sexual reproduction involves meiosis, during which parental alleles are reshuffled, and fertilization subsequently combines their genomes to produce novel genetic combinations (Fu et al. 2019). This process produces genetically unique offspring and has been observed across nearly all branches of the eukaryotic tree of life (Speijer et al. 2015). In contrast, strict asexuality is often regarded as evolutionarily disadvantageous because the absence of meiotic recombination leads to the accumulation of deleterious mutations in asexual species, a process known as Muller's ratchet (Hojsgaard and Hörandl 2015; Felsenstein 1974). Although Muller's ratchet predicts that strict asexuality is evolutionarily disadvantageous, many eukaryotic species lack documented evidence of sexual reproduction, even after extensive laboratory studies (Speijer et al. 2015; Seidl and Thomma 2014). It remains unclear whether many of these species are truly asexual and rely on alternative mechanisms to generate genetic diversity, or instead undergo rare, hidden sexual cycles in which reproduction occurs only under specific conditions (Speijer et al. 2015; Seidl and Thomma 2014). In addition to conventional sexual reproduction, some eukaryotes undergo alternative reproduction processes, such as parasexuality, which has been described in fungi (Seidl and Thomma 2014). Parasexuality involves the exchange of genetic material without meiosis, typically initiated by hyphal fusion between compatible individuals, followed by nuclear fusion and genetic recombination during mitotic divisions (Strom and Bushley 2016). Through this process, genetic variation is generated during vegetative growth (Strom and Bushley 2016).

Verticillium dahliae is a soilborne ascomycete fungus that causes vascular wilt disease in hundreds of plant species, including numerous crops (Acharya et al. 2020; Sayari et al. 2024; Bhat and Subbarao 1999). *V. dahliae* is considered strictly asexual because no sexual reproduction has been observed (Klosterman et al. 2011). Whole-genome sequencing of *V. dahliae* strains collected worldwide revealed high sequence similarity across their core genomes, with nucleotide diversity ranging from 0.02% to 0.5% among the sequenced strains, consistent with clonal reproduction (de Jonge et al. 2013; Torres et al. 2021). Pairwise whole genome alignments among *V. dahliae* strains revealed extensive chromosomal rearrangements, which are considered an alternative source of genomic variation in the absence of sexual reproduction (de Jonge et al. 2013; Faino et al. 2016). Chromosomal rearrangements can contribute to genome diversification independently of meiotic recombination by generating structural variation, including inversions, translocations, deletions, and duplications (Flot et al. 2013; Seidl and Thomma 2014). These rearrangements are thought to arise from double strand DNA breaks followed by unfaithful DNA repair involving

homologous sequences and repetitive elements, particularly transposable elements, which are proposed to provide ectopic substrates for homology-based unfaithful DNA repair (Faino et al. 2016). Because sexual reproduction relies on proper pairing of homologous chromosomes during meiosis, structural changes that disrupt chromosomal collinearity may impair homologous recombination, potentially reinforcing an asexual lifestyle in *V. dahliae* (Flot et al. 2013; de Jonge et al. 2013).

Intriguingly, despite the apparent absence of sexual reproduction, the *V. dahliae* genome retains highly conserved mating-type (*MAT*) genes (Klosterman et al. 2011; Zhang et al. 2024). In sexually reproducing ascomycete fungi, *MAT* genes define sexual compatibility through heterothallic (self-incompatible) or homothallic (self-fertile) reproductive strategies, and some species are capable of switching between these two modes of reproduction (Dyer et al. 2016). In heterothallic ascomycetes, mating requires interaction between individuals carrying opposite *MAT* idiomorphs, *MAT1-1* and *MAT1-2*, which are highly divergent sequences located at a single chromosomal site known as the *MAT* locus (Dyer et al. 2016). The *MAT* locus harbors either the *MAT1-1* or the *MAT1-2* idiomorph in heterothallic ascomycetes, and is typically flanked by conserved genomic regions shared among isolates of the same species (Dyer et al. 2016). The *MAT1-1* idiomorph encodes at least one transcription factor containing a conserved high-mobility group (HMG) DNA-binding domain, whereas *MAT1-2* encodes at least one transcription factor containing a conserved α-box DNA-binding domain (Dyer et al. 2016; Martin et al. 2010). Additional *MAT* genes may also reside within each idiomorph and are commonly designated sequentially, for example *MAT1-1-x* and *MAT1-1-y* (Dyer et al. 2016). In contrast, homothallic ascomycetes are self-fertile and harbor both HMG- and α-box–encoding *MAT* idiomorphs within a single genome, with the two idiomorphs positioned either closely linked to each other at a single *MAT* locus or located on separate chromosomes (Dyer et al. 2016). In sexually reproducing fungi, *MAT* transcription factors can coordinate the expression of genes involved in pheromone production and perception, thereby facilitating cell–cell recognition and fusion during sexual reproduction (Kim et al. 2015; Bobrowicz et al. 2002). Thus, deletion of *MAT* genes often results in reduced fertility or sterility (Paoletti et al. 2007; Wang et al. 2021; Kim et al. 2015).

V. dahliae strains carry either the *MAT1-1* or the *MAT1-2* idiomorph, a genomic organization consistent with a heterothallic mating-type system (Klosterman et al. 2011). The *MAT1-1* idiomorph comprises *MAT1-1-1*, encoding the α-box DNA-binding domain, and *MAT1-1-3*, encoding the HMG DNA-binding domain (Klosterman et al. 2011). The *MAT1-2* idiomorph comprises *MAT1-2-1*, encoding the HMG DNA-binding domain, and *MAT1-2-2*, which does not encode a domain indicative of a transcription factor (Klosterman et al. 2011). A highly skewed mating-type distribution was observed among 1,120 *V. dahliae* isolates sampled globally, with

the *MAT1-1* idiomorph detected in approximately 1% of isolates and *MAT1-2* present in the remaining 99% (Short et al. 2014). Such pronounced skew is expected to substantially reduce the likelihood of encounters between *V. dahliae* strains carrying opposite mating types in natural environments, thereby constraining opportunities for sexual reproduction. However, the possibility of sexual reproduction occurring at low frequency cannot be excluded if strains of opposite mating types encounter one another in nature (Short et al. 2014). In addition to harboring both *MAT* idiomorphs, *V. dahliae* exhibits several molecular characteristics that are commonly associated with sexual reproduction, raising the possibility of rare or cryptic sexual processes. Genome analyses identified 88 genes in *V. dahliae* as orthologs of 93 genes characterized for roles in sexual reproduction in other ascomycete fungi, including *Neurospora crassa*, *Saccharomyces cerevisiae*, and *Podospora anserina* (Short et al. 2014). At least ten of these sex-related genes, including the *MAT* genes, are transcriptionally active in *V. dahliae* (Short et al. 2014). Notably, transposable elements in *V. dahliae* exhibit signs of repeat-induced point mutations (RIP), a genome defense mechanism that is commonly associated with the sexual cycle in fungi (Kramer et al. 2021; Torres et al. 2021). RIP introduces numerous cytosine-to-thymine (C-to-T) point mutations into duplicated DNA sequences, including transposable elements, leading to their inactivation and limiting their proliferation, and it typically occurs prior to meiosis (Galagan and Selker 2004). However, the presence of RIP alone does not constitute definitive evidence of sexual reproduction, as RIP signatures may be retained as remnants of ancestral sexual reproduction if *V. dahliae* ancestors reproduced sexually. Moreover, similar RIP-like mutation patterns have been reported during mitotic growth in other fungal species, leaving unresolved whether the RIP signatures observed in the *V. dahliae* genome arise through sexual or alternative, nonsexual mechanisms (Clutterbuck 2011). Whether the presence of *MAT* idiomorphs, sex-related genes, and RIP signatures indicates rare sexual reproduction in *V. dahliae* remains unresolved. Given that *MAT* genes in many fungi encode transcription factors (Sun et al. 2025), we performed transcriptomic analyses to assess whether *MAT* genes in *V. dahliae* regulate the expression of other genes and, if so, whether such regulation is associated with sexual or nonsexual processes.

RESULTS

Predominance of the *MAT1-2* idiomorph among *Verticillium dahliae* strains

To identify the mating-type (*MAT*) loci in *V. dahliae*, we analyzed 52 strains for which whole-genome sequences were available in our laboratory (Figure 1; de Jonge et al. 2012; Faino et al. 2015; Fan et al. 2018; Gibriel et al. 2019). Using the *V. dahliae MAT1-2* idiomorph sequence and the *V. albo-atrum MAT1-1* idiomorph sequence (Klosterman et al. 2011), we searched for *MAT* loci across the 52 *V. dahliae* genomes. The analysis revealed that each strain harbored a single *MAT* locus with two *MAT* genes, either *MAT1-1-1* and *MAT1-1-3* that form the *MAT1-1* idiomorph, or *MAT1-2-1* and *MAT1-2-2* that form the *MAT1-2* idiomorph (Figure 1). No strain carried both idiomorphs (Figure 1). Most strains carried the *MAT1-2* idiomorph, with only 7 of the 52 genomes carrying the *MAT1-1* idiomorph, demonstrating a skewed mating-type distribution in our *V. dahliae* genome collection (Figure 1).

MAT mutants grow similar to wild-type *V. dahliae*

To further investigate the functional role of *MAT* idiomorphs, transformants of *V. dahliae* with identical karyotypes that differ solely in their *MAT* idiomorphs were generated. The *V. dahliae* strain JR2 (Faino et al. 2015), which naturally carries the *MAT1-2* idiomorph, was used as the parental strain for all transformations and is hereafter referred to as JR2_*MAT2*. First, the *MAT1-1* idiomorph from *V. dahliae* strain DvD-S29 (de Jonge et al. 2012) was inserted approximately 900 base pairs (bp) downstream of the *MAT1-2* idiomorph in JR2_*MAT2.* This modification resulted in a strain carrying both mating-type idiomorphs (JR2_*MAT1*_*MAT2*), equivalent to the genome composition typically found in homothallic ascomycetes. Second, The *MAT1-2* idiomorph was deleted from *V. dahliae* strain JR2, resulting in a strain that lacks any mating-type idiomorph (JR2 Δ*MAT*). Third, the *MAT1-1* idiomorph from strain DvD-S29 was inserted into the *MAT1-2* deletion strain (JR2 Δ*MAT*) approximately 900 bp downstream of the original *MAT1-2* idiomorph, generating a strain that carries only the *MAT1-1* idiomorph (JR2_*MAT1*). For each genotype, three independent transformants were selected for subsequent analyses (Supplementary Figure 1). To assess *in vitro* growth and development, 10^3 *V. dahliae* conidiospores of each genotype were deposited onto potato dextrose agar (PDA) and incubated at 22°C for 10 days. Colony diameters were measured and colony morphology was visually assessed. All genotypes exhibited comparable radial growth and mycelium development, with no measurable differences among the genotypes (Figure 2A, C). After 13 days of growth on PDA at 22°C, conidiospore production was quantified. Conidiospores were released from five 4 mm agar plugs collected from the edge of each colony by incubation of the agar plugs in 800 µL sterile water at 30 rpm for 1.5 h. Conidiospore concentrations were

subsequently determined in the suspension. No significant differences in conidiospore production were detected among the genotypes (Figure 2B). Thus, *MAT* mutants display *in vitro* growth comparable to wild-type *V. dahliae* on PDA at 22°C.

In Ascomycota, sexual reproduction is characterized by the formation of ascospores enclosed within sac-like structures called asci, which are organized into multicellular fruiting bodies (ascocarps) in some species, whereas in others the asci are produced without ascocarp formation (Wallen and Perlin 2018). In heterothallic ascomycetes, sexual reproduction occurs between strains carrying opposite mating types (Dyer et al. 2016). Such sexual reproduction is commonly established *in vitro* either by co-culturing mixed conidiospores of opposite mating types, or by placing both strains in close proximity on the same agar medium to allow colony interaction at the barrage zone (Dyer and O'Gorman 2012; Cai et al. 2025; Kim et al. 2012a; Ashton and Dyer 2019; Yeh et al. 2019). We applied both approaches to determine whether physical interaction between strains of opposite mating types induces visible signs of sexual development in *V. dahliae*. First, 10 µL conidiospore suspensions containing 10^3 *V. dahliae* conidiospores composed of equal proportions of JR2_*MAT1* and JR2_*MAT2* were deposited in the middle of PDA. The resulting colonies displayed morphology comparable to the control PDA containing JR2_*MAT2* alone after 10 days of growth at 22°C (Figure 2C). No visible signs of sexual development, such as ascocarp formation, were observed (Figure 2C). For the second approach, 10 µL conidiospore suspensions containing 10^3 *V. dahliae* conidiospores of JR2_*MAT1* and JR2_*MAT2*, respectively, were deposited 4 cm apart on PDA and incubated at 22°C for 10 and 13 days. The resulting colonies did not show preferential growth toward the neighboring colony, similar to the control PDA in which two JR2_*MAT2* were co-cultured in close proximity (Figure 2D). The colonies established a straight, narrow barrage zone, with neither colony extending into the other, and no differences in colony morphology were observed (Figure 2D). These results demonstrate that co-culturing *V. dahliae* strains of opposite mating type does not induce visible signs of sexual development on PDA at 22°C.

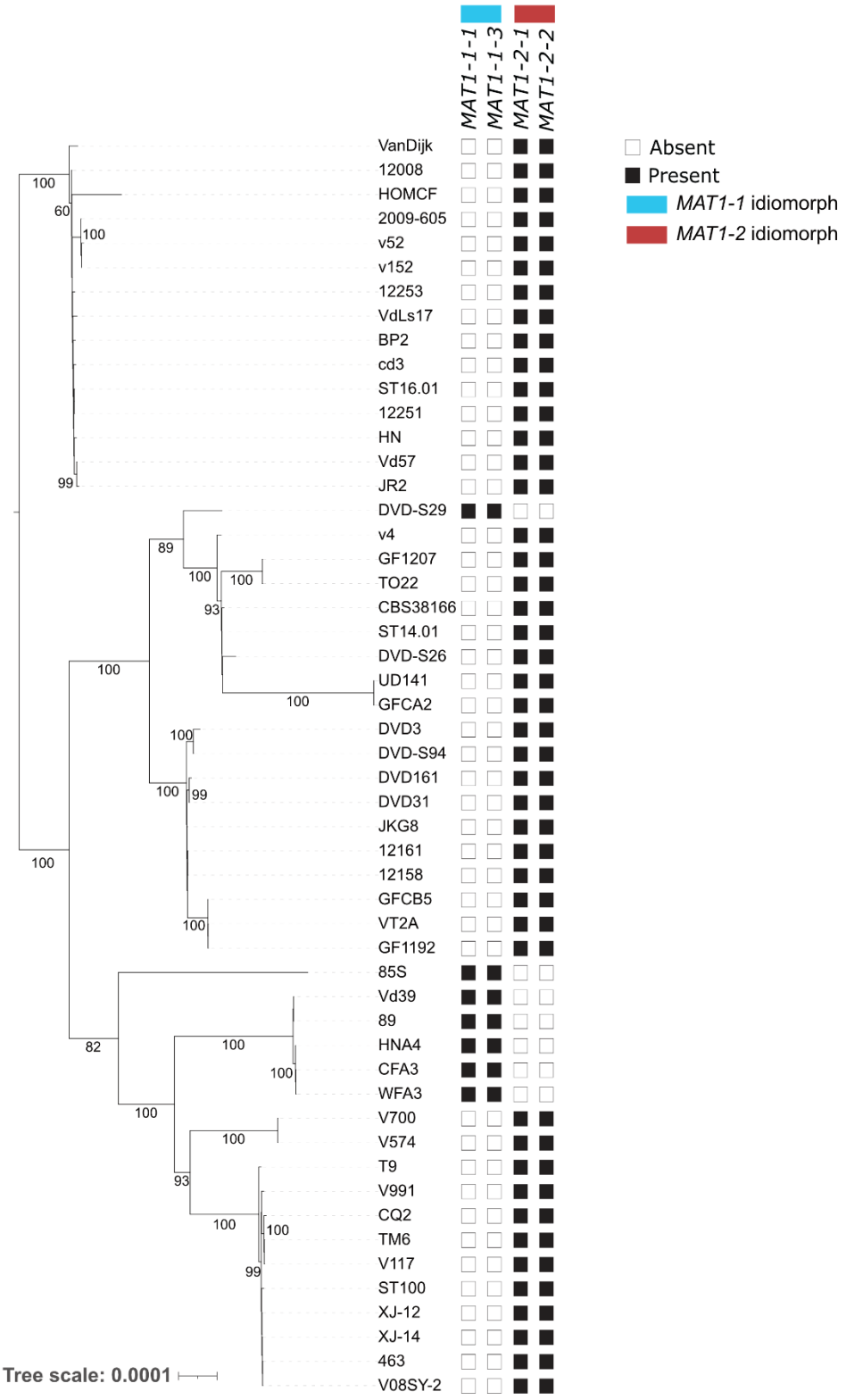


Figure 1. Phylogenetic relationships of *Verticillium dahliae* strains and presence-absence variation for the *MAT1-1* and *MAT1-2* idiomorphs. A maximum-likelihood phylogeny of 52 *V. dahliae* strains was constructed from whole-genome alignments using RealPhy (Bertels et al. 2014) . Bootstrap values (>50%) from 1000 iterations are shown at the nodes, and branch lengths represent the phylogenetic distance. Presence–absence information for the *MAT1-1* idiomorph and *MAT1-2* idiomorph is shown alongside the phylogenetic tree.

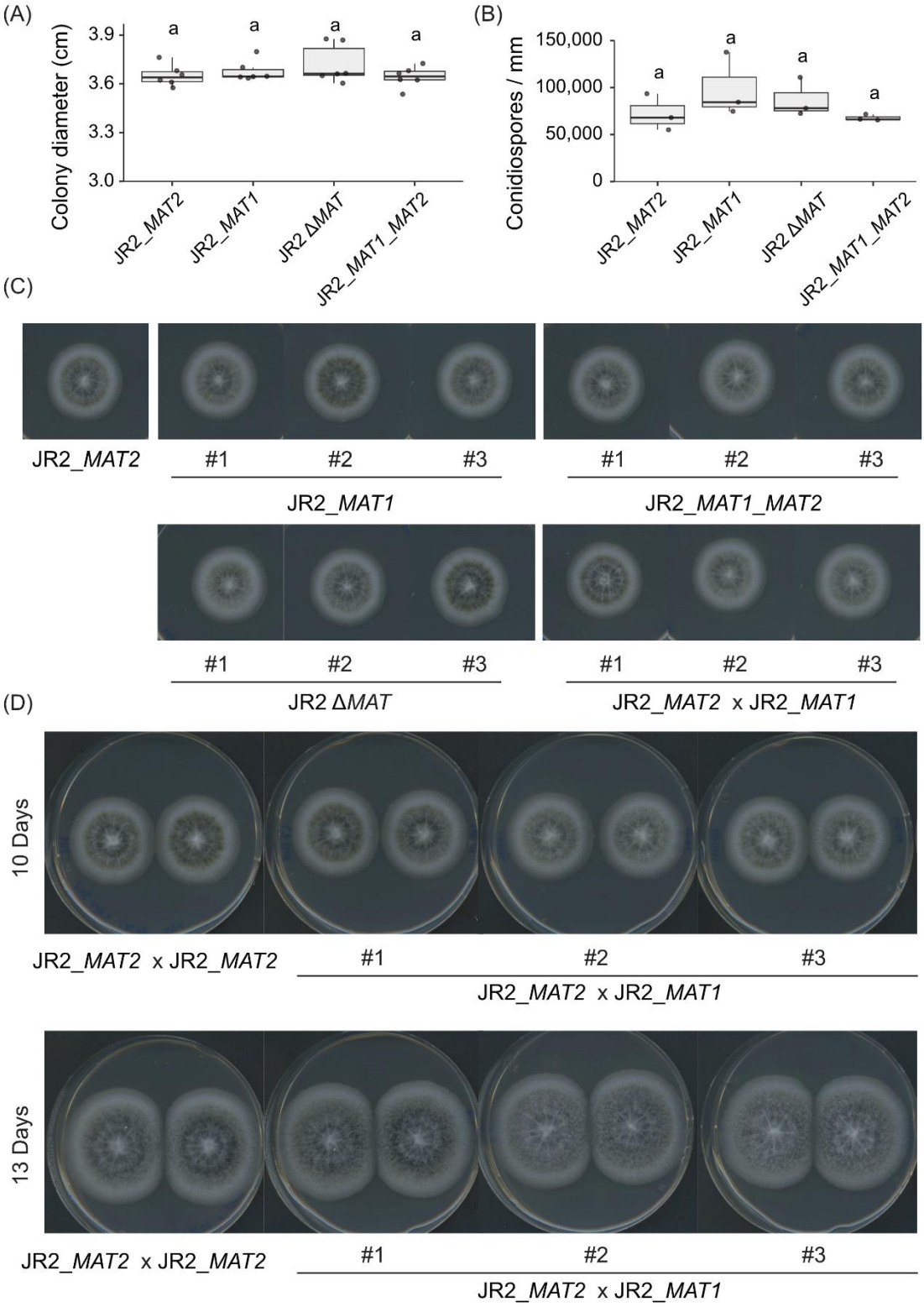


Figure 2. *MAT* mutants of *Verticillium dahliae* do not show altered growth *in vitro*. *In vitro* growth of wild-type *V. dahliae* (JR2_*MAT2*) and *MAT* mutants, including JR2_*MAT1* (harboring the *MAT1-1* idiomorph only), JR2 Δ*MAT* (lacking any *MAT* locus), and JR2_*MAT1_MAT2* (harboring both *MAT1-1* and *MAT1-2* idiomorphs). (A) Colony diameter after 10 days of growth on potato dextrose agar (PDA) (one-way ANOVA and Tukey's post-hoc test; $p < 0.05$; N = 6). (B) Conidiospore production per mm^2 colony area after 13 days of growth on PDA (one-way ANOVA and Tukey's post-hoc test; $p < 0.05$; N = 3). (C) Representative colony morphology after 10 days of growth on PDA. The co-culture (JR2_*MAT2* × JR2_MAT1) was established by depositing mixed conidiospore suspensions

containing equal proportions of both genotypes. Each *MAT* mutant is represented by three independent transformants (#1, #2, #3). (D) Representative colony morphology after 10 and 13 days of growth on PDA. Two conidiospore suspensions were deposited 4 cm apart on each plate to establish a co-culture of JR2_*MAT2* with JR2_*MAT1*. JR2_*MAT1* is represented by three independent transformants (#1, #2, #3).

MAT genes are transcriptionally active in *V. dahliae*

Since *MAT* genes in *V. dahliae* include transcription factor–encoding genes (Zhang et al. 2024), we hypothesized that genetic modifications of the *V. dahliae MAT* locus could alter the expression of *MAT*-regulated genes. To examine this, all *MAT* mutants and the wild-type strain JR2_*MAT2* were cultivated *in vitro* for RNA sequencing. For every genotype, three independent transformants were cultivated as biological replicates. To assess whether *MAT* genes and *MAT*-regulated genes show differential expression under different growth conditions, *V. dahliae* cultures were initiated from conidiospores in potato dextrose broth (PDB) and in Czapek–Dox broth (CDB) for up to 13 days. Furthermore, co-cultures were generated by co-cultivating strains with opposite mating types in both media and maintained for up to 13 days. All *V. dahliae* cultures were sampled at 0, 3, and 13 days and subjected to RNA sequencing.

We first assessed the expression of *MAT* genes across all transcriptome profiles generated in this study quantified as transcripts per million (TPM). Expression of *MAT* genes was detected in all genotypes harboring *MAT* genes and was absent only in JR2 Δ*MAT*, consistent with previous reverse transcription polymerase chain reaction (RT-PCR) analyses indicating that *MAT* genes are transcriptionally active in *V. dahliae* (Figure 3 and Supplementary Figure; Short et al. 2014; Zhang et al. 2024). The highest observed TPM values were 3.86 and 3.78 for genes within the *MAT1-1* and *MAT1-2* idiomorphs, respectively, corresponding to 18 reads mapped to *MAT1-1-3* in strain JR2_*MAT1* in CDB at day 13 and 26 reads mapped to *MAT1-2-1* in strain JR2_*MAT2* at day 0 (Figure 3 and Supplementary Figure 2). The low *MAT* expression is aligned with previous studies showing that fungal *MAT* genes are typically expressed at low levels during vegetative growth and early stages of sexual development (Wallen and Perlin 2018; Sikhakolli et al. 2012; Wang et al. 2014; Yu et al. 2016; Yu et al. 2018).

The expression of *MAT* genes observed across our transcriptome profiles prompted us to investigate whether *MAT* genes are generally expressed at low levels in *V. dahliae*. To this end, we analyzed published transcriptomic datasets (de Jonge et al. 2012; Cook et al. 2020; Shi-Kunne et al. 2019) of *V. dahliae* strain JR2, which harbors the *MAT1-2* idiomorph. Expression of genes within the *MAT1-2* idiomorph was detected in 12 out of 20 public *V. dahliae* transcriptome profiles, consistently at low expression levels (maximum TPM = 2.16 for *MAT1-2-1* and 1.74 for *MAT1-2-2*) (Figure 4). Thus, our analysis indicates that *V. dahliae MAT* genes are transcriptionally active and expressed at low levels across diverse transcriptome profiles.

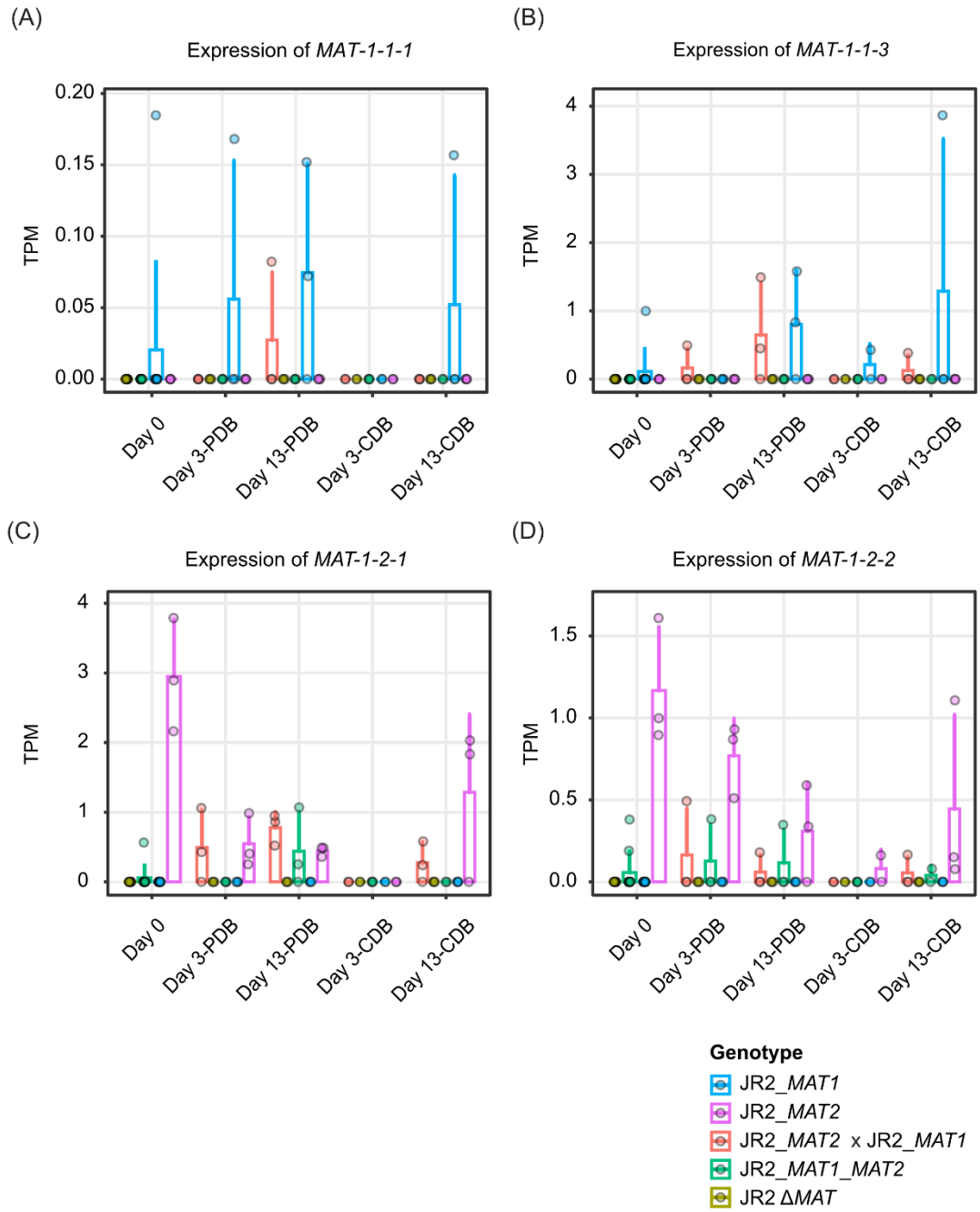


Figure 3. *MAT* genes are expressed in *Verticillium dahliae*. Transcriptome analysis shows the expression of (A) *MAT1-1-1*, (B) *MAT1-1-3*, (C) *MAT1-2-1*, and (D) *MAT1-2-2* across *V. dahliae* genotypes. Expression is shown as transcripts per million (TPM). Each dot represents an individual transformant, and three independent transformants were included per genotype. Error bars indicate mean values + SD. Transcriptomes were obtained from *V. dahliae* collected at day 0, 3 and 13 after cultivation in potato dextrose broth (PDB) or Czapek–Dox broth (CDB). *V. dahliae* strains comprise wild-type (JR2_*MAT2*), *MAT* mutants (JR2 Δ*MAT*, JR2_*MAT1*, and JR2_*MAT1_MAT2*), and a co-culture of JR2_*MAT2* and JR2_*MAT1* (JR2_*MAT2* × JR2_*MAT1*).

