Testing for adaptive evolution of floral scent using genomics, metabolomics, and phylogenetics

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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April 2023

Abstract

Floral volatiles are a key olfactory communication channel that influences plant interactions with pollinators and other organisms. Plants rely on their floral secondary metabolism to recruit pollinating insects – for plants which are incapable of self-fertilisation, pollinator recruitment is a critical life history function. However, the dynamics and drivers of floral scent evolution remain poorly understood. Research has highlighted environmental factors such as herbivory stress not only cause upregulation of leaf metabolites, but also flower metabolites, suggesting that floral chemical diversity may be shaped by a wider range of ecological interactions than is currently appreciated. The first chapter of my thesis demonstrates the strengths and limitations of past and current research on the evolution of floral scent. In chapters two and three, I focus on identifying adaptive variation in floral secondary metabolism in outcrossing and self-fertilizing plants. My thesis uses a comparative evolutionary approach, combining metabolomics with species-level phylogenetic data on the magnitude of induced responses in different plant tissues across 20 closely related wild potatoes (chapter 2), and with whole-genome sequence data to test the role of climatic variables and mating system transitions in driving the evolution of floral scents and the genes responsible for it, in populations of *Arabidopsis lyrata* (chapter 3). This work gives insight into the evolution of the remarkable diversity of plant reproductive phenotypes in concert with mating system transitions. Particularly, my results are among the first to show the repeated, parallel evolution of floral scent in plants that are either dependent on insects for pollination or can self-fertilise. I observed striking reductions in emission of floral scent compounds in self-fertilizing populations, suggesting the repeated evolution of an "olfactory selfing syndrome". I also propose a new conceptual model for the dynamics of floral trait evolution in selfers. Most plants also upregulate defensive secondary metabolites against microbial attack, and in the fourth chapter, I examined the interaction and dynamics of cellulose and callose synthase complexes at the site of pathogen interaction using super-resolution microscopy approach. My results lead to the discovery of currently uncharacterised migration mechanism of callose synthase complexes and associated callose polymer synthesis in the apoplast. The study helped to identify further research targets for possible modification and optimization of defence-related callose deposition at infection sites to increase pathogen resistance. Overall, my research findings have yielded novel insight into the evolution of flowering plant phenotypic traits, which may help in developing crops for optimal floral signalling and optimal defence.

Acknowledgements

I am extremely grateful to everyone that has guided and supported me during my PhD journey.

Most importantly, my profound gratitude to my supervisor, Dr Stuart Campbell, for accepting the responsibility to supervise my PhD research after the departure of my former supervisor from the university. I would like to thank him for being an invaluable mentor while providing me with incredible guidance and support throughout my PhD. He has been committed, encouraging, communicative and patient through every step of my project, providing extensive advice on data analysis, thesis writing, research presentation and professional development. I am extremely grateful for his time, professionalism, and enthusiasm towards the success of this dissertation.

I also extend special thanks to Dr Christian Voigt for a year of supervision on callose project before his departure from the university. I thank him for providing me with this incredible opportunity of a PhD research in his laboratory in the first place. I appreciate his commitments to the project, the dSTORM imaging and the assistance and advice he rendered on image data analysis generally. I am also appreciative of past and present members of the Campbell lab and Plant Biotic Interactions Lab group for their support, encouragement, and advice during my time at the University of Sheffield. Someone who merit mention include Nancy Muringai, for her help and advice in my first year of PhD that continues for me till date.

I would also like to thank my collaborators. My thesis was computational, and relied on diverse datasets accumulated over a number of years with the support of numerous collaborators from different institutions (most of whom I am yet to meet), particularly: Andre Kessler Lab (Cornell University), John Bamberg, Jesse Schartner (USDA-ARS Potato Genebank), Rayko Halitschke (Max Planck Institute for Chemical Ecology), Spencer Barrett and Stephen Wright Labs (University of Toronto), and also Barbara Mable (University of Glasgow), and Yvonne Willi (Université de Neuchâtel).

I am grateful to the University of Sheffield for University of Sheffield Faculty of Science University Post Graduate Research Committee (UPGRC) Fee Scholarship that have supported my research through a full tuition grant. I also thank the Science Graduate School for the publication scholarship that funded an additional term to reformat the most important outcomes of my study for publication. This allowed me to concentrate on finishing my thesis during the primary registration time and to look for publication opportunities after submission.

I thank my family members. To my parents (Oba Julius Oluropo Fatanmi and Olori Esther Fatanmi) and my siblings (Victor, Olamide and John), for all their support, encouragement, inspiration, prayers and for being the best friends anyone could ask for. I also specially thank my brother-in-law, Dr. Lanre Oladapo for his immense support that made it possible to start off with my PhD program.

I will be forever grateful to my husband, Seyi Oladapo for his unwavering support, patience, sense of humour, and being a constant source of motivation. I thank him for listening and giving me useful feedback every time I rehearsed my presentations. I appreciate his unconditional love towards me every day. When I felt discouraged, there he was lifting my spirits. I thank him for being my best friend and for being a caring and trustworthy partner. I feel blessed to have Deborah and Joshua as my children. I thank them for understanding when mummy needed some space and was not available to play hide and seek. Whenever I looked at their beautiful faces and saw how appreciative they were with the little gift, care, and time I was able to dedicate to them, I got motivated and excited. They made life bearable for me during my PhD program. They are part of my success story.

Ultimately, all thanks to the almighty God for giving me these gifts of invaluable people. I thank him for the strength to undertake a PhD program and for all the support I got. I never would have made it this far without the help of God.

COVID-19 impact statement

Firstly, due to the COVID-19 pandemic, laboratories were closed from March to November 2020, with restricted or no access, when typically, I would have been running experiments. During that period, I managed to do some writing of my research literature review. Prior to covid 19, I had started to generate and grow some transgenic plants which were all lost due to restrictions on use of the controlled environment facility. I also lost some data collection which meant increased data collection in the following year.

Secondly, my two school-age children were not in school at this period as schools were all closed leaving just a very tiny percentage of my time for my PhD, so I did most of the writings at night whenever I could.

More significantly, I am supported by the Faculty of Science for my tuition fee, but I do not receive a stipend. Prior to covid-19, I had a potential financial sponsorship of £20,000 per year to cover for my living expenses during my PhD program. Unfortunately, the sponsor withdrew from the grant due to the economic recession because of covid. Consequently, I have worked part-time for most of my PhD program to support myself and my young family.

Finally, my initial supervisor left the university while the university adjusted to the COVID19 impact at the time of my confirmation review. As a result, I was left seeking a new project, which led me to start work in a new research group in my year 2 (March 2021), on a completely different project, in a completely different field. Because of the COVID restrictions mentioned above, my new PhD needed to be computationally focused.

Despite these challenges, I quickly adapted to a new theoretical and technical background and carried out comprehensive analyses and I rapidly learnt a broad suite of new bioinformatics and coding skills for the new project. I managed to finish my PhD within my thesis submission deadline which allowed me to obtain a competitive 3-month publication scholarship. I will be writing up two manuscripts for publication in high impact journals following my thesis submission. Because of the variety of my topics, I appreciate the fact that I have learned broadly (three different projects) and might not be coming out of my PhD program as the single most knowledgeable expert in each one. However, I am particularly proud of having learned such broad range of technical and professional skills, which I know will benefit my future career, and having produced this thesis while enduring numerous intersecting challenges.

Declaration and co-author contributions

My thesis format depicts stand-alone research papers. I take ownership of the work in this thesis with contribution from co-authors which are acknowledged at the beginning of each chapter including the list of the contributions they made. I will be first author on two high-impact papers arising from my work. I initially plan to submit Chapter 2 to Nature Ecology and Evolution, one of the top journals in this field and Chapter 3 to Proceedings of the National Academy of Sciences (PNAS), one of the top general science journals.

List of Figures

List of Tables

Table of Contents

Chapter 1:

General Introduction

1.1 Background

Floral scent mediates interactions of plants with mutualists such as pollinators and antagonists such as herbivores, which then act as agents of selection. These interactions can in theory drive various evolutionary transitions including mating system transitions or pollination system transitions in plants, with consequences for the evolution of floral traits. For instance, an absence or limited abundance of pollinators can lead to the evolution of self-fertilization (Moeller *et al*., 2012) and can lead to evolutionary reductions in flower size and number. However, in contrast to morphological traits, empirical studies of floral scent evolution remain surprisingly limited, despite the extraordinary diversity of floral secondary metabolism. Analysing variation in floral scent at different evolutionary scales and in relation to transitions in plant reproductive systems, can contribute to a mechanistic understanding of how scent variation evolves. For this, I combined species-level phylogenetic data on the magnitude of both constitutive and induced floral scent in 20 closely related wild potatoes, together with whole-genome sequence data to test the role of climate variables and mating system transitions in driving the evolution of floral scents, in populations of *Arabidopsis lyrata*. These investigations allowed me to compare and contrast floral scent variation at two different evolutionary scales: within species (microevolutionary scale) and among species (macroevolution). This thesis focuses primarily on floral scent diversity in relation to mating system shifts.

Mating system transitions have significant impacts on the ecology, distribution, and genetic diversity of plants, particularly their interactions with other organisms (Campbell and Kessler, 2013; Evans and Jacquemyn, 2020; Benjamin *et al*., 2018). Mating system is the relative amount of inbreeding (e.g., self-fertilisation) vs. outcrossing for a population or a species (Karron *et al*., 2012). Mating systems occur on a continuum, from obligate outcrossing to various forms of inbreeding. When inbreeding occurs between separate but closely related individuals, it is termed biparental inbreeding (Levin, 1981; van Rossum and Triest, 2007). At the other end of the continuum self-fertilization, commonly referred to as "selfing" is another type of inbreeding exclusive to hermaphroditic species (Avise, 2009). Many angiosperms have hermaphroditic flowers that permits selfing, outcrossing, and mixed mating (Avise, 2009).

Selfed progeny are more homozygous and have lower allelic diversity in comparison to outcrossers (Neaves *et al*., 2015). The reduction in genetic diversity and the concomitant reduction in the efficacy of recombination can hinder the future adaptation of selfers (Neaves *et al*., 2015; Stebbins, 1974; Igic *et al*., 2006). Selfing also leads to changes in a set of floral traits, including smaller flowers, reduction in pollen–ovule ratio, formation of flowers that do not open (cleistogamy) and reduced distance between anther and stigma: this set of traits is termed the selfing syndrome (Duncan and Rausher, 2013). Selfing can be costly because of "inbreeding depression" (Darwin, 1876; Busch, 2005; Barrett, 2010; Goldberg *et al*., 2010). Inbreeding depression is the reduction in offspring fitness brought on by inbreeding. Hence, many plants possess characteristics that limit inbreeding (Charlesworth and Willis, 2009). Despite these drawbacks, flowering plants frequently go through the evolutionary shift from outcrossing to self-fertilization (Stebbins, 1974; Igic *et al*., 2006; Barrett, 2010; Goldberg *et al*., 2010) leading to a remarkable diversity of floral traits.

Frequent shifts to selfing are attributed to its beneficial effects, namely transmission advantage and reproductive assurance (Baker, 1955; Tsuchimatsu and Fujii, 2022). Selfing populations transmit two copies of their genes to their progenies, while outcrossing populations pass on only one copy (Fisher, 1941). This can result in a 2:1 ratio of transmission advantage for selfing genes compared with outcrossing genes, if selfing does not accompany pollen discounting (Nagylacki, 1976) and in the absence of strong inbreeding depression. Self-fertilization persistently displaces the apparent necessity of biparental sex in eukaryotes because a plant that can fertilise itself does not have to rely on pollinators or mates (Darwin, 1876). This is one of the most obvious theoretical benefits of self-fertilization over cross-fertilization. This benefit could help to explain why selfing is more prevalent in populations that have unreliable access to pollinators or mates (Darwin, 1876; Igic *et al*., 2006; Barrett, 2010; Goldberg *et al*., 2010; Moeller *et al*., 2012), such as those that are found in frequently disturbed areas, on the periphery of a species' range, or in places that can only be reached by long-distance dispersal. Pollen from "selfers" can fertilise their own ovules without having to compete with pollen from other parents, which is a more subdued benefit (Fisher, 1941).

Recent research has focused the role of mating systems in ecological traits in the context of environmental changes, particularly, the reduction in abundance of pollinators (Thomann *et al*., 2013; Hallmann *et al*., 2017) and the possibility of insect-pollinated plants being under threat. The work of Biesmeijer *et al*. (2006) demonstrated a reduction in the abundance of pollinators

and insect-pollinated plants thereby indicating a possible relationship between a decrease in pollinators and insect-pollinated plants extinction hence given rise to selfing. Eckert *et al*. (2010) identified environmental changes such as habitat fragmentation and an impoverished pollinator environment as impacting the mating system traits in plants. These findings led to studies of short-term adaptive dynamics in the context of changes in pollinators (Thomann *et al*., 2013; Campbell, 2015; Gervasi and Schiestl, 2017). A plant can avoid extinction in an isolated environment if it self-fertilizes, though the long-term expectations for selfing taxa remain uncertain: for example, Stebbins (1957), considered selfing over a long period of time to be an evolutionary stalemate, while Goldberg *et al*. (2010) showed a significantly higher diversification rate in outcrossing than selfing lineages thereby favouring obligate outcrossing over the short-term advantages of selfing. These findings may suggest an underlying explanation for mating system not being neutral in the context of population and interspecies interactions. This in turn suggests that the effects of mating system on reproductive traits may differ depending on the evolutionary timescale. Transitions to selfing may lead to selection for outcrossing related traits in population in the short term, but these traits may become detrimental (selected against) in the long term. This hypothesis is consistent with Igic and Busch (2013) who speculated that selective advantages at play at a microevolutionary level are not necessarily consistent at a macroevolutionary scale in the long term. Hence, analysing variation in mating system related floral traits at both microevolutionary and macroevolutionary scales will enhance our understanding of the trait evolution.

1.2 Dynamics of mating system related traits: micro- and macro- evolutionary studies

Mating system transitions have been much discussed in evolutionary ecology (Yampolsky and Yampolsky, 1922, Duncan and Rausher, 2013, Campbell and Kessler, 2013; Evans and Jacquemyn, 2020; Benjamin *et al*., 2018). Many studies have established the evolutionary patterns of mating systems (Levin, 1981; van Rossum and Triest, 2007; Avise, 2009; Neaves *et al*., 2015). Experimental work has demonstrated that selfing can evolve within a species or population over a short period of time (microevolution) as reproductive assurance in response to changes in the environments (for example, impoverished pollinator communities) (Thomann *et al*., 2013; Gervasi and Schiestl, 2017). Conversely, phylogenetic studies have shown repeated and independent evolution of selfing over a long period of time (macroevolution) among closely related species (Igic, *et al*., 2008). Research findings have indicated that

transition to selfing can have contrasting effects depending on the evolutionary scale (Cheptou, 2019).

A key method for revealing general trends of adaptive evolution is to focus on the repeated, parallel evolution of the same traits across diverse taxa or populations (Stern, 2013; Tsuchimatsu and Shimizu, 2013, Tsuchimatsu and Fujii, 2022). Selfing populations or taxa in flowering plants have provided a superb platform for studying parallel or convergent evolutionary patterns of selfing for many years (Campbell and Kessler, 2013). Adaptive traits of repeated independent evolution of selfing among populations have been intensively studied for decades. Ashman and Majetic (2006) found a significant floral trait heritability and variation in line with mating system (particularly changes in anther–stigma distance) and pollinator attraction (flower corolla width and nectar production) in a set of 41 hermaphroditic populations. Thus, traits related to mating system can respond to selection over a short period of time (microevolution). In support of the genetic basis of selfing traits, significant population-level variation can also be impacted by adaptation to local ecological conditions (Whitehead *et al*., 2018). Recent pollinator loss in America and Europe (Winfree *et al*., 2011) have yielded additional 'natural' experiments for testing for adaptation of selfing traits. Roels and Kelly (2011) studied this by performing a sophisticated experiment with the species *Mimulus guttatus* and discovered changes in the anther-stigma separartion (herkogamy) and a plant's capacity to produce seeds without pollinators, over a few generations. The evolutionary potential in this species was not constrained and notably, the heritability of the anther-stigma separation was much higher after selection. Brys and Jacquemyn (2012) have also noted this form of development towards selfing in wild populations of *Centaurium erythraea* in which populations with substantial pollinator activity were compared with populations with a scarcity of pollinators. They found reduced herkogamy and a higher capacity for independent selfing in isolated habitat. These findings show how mating systems can naturally be associated with rapid adaptation in response to environmental change.

Other studies indicate that the evolution of selfing may `not necessarily be an immediate effect of pollinator scarcity. For example, Thomann *et al*. (2015) reported a smaller capitula (flower heads size) and a lower floral display in old self-incompatible populations of *Centaurea cyanus* with pollinator abundance than 18 years newer populations which remained self-incompatible with pollinator scarcity. While this report may seem contrasting to previous studies, increase in attractiveness in the recent self-incompatible populations may indeed indicate a response to

pollinator limitation. Such findings may indicate the impact of time on the evolution of plant mating systems and the likelihood that evolution of selfing is a result of concurrent adaptation to fragmentated habitats, pollinator decline and climate change, over time. A study of selfing rate and flower size association among *Collinsia* species and among *Collinsia verna* populations by McElderry *et al*. (2022), suggests that there are no associations observed between selfing rate and floral traits across *Collinsia* species at the microevolutionary scales (Kalisz *et al*., 2012). The research indicated that floral trait evolution would possibly follow step-by-step from mating system transitions, if *Collinsia verna* continue to evolve in the direction of the selfing syndrome.

Beyond the evolutionary processes among or within populations (microevolutionary), adaptive traits of repeated independent evolution of selfing among closely related species (macroevolution) is considered a frequent occurrence in flowering plants. Some of the macroevolutionary studies features the genera *Capsella* and *Solanum*. Campbell and Kessler (2013) analysed the impact of selfing on plant resistance to herbivores in 56 wild species of Solanaceae and showed that the repeated, unidirectional evolution from ancestral outcrossing to selfing leads to the evolution of a reduced constitutive resistance to herbivores, but a shift to increased leaf defensive plasticity. Many other studies have demonstrated the same reduction pattern with selfing evolution. A series of studies on *Capsella* (Brassicaceae) have identified reductions in petal size, pollen, nectar, and scent emission associated with the evolution of selfing (Sicard and Lenhard, 2018) and identified loci associated with the selfing syndrome (Slotte *et al*., 2012). The work indicates that changes at a few genomic regions can be responsible for the reduction in flora traits in selfing *Capsella*. Goldberg *et al.* (2010) described the long-term disadvantage of selfing species showing that there was an increase in the extinction rate of SC lineages over time. However, there have been only a few studies linking the species-level and population-level variation in reproductive traits.

A major mechanism underlying the evolution of floral traits associated with selfing is the loss of key traits that enforce outcrossing. Gametophytic and sporophytic incompatibility systems allow plants to selectively prevent ovule fertilization by related pollen, and include complex male and female linked molecular traits that must both function to maintain an obligately outcrossing mating system (Wright *et al*., 2013; Cutter, 2019). The loss of self-incompatibility (SI) is therefore one of the primary mechanisms facilitating the transition to selfing in flowering plants. Shifts to selfing due to, e.g., loss-of-function mutations in the SI system, then often lead

to reduced allocation to outcrossing related traits, especially those involved in attracting pollinators, such as flower corolla size, count of flowers, nectar production, flower scent, and loss of herkogamy or dichogamy are some of the features of selfing (Barrett, 2002; Sicard and Lenhard, 2011; Shimizu and Tsuchimatsu, 2015). While visual cue reductions are well-studied, there have been relatively few studies on the emission of floral scent. The few studies that have been conducted on the impact of selfing evolution on floral scent emission have been on the level of macroevolutionary scale. In short, most studies on the patterns and underlying mechanisms of adaptive traits evolution have focused on the macroevolution of self-fertilization but not on the effect of selfing transitions within a species (microevolution). Overall, there is still limited understanding of patterns of floral scent variation within and among species.

1.3 Intraspecific and interspecific evolution of floral scent variation – overview

An estimated 200,000 or more secondary metabolites are usually produced by plants (Hartmann, 2007). Plant floral volatile organic compounds (VOCs) or floral scents are secondary metabolites with high vapor pressures and are not directly involved in plant growth and development (Pichersky *et al*., 2006). Interactions of plants with mutualists and antagonists, both of which might serve as selection agents, can be mediated by floral scent (Kessler *et al*., 2013). Recent research has improved our understanding of the complex plant VOC emissions by revealing that that floral scent blends frequently contain a core set of compounds which may mediate plant-insect interactions in generalised systems (Knudsen and Gershenzon, 2006; Schiestl *et al*., 2018; Kantsa *et al*., 2019). Nevertheless, besides a few systems, our understanding of the evolutionary drivers of floral scent variation is still limited (Kessler and Halitschke, 2009; Gervasi and Schiestl, 2017; Byers, 2021). One major way to determine how floral scent variation evolves is to focus on whether scent is variable at different evolutionary scales. Particularly, if scent evolution has occurred due to natural selection, we would predict scent to vary predictably among populations or species. Standing genetic variation in a population may be beneficial if the environment changes, forming the basis for adaptation to new environments (Barrett and Schluter, 2008), and variation within a species can change the outcome of plant interactions (Bolnick *et al*., 2011). There are a few studies on scent variation at different levels of biological organization. At a macroevolutionary scale, floral scent has been shown to vary among plant species that are adapted to different or similar pollinators (Raguso *et al*., 2003; Dobson, 2006; Waelti *et al*., 2008; Jürgens *et al*., 2013; Schiestl and

Johnson, 2013; Byers *et al*., 2014; Bischoff *et al*., 2015). At a microevolutionary scale, floral scent changes within a species that stretch across species' ranges where plants may associate with different antagonists and/or mutualists (Suinyuy *et al*., 2012; Souto-Vilarós *et al*., 2018; Friberg *et al*., 2019). Likewise, shifts in the abundance of pollinators which often drive mating system transitions have been related to increase and reduction of floral VOCs emissions (Raguso *et al*., 2007; Doubleday *et al*., 2013; Sas *et al*., 2016; Petrén *et al*., 2021).

Despite the few studies demonstrating the evidence for selection on floral scent at various levels, variation within a species has not been extensively studied relative to macroevolutionary scale. The interspecific studies usually focus on VOC involvement in reproductive isolation between two or more species (usually involves attraction of distinct pollinators) thereby reducing gene flow and may contribute to speciation (Byers *et al*., 2014; Schiestl, 2015). However, a prerequisite for this is the extent of genetic variation and how this variation is partitioned within and among populations (Knudsen, 2002; Raguso, 2008a). Therefore, the processes underlying variation in floral VOCs and the understanding of the microevolutionary processes involved in the dynamics of such variation, are important points to be investigated.

Overall, despite the growing literature on the functional, ecological, and evolutionary roles of floral scent, we still lack an evolutionary understanding, at both evolutionary scales, and importantly how these have evolved in concert with the mating system, and attendant reliance on pollinators. Microevolutionary study usually focus on one population or species and are important for understanding the functional roles of plant scent over a short period of time. However, plant VOCs have evolved together with plants-insect interactions for millions of years and these interactions usually mediates plant mating systems. As a result, we are better able to understand how plant VOCs affect plant-insect interactions. as well as mating system transitions by adding a comparative perspective. For instance, understanding whether some plant scents or their compound classes are phylogenetically or geographically constrained or whether floral scents profiles are determined more by plant identity or by mating system groups.

To the best of my knowledge, consequences of mating system transitions on traits important for reproduction such as floral scent, under microevolutionary and macroevolutionary (phylogenetic) timescales combined with phylogenetic framework, have not yet been fully addressed. This thesis aims to examine patterns of floral scent evolution and their genetic basis at both micro- and macro- evolutionary scales to analyse the dynamics of adaptation in selfing plants at genomic and phenotypic scales. **Chapter 2** of this dissertation focuses on the

correlation between the macroevolution of floral scent and plant mating system transitions. In particular, I was interested in the evolution of both constitutive scent expression, and the stability (plasticity) of floral scent under biotic stress. A phylogenetically controlled analysis in 7 self-compatible and 13 self-incompatible species of wild potatoes gives insight into the co-evolution of mating systems with constitutive and MeJA induced floral scent. Specifically, evolution of selfing is associated with reduced constitutive floral scent but strong and significant increases in the inducibility of floral scent.

In addition to the phenotypic consequences of selfing, the shift to selfing leads to several changes at a genome-wide scale affecting nucleotide diversity and the density of transposable elements, among others (Pereira *et al*., 2021; Arunkumarb *et al*., 2015). The genomic signatures of selfing have drawn considerable interest due to their ecological and evolutionary importance. However, studies that have successfully identified genes or mutations involved in the selfing syndrome are few (Tsuchimatsu and Fujii, 2022). This is largely due to the traits being typically quantitative and driven by many genes and environmental factors, thereby, making it challenging to precisely determine the causal genes and mutations. Nevertheless, there are now an improved number of studies on the genes and mutations involved in selfing syndrome traits (Tsuchimatsu and Fujii, 2022) thanks to the improvements in population genomics techniques combined with high-throughput sequencing technologies (Ellegren, 2014; Weigel and Nordborg, 2015; Bamba *et al*., 2019). Also, there are many studies on the population consequences of selfing evolution (Wright, 2013; Barrett *et al*., 2014; Cutter, 2019; Glémin *et al*., 2019; Mattila *et al*., 2020; Mattila *et al*., 2020), and these consistently show through decreased effective population size and changes in the efficacy and the mode of selection (Barrett *et al*., 2014; Cutter, 2019; Glémin *et al*., 2019). Such genomic footprints are also termed the genomic selfing syndrome (Cutter, 2019; Wang, 2021). **Chapter 3** of this dissertation studies the genomic signatures and phenotypic consequences of mating system transitions among populations of a single highly variable species. Among-population variation (microevolution) study may be important to understand the causal relationship between mating system transitions and floral scent, and whether these associations have developed immediately or in concert with selection shaping the evolution of one trait and then the other. To address this knowledge gap, I explored the loci, genes and the biological process associated with the evolution of selfing in multiple populations of North American *Arabidopsis lyrata* subsp. *lyrata*, and a large dataset of the floral scent bouquets of the same populations. Evolutionarily consistent differences in floral scent between populations from the two mating systems were

found and appeared to conform to a predicted outcome of pollinator-mediated selection. Striking reductions in emission of floral scent compounds in self-fertilize populations were observed, suggesting the repeated evolution of reduced floral display; however, there was also significant phenotypic divergence based on clade age and/or the abiotic environment.