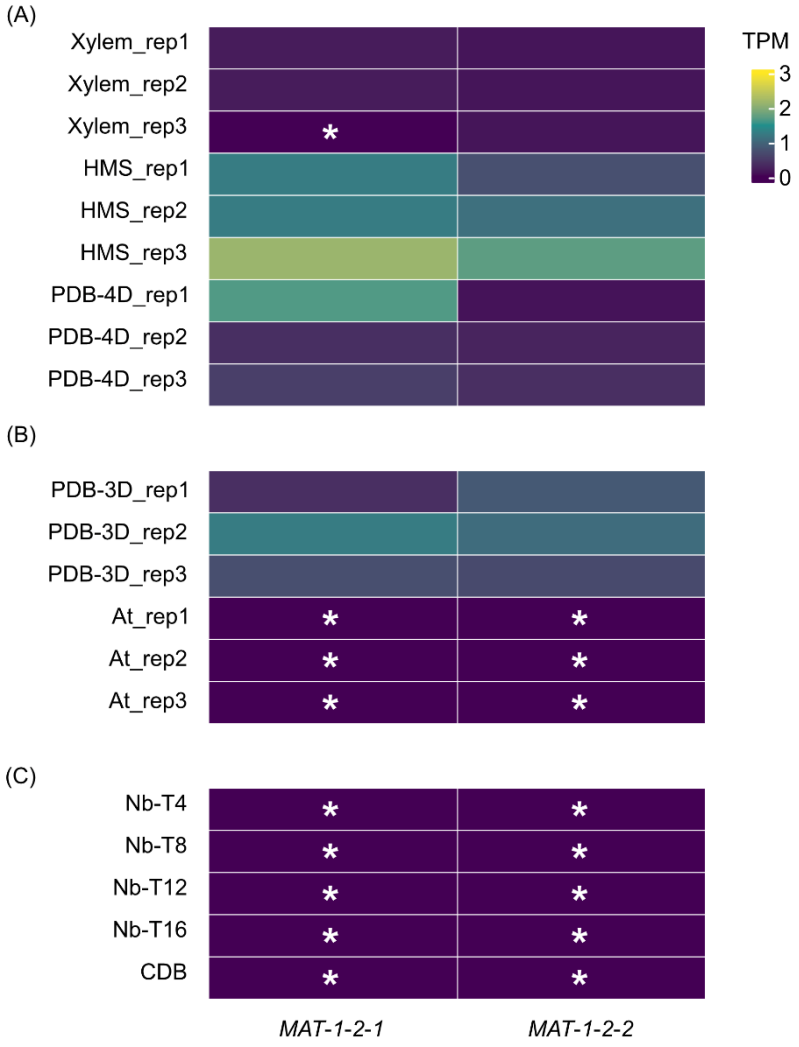


Figure 4. The *Verticillium dahliae MAT* genes exhibit low expression across transcriptome datasets. Expression of *MAT* genes is shown across three independent transcriptome datasets. Columns marked with an asterisk (*) indicate no detectable expression (transcripts per million, TPM = 0). (A) Heatmap of *MAT* genes expression in *V. dahliae* grown in xylem sap (Xylem), half-strength Murashige and Skoog medium supplemented with vitamins (HMS), and potato dextrose broth for 4 days (PDB-4D). Three replicates are shown (rep1, rep2, rep3) (Cook et al. 2020). (B) Heatmap of *MAT* genes expression in *V. dahliae* grown for 3 days in potato dextrose broth (PDB-3D) and in *V. dahliae* infecting *Arabidopsis thaliana* (At). Three replicates are shown (rep1, rep2, rep3) (Shi-Kunne et al. 2019). (C) Heatmap of *MAT* genes expression in *V. dahliae* obtained from *Nicotiana benthamiana* (Nb) inoculated with *V. dahliae* at 4, 8, 12, and 16 days post inoculation, and in *V. dahliae* grown in Czapek–Dox broth (CDB) (de Jonge et al. 2012).

Next, principal component analysis (PCA) was performed to evaluate variation in gene expression among the RNA-sequencing samples. PCA revealed significant separation (PERMANOVA, $P < 0.01$) of transcriptome profiles primarily according to growth media (20.3%), while separation by time points was less pronounced (12.4%), as RNA-sequencing samples of *V. dahliae* cultured in CDB at 3 and 13 days clustered (Figure 5). Rather surprisingly, no distinct clustering by genotype was observed, as the genotype explained only 1.7% of the variation in transcriptomic profiles (PERMANOVA, $P = 0.216$) (Figure 5). In addition, PCA

showed that the transcriptome profiles of co-culture samples clustered with single cultures, suggesting similar global transcriptome profiles between the co-cultures and single cultures (Figure 5). These results indicate that the *V. dahliae* genotype does not significantly contribute to global gene expression variation among the RNA-sequencing samples.

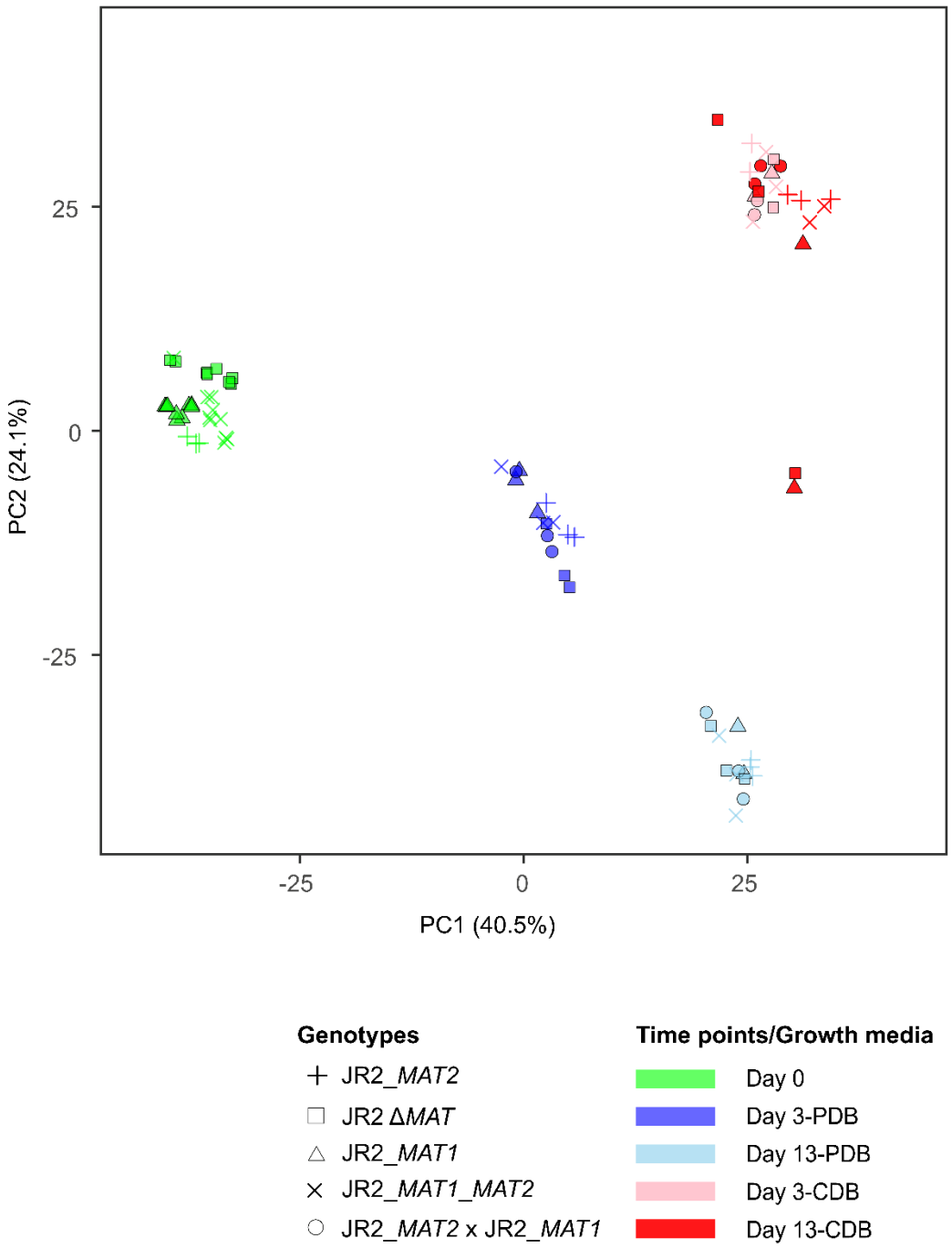


Figure 5. Genotype does not significantly affect gene expression variation among the RNA-sequencing samples. Principal component analysis (PCA) of *V. dahliae* transcriptomes from *MAT* mutants and the wild-type strain JR2_*MAT2*. Transcriptomes were obtained from *V. dahliae* collected at day 0, 3 and 13 after cultivation in potato dextrose broth (PDB) or Czapek–Dox broth (CDB). *V. dahliae* strains comprise wild-type (JR2_*MAT2*), *MAT* mutants (JR2 Δ*MAT*, JR2_*MAT1*, and JR2_*MAT1_MAT2*), and a co-culture of JR2_*MAT2* and JR2_*MAT1*

(JR2_*MAT2* × JR2_*MAT1*). For each *MAT* mutant, three independent transformants were included as biological replicates.

MAT mutants show shared and distinct transcriptional changes

Despite the observation that the genotype accounts for only 1.7% of the gene expression variation among the RNA-sequencing samples, the genotype may still influence the expression of certain genes. We therefore performed pairwise differential expression analyses comparing each *MAT* mutant with the wild-type strain *V. dahliae* JR2_*MAT2* to identify differentially expressed genes (DEGs). Differential expression analysis was conducted using DESeq2 (Love et al. 2014), with the three independent transformants of each *MAT* mutant included as biological replicates. Thus, each DEG reflects significant differential expression between the *MAT* mutant and JR2_*MAT2* after accounting for variation among the three independent transformants. DEGs were defined as genes with an absolute $\log_2$ fold change ($|\log_2 FC|$) ≥ 1 and an adjusted p-value < 0.05. For each *MAT* mutant, DEGs were identified relative to JR2_*MAT2* separately within each condition (day 0, day 3-PDB, day 13-PDB, day 3-CDB, and day 13-CDB).

To assess transcriptional changes associated with loss of *MAT1-2*, we first analyzed DEGs in JR2 Δ*MAT* (JR2 Δ*MAT* vs. JR2_*MAT2*), using three independent transformants of JR2 Δ*MAT* as biological replicates. Across DEGs identified for each condition (PDB or CDB at 0, 3, and 13 days), this yielded a total of 1358 unique DEGs in the JR2 Δ*MAT* vs. JR2_*MAT2* comparison (Figure 6A). We then examined DEG overlap across conditions in the JR2 Δ*MAT* vs. JR2_*MAT2* comparison to identify genes consistently affected by *MAT1-2* loss. None of the DEGs in the JR2 Δ*MAT* vs. JR2_*MAT2* comparison were consistently identified across all conditions (Figure 6A). However, a small proportion of DEGs in the JR2 Δ*MAT* vs. JR2_*MAT2* comparison were detected across more than one condition, accounting for 10% (147/1358) of DEGs in the JR2 Δ*MAT* vs. JR2_*MAT2* comparison (Figure 6A). A similarly high level of transcriptional variation across time points has also been reported in fungal *MAT* deletion mutants compared with the wild-type strain, likely because *MAT* genes regulate genetic networks that vary with fungal developmental stage and growth condition (Böhm et al. 2013). These results indicate that the loss of *MAT1-2* causes a similar transcriptional response across the three independent JR2 Δ*MAT* transformants relative to JR2_*MAT2*.

To assess transcriptional changes associated with different *MAT* genotypes, we examined DEGs in JR2_*MAT1* and JR2_*MAT1_MAT2*, using three independent transformants per genotype as biological replicates, with each genotype compared to JR2_*MAT2* (JR2_*MAT1* vs. JR2_*MAT2* and **J**R2_*MAT1_MAT2* vs. JR2_*MAT2*). Across DEGs identified for each condition (PDB or CDB at 0, 3, and 13 days), this yielded a total of 1279 and 998 unique DEGs in the

JR2_*MAT1* vs. JR2_*MAT2* and JR2_*MAT1*_*MAT2* vs. JR2_*MAT2* comparisons, respectively (Figure 6B, C). None of these DEGs were shared across all conditions in either comparison (Figure 6B, C). Even so, some transcriptional responses associated with different *MAT* genotypes were detected in more than one condition, comprising 11% (130/1,279) and 3% (21/998) of DEGs in the JR2_*MAT1* vs. JR2_*MAT2* and JR2_*MAT1*_*MAT2* vs. JR2_*MAT2* comparisons, respectively (Figure 6B, C). These results show that strains carrying *MAT1-1*, either alone or in combination with *MAT1-2*, differ transcriptionally from JR2_*MAT2*.

To assess potential transcriptional effects of co-culturing opposite mating types, we examined DEGs in co-cultures, each containing JR2_*MAT2* and one of three independent JR2_*MAT1* transformants used as biological replicates, relative to single-strain cultures of JR2_*MAT2* (co-cultures vs. JR2_*MAT2*). DEGs were identified for each condition (PDB or CDB at 0, 3, and 13 days), yielding a total of 410 unique DEGs in the co-cultures vs. JR2_*MAT2* comparison (Figure 6D). None of the DEGs in the co-cultures vs. JR2_*MAT2* comparison were shared across all conditions (Figure 6D). However, despite limited overall overlap, 7% (30/410) of the DEGs in the co-cultures vs. JR2_*MAT2* comparison were detected in more than one condition (Figure 6D). These results indicate that co-cultures differ transcriptionally from single-strain JR2_*MAT2* cultures.

Transcriptomic analyses in other fungi suggest that *MAT* genes from different idiomorphs in the same fungal species can regulate distinct target genes, while also often regulating the same genetic networks, in which the *MAT* genes exhibit antagonistic or overlapping functions (Dyer and Kück 2017; Dyer et al. 2016; Kim et al. 2015; Yu et al. 2018). To explore the potential regulatory roles of *MAT* genes in *V. dahliae*, we assessed DEGs overlap across all comparisons (JR2 Δ*MAT* vs. JR2_*MAT2*, JR2_*MAT1* vs. JR2_*MAT2*, JR2_*MAT1*_*MAT2* vs. JR2_*MAT2*, and co-cultures vs. JR2_*MAT2*) under each condition to determine whether transcriptional changes were shared or distinct. Overall, most DEGs were specific to individual comparisons with JR2_*MAT2*, suggesting genotype-specific and co-culture-specific transcriptional responses (Figure 7). However, some transcriptional responses overlapped, with DEGs shared across all comparisons with JR2_*MAT2* identified at day 0 and day 3, but not at day 13 (Figure 7).

At day 0, co-cultures were not included, and 16 DEGs were shared across all comparisons with JR2_*MAT2* (Figure 7A). Gene ontology (GO) enrichment analysis of the 16 DEGs revealed no significantly enriched GO terms, although the annotated genes were primarily associated with membrane transport, metabolic processes, catalytic activity, and oxidative stress responses, while six genes lacked functional annotation (Supplementary Table 2). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis further showed enrichment in the tyrosine metabolism pathway (3 genes with adjusted $P < 0.01$).

At day 3 in PDB, 33 DEGs were shared among all comparisons with JR2_*MAT2* (Figure 7B). GO enrichment analysis of these 33 DEGs revealed significant enrichment of genes involved in ribosome biogenesis (2 genes with adjusted P = 0.031) (Supplementary Table 3), whereas KEGG analysis did not identify any significantly enriched pathways. Notably, the 16 and 33 DEG sets contained entirely different genes, yet their GO annotations were associated with similar functions (Supplementary Table 2-3). The 33 DEGs were primarily associated with membrane transport, metabolic processes, catalytic activity, and ribosome biogenesis, while 17 genes lacked functional annotation (Supplementary Table 3). These results are consistent with transcriptomic studies in other fungi, which demonstrate that numerous transcriptional changes associated with different *MAT* genotypes are not directly linked to mating-related gene, but instead to genes associated with broader physiological functions, including general metabolic and cellular processes (Dyer and Kück 2017; Dyer et al. 2016; Kim et al. 2015; Yu et al. 2018).

In CDB at day 3, however, only a single DEG (VDAG_JR2_Chr7g02870a) without functional annotation was shared across all comparisons with JR2_*MAT2* (Figure 7D). The shared DEG sets identified across all comparisons with JR2_*MAT2* at day 0 (16 DEGs), day 3 in PDB (33 DEGs), and day 3 in CDB (1 DEG) showed no overlap in gene identity. The presence of genotype-specific DEGs in each condition likely reflects differences in the regulatory targets of *MAT1-1* and *MAT1-2* in *V. dahliae* (Figure 7). In addition, co-culture-specific DEGs under each condition suggest that co-cultivation triggers transcriptional responses associated with the presence of opposite mating types (Figure 7). However, the shared DEGs involved in general metabolic and cellular processes also support the presence of common regulatory networks between *MAT1-1* and *MAT1-2* in *V. dahliae*.

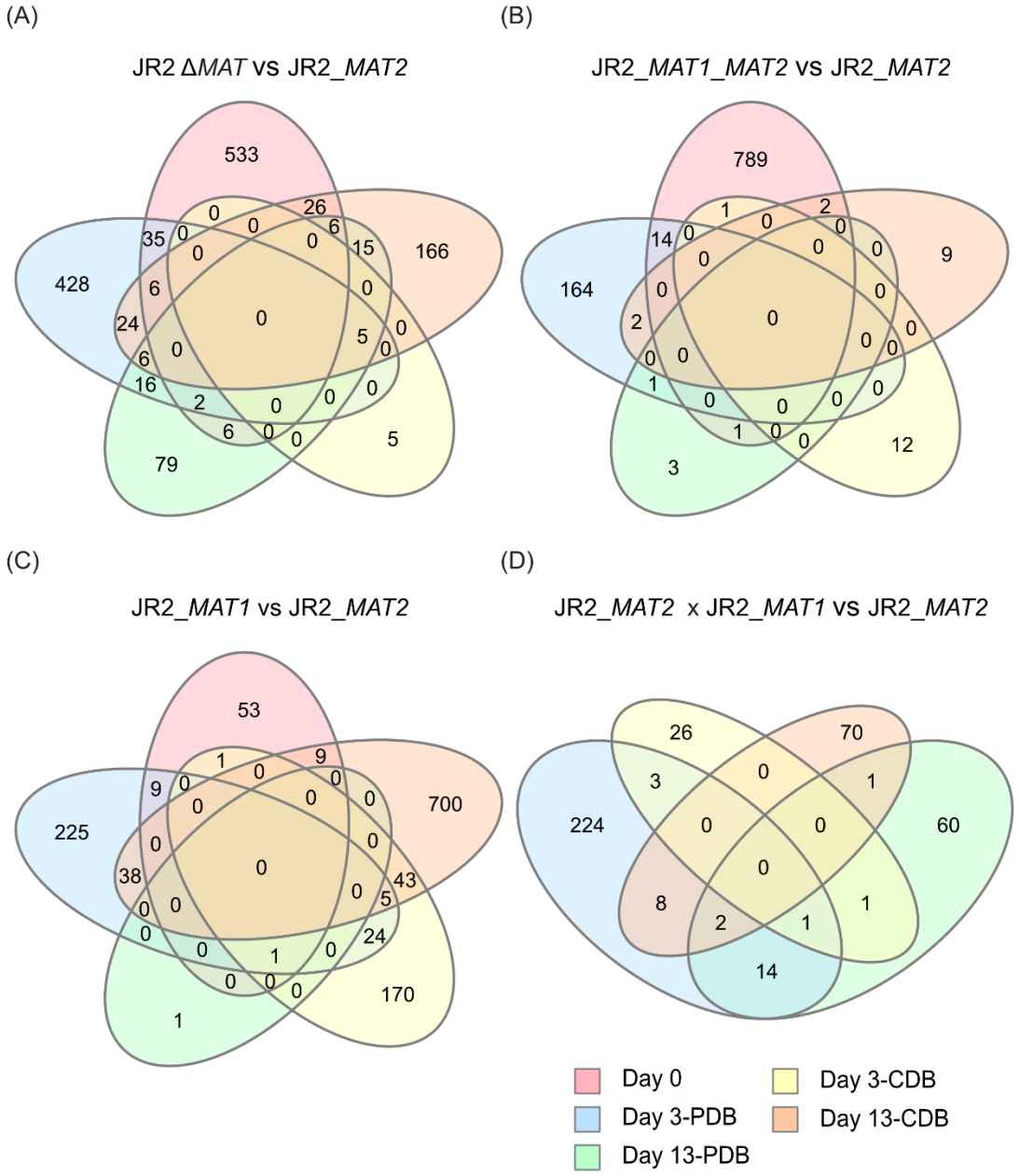


Figure 6. *MAT* mutants and co-cultures exhibit differential gene expression compared to wild-type *Verticillium dahliae*. Venn diagrams show the differentially expressed genes (DEGs) identified in each *V. dahliae* strain and in co-cultures of strains JR2_*MAT2* and JR2_*MAT1*, compared to the wild-type strain JR2_*MAT2*. Transcriptomes were obtained from *V. dahliae* collected at day 0, 3 and 13 after cultivation in potato dextrose broth (PDB) or Czapek–Dox broth (CDB). Differential expression was defined as an absolute $\log_2$ fold change ($|\log_2 FC|$) ≥ 1 and *p.adj* < 0.05. DEGs are shown for *V. dahliae MAT* mutants (A) JR2 Δ*MAT*, (B) JR2_*MAT1_MAT2,* (C) JR2_*MAT1*, and (D) the co-culture of JR2_*MAT2* and JR2_*MAT1* (JR2_*MAT2* × JR2_*MAT1*). For each *MAT* mutant, three independent transformants were used as biological replicates.

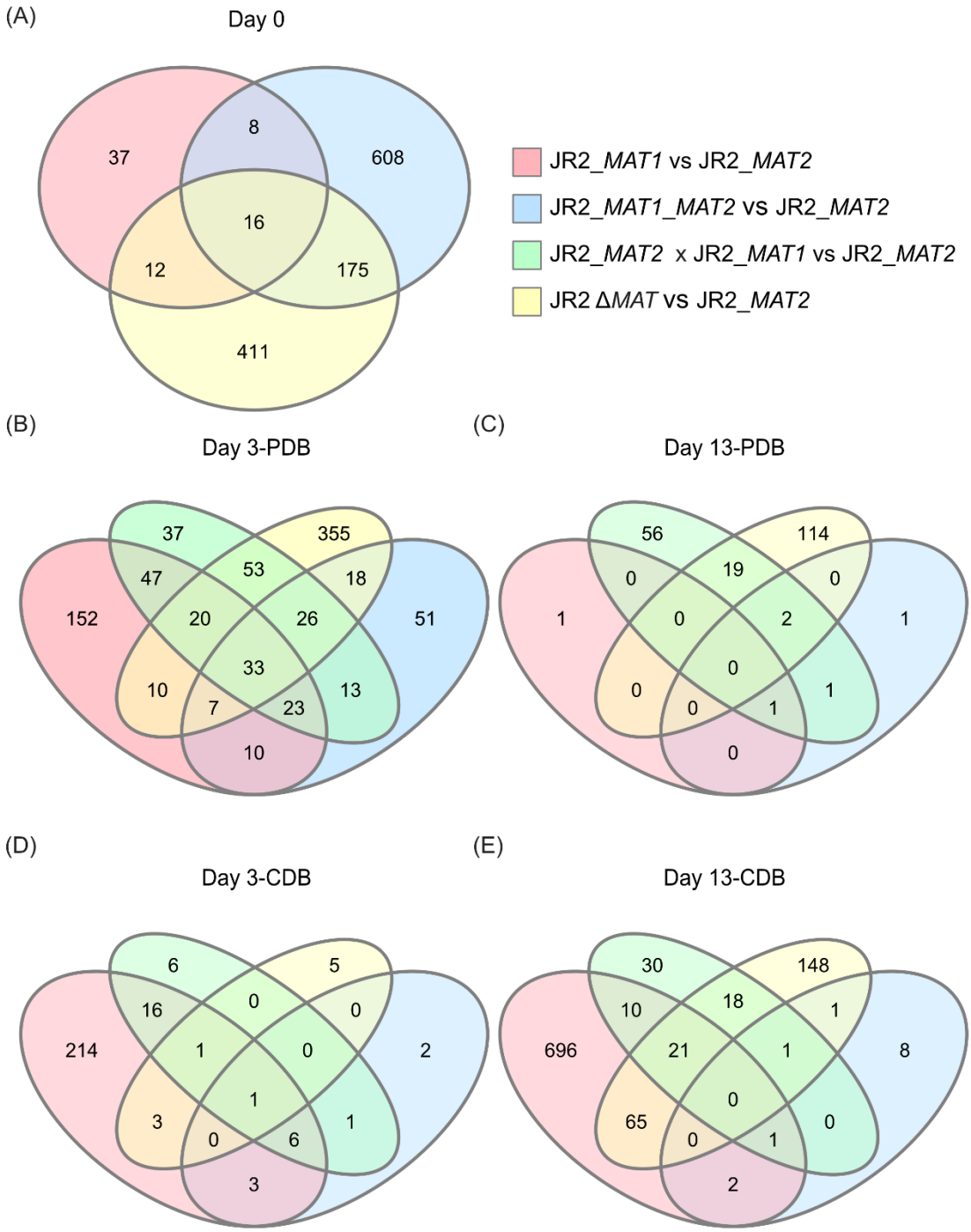


Figure 7. Overlap of differentially expressed genes (DEGs) among *Verticillium dahliae* strains and co-cultures. Venn diagrams show shared and distinct DEGs across *MAT* mutants and in co-cultures of strain JR2_*MAT2* and JR2_*MAT1,* compared to the wild-type strain JR2_*MAT2*. Transcriptomes were obtained from *V. dahliae* collected at day 0, 3 and 13 after cultivation in potato dextrose broth (PDB) or Czapek–Dox broth (CDB). Differential expression was defined as an absolute $\log_2$ fold change ($|\log_2 FC|$) ≥ 1 and *p.adj* < 0.05. *V. dahliae* strains comprise *MAT* mutants (JR2 Δ*MAT*, JR2_*MAT1*, and JR2_*MAT1_MAT2*), and co-cultures of JR2_*MAT2* and JR2_*MAT1* (JR2_*MAT2* × JR2_*MAT1*). DEGs shared across *V. dahliae* strains and co-cultures are shown for (A) day 0, (B) 3 days in PDB, (C) 13 days in PDB, (D) 3 days in CDB, and (E) 13 days in CDB (with co-cultures included only at days 3 and 13). For each *MAT* mutant, three independent transformants were used as biological replicates.

Sex-related genes are transcriptionally active in *V. dahliae* independent of *MAT* genotype

In ascomycetes, *MAT* genes typically encode transcription factors that regulate sexual reproduction (Wallen and Perlin 2018). Using previously reported sex-related genes from *V. dahliae* strain VdLs17 (Short et al. 2014) as references, BLAST search identified 85 sex-related genes in *V. dahliae* strain JR2, in addition to the *MAT* genes. We then investigated whether these sex-related genes were differentially expressed in *MAT* mutants compared to JR2_*MAT2* (JR2 Δ*MAT* vs. JR2_*MAT2*, JR2_*MAT1* vs. JR2_*MAT2*, and JR2_*MAT1*_*MAT2* vs. JR2_*MAT2*), using three independent transformants per mutant as biological replicates. The expression of the sex-related genes was assessed separately in each *MAT* mutant vs. JR2_*MAT2* comparison under each condition (PDB and CDB at 0, 3, and 13 days). Across all comparisons, 4, 6, and 8 sex-related genes were differentially expressed in JR2 Δ*MAT*, JR2_*MAT1*, and JR2_*MAT1*_*MAT2*, respectively, compared to JR2_*MAT2* (Figure 8 and Supplementary Table 4).

Most sex-related DEGs in the JR2 Δ*MAT* vs. JR2_*MAT2* comparison (3 out of 4 DEGs) and the JR2_*MAT1*_*MAT2* vs. JR2_*MAT2* comparison (7 out of 8 DEGs) were detected only at day 0 (Figure 8). These genes were mainly associated with meiotic chromosome organization, including synaptonemal complex assembly, chromosome condensation and segregation, sister chromatid separation, and DNA mismatch repair (Supplementary Table 4). Notably, two sex-related DEGs detected at day 0 were shared between JR2 Δ*MAT* and JR2_*MAT1*_*MAT2*, and both were upregulated relative to JR2_*MAT2* (Supplementary Table 4). These two genes were VDAG_JR2_Chr3g07390a and VDAG_JR2_Chr1g05610a, encoding predicted proteins homologous to MutL involved in meiotic DNA mismatch repair and Ste6 involved in mating pheromone export, respectively (Supplementary Table 4). For the JR2_*MAT1* vs. JR2_*MAT2* comparison, sex-related DEGs were detected only at later time points (2 in PDB at day 3, 1 in CDB at day 3, and 3 in CDB at day 13) (Figure 8). These genes were mainly associated with meiotic recombination and chromosome dynamics, including crossing over, chromosome cohesion, and cell-cycle regulation, as well as one gene involved in serine/threonine protein kinase signaling, which is linked to kinase-mediated signaling during sexual development (Supplementary Table 4).

We further examined whether co-cultures induced the expression of sex-related genes relative to single-strain cultures of JR2_*MAT2* (co-cultures vs. JR2_*MAT2*), as expected in heterothallic ascomycetes. Each co-culture contained JR2_*MAT2* and one of three independent JR2_*MAT1* transformants used as biological replicates. One sex-related gene, VDAG_JR2_Chr3g11340a, encoding a predicted protein involved in the resolution of meiotic recombination intermediates, was upregulated in the co-culture vs. JR2_*MAT2* comparison but detected only in PDB at day

3 (Figure 8; Supplementary Table 4). These results indicate that a small subset of sex-related genes is differentially expressed in both *MAT* mutants and co-cultures when compared to JR2_*MAT2*.

To determine whether the limited detection of sex-related DEGs reflects a lack of transcriptional activity of the sex-related genes under the tested conditions in *V. dahliae*, we assessed the expression of all 85 identified sex-related genes. All 85 sex-related genes were transcriptionally active across all *MAT* mutants, including JR2 Δ*MAT* (Figure 9). Hierarchical clustering further indicated that the expression of the 85 sex-related genes clustered independently of *MAT* genotype, but instead according to conditions (PDB and CDB at 0, 3, and 13 days) (Supplementary Figure 3). These results indicate that, although sex-related genes are broadly transcriptionally active in *V. dahliae*, their overall expression is largely independent of *MAT* genotype.