Overall, these two chapters of my thesis give novel insight into the ecological and genomic processes underlying the immense diversity of flowering plants. My results are among the first to show the repeated, parallel evolution of floral scent in plants that are dependent on insects for pollination versus self-fertilization. This work may also guide the development of optimal floral signalling systems for cultivated plants, particularly given the twin demands of growing human populations and rapidly changing climate. In addition to my work on floral traits, I also explored the molecular mechanisms of plant defence against pathogens; specifically, in **chapter 4**, I investigated the molecular mechanisms and dynamics of callose, and cellulose synthase and corresponding polymer interaction involved in plant defence against pathogens. Most prominently, the interaction and dynamics of single callose and cellulose synthase complexes at pathogen interaction sites. This research leads to the discovery of yet unknown transport mechanism of callose synthase complexes and associated callose polymer synthesis in the apoplast.

1.4 Molecular mechanisms of callose and cellulose synthase

The formation of defence-related papillae at sites of attempted and successful penetration from invading pathogens is one of the most interesting, universal defence responses in plants (Sherwood and Vance, 1976). The most abundant component of these papillae is the 1,3-beta-glucan polymer callose which strengthens and seals the cell wall at the plant-pathogen interaction site (Aspinall and Kessler, 1957). Hence, understanding the underlying mechanism of callose biosynthesis, transport and deposition is a useful tool to define new defence targets in plants and crops. Recent studies have suggested that in addition to callose synthases and the corresponding polymer callose, cellulose synthases and cellulose play an important role in the formation and success of defence-related papillae (Voigt, 2016). In chapter 4, I show the impact that super-resolution microscopy can have to decipher molecular mechanisms of callose and cellulose synthase and corresponding polymer interaction. Most prominently, I elucidate the interaction and dynamics of single callose and cellulose synthase complexes at pathogen interaction sites, leading to the discovery of yet unknown transport mechanism of callose synthase complexes and associated callose polymer synthesis in the apoplast.

Collectively, this thesis may guide the development of optimal floral signalling systems for cultivated plants and the understanding of defence-related interactions between callose and cellulose synthase for identifying new defence targets in plants and crops, particularly given the demands of growing human populations and rapidly changing climate.

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Chapter 2:

Plant mating system transitions influence the macroevolution of constitutive and methyl-jasmonate induced floral traits

To be submitted to Nature Ecology and Evolution

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2.1 Abstract

Mating strategies such as selfing and outcrossing have spectacular consequences on floral diversity in angiosperms. Mating systems shift to selfing mostly happen to ensure reproduction in isolated habitats where pollinators are scarce. Selfing evolution can also occur to reduce the susceptibility of plants to herbivores. Whichever way, mating systems are closely related to specific geographic conditions and thereby influence the evolution of floral traits. I tested the hypothesis that the evolutionary transition from an outcrossing to selfing should be accompanied by changes in functional floral traits in 20 species of wild potato (Solanaceae, Section Petota). I focused on important but less studied floral traits that are involved in pollination and defence: the diverse secondary metabolites used as insect attractants and repellents. Mating system transitions and trait evolution among species were inferred by reconstructing a molecular phylogenetic tree based on a panel of single nucleotide

polymorphisms (SNPs). Floral traits, including flower morphology and emissions of volatile organic compounds (VOCs) were measured under two different conditions; control, and methyl jasmonate (MeJA) treatment, to examine the evolution of floral trait plasticity. Phylogenetically controlled models of trait evolution revealed that transitions to selfing are associated with significant reductions in constitutive emissions of floral VOCs, corolla width and stigma-anther distance, providing support for a novel 'scent-based selfing syndrome' in species that no longer require animal pollinators. Self-compatible taxa also showed significantly greater floral scent plasticity in response to the MeJA elicitor compared to outcrossers, suggesting that the greater reliance on pollinators in outcrossers may have selected for more stable floral signalling. My study suggests that at macroevolutionary scales, mating systems and ecological interactions have shaped the repeated evolution of complex floral phenotypes.

2.2 Introduction

Plants exhibit outstanding variation in how they disperse their seeds or gametes, over distances ranging from the local (within same habitats as their relation) to new habitats far beyond the source population (Nathan, 2006). Typically, plants mate with nearby individuals, therefore, the evolution of seed dispersal distance may influence their systems of mating (Pannell *et al*., 2015; Pannell and Voillemot, 2017). Baker's law predicts that long-distance dispersal is usually associated with the shift to self-compatibility from self-incompatibility, for reproductive assurance in the absence of pollinators (Pannell *et al.*, 2015; Pannell and Voillemot, 2017). Self-incompatibility (SI) is a general name for several genetic mechanisms that prevent self-fertilization in hermaphroditic plants, and thus encourage outcrossing (Igic *et al*., 2008; Cutter, 2019). Shifts to self-compatibility tend to happen at range edges and/or in isolated populations where mates and pollen vectors may be low in abundance or absent (Griffin and Willi, 2014). In addition, plant defence theory predicts that in such habitats, unpredictable presence of herbivores will select for attack-induced defences over the constitutive defence normally exhibited where herbivory is predictable and/or likely (Campbell & Kessler, 2013, Campbell, 2015). Expressing these inducible traits would save the costs of constitutive defence in the absence of herbivores. These two theories together, posit an evolutionary correlation between self-compatibility and inducibility upon dispersal to isolated habitats where mates and enemies are lacking. While this correlation has been tested for defence traits in leaf, it has not been tested for floral traits. Moreover, research has highlighted that environmental factor such as

herbivory not only cause upregulation of leaf metabolites, but also flower metabolites (Rusman *et al*., 2022). In this study I hypothesize that transitions to self-compatibility would be accompanied by a shift to greater inducibility of floral volatile emissions and reductions in volatile emissions patterns.

Mating systems variations such as self-fertilization and cross-fertilization have profound impacts on the ecology, distribution and plant population genetic structure and even the macroevolution of traits (Ferrari *et al*., 2006; Campbell, 2015; Johnson *et al*., 2015; Whitehead, 2018). In general, cross-fertilizing plants exhibit larger, more genetically variable population, whereas self-fertilizing plants tend to exhibit smaller, less genetically variable populations (Charlesworth and Charlesworth, 1987; Campbell, 2015; Whitehead, 2018; Whitehead *et al.*, 2018). Although the two forms of mating system have their different advantages and disadvantages, plant mating system research largely focuses on the rate and consequences of selfing (Igic *et al*., 2008; Wright *et al*., 2008; Porcher *et al*., 2012; Campbell & Kessler, 2013). One of the most obvious theoretical benefits of selfing plants over outcrossers is that a plant that can self-fertilize avoids the need to depend on pollinators service. This benefit could explain why self-fertilization is frequent in isolated populations where there is no or low access to pollinators or compatible mates, such as sites that are frequently disturbed, species' range edges and areas reachable by long distance dispersal only (Campbell, 2015; Pannell and Voillemot, 2017).

Such environmental condition can impact on the phenotypic plasticity of plants. Variation in in plants traits in response to an environmental signal mostly evolve as a way to adapt in changing environments, thereby leading to fitness sustainability in plants (Schlichting and Smith 2002; Charlesworth and Charlesworth, 1987; Barrett, 2010; Campbell and Kessler, 2013; Johnson, Campbell, 2015; Campbell and Barrett, 2015; Gao and Gao, 2016). The transition of mating system from self-incompatibility (hereafter, referred to as outcrossing) to self-compatibility (hereafter, referred to as selfing) is a common shift in plant evolution. About 15% of flowering plants are predominantly self-fertilizing thereby, giving rise to inbreeding depression frequently (Markova *et al*., 2017; Nasrallah, 2017). Selfing plants usually overcome the inbreeding depression, in order to fill new niche and potentially speciate (Charlesworth and Charlesworth, 1987; Carr, Roulston and Hart, 2014; Johnson, Campbell and Barrett, 2015; Pannell and Voillemot, 2017). Thus, the variation in traits in selfing plants is likely always due to natural selection rather than a consequence of inbreeding depression. Many studies have

reported the impacts of self-fertilization on a large range of traits that are related to natural selection such as growth, reproductive and defence traits (Hull-Sanders and Eubanks, 2005; Delphia *et al*., 2009; Stefan & Jones, 2013; Kariyat *et al.*, 2012, 2013, 2021; Campbell & Kessler, 2013; Johnson, Campbell and Barrett, 2015; Markova *et al.*, 2017; Ansaldi *et al*., 2019; Nihranz *et al.*, 2019).

In plants, one of the frequently studied aspects of trait plasticity is the herbivore-induced changes in defence traits. Plants mainly cope with pest attack by upregulating defensive secondary metabolites when attacked; these metabolites act as toxins, deterrents, or digestion-inhibitors to leaf chewing herbivores, and thereby benefit the plant. In addition to the upregulation of toxic secondary metabolites such as VOCs, morphological traits in leaves, flower and other parts of the plants also changes in response to herbivore attack (Heil, 2010). Volatile organic compounds (VOCs) are released by all living organisms including plants and therefore, ubiquitous in nature (Junker *et al*., 2017; Lemfack *et al*., 2017). Plants exhibit incredible diversity of VOCs, which comprise thousands of compounds that constitute a significant portion of biogenic hydrocarbons in the atmosphere (Kunze and Gumbert, 2001; Raguso, 2008; Guenther *et al*., 2012). These VOCs are composed of numerous compound classes such as terpenoids, phenolics, benzenoids, green leaf volatiles, nitrogenous amines and many more (Dudareva *et al*., 2006, 2013; Knudsen and Gershenzon, 2006). Plants frequently store up VOCs in appropriate plant cells such as glands and ducts and emit them upon damage to tissue (Celedon and Bohlmann, 2019).

Composition of leaf volatiles and the magnitude of VOC emissions change dramatically in response to attack because VOCs are used by predators of herbivores to locate their prey (Skoczek *et al*., 2017; Knauer *et al*. 2018; Fincheira *et al*., 2021). Skoczek *et al*. (2017) showed that herbivore feeding generally increases volatile emissions from plant tissues, including altering the composition of the volatile blend. Induced volatiles are involved in recruitment of the natural enemies of feeding herbivores and hence, volatile induction is suggested to be an indirect form of plant defence (Paré and Tumlinson, 1999; Du *et al.*, 2008; Bello-Bedoy and Núñez-Farfán, 2011; Kariyat *et al*., 2012; Kariyat *et al.*, 2013). Plant VOCs serve as a vital foraging signal for arthropod herbivores (Runyon, Mescher and De Moraes, 2006; Pinto *et al*., 2010; Kariyat *et al*., 2012; Dudareva *et al*., 2013; Erb *et al*., 2015; Gray *et al.*, 2015; Paprocka *et al*., 2018; Brilli, Loreto and Baccelli, 2019; Hammerbacher, Coutinho and Gershenzon, 2019; Fincheira *et al.*, 2021). VOC emissions from damaged plants often contains varieties of

compounds not normally released by undamaged plant tissues (Lemfack *et al*., 2017; Smith and Beck, 2013). Theory on the evolution of defence predicts that unpredictable herbivory should favour inducible defences because the production of chemicals including VOCs can be costly to the plants. In contrast, constant and predictable herbivory should select for constitutive defence (i.e., constant emission of VOCs) (Heil 2010; Bixenmann *et al*. 2016).

Previous studies have demonstrated that insect herbivores can drive the evolution of defensive plant traits by imposing selection on such traits (Pichersky and Gershenzon, 2002; Bode and Kessler 2012; Carmona and Fornoni 2013). In addition, Campbell and Kesler (2013) supported the hypothesis that predictability of herbivory is a determinant of the extent of defence evolution toward being constitutive or inducible. The work of Scoville and Pfrender (2010) on *Daphnia* and that of Westra *et al*. (2015) on bacteria also showed that constant and predictable predation or attack favours the evolution of constitutive defences as opposed to inducible ones respectively. In plants, some experimental work where herbivore presence has been modified showed some evidence suggesting that the constant and abundant availability of insect herbivores can favour the evolution of higher constitutive production of chemical defences (Dicke, 1998; Stamp, 2003; Agrawal *et al*., 2012; Campbell and Kessler, 2013). Inducible chemical defences and morphological plasticity are exhibited in environments where attackers are scarce (Karban and Baldwin, 1997; Campbell, 2015; Pannell and Voillemot, 2017).

Plastic responses to herbivory does not only cause upregulation of defensive leaf metabolites, but also flower metabolites, thereby driving the evolution of floral and leaf chemical diversity (Barber *et al*. 2012; Schiestl *et al*. 2014; Levin, 2015; Moreira *et al*. 2019). Upregulation of defensive metabolites in flowers is usually detrimental for reproductive fitness through physiological or ecological costs (Rusman *et al*. 2019b). Plant induced responses usually involve jasmonic acid (JA), important phytohormone that plays a vital role in plant defence signalling in response to herbivorous insects by mediating the production of biochemical and physical defences. It also plays an important role in flower development (Wang, 2021) such as pollen fertility (Stintzi and Browse 2000), style elongation (Stitz *et al*. 2014), secretion of nectar (Radhika *et al*. 2010) and floral VOC production (Li *et al*. 2018). Research has provided evidence that herbivores can indirectly mediate selection on floral phenotypic traits and on mating system (Agren *et al*. 2013; Santangelo *et al*. 2019) which is also often mediated by changes in ecology (plant–pollinator interactions and abundance of pollinators). Therefore,

comparing the impact of herbivory between mating strategies, is an important consideration in determining the evolution of inducibility in the floral traits (e.g., floral VOC emissions).

Pollinators can also be affected by plant induced responses to herbivory. For example, pollinating insects and birds can be affected by accumulation of defensive compounds in the nectar caused by the induction of defences in leaf tissues (Ramos and Schiestl 2019). For example, Kessler *et al*. (2011) demonstrated a deterrent effect in flower fragrance due to increased emission of defensive terpenoids in vegetative tissues. These induced responses may be linked to mating system transitions such as shifts from outcrossing to selfing, and their effects on the defence strategy, as evidenced by the non-independent evolution of pollination and plant defences (Johnson, Smith and Rausher, 2009; Johnson, Smith and Rausher, 2009; Campbell and Kessler 2013; Campbell *et al*. 2013; Johnson *et al*. 2015; Campbell and Barrett, 2015; Levin, 2015; Ansaldi *et al.*, 2019). The effects of mating system variation on floral scent evolution and floral trait plasticity are less well studied (Byers, 2021). Across generations, the quantity and composition of VOCs emitted from leaves and insect attraction and feeding, are affected by inbreeding depression (Kariyat *et al.*, 2012, 2013; Campbell and Kessler, 2013; Campbell, 2015; Nihranz *et al.*, 2019), but the adaptive evolution of scent plasticity among populations or species remain less studied. Majetic *et al*. (2019) reported a decreased quantity of specific floral scents in selfing species of Phlox. In principle, outcrossers should exhibit lower scent plasticity than selfers, due to the pleiotropic effects of selection for defence inducibility in selfers (Campbell and Kessler, 2013) and the expected greater costs of stress-induced changes to scent for outcrossers. However, there is limited knowledge on how selfing and the induced leaf volatiles might impact floral VOCs and other floral phenotypic traits that influence floral visitation.

Within and among a species, floral scents have evolved as adaptive signals functioning as flower location and identity cues for insect pollinators. Previous works have characterized the floral odour of over a hundred angiosperm families plant species, identifying more than 1700 floral VOCs (Kunze and Gumbert, 2001; Raguso, 2008). These scents are composed of floral VOCs which are made up of a blend of individual volatile organic compounds (Pichersky and Dudareva, 2007; Majetic, Raguso and Ashman, 2009). In addition, flower colour, size and shape have also been considered significant floral signal (Chittka and Menzel, 1992; Kevan, Giurfa and Chittka, 1996). Utilization of floral cues seems to increase the efficiency of foraging by pollinators as search time is being reduced, thereby improving the efficiency of obtaining

floral rewards (Heinrich, 1976; Waser and Price, 1983, 1985). Despite the wealth of data on the evolution of morphological or visual traits in selfing species, little is known about the effects of selfing on floral volatiles evolution and its inducibility. Doubleday *et al*. (2013) documented significant reduction in all the analysed floral traits with *Abronia umbellate* (Nyctaginaceae) selfing populations where floral VOCs exhibited approximately ninety percent reduction in emission rate when compared to outcrossers. However, this study was limited in its replication, with only a small number of non-independent populations.

In this chapter, I investigated macroevolutionary patterns in floral scent in order to test the hypothesis that reproductive strategies of plants have played an important role in scent diversity. Specifically, I tested three hypotheses: First that floral scent will be reduced in selfing species as a result of directional selection to reduce the costs of VOC (scent) production; second, that the inducibility of floral scent in response to an herbivory signal will be less in obligately outcrossing species, to minimise disruption to pollination, while selfing species will be more inducible due to reduced ecological costs and/or selection for defensive plasticity; and third, that there will be a continuous negative relationship between the inducibility of floral scent and the constitutive expression of the scent when comparing multiple species. Getting a wider view on the variation of floral traits among closely related species will also be useful for resolving questions pertaining to the evolution of such variation. For instance, does the extent of closeness among related species tend to impact on the similarity in trait variation (i.e., do components of floral scent show evidence of phylogenetic conservatism/constraint)? This study focuses on the effect of mating system transitions on the evolution of constitutive and methyl-jasmonate induced VOC emission as well as the morphological phenotypes in the flowers of 20 closely related wild potato species while also accounting for their phylogenetic history.

2.3 Materials And Methods

Twenty closely related species (Figure 2.1) belonging to the genus Solanum section Petota (wild potatoes) were sampled, including 7 self-incompatible and 13 self-compatible species. In the Solanaceae, obligate outcrossing is ancestral, and is enforced by the presence of a genetic incompatibility (SI) system; repeated loss-of-function mutations in this system lead to the irreversible loss of obligate outcrossing and the shift to self-compatibility (SC). The wild potato sampling made use of these repeated, independent, and irreversible losses of self-incompatibility in the Solanaceae (Igic *et al* 2008), thereby testing whether the transitions from

obligate self-incompatibility to potentially increasing self-compatibility have independently led to the floral trait evolution. Self-compatibility by itself does not automatically guarantee a highly selfing mating system in natural populations, but making my analysis somewhat conservative with respect to mating system per se.

2.3.1 Sample collection and genotyping

Replicate wild populations (accessions) of 20 wild potato species, Solanum sect. Petota, used in this study were grown in a screenhouse environment (US Potato Genebank) at Sturgeon Bay Sturgeon Bay, WI (USA) as a part of a large-scale USDA germplasm screen (Hardigan *et al.*, 2015). Three populations from each wild potato species were sampled from fourteen self-incompatible (SI) with six closely related self-compatible (SC) wild potato species. Diploid species in both mating systems were mostly selected to avoid confounding any polyploidisation impact on mating system transitions due to polyploidization being a common route to self-compatibility (Bachmann *et al.*, 2021). However, three taxa in the sampling were polyploids: *Solanum demissum, S. hjertingii* and *S. stoloniferum*. Removal of these three taxa from the analyses did not show qualitatively different results. SNP data were obtained from a published study on the same accessions (Hardigan *et al*., 2015) and used to generate high confidence SNPs that were then used to reconstruct the phylogenetic history of the investigated species.

2.3.2 Plant Treatment with Methyl Jasmonate (MeJA)

The induced defence responses of plants to attacks from herbivores are greatly mediated by the phytohormone jasmonic acid (JA) and its derivatives (Howe and Jander, 2008). Methyl jasmonate (MeJA) is a derivative of JA and the reaction is catalysed by S-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (Andreou and Feussner, 2009). Jasmonic acid and methyl jasmonate are produced by plants in response to stresses most especially to herbivory and wounding, thereby building up in the damaged plant (Andreou and Feussner, 2009). Herbivory causes plants to produce MeJA as a volatile organic compound emission for defence (Farmer and Ryan, 1990), in addition to its role in internal plant signalling. In this context, methyl jasmonate was used in this work to mimic the physiological effects of herbivory; while such an application does not mimic all the potential impacts of herbivore feeding and defence elicitation, it importantly avoids the confounding effects of variation in consumption (tissue removal) which is unavoidable in comparative studies using a real herbivore. For each of the three accessions of the 20 wild potato species, three to four replicate individual plants were treated with 100µg of a defence elicitor, methyl jasmonate, MeJA which

was dissolved in 20µl of lanolin (Halitschke *et al.*, 2000) and applied in a 2 cm band around the stem base of each plant. Control plants were similarly treated with 20µl of pure lanolin paste alone.

2.3.3 Phenotypic trait measurements

The morphology of flower such as corolla size, cone width and stigma-anther distance as well as floral volatiles across 20 species of wild potatoes that were grown in a screenhouse were measured. The number of replicate populations per species ranges from 1 to 3 with 2–5 individuals per accession.

2.3.3.1 Volatile collections and measurements

Volatiles were collected from the inflorescence of 20 closely related wild potato species. We collected from six to eight individuals per species. Half was randomly selected to be treated with MeJA and the other half served as untreated control. After 72 hours, single inflorescences were enclosed in 500 mL polyethylene cups in-situ, ensuring that leaves were not part of the enclosed sample. It was acknowledged that the inflorescence might contain vegetative tissues, like the calyx and petiole, which could also potentially emit volatile compounds. However, since the calyx and petiole are all considered part of the inflorescence structure (Gagliardi *et al*., 2018), the volatile emissions from the vegetative tissue, were not quantified separately. The air collected in the headspace was pulled through ORBO-32 charcoal adsorbent tubes (Supelco, Bellefonte, PA, USA) using a 12 V vacuum pump at approximately 11.5 l/min (Gast Manufacturing Inc., Benton Harbor, MI, USA). Floral volatiles were collected over a period of 8-10 hours. Since the duration of volatile collection was variable among the plants, we quantified volatile emission per hour of collection. The analyses were conducted in a screenhouse to minimize the likelihood of contaminants, ensuring the integrity of our samples, and avoiding any potential interference. Nevertheless, we included an air control (empty chamber) to allow identification of environmental contaminants and background VOC levels.

After collecting the VOC (Volatile Organic Compound) traps, they were spiked with 5 µL of a 90 ng/µL tetraline internal standard, eluted in 250 µL of dichloromethane solvent. Subsequently, the samples were subjected to analysis through Gas Chromatography-Mass Spectrometry (GC-MS) utilizing a Varian Saturn 2200 GC/MS/MS instrument equipped with a CP-8400 autosampler. The GC analysis was performed in splitless mode on a DB-5 column, specifically an Agilent J&W GC Column with dimensions of 30 m length, 0.25 mm inner

diameter, and a film thickness of 0.25 μm. The temperature program commenced with an initial injection temperature of 225 °C. The column temperature was then ramped from 40 °C to 180 °C at a rate of 10 °C per minute. The analysis concluded with a final hold at 180 °C for a duration of 10 minutes. The identification process involved comparing the mass spectra of the compound peaks with the NIST 2008 and 2014 mass spectral libraries (National Institute of Standards and Technology). When possible, identities were confirmed by comparing mass spectra and retention times with those of known standards.

Individual-level VOC analysis lacks consistent patterns due to the significant diversity among the studied species. Consequently, it's not anticipated that the same individual VOCs would serve identical functions across all species. However, we anticipate that groups of VOCs within the same class would have shared functions. Therefore, to examine functional classes across species, considering the variations in individual compounds, we combined the volatile compounds into suitable classes before proceeding with subsequent investigations. Total amount of VOC emission and the emission of major groups of VOCs were considered separately. Inducibility in floral signal was estimated as the relative difference between the floral signal in control and MeJA-treated plants.

2.3.3.2 Floral morphological trait measurements

In addition to volatile collections and analysis, flower morphology such as corolla width, anther cone width, and stigma-anther separation were measured using a digital vernier calliper. Flower size is a vital trait that influences pollinator visitation (Harder and Johnson, 2009). Corolla width was measured from newly and fully opened flowers randomly chosen from the mature flowers at mid-height of mature inflorescences. One flower on a whole inflorescence per individual plant was measured. There were no apparent intra-inflorescence differences in the width of the anthers within the individual flowers, hence, the width of one anther was measured. The diameter of the anther was used to calculate the anther cone width. Stigma-anther separation was measured by subtracting the height of the low reproductive organ (distance from the base of the ovary to the top of the reproductive organ; anther or stigma) from the high reproductive organ height in one flower.

2.3.4 Phylogenetic Comparative Analysis

Phylogenetic relatedness for each of the traits measured was performed using the R package, ‘ape 5.5’, to analyse whether trait differences among species were predicted by their shared

evolutionary history. A coalescent-based phylogenetic tree of the closely related wild potato species (Figure 2.1) was reconstructed in a Bayesian framework using a data matrix containing 3071 high confidence biallelic SNPs for a subset of 20 samples using the SNAPP package in the program BEAST2 version 2.5.1 (Bryant *et al.*, 2012; Bouckaert *et al.*, 2014; Leaché *et al.*, 2014). A 'log-normal' prior model was set for lambda with MCMC chain length of 2,000,000 postburn-in generations and pre-burnin set to 5000. The remaining options were left at their default values. Three independent analyses using different starting seeds were performed and compared to ensure MCMC convergence. This analysis yielded a defined phylogeny that was largely in agreement with the published phylogeny of Solanum section Petota (Huang *et al.*, 2019). The posterior distribution of species trees was summarised and the topology with the best posterior support was identified thereby, generating a maximum clade credibility tree using the tree annotator in BEAST.