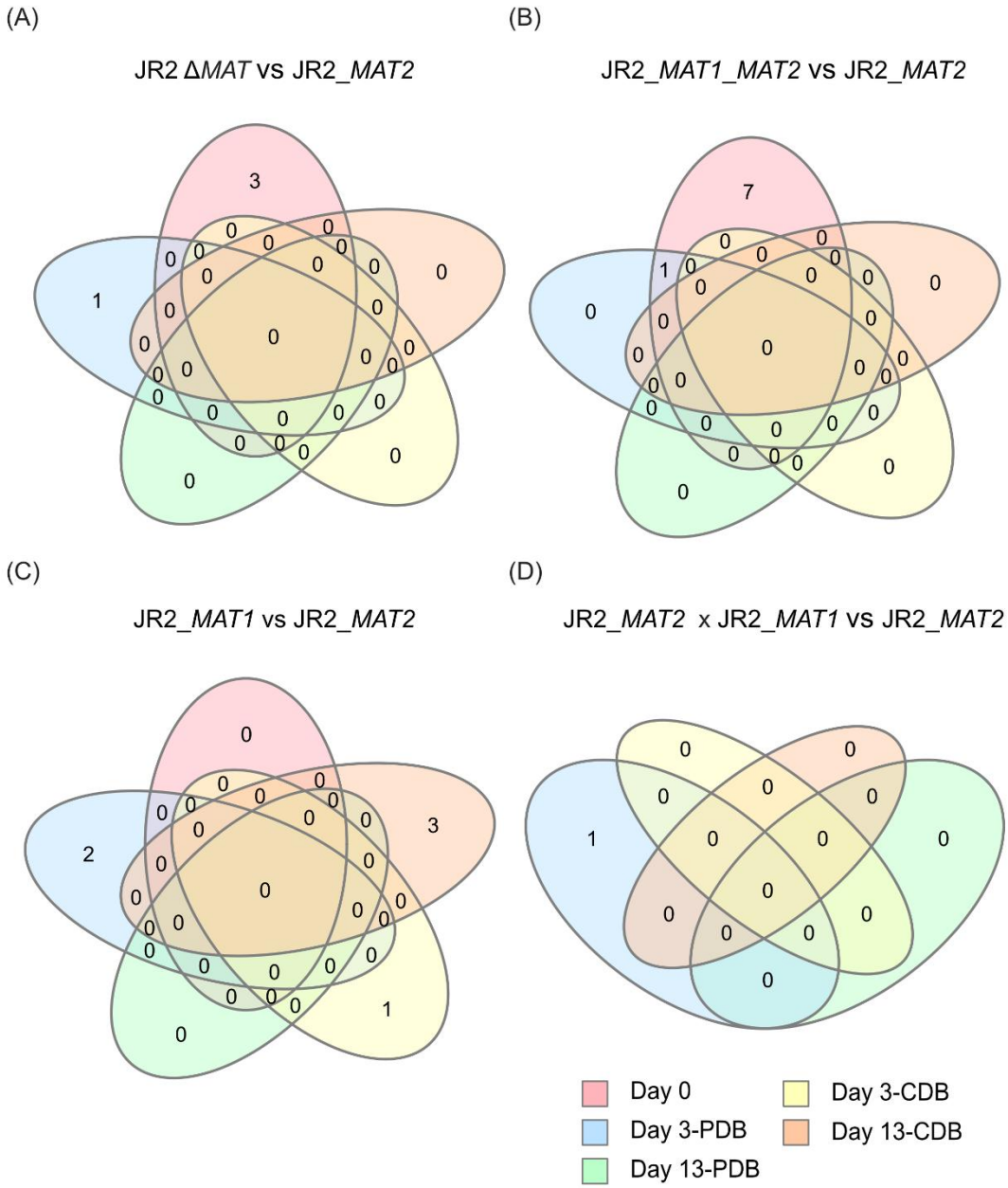


Figure 8. Few sex-related genes are differentially expressed in *Verticillium dahliae* strains and co-cultures. Venn diagrams show sex-related differentially expressed genes (DEGs) within each *Verticillium dahliae* strain and in co-cultures of strain JR2_*MAT2* and JR2_*MAT1*, compared to the wild-type strain JR2_*MAT2*. Transcriptomes were obtained from *V. dahliae* collected at day 0, 3 and 13 after cultivation in potato dextrose broth (PDB) or Czapek–Dox broth (CDB). Differential expression was defined as an absolute $\log_2$ fold change ($|\log_2 FC|$) ≥ 1 and *p.adj* < 0.05. Sex-related DEGs are shown for *V. dahliae MAT* mutants (A) JR2 Δ*MAT*, (B) JR2_*MAT1_MAT2,* (C) JR2_*MAT1*, and (D) the co-culture of JR2_*MAT2* and JR2_*MAT1* (JR2_*MAT2* × JR2_*MAT1*). For each *MAT* mutant, three independent transformants were included as biological replicates.

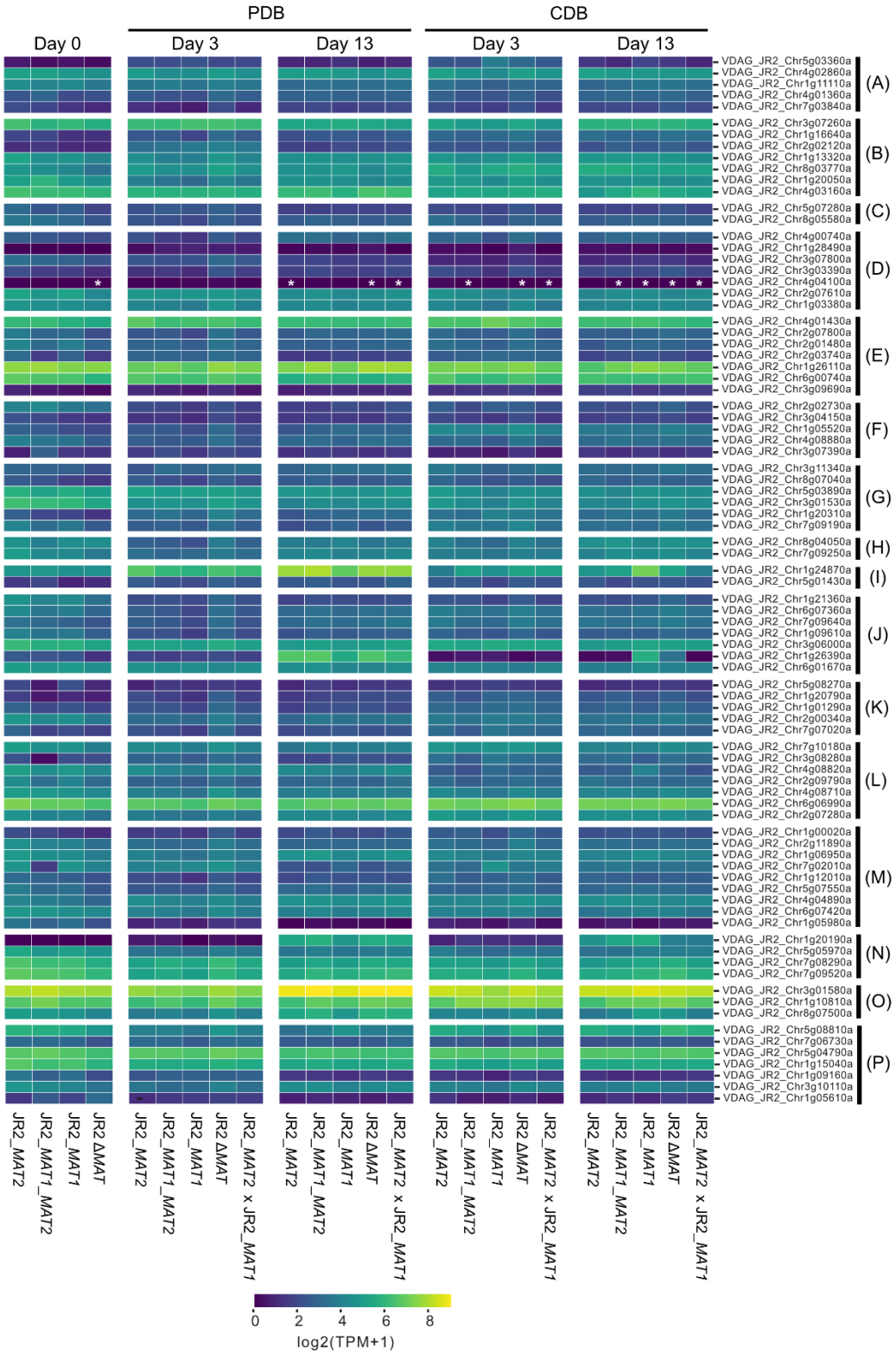


Figure 9. Sex-related genes are transcriptionally active across *Verticillium dahliae* strains. Heatmap shows the expression of sex-related genes in *V. dahliae*. Columns marked with an asterisk (*) indicate no detectable expression (transcripts per million = 0). Transcriptomes were obtained from *V. dahliae* collected at day 0, 3 and 13 after cultivation in potato dextrose broth (PDB) or Czapek–Dox broth (CDB). *V. dahliae* strains comprise wild-type (JR2_*MAT2*), *MAT* mutants (JR2 Δ*MAT*, JR2_*MAT1*, and JR2_*MAT1*_*MAT2*), and a co-culture of JR2_*MAT2* and JR2_*MAT1* (JR2_*MAT2* × JR2_*MAT1*). For each *MAT* mutant, three independent transformants were included as biological replicates. Genes are ordered according to their functional annotation described in (Short et al. 2014).

Functional categories are as follows: (A) Meiosis double-strand DNA break formation & processing; (B) Meiosis single-strand invasion; (C) Meiosis DNA damage checkpoint; (D) Genes involved in crossing over; (E) Synaptonemal complex; (F) Mismatch repair; (G) Resolution of recombination intermediates; (H) Non-homologous end joining; (I) Meiotic recombination and repeat-induced point mutation genes; (J) Chromosome cohesion; (K) Genes encoding separase and condensin complexes; (L) Chromosome segregation; (M) Anaphase-promoting complex; (N) Meiosis-related transcription & chromatin regulation; (O) Signal transduction; and (P) Pheromone & mating-related genes.

Expression of genome maintenance-related genes clustered independently of *V. dahliae MAT* genotype

The genome maintenance pathway comprises genes that mediate DNA repair, regulate chromatin organization, ensure accurate DNA replication and chromosome segregation, and coordinate cell-cycle checkpoints (Bansbach and Cortez 2011). Disruption of these processes can destabilize the genome and promote chromosomal rearrangements (Chen and Kolodner 1999; Kim et al. 2012b; Burssed et al. 2022). Given the extensive chromosomal rearrangements observed in *V. dahliae*, and evidence that sex-related genes in fungi can be repurposed to mediate genetic recombination outside sexual reproduction (Forche et al. 2008), we asked whether *MAT* genes in *V. dahliae* may contribute to the formation of chromosomal rearrangements. We hypothesized that, if *MAT* genes influence chromosomal rearrangements, their effects may be mediated through altered expression of genome maintenance-related genes.

To explore this hypothesis, we performed gene ontology (GO) annotation of all DEGs identified in each comparison (JR2 Δ*MAT* vs. JR2_*MAT2*, JR2_*MAT1* vs. JR2_*MAT2*, JR2_*MAT1*_*MAT2* vs. JR2_*MAT2,* and co-culture vs. JR2_*MAT2*), using three independent transformants per mutant as biological replicates. We then selected DEGs related to genome maintenance for further analysis, identifying them separately for each comparison to JR2_*MAT***2** under each condition (PDB or CDB at 0, 3, and 13 days). In total, we detected 28 genome maintenance–related DEGs in JR2 Δ*MAT*, 21 in JR2_*MAT1*, 22 in JR2_*MAT1*_*MAT2,* and 3 in the co-culture, respectively, compared to JR2_*MAT2* (Figure 10). Both up- and downregulated genome maintenance–related genes were identified in each *comparison* (Supplementary Figure 4-6). Most genome maintenance-related DEGs in the JR2 Δ*MAT* vs. JR2_*MAT2* comparison (16 out of 28 DEGs) and the JR2_*MAT1*_*MAT2* vs. JR2_*MAT2* comparison (16 out of 22 DEGs) were detected only at day 0 (Figure 10). In contrast, genome maintenance-related DEGs in the JR2_*MAT1* vs. JR2_*MAT2* comparison were distributed across different conditions, and all 3 genome maintenance-related DEGs in the co-culture vs. JR2_*MAT2* comparison were detected in PDB at day 3 (Figure 10. Despite the identification of genome maintenance–related DEGs in each comparison to JR2_*MAT2*, hierarchical clustering indicated that the expression of these genome maintenance–related genes was primarily clustered according to conditions,

independently of *MAT* genotype (Figure 11). These results indicate that the overall expression of genome maintenance–related genes is independent of *MAT* genotype.

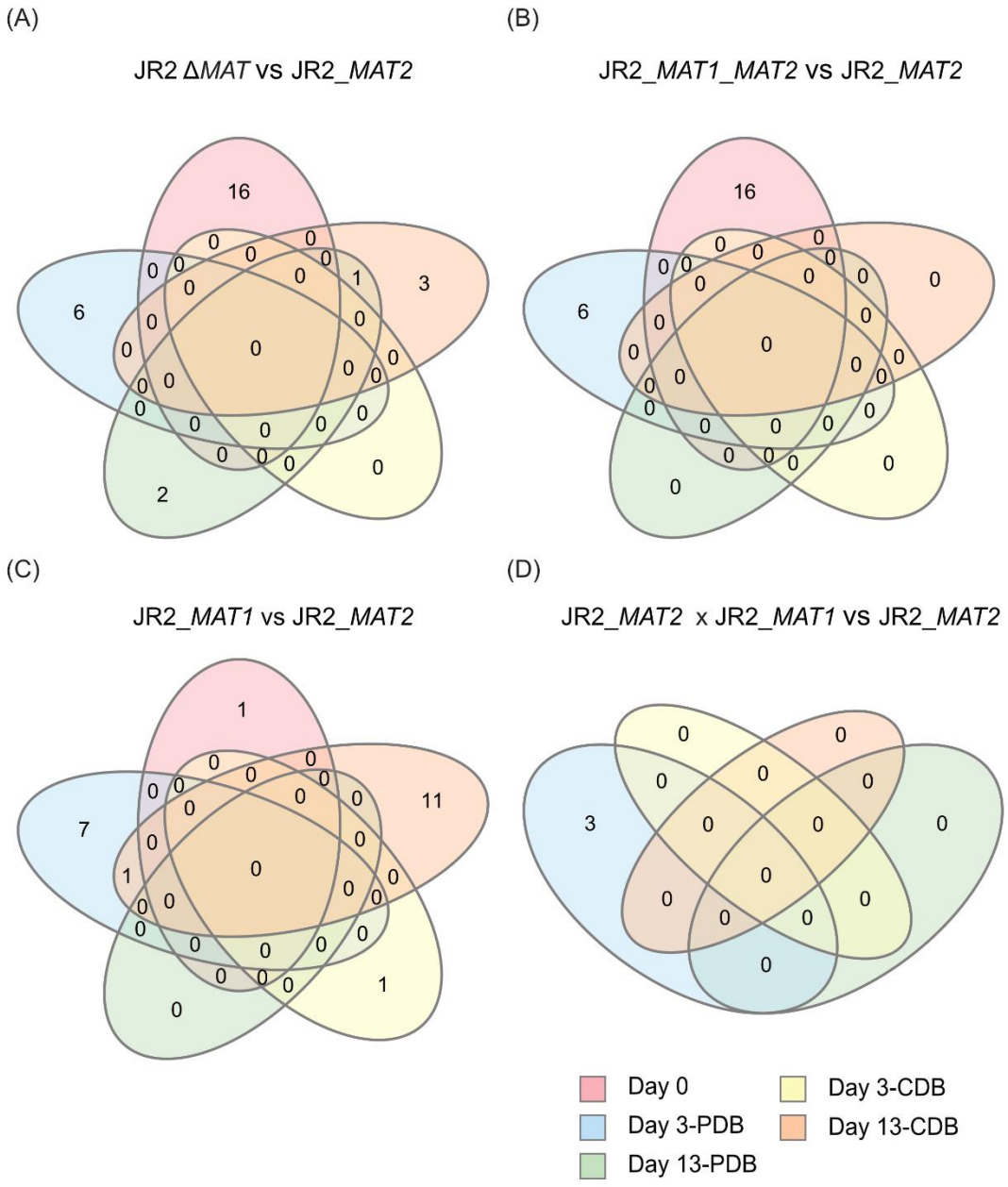


Figure 10. Few genome maintenance-related genes are differentially expressed in *Verticillium dahliae* strains and co-cultures. Venn diagrams show genome maintenance-related differentially expressed genes (DEGs) within each *Verticillium dahliae* strain and in co-cultures of strain JR2_*MAT2* and JR2_*MAT1*, compared to wild-type JR2_*MAT2.* Transcriptomes were obtained from *V. dahliae* collected at day 0, 3 and 13 after cultivation in potato dextrose broth (PDB) or Czapek–Dox broth (CDB). Differential expression was defined as an absolute $\log_2$ fold change ($|\log_2 FC| \geq 1$ and *p.adj* < 0.05. Genome maintenance-related DEGs are shown for *V. dahliae MAT* mutants (A) JR2 Δ*MAT*, (B) JR2_*MAT1_MAT2,* (C) JR2_*MAT1*, and (D) the co-culture of JR2_*MAT2* and JR2_*MAT1* (JR2_*MAT2* × JR2_*MAT1*). For each *MAT* mutant, three independent transformants were included as biological replicates.

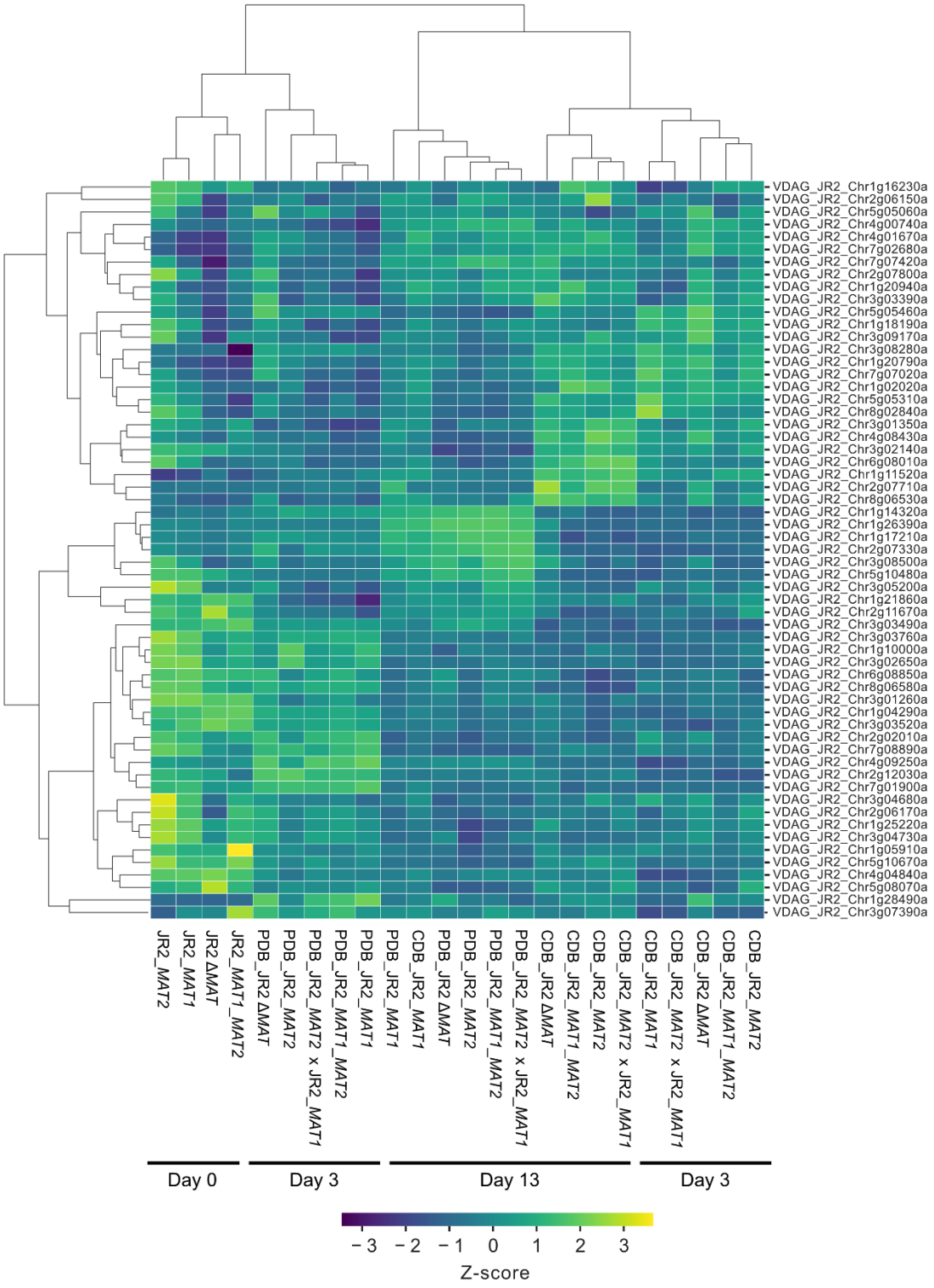


Figure 11. Hierarchical clustering of genome maintenance–related differentially expressed genes (DEGs) shows clustering independent of *MAT* genotypes. Genes annotated as genome maintenance–related and identified as differentially expressed in at least one comparison with the wild-type *Verticillium dahliae* strain JR2_*MAT2* were selected. Heatmap shows the expression these genes in *V. dahliae* strains and in co-cultures of strain JR2_*MAT2* and JR2_*MAT1*. Columns are arranged based on similarity in gene expression patterns. Transcriptomes were obtained from *V. dahliae* collected at day 0, 3 and 13 after cultivation in potato dextrose broth (PDB) or Czapek–Dox broth (CDB). *V. dahliae* strains comprise wild-type (JR2_*MAT2*), *MAT* mutants (JR2 Δ*MAT*,

JR2_*MAT1*, and JR2_*MAT1*_*MAT2*), and a co-culture of JR2_*MAT2* and JR2_*MAT1* (JR2_*MAT2* × JR2_*MAT1*). For each *MAT* mutant, three independent transformants were included as biological replicates.

DISCUSSION

The persistence of sex-related genes in fungi that lack an observable sexual cycle raises the intriguing questions why these genes are maintained and what functions they serve (Wallen and Perlin 2018). *Verticillium dahliae* is presumed to be asexual, yet its genome retains mating-type (*MAT*) genes, which typically function as master regulators of sexual reproduction in fungi (Zhang et al. 2024). Here, we used transcriptomic analysis to reveal that *MAT* genes, along with 85 sex-related genes in *V. dahliae,* are transcriptionally active *in vitro*. Hierarchical clustering showed that the expression of sex-related genes is independent of *MAT* genotype across *V. dahliae MAT* deletion strains and strains carrying either *MAT* idiomorph (*MAT1-1* or *MAT1-2*) or both idiomorphs. Moreover, genes differentially expressed across all strains were not enriched for the 85 sex-related genes, but instead were primarily associated with general metabolic and cellular processes. Thus, *MAT* genes are expressed in *V. dahliae* and are associated with transcriptional changes in general metabolic and cellular processes, while sex-related genes are transcriptionally active but their overall expression is not affected by *MAT* genotype under the tested conditions. These findings may help explain the paradox of the retention of *MAT* genes in *V. dahliae*, because *MAT* genes appear to influence transcriptional processes beyond sexual reproduction.

In fungi, bipolar heterothallism is characterized by a single *MAT* locus with two alternative idiomorphs, and mating occurs exclusively between strains of opposite mating types (Dyer et al. 2016). *V. dahliae* possesses a genomic organization resembling bipolar heterothallism, with individual strains carrying either the *MAT1-1* or *MAT1-2* idiomorph (Klosterman et al. 2011). Previous population studies based on polymerase chain reaction (PCR) assays reported that *MAT1-2* accounts for 99% of 1,120 *V. dahliae* isolates sampled globally (Short et al. 2014). Consistent with this skewed mating-type distribution, our analysis of 52 previously assembled *V. dahliae* genomes from strains collected from diverse hosts and geographic regions, including Europe, China, Canada, and the United States (de Jonge et al. 2012; Faino et al. 2015; Fan et al. 2018; Gibriel et al. 2019) showed that *MAT1-2* predominates, with only 7 of 52 strains carrying *MAT1-1*. The distribution of *MAT* idiomorphs in *V. dahliae* suggests that, if sexual reproduction exists in *V. dahliae*, it is likely infrequent in natural populations. Accordingly, occurrences of sexual reproduction in *V. dahliae* cannot be ruled out, as strongly skewed mating-type distributions have also been documented in sexual fungi with infrequent reproduction (Nieuwenhuis and James 2016; Yan et al. 2002). Additional evidence for the functional importance of *MAT* genes in *V. dahliae* comes from analyses of nonsynonymous and synonymous nucleotide substitution rates, which indicate that *V. dahliae MAT* genes are under strong purifying selection (Short et al. 2014). Purifying selection is generally interpreted as evidence that genes remain functional, rather than undergoing neutral decay leading to

pseudogenization (Balakirev and Ayala 2003; Yang and Nielsen 2000). The strength of purifying selection on *V. dahliae MAT* genes (approximately 34–35%) is comparable to that observed for *MAT* genes in sexually reproducing fungi, further supporting the idea that *MAT* genes have functional roles in *V. dahliae* (Short et al. 2014).

It remains unclear whether any fungal species is truly asexual, as it is increasingly recognized that sexual reproduction in supposedly asexual fungi may be triggered only under specific conditions (Seidl and Thomma 2014; Nieuwenhuis and James 2016; Dyer and Kück 2017). This is demonstrated for the human fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*, which complete their sexual cycle on plant surfaces, where this process is promoted by plant-derived molecules such as myo-inositol and indole-3-acetic acid (IAA) (Xue et al. 2007). Moreover, abiotic stresses such as nutrient starvation and oxidative stress can also trigger mating in fungi (Wallen and Perlin 2018). In our study, both the *in vitro* growth assay using *V. dahliae* strain carrying both mating types (resembling a homothallic system), and co-culture experiments between *V. dahliae* strains of opposite mating types (resembling a heterothallic system) were performed in potato dextrose broth and Czapek–Dox broth. Future studies incorporating a broader range of growth conditions, including plant-derived molecules and stress treatments, could help determine whether rare sexual reproduction occurs in *V. dahliae* under specific conditions. Furthermore, future studies incorporating detailed microscopic analyses of sexual structures, such as ascospores, which are the sexual spores of ascomycetes, will help determine whether *V. dahliae* undergoes sexual development in the tested conditions.

One key finding of this study is that 85 sex-related genes, including genes typically involved in pheromone-signaling and meiotic processes in sexual fungi, are transcriptionally active in *V. dahliae*, which raises questions about their functional roles in *V. dahliae*. The expression of sex-related genes is often taken as indirect evidence for the potential occurrence of sexual reproduction in fungi (Dyer and Kück 2017; Dyer et al. 2016). Notably, active expression of sex-related genes has also been reported in other fungi traditionally regarded as asexual. For example, the arbuscular mycorrhizal fungus *Glomus intraradices*, previously considered an ancient asexual species, was later found to express core meiosis-specific genes, including those encoding homologous pairing protein 2 (HOP2) and meiotic nuclear division protein 1 (MND1) (Rosendahl 2012; Tisserant et al. 2012). Such observations have led to the hypothesis that arbuscular mycorrhizal fungi are not strictly ancient asexual organisms (Taylor et al. 2015; Milgroom et al. 2014; Oliveira et al. 2024; Rosendahl 2012; Nieuwenhuis and James 2016). Thus, the transcriptional activity of 85 sex-related genes in *V. dahliae* suggests the possibility of an unobserved, rare sexual stage. However, the association between the expression of sex-related genes and sexual reproduction should be interpreted with caution, because sex-related

genes may serve functions beyond sexual reproduction (Dyer and Kück 2017). For example, the sporulation protein SPO11, classically known to initiate meiotic double-strand breaks (Keeney et al. 1997), has also been shown to mediate non-meiotic genetic recombination during the parasexual cycle of the human fungal pathogen *Candida albicans* (Forche et al. 2008). Moreover, in the asexual fungus *Fusarium oxysporum*, the seven-pass transmembrane pheromone receptor STE2 detects plant exudates and facilitates fungal growth toward suitable hosts, indicating functions beyond sexual reproduction (Turrà et al. 2015). These findings underscore the need for further functional characterization of the 85 sex-related genes in *V. dahliae.*

The expression of *MAT* genes in *V. dahliae* suggests that they play functional roles. By comparing *V. dahliae* strains differing in their *MAT* idiomorphs, including *MAT* deletion strains, we identified genes that were differentially expressed across all strains. However, these genes were not enriched for the 85 sex-related genes but were primarily associated with general metabolic and cellular processes. Our findings indicate that *V. dahliae MAT* genes are associated with transcriptional changes in general metabolic and cellular processes, independent of sexual processes under the tested *in vitro* conditions. This finding is in line with accumulating evidence from transcriptomic analyses in different fungi and chromatin immunoprecipitation sequencing (ChIP-seq) studies in the ascomycete *Penicillium chrysogenum*, indicating that the functions of *MAT* genes extend beyond sexual reproduction, even in sexual fungi (Dyer and Kück 2017; Dyer et al. 2016; Kim et al. 2015; Yu et al. 2018; Becker et al. 2015). A large number of *MAT*-regulated genes are not directly involved in mating or sexual reproduction, but are instead associated with general metabolism, cellular processes, and developmental processes (Becker et al. 2015; Dyer and Kück 2017). Moreover, in *F. oxysporum*, *MAT* genes regulate cell–cell communication independently of sexual reproduction by controlling an autocrine pheromone-signaling network involved in population-density sensing (APS), enabling cells to sense and respond to population density (Vitale et al. 2026). These findings indicate that *MAT* genes have functional roles beyond conventional sexual processes in fungi.

In conclusion, our study demonstrates the transcriptional activity of 85 sex-related genes along with *MAT* genes in *V. dahliae*. We proposed that *MAT* genes in *V. dahliae* have functional roles and are associated with transcriptional changes in metabolic and cellular processes independent of sexual processes. By revealing widespread expression of 85 sex-related genes, this study raises new questions about how these genes may function in *V. dahliae*.