Relatedness between mating systems and the measures of both constitutive and induced floral phenotypic traits were analysed in a phylogenetic context by estimating the Pagel's "lambda" correlation structure, λ (Freckleton, Harvey and Pagel, 2002, 2015) available in "nlme" libraries in R package, ape. For each of the floral phenotypic traits (volatile emissions, corolla width, cone height, cone width, and stigma-anther distance), I asked whether trait variance between mating system types was predicted by the phylogenetic relatedness of those closely related wild potato species using the corPagel framework (Freckleton, Harvey and Pagel, 2002; Münkemüller *et al.*, 2012). Normally, a Brownian model of evolution which accounts for the phylogenetic relatedness of species defines the expected (random) covariance matrix of their phenotypes. Nevertheless, whenever additional factors, apart from their phylogenetic history, influence trait evolution, the influence of the phylogeny is relatively down weighted. This weight is defined by the coefficient λ (lambda) and is fitted to observed trait data, scaling the Brownian phylogenetic covariances down to the observed ones. In other words, λ assesses any deviations from the Brownian model such that a lambda value of 1 reflects a pattern where closely related species are like the Brownian motion expectation, and 0 reflects the opposite. In summary, lambda estimate of 0, indicates that the traits have no phylogenetic signal while lambda estimate of 1 indicates very strong phylogenetic signal (trait variance explained by shared evolutionary history).

2.3.5 Statistical analyses

Phylogenetic generalized least squares (PGLS) models were utilized to analyse whether the evolution of self-compatibility is correlated with the evolution of flower morphological traits, volatile emissions and MeJA treatment effects, using the gls and corpagel function in the R package "ape". PGLS models tested whether the closely related species differs in their phenotypic traits between the two mating systems while also accounting for the evolutionary history of the wild potatoes using Pagel's "lambda" correlation structure from the corpagel function in R package, ape. Corpagel was set to estimate the phylogenetic signal parameter called lambda, λ, for the phenotypic traits. In the model, mating systems were used as the fixed/independent variable while the other phenotypic traits were response/dependent variables. Interaction effects of MeJA treatment with mating system was also tested. Significance was assessed using F-tests. Effects were considered significant if $P < 0.05$. The mean values of the traits were log-transformed for normality prior to statistical analyses. Post hoc comparisons of the treatment*mating system combinations employed the Ryan-Einot-Gabriel-Welsch Multiple Q (REGWQ) test to examine differences among means for the biologically relevant mating system by treatment interaction. Most of these investigated species are diploid and some are tetraploids. To avoid confounding polyploidization with mating system transition, the effects of polyploidy on traits evolution analysis were tested by comparing analyses when tetraploids were included and excluded. Polyploidy did not have any qualitative effect on the analysis.

2.4 Results

2.4.1 Macroevolution of MeJA-inducible floral scent

The reconstructed phylogenetic tree (Figure 2.1) indicates at least four independent evolutionary transitions from outcrossing to selfing occurring in four distinct clades among the species studied. One selfing species (*S. candolleanum*), another one (*S. brevicaule*) and yet another (*S. verrucosum*) and four others (*S. stoloniferum, S. hjertingii, S. demissum, S. acaule*) occur in the four clades separately. The phylogeny provides the framework for testing our hypotheses. With the phylogenetic resolution, I have greater certainty of floral scent evolution. Ancestral wild potato species produced higher amount of floral scent with evolutionary transitions to lesser floral scent which coincides with shifts to a selfing mode of mating. Analysis of total VOC emission on the tree shows that the measured floral scents were significantly reduced with selfing evolution (Table 2.1; Figure 2.1). MeJA treatment effect on

floral scent showed a contrast between the selfing and outcrossing relatives. Reconstruction of the phylogeny of the related wild potato species together with their floral signal inducibility indicates that the evolution of selfing is accompanied by increase in plasticity (Figure 2.1).

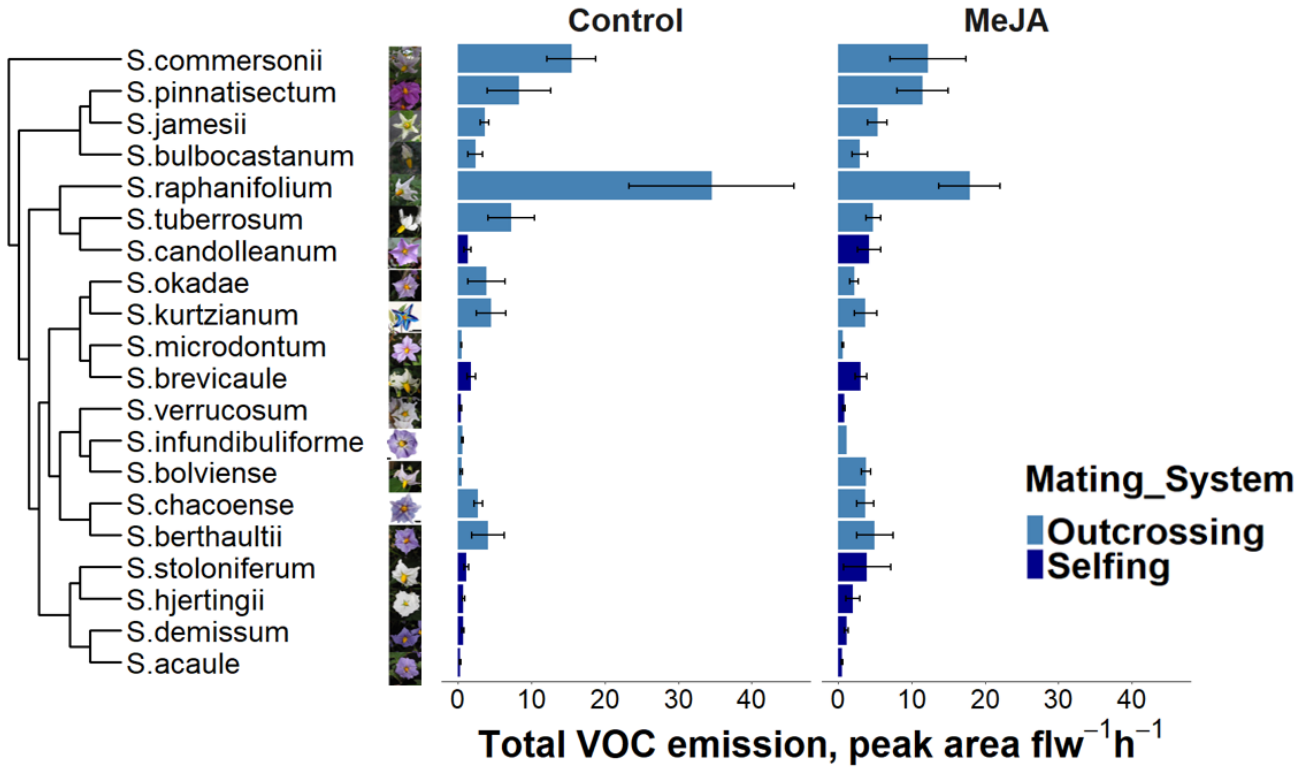

Figure 2.1 Maximum likelihood phylogenetic tree inferred directly from single nucleotide polymorphism of multiple individuals of the wild potato species. Total VOC emissions (mean ± SE) of the species are mapped on the phylogeny and represented by the bars. Dark blue bars indicate self-compatible plants and light blue bars indicate self-incompatible species. Bar graphs show the mean difference between mating systems in constitutive and MeJA-induced floral VOC emissions.

2.4.2 Floral VOCs released by wild potatoes.

To determine how floral scents vary with mating system transitions, constitutive VOC emissions and MeJA-induced VOC emissions were analysed. The results of the analysis of total volatiles are displayed in Figure 2.2, with details of the statistical analyses depicted above each panel in Table 2.1. Generally, floral volatiles varied by mating system and by treatment,

indicating that selfing contributes to the cause of floral scents variation. Self-incompatible species emitted on average 100% more constitutive total volatiles but exhibited weaker induction of the total VOCs in response to MeJA treatment relative to self-compatible species (Figure 2.2a). In other words, selfing taxa exposed to MeJA treatment had more than double of total VOC emission from the control plants (selfing = 0.89 ± 0.2, outcrossing = 2.22 ± 0.6; Figure 2.2a). Self-incompatible plants had over 100% increase in the major classes of floral scent compared to self-compatible except the green leaf volatiles (Figure 2.2). There is also a significant treatment × mating system interaction with MeJA-treated, outcrossing plants having a lower green leaf volatile emission than MeJA-treated, selfing plants (Table 2.1; Figure 2.2d). No significant effects were detected for treatment × mating system interaction for other compound classes. All compound classes except benzenoids were significantly up regulated in response to MeJA treatment in the self-compatible plants. Estimates of the extent of inducibility of floral scents reveal that trait inducibility was influenced by mating system (Table 2.1; Figure 2.2). The transition from outcrossing to selfing is accompanied by a shift to approximately three times as more inducible floral signal as outcrossers in all floral scents categories except benzenoids.

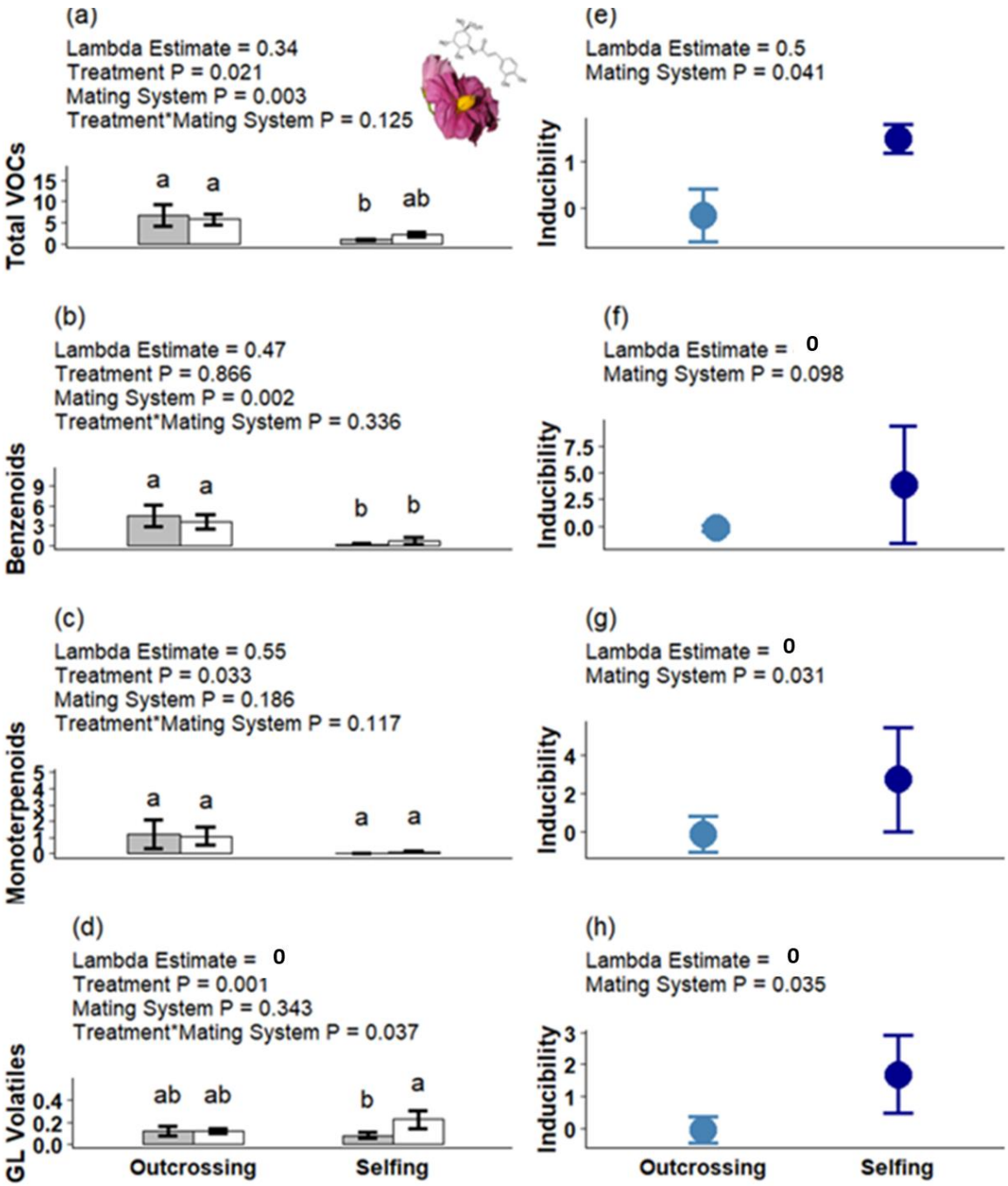

Figure 2.2 Constitutive and MeJA-induced emission rates (normalised peak areas per flower per hour) of major classes of volatile organic compounds (VOCs) by single individual inflorescence of the closely related wild potatoes. Outcrossing and selfing plants were either treated with methyl jasmonate (MJ, grey bars) or untreated (C, white bars). Values in left panels (a: total VOCs, b: benzenoids, c: monoterpenoids, and d: green leaf volatiles) represent absolute amounts of VOC emissions and values in the right panels represent the corresponding inducibility of the major groups of VOCs. Bars represent means ± SE, n = 20. The results of

the statistical analyses are depicted above each panel and in Table 2.1. Different letters above the bars indicate significant differences among mating system × MeJA treatment combinations determined by post hoc analysis using Ryan-Einot-Gabriel-Welsh multiple range test ($P < 0.05$). The results of the PGLS analyses with corPagel lambda (λ), a parameter that account for phylogenetic relationships among the species, are depicted above each panel. The analyses tests whether traits have been shaped by mating system transitions, MeJA treatment and shared phylogenetical history. λ ranges from zero (no phylogenetic signal), to one (strong phylogenetic signal). Intermediate values imply average phylogenetic support (Pagel, 1999).

Table 2.1 Statistical results for the analysis of floral traits from VOC from individual inflorescence of the closely related wild potatoes (Figure 2.5, 2.2, 2.3 and 2.4). The table shows effects of mating systems (outcrossing and selfing), treatment (MeJA treated and control) and their interaction on floral traits. Statistical analyses were performed with constitutive and MeJA-induced traits and trait inducibility (relative difference between MeJA treated and control). The second column in both panels (Floral morphological traits, A and floral VOC emissions, B) reports the statistical results for the effect of MeJA-mimicked herbivory. Interactions between mating system and herbivory are denoted in the column "interactions". Asterisks indicating level of significance (***P < 0.001, **P < 0.01, *P ≤ 0.05) depicts statistically significant results and "ns" denotes "no significance". Degrees of freedom are stated at the table headings as F (df1, df2).

A (Floral morphological traits)

Traits				Inducibility
Traits	Treatment F(1, 160)	Mating_System F(1, 160)	Interaction	Mating_System F(1, 14)
Corolla width	1.3205 ns	1e-04 ns	0.5487 ns	14.7863 **
Cone width	1.2173 ns	1.2217 ns	0.1055 ns	0.3412 ns
S_A distance	0.023 ns	0.1889 ns	1.6832 ns	0.481 ns

B (Floral scents)

Traits				Inducibility	Inducibility vs Constitutive	Count		
VOCs	Treatment F(1, 36)	Mating_System F(1, 36)	Interaction	Mating_System F(1, 18)	Constitutive resistance F(1, 18)	Treatment F(1, 261)	Mating_System F(1, 261)	Interactions
Benzenoids	0.0289 ns	10.8652 **	0.9525 ns	3.0381 ns	46.1604 ***	0.3918 ns	6.5082 *	0.0022 ns
GL_Volatiles	14.0328 ***	0.9229 ns	4.7007 *	5.1674 *	7.7623 *	-	-	-
Monoterpenoids	4.9169 *	1.82 ns	2.5722 ns	5.4802 *	49.3805 ***	-	-	-
Other_VOCs	-	-	-	-	17.2293 ***	4.25 *	0.0831 ns	0.3292 ns
Sesquiterpenes	-	-	-	-	0.8497 ns	-	-	-
Terpenoids.GLV	-	-	-	-	-	1e-04 ns	1.1307 ns	0.2815 ns
Total_VOCs	5.8009 *	10.2676 **	2.4691 ns	4.8622 *	66.3972 ***	5.8302 *	3.6407 ns	4.5037 *

2.4.3 Diversity of VOC emission

On average, 45 different identified VOCs (Table S1) were emitted from across all outcrossing plant species, and compound richness was affected by both mating system transitions and MeJA treatment (MeJA-treated, Figure 2.3). The composition of total VOCs and the VOC compound classes was 50 – 100% lower in selfing species than in outcrossers. Similarly, MeJA treatment had a significant effect on the number of total VOCs and other VOCs emitted: control plants released about 50% fewer VOCs than the MeJA-treated species. The emission of terpenoids and GLVs (monoterpenoids, sesquiterpenes and green leaf volatiles) was not

affected by MeJA but was reduced as a result of the transition to a self-compatible mating system. The statistical results of the analysis of volatiles count are displayed in Table 2.1 and depicted above each panel in Figure 2.3.

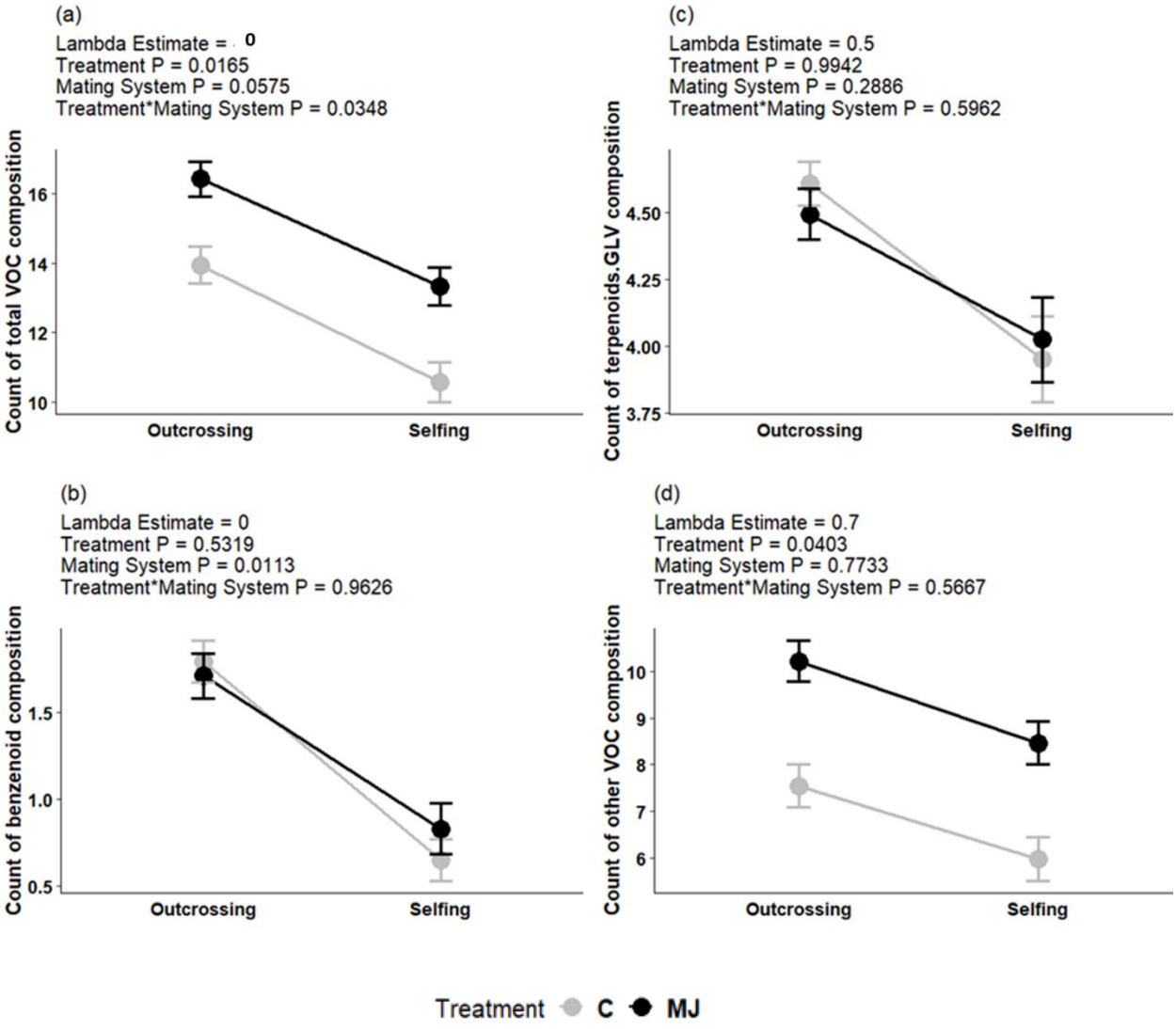

Figure 2.3 The numbers of compounds in the constitutive and MeJA-induced VOC emission of four groups of volatile organic compounds (VOCs) emitted from the the selfing and outcrossing wild potato species. Individual plants were either exposed to treatment with methyl jasmonate (MeJA treatment, dark coloured bars) or had no MeJA treatment (Control, grey bars). Values in panels represent average counts of VOCs. Bars represent means ± SEM, n = 16. The results of the statistical analyses are depicted above each panel and in Table 1. The results of the PGLS analyses with corPagel lambda (λ), a parameter that account for phylogenetic relationships among the species, are depicted above each panel. The analyses tests whether

traits variation is influenced by mating system transitions, MeJA treatment, their interaction and shared phylogenetical history. λ ranges from zero (no phylogenetic signal), to one (strong phylogenetic signal). Intermediate values imply average phylogenetic support (Pagel, 1999). Terpenoids.GLV denotes the pooled values for sesquiterpenes, monoterpenoids and green leaf volatiles.

2.4.4 Trade-off between constitutive floral signal and floral signal plasticity

In support of our hypothesis, across the closely related wild potato species, I found a highly significant negative relationship between the constitutive signal and inducibility of signal in all the compound classes investigated except sesquiterpenes (Figure 2.4). Selfing plants have significantly higher inducibility and lower constitutive floral trait values and outcrossing taxa have lower inducibility and higher constitutive floral signals (Figure 2.4).

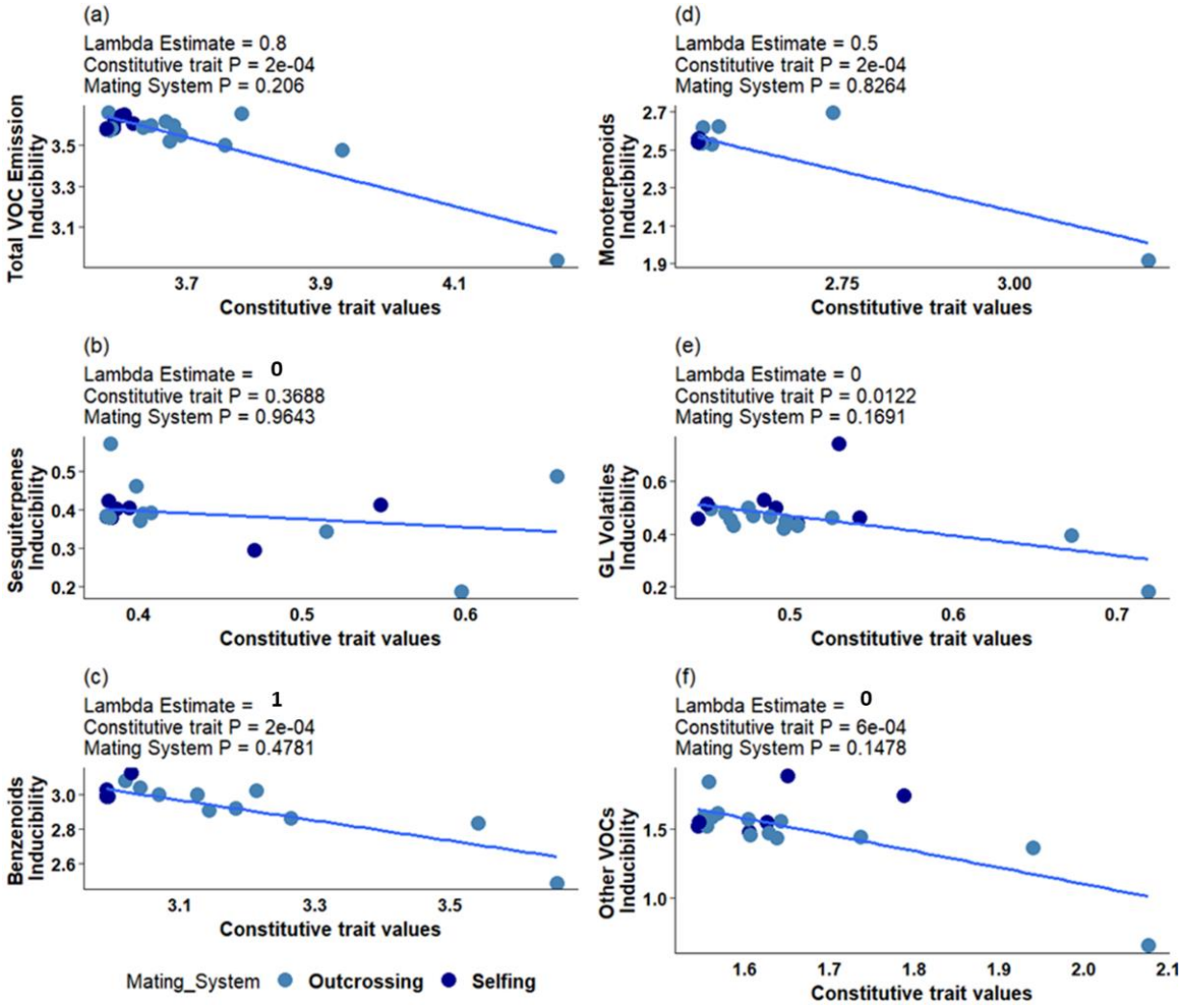

Figure 2.4 Inducible and constitutive traits trade-offs are evolutionary alternatives. The relationship between the inducibility of floral signals and the constitutive expression of the signal across multiple species. Trait inducibility and constitutive trait values for selfing (dark blue) and outcrossing (light blue) species. Data are phenotypic trait mean values and statistics are corrected for phylogenetic relationships among taxa using phylogenetic generalized least squares (PGLS). The results of the phylogenetically controlled statistical analyses are depicted above each panel and in Table 2.1. PGLS tests for both outcrossing and selfing taxa support a model of a negative phylogenetic relationship between traits trade-offs. The results of corPagel lambda (λ) in pgls, a parameter that account for phylogenetic relationships among the species, are depicted above each panel. The analyses tests whether there is a positive covariance between trait values and trait homeostasis and if this covariances are of correlated evolution. λ ranges from zero (no phylogenetic signal), to one (strong phylogenetic signal). Intermediate values imply average phylogenetic support (Pagel, 1999). Data points represent the mean values for species. Mating systems are over laid in the plot (Figure 2.4) and data points are the mean values for species.