MATERIALS AND METHODS

Phylogenetic tree construction and mating-type idiomorph identification

Phylogenetic analysis was conducted using RealPhy (version 1.12; Bertels et al. 2014) to generate the sequence alignment, followed by maximum-likelihood inference in RAxML-NG (version 1.2.2; Kozlov et al. 2019) under the GTR substitution model. The resulting phylogeny was visualized with iTOL (version 7; Letunic and Bork 2024). The presence of *MAT1-1* and *MAT1-2* idiomorphs was assessed in 52 *V. dahliae* whole-genome sequences (de Jonge et al. 2012; Faino et al. 2015; Fan et al. 2018; Gibriel et al. 2019). *MAT1-1* and *MAT1-2* idiomorphs were identified by sequence similarity searches using tblastn (version 2.5.0+; default parameters), with the *V. dahliae MAT1-2* and the *V. albo-atrum MAT1-1* idiomorphs (Klosterman et al. 2011) as query sequences. The *MAT1-1* idiomorph corresponds to *MAT1-1-1* (VDBG_01985.1) and *MAT1-1-3* (VDBG_01986.1), whereas the *MAT1-2* idiomorph corresponds to *MAT1-2-1* (VDAG_02444.1) and *MAT1-2-2* (VDAG_02443.1) (Klosterman et al. 2011).

Generation of MAT-engineered *V. dahliae* strains

JR2 Δ*MAT*, JR2_*MAT1*, and the JR2_*MAT1_MAT2 V. dahliae* strains were constructed by CRISPR-Cas9-mediated genome editing in the *V. dahliae* strain JR2 (Faino et al. 2015). Genome editing followed Leisen et al. (2020) with modifications. Single-guide RNAs (sgRNAs) targeting the mating-type locus were designed (Supplementary Table 1). Templates for single-guide RNA (sgRNA) synthesis were generated by annealing an oligonucleotide encoding the trans-activating CRISPR RNA (tracrRNA) with each protospacer-specific oligonucleotide (Supplementary Table 1). Annealed products were converted into double-stranded transcription templates through overhang extension using T4 DNA polymerase (New England Biolabs, Ipswich, USA) and subsequently purified with the Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, USA). sgRNAs were produced by *in vitro* transcription using the HiScribe™ T7 High Yield RNA Synthesis Kit (New England Biolabs, Ipswich, USA) and purified with the RNA Clean & Concentrator 25 (Zymo Research, Irvine, CA, USA). Ribonucleoprotein (RNP) complexes were assembled by combining EnGen® Spy Cas9 HF1 (New England Biolabs, Ipswich, USA) with the purified sgRNAs. Donor DNA fragments for homology-directed repair were PCR-amplified using primers listed in Supplementary Table 1. Donor DNA for JR2 Δ*MAT* was generated by PCR amplification of the 1 kb upstream and 1 kb downstream regions flanking the *MAT2* locus from strain JR2 (Supplementary Table 1). Donor DNA for JR2_*MAT1* and the JR2_*MAT1_MAT2 V. dahliae* was generated by PCR amplification of the *MAT1-1* locus from strain DvD-S29 (de Jonge et al. 2012) and subsequent fusion to JR2 genomic sequences adjacent to the native *MAT2* locus (Supplementary Table 1).

Protoplast transformation was conducted as described by Rehman et al. (2016) with minor modifications. Protoplasts for generating JR2 Δ*MAT* and JR2_*MAT1_MAT2 V. dahliae* were prepared from *V. dahliae* strain JR2. Protoplasts for generating JR2_*MAT1 V. dahliae* were prepared from the JR2 Δ*MAT V. dahliae* generated in *V. dahliae* strain JR2. *V. dahliae* conidiospores were harvested from potato dextrose agar (PDA; Carl Roth, Karlsruhe, Germany) and washed three times with sterile Milli-Q water. Subsequently, 3×10^7 conidiospores were cultured for 20 h at 28°C in 100 mL Complete Medium (CM; yeast extract 6 g, casein acid hydrolysate 6 g and 10 g sucrose in 1L H_20) while shaking at 150 rpm. Mycelium was washed with 0.7 M NaCl and incubated in 10 mL of 2% Driselase solution (Sigma-Aldrich, St. Louis, MO, USA) for 2.5 h at 33°C while shaking at 60 rpm. Released protoplasts were filtered through a 40 µm cell strainer (Corning Inc., Corning, NY, USA), washed with STC buffer, and resuspended in STC buffer (20% sucrose, 10 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$) to a final concentration of 10^6 to 5×10^6 protoplasts/mL. For each transformation, protoplasts were co-incubated with assembled Cas9 ribonucleoprotein (RNP) complexes (20-40 pmol), donor DNA (20 pmol), and pTEL-Hyg (0.1 pmol). Protoplasts were incubated on ice for 30 min prior to treatment with PEG-STC (60 g PEG 4000, 200 g sucrose, 10 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$ per L water). Following treatment, the protoplasts were recovered in TB3 broth (30 g yeast extract, 30 g casein acid hydrolysate, 200 g sucrose per L water) for 18 h at room temperature. The regenerated cells were plated on PDA plates supplemented with hygromycin B (50 µg/mL) and incubated at room temperature until single colonies developed. Transformants were validated by PCR and sequencing using the primers listed in Supplementary Table 1. Marker-free mutants were identified by the loss of hygromycin resistance, as determined by comparative growth of the mutants on PDA plates in the presence and absence of hygromycin B (50 µg/mL).

In vitro growth assays

In vitro growth assays were performed with the wild-type strain JR2 (Faino et al. 2015), JR2 Δ*MAT*, JR2_*MAT1*, and the JR2_*MAT1_MAT2 V. dahliae*. *V. dahliae* conidiospores were harvested from PDA plates after 5 days of growth. Conidiospores were washed three times with sterile Milli-Q water and resuspended in sterile Milli-Q water. Suspensions were adjusted to 10^5 conidiospores/mL. For assessment of colony morphology and diameter, 10 µL drops containing 10^3 conidiospores were deposited in the middle of 90 mm Petri dishes with 25 mL PDA and incubated at 22°C in darkness. After 10 days of incubation, colony diameters were measured and colony morphology was documented using a flatbed scanner (Epson Perfection V700, Long Beach, USA). For conidiospore production, five 4 mm agar plugs were collected from the edge of the 13-day-old colonies using a hole puncher (KAI Medical, Solingen, Germany), and suspended in 800 µL sterile water. Suspensions were vortexed for 2 min and

incubated on a rotator (Intelli-Mixer RM-2M; ELMI, Riga, Latvia) at 30 rpm for 1.5 h to release conidiospores, after which 50 µL of the conidiospores suspensions was used for conidiospore quantification using a haemocytometer. To assess interactions between wild-type strain JR2 and JR2_*MAT1*, conidiospore suspensions of each strain were adjusted to 10^5 conidiospores/mL. Equal volumes of conidiospore suspensions from both genotype were mixed, and 10 µL aliquots were deposited in the middle of 90 mm Petri dishes with 25 mL PDA. Plates were incubated at 22°C in darkness for 10 days, and colony morphology was documented using the flatbed scanner. For co-culture of the wild-type strain JR2 and the JR2_*MAT1* genotype in close proximity on PDA plates, 10 µL conidiospore suspensions from each genotype were deposited in the middle of 90 mm Petri dishes (containing 25 mL PDA), with the suspensions positioned 4 cm apart. Plates were incubated at 22°C in darkness for 10 and 13 days. Colony morphology was documented using the flatbed scanner.

RNA extraction, sequencing

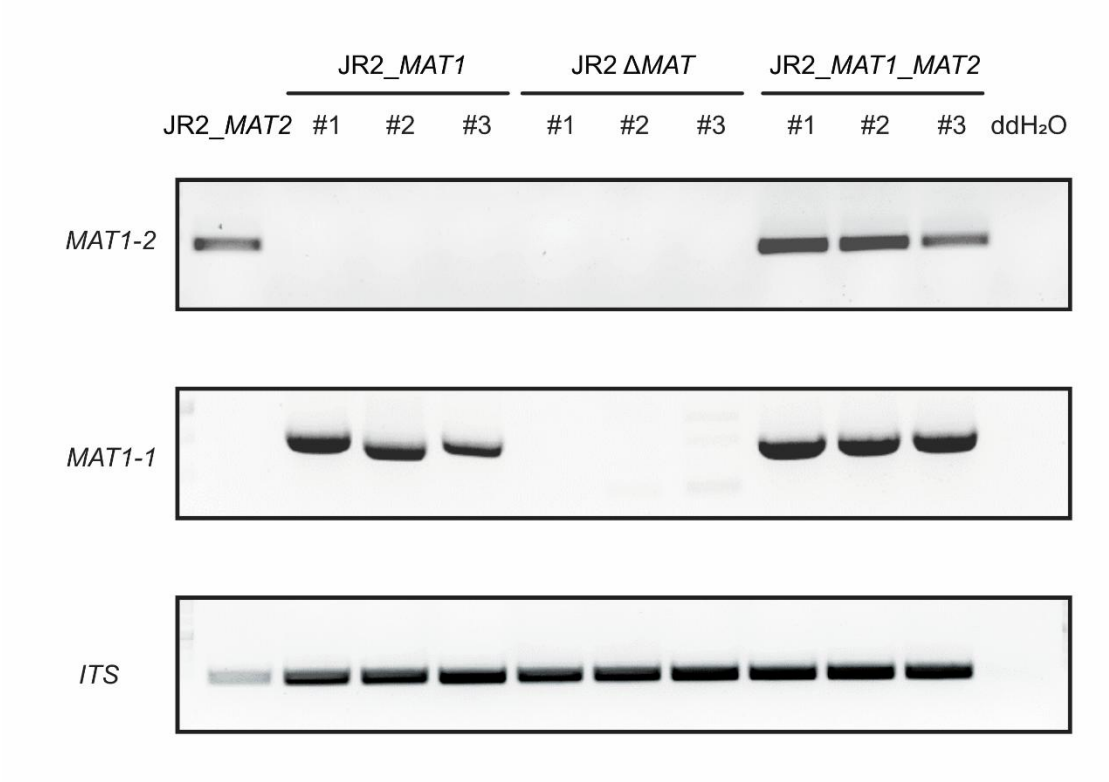
The conidiospores of *V. dahliae* wild-type strain JR2 (Faino et al. 2015), JR2 Δ*MAT*, JR2_*MAT1*, and the JR2_*MAT1_MAT2 V. dahliae strains* were harvested from PDA plates after 5 days of growth. Conidiospores were washed three times with sterile Milli-Q water and resuspended in sterile Milli-Q water. All *V. dahliae* strains were cultured in Potato Dextrose Broth (PDB; VWR, Radnor, PA, USA) and Czapek–Dox broth (CDB; Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 10^5 conidiospores/mL and incubated at 22°C and 130 rpm for 3 and 13 days. For each *MAT*-engineered *V. dahliae* genotype, three independently generated transformants were culture per growth condition. For day 0, conidiospores were collected by centrifugation at 10,000 rpm for 5 minutes. For later time points, fungal biomass was harvested on sterilized miracloth (Merck, Darmstadt, Germany). All samples were snap-frozen in liquid nitrogen and homogenized with 2.4 mm metal beads (Omni International, Kennesaw, GA, USA) using a Tissue-Lyser (Retsch, Haan, Germany). RNA was extracted using the RSC Plant RNA Kit (Promega, Madison, WI, USA) on the Maxwell RSC device (Promega, Madison, WI, USA). RNA integrity assessment and library preparation and was performed by BGI (BGI, Hong Kong, China) and sequencing was conducted on the DNBSEQ™ platform (MGI, Shenzhen, China) with paired-end 150 bp reads.

Transcriptome analysis

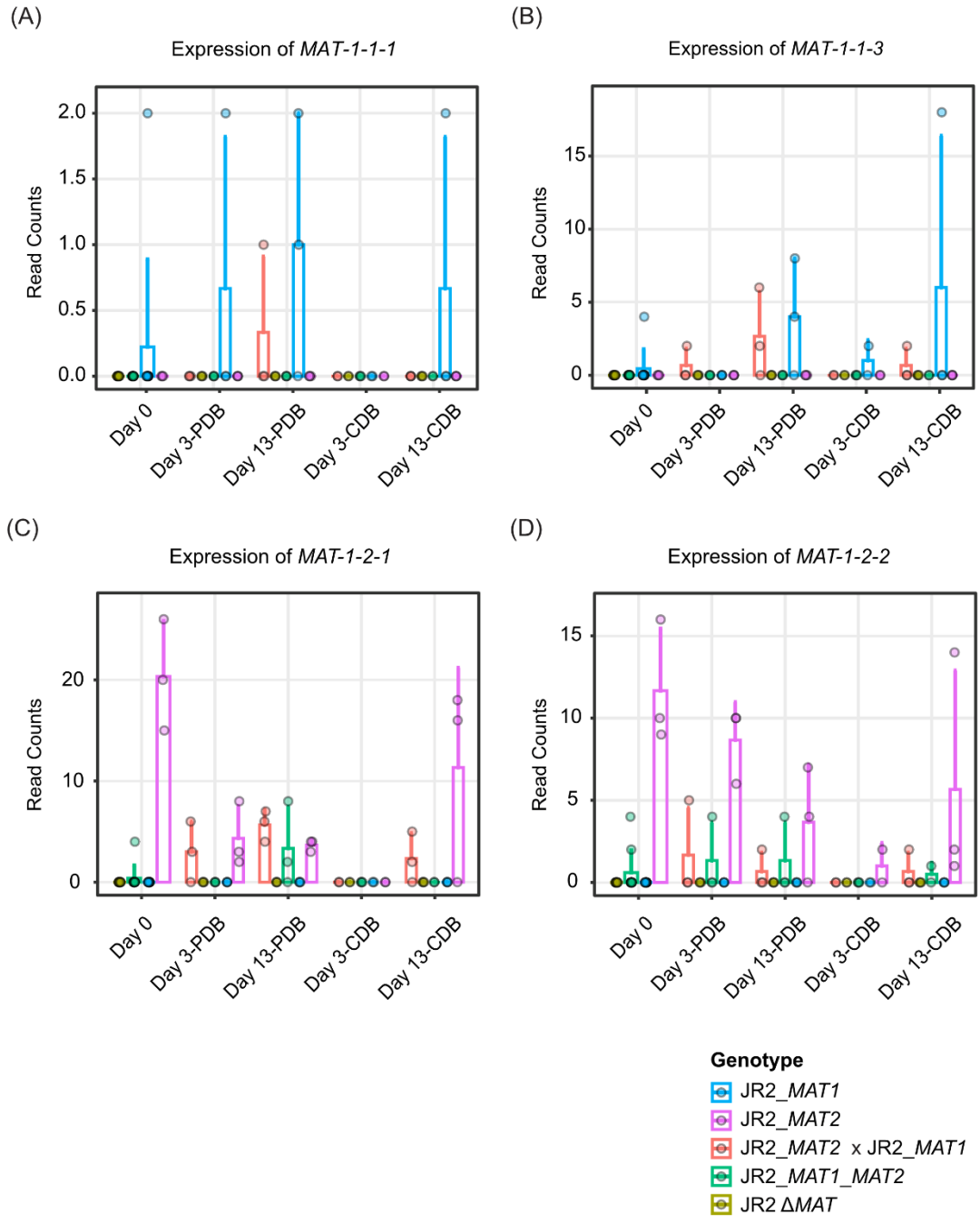
Published transcriptome datasets were obtained from the NCBI Sequence Read Archive under accession numbers PRJNA592220, PRJNA473305, and PRJNA169154 (de Jonge et al. 2012; Cook et al. 2020; Shi-Kunne et al. 2019). RNA-seq reads were mapped onto the *V. dahliae* strain JR2 genome (Faino et al. 2015) using STAR (version 2.7.9a; Dobin et al. 2013). Gene expression was quantified using uniquely mapped and paired reads with FeatureCounts (version 2.0.4; Liao et al. 2014). Transcripts per million (TPM) values were calculated as

previously described (Wagner et al. 2012) and transformed as log2(TPM + 1). The top 2,000 most variable genes were selected for principal component analysis (PCA). PCA was performed using the prcomp function in R (R Core Team 2014), and results were visualized with ggplot2 (Wickham 2016). Differentially expressed genes (DEGs) between mutants and wild-type were identified using DESeq2 (version 1.42.1; Love et al. 2014). Genes were considered differentially expressed when they displayed $|\log_2 FC| \geq 1$ and $p.adj < 0.05$. Venn diagrams were drawn using VennDiagram (version 1.7.3; Chen and Boutros 2011). Gene ontology (GO) analyses were performed using clusterProfiler (version 4.10.1; Wu et al. 2021), with $p.adj < 0.05$ used as the threshold for enriched terms. KEGG pathway enrichment analysis was performed using clusterProfiler (version 4.18.4; Yu et al. 2012), with an adjusted *p*-value (p.adj) < 0.05 as the threshold for significant enrichment. Sex-related genes in *V. dahliae* strain JR2 were identified by protein sequence similarity searches using blastp (version 2.17.0), with sex-related genes from *V. dahliae* strain VdLs17 (Short et al. 2014) as query sequences. Hierarchical clustering was performed using SciPy (version 1.14.1; Virtanen et al. 2020), and heatmaps were visualized using Seaborn (version 0.13.2; Michael Waskom et al. 2017), and ComplexHeatmap (version 2.18.0; Gu et al. 2016).

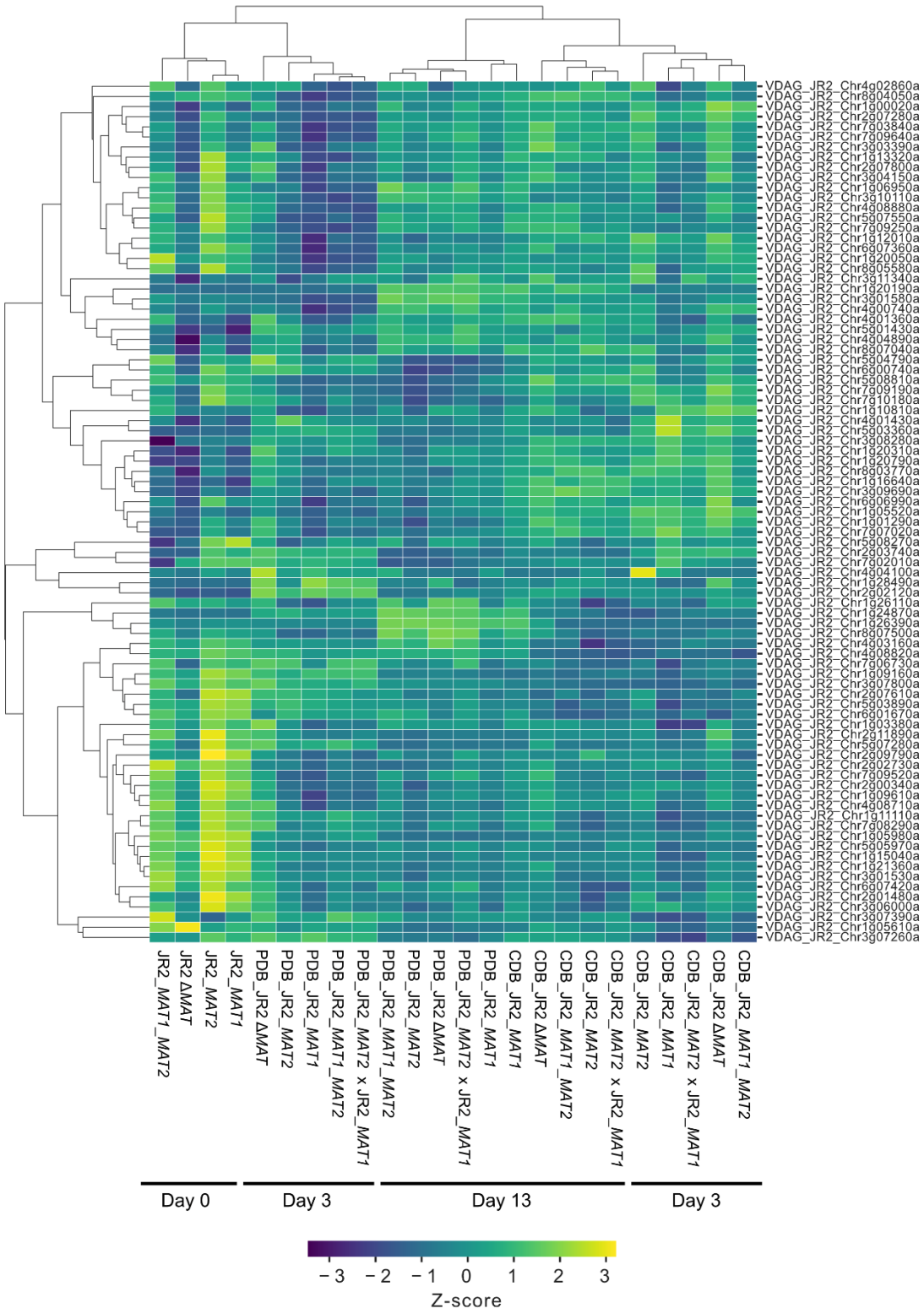
SUPPLEMENTARY DATA



Supplementary Figure 1. PCR validation of mating-type locus composition in *MAT* mutants of *Verticillium dahliae*. Genomic DNA was extracted from wild-type *Verticillium dahliae* (JR2_*MAT2*) and *MAT* mutant strains, including JR2_*MAT1* (harboring *MAT1-1* idiomorph only), JR2 Δ*MAT* (lacking the *MAT* locus), and JR2_*MAT1_MAT2* (harboring both *MAT1-1* and *MAT1-2* idiomorphs). Three independent transformants (#1, #2, and #3) are shown for each *MAT* mutant. PCR using gene-specific primers for *MAT1-1* and *MAT1-2* to assess locus presence. Amplification of the internal transcribed spacer (ITS) region was used as an endogenous control, and ddH_2O was used as a negative control.

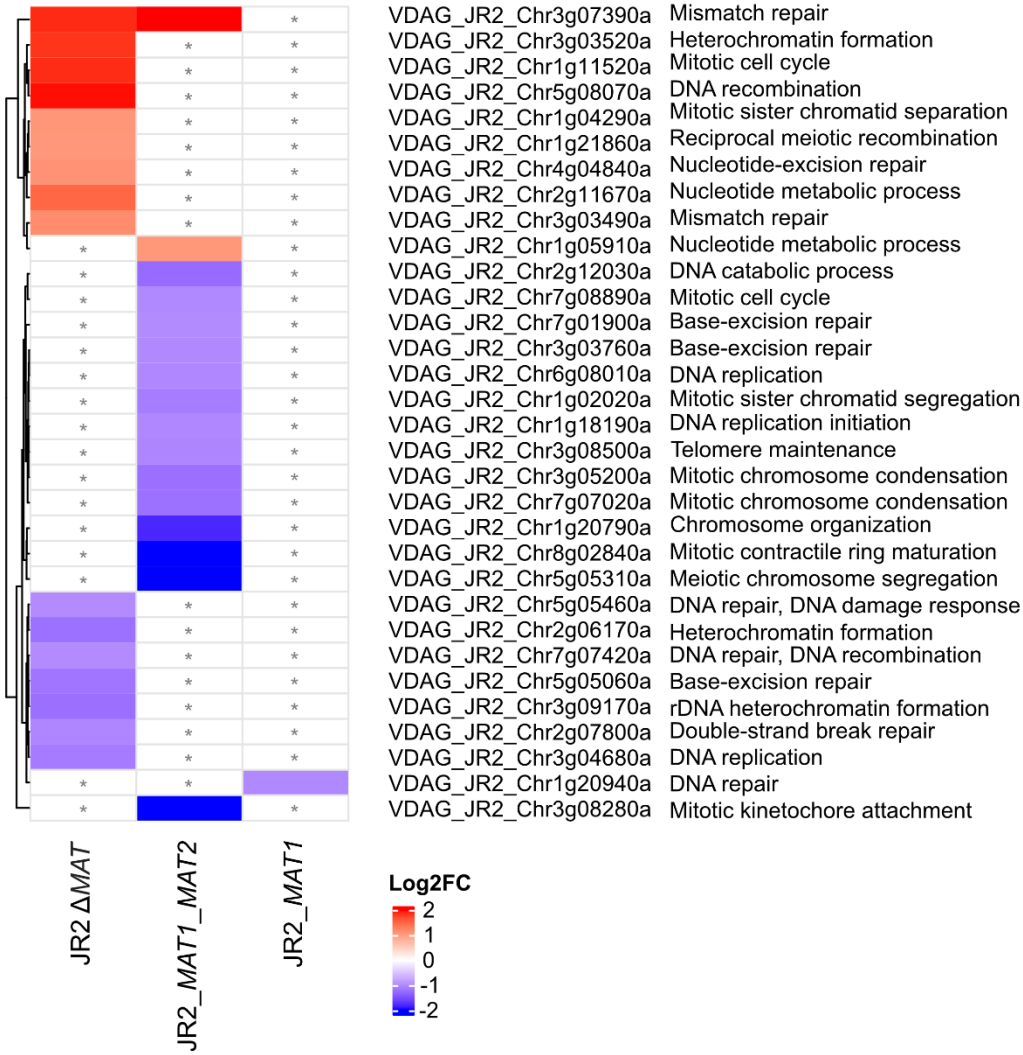


Supplementary Figure 2. *MAT* genes are expressed in *Verticillium dahliae*. Transcriptome analysis shows the expression of (A) *MAT1-1-1*, (B) *MAT1-1-3*, (C) *MAT1-2-1*, and (D) *MAT1-2-2* across *V. dahliae* genotypes. Gene expression is presented as RNA-seq read counts mapped to each gene. Each dot represents an individual transformant, and three independent transformants were included per genotype. Error bars indicate mean values + SD. Transcriptomes were obtained from *V. dahliae* collected at day 0, 3 and 13 after cultivation in potato dextrose broth (PDB) or Czapek–Dox broth (CDB). *V. dahliae* strains comprise wild-type (JR2_*MAT2*), *MAT* mutants (JR2 Δ*MAT*, JR2_*MAT1*, and JR2_*MAT1_MAT2*), and a co-culture of JR2_*MAT2* and JR2_*MAT1* (JR2_*MAT2* × JR2_*MAT1*).

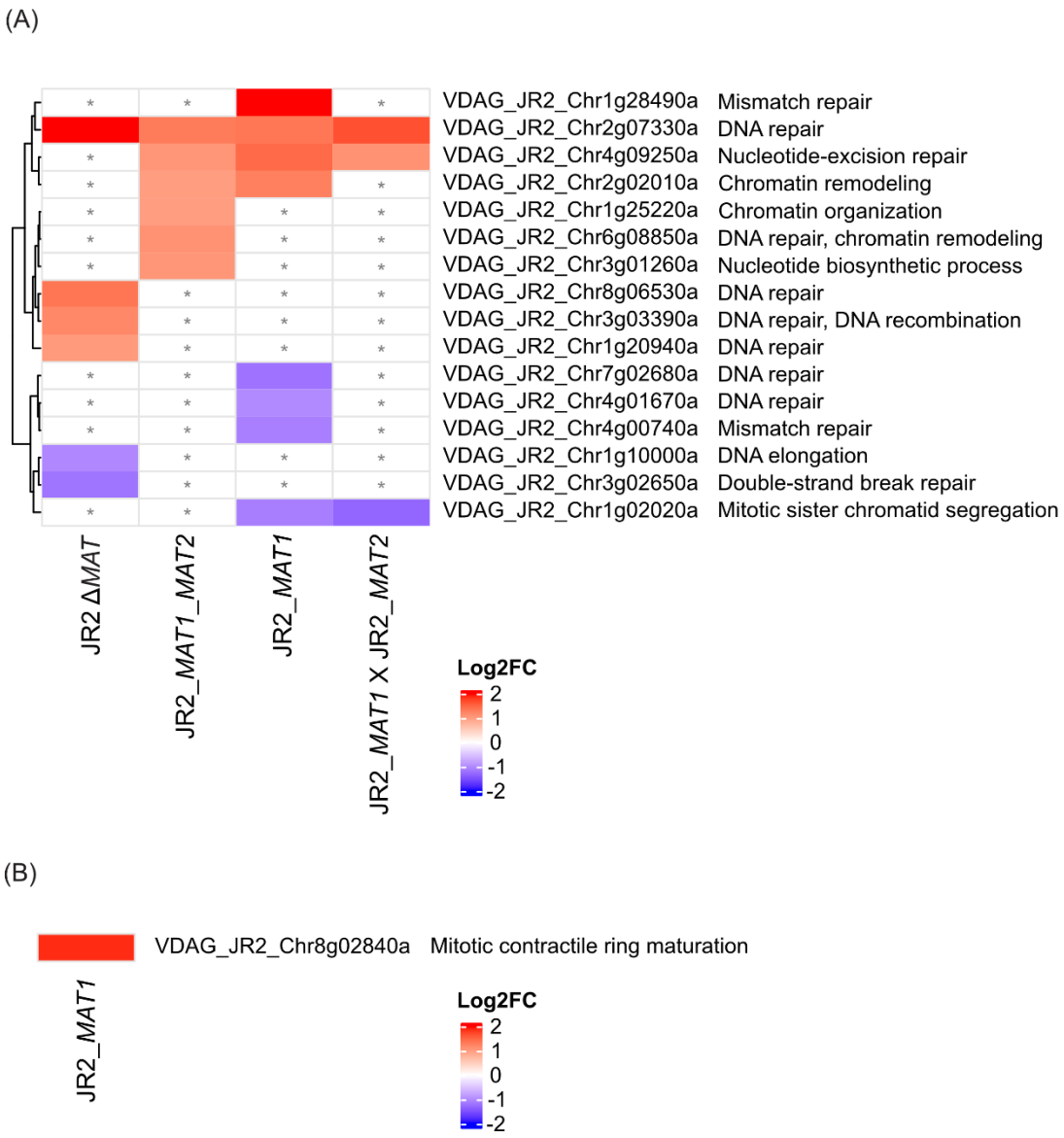


Supplementary Figure 3. Hierarchical clustering of sex-related gene expression reveals clustering independent of *MAT* genotype. Heatmap shows the expression of sex-related genes in *Verticillium dahliae* strains and in co-cultures of strain JR2_*MAT2* and JR2_*MAT1*. Columns are arranged based on similarity in gene expression patterns. Transcriptomes were obtained from *V. dahliae* collected at day 0, 3 and 13 after cultivation in potato dextrose broth (PDB) or Czapek–Dox broth (CDB). *V. dahliae* strains comprise wild-type (JR2_*MAT2*), *MAT* mutants (JR2 Δ*MAT*, JR2_*MAT1*, and JR2_*MAT1_MAT2*), and a co-culture of JR2_*MAT2* and JR2_*MAT1*

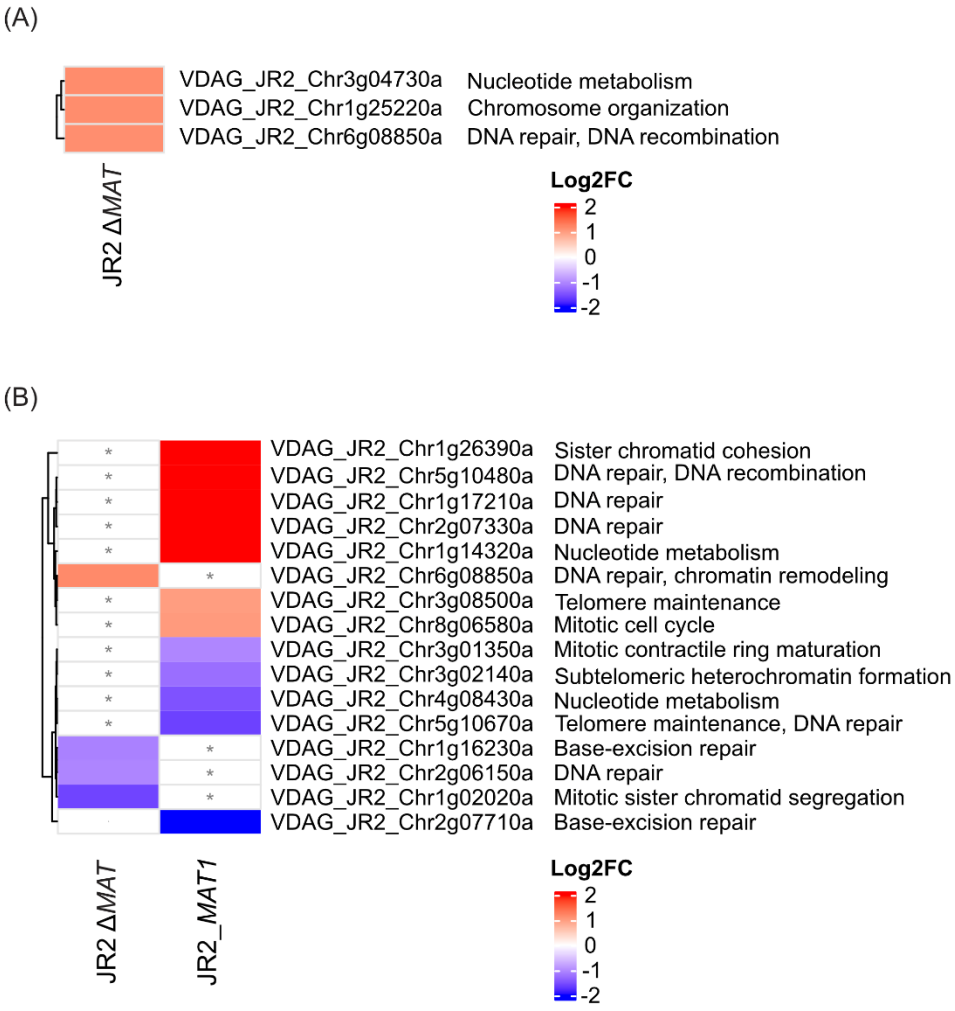
(JR2_*MAT2* × JR2_*MAT1*). For each *MAT* mutant, three independent transformants were included as biological replicates.