2.4.5 Morphological floral traits

Outcrossing plants produced significantly larger flower size than selfers. Self-incompatible plants had approximately 10% wider corolla (outcrossing = 29.1 mm ± 0.8; selfing = 25.2 mm ± 1.2; log-transformed least square means ± SE; LSMeans ± SE; Table 2.1) and cones (outcrossing = 3.9 mm ± 0.1; selfing = 3.2 mm ± 0.15; log-transformed least square means ± SE; LSMeans ± SE; Table 2.1) than the self-compatible plants in the control plants (Figure 2.5). Compared to the MeJA treatment, selfing and outcrossing control plants had 12% and 0% smaller flower corolla (Figure 2.5a) and 10% and 0% smaller flower cone, respectively (Figure 2.5b). Selfing plants exhibited approximately three-fold increase in corolla width plasticity compared to outcrossing taxa (Figure 2.5d). There was also some effect of treatment × mating system interaction (Figure 2.5a and b) on the flower size (corolla width and cone width) but it is not statistically significant. In the case of stigma-anther distance, neither the interaction of mating system with treatment nor the effect of mating system and MeJA treatment were significant (Figure 2.5 c and f).

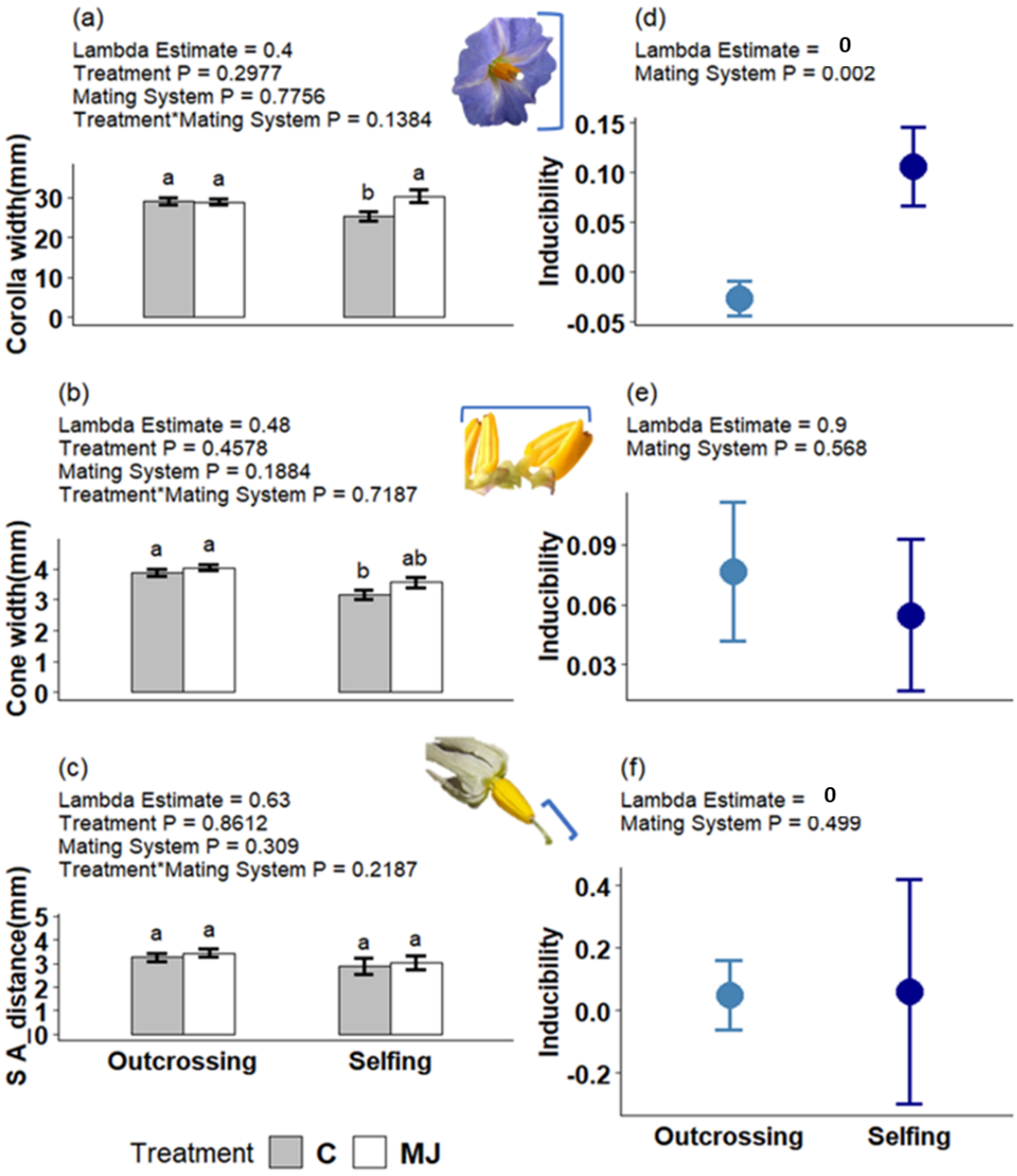

Figure 2.5 Flower morphology measurements of self-compatible and self-incompatible wild potato species. Values in panels represent absolute constitutive and MeJA-induced traits measurements (left): corolla width (a), cone width (b) and stigma-anther distance (c), and corresponding trait inducibility (right): d, e, f. Bars represent means ± SE n = 6 * 3 for outcrossing and n = 14 * 3 for selfing. The results of the PGLS analyses with corPagel lambda (λ), a parameter that account for phylogenetic relationships among the species, are depicted above each chart. The analyses test whether traits have been shaped by mating system

transitions, MeJA treatment and shared phylogenetical history. λ ranges from zero (no phylogenetic signal), to one (strong phylogenetic signal). Intermediate values imply average support (Pagel, 1999). Different letters above the bars indicate significant differences among mating system × MeJA treatment combinations determined by post hoc analysis using Ryan-Einot-Gabriel-Welsh multiple range test ($P < 0.05$).

2.5 Discussion

Many studies have focussed on the role of inbreeding depression in the variation in plant mating systems (Charlesworth and Charlesworth, 1990; Barrett, 2003; Kariyat *et al*., 2021). Contrastingly, few studies have investigated the evolutionary consequences of the adaptive advantages of selfing on floral trait evolution (Karron *et al*., 2012). Transition to selfing is a common evolutionary transition within the angiosperms (Igic *et al*., 2006) and it is an important evolutionary process influencing the diversity of plant reproductive traits (Karron *et al*., 2012; Busch and Delph, 2012). In this study I investigated how the evolution of selfing affects the constitutive and induced adaptive floral traits in twenty closely related wild potato species. In the experimentally grown species in a screenhouse environment, I found that both the constitutive and herbivore induced VOC emission from outcrossing plants were higher compared to selfers, indicating that mating system transition strongly impacts the VOC emissions of wild potatoes. No prior phylogenetic studies have examined the evolution of induced floral signals. Our mapping of constitutive and inducible floral scent along the phylogeny of closely related wild potato species (Figure 2.1) showed independent and repeated reduction of floral traits, particularly scent and scent regulation, indicating evolutionary variation and independence in the mode of floral traits investment.

2.5.1 Consistent reductions in floral trait investment accompany the repeated evolution of selfing

I detected differences in floral signals between selfing and outcrossing wild potato species that may suggest that the presence or absence of pollinators might influence the variation of these species and/or populations. However, not all the flower trait studied show the same pattern of variation. For instance, flower size (corolla width and cone width) did not differ substantially between the two mating systems, though there was a trend that flowers in outcrossing taxa were generally bigger in comparison to flowers of selfers (Figure 2.5). Significant flower size differences were also found in other studies on the impact of abundance or predictability of pollinators on floral signal (Elle and Carney, 2003; Carr *et al*; Porturas *et al*., 2019). Notably,

anther–stigma distance was not higher in outcrossing taxa, though this trait may be redundant to an effective SI system. While floral corolla size and anther cone width showed a moderately significant statistical difference between mating systems, these changes are relatively small compared to massive variation in floral scent (Figure 2.2; Figure 2.3). A study on *Abronia umbellate* showed that floral scent is much more significantly reduced in selfers compared to outcrossers than other floral visual cues (Doubleday *et al*., 2013). Our results suggest that floral scent may be the most vital floral signal to differentiate between plants exhibiting diverse mating strategies.

Selfing and outcrossing wild potato species differed in their diversity of floral volatiles (Figure 2.3) suggesting that these two groups differ in their scent emission. Both total amount of floral VOC production and scent diversity were reduced in selfing species compared with outcrossing species of the wild potatoes. This finding supports our hypothesis that evolutionary shifts from outcrossing to selfing species should minimize the investment in floral scent production by reducing floral VOC emission. Higher VOC emissions have been linked to pollinator abundance which is compromised in selfing species habitat (Raguso, 2008; Majetic *et al*., 2009). These distinctions have been characterized as adaptive adjustments to save the cost of resources in selfing species (Sicard and Lenhard, 2011). Previous studies have associated the significant variation in mating system and floral scent with ecological traits relating to circumstances selfing evolution (Yuan *et al*., 2017). Lack of sufficient pollinator service is a major driver of selfing evolution in angiosperms due to the benefits of reproductive assurance (Busch and Delph, 2012). In environments with a dearth of pollinators, selfers would likely have a reproductive advantage over outcrossers and thereby lead to the evolution of reduced investment in scent production that are important for pollinator attraction.

I found significant differences in the specific compound classes in the volatile bouquets (Figure 2.2, 2.3). Benzenoids were emitted in over three times higher amounts from outcrossers compared to selfers and are common floral scents that are important for pollinator attraction (Pichersky and Gershenzon, 2002; Knudsen *et al*., 2006). The emission of an important floral compound class, benzenoids which are involved in pollinator attraction (Pichersky and Gershenzon, 2002), were reduced in selfers. This suggests the possibility that aromatic/benzenoid compounds (Shi *et al*., 2019) may serve as reliable but costly attractants for pollinators (Pichersky and Gershenzon, 2002; Hoballah *et al*., 2005; Kessler *et al*., 2013). Therefore, plant species that do not necessarily require a mate (such as selfing taxa) for

reproductive assurance, may save the physiological cost of VOC production in the absence of pollinators by exhibiting a reduction in scent emission as observed in this study. Consistent with my findings, trans methyl cinnamate, a significant component of benzenoids, has been found to be an essential signalling compound for euglossine bee pollinators of orchid species (Ayasse *et al*., 2003; Schiestl and Roubik, 2003; Eltz and Lunau, 2005). In addition, benzenoids like many other VOCs, may also be involved in attracting herbivores' parasitoids or predators (Dicke and Baldwin, 2010). Thus, outcrossers showing increased amount of constitutive total VOCs may indicate a way to attract pollinators to further encourage obligate outcrossing but may also be involved in attracting predators of natural enemies or even repel herbivores in an environment with an abundance of insects overall. Conversely, selfers showing a decrease in total VOC emission may reflect the need to conserve investing in defence or pollination attraction in an isolated range where herbivores and pollinators are either absent, low in abundance, or variable (Campbell, 2015).

Interestingly, I did not observe a difference in the emission of sesquiterpenes between the selfers and the outcrossers (Figure S1). Research has shown that benzenoids and monoterpenes are involved in attracting a diversity of insects to flowers especially bumblebees (Farré-Armengol *et al*., 2017). Significantly, bumblebees and flies are the most abundant buzz-pollinators in environments with wild potatoes (Buchanan *et al*., 2017). Conversely, sesquiterpenes might play a vital role in attracting less effective moth pollinators (Dobson, 2006). Therefore, the significant reduction in the emission of benzenoids and monoterpenes as against sesquiterpenes (Figure S1), from flowers of selfing wild potatoes seem likely to be related to the loss of these types of pollinators from isolated habitats where selfing plants occur. The reduction I found are strong evidence for the physiological costs associated to VOC emissions. While VOCs have been long proposed as adaptive signals to predators and parasitoids, evidence for their effects on plant's fitness remains surprisingly limited. My results showing adaptive reductions therefore have important implications for our understanding of plant's VOCs more generally.

2.5.2 Evolution of floral trait plasticity

A significant effect of mimicked herbivory on VOC emission from many of our experimental species was observed, with significant overall treatment effects after correcting for phylogeny. There were also numerous interactions of mating system and treatment, with contrasting effects of MeJA on floral VOCs depending on the mating system. Analysis of inducibility of total

floral scents supported my hypothesis that the evolution of selfing from outcrossing has been accompanied by a transition to a more inducible floral VOC emission. Total floral VOCs is on average more than 50% more inducible in selfers compared with outcrossing species (Figure 2.2). Similar patterns were observed when green leaf volatiles and monoterpenoids were separately analysed. Interestingly, a significant difference between mating systems was not observed for benzenoid inducibility. This is consistent with their canonical role as attractants: i.e., a lack of an ecological cost for benzenoid induction in outcrossers may explain a lack of adaptive divergence in benzenoid regulation. This is further evidence that floral phenotypic inducibility in response to biotic stress has coevolved with plant mating transitions (Figure 2.2 and 2.4). Similarly, green leaf volatiles (GLVs) which are particularly involved in repelling herbivores and florivores (Stenberg *et al.,* 2015; Picket and Khan, 2016; Brilli, 2019), showed the highest relative inducibility in emission after MeJA treatment (Figure 2.2d).

Herbivory can influence floral traits and adversely affect pollinators by inducing toxic compounds in floral rewards such as pollens which could be costly to obligate outcrossers (Kessler and Halitschke, 2009; Kessler, 2011, James *et al*., 2018). This theory may explain why the green leaf volatiles involved in repelling herbivores which can potentially repel useful pollinators, are not induced in outcrossers (Figure 2.2), simply to avoid the cost of inducibility (deterring pollinators). However, in selfing plants, herbivory induced bigger flowers which are important for attracting insects, and total VOCs including green leaf volatiles (GLVs) which are produced in response to stress (Figure 2.5). Green leaf volatiles (GLVs) such as Z-3-hexenyl acetate, were rapidly emitted from wheat plants upon biotic attack (Ameye *et al.,* 2015) and have also been reported to be ubiquitously released following damage of leaf tissues (Brilli *et al.,* 2011; Cofer *et al.*, 2018). The selection on induced bigger flowers and increased VOCs may suggest an additional defence mechanism of selfing taxa devising a way to attract herbivores' parasitoids or predators. On the other hand, James *et al* (2018) suggested that large displays can be costly in the presence of herbivores and that herbivores weakened selection on flower size. Hence, outcrossers which are usually found in environments with abundance of herbivores (Moeller, 2005; Campbell, 2015; Alexandra and Hans, 2020), may avoid inducing large floral display (Figure 2.5) to reduce being attractive to more herbivores.

Increased number of total VOC diversity with MeJA mimicked herbivory in both selfing and outcrossing taxa were observed (Figure 2.3) but this was not the case when major compound classes were analysed separately. One possible explanation for a change in the number of total

VOCs might be the need to increase VOC emission blend, to be easily sensed by beneficial insects such as parasitoids or predators of their natural enemies (in case of aromatic metabolites) or to repel the natural enemies (in case of toxic metabolites). Although neither the number of the aromatic VOCs nor the green leaf volatiles was increased with herbivory, the total VOC blend contains other unknown or unidentified VOCs that may be involved in attraction or repulsion of insects. Further analysis, particularly in the field, are required to analyse the effectiveness of parasitoid attraction or repulsion of analysis to confirm or to further test these hypotheses. Nevertheless, I hypothesize that mating system transitions could have a wide range of impacts on plant antagonistic and mutualistic interactions, especially when mating system evolution is accompanied by changes in ecological factors. Such widespread impacts of mating system directly or indirectly affect both constitutive and inducible floral scents. Induced floral signal as responses to MeJA treatment (herbivory) are believed to have evolved based on changes in the abundance of herbivory and the costs of expressing such floral signals (Karban, 1997; Campbell and Kessler, 2013; Campbell, 2015, James *et al*, 2018).

2.5.3 Trade-off between floral trait values and trait inducibility

Overall, the effect of mating system on constitutive and inducible floral traits are contrasting, thereby prompting us to investigate the relationship between the two strategies. The hypothesis that constitutive resistance and inducibility should trade-off has been a longstanding postulation in the study of plant defence. This trade-off should exist because plants should benefit from inducible defences as a cost-saving strategy (Karban *et al*., 1997) in the absence of herbivores (Stamp, 2003). Several studies have supported this hypothesis by showing trade-offs between constitutive resistance and inducibility, both within species (Gianoli, 2002; Koricheva *et al*., 2004; Rasmann *et al*., 2014) and across species (Zhang *et al*., 2008; Kempel *et al*., 2011; Rasmann and Agrawal, 2010; Campbell and Kessler, 2013; Moreira *et al*., 2014; Rasmann *et al*., 2015). Prior studies have not examined the hypothesis in floral signals among species. Across the closely related wild potatoes, a highly significant, negative relationship between the inducibility of floral signals and the constitutive (uninduced) expression of the signal was found except for monoterpenoids and benzenoids (Figure 2.4).

Our findings indicate that those species with lower reliance on pollinators such as the selfers, which tend to be in isolated environments, will be under directional selection to minimise the costs of the floral signal and relaxed selection for inducibility, that is, lower constitutive values and higher inducibility. Likewise, species with a greater reliance on pollinators such as the

outcrossers in an environment with abundance of herbivores and pollinators, will be under strong natural selection to maximise the strength and stability of the signal, that is, high control values, low inducibility. These findings suggest further that pollinators may play an important but poorly appreciated role in the evolution of plant inducibility not just in flowers, but also in leaves, and highlight the importance of testing for trade-offs in leaf VOC inducibility between mating systems in the future.

2.6 Conclusions

My study indicates that mating system transitions have broadly shaped the macroevolution of floral traits across the closely related wild potatoes studied, including the evolution of inducible floral traits. Consistent with my hypotheses, a consistent negative relationship was observed between the constitutive total floral scent and inducibility. Further research needs to be performed to explain these discrepancies better. For instance, the absence of a negative relationship between the two floral trait strategies when examining specific major compound classes may be explained by the differences in VOC compositions of each compound class across different populations. Molecules making up such compound class may have different deterrent properties: some may be involved in attraction while some tends towards repulsion. Careful research into functions of each VOC compound may be needed for a full understanding of scent evolution. In addition, my study has utilized MeJA treatment to mimic herbivory, but additional factors may contribute to the floral traits inducibility (which may also affect how mating system impacts these floral trait evolution) when an in-situ study is performed using natural herbivory. For instance, insect herbivory does not only induce jasmonic acid in plants but also generates ion fluxes which provoke the initiation of signal transduction and activates other various hormones such as salicylic acid and ethylene (Mostafa *et al*., 2022). Other factors such as species composition of neighbouring plant community (Kigathi *et al*., 2019), priming (van Hulten *et al*., 2006; Ahmad *et al*., 2011) may also contribute to such inducibility. Furthermore, a study accounting for differences in accessions and floral traits might be needed for the full understanding of effects of mating systems. Nevertheless, my results are robust because my analyses are phylogenetically controlled and are based on repeated, independent evolutionary mating system transitions which is an important evolutionary occurrence. Lastly, my phylogenetic comparative study of inducible and constitutive floral VOCs is a unique and rare study which has provide strong evidence of the effects of mating system on the evolution of plant floral traits which can be partly explained to be driven by their role in altering

interactions with insects and possible production costs associated with the plant's investment in chemical floral cues. In general, understanding how ongoing variation in plant reproductive strategies affects biotic selection by herbivores and pollinators on plant floral traits in a wider range of systems would be an interesting field for future research.

2.7 Supplementary information

Table S1 Constitutive and MeJA-induced VOCs released from closely related wild potatoes grown in a screenhouse environment. Both selfing and outcrossing plants were either treated with MeJA (Herbivory) or untreated (Control). Values represent mean VOC emissions (peak area/flower/hour) ± SEM of the experimental plants.

VOCs	Control (C)		MeJA-treatment (M)	
	Outcrossing.C	Selfing.C	Outcrossing.M	Selfing.M
Sesquiterpenes	**0.0899 ± 0.0426**	**0.06316 ± 0.0387**	**0.11522 ± 0.0496**	**0.07275 ± 0.0417**
ST14.42min	0.00087 ± 4e-04	0.00207 ± 0.0016	0.00134 ± 5e-04	0.00238 ± 0.002
ST14.72min	2e-05 ± 0	7e-05 ± 1e-04	0.00032 ± 3e-04	3e-05 ± 0
ST14.95min	0.01259 ± 0.0074	0.00219 ± 0.0013	0.00849 ± 0.0041	0.00355 ± 0.0018
ST17.36min	0.00291 ± 0.0022	0.00223 ± 0.0022	0.00626 ± 0.0044	0.00452 ± 0.004
germacrene.d...	0.00606 ± 0.0033	0.02836 ± 0.0248	0.0172 ± 0.009	0.03553 ± 0.0273
ST15.58min	0.00815 ± 0.0054	0.00708 ± 0.005	0.00962 ± 0.004	0.00877 ± 0.0069
ST16.15min	2e-04 ± 1e-04	0.00157 ± 0.0014	0.00102 ± 6e-04	0.00218 ± 0.0015
b.elemene	0.00031 ± 3e-04	1e-05 ± 0	0.00064 ± 3e-04	0.00026 ± 2e-04
caryophyllene	0.00368 ± 0.0019	0.00103 ± 5e-04	0.00479 ± 0.0015	0.00371 ± 0.0024
ST24.56min..guaiol..	0.00214 ± 0.0014	0.00272 ± 0.0022	0.00175 ± 0.0011	0.00507 ± 0.0029
ST20.08min	0.00471 ± 0.0047	0.00035 ± 2e-04	0.00029 ± 2e-04	0.00151 ± 0.0015
a.humulene	0.00149 ± 0.001	0.00056 ± 3e-04	0.00388 ± 0.0017	0.00122 ± 8e-04
b.bisabolene	0.00106 ± 5e-04	0.00019 ± 1e-04	0.00356 ± 0.0017	0.00176 ± 0.0011
ST18.62min	0.00018 ± 1e-04	0.00041 ± 3e-04	0.00116 ± 9e-04	7e-04 ± 5e-04
ST23.65min	0.03574 ± 0.0352	0.00012 ± 1e-04	0.04549 ± 0.0451	4e-04 ± 4e-04
cadinene	0.0098 ± 0.0053	0.0142 ± 0.0134	0.0094 ± 0.0048	0.00116 ± 6e-04
Benzenoids	**4.49634 ± 1.6233**	**0.21987 ± 0.1921**	**3.55648 ± 1.0565**	**0.65518 ± 0.5043**
trans.methyl.cinnamate	1.48805 ± 1.1058	0.0168 ± 0.0047	1.41833 ± 0.8776	0.01558 ± 0.0147
methoxybenzoic.acid	0.03159 ± 0.0298	1e-04 ± 1e-04	0.00478 ± 0.003	0.00017 ± 2e-04
mandelic.acid.methyl.ester	2.22793 ± 1.1056	0.13526 ± 0.1284	1.53523 ± 0.635	0.46208 ± 0.3749
benzeneacetic.acid.methyl.ester	0.22133 ± 0.1037	0.01742 ± 0.0168	0.27748 ± 0.1177	0.07071 ± 0.0581
cis.methyl.cinnamate	0.03743 ± 0.0205	0.00066 ± 7e-04	0.02303 ± 0.0109	0.0091 ± 0.007
a.hydroxyacetophenone	0.49001 ± 0.2338	0.04964 ± 0.0481	0.29763 ± 0.141	0.09754 ± 0.0758
Monoterpenoids	**1.1896 ± 0.8974**	**0.03508 ± 0.0105**	**1.06974 ± 0.5473**	**0.13197 ± 0.0425**
linalool	0.00547 ± 0.0034	0.00033 ± 3e-04	0.00688 ± 0.0022	0.00162 ± 8e-04
geranyl.acetone	0.03273 ± 0.0079	0.02581 ± 0.0065	0.02832 ± 0.0052	0.03395 ± 0.0065
geraniol	1.1514 ± 0.8888	0.00894 ± 0.0062	1.03454 ± 0.5427	0.0964 ± 0.041
limonene	0.00483 ± 0.0019	0.00297 ± 0.0015	0.0036 ± 0.0011	0.0068 ± 0.0028
p.cymene	0.01945 ± 0.006	0.01112 ± 0.0049	0.014 ± 0.005	0.03224 ± 0.0199
GL_Volatiles	**0.12651 ± 0.0415**	**0.08971 ± 0.0285**	**0.1232 ± 0.0211**	**0.22563 ± 0.0821**
Z3.hexenyl.acetate	0.03126 ± 0.0216	0.00519 ± 0.0044	0.0192 ± 0.0121	0.07957 ± 0.0557
Z3.hexenyl.formate	0.09525 ± 0.0221	0.08451 ± 0.0262	0.104 ± 0.0127	0.14605 ± 0.0344
Other_VOCs	**0.8786 ± 0.2743**	**0.56697 ± 0.1717**	**0.85896 ± 0.1519**	**1.13773 ± 0.4223**
ethyl.hexanoate	0.00984 ± 0.0042	0.00928 ± 0.0042	0.01139 ± 0.0046	0.01847 ± 0.0103
X6.methyl.5.hepten.2.one	0.28029 ± 0.0601	0.16354 ± 0.0233	0.24756 ± 0.0439	0.17327 ± 0.017
unknown22.98min	0.19582 ± 0.1423	0.0065 ± 0.0065	0.01304 ± 0.0105	0.07069 ± 0.0658
X2.heptanone	0.03712 ± 0.0255	0.14802 ± 0.1337	0.16052 ± 0.0868	0.29171 ± 0.2682
X2.octanone	0.02927 ± 0.0245	0.00081 ± 5e-04	0.04525 ± 0.0414	0.00558 ± 0.0034
X2.nonanone	0.0182 ± 0.0118	0.01605 ± 0.0103	0.02881 ± 0.0159	0.02113 ± 0.0153
unknown18.43min	0.01446 ± 0.0057	0.00823 ± 0.0054	0.01961 ± 0.0092	0.02854 ± 0.0201
pentadecane	0.07426 ± 0.0382	0.01164 ± 0.0083	0.03649 ± 0.0165	0.08033 ± 0.068
unknown10.56min	0.01652 ± 0.0068	0.01537 ± 0.0068	0.01903 ± 0.0073	0.03061 ± 0.017
octanoic.acid	0.01287 ± 0.0052	0.08589 ± 0.0486	0.08582 ± 0.0402	0.13951 ± 0.0665
heptadecene	0.06396 ± 0.0227	0.02376 ± 0.0143	0.05995 ± 0.0276	0.09725 ± 0.0707
unknown11.93min	0.01312 ± 0.0067	0.00761 ± 0.0061	0.00678 ± 0.0029	0.01617 ± 0.0149
unknown.17.20min	0.01949 ± 0.0153	0.0016 ± 0.0011	0.01245 ± 0.0095	0.03138 ± 0.029
unknown9.43min	0.02484 ± 0.0076	0.01734 ± 0.0061	0.03018 ± 0.0043	0.04169 ± 0.0086
decanal	0.02942 ± 0.0077	0.03214 ± 0.0077	0.02925 ± 0.0055	0.04318 ± 0.0118
methyl.hexanoate	0.01485 ± 0.0084	0.00507 ± 0.0045	0.03522 ± 0.0149	0.00918 ± 0.0059

Texts and values in bold font designate compound classes

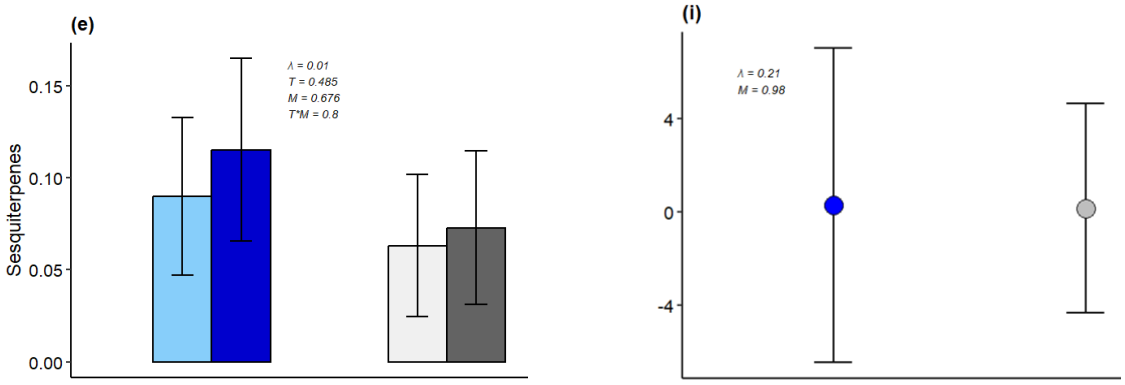

Figure S1 Constitutive and MeJA-induced emission of sesquiterpenes (normalised peak areas per flower per hour) by single individual inflorescence of the closely related wild potatoes. Outcrossing and selfing plants were either treated with methyl jasmonate (MJ, grey bars) or untreated (C, white bars). Values in left panels represent absolute amount of sesquiterpene production and values in the right panels represent the sesquiterpenes inducibility. Bars represent means ± SE, n = 20. The results of the statistical analyses are depicted in each panel and in Table 2.1. The results of the PGLS analyses with corPagel lambda (λ), a parameter that account for phylogenetic relationships among the species, are depicted above each panel. The analyses tests whether sesquiterpenes emission have been shaped by mating system transitions, MeJA treatment and shared phylogenetical history. λ ranges from zero (no phylogenetic signal), to one (strong phylogenetic signal). Intermediate values imply average phylogenetic support (Pagel, 1999).

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Chapter 3:

Genomic signatures and phenotypic consequences of mating system transitions in a geographically widespread plant species

To be submitted to The Proceedings of the National Academy of Sciences (PNAS)

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3.1 Abstract

Plant diversity is associated to environmental conditions and to a wide range of species interactions that impact the dispersal, establishment, and evolutionary success of a species. However, an enduring challenge in evolutionary biology is to show that adaptations arise predictably based on specific ecological conditions and show that evolutionary convergence in phenotype is linked with genotypic convergence. Mating systems are an important driver of population variation in plants. Cross-fertilization is ancestral in angiosperms but shifts to self-fertilization are common and are linked with reductions in the availability of pollinators, particularly at range edges. Repeated shifts from outcrossing to selfing therefore offer a natural system to study the basis of adaptive convergence, particularly for floral traits. Here, I examined the genomic and phenotypic consequences of intraspecific mating system variation

in fourteen populations of selfing and outcrossing *Arabidopsis lyrata* ssp *lyrata* from across eastern North America. Analysis of SNP frequencies from pooled whole-genome sequence data indicate that a wide range of shared SNPs and genes are under selection during independent transitions to selfing, particularly after accounting for divergence between the older, southern clade and the younger northern clade. GO analysis of SNPs associated with mating system transitions revealed significant enrichment for secondary metabolism. Analysis of floral scent emission using GC-MS revealed consistent reductions in floral volatile emissions in more selfing populations; the magnitude of the reduction was partly explained by the class of secondary metabolite, geographical location and the predicted age of populations. Younger, northern selfing populations exhibit an enrichment in aromatic compounds known to be pollinator attractants, while more established southern populations showed reduced aromatic emissions. All populations exhibited reductions in the floral emissions of so-called green leaf volatiles, and terpenoids. Vestigialisation of floral scent in selfers is consistent with a long standing, but poorly evidenced hypothesis that volatile secondary metabolites carry intrinsic physiological costs to plants. I conclude that mating system transitions lead to predictable evolutionary responses and highlight the significance of coupled genomic and phenotypic studies to understand plant adaptation.

3.2 Introduction

A striking feature of natural populations is their adaptation to local environmental conditions, with consequences for the diversity of plant and animal species (Brown *et al*., 1996; Turner, Levine, Eckert and Begun, 2008; Fournier-Level *et al*., 2011; Hancock *et al*., 2011; Keller & Seehausen, 2012; Savolainen, Lascoux and Merilä, 2013; Sexton *et al*., 2017). Divergent selection due to environmental variables can generate distinct ecological specialization within single species (Seehausen *et al*., 2014), but an enduring challenge in evolutionary biology is to test whether such ecological adaptation is predictable and repeatable. For example, postglacial range expansions after the Pleistocene, led to ecotype formation at expanding range limits in both plants and animals (Baack *et al*., 2015; Cutter and Gray, 2016; Forbes *et al*., 2017; Willi *et al*., 2018; Sánchez-Castro, Perrier and Willi, 2022). In plants, the diversity of a species is particularly influenced by traits that affect its dispersal and successful establishment in new habitats (Gaston, 2003). Within a single species, traits that facilitate reproduction, defence against attack, pollinator attraction, and colonization of novel environments or that have high genetic variation, and hence high evolutionary potential, can be expected to rapidly adapt to

specific environments (Anacker and Strauss, 2014; Walden, Lucek and Willi, 2020; Sánchez-Castro, Perrier and Willi, 2022).

Plant mating systems (i.e., the relative amount of mating between unrelated individuals) have a large impact on the geographical distribution and adaptation of plant species (Barrett and Harder, 1996; Campbell, 2015; Hartfield, Bataillon and Glémin, 2017; Grant and Kalisz, 2020). Reproduction methods, pollinators and mating systems play important roles in the processes of diversification (Barrett, 2002; Kay and Sargent, 2009; Goldberg *et al.*, 2010). In most species of angiosperms, flowers are hermaphroditic (i.e., same flower contain both the male and female floral organs: pistils and stamens). Self-fertilization occurs easily in hermaphrodites which may lead to inbreeding depression and can decrease genetic variation. Therefore, plants have evolved several mechanisms to avoid self-fertilization (Raduski *et al.*, 2012), thereby making them obligate outcrossers (Igic, Lande and Kohn, 2008). Genetic self-incompatibility (SI), a mechanism that allows plants to recognise and reject closely related pollen, is defined as the inability of a fertile hermaphroditic plant to produce zygotes after self-pollination (Watanabe *et al.*, 2012). This recognition system has arisen multiple times in the history of flowering plants (Raduski, Haney and Igić, 2012). Despite the ubiquity of molecular SI mechanisms, its breakdown due to loss of function mutations occurs frequently, leading to the evolution of self-compatibility (SC). Loss of SI is apparently unidirectional/irreversible, perhaps due to the complexity of the trait (Dollo's Law). The transition from obligate outcrossing to self-fertilization is therefore one of the most common major evolutionary transitions in angiosperms (Igic, Lande and Kohn, 2008).

The evolution of selfing has profound evolutionary consequences. Selfing decreases effective population size and thus changes the efficacy of recombination and the response to selection, leaving various genomic signatures (Hartfield, Bataillon and Glémin, 2017; Cutter, 2019; Mattila, Laenen and Slotte, 2020; Fujii and Tsuchimatsu, 2022). In self-fertilization, two gametes are passed on to the offspring from the same parent, thereby reducing haploid gene flow while also increasing homozygosity (Hartfield *et al.*, 2017). In contrast, outcrossing offspring inherits only one copy of an individual parent's chromosomes which can sometimes lead to the breakdown of alleles that are important for the evolution of adaptive traits (Grossenbacher *et al.*, 2015). Self-fertilization can change population allele frequencies, standing genetic variation (SGV) (Wright, Kalisz and Slotte, 2013), and the likelihood of genetic drift (Nordborg, 2000, Charlesworth, 1992), which may limit their capacity to adapt to

changing environmental conditions (Robinson *et al.,* 1992). Conversely, influx of maladaptive genes is also limited and the proportion of parent genes that get passed on to the offspring increases (a process termed 'transmission advantage'), potentially increasing the rate of adaptive evolution (Álvarez-Castro and Yang, 2012). Selfing species also have an advantage of reproductive assurance because they do not depend on the presence of pollinators and compatible mates to reproduce in a new habitat, thereby facilitating colonisation of new habitats beyond the range margin (Baker, 1955; Charlesworth, 1989; Barrett and Harder, 1996; (Charlesworth, 2006; Griffin and Willi, 2014; Campbell, 2015). However, the balance of the genomic and ecological costs and benefits of selfing remains relatively poorly understood.

In addition to influencing genetic variation, evolution to selfing may also facilitate adaptive phenotypic evolution, and in some cases, speciation. Transition to selfing frequently results in reduced floral display (Baker, 1955; Sicard and Lenhard, 2011; Wright, Kalisz and Slotte, 2013), because reduced investment in floral display should decrease costs of floral signalling, whereas reduced stigma–anther distance may increase the capacity for self-fertilization (Johnson, Campbell and Barrett, 2015; Shimizu and Tsuchimatsu, 2015; Toräng *et al.*, 2017). The convergent vestigialization of floral traits in many selfing species is occasionally termed the "selfing syndrome" (Doubleday *et al*., 2013), but research on this phenomenon has focused almost exclusively on morphological or colour traits that act as visual cues to pollinators (Byers, 2021). Plants also produce a stunning diversity of scent cues that are particularly important for insect pollinators, including bees, flies, beetles and particularly moths and butterflies but generally, floral scent evolution has received less attention (Byers, 2021). Majetic *et al*. (2019) found decreased emission of VOCs that may be involved in pollinator attraction, in the self-fertile inbred phlox (*Phlox cuspitata*) when compared to the outcrossing *Phlox drummondii*, but only a handful of studies have examined the evolution of scent. A study on *Abronia umbellate* showed that floral scent is much more significantly reduced in selfing compared to outcrossing populations (Doubleday *et al*., 2013). Likewise, selfing populations of geographically widespread *Arabis alpina* (Brassicaceae) produced less scented flowers than the outcrossing populations (Petren *et al*., 2021). However, both studies were limited by a lack of repeated independent transitions to selfing in these particular study systems.

The reproductive assurance provided by selfing allows self-compatible species and genotypes to colonise habitats in which mates and/or pollinators may be limited, and thereby facilitate range expansion. A considerable body of research has documented the association of selfing

with islands and other isolated habitats (i.e., Baker's Law; Baker,1955, 1967), and at range margins (Griffin and Willi 2014). Colonisation beyond the established range margin may lead to increased abiotic stress, and reductions in visitation by both pollinators and herbivores/predators, leading to the evolution of reduced allocation to floral (McNichol *et al*., 2023) and defence traits (Campbell and Kessler 2013). However how selection from these altered ecological interactions shapes the evolution of selfers across a species range remains poorly understood; few studies have tried to explain the relationship between plant mating system and adaptation to habitat in part due to the difficulty in accounting for evolutionary history, ecological factors, and geography (Caswell 2001, Metcalf *et al.*, 2009 and Caswell, 2012). For example, studies on *A. lyrata* showed that mating system shifts to selfing was associated with decreased effective population size toward range edges (Griffin and Willi, 2014; Willi *et al*., 2018), though these shifts were not associated with adaptation to a particular substrate (Lucek *et al*., 2019). Mating systems should theoretically influence adaptation at range limits, but empirical studies based on replicate transitions to selfing remain limited, and studies linking genomic and phenotypic evidence are virtually non-existent.

Here, I focused on the effect of mating system on the evolution of adaptive traits across the latitudinal gradient of a species' distribution, using the short-lived perennial plant *Arabidopsis lyrata spp. Lyrata.* This nominally SI species is widely distributed across North America and is characterised by numerous independent losses of effective SI during its postglacial range expansion (Mable *et al*., 2005, Griffin and Willi, 2014; Willi *et al*., 2018; Sánchez-Castro, Perrier, and Willi, 2022). Using a genome-wide association study (GWAS), I asked three questions: 1) Is there genomic evidence for convergent selection across independent transitions to selfing? 2) Are there shared polymorphisms or loci linked with repeated mating system transitions? and 3) Is there a correspondence between genomic signatures of selection and reproductive phenotypes, specifically floral scent?

3.3 Materials and Methods

3.3.1 Study system

Arabidopsis lyrata is an hermaphroditic flowering plant belonging to the family Brassicaceae and a close relative of the model organism *A. thaliana* (Novikova *et al.*, 2016). *Arabidopsis lyrata* is found across northern and central Europe, Asia, and North America (Ross-Ibarra *et al.*, 2008). The North American *A. lyrata subsp. lyrata* (hereafter referred to as *A. lyrata*) is

distributed in the US and Canada from North Carolina to the state of New York in the eastern United States, and from Missouri to south-western Ontario in the Midwest (Willi *et al.*, 2010, 2018; Griffin and Willi, 2014). *A. lyrata* generally grows on outcrops of limestone, dolomite, siltstone, metasiltstone, amphibolite, sandy riverbanks, crevices, and thinly soiled ledges, and other igneous and metamorphic rocks (*Digital Atlas of the Virginia Floral | Arabidopsis lyrata (L.) O'Kane & Al-Shehbaz ssp. lyrata*; Sánchez-Castro, Armbruster and Willi, 2022). In my work, fourteen populations of *A. lyrata* were selected across a wide range of latitudes, from Canada to the southern US and including the two types of mating system (Figure 3.1, Table 3.1). Populations from across the latitudes of 41°N to 50°N are hereafter referred to as Northern region and those within the latitudes of 35°W to 40°W are addressed as Southern region. Generally, the main system of mating in *A. lyrata* is outcrossing (Mable, Schierup and Charlesworth, 2003; Willi and Maattanen, 2011; Mable *et al.*, 2017). Nevertheless, predominantly selfing populations occur at range edges (Griffin and Willi, 2014). The type of mating system of the populations investigated was determined from the study by Mable *et al.* (2005); Willi and Maattanen (2010); Willi and Maattanen (2011); Griffin and Willi (2014) and Mable *et al.* (2017) where they analysed the progeny and estimated the multi-locus outcrossing rate. Populations with outcrossing rate lesser than 0.3 were assessed as selfers while population with outcrossing rate greater than 0.8 were considered as outcrossers (Table 3.1).

Fourteen populations of *Arabidopsis lyrata* covering the geographical range of the species in the eastern North America, from Canada to the southern United States were studied. These comprise the two categories of mating systems: outcrossing and selfing (Figure 3.1 and Table 3.1), selected as clusters (typically pairs) of geographically similar selfing and outcrossing populations. Ten populations were in the north of the distribution (northern clade). Six of these were self-fertilizing and the other four were outcrossers. Four populations were in the south of the distribution (southern clade), two of which were self-fertilizing and the other two, outcrossing (Figure 3.1 and Table 3.1).

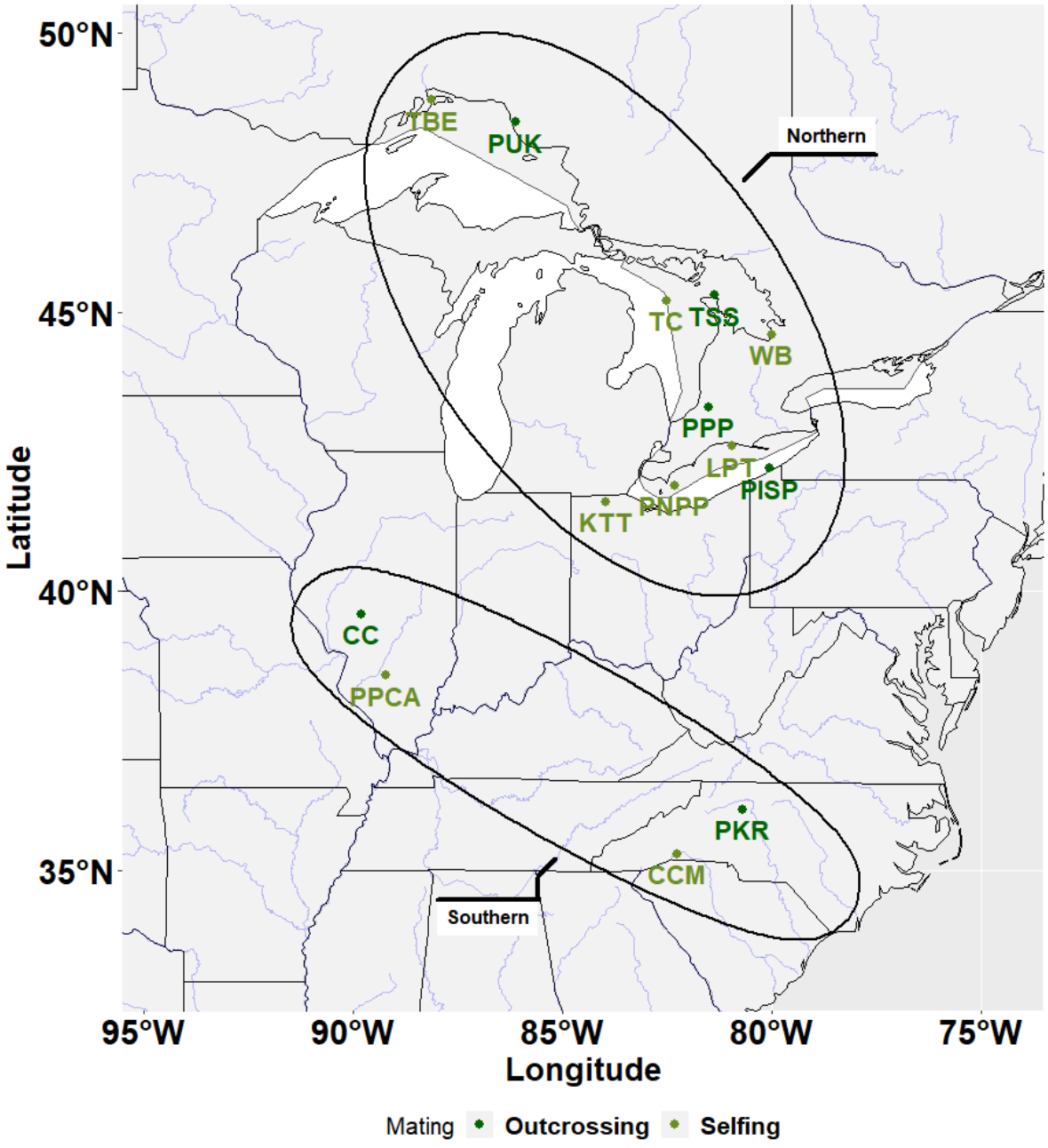

Figure 3.1 Site locations of the fourteen *Arabidopsis lyrata* populations in this study. The dark-green coloured dots indicate the obligate outcrossing and the light green, selfing. The two circles with four and ten populations denotes southern and northern clades respectively. The population abbreviation notation is described in Table 3.1.

3.3.2 Data collection and processing

I used a pooled sequencing approach to study the population genomics of selfing transitions. Pooled sequencing approach is an alternative option to sequencing many individuals, and it involves sequencing pooled DNA from several individuals. Pooled sequencing has numerous

benefits like reduced cost, less time for sequencing experiments and requiring less DNA from each single individual (Anand *et al*., 2016). In addition, due to the larger sample size Pool-seq presents, it gives a more robust estimate of allele frequencies which allows decreasing the overall variance of the estimated allele frequency (Schlötterer *et al*., 2014); however, individual haplotype information is lost. For each population, fresh leaf tissue from 15 to 56 individual plants was collected, and rapidly frozen on dry ice. DNA was extracted from each individual, using the QIAGEN DNeasy Plant Mini-Pro kit. Genomic libraries based on individual equimolar pools from each population were then sequenced on four Illumina HiSeq 2000 lanes using standard chemistry. Twenty-five percent of each lane was used to account for potential artefacts between runs. Paired-end sequencing was performed with average read length ca. 150 base pairs. Two technical sequencing experiments were conducted (N = 2 for each population) to protect against sequencing error or instrumental failure.

I developed a similar bioinformatics and analysis workflow for the pool-seq data analysis based on Popoolation (Kofler *et al*., 2011) and as described by Lucek, Hohmann and Willi (2019). The adapter sequence was clipped and the nucleotides with bad quality were trimmed using fastp v0.23.1 (Chen *et al.*, 2018, https://github.com/OpenGene/fastp). A minimum base quality threshold of 20 and a minimum length of 80 bp was used. Subsequently, all retained reads were mapped to *A. lyrata* reference genome v2.1 (Hu *et al.*, 2011; Rawat *et al.*, 2015) retrieved from JGI's PHYTOZOME portal (https://phytozome-next.jgi.doe.gov/info/Alyrata_v2_1). Duplicate reads were filtered out using Picard 2.0.1 (https://github.com/broadinstitute/picard) while also retaining only properly mapped reads with mapping quality greater than 20 using SAMtools 1.7 (Li *et al*., 2009). A mpileup file combining all the fourteen populations was generated using SAMtools and mpileup2snp command in VarScan 2.4.4 (Koboldt *et al.*, 2012) was used to call SNPs with a minimal read depth of 50 and minimum of 4 reads per allele. A minimal variant allele frequency threshold of 0.03 was also applied. SNPs with a strand bias of more than 90% were filtered out and the percentage missing SNPs was calculated for the fourteen samples using PLINK v2.0 (Chang *et al.*, 2015). All samples have less than 10% missing data, hence, SNPs with missing data were not filtered out. Overall, the filtering processes resulted in a total number of 3,288,707 high quality SNPs.

Test of genome wide selection can be influenced by SNP co-localisation; to avoid this, I adopted a sub-sampling strategy (Gautier *et al.*, 2015) by using the function "pooldata.subset()" available from the R package poolfstat (Hivert *et al.*, 2018). I generated 10 sub-data sets by

sampling every 10th-SNPs in an ordered map. The 10 sub-data sets were then analysed separately and in parallel. After ensuring that the different estimated covariance matrices of the sub-data sets were similar, one sub-data set was randomly chosen for further analysis. The subsampling strategy led to data sets comprising 328,871 polymorphic SNPs each for northern, southern and all populations combined.

Table 3.1 List of the populations studied, with information on population name, date of sampling, location, the latitude recorded in the field, mating system as well as the diploid number of individuals per population.