Supplementary Figure 4. Heatmap showing differential expression of genome maintenance–related genes in *Verticillium dahliae* strains at day 0 before cultivation in media. Differential expression was defined as an absolute $\log_2$ fold change ($|\log_2 FC| \geq 1$ and *p.adj* < 0.05 relative to the wild-type strain JR2_*MAT2*. *V. dahliae* strains comprise JR2 Δ*MAT*, JR2_*MAT1*, and JR2_*MAT1*_*MAT2*. For each *MAT* mutant, three independent transformants were included as biological replicates.



Supplementary Figure 5. Heatmap showing differential expression of genome maintenance–related genes in *Verticillium dahliae* strains after 3 days of cultivation. Differential expression was defined as an absolute $\log_2$ fold change ($|\log_2 FC|$) ≥ 1 and *p.adj* < 0.05 relative to the wild-type strain JR2_*MAT2*. *V. dahliae* strains comprise the *MAT* mutants (JR2 Δ*MAT*, JR2_*MAT1*, and JR2_*MAT1*_*MAT2*), and the co-cultures of JR2_*MAT2* and JR2_*MAT1* (JR2_*MAT2* × JR2_*MAT1*). Genome maintenance-related DEGs in *V. dahliae* strains are shown for (A) 3 days of cultivation in potato dextrose broth (PDB) and (B) 3 days of cultivation in Czapek–Dox broth (CDB). For each *MAT* mutant, three independent transformants were included as biological replicates.



Supplementary Figure 6. Heatmap showing differential expression of genome maintenance–related genes in *Verticillium dahliae* strains after 13 days of cultivation. Differential expression was defined as an absolute $\log_2$ fold change ($|\log_2 FC| \geq 1$ and *p.adj* < 0.05 relative to the wild-type strain JR2_*MAT2*. *V. dahliae* strains comprise JR2 Δ*MAT* and JR2_*MAT1*. Genome maintenance-related DEGs in *V. dahliae* strains are shown for (A) 13 days of cultivation in potato dextrose broth (PDB) and (B) 13 days of cultivation in Czapek–Dox broth (CDB). For each *MAT* mutant, three independent transformants were included as biological replicates.

Supplementary Table 1. Primers used in this study.

*MAT1*_F1	GCTGGCGAAATTTGTGAGTGCT	Verification of *MAT1-1* presence
*MAT1*_R1	GTTGGCGTAATTCCCTTTGACCTT	Verification of *MAT1-1* presence
*MAT2*_F1	GATTATTGGTGTATGCGGGGTTCG	Verification of *MAT1-2* presence
*MAT2*_R1	TGGAAACCGCATGCTCGTCAG	Verification of *MAT1-2* presence
VdITS1_F	AAAGTTTTAATGGTTCGCTAAGA	ITS-specific primer
VdITS1_R	CTTGGTCATTTAGAGGAAGTAA	ITS-specific primer
*MAT1*_F2	TACCATGAGGGCTAAAACGAGGTA	Amplify the entire *MAT1* locus from strain DvD-S29
*MAT1*_R2	AAGATCCTTTCGCTCTGCTTCAACC	Amplify the entire *MAT1-1* locus from strain DvD-S29
Δ*MAT*_F1	CATCAGTTATGATACCTGCAATGATGG	To amplify a 1 kb fragment downstream of the *MAT1-2* locus
Δ*MAT*_R1	AGGAGATCTTCTAGAAAGATTGTAGCATTTGGCGTCAATACTTT	To amplify a 1 kb fragment downstream of the *MAT1-2* locus
Δ*MAT*_F2	CTCGAGTTTTTCAGCAAGATAAAGCAGAGTATTGGTCATGGATG	To amplify a 1 kb fragment upstream of the *MAT1-2* locus
Δ*MAT*_R2	CAGCGGTCGATGAAACCAGCGAAT	To amplify a 1 kb fragment upstream of the *MAT1-2* locus
Donor_*MAT1*_F1	TGCTGAGCAATGATCTACTCAAGAGCGGGGAGTTGACGCCCGTCGTACCATGAGGGCTAAAACGA	To create donor DNA for JR2_*MAT1_MAT2* and JR2_*MAT1*
Donor_*MAT1*_R1	CTTTATGGAGCAATCGAGCGATCATCTTGGTATTGCAACCAACAGAAGATCCTTTCGCTCTGCTT	To create donor DNA for JR2_*MAT1_MAT2* and JR2_*MAT1*
Donor_Δ*MAT*_F1	AAAGCAGAGTATTGGTCATGGATG	To create donor DNA for JR2 Δ*MAT*
Donor_Δ*MAT*_R1	TGTAGCATTTGGCGTCAATACTTT	To create donor DNA for JR2 Δ*MAT*
SgRNA_constant oligo	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	sgRNA constant oligo
sgRNA_Δ*MAT*_1	AAGCTAATACGACTCACTATAGGAGGTATCATAACTGATGCGAGTTTTAGAGCTAGAAATAGCAAG	sgRNA protospacer oligo for deleting the *MAT1-2* locus

sgRNA_Δ*MAT*_2	AAGCTAATACGACTCACTATAGGCGTAGTGCCTAAGGATCAGGTTTTAGAGCTAGAAATAGCAAG	sgRNA protospacer oligo for deleting the *MAT1-2* locus
sgRNA_*MAT2*_Down	AAGCTAATACGACTCACTATAGGTATTGCAACCAACAGCGAGTTTTAGAGCTAGAAATAGCAAG	sgRNA protospacer oligo targeting the genomic region adjacent to the *MAT1-2* locus

Supplementary Table 2. Gene Ontology (GO) annotations of differentially expressed genes shared by all *MAT* mutants at day 0 (n = 16)

Gene_ID	Biological Process (BP)	Molecular Function (MF)	Cellular Component (CC)
VDAG_JR2_Chr1g05600a	X	X	X
VDAG_JR2_Chr1g17390a	transmembrane transport	transmembrane transporter activity	X
VDAG_JR2_Chr1g27660a	X	ATP binding; metal ion binding	X
VDAG_JR2_Chr2g02450a	X	protein binding	X
VDAG_JR2_Chr3g02430a	X	X	X
VDAG_JR2_Chr3g07310a	monoatomic cation transport; transmembrane transport	monoatomic cation transmembrane transporter activity	membrane
VDAG_JR2_Chr3g12780a	X	catalytic activity	X
VDAG_JR2_Chr4g10730a	X	X	X
VDAG_JR2_Chr4g10740a	aromatic amino acid metabolic process	catalytic activity; fumarylacetoacetase activity	X
VDAG_JR2_Chr4g10750a	L-phenylalanine catabolic process; tyrosine metabolic process	homogentisate 1,2-dioxygenase activity	X
VDAG_JR2_Chr4g10770a	amine metabolic process	copper ion binding; primary amine oxidase activity; quinone binding	X
VDAG_JR2_Chr6g04150a	X	X	X
VDAG_JR2_Chr6g08910a	X	X	X
VDAG_JR2_Chr6g10280a	response to oxidative stress	catalase activity; heme binding	X
VDAG_JR2_Chr7g00850a	X	X	X
VDAG_JR2_Chr7g01620a	X	lyase activity	periplasmic space

Supplementary Table 3. Gene Ontology (GO) annotations of differentially expressed genes shared by all *MAT* mutants at day 3 in PDB (n = 33)

Gene_ID	Biological Process (BP)	Molecular Function (MF)	Cellular Component (CC)
VDAG_JR2_Chr1g02470a	carbohydrate metabolic process	chitin binding	X
VDAG_JR2_Chr1g04650a	X	X	X
VDAG_JR2_Chr1g10090a	maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA); ribosome biogenesis	nucleic acid binding; RNA helicase activity; helicase activity; ATP binding	nucleolus; small-subunit processome
VDAG_JR2_Chr1g13180a	X	X	X
VDAG_JR2_Chr1g24360a	X	X	X
VDAG_JR2_Chr1g29030a	transmembrane transport	ATP binding; ATP hydrolysis activity; ATPase-coupled transmembrane transporter activity; ABC-type transporter activity	membrane
VDAG_JR2_Chr2g02980a	X	X	X
VDAG_JR2_Chr2g04660a	amino acid transport; transmembrane transport	X	membrane
VDAG_JR2_Chr2g07330a	DNA repair	X	X
VDAG_JR2_Chr2g07620a	X	X	X
VDAG_JR2_Chr2g10010a	X	X	X
VDAG_JR2_Chr3g00880a	X	X	X
VDAG_JR2_Chr3g10230a	protein phosphorylation	protein kinase activity; ATP binding	X
VDAG_JR2_Chr3g10620a	X	X	X
VDAG_JR2_Chr4g00860a	X	ATP binding; ABC-type transporter activity	membrane
VDAG_JR2_Chr4g03070a	X	X	X
VDAG_JR2_Chr4g03990a	X	X	X
VDAG_JR2_Chr4g04660a	X	FMN binding; oxidoreductase activity	X
VDAG_JR2_Chr4g11910a	transmembrane transport	transmembrane transporter activity	X
VDAG_JR2_Chr4g11920a	X	X	X
VDAG_JR2_Chr5g01220a	X	X	X
VDAG_JR2_Chr5g04480a	X	X	X
VDAG_JR2_Chr5g05670a	DNA-templated transcription	DNA binding; DNA-directed 5'-3' RNA polymerase activity	X
VDAG_JR2_Chr5g09890a	carbohydrate metabolic process	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	X
VDAG_JR2_Chr5g10150a	fatty acid biosynthetic process	3-oxoacyl-[acyl-carrier-protein] synthase activity; transferase activity; acyltransferase activity	X

Supplementary Table 3. Gene Ontology (GO) annotations of differentially expressed genes shared by all *MAT* mutants at day 3 in PDB (n = 33)

Gene_ID	Biological Process (BP)	Molecular Function (MF)	Cellular Component (CC)
VDAG_JR2_Chr6g10480a	X	X	X
VDAG_JR2_Chr6g10830a	carbohydrate metabolic process	catalytic activity; carbon-oxygen lyase activity, acting on polysaccharides; carbohydrate binding	X
VDAG_JR2_Chr7g01910a	ribosome biogenesis	X	nucleolus
VDAG_JR2_Chr7g02450a	X	protein binding	X
VDAG_JR2_Chr7g04200a	X	X	X
VDAG_JR2_Chr7g07330a	X	X	X
VDAG_JR2_Chr8g05190a	X	X	X
VDAG_JR2_Chr8g09320a	X	X	X

Supplementary Table 4. Differentially expressed genes (DEGs) associated with sexual reproduction in *Verticillium dahliae MAT* mutants.

Gene ID	log2FC[a]	*MAT* mutants	Time points / Growth media[b]	Sexual-related function[c]
VDAG_JR2_Chr2g07800a	-1.045	JR2 Δ*MAT*	Day 0	Synaptonemal complex
VDAG_JR2_Chr3g03390a	1.210	JR2 Δ*MAT*	Day 3_PDB	Crossing over
VDAG_JR2_Chr3g07390a	1.893	JR2 Δ*MAT*	Day 0	Mismatch repair
	3.033	JR2_*MAT1*_*MAT2*		
VDAG_JR2_Chr1g05610a	2.861	JR2 Δ*MAT*	Day 0	Pheromone & mating-related genes
	1.532	JR2_*MAT1*_*MAT2*		
VDAG_JR2_Chr4g00740a	-1.104	JR2_*MAT1*	Day 3_PDB	Crossing over
VDAG_JR2_Chr1g28490a	2.061	JR2_*MAT1*	Day 3_PDB	Crossing over
VDAG_JR2_Chr1g24870a	2.178	JR2_*MAT1*	Day 13_CDB	Meiotic recombination
VDAG_JR2_Chr1g26390a	7.573	JR2_*MAT1*	Day 13_CDB	Cohesion & chromosome dynamics
VDAG_JR2_Chr7g02010a	1.683	JR2_*MAT1*	Day 3_CDB	Anaphase-promoting complex
	-3.438	JR2_*MAT1*_*MAT2*	Day 0	
VDAG_JR2_Chr8g07500a	1.421	JR2_*MAT1*	Day 13_CDB	Signal transduction
VDAG_JR2_Chr2g03740a	-1.649	JR2_*MAT1*_*MAT2*	Day 0	Synaptonemal complex
VDAG_JR2_Chr5g08270a	-2.234	JR2_*MAT1*_*MAT2*	Day 0	Separin & condensins
	1.212		Day 3_PDB	
VDAG_JR2_Chr1g20790a	-1.827	JR2_*MAT1*_*MAT2*	Day 0	Separin & condensins
VDAG_JR2_Chr7g07020a	-1.231	JR2_*MAT1*_*MAT2*	Day 0	Separin & condensins
VDAG_JR2_Chr3g08280a	-3.853	JR2_*MAT1*_*MAT2*	Day 0	Chromosome segregation
VDAG_JR2_Chr3g11340a	1.019	JR2_*MAT2* × JR2_*MAT1*	Day 3_PDB	Resolution of recombination intermediates

[a] Differential expression was defined as an absolute $\log_2$ fold change ($|\log_2 FC| \geq 1$ and $p.adj < 0.05$ relative to the wild-type strain JR2_*MAT2*. For each *MAT* mutant, three independent transformants were included as biological replicates.

[b] Sex-related DEGs were identified in *MAT* mutants at days 0, 3, and 13 after cultivation in potato dextrose broth (PDB) or Czapek–Dox broth (CDB).s

[c] Functional annotations of each gene are as described in Short et al. (2014).

Chapter 5: General discussion

Introduction

Fungi constitute one of the oldest and most diverse eukaryotic kingdoms, with an evolutionary history dating back more than one billion years (Naranjo-Ortiz and Gabaldón 2019; Berbee et al. 2017). Over this long evolutionary timescale, fungi have diversified into millions of species with multiple phyla and accumulated extensive genomic variation (Hawksworth and Lücking 2017). Genome sequence divergence can be exceptionally high even within a single fungal subphylum (Shen et al. 2018). For example, genome-wide analyses have shown that Saccharomycotina, the budding yeast subphylum within Ascomycota, exhibits extraordinary sequence divergence exceeding that observed between genetically distant animals such as humans and roundworms (Shen et al. 2018). The extensive genomic diversity of fungi is reflected in their wide range of phenotypes, lifestyles, and habitats (Blackwell 2011). Although fungal genome sizes vary widely, many are relatively small, and advances in sequencing technology now enable researchers to efficiently generate fungal genome assemblies using the Oxford Nanopore MinION, a pocket-sized long-read sequencing device (Petersen et al. 2022). The combination of relatively compact genomes, eukaryotic cellular complexity, remarkable diversity, and rapid growth makes fungi powerful models for studying the evolutionary dynamics of eukaryotic genomes (Fisher and Lang 2016). Yet, despite the central role of sexual reproduction in driving eukaryotic genome evolution, approximately 20% of fungal species lack documented sexual reproduction (Dyer and Kück 2017). This raises the intriguing question of how these fungi generate and maintain genomic diversity over evolutionary time in the absence of apparent sexual reproduction (Seidl and Thomma 2014). My doctoral research addressed this question by investigating genome evolution in the presumed asexual plant-pathogenic fungus *Verticillium dahliae*, a broad host-range pathogen that causes substantial agricultural losses (Fradin and Thomma 2006).

Through my doctoral research, I established a collection of *V. dahliae* lineages generated under diverse conditions, including exposure to abiotic pressures and repeated passage through the plant host *Arabidopsis thaliana* (Chapter 2). Among the established lineages, I identified a *de novo* inter-chromosomal rearrangement in a *V. dahliae* lineage after a single passage through *A. thaliana* and showed that this rearrangement confers a competitive advantage during host colonization (Chapter3). This chapter provides the first direct evidence that large-scale chromosomal rearrangements are involved in ongoing genome evolution and host adaptation in *V. dahliae* (Chapter3). Finally, I demonstrated that 85 sex-related genes, along with *MAT* genes, are transcriptionally active in *V. dahliae*, suggesting the possibility of an unidentified and rare sexual stage in *V. dahliae* (Chapter 4). I further show that *MAT* genes

have noncanonical regulatory roles, influencing the transcription of genes outside the set of 85sex-related genes, suggesting broader roles beyond sexual reproduction (Chapter 4). In the following sections, I will discuss the findings from my doctoral research and position them within a broader scientific context.

Chromosomal rearrangements as drivers of fungal adaptation and genome innovation

Many fungal species reproduce predominantly asexually, yet their genome plasticity is not necessarily constrained by the absence of frequent sexual recombination (Seidl and Thomma 2014). In *V. dahliae*, comparative genomic analyses have revealed widespread large-scale chromosomal rearrangements among strains isolated from diverse hosts and environments, with these rearrangements contributing to host adaptation (Torres et al. 2021; de Jonge et al. 2013; Faino et al. 2016). Ancestral genome reconstruction within the *Verticillium* genus further revealed that large-scale chromosomal rearrangements have occurred repeatedly during evolution, suggesting a role in *Verticillium* speciation (Shi-Kunne et al. 2018). However, direct experimental evidence for the *de novo* formation of chromosomal rearrangements in *V. dahliae* has been lacking.

In Chapter 3, we extend our understanding of chromosomal rearrangements in *V. dahliae* by demonstrating that they can arise *de novo*. This is demonstrated by the formation of ICR1.5, an inter-chromosomal rearrangement between chromosomes 1 and 5, which was detected in the evolved strain P1 after a single passage through *A. thaliana* (Chapter 2 & 3). Through co-inoculation assays in *A. thaliana*, we further showed that the proportion of strain P1 increased *in planta* when it was co-inoculated with either the parental strain or transformants in which ICR1.5 had been restored to the parental chromosomal configuration (Chapter 3). This result indicates that ICR1.5 confers a competitive advantage to *V. dahliae*. However, this competitive advantage was not observed in *in vitro* assays, indicating that the advantage conferred by ICR1.5 is specifically associated with colonization of the plant host (Chapter 3). These results provide direct evidence that large-scale chromosomal rearrangements contribute to ongoing genome evolution and host adaptation in *V. dahliae* (Chapter 3). In addition to our study, accumulating evidence indicates that chromosomal rearrangements are important drivers of adaptation across diverse fungi, as demonstrated by genome sequencing studies of natural isolates and experimentally evolved lineages, including human-pathogenic fungi, plant-pathogenic fungi, and yeasts (Gorkovskiy and Verstrepen 2021; Langner et al. 2021; Díaz et al. 2025; de Jonge et al. 2013). Furthermore, the SCRaMbLE (Synthetic Chromosome Rearrangement and Modification by LoxP-mediated Evolution) system has been used in *Saccharomyces cerevisiae* to generate synthetic chromosome rearrangements and assess their adaptive potential (Luo et al. 2018; Lu et al. 2025). Strains derived from the SCRaMbLE

system have shown improved performance under diverse conditions. Several of these adaptive phenotypes, such as ethanol tolerance, improved growth under high alkalinity, auxotrophic adaptation, and altered violacein yield, have been directly linked to chromosomal rearrangements (Dymond et al. 2011; Liu et al. 2018; Luo et al. 2018; Ma et al. 2019; Lu et al. 2025). Although SCRaMbLE represents an artificial system, it provides experimental evidence that chromosomal rearrangements alone can generate diverse phenotypic variation, including adaptive traits (Luo et al. 2018; Lu et al. 2025). These findings support the view that chromosomal rearrangements can serve as powerful drivers of adaptation and fitness innovation in fungi.

Stressful environments can increase genome instability, leading to diverse chromosomal aberrations (Li et al. 2025; Vande Zande et al. 2023). Studies have shown that heat stress is associated with an increased frequency of chromosomal rearrangements in fungi, including the yeast *Saccharomyces cerevisiae* and the plant-pathogenic fungus *Zymoseptoria tritici* (Shen et al. 2020; Möller et al. 2018). For example, Shen et al. (2020) showed that exposing *S. cerevisiae* to an extreme but brief heat shock at 52 °C caused an almost 100-fold increase in chromosomal aberrations, which were mainly whole-chromosome amplifications but also included chromosomal rearrangements (Shen et al. 2020). Similarly, when *Z. tritici* was exposed to elevated temperatures, there was a substantial increase in the loss of dispensable chromosomes, as well as increased chromosome breakage, fusions, and duplications (Möller et al. 2018). These observations suggest that stress can increase genome instability, thereby increasing the likelihood that chromosomal rearrangements arise. Studies across diverse organisms have shown that chromosomal rearrangements can alter gene dosage, reposition genes relative to regulatory elements, or create novel gene structures, thereby generating genetic variation that facilitates the emergence of adaptive phenotypes (Gorkovskiy and Verstrepen 2021; Hayday et al. 1984; El-Tanani et al. 2024). However, chromosomal rearrangements are not universally beneficial, as their potential to compromise genome function is evident from their frequent association with human disease (Federico et al. 2021; Gorkovskiy and Verstrepen 2021; Zhang et al. 2013). Thus, while some chromosomal rearrangements confer adaptive advantages, many are likely to be deleterious or neutral with no functional effect (Gorkovskiy and Verstrepen 2021; Figure 1). Consequently, we speculate that additional chromosomal rearrangements may have occurred during our experimental evolution experiments (Chapter 2 & 3), but remained undetected because they were either deleterious or neutral and therefore present at low abundance in *V. dahliae* lineages, below the sequencing detection limit (Figure 1).

Among beneficial chromosomal rearrangements, some contribute to long-term adaptation and speciation (Nosil et al. 2023), whereas others may represent temporary evolutionary solutions

because their effects involve evolutionary trade-offs (Gorkovskiy and Verstrepen 2021). For example, rearrangements can simultaneously alter the expression of multiple genes located within or near the rearranged regions, including genes whose altered expression may impose fitness costs (Gorkovskiy and Verstrepen 2021; Jeffares et al. 2017; Federico et al. 2021). It has therefore been proposed that chromosomal rearrangements can act as short-term evolutionary "quick fixes," providing rapid benefits under specific selective conditions but later being replaced by more precise, less costly gene-level mutations (Vande Zande et al. 2023; Gorkovskiy and Verstrepen 2021). In Chapters 2 and 3, sequencing was performed only at the final time point for *V. dahliae* lineages exposed to prolonged heat stress or sustained co-cultivation with a bacterial antagonist. As a result, we cannot exclude the possibility that beneficial chromosomal rearrangements arose transiently during experimental evolution but were subsequently lost when lineages carrying less costly or more refined mutations outcompeted them.

In *V. dahliae*, chromosomal rearrangements have been attributed to double-strand DNA breaks followed by unfaithful DNA repair (Faino et al. 2016; Figure 1). These rearrangements often occur between homologous sequences and repetitive elements, particularly transposable elements (TEs), which are thought to act as ectopic substrates for unfaithful repair (Faino et al. 2016). Recently characterized giant transposons termed "Starships" have also been identified as major hotspots for chromosomal rearrangements in *V. dahliae*, likely because of their repetitive sequence features (Sato et al. 2025). In addition to sequence-level features, it has been proposed that genome organization within the nucleus may influence the formation of chromosomal rearrangements by bringing distant genomic regions into spatial proximity, thereby facilitating erroneous DNA repair events (Torres et al. 2024). While these studies highlight genomic features that facilitate chromosomal rearrangements in *V. dahliae*, the molecular machinery underlying the formation of chromosomal rearrangements remains unclear. Evidence from yeast indicates that mutations in individual genes that function in DNA double-strand break (DSB) repair can increase chromosomal rearrangement rates by hundreds- to thousands-fold (Chen and Kolodner 1999; Figure 1). These include genes encoding components of the MRE11-RAD50-XRS2 (MRX) complex, which functions in DSB repair, as well as genes encoding DNA replication-associated proteins such as RFA1 and RAD27 (Chen and Kolodner 1999). Mutations that impair genes encoding proteins involved in homologous recombination repair (RAD51, RAD54, and RAD57) or non-homologous end joining (YKU70, YKU80, and LIG4) also increase chromosomal rearrangements in yeast, although to a lesser extent, approximately two- to ten-fold (Chen and Kolodner 1999). In Chapter 4, we showed that *V. dahliae* actively expresses 85 genes associated with sexual development and meiotic recombination, including RAD50, RAD51, RAD54, and RPA1, with RPA1 being the ortholog of yeast RFA1. Because the orthologs of these genes influence

chromosomal rearrangements in yeast, they may also contribute to chromosomal rearrangements in *V. dahliae* and therefore represent promising candidates for future functional analysis (Chen and Kolodner 1999; Chapter 4).

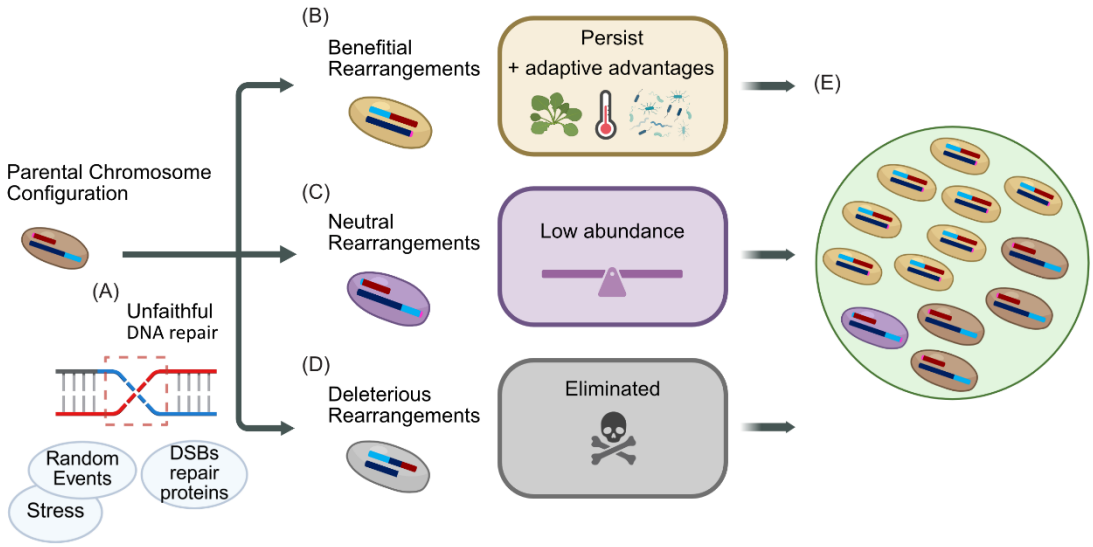


Figure 1. Proposed model for the generation, selection, and persistence of chromosomal rearrangements in *Verticillium dahliae*. (A) Chromosomal rearrangements arise from random DNA double-strand breaks (DSBs), followed by unfaithful repair mediated by DSB repair proteins, particularly when repair occurs between repeated sequences. These rearrangements may also be induced under stress conditions. (B) When chromosomal rearrangements confer adaptive advantages, such as improved host adaptation, increased stress resistance, or enhanced tolerance to antagonistic microorganisms, *V. dahliae* strains carrying these rearrangements may increase in abundance within the population. (C) If chromosomal rearrangements are neutral and do not provide adaptive benefits, strains carrying them are likely to remain at low abundance and may eventually be lost from the population. (D) Many chromosomal rearrangements may be deleterious if they disrupt genome organization or damage genes required for survival. Consequently, *V. dahliae* strains carrying such rearrangements are likely to be eliminated. (E) Over time, the *V. dahliae* population is therefore expected to become enriched for strains carrying beneficial chromosomal rearrangements, unless these strains are subsequently outcompeted by strains carrying alternative advantageous genomic changes.