Location	Population	Mating_System	Clade	Latitude(°N)	Longitude(°W)	SSGD	SSPD	Outcrossing_rate
Presqu'Ile State Park (PRI) PA	PISP	Outcrossing	Northern	42.20	80.04	56	38	0.89
Tobermory Singing Sands Ontario	TSS	Outcrossing	Northern	45.30	81.35	19	-	0.91
Pinery Provincial Park (PIN) Ontario	PPP	Outcrossing	Northern	43.30	81.49	35	8	0.84
Pukaskwa National Park	PUK	Outcrossing	Northern	48.40	86.11	25	27	0.96
Crooked Creek	CC	Outcrossing	Southern	39.60	89.82	32	12	> 0.8
Pore's Knob Road	PKR	Outcrossing	Southern	36.10	80.71	33	29	> 0.9
Long Point Provincial Park	LPT	Selfing	Northern	42.60	80.93	27	14	0.13
Point Pelee National Park Ontario	PNPP	Selfing	Northern	41.90	82.31	18	4	0.09
Wasaga Beach, Blueberry Trails	WB	Selfing	Northern	44.60	80.01	32	28	0.25
Kitty Todd Nature Preserve, Toledo, Ohio	KTT	Selfing	Northern	41.60	83.97	33	11	< 0.3
Tobermory Cliff	TC	Selfing	Northern	45.20	82.51	26	-	0.18
Terrace Bay East	TBE	Selfing	Northern	48.80	88.12	15	-	< 0.3
Pacific Palisades Conservation Area	PPCA	Selfing	Southern	38.50	88.81	24	16	< 0.3
Cedar Cliff MTN	CCM	Selfing	Southern	35.30	82.25	34	27	< 0.3
Fort Leonard Wood	FLW	Outcrossing	Southern	37.72	-	-	26	> 0.8

Keys: SSGD - sample size for genotypic analysis; SSPD - sample size for phenotypic trait analysis

3.3.3 SNP association analysis

Natural selection can favour alleles in a population thereby leading to increase in frequency of that allele over generations and eventually causing microevolution or change in allele frequency (Chen *et al*., 2019). For instance, a selfing population may have more of one fitness-increasing allele of a gene relative to outcrossing population, while outcrossers may have more of another beneficial allele. If such a pattern is found for many different selfing or outcrossing populations, then I can infer that the alleles are adaptive for being a selfer or outcrosser respectively. Loci with significantly overly differentiated alleles can then be identified as being subjected to selection and associated with strategies of mating. In this study, association of mating system with SNP frequency sets for each geographical clade was tested by the auxiliary covariate model in BayPass version 2.2 (Gautier, 2015). BayPass estimates and corrects for underlying population structure by using the scaled covariance matrix of population allele frequencies while also dealing with multiple testing issues (Gautier, 2015). Because our initial analysis revealed a deep divergence between southern and northern clades (Figure 3.2 and Figure 3.1), I proceeded to analyse our data for three SNP data sets: (a) only northern populations (N = 10), (b) only southern populations N = (4) and (c) all 14 populations combined.

The mating system (outcrossing vs selfing) was treated as a binary variable in the association study. In BayPass, prior to using the auxiliary covariate model, the core model was used with default parameters to generate the population covariance matrix from corresponding SNP sets. The population structure was then adjusted for, in the auxiliary covariate model using the generated covariance matrix. The auxiliary covariate model was run independently five times using different random seeds (Gautier, 2015), to reduce artefacts due to instability between runs for small numbers of populations. The mean correlation co-efficient computed over the five independent runs was then used as the estimate of association. Each independent auxiliary covariate run was performed with twenty pilot runs (1,000 steps each) followed by 5,000 burn-in iterations and then 25,000 post-burnin steps with a thinning factor of 25. The Bayes factor (BF) expressed in deciban units (dB) was calculated for each SNP quantifying the extent to its frequency is related with mating system. Outlier SNPs were defined as those SNPs whose mean Bayes Factor (BF) over five runs was among the top 0.8% for each clade and mating system variable while also ensuring that the defined outlier SNP were also not below the minimum threshold of evidence, BF = 10 (Gautier, 2015). The number of outlier SNPs found was 2,646,

2,665 and 2659 SNPs for the data sets of northern, southern, and combined population respectively. The number of genes containing the BF outlier SNPs was analysed for each clade.

To confirm the mating system associated allele frequencies differentiation among the outlier SNPs, I used pcadapt to perform a PCA (principal component analysis) (Luu *et al*., 2017), respectively, for Northern and southern populations. pcadapt was developed to detect genetic markers that are involved in biological adaptation. Next, I utilized a linear model with the mean PC scores per population group to examine segregation between selfing and outcrossing populations along the two leading PC axes.

3.3.4 Outlier gene annotation and GO enrichment analysis.

The number of genes in which the discovered outlier SNPs were located was analysed. The number of overlapping outlier SNPs-containing genes (hereafter, referred to as outlier genes) between northern and southern clade data sets was tested for being lower or higher than expected by chance. This was assessed by conducting a resampling analysis based on 10,000 replicates. The number of outlier genes was randomly sampled from the total pool of covered genes separately for northern and southern clades, calculating the overlap for the 10000 replicates and comparing the results with my observed values. Gene ontology (GO) enrichment analysis was performed to identify the biological processes associated with outlier genes. The functional enrichment analyses were restricted to exonic regions and performs over-representation analysis with hypergeometric test using gprofiler2 (Peterson *et al.*, 2020). The gprofiler2 provides access to various gene identifier conversion functionality which enables mapping between hundreds of distinct identifier types or orthologous species. Also, it enables analysis of genes from numerous organisms using high-quality updated annotation databases while archiving the previous versions for reproducibility. The *A. thaliana* orthologues were obtained from the updated *A. lyrata* genome annotation (Rawat *et al*., 2015). The GO enrichment analysis that was conducted in gprofiler2 used the most recently updated annotation of *A. thaliana*, TAIR10 from TAIR (The *Arabidopsis* Information Resource, www.*Arabidopsis*.org) accessed via gprofiler2. The GO analysis was conducted for the three datasets using a False Discovery Rate (FDR) of 0.05 and restricting analysis to terms of biological processes.

3.3.5 Floral volatile metabolic analysis

Floral scent was collected in-situ from a few to many individuals from twelve populations of *Arabidopsis lyrata* (Table 3.1), using a dynamic headspace trapping. Measurements of floral VOCs and genomic resequencing were from the same populations and same individual plants except for the FLW population which was included in the floral VOCs measurements but was not re-sequenced. Single inflorescences were enclosed in 500 mL polyethylene cups in-situ. We ensured that the leaves were not enclosed as part of the single inflorescence. We also appreciate that the inflorescence has vegetative tissues such as calyx that may also be emitting volatile compounds. Calyx is considered as part of the flower bouquet (Gagliardi *et al*., 2018) and therefore we did not separately quantify the VOC emissions from the vegetative tissue, calyx. The air collected in the headspace was pulled through ORBO-32 charcoal adsorbent tubes (Supelco, Bellefonte, PA, USA), using a 12 V vacuum pump at ca. 11.5 l/min (Gast Manufacturing Inc., Benton Harbor, MI, USA). Floral volatiles were collected between 10:00 and 20:00 hours (6-8 hours collection). Due to variations in the duration of volatile collection among the plants, we assessed the volatile emission rate per hour of collection. The analyses took place within a screenhouse, chosen to reduce the risk of contaminants. This measure was taken to safeguard the reliability of our samples and prevent any possible disruptions. However, we introduced an air control, represented by an empty chamber, to facilitate the identification of environmental contaminants and establish baseline levels of VOC in the background.

After collecting the VOC (Volatile Organic Compound) traps, they were spiked with 5 µL of a tetraline internal standard, which had a concentration of 90 ng/µL, in a solvent of 250 µL of dichloromethane. Following this, the samples underwent analysis using Gas Chromatography-Mass Spectrometry (GC-MS) with the utilization of a Varian Saturn 2200 GC/MS/MS instrument, which was equipped with a CP-8400 autosampler. The GC analysis was carried out in splitless mode, employing a DB-5 column (an Agilent J&W GC Column, characterized by dimensions of 30 meters in length, an inner diameter of 0.25 mm, and a film thickness of 0.25 µm). The temperature program was initiated with an initial injection temperature set at 225 °C. Subsequently, the column temperature underwent a gradual increase from 40 °C to 180 °C, at a rate of 10 °C per minute. The analysis culminated with a final temperature hold at 180 °C, maintained for a duration of 10 minutes. The identification process involved comparing the mass spectra of the compound peaks with the NIST 2008 and 2014 mass spectral libraries (National Institute of Standards and Technology). When possible, identities were confirmed by comparing mass spectra and retention times with those of known standards.

Individual-level VOC analysis lacks consistent patterns due to the significant diversity among the studied species. Consequently, it's not anticipated that the same individual VOCs would serve identical functions across all species. However, we anticipate that groups of VOCs within the same class would have shared functions. Therefore, to examine functional classes across species, considering the variations in individual compounds, we combined the volatile compounds into suitable classes before proceeding with subsequent investigations. Total amount of VOC emission and the emission of major groups of VOCs were analysed separately.

3.3.6 Statistical analysis of phenotypic data

To examine the effect of mating system and latitude or population clade and the interaction between the two independent variables, on floral scent variation of *A. lyrata*, I performed a two-way analysis of variance (ANOVA) for each compound class of the floral scent using lm() function in in R version 4.1.3 (R Core Team, 2022), followed by the REGWQ (Ryan-Einot-Gabriel-Welsch and Quiot) post-hoc test using REGW.test() in R version 4.1.3 (R Core Team, 2022). The model included mating system and latitude or clade together with their interaction as independent factors or predictor and emission (peak area flower^{-1} h^{-1}) of the various floral volatiles (total VOCs, benzenoids, terpenoids, green leaf volatile (GLV), or other VOCs) as the dependent variable. Significance was assessed using F-tests. Effects were considered significant if $P < 0.05$. The mean values of the traits were log-transformed for normality prior to the analyses.

3.4 Results

3.4.1 Population covariance structure *A. lyrata*

The population structure of North American *A. lyrata* populations was analysed by using pooled whole-genome sequence data from across the *A. lyrata* populations distribution. Two well separated genetic clades were confirmed by hierarchical demography estimated by BayPass under the core model, based on the overall SNPs (Figure 3.2). BayPass estimates of covariance structure agree with the geographic origins of the sampled *A. lyrata* populations (Figure 3.1). The dendrogram shows two distinct lineages and 6 independent transitions of mating systems thereby justifying our population pair choices.

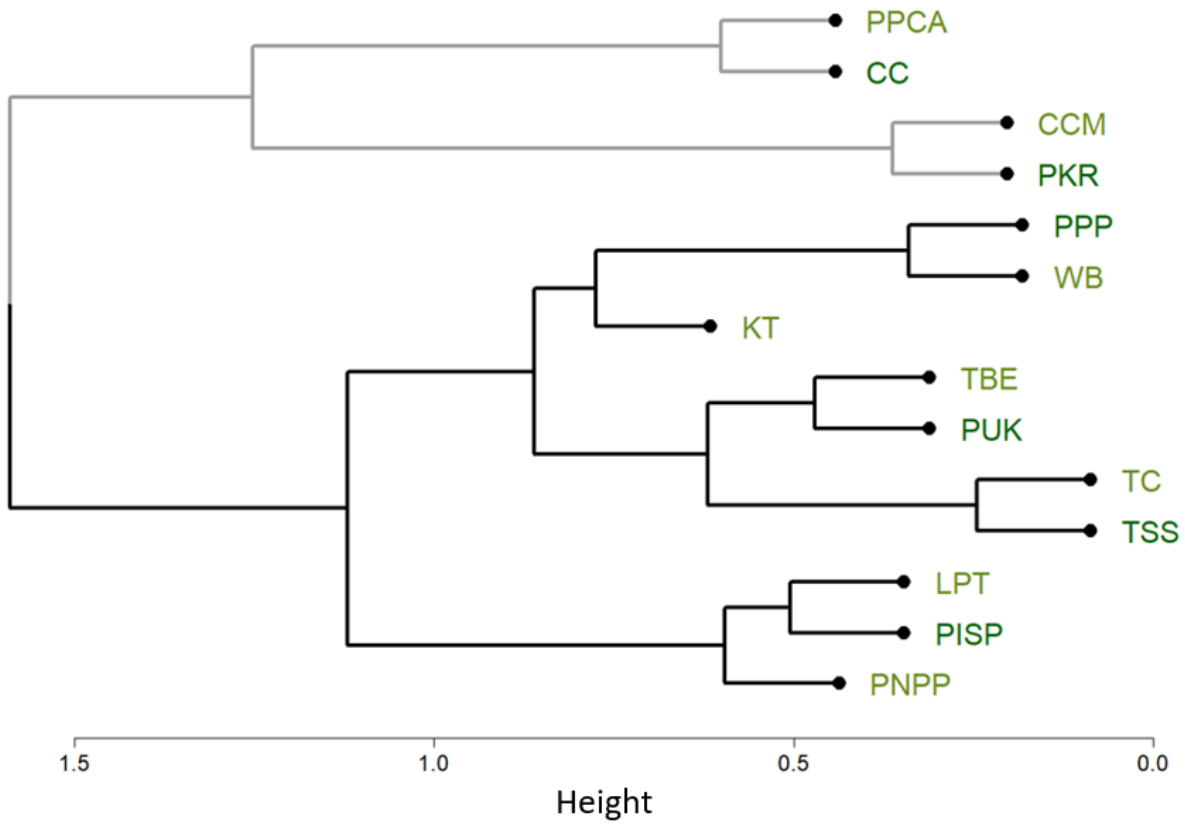

Figure 3.2 Hierarchical clustering tree using the average agglomeration method from the scaled covariance matrices among 14 *A. lyrata* populations estimated from BayPass under the core model (Gautier, 2015). Estimates are based on the analysis of the analysis of the overall SNP data set consisting of 32,887 SNPs. Hierarchical clustering tree revealed two distinct clades with southern *A. lyrata* populations (grey coloured branches) possibly evolved earlier than the northern ones (black coloured branches). Population codes are colored according to their mating systems; outcrossing populations in dark green and selfing populations in light green.

3.4.2 Mating system association, outlier SNPs and GO functional enrichment analysis.

The 328,871 single nucleotide polymorphisms detected in the combined set (both clades) were distributed across *A. lyrata* genome sequence (Figure 3.3). Thirty-six percent of the SNPs (116,613) were found within genes. The top 0.8% outlier SNPs (SNPs associated with mating systems) reported by Baypass for the northern population ($N_{Northern}$= 2,646) and southern population ($N_{Southern}$ = 2,665) data sets were distributed across the genome (Figure 3.3). Of those, between 24% and 35% were located within genes for the two clades.

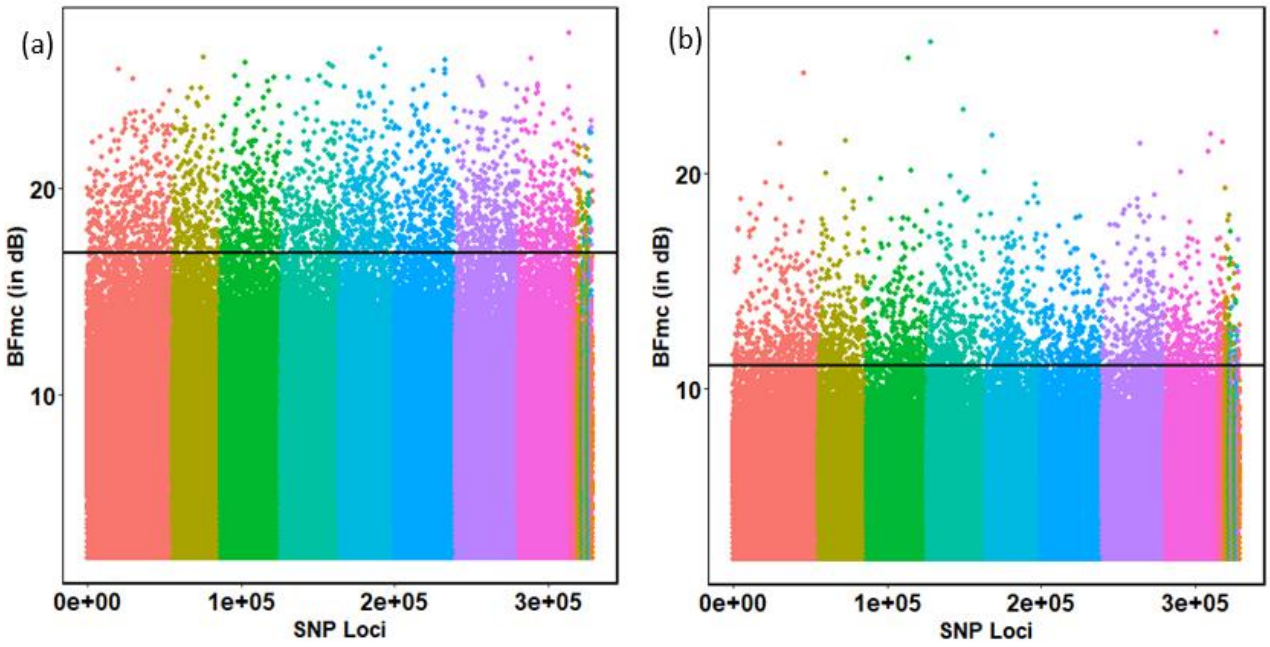

Figure 3.3 The genomic footprint of mating system in *Arabidopsis lyrata* populations. Manhattan plots shows the distribution of mean Bayes Factor (BF_{mc}) derived from five Baypass auxiliary covariate model runs for analysing the association of SNPs with independent mating system variable for (a) 328,871 biallelic SNPs of northern populations and (b) 328,871 SNPs of southern populations. The 0.8% SNPs with the top Bayes Factor are at the threshold line and above. The major scaffolds, scaffolds I-VIII are highlighted in colour, corresponding to the eight chromosomes of *A. lyrata*.

The principal component analysis performed with the outlier SNPs (Figure S2a) confirmed the SNP association study outliers by separating populations of different mating systems in the northern clade along the first axes (northern: F1,8 = 36.36, p = 0.0003; southern: F 1,2 = 1.5721, p = 0.3366) and second PC (northern: F1,8 = 108.17, p = 6.326e-06; southern: F 1,2 = 0.0059, p = 0.9457) for northern clade. For nonoutlier SNPs (Figure S2b), no association with mating system was found, indicating that the top 1% outlier SNPs are indeed the SNP that are associated with different mating systems (PC1: northern: F1,8 = 0.0045, p = 0.9481; southern: F1,2 = 0.0216, p = 0.8965; PC2: northern: F1,8 = 0.1081, p = 0.7507; southern: F1,2 = 3.715, p = 0.1937).

To evaluate parallelism in the association of genetic variation with mating system, both within and across the northern and southern population clusters, the outlier SNPs and outlier genes, from the two clades were compared. Among all the outlier SNPs, 28 SNPs (0.5%) overlap between the northern and southern dataset and 33 genes (2.5%) were affected in common by

at least one outlier SNP (Figure 3.4). I compared the number of overlap outlier SNPs and genes with the expected number of shared SNPs and genes calculated by resampling analysis. Observed values were higher than expected by chance for the outlier SNPs and overlap genes were in the range of random expectation (Figure 3.5). In comparison to the outlier SNPs and genes overlap, a higher proportion of enriched GO terms (10.5%) was shared between clades (Figure 3.4). Of the enriched GO terms for northern, southern, and combined set populations, 20%, 12% and 18% respectively were associated with "response to stress" while 11%, 16% and 3% were associated with "organic substance biosynthesis or metabolism" respectively.

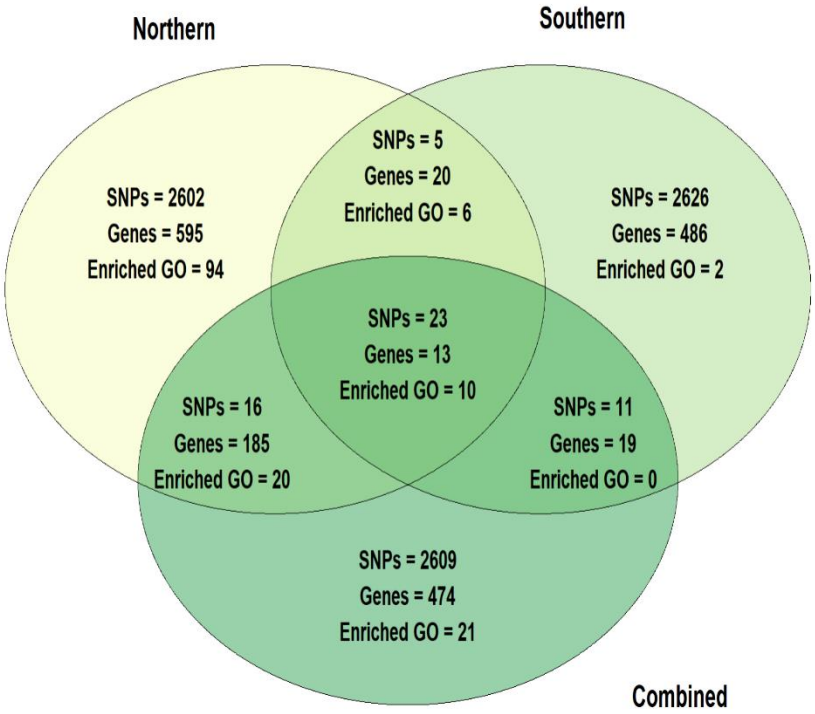

Figure 3.4 Venn diagram showing the overlap in the 0.8% outlier SNPs, the number of genes mapped to by outliers SNPs, and the number of enriched Gene Ontology terms for northern populations, southern populations, and combined populations.

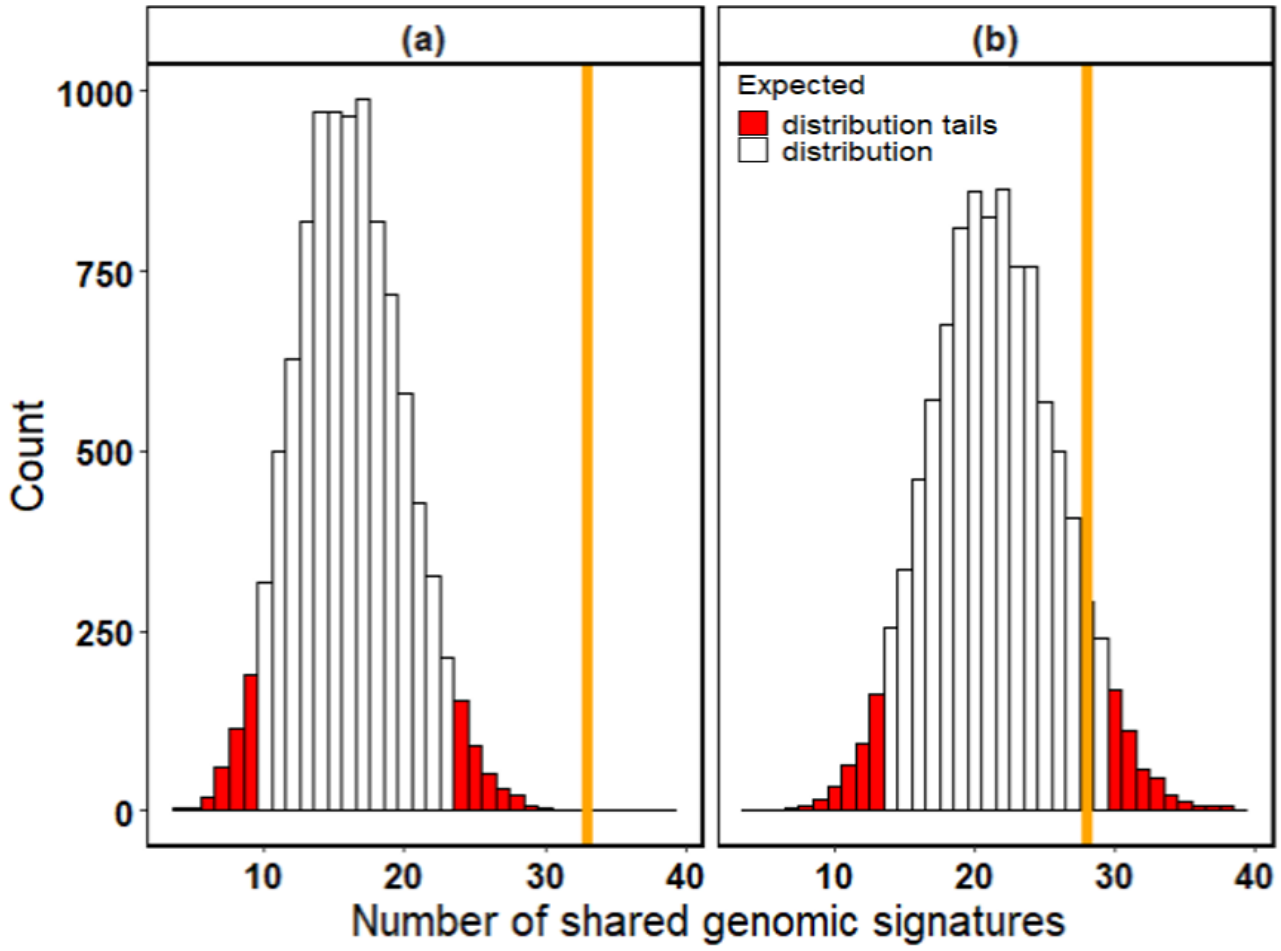

Figure 3.5 Distribution of expected count values of shared outlier SNPs (a) and shared outlier genes (b) between the northern and southern clade of *Arabidopsis lyrata*. Expected values were calculated using a random resampling analysis with 10,000 iterations and are indicated in white bars (the red-coloured tails represent the 2.5% of the distribution). Observed values for the number of features are indicated in orange.

To remove redundant GO terms, term-size greater than 5700 were filtered out. Term size is the number of domain genes associated with a given GO term. GO terms that have over 6000 genes associated with them were considered as too broad (e.g., housekeeping processes) and were removed. After removing the redundant terms, 6 – 39 Go terms remained (Figure 3.6). Of the six filtered GO terms that were enriched in both northern and southern clades, two terms were related to "response to stress" and four terms were linked "organic substance biosynthesis/metabolism" (Figure 3.6). For northern populations, "aromatic compound biosynthetic process" was specifically enriched (Figure 3.6). The combined data s*et al*so has some GO terms linked to "response to stress" and precisely, "response to other organisms" as well as "organic compound metabolic process" (Figure 3.6).

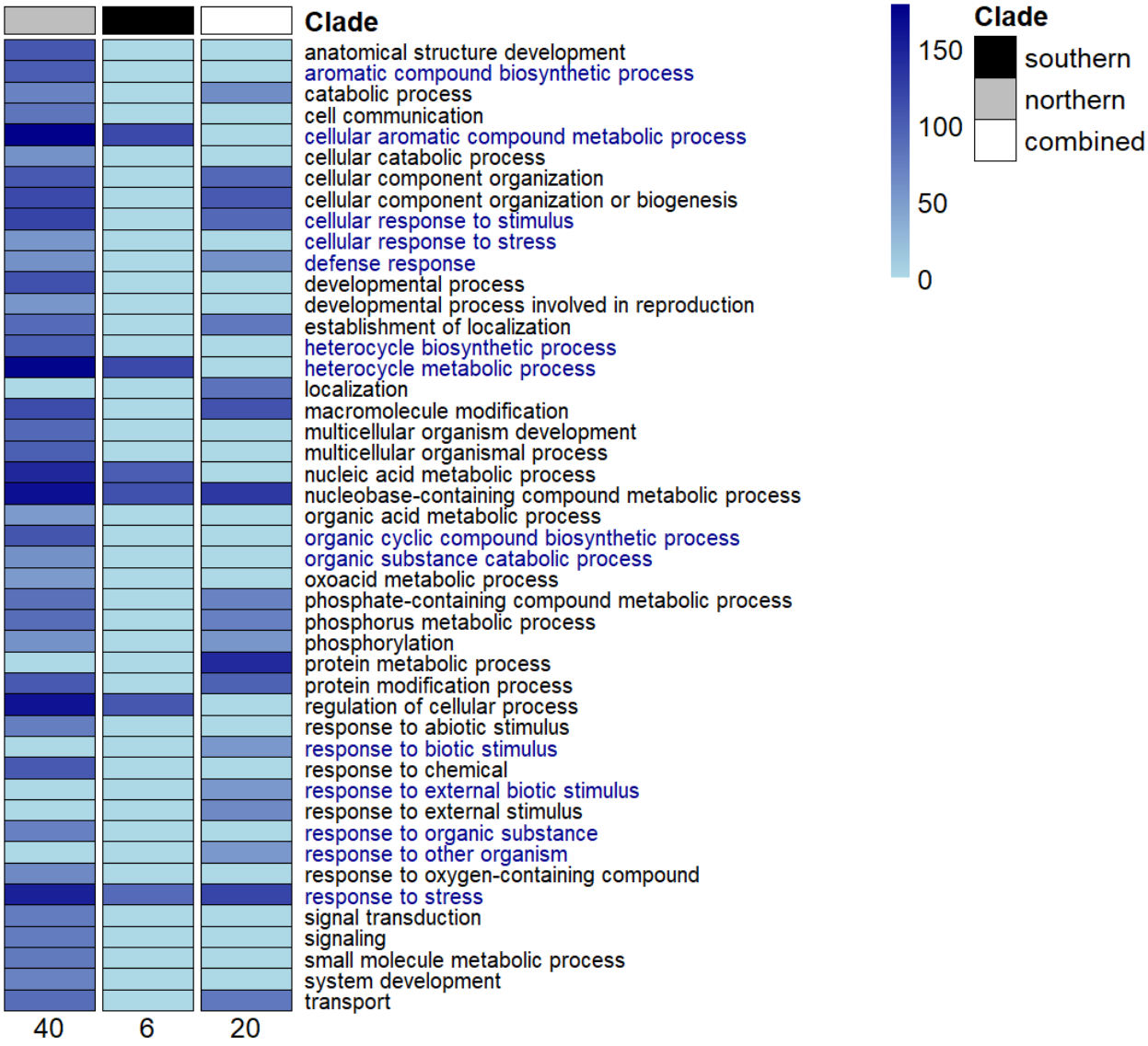

Figure 3.6 Enriched GO terms of northern clade (grey), southern clade (black) and combined dataset (white). X-axis values labels represent the number of GO terms in each population set. Y-axis represents the list of enriched GO functional annotations. Dark blue indicates the highest number of genes annotated on the GO and light blue indicates lowest gene count involve in the corresponding GO annotation (The darker the blueness, the higher gene count involved in GO term). Blue highlighted annotations correspond to the phenotypic traits of interest.

3.4.3 Floral VOC emissions

To determine the degree to which variation in floral VOC emission are related to selfing evolution, I analysed the 42 floral volatiles (Table S2) of 8 representative selfing populations and 12 outcrossing populations of *A. lyrata*. Hierarchical clustering based on genetic distances,

using available SNP datasets, distinctively clustered *A. lyrata* populations into southern and northern clades from which selfers have evolved (Figure 3.2). Flowers of *A. lyrata* growing in the field emitted various volatile organic compounds (VOCs) which included terpenoids, benzenoids, green leaf volatiles, and other unknown or unclassified VOCs (Table S2). The amount of VOC emissions varies with mating system transitions. There is a significant interaction between mating system and clade for the total amount of VOC, terpenoid, green leaf volatile (GLVs) and benzenoid emissions (Table 3.2; Figure 3.7). Transition to selfing reduced the total VOC, benzenoid and other VOC emissions by 35 - 45% in the southern clade enhanced the total VOC, benzenoid and other VOC emissions by 25 - 45% in the northern clade (Table 3.2; Figure 3.7a, c and e). Selfing evolution reduced the terpenoid emissions by 40% in the southern clade and had no significant effect on terpenoids production in the northern clade (Table 3.2; Figure 3.7b). Shift to selfing decreased the green leaf volatile emissions by 50% in the southern and 55% in the northern clade (Table 3.2; Figure 3.7d). Effect of mating system on floral scent production is not correlated with latitude (Figure 3.8) as opposed to the significant changes observed with clades (Figure 3.7). All the results from latitude effect analysis receive no statistical support (Figure 3.8) except green leaf volatiles (Figure 3.8d) thereby highlighting a lack of floral scent variation linked latitude.

Table 3.2 Statistical results for the ANOVA analysis of VOC compound group emissions from *A. lyrata* populations in-situ (Figure 3.7). The table shows effects of region clades, mating system and their interactions (A) and the effects of latitudes, mating system and their interactions (B). Statistical analyses were performed with the absolute amounts ($ng^{-1}\ h^{-1}$) of VOCs emitted. The first column indicates the statistical results for the effect of region/clade. Interactions between region clade and mating system, are reported in the last column. Data were log-transformed to meet assumption of normality. Level of statistical significance are indicated with asterisks (***P < 0.001, **P < 0.01, *P ≤ 0.05 and ns (not significant) P >0.05). Values are F-statistics and degrees of freedom are stated at the heading of the table as F (df1, df2), sample sizes are shown in table 3.1.

A

Floral scent	Clade F(1, 236)	Mating System F(1, 236)	Clade:Mating System
Total VOCs	3.9817 *	0.2113 ns	10.6483 **
Terpenoids	10.6194 **	1.2437 ns	3.6865 ns
Benzenoids	3.6235 ns	0.1179 ns	16.1791 ***
Green Leaf VOCs	0.0312 ns	11.1727 ***	0 ns
Other VOCs	5.7213 *	1.7535 ns	17.0474 ***

B

Foral scent	Latitude F(1, 236)	Mating System F(1, 236)	Latitude:Mating System
Total VOCs	1.2474 ns	0.0265 ns	2.1195 ns
Terpenoids	0.1494 ns	0.9038 ns	0.0853 ns
Benzenoids	0.0192 ns	0.2259 ns	6.5018 *
Green Leaf VOCs	22.2924 ***	8.0186 **	7.5721 **
Other VOCs	0.0958 ns	1.2258 ns	9.4606 **

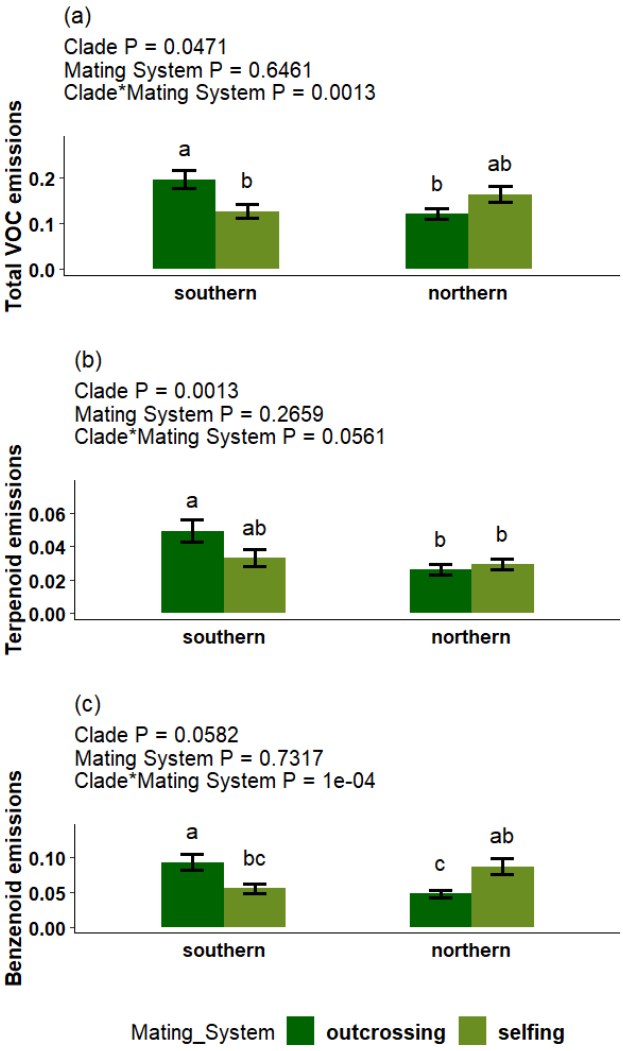

Figure 3.7 Emission of major groups of floral VOCs (Volatile organic compounds) by individuals from northern and southern region in-situ. The two region clades contains both outcrossing and selfing individuals. Bars represent means ± SEM, sample sizes are shown in table 2. 1.GL VOCs denotes Green Leaf VOCs. Different letters indicate significant differences among mating system × clade determined by post hoc analysis using Ryan-Einot-Gabriel-Welsh multiple range test ($P < 0.05$).

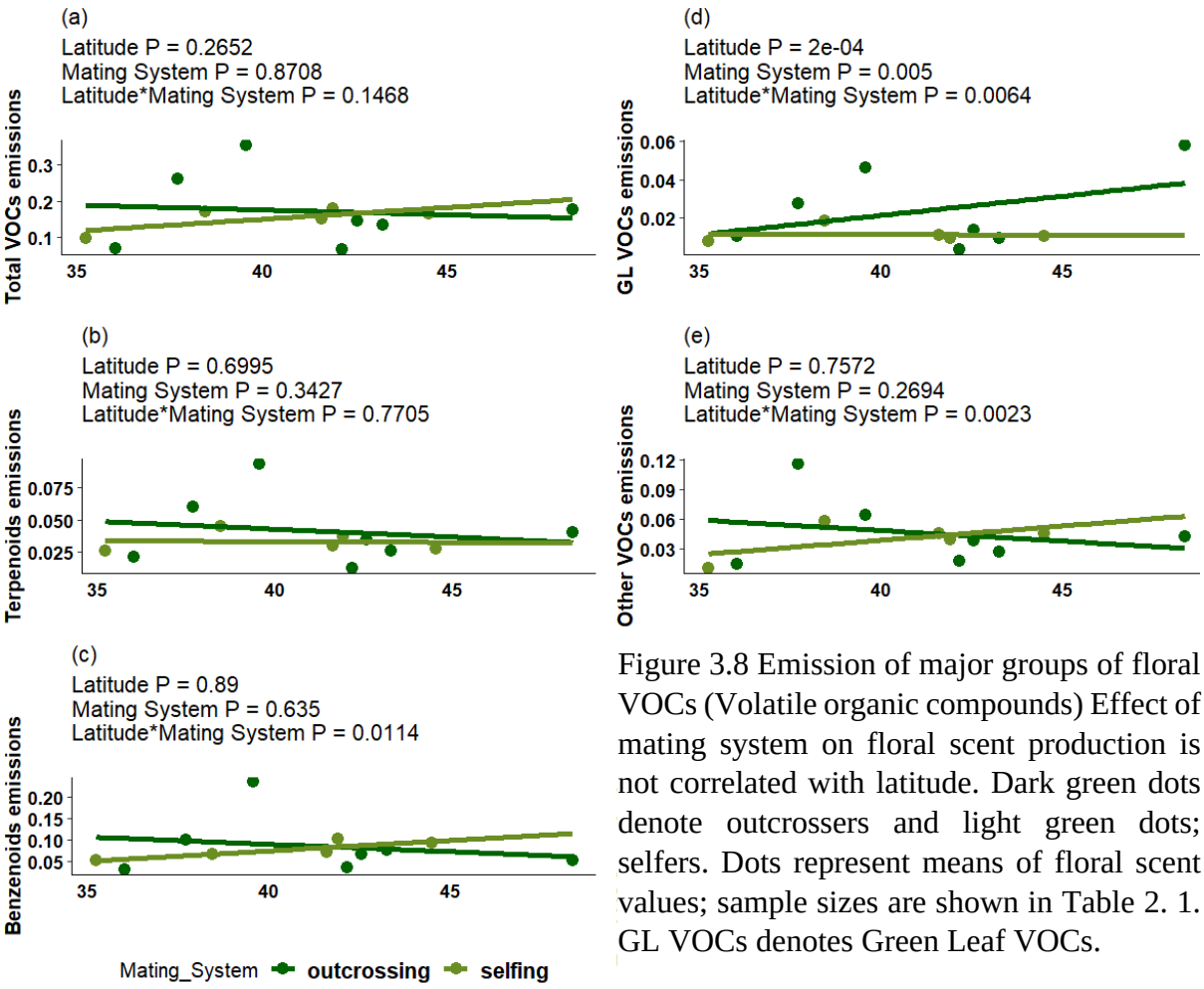

Figure 3.8 Emission of major groups of floral VOCs (Volatile organic compounds) Effect of mating system on floral scent production is not correlated with latitude. Dark green dots denote outcrossers and light green dots; selfers. Dots represent means of floral scent values; sample sizes are shown in Table 2. 1. GL VOCs denotes Green Leaf VOCs.

3.5 Discussion

3.5.1 Genetic evidence of mating system transitions

The first goal of this research was to detect genomic evidence for convergent selection across independent transitions to selfing. Our SNP-association study identified a significant number of SNPs, genes and enriched gene ontology terms associated with replicate, independent mating system transitions in *Arabidopsis lyrata*. Our study detected the genetic signatures for mating system related to the observed floral phenotypic traits. I used principal component analyses as a verification approach for outlier SNPs. The principal component analyses on both outlier and nonoutlier SNPs suggest that genetic differentiation at outlier SNPs in the northern clade but not at nonoutlier SNPs, was strongly associated with mating system (Figure S2a). The gene ontology enrichment analysis shows that the outlier genes detected for mating system are candidates of various biological processes including aromatic compound biosynthesis

(Figure 3.6). This suggests that some of these genes are regulators of floral scent emissions. This is congruent with our prediction that that the evolution of floral scent would vary with mating system because floral scent plays a role in adaptation of outcrossing plants to environment with abundance of pollinators or herbivores and subsequentially lead to evolution of selfers by ecological changes (Petrén *et al*., 2021). On the other hand, the outlier genes which are the genes under selection for repeated evolution of selfing suggests that some of these genes might be directly involved in preventing self-compatibility and in parallel, may cause selfing to evolve by disruption of their function. To the best of my knowledge this is the first study to show nature of genetic selection associated to mating system transition that includes multiple populations of a *A. lyrata.*

Regulation of aromatic compound biosynthesis is polygenic (Ramya *et al*., 2020) with 170 to 489 genes in *A. lyrata* populations being annotated with GO terms linked to organic substance synthetic and metabolic processes in this work. Furthermore, the enriched gene ontology (GO) identified candidates of mating system transitions to be genes that are involved in some other biological processes such as signal transduction and defence response (Figure 3.6). Defence responses to herbivores have been shown to be mediated by mating system transitions. For instance, the reduction in abundance of herbivores in an environment where selfers are usually found will also decrease the attraction of pests, the transition to selfing relieves the requirement for strong defence response against herbivores (Jacobsen and Raguso, 2018; Parachnowitsch and Kessler, 2010; Egan *et al*., 2021). Other significant terms included system and flower development. Some of these processes such as flower development have been shown to be important factors in trait evolutionary histories of outcrossing and selfing populations within and among plant species (Broz *et al*., 2017; Dudley *et al*., 2006; Gorman, 2021). These convergent selection on the afore-mentioned phenotypic traits suggests differences in environmental selection between selfing and outcrossing populations.

Our second aim was to determine the shared polymorphisms or loci linked with repeated mating system transitions in both lineages: northern and southern clade. The extent to which similar SNPs, genes or enriched GO terms were associated with mating systems in both clades was analysed and suggests differences in the targets of selection between north and south. Only few overlapped SNPs and genes associated with mating systems between the two clades were detected. Shared SNPs were hardly more than expected by chance and shared genes were in the range of expectation (Figure 3.5). The level of selection for outlier genes were lower in

southern populations compared with northern population, thereby indicating the significance of lineage-specific mating system transition in *A. lyrata* on the genomic level. In contrast to the low level of shared outlier SNPs and genes, similar evolution was higher at the level of enriched GO terms, signifying the involvement of similar biological processes in relation to mating system transitions across *A. lyrata* distribution despite the prevailing clade-specific mating system evolution on the genomic level. The large number of secondary metabolism-related outlier genes in north and south suggests that pathways of volatile organic compounds biosynthesis and metabolism are important target of selection during mating system evolution. Walden *et al*. (2020) showed that the absence or presence of standing genetic variation can impact on the amount of similar mating system evolution in different lineages of *A. lyrata*. The Pleistocene event caused many species to colonize new and heterogeneous habitats while also experiencing divergent selection (Hewitt, 2000; Willi *et al.*, 2018; Sánchez-Castro, Perrier and Willi, 2022). Isolated refuge areas, range expansion and subsequent recolonization can often lead to reduction in population sizes and standing genetic variation available for selection (Jones *et al*., 2012; Willi *et al*., 2018). The allele frequencies in *A. lyrata* populations may have been affected during demographic bottlenecks caused by the glaciation history which may have led to the clade-specific patterns of mating system evolution. This seems to be the case here, where populations in the southern clade were shown to have evolved first (Figure 3.2), likely with a low genetic diversity (Mattila *et al*., 2017; Willi *et al*., 2018). Accordingly, I detected fewer SNPs and genes in the populations of the southern clade in those SNPs and genes identified as outliers in the northern clade, which may be reflecting a bottleneck impact in the southern populations.

In summary, genes and biological processes involved in mating system evolution have been identified. Unlike the significant parallelism found within each clade, only little evidence for intraspecific similar mating system transitions on the level of SNPs and genes between two clades were identified. Nevertheless, this study identified the genetic signatures of mating system evolution. Gene ontology terms related to synthesis of secondary metabolites and specifically aromatic compound biosynthesis and response to stress were found, signifying the changes in organic compound biosynthesis and defence response with mating systems transitions.

3.5.2 Variation in floral scent emission between selfing and outcrossing population

The third aim of this study was to assess whether selection on floral scent, a reproductive phenotype corresponds to the genomic signatures of mating system. I predicted that floral scent would be reduced with mating system transitions to selfing because floral scent comprises a blend of chemical signals involved in pollinator attraction and potentially an indirect mechanism against insect herbivore attack (Wright and Schiestl, 2009; Parachnowitsch and Kessler, 2010; Egan *et al*., 2021) which mediates mating system transitions. Indeed, in the North American *Arabidopsis lyrata* populations, the transitions to selfing did not only lead to changes in the total amount of volatiles emitted but also affected the production of specific compound classes (Figure 3.7). The variation in total VOC emission between selfing and outcrossing *A. lyrata* populations is consistent with previous studies of other plant species within the same family with *A. lyrata*, comparing scent emission of selfers and outcrossers (Doubleday *et al*., 2013; Tedder *et al*., 2015; Sas *et al*., 2016; Hampus *et al*., 2021) but contrasting with the lack of significant support for reductions of total scent emission in selfing *P. cuspidata* compared to outcrossing *P. drummondii* (Majetic *et al*., 2019). The similarity among the flower scent evolution in the selfing populations is such that it can accurately predict the mating strategies. All floral VOC compound classes were similarly affected in all the selfing populations. The emission of the total VOC emission was decreased after the shift to selfing in the southern clade, which is in line with a loss of attractiveness to pollinators triggered by selfing evolution. Among the floral scent, three specific compound classes, including terpenoids, benzenoids, and green leaf volatiles, were annotated. These floral scents are common volatile compounds in floral signals and are believed to promote pollinator attraction (Knudsen and Tollsten, 1993; Knudsen and Gershenzon, 2006; Weiss *et al*., 2016; Qian *et al*., 2019).

Benzenoids have been reported to act as a common pollinator attractant due to its emission by more than half of angiosperms families, and research has shown that it increases the rate of pollinator visits (Theis, 2006, Filella *et al*., 2013; Byers *et al*., 2014; Farré-Armengol *et al*., 2017; Peng *et al.*, 2017). Like the other floral scents, the amount of benzenoid emitted differed most notably between selfing and outcrossing *A. thaliana* populations. One major difference between selfing and outcrossing populations in the southern clade was the high emission of benzenoids in the outcrossers (Figure 3.7), but reductions in selfers, but the opposite pattern in northern populations. The role of benzenoids in floral scent has been documented in two recent works. Shift from hawkmoth pollination in *Petunia axillaris* to hummingbird pollination in *P.*

exserta was accompanied by loss of benzaldehyde, a benzenoid compound (Amrad *et al*., 2016). Likewise, outcrossing *C. grandiflora* emitted a benzenoid compound which was absent in emissions from selfing *C. rubella* (Sas *et al*., 2016). However, our results also showed that compounds involved in defence mechanisms are also influenced by the shift to selfing. Green leaf volatiles (GLVs) have been shown to be rapidly released after attack by insect herbivores, pathogens, and abiotic stress (Brilli *et al*., 2011; Kim and Felton, 2013; Ameye *et al*., 2015; Cofer *et al*.,2 018). Notably, its emission is also reduced in all selfing populations which suggests that the reproduction changes in plants that rely on pollinators, depends on a balance between attraction and defence mechanism. Outcrossers may emit a complex blend of floral VOCs that enhances attraction to pollinators while also restricting attack from insect pests (Jacobsen and Raguso, 2018). As a result, I hypothesize that selfing evolution alleviates or reduced the necessity for robust defence strategy against pests because the reduced pollinator attractiveness also leads to the reduction in the attraction of herbivores.

Interestingly, the northern populations which are the more recent expansion geographically, showed an opposite selection on aromatic compound with selfing evolution compared to southern populations. There are at least three non-exclusive explanations for the divergent response of benzenoid emissions in northern and southern population clusters. First, latitudinal and longitudinal gradients have been shown to impact species diversification in some plant systems. For example, these geographical variables affected floral scent diversification in alpine populations of *Lavandula angustifolia* (Demasi *et al*., 2018). The study showed substantial intra-specific variations in secondary metabolites along the latitudinal gradient. However, geographical distance such as the latitude and longitude are not likely to be a major determinant in this system because the variation of floral emission in our study was generally not explained by the latitude (Figure 3.8). Second, divergent selection on floral VOC emissions in northern and southern mating system transitions could be due to spatial differences in pollinator and herbivore abundance and diversity as these are likely to play key roles as agents of selection on floral scents emissions (Gross *et al*., 2016; Chapurlat *et al*., 2019; Friberg *et al*., 2019). For example, benzenoid emissions are known to be particularly attractive to bee pollinators, rather than flies, and the relative importance of different pollinators may differ in the two clusters. However, if true, this hypothesis would predict that benzenoid emission would also differ between northern and southern outcrossing populations, however this was not the case (Figure 3.7). Third, there may be a temporal dynamic to the evolution of floral scent in selfing populations. Northern selfers are the younger selfing populations and may represent

more recent losses of SI at the expanding range front (Willi *et al*., 2022). Younger selfing populations may exhibit an enrichment in aromatic compounds known to be pollinator attractants (Theis, 2006; Jakubska-Busse *et al*., 2022), due to a period of initially strong selection to maximise pollinator recruitment at range margins. Over time, as self-fertilizing continues, I hypothesize that there may be selection against increased benzenoids emission, as observed for the other non-aromatic compounds (terpenoids and green leaf volatiles) in this study, and for the older, southern populations. Parallel reduction in green leaf volatiles with selfing evolution in the two distinct geographical clades were observed. GLVs are implicated in indirect defence against herbivores, potentially suggesting a reduction in herbivore abundance in selfing populations, as posited by Campbell and Kessler (2013). Based on my results, I propose a two-step process in the transitions from self-incompatibility to self-compatibility for changes in floral signalling, with initial strong selection to improve signalling prior to becoming more fully selfing, after which the costs associated with VOC emissions lead to purifying selection and reductions in scent investment in established selfing populations. This model is also consistent with the elevated numbers of mating system associated genes in northern populations (Figure 3.5), which also suggest a more dynamic selection environment in more recent transitions.

3.6 Conclusions

This study is an example of how genome-wide association analysis (GWAS), and subsequent gene ontology analysis can be coupled with phenotypic trait analysis to investigate the predictability of adaptive evolution. Evidence for mating strategies in *A. lyrata* populations between two broadly categorized clades were detected. Gene ontology analyses on outlier SNPs identified a common garden study, which confirmed that outcrossing populations differed from selfing populations with higher VOC emissions from outcrossers compared with selfers (Figure 3.7). The mating system age, geographical location and ancestry may all impact on the direction of selection on floral scents. Our results suggest that the evolution of floral aromatic compounds such as benzenoids in the North American *A. lyrata* populations is largely driven by mating system transitions and partly explained a complex set of factors including geographical location and the predicted age of populations. Even though our results suggest a two-step process in the evolution of selfers and changes in VOC emissions, future studies should further investigate when changes in floral chemical signal occurs during transition from outcrossing to selfing and examine how other factors can interact to generate intraspecific

variation in floral scent among angiosperms. Such information can be important in predicting how changes in environment or population history can affect selection regimes of populations of a plant species.