Conserved and transcriptionally active sex-related genes point to cryptic fungal sexuality

Sexual reproduction offers fungi a range of evolutionary benefits (Nieuwenhuis and James 2016). By combining genetic material from two parental genomes and reshuffling genomes through meiotic recombination, sexual reproduction generates novel allelic combinations and increases genetic diversity (Drenth et al. 2019). Meiotic recombination can also reduce the accumulation of deleterious mutations, thereby helping to counteract Muller's ratchet, a process in which deleterious mutations progressively accumulate under strictly asexual reproduction (Drenth et al. 2019). In some fungal species, sexual reproduction is associated with repeat-induced point mutation (RIP), a premeiotic genome-defense mechanism that

targets duplicated DNA sequences, including transposable elements (Nieuwenhuis and James 2016). By introducing extensive C-to-T or G-to-A mutations, RIP can inactivate transposable elements and reduce their potential to cause genetic damage, such as disrupting genes and destabilizing the genome (Nieuwenhuis and James 2016). Sexual reproduction can also provide ecological advantages (Sun et al. 2025). For example, ascospores of some filamentous fungi tend to be more heat-resistant than mycelia and conidia, making them well suited for survival under heat stress and potentially facilitating persistence and dispersal from unfavorable environments (Dijksterhuis 2007; Sun et al. 2025). Even though sexual reproduction provides substantial evolutionary benefits, approximately 20% of fungal species have no observed sexual stage and have traditionally been classified as asexual fungi (Dyer and Kück 2017). Historically, these species were regarded as “evolutionary scandals” (Oliveira et al. 2024). However, growing evidence suggests that many fungi previously considered asexual possess the genetic features required for sexual reproduction and may be capable of cryptic sexual reproduction, a rare and hidden sexual cycle (Dyer and O'Gorman 2012; Dyer and Kück 2017; Chapter 4). Cryptic sexuality is difficult to observe, but it has now been detected in some fungal species once considered asexual (Dyer and Kück 2017). Thus, the absence of an observed sexual stage may reflect limitations in observation rather than a genuine lack of sexual capability (Dyer and O'Gorman 2012; Dyer and Kück 2017). This perspective has led researchers to question whether any fungi are permanently asexual (Hofstatter and Lahr 2019).

The fungal mating-type (*MAT*) locus is a genomic region that determines sexual identity and is widely used as an indicator for assessing the potential for sexual reproduction in fungi (Sun et al. 2025; Dyer and Kück 2017; Dyer et al. 2016). Although the structure and gene content of the *MAT* locus vary considerably among fungal phyla, the organization of the *MAT* locus in ascomycetes is relatively well characterized, typically comprising a single locus with two alternative idiomorphs, *MAT1-1* and *MAT1-2* (Dyer et al. 2016; Sun et al. 2025). The *MAT1-1* idiomorph typically contains at least one *MAT* gene encoding an α-box-domain transcription factor, whereas the *MAT1-2* idiomorph contains at least one *MAT* gene encoding an HMG-domain transcription factor (Dyer et al. 2016; Martin et al. 2010). In heterothallic ascomycetes, each strain carries only one idiomorph, either *MAT1-1* or *MAT1-2*, and therefore requires a compatible partner carrying the opposite idiomorph for sexual reproduction (Dyer et al. 2016). In contrast, homothallic ascomycetes contain both *MAT* idiomorphs within a single genome, enabling self-fertility (Dyer et al. 2016). Surprisingly, many ascomycetes, including *V. dahliae*, have no documented sexual stage, yet individual strains carry either the *MAT1-1* or *MAT1-2* idiomorph (Dyer and Kück 2017; Dyer et al. 2016; Chapter 4). This genome organization resembles the mating-type locus structure typically found in heterothallic ascomycetes (Dyer and Kück 2017). Moreover, strong purifying selection on *MAT* genes has been observed in

several fungal pathogens without a documented sexual stage, including *Fusarium* species and *V. dahliae*, suggesting that *MAT* genes maintain functional roles in these fungi (Short et al. 2014; O'Donnell et al. 2004). Furthermore, expression of *MAT* genes has been reported in many of these fungi, and our analyses in Chapter 4 also show that *MAT* genes are expressed in *V. dahliae* across multiple transcriptomic profiles (Dyer and Kück 2017; Chapter 4). These findings indicate that many fungi without a documented sexual stage may nevertheless possess *MAT* loci organized similarly to those of known heterothallic fungi, along with intact and transcriptionally active *MAT* genes (Dyer and Kück 2017; Chapter 4). These observations suggest that *MAT* genes may remain functional in these fungi and contribute to cryptic sexual reproduction, although their involvement in other regulatory networks cannot be excluded.

Another important line of evidence supporting the possible occurrence of cryptic sexual reproduction in fungi is the presence and expression of meiotic genes (Dyer and Kück 2017). Previous genome analyses showed that, in addition to the *MAT* genes, *V. dahliae* possesses 85 sex-related genes orthologous to 91 genes previously identified in sexual ascomycete fungi, including many genes that typically function in meiosis in sexual fungi (Short et al. 2014). This high degree of conservation demonstrates that *V. dahliae* harbors a substantial number of genes associated with sexual reproduction (Short et al. 2014). The difference in gene number reflects cases in which two sex-related genes in *N. crassa* correspond to a single ortholog in *V. dahliae*, as well as the absence of five sex-related genes in *V. dahliae* (Short et al. 2014). Notably, three of the absent genes encode orthologs of HOP1, HOP2, and MND1, which typically function in meiotic recombination (Short et al. 2014). However, these genes have likewise not been detected in other sexual fungi within the Sordariomycetes, the class to which *V. dahliae* belongs (Short et al. 2014). Researchers have therefore speculated that these three genes may not be essential for a fully functional sexual cycle in Sordariomycetes (Short et al. 2014). Reverse transcription polymerase chain reaction (RT-PCR) analyses previously showed that at least 8 of the 85 sex-related genes are transcriptionally active in *V. dahliae* (Short et al. 2014). The transcriptomic analysis presented in Chapter 4 extends these findings by showing that all 85 identified sex-related genes are expressed in *V. dahliae*, indicating that the molecular machinery associated with sexual reproduction is largely transcriptionally active. Although gene expression alone does not demonstrate that sexual reproduction occurs, these findings raise important questions about the functional roles of sex-related genes in *V. dahliae*, and support the hypothesis that *V. dahliae* is capable of cryptic sexual reproduction (Short et al. 2014; Chapter 4). Similar inferences have been drawn for other fungi lacking a documented sexual stage, such as arbuscular mycorrhizal fungi, in which the presence of core meiotic genes has been interpreted as evidence for potential cryptic sexuality (Taylor et al. 2015; Oliveira et al. 2024; Rosendahl 2012; Nieuwenhuis and James 2016). These findings suggest

that fungi lacking an observed sexual stage may nevertheless have genetic components required for a complete sexual cycle and thus may be capable of cryptic sexual reproduction.

The discovery of structurally conserved mating-type loci and transcriptionally active meiotic genes has enabled researchers to detect cryptic sexual reproduction in several fungi previously considered strictly asexual (Dyer and Kück 2017). A key example is the opportunistic fungal pathogen *Aspergillus fumigatus*, which, despite its long-standing classification as an asexual species, was shown by genome sequencing to carry a complete repertoire of sex-related genes (Dyer and O'Gorman 2012; Galagan et al. 2005). Subsequent studies demonstrated that *A. fumigatus* is capable of sexual reproduction *in vitro* (O'Gorman et al. 2009). Using environmental isolates, researchers established 36 crosses between strains of opposite mating types (O'Gorman et al. 2009). After six months of cultivation on Parafilm-sealed oatmeal agar in the dark at 30°C, cleistothecia, the sexual fruiting bodies, were observed in 34 crosses. (O'Gorman et al. 2009). These cleistothecia contained sexual spores, ascospores, and analysis of the resulting progeny confirmed that genetic recombination had occurred (O'Gorman et al. 2009). These findings showed that *A. fumigatus* possesses functional machinery for completing the sexual cycle. Moreover, population genetic evidence suggests that sexual reproduction may also occur in nature, as Klaassen et al. (2012) detected recombination indicative of sexual reproduction in a natural population. These findings demonstrate that cryptic sexual reproduction can occur in fungi, although it may be rare, restricted to compatible strain combinations, and dependent on specific conditions. They also highlight a major challenge in identifying cryptic sexual reproduction, namely that researchers must first obtain compatible strains capable of mating before screening appropriate conditions which allow sexual reproduction to occur. Accordingly, two *A. fumigatus* crosses failed to undergo sexual reproduction, despite involving strains of opposite mating types and being cultured under conditions that supported sexual reproduction in other crosses (O'Gorman et al. 2009). This challenge may be particularly pronounced in *V. dahliae*, because different strains with opposite mating type typically exhibit extensive chromosomal rearrangements (de Jonge et al. 2013; Torres et al. 2021; Chapter 4). Such rearrangements are considered incompatible with chromosome pairing during meiosis, thereby reducing the likelihood of successful sexual reproduction (Flot et al. 2013). In Chapter 4, we generated strains that differ only in mating type but have an otherwise identical karyotype. By eliminating incompatibility caused by chromosomal rearrangements, these strains provide a valuable resource for large-scale mating screens under diverse conditions to test whether cryptic sexual reproduction can occur in *V. dahliae* (Chapter 4).

Sex-related genes have broader functions beyond sexual reproduction in fungi

Transcriptionally active sex-related genes suggest the potential for sexual reproduction, but their biological functions should not be interpreted as being restricted solely to sexual processes, as some meiotic genes have been shown to contribute to non-sexual processes (Dyer and Kück 2017; Maciver et al. 2019; Terwagne et al. 2022). For example, SPO11 initiates meiotic recombination by generating double-strand breaks, but in the human fungal pathogen *Candida albicans*, it also mediates non-meiotic genetic recombination during parasexuality, during which genetic variation arises during vegetative growth through mitotic rather than meiotic recombination (Forche et al. 2008). This non-meiotic function is consistent with the evolutionary origin of meiotic genes, as SPO11 and several other meiotic genes are derived from ancestral DNA repair systems in archaea, which existed long before the emergence of true eukaryotic meiosis (Hofstatter and Lahr 2019). Sexual recombination has therefore been proposed to have originated as a “fortunate byproduct” of ancient DNA repair systems that helped organisms cope with environmental stress (Wallen and Perlin 2018). Thus, some meiotic genes may continue to participate in non-sexual DNA recombination processes because of their evolutionary origin (Hofstatter and Lahr 2019). In Chapter 4, we detected the expression of numerous *V. dahliae* genes orthologous to meiotic genes under conditions in which sexual reproduction has not been observed. This suggests that *V. dahliae* may undergo cryptic sexual reproduction under conditions yet to be identified, but also raises the possibility that these genes have additional functions when cryptic sexual processes are not activated (Chapter 4). Thus, the expression of sex-related genes may reflect not only potential participation in cryptic sexual processes, but also possible roles in non-sexual processes.

Similar to meiotic genes, accumulating evidence indicates that fungal *MAT* genes can have functions beyond sexual reproduction, including in species with an observable sexual stage (Dyer and Kück 2017; Dyer et al. 2016; Kim et al. 2015; Yu et al. 2018; Becker et al. 2015). In Chapter 4, we found that both the *MAT* genes and 85 sex-related genes are transcriptionally active in *V. dahliae*. However, under the tested conditions, transcriptomic analyses showed that the *MAT* genes did not regulate these 85 sex-related genes (Chapter 4). Instead, they affected the expression of other genes in broader metabolic and cellular processes, outside these 85 sex-related genes (Chapter 4). These findings do not exclude the possibility that *MAT* genes regulate core sex-related genes under conditions yet to be identified, but they suggest that *MAT* genes in *V. dahliae* may also function in non-sexual biological processes (Chapter 4). This interpretation is consistent with chromatin immunoprecipitation sequencing (ChIP-seq) studies in the sexual ascomycete *Penicillium chrysogenum*, which showed that many *MAT*-regulated genes are not directly involved in sexual reproduction, but are instead associated with fungal asexual development and broader metabolic, cellular processes (Becker et al.

2015). Such broad regulatory effects may arise because *MAT* genes typically encode transcription factors that can bind DNA and regulate other genes (Dyer et al. 2016). Fungal genomes naturally harbor numerous potential transcription-factor binding sites, and *MAT* transcription factors may have initially associated by chance with sites that contained compatible *MAT*-binding motifs (Dyer et al. 2016). If regulation of nearby genes provides a selective advantage, even when those genes are unrelated to sexual reproduction, natural selection can maintain these new *MAT*-regulated networks (Dyer et al. 2016). Over evolutionary time, this process may allow *MAT* genes to acquire increasingly broad regulatory roles in fungal metabolism, development, and adaptation (Dyer et al. 2016). Consistent with this idea, phenotypic analyses in diverse fungi have shown that *MAT* genes influence asexual development, morphogenesis, stress tolerance, and virulence (Zhang et al. 2024; Vitale et al. 2026; Dyer and Kück 2017; Yong et al. 2020; Liu et al. 2022; Böhm et al. 2013; Zheng et al. 2013). These findings suggest that *MAT* genes have been evolutionarily repurposed as master regulators capable of coordinating extensive non-sexual genetic networks, even in sexual fungi.

Concluding remarks

Conceptually, this thesis supports the view that large-scale genomic restructuring independent of sexual reproduction represents an important route by which *V. dahliae* adapts to plant hosts (de Jonge et al. 2013; Faino et al. 2016; Chapter 2 & 3). Specifically, we showed that an inter-chromosomal rearrangement emerged after a single passage of *V. dahliae* through *A. thaliana* and contributed to ongoing genome evolution and host adaptation (Chapter 2 & 3). In addition to investigating non-sexual mechanism of genome evolution, this thesis examines molecular components typically associated with fungal sexual reproduction and shows that they are broadly transcriptionally active in *V. dahliae* (Chapter 4). These include the *MAT* genes and 85 additional sex-related genes, despite the absence of apparent sexual reproduction in *V. dahliae* (Chapter 4). This finding raises the possibility that *V. dahliae* may have cryptic sexuality, consistent with the hypothesis that most fungi possess sexual machinery and that truly asexual fungi may be rare or absent (Hofstatter and Lahr 2019; Dyer and Kück 2017; Chapter 4). Moreover, previous studies, together with the findings of this thesis, support the idea that *MAT* genes can influence regulatory networks not directly associated with sexual reproduction, irrespective of whether they also participate in sexual processes (Hofstatter and Lahr 2019; Dyer and Kück 2017; Chapter 4). Such broader functionality may help explain the conservation and widespread distribution of *MAT* genes across the fungal kingdom, as these genes may contribute not only to fungal sexuality but also to biological processes beyond sexual reproduction (Dyer et al. 2016; Dyer and Kück 2017).

References

Acharya, Bhupendra; Ingram, Thomas W.; Oh, YeonYee; Adhikari, Tika B.; Dean, Ralph A.; Louws, Frank J. (2020): Opportunities and Challenges in Studies of Host-Pathogen Interactions and Management of *Verticillium dahliae* in Tomatoes. In Plants (Basel, Switzerland) 9 (11). DOI: 10.3390/plants9111622.

Alkher, H.; El Hadrami, A.; Rashid, K. Y.; Adam, L. R.; Daayf, F. (2009): Pathogenic variation of *Verticillium dahliae* after serial passages through potato and sunflower. In Canadian Journal of Plant Pathology 31 (4), pp. 427–438. DOI: 10.1080/07060660909507617.

Angelini, Christine; Altieri, Andrew H.; Silliman, Brian R.; Bertness, Mark D. (2011): Interactions among Foundation Species and Their Consequences for Community Organization, Biodiversity, and Conservation. In BioScience 61 (10), pp. 782–789. DOI: 10.1525/bio.2011.61.10.8.

Angourakis, Andreas; Alcaina-Mateos, Jonas; Madella, Marco; Zurro, Debora (2022): Human-Plant Coevolution: A modelling framework for theory-building on the origins of agriculture. In PloS One 17 (9), e0260904. DOI: 10.1371/journal.pone.0260904.

Ashton, George D.; Dyer, Paul S. (2019): Culturing and Mating of *Aspergillus fumigatus*. In Current Protocols In Microbiology 54 (1), e87. DOI: 10.1002/cpmc.87.

Ashu, Eta Ebasi; Xu, Jianping (2015): The roles of sexual and asexual reproduction in the origin and dissemination of strains causing fungal infectious disease outbreaks. In Infection, Genetics and Evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases 36, pp. 199–209. DOI: 10.1016/j.meegid.2015.09.019.

Avalos, Javier; Carmen Limón, M. (2015): Biological roles of fungal carotenoids. In Current Genetics 61 (3), pp. 309–324. DOI: 10.1007/s00294-014-0454-x.

Balakirev, Evgeniy S.; Ayala, Francisco J. (2003): Pseudogenes: are they "junk" or functional DNA? In Annual Review of Genetics 37, pp. 123–151. DOI: 10.1146/annurev.genet.37.040103.103949.

Bar-On, Yinon M.; Phillips, Rob; Milo, Ron (2018): The biomass distribution on Earth. In Proceedings of the National Academy of Sciences of the United States of America 115 (25), pp. 6506–6511. DOI: 10.1073/pnas.1711842115.

Barrick, Jeffrey E.; Yu, Dong Su; Yoon, Sung Ho; Jeong, Haeyoung; Oh, Tae Kwang; Schneider, Dominique et al. (2009): Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. In Nature 461 (7268), pp. 1243–1247. DOI: 10.1038/nature08480.

Bateson, Patrick (2017): Adaptability and evolution. In Interface Focus 7 (5), p. 20160126. DOI: 10.1098/rsfs.2016.0126.

Becker, Kordula; Beer, Christina; Freitag, Michael; Kück, Ulrich (2015): Genome-wide identification of target genes of a mating-type α-domain transcription factor reveals functions beyond sexual development. In Molecular Microbiology 96 (5), pp. 1002–1022. DOI: 10.1111/mmi.12987.

Bell, A. A.; Stipanovic, R. D.; Puhalla, J. E. (1976): Pentaketide metabolites of *verticillium dahliae*. In Tetrahedron 32 (12), pp. 1353–1356. DOI: 10.1016/0040-4020(76)85009-0.

Berbee, Mary L.; James, Timothy Y.; Strullu-Derrien, Christine (2017): Early Diverging Fungi: Diversity and Impact at the Dawn of Terrestrial Life. In Annual Review of Microbiology 71, pp. 41–60. DOI: 10.1146/annurev-micro-030117-020324.

Bertels, Frederic; Silander, Olin K.; Pachkov, Mikhail; Rainey, Paul B.; van Nimwegen, Erik (2014): Automated reconstruction of whole-genome phylogenies from short-sequence

reads. In Molecular Biology and Evolution 31 (5), pp. 1077–1088. DOI: 10.1093/molbev/msu088.

Bhat, R. G.; Subbarao, K. V. (1999): Host Range Specificity in *Verticillium dahliae*. In Phytopathology 89 (12), pp. 1218–1225. DOI: 10.1094/PHYTO.1999.89.12.1218.

Blackwell, Meredith (2011): The fungi: 1, 2, 3 … 5.1 million species? In American Journal of Botany 98 (3), pp. 426–438. DOI: 10.3732/ajb.1000298.

Blount, Zachary D.; Borland, Christina Z.; Lenski, Richard E. (2008): Historical contingency and the evolution of a key innovation in an experimental population of *Escherichia coli*. In Proceedings of the National Academy of Sciences of the United States of America 105 (23), pp. 7899–7906. DOI: 10.1073/pnas.0803151105.

Blount, Zachary D.; Lenski, Richard E.; Losos, Jonathan B. (2018): Contingency and determinism in evolution: Replaying life's tape. In Science (New York, N.Y.) 362 (6415). DOI: 10.1126/science.aam5979.

Bobrowicz, Piotr; Pawlak, Rebecca; Correa, Alejandro; Bell-Pedersen, Deborah; Ebbole, Daniel J. (2002): The *Neurospora crassa* pheromone precursor genes are regulated by the mating type locus and the circadian clock. In Molecular Microbiology 45 (3), pp. 795–804. DOI: 10.1046/j.1365-2958.2002.03052.x.

Böhm, Julia; Hoff, Birgit; O'Gorman, Céline M.; Wolfers, Simon; Klix, Volker; Binger, Danielle et al. (2013): Sexual reproduction and mating-type-mediated strain development in the penicillin-producing fungus *Penicillium chrysogenum*. In Proceedings of the National Academy of Sciences of the United States of America 110 (4), pp. 1476–1481. DOI: 10.1073/pnas.1217943110.

Bolger, Anthony M.; Lohse, Marc; Usadel, Bjoern (2014): Trimmomatic: a flexible trimmer for Illumina sequence data. In Bioinformatics (Oxford, England) 30 (15), pp. 2114–2120. DOI: 10.1093/bioinformatics/btu170.

Braga, Gilberto U. L.; Rangel, Drauzio E. N.; Fernandes, Éverton K. K.; Flint, Stephan D.; Roberts, Donald W. (2015): Molecular and physiological effects of environmental UV radiation on fungal conidia. In Current Genetics 61 (3), pp. 405–425. DOI: 10.1007/s00294-015-0483-0.

Braga, Gilberto U. L.; Rangel, Drauzio E. N.; Flint, Stephan D.; Anderson, Anne J.; Roberts, Donald W. (2006): Conidial pigmentation is important to tolerance against solar-simulated radiation in the entomopathogenic fungus *Metarhizium anisopliae*. In Photochemistry and Photobiology 82 (2), pp. 418–422. DOI: 10.1562/2005-05-08-RA-52.

Broad Institute (2019): Picard toolkit. GitHub repository: Broad Institute. Available online at https://broadinstitute.github.io/picard/.

Broad Institute. (2023): Genome Analysis Toolkit (GATK) v4.5.0.0. Available online at https://gatk.broadinstitute.org/.

Bucknell, Angus H.; McDonald, Megan C. (2023): That's no moon, it's a Starship: Giant transposons driving fungal horizontal gene transfer. In Molecular Microbiology 120 (4), pp. 555–563. DOI: 10.1111/mmi.15118.

Bürger, R. (1999): Evolution of genetic variability and the advantage of sex and recombination in changing environments. In Genetics 153 (2), pp. 1055–1069. DOI: 10.1093/genetics/153.2.1055.

Burssed, Bruna; Zamariolli, Malú; Bellucco, Fernanda Teixeira; Melaragno, Maria Isabel (2022): Mechanisms of structural chromosomal rearrangement formation. In Molecular Cytogenetics 15 (1), p. 23. DOI: 10.1186/s13039-022-00600-6.

Cai, Yingying; Wang, Jing; Noman, Muhammad; Hao, Zhongna; Zhang, Zhen; Qiu, Haiping et al. (2025): Establishment of novel and efficient methods for investigating sexual reproduction in *Magnaporthe oryzae*. In Journal of Fungi (Basel, Switzerland) 11 (8). DOI: 10.3390/jof11080604.

Catara, Vittoria (2007): *Pseudomonas corrugata*: plant pathogen and/or biological resource? In Molecular Plant Pathology 8 (3), pp. 233–244. DOI: 10.1111/j.1364-3703.2007.00391.x.

Chavarro-Carrero, Edgar A.; Vermeulen, Jasper P.; E Torres, David; Usami, Toshiyuki; Schouten, Henk J.; Bai, Yuling et al. (2021): Comparative genomics reveals the in planta-secreted *Verticillium dahliae* Av2 effector protein recognized in tomato plants that carry the V2 resistance locus. In Environmental Microbiology 23 (4), pp. 1941–1958. DOI: 10.1111/1462-2920.15288.

Chen, C.; Kolodner, R. D. (1999): Gross chromosomal rearrangements in *Saccharomyces cerevisiae* replication and recombination defective mutants. In Nature Genetics 23 (1), pp. 81–85. DOI: 10.1038/12687.

Chen, Hanbo; Boutros, Paul C. (2011): VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. In BMC Bioinformatics 12, p. 35. DOI: 10.1186/1471-2105-12-35.

Chisholm, Stephen T.; Coaker, Gitta; Day, Brad; Staskawicz, Brian J. (2006): Host-microbe interactions: shaping the evolution of the plant immune response. In Cell 124 (4), pp. 803–814. DOI: 10.1016/j.cell.2006.02.008.

Cingolani, Pablo; Platts, Adrian; Le Wang, Lily; Coon, Melissa; Nguyen, Tung; Wang, Luan et al. (2012): A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. In Fly 6 (2), pp. 80–92. DOI: 10.4161/fly.19695.

Cleere, Matthew M.; Novodvorska, Michaela; Geib, Elena; Whittaker, Jack; Dalton, Heather; Salih, Nadhira et al. (2024): New colours for old in the blue-cheese fungus *Penicillium roqueforti*. In NPJ Science of Food 8 (1), p. 3. DOI: 10.1038/s41538-023-00244-9.

Clutterbuck, A. John (2011): Genomic evidence of repeat-induced point mutation (RIP) in filamentous ascomycetes. In Fungal Genetics and Biology: FG & B 48 (3), pp. 306–326. DOI: 10.1016/j.fgb.2010.09.002.

Collemare, Jérôme; O'Connell, Richard; Lebrun, Marc-Henri (2019): Nonproteinaceous effectors: the terra incognita of plant-fungal interactions. In The New Phytologist 223 (2), pp. 590–596. DOI: 10.1111/nph.15785.

Cook, David E.; Kramer, H. Martin; Torres, David E.; Seidl, Michael F.; Thomma, Bart P. H. J. (2020): A unique chromatin profile defines adaptive genomic regions in a fungal plant pathogen. In ELife 9. DOI: 10.7554/eLife.62208.

Cook, David E.; Mesarich, Carl H.; Thomma, Bart P. H. J. (2015): Understanding plant immunity as a surveillance system to detect invasion. In Annual Review of Phytopathology 53, pp. 541–563. DOI: 10.1146/annurev-phyto-080614-120114.

Cooper, V. S.; Schneider, D.; Blot, M.; Lenski, R. E. (2001): Mechanisms causing rapid and parallel losses of ribose catabolism in evolving populations of *Escherichia coli* B. In Journal of Bacteriology 183 (9), pp. 2834–2841. DOI: 10.1128/jb.183.9.2834-2841.2001.

Cordero, Radames J. B.; Robert, Vincent; Cardinali, Gianluigi; Arinze, Ebuka S.; Thon, Susanna M.; Casadevall, Arturo (2018): Impact of yeast pigmentation on heat capture and latitudinal distribution. In Current Biology 28 (16), 2657-2664.e3. DOI: 10.1016/j.cub.2018.06.034.

Coster, Wouter de; D'Hert, Svenn; Schultz, Darrin T.; Cruts, Marc; van Broeckhoven, Christine (2018): NanoPack: visualizing and processing long-read sequencing data. In Bioinformatics (Oxford, England) 34 (15), pp. 2666–2669. DOI: 10.1093/bioinformatics/bty149.

Crecy, Eudes de; Jaronski, Stefan; Lyons, Benjamin; Lyons, Thomas J.; Keyhani, Nemat O. (2009): Directed evolution of a filamentous fungus for thermotolerance. In BMC Biotechnology 9, p. 74. DOI: 10.1186/1472-6750-9-74.

Croll, Daniel; McDonald, Bruce A. (2012): The accessory genome as a cradle for adaptive evolution in pathogens. In PLoS Pathogens 8 (4), e1002608. DOI: 10.1371/journal.ppat.1002608.

Danecek, Petr; Bonfield, James K.; Liddle, Jennifer; Marshall, John; Ohan, Valeriu; Pollard, Martin O. et al. (2021): Twelve years of SAMtools and BCFtools. In GigaScience 10 (2). DOI: 10.1093/gigascience/giab008.

Davey, John W.; Davis, Seth J.; Mottram, Jeremy C.; Ashton, Peter D. (2020): Tapestry: validate and edit small eukaryotic genome assemblies with long reads. In BioRxiv DOI: 10.1101/2020.04.24.059402

de Jonge, Ronnie; Bolton, Melvin D.; Kombrink, Anja; van den Berg, Grardy C. M.; Yadeta, Koste A.; Thomma, Bart P. H. J. (2013): Extensive chromosomal reshuffling drives evolution of virulence in an asexual pathogen. In Genome Research 23 (8), pp. 1271–1282. DOI: 10.1101/gr.152660.112.

de Jonge, Ronnie; van Esse, H. Peter; Maruthachalam, Karunakaran; Bolton, Melvin D.; Santhanam, Parthasarathy; Saber, Mojtaba Keykha et al. (2012): Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. In Proceedings of the National Academy of Sciences of the United States of America 109 (13), pp. 5110–5115. DOI: 10.1073/pnas.1119623109.

Díaz, Cristina López; Ayhan, Dilay Hazal; Rodríguez López, Ana; Gil, Lucía Gómez; Ma, Li-Jun; Di Pietro, Antonio (2025): Transposons and accessory genes drive adaptation in a clonally evolving fungal pathogen. In Nature Communications 16 (1), 6982. DOI: 10.1038/s41467-025-62213-y

Dijksterhuis, Jan (2007): Heat-resistant ascospores. Food mycology: A multifaceted approach to fungi and food: CRC Press.

Dobin, Alexander; Davis, Carrie A.; Schlesinger, Felix; Drenkow, Jorg; Zaleski, Chris; Jha, Sonali et al. (2013): STAR: ultrafast universal RNA-seq aligner. In Bioinformatics (Oxford, England) 29 (1), pp. 15–21. DOI: 10.1093/bioinformatics/bts635.

Dong, Suomeng; Raffaele, Sylvain; Kamoun, Sophien (2015): The two-speed genomes of filamentous pathogens: waltz with plants. In Current Opinion in Genetics & Development 35, pp. 57–65. DOI: 10.1016/j.gde.2015.09.001.

Drenth, André; McTaggart, Alistair R.; Wingfield, Brenda D. (2019): Fungal clones win the battle, but recombination wins the war. In IMA Fungus 10, p. 18. DOI: 10.1186/s43008-019-0020-8.

Dyer, P. S.; Inderbitzin, P.; Debuchy, R. (2016): 14 Mating-Type Structure, Function, Regulation and Evolution in the Pezizomycotina. In Jürgen Wendland (Ed.): Growth, Differentiation and Sexuality, vol. 1. Cham: Springer International Publishing (The Mycota), pp. 351–385.

Dyer, P. S.; Paoletti, M. (2005): Reproduction in Aspergillus fumigatus: sexuality in a supposedly asexual species? In Medical Mycology 43 Suppl 1, S7-14. DOI: 10.1080/13693780400029015.

Dyer, Paul S.; Kück, Ulrich (2017): Sex and the Imperfect Fungi. In Microbiology Spectrum 5 (3). DOI: 10.1128/microbiolspec.funk-0043-2017.

Dyer, Paul S.; O'Gorman, Céline M. (2012): Sexual development and cryptic sexuality in fungi: insights from Aspergillus species. In FEMS Microbiology Reviews 36 (1), pp. 165–192. DOI: 10.1111/j.1574-6976.2011.00308.x.

Dymond, Jessica S.; Richardson, Sarah M.; Coombes, Candice E.; Babatz, Timothy; Muller, Héloïse; Annaluru, Narayana et al. (2011): Synthetic chromosome arms function in yeast and generate phenotypic diversity by design. In Nature 477 (7365), pp. 471–476. DOI: 10.1038/nature10403.

Ellendorff, Ursula; Fradin, Emilie F.; Jonge, Ronnie de; Thomma, Bart P. H. J. (2009): RNA silencing is required for Arabidopsis defence against Verticillium wilt disease. In Journal of Experimental Botany 60 (2), pp. 591–602. DOI: 10.1093/jxb/ern306.

El-Tanani, Mohamed; Nsairat, Hamdi; Matalka, Ismail I.; Lee, Yin Fai; Rizzo, Manfredi; Aljabali, Alaa A. et al. (2024): The impact of the BCR-ABL oncogene in the pathology and treatment of chronic myeloid leukemia. In Pathology, Research and Practice 254, p. 155161. DOI: 10.1016/j.prp.2024.155161.

Faino, Luigi; Seidl, Michael F.; Datema, Erwin; van den Berg, Grardy C. M.; Janssen, Antoine; Wittenberg, Alexander H. J.; Thomma, Bart P. H. J. (2015): Single-molecule real-time sequencing combined with optical mapping yields completely finished fungal genome. In MBio 6 (4). DOI: 10.1128/mBio.00936-15.

Faino, Luigi; Seidl, Michael F.; Shi-Kunne, Xiaoqian; Pauper, Marc; van den Berg, Grardy C. M.; Wittenberg, Alexander H. J.; Thomma, Bart P. H. J. (2016): Transposons passively and actively contribute to evolution of the two-speed genome of a fungal pathogen. In Genome Research 26 (8), pp. 1091–1100. DOI: 10.1101/gr.204974.116.

Fan, Rong; Cockerton, Helen M.; Armitage, Andrew D.; Bates, Helen; Cascant-Lopez, Emma; Antanaviciute, Laima et al. (2018): Vegetative compatibility groups partition variation in the virulence of *Verticillium dahliae* on strawberry. In PloS One 13 (2), e0191824. DOI: 10.1371/journal.pone.0191824.

Fan, Rong; Klosterman, Steven J.; Wang, Conghao; Subbarao, Krishna V.; Xu, Xiangming; Shang, Wenjing; Hu, Xiaoping (2017): Vayg1 is required for microsclerotium formation and melanin production in *Verticillium dahliae*. In Fungal Genetics and Biology 98, pp. 1–11. DOI: 10.1016/j.fgb.2016.11.003.

Federico, Concetta; Bruno, Francesca; Ragusa, Denise; Clements, Craig S.; Brancato, Desiree; Henry, Marianne P. et al. (2021): Chromosomal rearrangements and altered nuclear organization: Recent mechanistic models in cancer. In Cancers 13 (22). DOI: 10.3390/cancers13225860.

Felsenstein, J. (1974): The evolutionary advantage of recombination. In Genetics 78 (2), pp. 737–756. DOI: 10.1093/genetics/78.2.737.

Fernandes, Patrícia; Colavolpe, Maria Belén; Serrazina, Susana; Costa, Rita Lourenço (2022): European and American chestnuts: an overview of the main threats and control efforts. In Frontiers in Plant Science 13, p. 951844. DOI: 10.3389/fpls.2022.951844.

Fisher, Kaitlin J.; Lang, Gregory I. (2016): Experimental evolution in fungi: an untapped resource. In Fungal Genetics and Biology: FG & B 94, pp. 88–94. DOI: 10.1016/j.fgb.2016.06.007.

Flot, Jean-François; Hespeels, Boris; Li, Xiang; Noel, Benjamin; Arkhipova, Irina; Danchin, Etienne G. J. et al. (2013): Genomic evidence for ameiotic evolution in the bdelloid rotifer *Adineta vaga*. In Nature 500 (7463), pp. 453–457. DOI: 10.1038/nature12326.

Forche, Anja; Alby, Kevin; Schaefer, Dana; Johnson, Alexander D.; Berman, Judith; Bennett, Richard J. (2008): The parasexual cycle in *Candida albicans* provides an alternative pathway to meiosis for the formation of recombinant strains. In PLoS Biology 6 (5), e110. DOI: 10.1371/journal.pbio.0060110.

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

Fradin, Emilie F.; Zhang, Zhao; Juarez Ayala, Juan C.; Castroverde, Christian D. M.; Nazar, Ross N.; Robb, Jane et al. (2009): Genetic dissection of Verticillium wilt resistance mediated by tomato Ve1. In Plant Physiology 150 (1), pp. 320–332. DOI: 10.1104/pp.109.136762.

Fu, Ci; Coelho, Marco A.; David-Palma, Márcia; Priest, Shelby J.; Heitman, Joseph (2019): Genetic and genomic evolution of sexual reproduction: echoes from LECA to the fungal kingdom. In Current opinion in Genetics & Development 58-59, pp. 70–75. DOI: 10.1016/j.gde.2019.07.008.

Galagan, James E.; Calvo, Sarah E.; Cuomo, Christina; Ma, Li-Jun; Wortman, Jennifer R.; Batzoglou, Serafim et al. (2005): Sequencing of *Aspergillus nidulans* and comparative analysis with A. fumigatus and A. oryzae. In Nature 438 (7071), pp. 1105–1115. DOI: 10.1038/nature04341.

Galagan, James E.; Selker, Eric U. (2004): RIP: the evolutionary cost of genome defense. In Trends in Genetics 20 (9), pp. 417–423. DOI: 10.1016/j.tig.2004.07.007.

Gibriel, Hesham A.Y.; Li, Jinling; Zhu, Longfu; Seidl, Michael F.; Thomma, Bart P.H.J. (2019): Verticillium dahliae strains that infect the same host plant display highly divergent effector catalogs. In BioRxiv DOI: 10.1101/528729

Gibson, Amanda K.; Delph, Lynda F.; Lively, Curtis M. (2017): The two-fold cost of sex: experimental evidence from a natural system. In Evolution Letters 1 (1), pp. 6–15. DOI: 10.1002/evl3.1.

Gilbert, Gregory S.; Parker, Ingrid M. (2010): Rapid evolution in a plant-pathogen interaction and the consequences for introduced host species. In Evolutionary Applications 3 (2), pp. 144–156. DOI: 10.1111/j.1752-4571.2009.00107.x.

Gompert, Zachariah; Feder, Jeffrey L.; Parchman, Thomas L.; Planidin, Nicholas P.; Whiting, Frederick J. H.; Nosil, Patrik (2025): Adaptation repeatedly uses complex structural genomic variation. In Science (New York, N.Y.) 388 (6744), eadp3745. DOI: 10.1126/science.adp3745.

Good, Benjamin H.; McDonald, Michael J.; Barrick, Jeffrey E.; Lenski, Richard E.; Desai, Michael M. (2017): The dynamics of molecular evolution over 60,000 generations. In Nature 551 (7678), pp. 45–50. DOI: 10.1038/nature24287.

Gorkovskiy, Anton; Verstrepen, Kevin J. (2021): The role of structural variation in adaptation and evolution of yeast and other fungi. In Genes 12 (5). DOI: 10.3390/genes12050699.

Gu, Zuguang; Eils, Roland; Schlesner, Matthias (2016): Complex heatmaps reveal patterns and correlations in multidimensional genomic data. In Bioinformatics (Oxford, England) 32 (18), pp. 2847–2849. DOI: 10.1093/bioinformatics/btw313.

Guo, Xiaoxuan; Zhang, Xiaoning; Qin, Yuan; Liu, Yong-Xin; Zhang, Jingying; Zhang, Na et al. (2020): Host-associated quantitative abundance profiling reveals the microbial load variation of root microbiome. In Plant Communications 1 (1), p. 100003. DOI: 10.1016/J.XPLC.2019.100003.

Hackl, Thomas; Ankenbrand, Markus; van Adrichem, Bart; Wilkins, David; Haslinger, Kristina (2024): gggenomes: effective and versatile visualizations for comparative genomics. ArXiv DOI: 10.48550/arXiv.2411.13556

Hamilton, W. D.; Zuk, M. (1982): Heritable true fitness and bright birds: a role for parasites? In Science (New York, N.Y.) 218 (4570), pp. 384–387. DOI: 10.1126/science.7123238.

Harewood, Louise; Fraser, Peter (2014): The impact of chromosomal rearrangements on regulation of gene expression. In Human Molecular Genetics 23 (R1), R76-82. DOI: 10.1093/hmg/ddu278.

Hawksworth, David L.; Lücking, Robert (2017): Fungal Diversity Revisited: 2.2 to 3.8 Million Species. In Microbiology Spectrum 5 (4). DOI: 10.1128/microbiolspec.funk-0052-2016.

Hayday, A. C.; Gillies, S. D.; Saito, H.; Wood, C.; Wiman, K.; Hayward, W. S.; Tonegawa, S. (1984): Activation of a translocated human c-myc gene by an enhancer in the immunoglobulin heavy-chain locus. In Nature 307 (5949), pp. 334–340. DOI: 10.1038/307334a0.

Heitman, Joseph (2015): Evolution of sexual reproduction: a view from the fungal kingdom supports an evolutionary epoch with sex before sexes. In Fungal Biology Reviews 29 (3-4), pp. 108–117. DOI: 10.1016/j.fbr.2015.08.002.

Hoffmann, Ary A.; Sgrò, Carla M. (2011): Climate change and evolutionary adaptation. In Nature 470 (7335), pp. 479–485. DOI: 10.1038/nature09670.

Hofstatter, Paulo G.; Lahr, Daniel J. G. (2019): All eukaryotes are sexual, unless proven otherwise: many so-called asexuals present meiotic machinery and might be able to have sex. In BioEssays: news and reviews in molecular, cellular and developmental biology 41 (6), e1800246. DOI: 10.1002/bies.201800246.

Hojsgaard, Diego; Hörandl, Elvira (2015): A little bit of sex matters for genome evolution in asexual plants. In Frontiers in Plant Science 6, p. 82. DOI: 10.3389/fpls.2015.00082.

Huang, Jun; Cook, David E. (2022): The contribution of DNA repair pathways to genome editing and evolution in filamentous pathogens. In FEMS Microbiology Reviews 46 (6). DOI: 10.1093/femsre/fuac035.

Inderbitzin, Patrik; Bostock, Richard M.; Davis, R. Michael; Usami, Toshiyuki; Platt, Harold W.; Subbarao, Krishna V. (2011): Phylogenetics and taxonomy of the fungal vascular wilt pathogen Verticillium, with the descriptions of five new species. In PLOS One 6 (12), e28341. DOI: 10.1371/journal.pone.0028341.

Jacobs, Stef; Boccarella, Giorgio; van den Berg, Pieter; van Dijck, Patrick; Carolus, Hans (2024): Unlocking the potential of experimental evolution to study drug resistance in pathogenic fungi. In NPJ Antimicrobials and Resistance 2 (1), p. 48. DOI: 10.1038/s44259-024-00064-1.

Jeffares, Daniel C.; Jolly, Clemency; Hoti, Mimoza; Speed, Doug; Shaw, Liam; Rallis, Charalampos et al. (2017): Transient structural variations have strong effects on quantitative traits and reproductive isolation in fission yeast. In Nature Communications 8, p. 14061. DOI: 10.1038/ncomms14061.

Johnson, Milo S.; Gopalakrishnan, Shreyas; Goyal, Juhee; Dillingham, Megan E.; Bakerlee, Christopher W.; Humphrey, Parris T. et al. (2021): Phenotypic and molecular evolution across 10,000 generations in laboratory budding yeast populations. In ELife 10. DOI: 10.7554/eLife.63910.

Johnson Pokorná, Martina; Reifová, Radka (2021): Evolution of B chromosomes: from dispensable parasitic chromosomes to essential genomic players. In Frontiers in Genetics 12, p. 727570. DOI: 10.3389/fgene.2021.727570.

Jones, Jonathan D. G.; Dangl, Jeffery L. (2006): The plant immune system. In Nature 444 (7117), pp. 323–329. DOI: 10.1038/nature05286.

Jones, R. Neil; Viegas, Wanda; Houben, Andreas (2008): A century of B chromosomes in plants: so what? In Annals of Botany 101 (6), pp. 767–775. DOI: 10.1093/aob/mcm167.

Joosten, M. H.; Cozijnsen, T. J.; Wit, P. J. de (1994): Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. In Nature 367 (6461), pp. 384–386. DOI: 10.1038/367384a0.

Judson, O. P.; Normark, B. B. (1996): Ancient asexual scandals. In Trends in Ecology & Evolution 11 (2), pp. 41–46. DOI: 10.1016/0169-5347(96)81040-8.

Kawecki, Tadeusz J.; Lenski, Richard E.; Ebert, Dieter; Hollis, Brian; Olivieri, Isabelle; Whitlock, Michael C. (2012): Experimental evolution. In Trends in Ecology & Evolution 27 (10), pp. 547–560. DOI: 10.1016/j.tree.2012.06.001.

Keeney, S.; Giroux, C. N.; Kleckner, N. (1997): Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. In Cell 88 (3), pp. 375–384. DOI: 10.1016/s0092-8674(00)81876-0.

Kim, Hee-Kyoung; Jo, Seong-Mi; Kim, Gi-Yong; Kim, Da-Woon; Kim, Yeon-Ki; Yun, Sung-Hwan (2015): A large-scale functional analysis of putative target genes of mating-type loci provides insight into the regulation of sexual development of the cereal pathogen *Fusarium graminearum*. In PLoS Genetics 11 (9), e1005486. DOI: 10.1371/journal.pgen.1005486.

Kim, Hyojeong; Wright, Sara J.; Park, Gyungsoon; Ouyang, Shouqiang; Krystofova, Svetlana; Borkovich, Katherine A. (2012a): Roles for receptors, pheromones, G proteins, and mating type genes during sexual reproduction in *Neurospora crassa*. In Genetics 190 (4), pp. 1389–1404. DOI: 10.1534/genetics.111.136358.

Kim, Tae Moon; Ko, Jun Ho; Hu, Lingchuan; Kim, Sung-A; Bishop, Alexander J. R.; Vijg, Jan et al. (2012b): RAD51 mutants cause replication defects and chromosomal instability. In Molecular and Cellular Biology 32 (18), pp. 3663–3680. DOI: 10.1128/MCB.00406-12.

Klaassen, Corné H. W.; Gibbons, John G.; Fedorova, Natalie D.; Meis, Jacques F.; Rokas, Antonis (2012): Evidence for genetic differentiation and variable recombination rates among Dutch populations of the opportunistic human pathogen *Aspergillus fumigatus*. In Molecular Ecology 21 (1), pp. 57–70. DOI: 10.1111/j.1365-294X.2011.05364.x.

Klosterman, Steven J.; Atallah, Zahi K.; Vallad, Gary E.; Subbarao, Krishna V. (2009): Diversity, pathogenicity, and management of Verticillium species. In Annual Review of Phytopathology 47, pp. 39–62. DOI: 10.1146/annurev-phyto-080508-081748.

Klosterman, Steven J.; Subbarao, Krishna V.; Kang, Seogchan; Veronese, Paola; Gold, Scott E.; Thomma, Bart P. H. J. et al. (2011): Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. In PLoS Pathogens 7 (7), e1002137. DOI: 10.1371/journal.ppat.1002137.

Konkel Neabore, Lindsey (2024): Wake-up call: rapid increase in human fungal diseases under climate change. In Environmental Health Perspectives 132 (4), p. 42001. DOI: 10.1289/EHP14722.

Koren, Sergey; Walenz, Brian P.; Berlin, Konstantin; Miller, Jason R.; Bergman, Nicholas H.; Phillippy, Adam M. (2017): Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. In Genome Research 27 (5), pp. 722–736. DOI: 10.1101/gr.215087.116.

Kozlov, Alexey M.; Darriba, Diego; Flouri, Tomáš; Morel, Benoit; Stamatakis, Alexandros (2019): RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood

phylogenetic inference. In Bioinformatics (Oxford, England) 35 (21), pp. 4453–4455. DOI: 10.1093/bioinformatics/btz305.

Kraege, Anton; Chavarro-Carrero, Edgar; Schnell, Eva; Heilmann-Heimbach, Stefanie; Becker, Kerstin; Köhrer, Karl et al. (2025): High quality genome assembly and annotation (v1) of the eukaryotic freshwater microalga *Coccomyxa elongata* SAG 216-3b. In G3 (Bethesda, Md.) 15 (2). DOI: 10.1093/g3journal/jkae294.

Kramer, H. Martin; Cook, David E.; van den Berg, Grardy C. M.; Seidl, Michael F.; Thomma, Bart P. H. J. (2021): Three putative DNA methyltransferases of *Verticillium dahliae* differentially contribute to DNA methylation that is dispensable for growth, development and virulence. In Epigenetics & Chromatin 14 (1), p. 21. DOI: 10.1186/s13072-021-00396-6.

Laine, Anna-Liisa; Leino, Sara (2025): Plant microbiomes feel the heat. In Science (New York, N.Y.) 388 (6752), pp. 1150–1152. DOI: 10.1126/science.adw3659.

Langner, Thorsten; Harant, Adeline; Gomez-Luciano, Luis B.; Shrestha, Ram K.; Malmgren, Angus; Latorre, Sergio M. et al. (2021): Genomic rearrangements generate hypervariable mini-chromosomes in host-specific isolates of the blast fungus. In PLoS Genetics 17 (2), e1009386. DOI: 10.1371/journal.pgen.1009386.

Lee, Soo Chan; Ni, Min; Li, Wenjun; Shertz, Cecelia; Heitman, Joseph (2010): The evolution of sex: a perspective from the fungal kingdom. In Microbiology and Molecular Biology Reviews: MMBR 74 (2), pp. 298–340. DOI: 10.1128/MMBR.00005-10.

Leisen, Thomas; Bietz, Fabian; Werner, Janina; Wegner, Alex; Schaffrath, Ulrich; Scheuring, David et al. (2020): CRISPR/Cas with ribonucleoprotein complexes and transiently selected telomere vectors allows highly efficient marker-free and multiple genome editing in *Botrytis cinerea*. In PLoS Pathogens 16 (8), e1008326. DOI: 10.1371/journal.ppat.1008326.

Lenormand, Thomas; Engelstädter, Jan; Johnston, Susan E.; Wijnker, Erik; Haag, Christoph R. (2016): Evolutionary mysteries in meiosis. In Philosophical Transactions of The Royal Society of London. Series B, Biological Sciences 371 (1706). DOI: 10.1098/rstb.2016.0001.

Lenski, Richard E.; Rose, Michael R.; Simpson, Suzanne C.; Tadler, Scott C. (1991): Long-term experimental evolution in *Escherichia coli*. I. adaptation and divergence during 2,000 generations. In The American Naturalist 138 (6), pp. 1315–1341. DOI: 10.1086/285289.

Leonard, Kurt J.; Szabo, Les J. (2005): Stem rust of small grains and grasses caused by *Puccinia graminis*. In Molecular Plant Pathology 6 (2), pp. 99–111. DOI: 10.1111/j.1364-3703.2005.00273.x.

Letunic, Ivica; Bork, Peer (2024): Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool. In Nucleic Acids Research 52 (W1), W78-W82. DOI: 10.1093/nar/gkae268.

Li, Heng (2013): Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. ArXiv DOI: 10.48550/arXiv.1303.3997

Li, Ke-Jing; Qi, Lei; Zhu, Ying-Xuan; He, Min; Xiang, Qian; Zheng, Dao-Qiong (2025): Spontaneous and environment induced genomic alterations in yeast model. In Cell Insight 4 (1), p. 100209. DOI: 10.1016/j.cellin.2024.100209.

Liao, Yang; Smyth, Gordon K.; Shi, Wei (2014): FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features. In Bioinformatics (Oxford, England) 30 (7), pp. 923–930. DOI: 10.1093/bioinformatics/btt656.

Liu, K. X.; Jia, J. Q.; Chen, N.; Fu, D. D.; Sun, J. Y.; Zhao, J. M. et al. (2022): Mating-type genes control sexual reproduction, conidial germination, and virulence in *Cochliobolus lunatus*. In Phytopathology 112 (5), pp. 1055–1062. DOI: 10.1094/PHYTO-02-21-0063-R.

Liu, Wei; Luo, Zhouqing; Wang, Yun; Pham, Nhan T.; Tuck, Laura; Pérez-Pi, Irene et al. (2018): Rapid pathway prototyping and engineering using *in vitro* and *in vivo* synthetic genome SCRaMbLE-in methods. In Nature Communications 9 (1), p. 1936. DOI: 10.1038/s41467-018-04254-0.

Love, Michael I.; Huber, Wolfgang; Anders, Simon (2014): Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. In Genome Biology 15 (12), p. 550. DOI: 10.1186/s13059-014-0550-8.

Lu, Xinyu; Ciurkot, Klaudia; Gowers, Glen-Oliver F.; Shaw, William M.; Ellis, Tom (2025): Iterative SCRaMbLE for engineering synthetic genome modules and chromosomes. In Nature Communications 16 (1), p. 7278. DOI: 10.1038/s41467-025-62356-y.

Luijckx, Pepijn; Ho, Eddie Ka Ho; Gasim, Majid; Chen, Suyang; Stanic, Andrijana; Yanchus, Connor et al. (2017): Higher rates of sex evolve during adaptation to more complex environments. In Proceedings of the National Academy of Sciences of the United States of America 114 (3), pp. 534–539. DOI: 10.1073/pnas.1604072114.

Luo, Zhouqing; Wang, Lihui; Wang, Yun; Zhang, Weimin; Guo, Yakun; Shen, Yue et al. (2018): Identifying and characterizing SCRaMbLEd synthetic yeast using ReSCuES. In Nature communications 9 (1), p. 1930. DOI: 10.1038/s41467-017-00806-y.

Lyons, Timothy W.; Tino, Christopher J.; Fournier, Gregory P.; Anderson, Rika E.; Leavitt, William D.; Konhauser, Kurt O.; Stüeken, Eva E. (2024): Co-evolution of early Earth environments and microbial life. In Nature Reviews. Microbiology 22 (9), pp. 572–586. DOI: 10.1038/s41579-024-01044-y.

Ma, Li-Jun; van der Does, H. Charlotte; Borkovich, Katherine A.; Coleman, Jeffrey J.; Daboussi, Marie-Josée; Di Pietro, Antonio et al. (2010): Comparative genomics reveals mobile pathogenicity chromosomes in Fusarium. In Nature 464 (7287), pp. 367–373. DOI: 10.1038/nature08850.

Ma, Lu; Li, Yunxiang; Chen, Xinyu; Ding, Mingzhu; Wu, Yi; Yuan, Ying-Jin (2019): SCRaMbLE generates evolved yeasts with increased alkali tolerance. In Microbial Cell Factories 18 (1), p. 52. DOI: 10.1186/s12934-019-1102-4.

Maciver, Sutherland K.; Koutsogiannis, Zisis; Del Obeso Fernández Valle, Alvaro de (2019): 'Meiotic genes' are constitutively expressed in an asexual amoeba and are not necessarily involved in sexual reproduction. In Biology Letters 15 (3), p. 20180871. DOI: 10.1098/rsbl.2018.0871.

Mahrt, Niels; Tietze, Alexandra; Künzel, Sven; Franzenburg, Sören; Barbosa, Camilo; Jansen, Gunther; Schulenburg, Hinrich (2021): Bottleneck size and selection level reproducibly impact evolution of antibiotic resistance. In Nature Ecology & Evolution 5 (9), pp. 1233–1242. DOI: 10.1038/s41559-021-01511-2.

Marçais, Guillaume; Delcher, Arthur L.; Phillippy, Adam M.; Coston, Rachel; Salzberg, Steven L.; Zimin, Aleksey (2018): MUMmer4: A fast and versatile genome alignment system. In PLoS Computational Biology 14 (1), e1005944. DOI: 10.1371/journal.pcbi.1005944.

Martin, Tom; Lu, Shun-Wen; van Tilbeurgh, Herman; Ripoll, Daniel R.; Dixelius, Christina; Turgeon, B. Gillian; Debuchy, Robert (2010): Tracing the origin of the fungal α1 domain places its ancestor in the HMG-box superfamily: implication for fungal mating-type evolution. In PLOS One 5 (12), e15199. DOI: 10.1371/journal.pone.0015199.

Mehrabi, Rahim; Mirzadi Gohari, Amir; Kema, Gert H. J. (2017): Karyotype variability in plant-pathogenic fungi. In Annual Review of Phytopathology 55, pp. 483–503. DOI: 10.1146/annurev-phyto-080615-095928.

Michael Waskom; Olga Botvinnik; Drew O'Kane; Paul Hobson; Saulius Lukauskas; David C Gemperline et al. (2017): mwaskom/seaborn: v0.8.1 (September 2017) Zenodo DOI: 10.5281/zenodo.8393472.

Milgroom, Michael G.; Del Jiménez-Gasco, María Mar; Olivares García, Concepción; Drott, Milton T.; Jiménez-Díaz, Rafael M. (2014): Recombination between clonal lineages of the asexual fungus *Verticillium dahliae* detected by genotyping by sequencing. In PLOS One 9 (9), e106740. DOI: 10.1371/journal.pone.0106740.

Möller, Mareike; Habig, Michael; Freitag, Michael; Stukenbrock, Eva H. (2018): Extraordinary genome instability and widespread chromosome rearrangements during vegetative growth. In Genetics 210 (2), pp. 517–529. DOI: 10.1534/genetics.118.301050.

Mondo, Stephen J.; Grigoriev, Igor V. (2025): A genomic perspective on fungal diversity and evolution. In Nature Reviews. Microbiology 23 (11), pp. 718–733. DOI: 10.1038/s41579-025-01195-6.

Mosquera, Sandra; Leveau, Johan H. J.; Stergiopoulos, Ioannis (2021): Repeated exposure of *Aspergillus niger* spores to the antifungal bacterium collimonas fungivorans Ter331 selects for delayed spore germination. In Applied and Environmental Microbiology 87 (12), e0023321. DOI: 10.1128/AEM.00233-21.

Muller, H. J. (1964): the relation of recombination to mutational advance. In Mutation Research 106, pp. 2–9. DOI: 10.1016/0027-5107(64)90047-8.

Naranjo-Ortiz, Miguel A.; Gabaldón, Toni (2019): Fungal evolution: major ecological adaptations and evolutionary transitions. In Biological Reviews of The Cambridge Philosophical Society 94 (4), pp. 1443–1476. DOI: 10.1111/brv.12510.

Newman, Toby E.; Derbyshire, Mark C. (2020): The evolutionary and molecular features of broad host-range necrotrophy in plant pathogenic fungi. In Frontiers in Plant Science 11, p. 591733. DOI: 10.3389/fpls.2020.591733.

Ni, Min; Feretzaki, Marianna; Sun, Sheng; Wang, Xuying; Heitman, Joseph (2011): Sex in fungi. In Annual Review of Genetics 45, pp. 405–430. DOI: 10.1146/annurev-genet-110410-132536.

Nielsen, Rasmus (2005): Molecular signatures of natural selection. In Annual Review of Genetics 39, pp. 197–218. DOI: 10.1146/annurev.genet.39.073003.112420.

Nieuwenhuis, Bart P. S.; James, Timothy Y. (2016): The frequency of sex in fungi. In Philosophical Transactions of The Royal Society of London. Series B, Biological Sciences 371 (1706). DOI: 10.1098/rstb.2015.0540.

Nosil, Patrik; Soria-Carrasco, Victor; Villoutreix, Romain; De-la-Mora, Marisol; Carvalho, Clarissa F. de; Parchman, Thomas et al. (2023): Complex evolutionary processes maintain an ancient chromosomal inversion. In Proceedings of the National Academy of Sciences of the United States of America 120 (25), e2300673120. DOI: 10.1073/pnas.2300673120.

O'Donnell, Kerry; Ward, Todd J.; Geiser, David M.; Corby Kistler, H.; Aoki, Takayuki (2004): Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. In Fungal Genetics and Biology 41 (6), pp. 600–623. DOI: 10.1016/j.fgb.2004.03.003.

O'Gorman, Céline M.; Fuller, Hubert T.; Dyer, Paul S. (2009): Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. In Nature 457 (7228), pp. 471–474. DOI: 10.1038/nature07528.

Oliveira, Jordana; Yildirir, Gokalp; Corradi, Nicolas (2024): From chaos comes order: genetics and genome biology of arbuscular mycorrhizal fungi. In Annual Review of Microbiology 78 (1), pp. 147–168. DOI: 10.1146/annurev-micro-041522-105143.

Osayande, Ivie Sonia; Han, Xiaowei; Tsuda, Kenichi (2025): Dynamic shifts in plant-microbe relationships. In Plant Biotechnology (Tokyo, Japan) 42 (3), pp. 271–277. DOI: 10.5511/plantbiotechnology.25.0428a.

Otto, Sarah P.; Lenormand, Thomas (2002): Resolving the paradox of sex and recombination. In Nature Reviews. Genetics 3 (4), pp. 252–261. DOI: 10.1038/nrg761.

Öztoprak, Hüsna; Gao, Shan; Guiglielmoni, Nadège; Brandt, Alexander; Zheng, Yichen; Errbii, Mohammed et al. (2025): Chromosome-scale genome dynamics reveal signatures of independent haplotype evolution in the ancient asexual mite *Platynothrus peltifer*. In Science Advances 11 (4), eadn0817. DOI: 10.1126/sciadv.adn0817.

Paoletti, Mathieu; Seymour, Fabian A.; Alcocer, Marcos J. C.; Kaur, Navgeet; Calvo, Ana M.; Archer, David B.; Dyer, Paul S. (2007): Mating type and the genetic basis of self-fertility in the model fungus *Aspergillus nidulans*. In Current Biology 17 (16), pp. 1384–1389. DOI: 10.1016/j.cub.2007.07.012.

Pegg, Kenneth G.; Coates, Lindel M.; O'Neill, Wayne T.; Turner, David W. (2019): The epidemiology of Fusarium wilt of banana. In Frontiers in Plant Science 10, p. 1395. DOI: 10.3389/fpls.2019.01395.

Peng, Yan; Li, Shi J.; Yan, Jun; Tang, Yong; Cheng, Jian P.; Gao, An J. et al. (2021): Research progress on phytopathogenic fungi and their role as biocontrol agents. In Frontiers in Microbiology 12, p. 670135. DOI: 10.3389/fmicb.2021.670135.

Petersen, Celine; Sørensen, Trine; Westphal, Klaus R.; Fechete, Lavinia I.; Sondergaard, Teis E.; Sørensen, Jens L.; Nielsen, Kåre L. (2022): High molecular weight DNA extraction methods lead to high quality filamentous ascomycete fungal genome assemblies using Oxford Nanopore sequencing. In Microbial Genomics 8 (4). DOI: 10.1099/mgen.0.000816.

Peterson, P. D.; Leonard, K. J.; Roelfs, A. P.; Sutton, T. B. (2005): Effect of barberry eradication on changes in populations of *Puccinia graminis* in Minnesota. In Plant Disease 89 (9), pp. 935–940. DOI: 10.1094/PD-89-0935.

Pironon, S.; Ondo, I.; Diazgranados, M.; Allkin, R.; Baquero, A. C.; Cámara-Leret, R. et al. (2024): The global distribution of plants used by humans. In Science (New York, N.Y.) 383 (6680), pp. 293–297. DOI: 10.1126/science.adg8028.

R Core Team (2014): R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Available online at https://www.R-project.org/.

Rastogi, Rajesh P.; Richa; Kumar, Ashok; Tyagi, Madhu B.; Sinha, Rajeshwar P. (2010): Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. In Journal of Nucleic Acids 2010, p. 592980. DOI: 10.4061/2010/592980.

Rehman, Latifur; Su, Xiaofeng; Guo, Huiming; Qi, Xiliang; Cheng, Hongmei (2016): Protoplast transformation as a potential platform for exploring gene function in *Verticillium dahliae*. In BMC Biotechnology 16 (1), p. 57. DOI: 10.1186/s12896-016-0287-4.

Richards, Thomas A.; Leonard, Guy; Soanes, Darren M.; Talbot, Nicholas J. (2011): Gene transfer into the fungi. In Fungal Biology Reviews 25 (2), pp. 98–110. DOI: 10.1016/j.fbr.2011.04.003.

Ristaino, Jean B.; Anderson, Pamela K.; Bebber, Daniel P.; Brauman, Kate A.; Cunniffe, Nik J.; Fedoroff, Nina V. et al. (2021): The persistent threat of emerging plant disease pandemics to global food security. In Proceedings of the National Academy of Sciences of the United States of America 118 (23). DOI: 10.1073/pnas.2022239118.

Robbins, W. J. (1944): The importance of plants. In Science (New York, N.Y.) 100 (2603), pp. 440–443. DOI: 10.1126/science.100.2603.440.

Rolfe, Stephen A.; Griffiths, Joseph; Ton, Jurriaan (2019): Crying out for help with root exudates: adaptive mechanisms by which stressed plants assemble health-promoting soil microbiomes. In Current Opinion in Microbiology 49, pp. 73–82. DOI: 10.1016/j.mib.2019.10.003.

Rosendahl, Søren (2012): The first glance into the Glomus genome: an ancient asexual scandal with meiosis? In The New Phytologist 193 (3), pp. 546–548. DOI: 10.1111/j.1469-8137.2011.04028.x.

Rovenich, Hanna; Boshoven, Jordi C.; Thomma, Bart P H J (2014): Filamentous pathogen effector functions: of pathogens, hosts and microbiomes. In Current Opinion in Plant Biology 20, pp. 96–103. DOI: 10.1016/j.pbi.2014.05.001.

Sato, Yukiyo; Bex, Roos; van den Berg, Grardy C. M.; Santhanam, Parthasarathy; Höfte, Monica; Seidl, Michael F.; Thomma, Bart P. H. J. (2025): Starship giant transposons dominate plastic genomic regions in a fungal plant pathogen and drive virulence evolution. In Nature Communications 16 (1), p. 6806. DOI: 10.1038/s41467-025-61986-6.

Savary, Serge; Willocquet, Laetitia; Pethybridge, Sarah Jane; Esker, Paul; McRoberts, Neil; Nelson, Andy (2019): The global burden of pathogens and pests on major food crops. In Nature Ecology & Evolution 3 (3), pp. 430–439. DOI: 10.1038/s41559-018-0793-y.

Sayari, M.; Dolatabadian, A.; El-Shetehy, M.; Daayf, F. (2024): Genomic insights into Verticillium: a review of progress in the genomics era. In Frontiers in Microbiology 15, p. 1463779. DOI: 10.3389/fmicb.2024.1463779.

Sbeiti, Abed Al Latif; Mazurier, Mélanie; Ben, Cécile; Rickauer, Martina; Gentzbittel, Laurent (2023): Temperature increase modifies susceptibility to Verticillium wilt in Medicago spp and may contribute to the emergence of more aggressive pathogenic strains. In Frontiers in Plant Science 14, p. 1109154. DOI: 10.3389/fpls.2023.1109154.

Schneider, Caroline A.; Rasband, Wayne S.; Eliceiri, Kevin W. (2012): NIH Image to ImageJ: 25 years of image analysis. In Nature Methods 9 (7), pp. 671–675. DOI: 10.1038/nmeth.2089.

Sedlazeck, Fritz J.; Rescheneder, Philipp; Smolka, Moritz; Fang, Han; Nattestad, Maria; Haeseler, Arndt von; Schatz, Michael C. (2018): Accurate detection of complex structural variations using single-molecule sequencing. In Nature methods 15 (6), pp. 461–468. DOI: 10.1038/s41592-018-0001-7.

Seidl, Michael F.; Kramer, H. Martin; Cook, David E.; Fiorin, Gabriel L.; van den Berg, Grardy C. M.; Faino, Luigi; Thomma, Bart P. H. J. (2020): Repetitive elements contribute to the diversity and evolution of centromeres in the fungal genus Verticillium. In MBio 11 (5). DOI: 10.1128/mbio.01714-20.

Seidl, Michael F.; Thomma, Bart P. H. J. (2014): Sex or no sex: evolutionary adaptation occurs regardless. In BioEssays: news and reviews in molecular, cellular and developmental biology 36 (4), pp. 335–345. DOI: 10.1002/bies.201300155.

Seidl, Michael F.; Thomma, Bart P. H. J. (2017): Transposable elements direct the coevolution between plants and microbes. In Trends in Genetics 33 (11), pp. 842–851. DOI: 10.1016/j.tig.2017.07.003.

Shen, Li; Wang, Yu-Ting; Tang, Xing-Xing; Zhang, Ke; Wang, Pin-Mei; Sui, Yang; Zheng, Dao-Qiong (2020): Heat shock drives genomic instability and phenotypic variations in yeast. In AMB Express 10 (1), p. 146. DOI: 10.1186/s13568-020-01091-7.

Shen, Xing-Xing; Opulente, Dana A.; Kominek, Jacek; Zhou, Xiaofan; Steenwyk, Jacob L.; Buh, Kelly V. et al. (2018): Tempo and mode of genome evolution in the budding yeast subphylum. In Cell 175 (6), 1533-1545.e20. DOI: 10.1016/j.cell.2018.10.023.

Shi-Kunne, Xiaoqian; Faino, Luigi; van den Berg, Grardy C. M.; Thomma, Bart P. H. J.; Seidl, Michael F. (2018): Evolution within the fungal genus Verticillium is characterized by chromosomal rearrangement and gene loss. In Environmental Microbiology 20 (4), pp. 1362–1373. DOI: 10.1111/1462-2920.14037.

Shi-Kunne, Xiaoqian; Jové, Roger de Pedro; Depotter, Jasper R. L.; Ebert, Malaika K.; Seidl, Michael F.; Thomma, Bart P. H. J. (2019): *In silico* prediction and characterisation of secondary metabolite clusters in the plant pathogenic fungus *Verticillium dahliae.* In FEMS Microbiology Letters 366 (7). DOI: 10.1093/femsle/fnz081.

Short, Dylan P. G.; Gurung, Suraj; Hu, Xiaoping; Inderbitzin, Patrik; Subbarao, Krishna V. (2014): Maintenance of sex-related genes and the co-occurrence of both mating types in *Verticillium dahliae*. In PLOS One 9 (11), e112145. DOI: 10.1371/journal.pone.0112145.

Showpnil, Iftekhar A.; Selich-Anderson, Julia; Taslim, Cenny; Boone, Megann A.; Crow, Jesse C.; Theisen, Emily R.; Lessnick, Stephen L. (2022): EWS/FLI mediated reprogramming of 3D chromatin promotes an altered transcriptional state in Ewing sarcoma. In Nucleic Acids Research 50 (17), pp. 9814–9837. DOI: 10.1093/nar/gkac747.

Smith, Amanda C.; Rizvi, Hassan; Hickman, Meleah A.; Morran, Levi T. (2022): Increased virulence and large-scale reduction in genome size of tetraploid candida albicans evolved in nematode hosts. In Frontiers in Fungal Biology 3, p. 903135. DOI: 10.3389/ffunb.2022.903135.

Smolka, Moritz; Paulin, Luis F.; Grochowski, Christopher M.; Horner, Dominic W.; Mahmoud, Medhat; Behera, Sairam et al. (2024): Detection of mosaic and population-level structural variants with Sniffles2. In Nature Biotechnology 42 (10), pp. 1571–1580. DOI: 10.1038/s41587-023-02024-y.

Snelders, Nick C.; Rovenich, Hanna; Petti, Gabriella C.; Rocafort, Mercedes; van den Berg, Grardy C. M.; Vorholt, Julia A. et al. (2020): Microbiome manipulation by a soil-borne fungal plant pathogen using effector proteins. In Nature Plants 6 (11), pp. 1365–1374. DOI: 10.1038/s41477-020-00799-5.

Snelders, Nick C.; Rovenich, Hanna; Thomma, Bart P. H. J. (2022): Microbiota manipulation through the secretion of effector proteins is fundamental to the wealth of lifestyles in the fungal kingdom. In FEMS Microbiology Reviews 46 (5). DOI: 10.1093/femsre/fuac022.

Sokol, Noah W.; Slessarev, Eric; Marschmann, Gianna L.; Nicolas, Alexa; Blazewicz, Steven J.; Brodie, Eoin L. et al. (2022): Life and death in the soil microbiome: how ecological processes influence biogeochemistry. In Nature Reviews. Microbiology 20 (7), pp. 415–430. DOI: 10.1038/s41579-022-00695-z#.

Speijer, Dave; Lukeš, Julius; Eliáš, Marek (2015): Sex is a ubiquitous, ancient, and inherent attribute of eukaryotic life. In Proceedings of the National Academy of Sciences of the United States of America 112 (29), pp. 8827–8834. DOI: 10.1073/pnas.1501725112.

Spooren, Jelle; van Bentum, Sietske; Thomashow, Linda S.; Pieterse, Corné M. J.; Weller, David M.; Berendsen, Roeland L. (2024): Plant-driven assembly of disease-suppressive soil microbiomes. In Annual Review of Phytopathology 62 (1), pp. 1–30. DOI: 10.1146/annurev-phyto-021622-100127.

Strom, Noah B.; Bushley, Kathryn E. (2016): Two genomes are better than one: history, genetics, and biotechnological applications of fungal heterokaryons. In Fungal Biology and Biotechnology 3, p. 4. DOI: 10.1186/s40694-016-0022-x.

Strotz, Luke C.; Simões, Marianna; Girard, Matthew G.; Breitkreuz, Laura; Kimmig, Julien; Lieberman, Bruce S. (2018): Getting somewhere with the Red Queen: chasing a biologically modern definition of the hypothesis. In Biology Letters 14 (5). DOI: 10.1098/rsbl.2017.0734.

Stroud, James T.; Ratcliff, William C. (2025): Long-term studies provide unique insights into evolution. In Nature 639 (8055), pp. 589–601. DOI: 10.1038/s41586-025-08597-9.

Sun, Sheng; Coelho, Marco A.; David-Palma, Márcia; Huang, Jun; Bian, Zhuyun; Heitman, Joseph (2025): Fungal sexual reproduction and mating-type loci. In Current Biology 35 (11), R496-R503. DOI: 10.1016/j.cub.2025.04.061.

Tarkka, Mika T.; Sarniguet, Alain; Frey-Klett, Pascale (2009): Inter-kingdom encounters: recent advances in molecular bacterium-fungus interactions. In Current Genetics 55 (3), pp. 233–243. DOI: 10.1007/s00294-009-0241-2.

Taylor, John W.; Hann-Soden, Christopher; Branco, Sara; Sylvain, Iman; Ellison, Christopher E. (2015): Clonal reproduction in fungi. In Proceedings of the National Academy of Sciences of the United States of America 112 (29), pp. 8901–8908. DOI: 10.1073/pnas.1503159112.

Tenaillon, Olivier; Barrick, Jeffrey E.; Ribeck, Noah; Deatherage, Daniel E.; Blanchard, Jeffrey L.; Dasgupta, Aurko et al. (2016): Tempo and mode of genome evolution in a 50,000-generation experiment. In Nature 536 (7615), pp. 165–170. DOI: 10.1038/nature18959.

Terwagne, Matthieu; Nicolas, Emilien; Hespeels, Boris; Herter, Ludovic; Virgo, Julie; Demazy, Catherine et al. (2022): DNA repair during nonreductional meiosis in the asexual rotifer Adineta vaga. In Science Advances 8 (48), eadc8829. DOI: 10.1126/sciadv.adc8829.

Tisserant, E.; Kohler, A.; Dozolme-Seddas, P.; Balestrini, R.; Benabdellah, K.; Colard, A. et al. (2012): The transcriptome of the arbuscular mycorrhizal fungus Glomus intraradices (DAOM 197198) reveals functional tradeoffs in an obligate symbiont. In The New phytologist 193 (3), pp. 755–769. DOI: 10.1111/j.1469-8137.2011.03948.x.

Torres, David E.; Kramer, H. Martin; Tracanna, Vittorio; Fiorin, Gabriel L.; Cook, David E.; Seidl, Michael F.; Thomma, Bart P. H. J. (2024): Implications of the three-dimensional chromatin organization for genome evolution in a fungal plant pathogen. In Nature Communications 15 (1), p. 1701. DOI: 10.1038/s41467-024-45884-x.

Torres, David E.; Oggenfuss, Ursula; Croll, Daniel; Seidl, Michael F. (2020): Genome evolution in fungal plant pathogens: looking beyond the two-speed genome model. In Fungal Biology Reviews 34 (3), pp. 136–143. DOI: 10.1016/j.fbr.2020.07.001.

Torres, David E.; Thomma, Bart P. H. J.; Seidl, Michael F. (2021): Transposable elements contribute to genome dynamics and gene expression variation in the fungal plant pathogen *Verticillium dahliae*. In Genome Biology and Evolution 13 (7). DOI: 10.1093/gbe/evab135.

Turrà, David; El Ghalid, Mennat; Rossi, Federico; Di Pietro, Antonio (2015): Fungal pathogen uses sex pheromone receptor for chemotropic sensing of host plant signals. In Nature 527 (7579), pp. 521–524. DOI: 10.1038/nature15516.

Urquhart, Andrew; Vogan, Aaron A.; Gluck-Thaler, Emile (2024): Starships: a new frontier for fungal biology. In Trends in Genetics 40 (12), pp. 1060–1073. DOI: 10.1016/j.tig.2024.08.006.

Urquhart, Andrew S.; Forsythe, Adrian; Vogan, Aaron A. (2025): Are Fungal Disease Outbreaks Instigated by Starship Transposons? In Molecular Plant Pathology 26 (7), e70124. DOI: 10.1111/mpp.70124.

van Valen, Leigh (1973): A new evolutionary law. In Evolutionary Theory 1:1–30.

Vande Zande, Pétra; Zhou, Xin; Selmecki, Anna (2023): The dynamic fungal genome: polyploidy, aneuploidy and copy number variation in response to stress. In Annual Review of Microbiology 77, pp. 341–361. DOI: 10.1146/annurev-micro-041320-112443.

Vandenkoornhuyse, Philippe; Quaiser, Achim; Duhamel, Marie; Le Van, Amandine; Dufresne, Alexis (2015): The importance of the microbiome of the plant holobiont. In The New Phytologist 206 (4), pp. 1196–1206. DOI: 10.1111/nph.13312.

Virtanen, Pauli; Gommers, Ralf; Oliphant, Travis E.; Haberland, Matt; Reddy, Tyler; Cournapeau, David et al. (2020): SciPy 1.0: fundamental algorithms for scientific computing in Python. In Nature Methods 17 (3), pp. 261–272. DOI: 10.1038/s41592-019-0686-2.

Vitale, Stefania; Barberio, Antonia; Cantelli, Riccardo; Ranesi, Marta; Curtis, Filippo de; Di Pietro, Antonio; Turrà, David (2026): Mating-type loci modulate pathogenicity and non-sexual development through autocrine pheromone signalling in the asexual fungus *Fusarium oxysporum*. In Molecular Plant Pathology 27 (3), e70248. DOI: 10.1111/mpp.70248.

Wagner, Günter P.; Kin, Koryu; Lynch, Vincent J. (2012): Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. In Theory in Biosciences 131 (4), pp. 281–285. DOI: 10.1007/s12064-012-0162-3.

Wallen, R. M.; Perlin, Michael H. (2018): An overview of the function and maintenance of sexual reproduction in dikaryotic fungi. In Frontiers in Microbiology 9, p. 503. DOI: 10.3389/fmicb.2018.00503.

Wang, Jiao-Yu; Wang, Shi-Zhen; Zhang, Zhen; Hao, Zhong-Na; Shi, Xiao-Xiao; Li, Ling et al. (2021): *MAT* loci play crucial roles in sexual development but are dispensable for asexual reproduction and pathogenicity in rice blast fungus *Magnaporthe oryzae*. In Journal of Fungi (Basel, Switzerland) 7 (10). DOI: 10.3390/jof7100858.

Wick, Ryan (2018): Porechop. Version 0.2.4: GitHub. Available online at https://github.com/rrwick/Porechop, checked on 1/31/2022.

Wickham, Hadley (Ed.) (2016): ggplot2. Cham: Springer International Publishing (Use R!).

Wiser, Michael J.; Ribeck, Noah; Lenski, Richard E. (2013): Long-term dynamics of adaptation in asexual populations. In Science (New York, N.Y.) 342 (6164), pp. 1364–1367. DOI: 10.1126/science.1243357.

Wu, Tianzhi; Hu, Erqiang; Xu, Shuangbin; Chen, Meijun; Guo, Pingfan; Dai, Zehan et al. (2021): clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. In Innovation (Cambridge (Mass.)) 2 (3), p. 100141. DOI: 10.1016/j.xinn.2021.100141.

Xiao, Jordan Yupeng; Hafner, Antonina; Boettiger, Alistair N. (2021): How subtle changes in 3D structure can create large changes in transcription. In ELife 10. DOI: 10.7554/eLife.64320.

Xue, Chaoyang; Tada, Yasuomi; Dong, Xinnian; Heitman, Joseph (2007): The human fungal pathogen Cryptococcus can complete its sexual cycle during a pathogenic association with plants. In Cell Host & Microbe 1 (4), pp. 263–273. DOI: 10.1016/j.chom.2007.05.005.

Yan, Zhun; Li, Xiaogang; Xu, Jianping (2002): Geographic distribution of mating type alleles of *Cryptococcus neoformans* in four areas of the United States. In Journal of Clinical Microbiology 40 (3), pp. 965–972. DOI: 10.1128/JCM.40.3.965-972.2002.

Yang, Z.; Nielsen, R. (2000): Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. In Molecular Biology and Evolution 17 (1), pp. 32–43. DOI: 10.1093/oxfordjournals.molbev.a026236.

Yeh, Chung-Shu; Wang, Zhifeng; Miao, Fang; Ma, Hongyan; Kao, Chung-Ting; Hsu, Tzu-Shu et al. (2019): A novel synthetic-genetic-array-based yeast one-hybrid system for high discovery rate and short processing time. In Genome Research 29 (8), pp. 1343–1351. DOI: 10.1101/gr.245951.118.

Yong, Mingli; Yu, Junjie; Pan, Xiayan; Yu, Mina; Cao, Huijuan; Song, Tianqiao et al. (2020): Two mating-type genes *MAT1-1-1* and *MAT1-1-2* with significant functions in conidiation, stress response, sexual development, and pathogenicity of rice false smut fungus *Villosiclava virens*. In Current Genetics 66 (5), pp. 989–1002. DOI: 10.1007/s00294-020-01085-9.

Yoshida, Kentaro; Schuenemann, Verena J.; Cano, Liliana M.; Pais, Marina; Mishra, Bagdevi; Sharma, Rahul et al. (2013): The rise and fall of the Phytophthora infestans lineage that triggered the Irish potato famine. In ELife 2, e00731. DOI: 10.7554/eLife.00731.

Yu, Guangchuang; Wang, Li-Gen; Han, Yanyan; He, Qing-Yu (2012): clusterProfiler: an R package for comparing biological themes among gene clusters. In Omics: a journal of integrative biology 16 (5), pp. 284–287. DOI: 10.1089/omi.2011.0118.

Yu, Stephanie C. Y.; Deng, Jiaen; Qiao, Rong; Cheng, Suk Hang; Peng, Wenlei; Lau, So Ling et al. (2023): Comparison of single molecule, real-time sequencing and nanopore sequencing for analysis of the size, end-motif, and tissue-of-origin of long cell-free dna in plasma. In Clinical Chemistry 69 (2), pp. 168–179. DOI: 10.1093/clinchem/hvac180.

Yu, Yidong; Blachowicz, Adriana; Will, Cornelia; Szewczyk, Edyta; Glenn, Steven; Gensberger-Reigl, Sabrina et al. (2018): Mating-type factor-specific regulation of the fumagillin/pseurotin secondary metabolite supercluster in *Aspergillus fumigatus*. In Molecular Microbiology 110 (6), pp. 1045–1065. DOI: 10.1111/mmi.14136.

Zhang, Cheng-Zhong; Leibowitz, Mitchell L.; Pellman, David (2013): Chromothripsis and beyond: rapid genome evolution from complex chromosomal rearrangements. In Genes & Development 27 (23), pp. 2513–2530. DOI: 10.1101/gad.229559.113.

Zhang, Jianhua; van den Heuvel, Joost; Debets, Alfons J. M.; Verweij, Paul E.; Melchers, Willem J. G.; Zwaan, Bas J.; Schoustra, Sijmen E. (2017): Evolution of cross-resistance to medical triazoles in *Aspergillus fumigatus* through selection pressure of environmental fungicides. In Proceedings. Biological sciences 284 (1863). DOI: 10.1098/rspb.2017.0635.

Zhang, Ya-Duo; Ji, Xiao-Bin; Zong, Juan; Dai, Xiao-Feng; Klosterman, Steven J.; Subbarao, Krishna V. et al. (2024): Functional analysis of the mating type genes in *Verticillium dahliae*. In BMC Biology 22 (1), p. 108. DOI: 10.1186/s12915-024-01900-6.

Zheng, Qian; Hou, Rui; Juanyu; Zhang; Ma, Jiwen; Wu, Zhongshou et al. (2013): The *MAT* locus genes play different roles in sexual reproduction and pathogenesis in *Fusarium graminearum*. In PLOS One 8 (6), e66980. DOI: 10.1371/journal.pone.0066980.

Acknowledgements

Finally, my PhD journey is coming to an end. More than 13 years ago, I came from a family that had nothing to do with farming, yet somehow, I dreamed of becoming a farmer, simply because I loved working with plants and was fascinated by them. Back then, I thought a world filled with green would be enough for me, or perhaps with a little yellow as well, since I loved growing maize. My path eventually took a different direction and led me into the world of plant-pathogenic fungi and genome research. Along the way, I realized that what truly drives me is the excitement of finding intriguing questions and the desire to follow them wherever they lead. I would like to thank all the amazing people who helped me through this journey and made it more colorful than I could ever have dreamed.

First, I would like to thank Bart Thomma. It still feels almost like a miracle that I ended up in your lab after knocking on your door without even bringing a CV. Somehow, that knock led to this PhD thesis. Thank you for sharing so much scientific knowledge and for building a lab full of talented people who are always willing to help one another. I truly appreciated meeting all the AG Thomma members who became part of my journey.

Second, I would like to thank all the collaborators who joined me in exploring the scientific questions that intrigued me. I would like to thanks Edgar and Andrea, you two were my first close collaborators, and I learned a lot from you, from how to work as a team and interact with students to how to enjoy doing research together rather than alone. I would like to thank Julie, who joined me toward the end of my PhD. You were always very positive about our research progress and willing to try again and again. I admire your passion for science and your kindness in helping others. I would also like to thank Jiyeun. You joined me during the final stage of my thesis writing, and without me having to beg too much, you always said yes whenever I asked for help. You generously carried out a great deal of analysis, joined all my Zoom meetings, and responded to my many messages. Discussing problems with you gave me motivation to get up in the morning and keep going, especially toward the end, when we were working across a time difference! Your support made this difficult final stage feel far less lonely. Lastly, I would like to thank all the students who have worked with me, Gabin, Alexander, Ciara, Maike, and Severin. I had a lot of fun interacting and working with you all, especially when you guys filled the entire sterile bench room, and I also learned a lot throughout the process.

Next, I would like to thank my ‘secret supervisors’, who were always there to guide me whenever I needed help. First, 卉 (Hui), there are so many things I need to thank you for that I do not think it makes sense to list them all here. I truly appreciate your guidance, your friendship, and everything you have taught me, both scientifically and in life. This thesis would not have been completed without your support, even until the very end of the writing process. I would like to thank Anton. For me, it is very important to have someone to discuss my thoughts

with during the writing process, and you were there when I needed it. I really appreciate your kindness! I would like to thank Daniele. I actually do not know how we ended up being on the same wavelength, especially because you always replied to my messages at a speed beyond my imagination of modern technology. But clearly, something brought us closer. I admire your passion for science and for life, and I appreciate everything I learned from you. When I turned 30, you gave me a bookmark that said, “Patience is an art; perseverance is a kind of hope. With patience and perseverance, one can achieve success in doing things and attain harmony in life.” I kept it on my desk while finishing this PhD thesis. Thank you for cheering me on and believing that I could finish this journey. Lastly, Tanya, thank you for being my rest hold throughout this PhD journey, although I guess you may never go bouldering again. Whenever I came to you with excitement or confusion, you were always there to listen, share my happiness, and keep me going. I think it is a luxury to have a friend like you in Europe, someone with such a different background from mine, yet with whom I could share so much fun and laughter. It has also been a true joy to realize how much humor I could find in our WhatsApp conversations. Somehow, you always managed to bring out my ability to joke around, and I still do not quite know how you did it.

I am also very thankful to the people in the “cool office.” Starting with my bro Wilko, you are one of the most positive people I have met, and I learned a lot from you. Without you, my PhD time would not have been nearly as fun. Gabbo, although you are not per se the most relaxed person, I always feel more relaxed with you. Around you, things do not always need to be planned, and I do not feel that I need to prove myself. Visiting your hometown is one of the most memorable experiences of my PhD journey! I am also truly thankful that whenever I shared a problem with you, you always calmly tried to help me find a solution. I also need to thank Gerlind. Somehow, you became my emergency line toward the end of my PhD whenever I needed help, and you always managed to help me. I feel very lucky to have received your help! And Ciaran, I think you are a very creative person. We did not cross paths that often, but I admire your artistic spirit in both science and life.

Of course, my PhD journey would not have been complete without the many people I met in AG Thomma, especially because the large-scale *in planta* inoculations were supported by so many people. I would like to thank all the employees and visiting researchers in AG Thomma. Special thanks to Heidrun and Eva, who were always calmly helping to keep the lab running. Whenever I arrived early in the morning, you were also the people I could have a short coffee chat with. Also, thank you Ana! I enjoyed eating good food with you, having special cooking sessions together, and playing with your cat. I was often amazed by your hospitality.

Besides all the people who were scientifically associated with me, I also need to thank my friends who helped me enjoy life. Thank you, Esperanza, Karo, and 航 (Hang). You three are such positive and relaxed people, and I always regained energy for both research and life after spending time with you, maybe because we were always talking about good food. Thank you,

Leia. You are a very warm person, and I truly appreciate all the support you gave me from the very beginning of my PhD. From you, I learned that there are so many important things in the world worth caring about, and I wish more people could be a little bit like you in caring for the environment and society. Thank you, Harim eon-ni! Besides Kai, you are really my only close friend in Europe with no connection to academia, and you showed me how many things there still are to learn in life. You always inspired me with new ideas and interests, and I see you as someone truly passionate about living. I also appreciate that you answered my very first call to play badminton, even though in the end we mostly just ate together.

I would also like to thank my Taiwanese teachers, 穎佳 and 淳卉. You two have always supported my decisions since high school and have seen me grow in many different directions. From enjoying eating the crops I grew to listening to whatever academic accomplishments I was excited about, even when you might not have been sure why they mattered so much to me, you always smiled. Finally, I can proudly tell you that I am getting a PhD. Also, 子瑩, I am very lucky to have you as my long-term friend. I am grateful that my wall is filled with postcards from you, even though I have always been busy and have not sent you many in return so far. I am glad to have a friend who sees life so differently from me, but who has supported and listened to me over the years, no matter how much I have changed. I would like to thank my family, as they have always respected my decisions and my time, and supported what I wanted to try, no matter whether my decision was to continue on the original path, end a path, or pursue something new. I used to think the freedom and trust I had were normal, but slowly I realized that not everyone has them. Therefore, I truly appreciate being able to live with a free spirit.

Last, here you are, my chef and husband, Kai. We made it! You were my closest companion throughout this writing journey, even though you still asked whether Gabin was actually Gabbo, or whether I studied molecular biology after I had already finished writing all the thesis chapters. I know this journey was not easy for you either. I often kept my work thoughts among my colleagues, while you were left waiting beside me, probably wondering when your wife would finally return from the mysterious world of fungi. I truly appreciate how you tried to understand my research and struggled with me to find appropriate academic German terminology for my thesis summary. Thank you for all the amazing food that keeps me healthy, comforted, and much less homesick while I am far away from my hometown. Most of all, thank you for supporting what I wanted to pursue, giving me the space to become who I wanted to become, and waiting for me throughout this very, very long marathon. In the end, I realized that what motivates my life is not only endless scientific questions, but also the many questions in life that I want to explore with you. I am looking forward to exploring this world more with you, but maybe we should first survive the 50 km one-day walk in August.