3.7 Supplementary Information

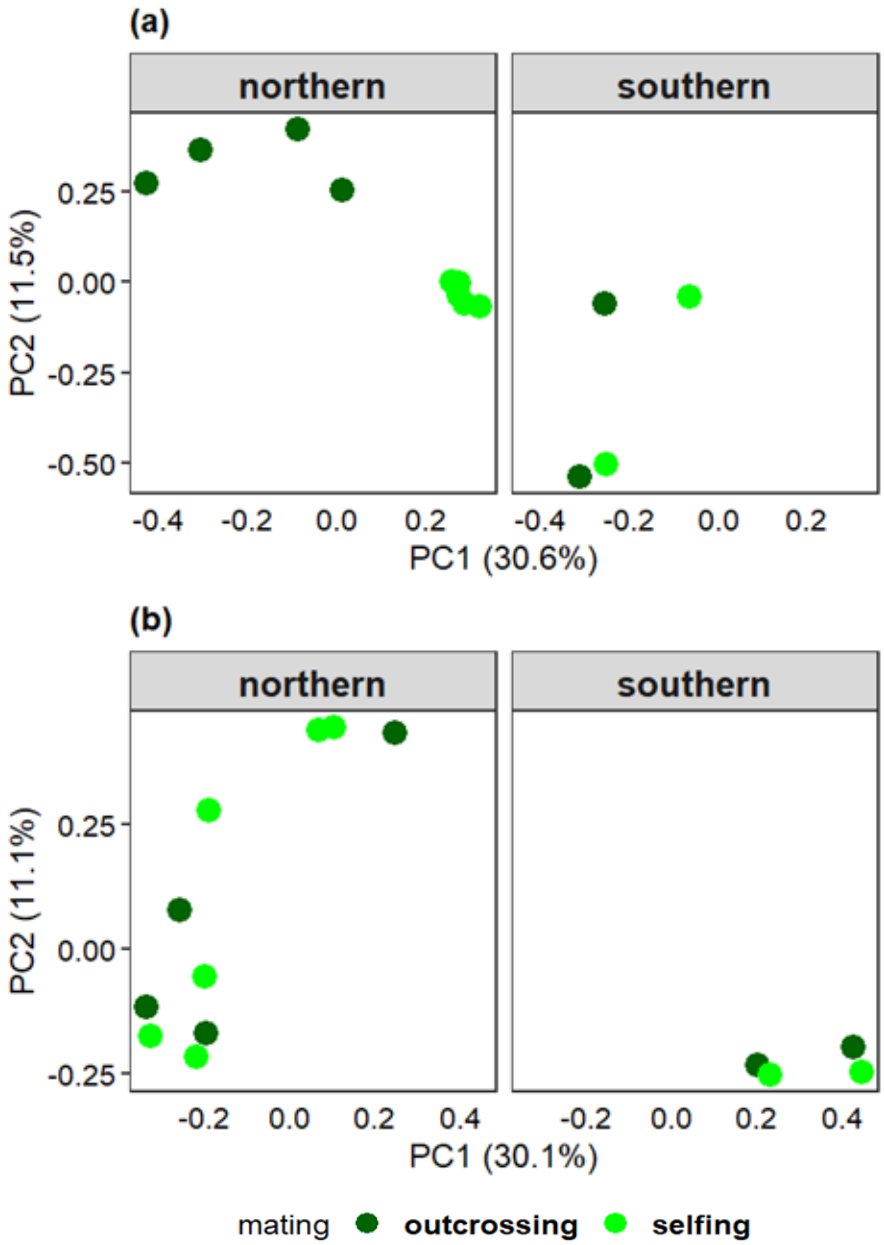

Figure S2 Principal component (PC) analyses using the top 0.8% outlier SNPs detected by our SNP association analysis (a) and non-outlier SNPs (b). The colour of the dots indicates the type of mating system, outcrossing in dark green and selfing in light green. The two first PC axes are presented.

Table S2 VOCs released from the populations of *Arabidopsis lyrata* in-situ.

Terpenoids	**Benzenoids**	**GL VOC**	**Other VOCs**
Myrcene	E-2-Hexenol	Z-3-Henxenyl-Acetate	Phenylacetaldehyde
Limonene	Unknown benzenoid	Z-3-Hexenol	Unknown
B-Ocimene	Benzaldehyde	Unknown GLV	Unknown
Unknown terpenoid	Unknown benzenoid	Unknown GLV	Unknown
Unknown terpenoid	Unknown benzenoid	Unknown GLV	Unknown
E-B-Caryophellene	Unknown benzenoid	Unknown GLV	E2 Nonanal
Linalool	Acetophenone benzenoid	Unknown GLV	E2 Decanal
A-Humulene		Z3-Hexenyl Butyrate	Unknown
Unknown terpenoid			Unknown
A-Ionone			Unknown
Nerolidol			Unknown
Unknown terpenoid			Unknown
Geranyl Acetate			Unknown
			Unknown

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Chapter 4:

Super-resolution imaging of cellulose and callose synthase motility in the living cell of *Arabidopsis thaliana* during microbial attack

To be submitted to International Journal of Plant Biology

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4.1 Abstract

Plant pathogens represent a major challenge for global food security. For invading pathogens, the plant cell wall represents the first physical barrier against penetration. Therefore, modification and strengthening of the plant cell wall has become a major focus of current research to improve resistance in crops to attacking plants pathogens. Recent studies have shown interaction networks between cellulose and callose in the formation and success of defence-related cell wall thickenings in plants, so-called callosic papillae. Therefore, understanding the mechanisms underlying enzymes complexes for cellulose and callose formation during microbial attack, may help to define new defence targets in plants and crops. The knowledge of cellulose and callose synthases have increased in recent years with the

imaging by conventional microscopy such as confocal imaging. However, the maximum resolution of confocal microscopy limits what can be discovered about these tiny biosynthetic molecules. Here, I applied a direct stochastic optical reconstruction microscopy (dSTORM) which images single-molecules and improves resolution two-fold more than confocal imaging, to explore the motility of cellulose synthase (35S::tdTomato-*CESA*6) particles and callose synthase (*35S::PMR4-GFP*) in the living cells in a model plant, *Arabidopsis thaliana* upon microbial inoculation with powdery mildew. Measured with a particle tracking algorithm, dSTORM imaging revealed a faster mean particle speed of 493.457±11.909nm/min (n = 1396 particles) of *CESA*6 compared to an average speed of 281.579±4.854nm/min (n = 1854 particles) for *PMR4* particles. Callose interaction reduced mean speed of *PMR4* by approximately 25% and had no significant effect on *CESA6*. I also revealed that *CESA*6 tracks are less distributed than *PMR4* tracks which also increased its distribution in the presence of callose, thus, implying possible migration to the site of attack. The interaction, patterning, and speed of *CESA*6 and *PMR4* varied widely among different regions of the leaf tissues, suggesting that speed and patterning together with interactions between these proteins might be one of the determinants of cellular response to microbial attack. Overall, my results show the impact that super-resolution microscopy can have to decipher molecular mechanisms of callose and cellulose synthases. Most prominently, the interaction and dynamics of single callose and cellulose synthase complexes at pathogen interaction sites, leading to the discovery of yet unknown migration mechanism of callose synthase complexes and associated callose polymer synthesis in the apoplast.

4.2 Introduction

Plant pathogens represent a major challenge for global food security. For invading pathogens such as fungi and oomycetes, the plant cell wall represents the first physical barrier against penetration (Underwood, 2012; Ristaino *et al*., 2021; Wan *et al*., 2021). Therefore, modification and strengthening of the plant cell wall has become a major focus of current research to improve resistance in crops to attacking plants pathogens (Ellinger *et al*., 2013; Bacete *et al*., 2018). The cell wall polymer callose, a (1,3)-β-glucan polymer with (1,6)-branches (Aspinall and Kessler, 1957) is the most abundant chemical constituent in defence-related cell wall thickenings in plants (Vance, Anderson, and Sherwood, 1976), These occur as papillae, at sites of microbial attack and form a critical part of the plant's early defence response. Callose is the earliest description of a plant defence mechanism against pathogens

(Wang, 2021). Papillae have been regarded as an early defence reaction which may not completely stop the pathogen, but act as a physical barrier to slow pathogen invasion (Voigt, 2016) and contribute to the plant's innate immunity (Schwessinger and Ronald, 2012). In addition, recent studies have suggested that besides callose synthases and the corresponding polymer callose, cellulose synthases and cellulose play an important role in the formation and success of defence-related papillae (Schneider *et al.*, 2016; Voigt, 2016; Wang *et al*., 2022). Cellulose has a similar primary structure as callose: it is a polysaccharide of glucose mainly linked by β-1,4-glycosidic bonds (Galatis and Apostolakos, 2010; Barghahn *et al.*, 2021). The two glucan synthase complexes, cellulose synthase (*CESA*) and callose synthase, *Powdery Mildew Resistance 4* (*PMR4*) also known as GSL5 (GLUCAN SYNTHASE-LIKE 5) which synthesizes cellulose and callose respectively, share UDP-glucose as the initial substrate (Galatis and Apostolakos, 2010; Barnes and Anderson, 2018; Verbančič *et al.*, 2018). Hence, callose and cellulose may have similar synthesis mechanisms. Recent studies have shown that the induction and optimisation of callose biosynthesis is promising target in plant breeding (Chen and Kim, 2009; Blümke, Somerville and Voigt, 2013; Ellinger *et al.*, 2013; Wang, Andargie and Fang, 2022).

Glucans such as cellulose and callose have recently been the subject of intensive research. Studies on the biochemistry and the structure of cellular molecules have revealed new approaches in the use of super-resolution fluorescence microscopy to generate tremendous details about subcellular structures in plant cells (George *et al.*, 2018). The mechanism of cellulose synthesis has been studied by characterizing the dynamics of cellulose synthase complexes tagged with a fluorescent protein in *Brachypodium distachyon* (Liu, 2017); however, this motility has been characterized using a confocal microscopy and other microscopy techniques like electron microscopy to achieve high resolution imaging. However, the maximum resolution of confocal microscopy limits what can be discovered about these tiny biosynthetic molecules. In this work, I imaged fluorescently tagged *CESA* and *PMR4* proteins in the leaf tissues of *Arabidopsis thaliana* using a super-resolution microscopy (dSTORM) with a focus on quantifying the speed, distribution, and pattern of *CESA* and *PMR4* molecules in response to pathogen infection. The study uses *Arabidopsis thaliana* following treatment with *Podosphaera xanthii*, a fungal pathogen that causes powdery mildew diseases in plants. The study investigates the pattern and motility of callose synthase *PMR4* and cellulose synthase *CESA*6 in *Arabidopsis thaliana* leaf tissues thereby addressing its potential defence

mechanism. This new knowledge may help to identify new targets for modification and optimization of defence-related papillae deposition at sites of attempted microbial penetration.

4.2.1 Plant pathogens - a major challenge for global food security

Global food security remains one of the most challenging issues for humans as significant crop loss continue to increase yearly (Voss-Fels *et al.*, 2019). Plants represent over eighty percent of the human food and therefore, essential for food security. Plant diseases pose a threat to global food security because they destroy crops, thereby reducing food availability and accessibility and increasing food expenses. Plant diseases can also adversely affect the flavour and palatability and lead to changes to the native food preferences of populations (McDonald and Stukenbrock, 2016). Plant pathogens have caused massive shortage of food over the years, thereby causing famine. In 1964, India was faced with a severe outbreak of Brown spot fungus that resulted in the destruction of approximately 90% of the rice crops in the region of Bengal (Padmanabhan, 1973), leading to two million deaths. In Ireland in 1845, a Central American pathogen, *Phytophtora infestans* caused a disease known as Potato late blight disease which led to a famine that killed over one million people (Padmanabhan, 1973). Powdery mildew disease caused by a wide variety of fungal pathogens including *Podosphaera xanthii* is also known to have reduced productivity of cucumber and wheat by infecting the healthy leaves leading to reduced income for growers (Jain *et al.*, 2019; Atoum *et al.*, 2016; Barbedo, 2016). Currently, with current estimates of a global population set to reach 9.1 billion people by 2050 (Voss-Fels *et al.*, 2019), increasing world trade and climatic changes, plant diseases present an unprecedented threat to food security. Around forty percent of global food production is affected by plant diseases. Chemical control methods have proven some effectiveness but are very expensive and environmentally not sustainable. Pathogens always develop immunity to them overtime (Kaur and Garg, 2014). There is now the need to develop optimization methods for natural defence optimisation, against pathogens in plants. In view of enhancing sustainable food crop protection, investigation into the mechanisms of plant defence from pathogen attack has led to an increased focus on the role of callose and cellulose synthases, as the first innate line of defence against pathogen attack. Having such knowledge can lead to the discovery of further interaction mechanism of the synthase complexes and how it can be optimized for enhanced disease resistance in the plant cell.

4.2.2 The plant cell wall - a first line of defence

Plant cell walls are tough layers of polysaccharides situated as a barrier outside the plasma membrane of the cells in plants, consisting of a mixture of various glucose polymers such as cellulose, forming a rigid network (Keegstra 2010; Vorwerk *et al.* 2004 and Somerville *et al.* 2004). Cell walls serve as the first line of defence in a plant cell providing protection to plant against environmental conditions and serving as a channel of communication between the symplast and apoplast. They regulate cell expansion needed for plant morphology support while also allowing for solute transport between cells and root-shoot molecule transport (Zhang *et al.*, 2021). There are two different types of plant cell walls: the primary cell wall is found in almost all the plant cells still undergoing growth and division, and secondary cell wall which is found in plant cells that are already in a constant state and contributes to the thickening of the cell (Keegstra 2010; McCann *et al.* 2001 and Somerville *et al.* 2004). There are two major classes of polysaccharides that provide the basic cell wall structure: the homopolysaccharides which are cellulose and callose, and the heteropolysaccharides which are pectins and hemicelluloses (Mohnen, 2008; Scheller and Ulvskov, 2010). The heteropolysaccharides are known as matrix polysaccharides and are secreted in the Golgi apparatus and apoplast where they sometimes get modified and incorporated into the growing cell wall (Mohnen, 2008; Scheller and Ulvskov, 2010). In contrast, callose and cellulose are produced at the plasma membrane by large glucan synthase complexes that are referred to as callose synthases (CalS or GSL for GLUCAN SYNTHASE-LIKE) and cellulose synthases (*CESA*s) respectively (Ellinger and Voigt, 2014; McFarlane, Döring and Persson, 2014; Xu *et al.*, 2022). Cellulose can be found in the cell walls of almost all the land plants while callose is found only in specialized walls which support cell division, pollen and pollen tube development, functioning of the plasmodesmata, tip growth as well as in response to biotic or abiotic stress (Schneider *et al.*, 2016).

4.2.3 Cellulose Synthases

Cellulose synthase (*CESA*) complexes (CSCs) are assembled in the golgi apparatus endoplasmic reticulum (ER) and are then deposited at the plasma membrane (PM), where they synthesize cellulose polymer (McFarlane, Döring and Persson, 2014; Polko and Kieber, 2019). Cellulose is a 1,4-β-linked glucan polymer formed by microfibrils (McFarlane, Döring and Persson, 2014; Schneider *et al.*, 2016). Three distinct cellulose synthases (*CESA*) in equal ratios, form a cellulose synthase complex (CSC) which is involved in cellulose microfibril

synthesis (Venu *et al*., 2016; Turner and Kumar, 2018). The CSC travels along cortical microtubule paths and impact the orientation of cellulose microfibril synthesis (Polko and Kieber, 2019). The *CESA* proteins contain multiple transmembrane domains that are assembled into two domains: cytosolic C-terminal domain and intracellular C-terminal domain with a large hydrophilic cytoplasmic central loop that contains the putative catalytic domain which is divided into two regions (Kurek *et al*., 2002; Sethaphong *et al.*, 2013). The two regions are the UDP-glucose-binding domain and the glycosyltransferase domain which are characterized by the presence of multiple aspartic acid triplets - D,D,D and a motif designated as QXXRW and conserved in the *CESA* superfamily (Verma and Hong, 2001; Dong *et al.*, 2005). *CESA* is an enigmatic enzyme which becomes inactive when purified from plant tissues (Cifuentes, *et al.*, 2010; Lai-kee-Him, *et al.*, 2002). *CESA* gene family encodes cellulose synthase catalytic subunits from three distinct clades which are *CESA*1, *CESA6*, and *CESA6*-like in *A. thaliana* (Bashline, Li and Gu, 2014; Liu *et al.*, 2017). To study the mechanism of callose and cellulose mediated responses, in this study, *A. thaliana* plants in which *CESA6* proteins are tagged with a bright red tdTomato fluorescent protein were used to track the *CESA* motility in the leaf in response to pathogen attack. Cellulose synthase has many similar features with callose synthases which synthesizes callose, the only other plant cell wall glucan that is directly synthesized at the plasma membrane.

4.2.4 Callose Synthases

Callose synthases in *Arabidopsis* are involved in callose deposition (Sherwood and Vance, 1976; Stone and Clarke, 1992; Verma and Hong, 2001; Jacobs *et al.*, 2003; Xiao *et al*., 2016). Callose is a cell wall polymer and is composed of glucose residues that are linked together through β-1,3-linkages with some (1,6)-branches and termed β-glucan (Xiao *et al*., 2016). It was identified in plants in the late 19th century (Aspinall and Kessler, 1957 and Stone and Clarke, 1992), as a gel-like structure produced as helical chains by a class of enzymes and used to plug and seal ruptured cell walls (Aist, 1976; Verma and Hong, 2001). Callose is involved in many essential plant developmental processes in angiosperms (Xiao *et al*., 2016) such as deposition at cell plates during cytokinesis (Davis *et al*., 2020); deposition at plasmodesmata (PD) for controlling the SEL (Size Exclusion Limit) of plasmodesmata thereby, regulating the cell-to-cell movement of molecules (Shu-Wei *et al*., 2018). Studies have shown that callose is involved in the callose wall formation that surrounds fertilized zygote during plant development (Williams *et al.*, 1984), phloem transport and development, inflorescence growth,

development of root hairs, and cytokinesis during somatic tissue development (Somssich *et al.* 2016). In bryophytes, callose has been identified around the spore mother cell (Ks *et al*., 2020) and can be linked to cell-wall development, however the mechanisms are not well understood. The role of callose in cell-wall development in many other plant groups is similarly unclear (Wang *et al.*, 2022). A relationship between callose reaction and abiotic stress was shown in mechanically stressed plants (Rejeb *et al*., 2014) and in *A. thaliana* plants stressed by wind, rain, and mechanical substances (Jaffe and Telewski; Jacobs *et al*. 2003). In addition, biotic stress such as pathogens can also induce formation and deposition of callose. In wheat and *A. thaliana*, callose response was found after aphid transfections (Botha *et al*., 2004; Will and van de Ven, 2006; Kempema *et al*., 2007). Callose is found only in relatively low amounts in plant cell walls representing approximately 5% of the total cell-wall content in *Arabidopsis* and Miscanthus (Falter *et al.*, 2015), yet it is the most abundant chemical constituent in defence-related cell wall thickenings in plants called papillae (Sherwood and Vance, 1976; Aist 1976; Nishimura 2008; Stone and Clarke, 1992), at sites of microbial attack and part of the plant's early defence response.

Biochemical, molecular, and genetic studies using *Arabidopsis thaliana* as model plant, have identified a set of genes involved in callose biosynthesis and deposition (Xiong-Yan and Jae-Yean, 2009). Twelve genes which encode putative glucan synthase-likes have been identified in *Arabidopsis thaliana* by two different research groups (Verma and Hong, 2001; Richmond and Somerville, 2000). These two groups named the genes differently and independently, however, the use of glucan synthase-like, GSL gene nomenclature and hence, AtGSL1 to AtGSL12 (Richmond and Somerville, 2000), is widely used by glucan synthase-like researchers (Ellinger and Voigt, 2014; Jacobs *et al.*, 2003; Nishimura *et al.*, 2003; Enns *et al.*, 2005; Nishikawa *et al.*, 2005; Töller *et al.*, 2005; Huang *et al.*, 2009 and Thiele *et al.*, 2008). The GSLs have been classified into two major groups: CalS1(GSL6), CalS5(GSL2), CalS9(GSL10), CalS10(GSL8) and CalS11(GSL1) contribute to fertility and cell division; CalS3(GSL12), CalS7(GSL7), CalS8(GSL4), CalS12(GSL5) provide the reinforcement of structural cell walls (Záveská and Honys, 2017).

Single GSL gene can have diverse functions. CalS1 (GLS6) forms a complex with a UDP-glucose transferase, which is localized at the cell plate during cytokinesis (Chen and Kim, 2009; Hong *et al.*, 2001) and regulates plasmodesmal permeability under biotic and mechanical stress (Cui and Lee, 2016). CalS3 (GLS12) is involved in callose accumulation in

plasmodesmata (Chen and Kim, 2009), while CalS5 (GLS2) deposits callose in the wall, is involved in formation of callose plugs in germinated pollen tubes and is required for exine formation during microsporogenesis and hence pollen viability (Nishikawa *et al.*, 2005; Dong *et al.*, 2005; Chen and Kim, 2009; Töller *et al.*, 2008). CalS7 (GLS7) is important for callose deposition at the sieve plate (Xie *et al.*, 2011). CalS8 (GLS4) is required for the regulation of plasmodesmata permeability under biotic and mechanical stress (Cui and Lee, 2016). CalS9 (GLS10) is involved in the entry of microspores into mitosis (Chen and Kim, 2009; Töller *et al.*, 2008; Huang *et al.*, 2009) and essential for the microspore asymmetric division (Toller *et al.*, 2008). CalS10 (GLS8) is involved in pollen development, is required for callose synthesis at the cell plate and is also important in stomatal pattering and callose deposition at the plasmodesmata (Töller *et al.*, 2008; Thiele *et al.*, 2008). CalS11 (GLS1) is crucial for cell plate formation in sporophytic tissues and prevents callose wall degradation in microspores early in development (Chen and Kim, 2009; Verma and Hong, 2001; Enns *et al.*, 2005; Xu *et al.*, 2016). CalS12 (GLS5) also known as AtGLS5, AT4G03550, At4g03550, AtGSL05, POWDERY MILDEW RESISTANT 4 and *PMR4* (Ellinger *et al.*, 2013; Blümke *et al.*, 2013; Nishimura *et al.*, 2003), is involved in the biosynthesis of stress-inducible callose in leaf tissue (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003; Enns *et al.*, 2005). In addition, it plays a crucial role in the synthesis of callose wall separating microspores within tetrads (Enns *et al.*, 2005) thereby preventing callose wall degradation in microspores early in development (Chen and Kim, 2009; Enns *et al.*, 2005; Xu *et al.*, 2016). It is also vital for exine formation, pollen wall pattering, cell plate formation in sporophytic tissues (Enns *et al.*, 2005; Chen and Kim, 2009). In *Arabidopsis thaliana*, callose synthase *PMR4* in plasma membrane migrated to sites of papillae formation through vesicle-like bodies (Ellinger *et al.*, 2013).

Using electron microscopy, Nishimura *et al.* (2003) analysed callose deposition at forming papillae where callosic papillae extended to the intracellular cytosol: glucan deposition was localized in the space between the pre-existing cellulosic cell wall and the cell membrane. Confocal microscopy successfully imaged an explicit, layered cellulose-callose structure in cell walls of epidermal leaf cell directly exposed to both biotic and abiotic stress (Falter, *et al.*, 2015). However, the resolution limit (approximately 250 nm) of light microscopy is bigger than both the size of individual polymer molecule and their typical spacing in the cell membrane (Reiss *et al.*, 1984). This limitation has been overcome in the studies of glucan polymer networks at sites of attempted fungal penetration using a form of super-resolution microscopy called, dSTORM (Direct stochastic optical reconstruction microscopy). dSTORM

was found to improve the resolution of the light microscopy by three-fold to four-fold, differentiating objects spaced as little as 50 nm apart for super-resolution, in contrast to the 200 nm for conventional confocal microscopy (Anderson *et al.*, 2010; Ellinger *et al.*, 2013 and Evans *et al.*, 1984) in *Arabidopsis* leaves.

STORM application has revealed cellulose-callose microfibrils direct interaction within the region of callosic papillae formation, in response to pathogenic powdery mildew (Eggert *et al.*, 2014). Here, a penetration of callose microfibrils via the internal cell-wall nanopores which requires a gel-like condition of callose that may be dependent on the pH was indicated (Voigt, 2014). These results suggest an overexpression of *PMR4* enhancing callose deposition at such interaction sites (Ellinger, 2013), leading to the expansion of the cellulose-callose complexes, and finally to a callose layer formation at the top of the pre-existing cellulose complexes (Eggert *et al.*, 2014 and Voigt, 2014). Despite the success in using super-resolution microscopy to image polymers in plant cell, it has not yet been applied to enzymes that synthesizes the glucan polymers. Here, I used the super-resolution microscopy, dSTORM, to image and quantitatively analyse the motility of the enzymes: callose synthase and cellulose synthases, that are involve in synthesizing the glucan callose and cellulose, in challenged leaf cells of *Arabidopsis thaliana*.

4.2.5 Super-resolution fluorescence microscopy

For the past few years, confocal microscopy has been used for analysing the density, behaviour, trafficking, motility, speed, and interactions of fluorescently tagged *CESA* molecules in living plant tissues (Duncombe *et al*., 2020; Allen *et al*., 2021). However, the diffraction limitations which define the resolution limit of a conventional confocal microscopy despite a slight gain of resolution restrict its capacity for a super-resolution imaging (Reiss *et al*., 1984; Yang, 2016; Schubert, 2017; George *et al.*, 2018). Light diffraction limits the resolution of light microscopy to about 250 nm, making it impossible to resolve the intracellular structure of cells below 250 nm (Schubert, 2017). This limitation prevents super-resolution imaging of a single or individual protein particle, observed by confocal or other light microscopy as fluorophore spots in plant tissues (Chen *et al*., 2014; Sydney *et al*., 202). Gutierrez *et al.* (2009) indicated that cellulose synthases might moves in closely spaced clusters that cannot possibly be resolved by a conventional confocal or electron microscopy imaging. Another example of a confocal imaging limitations is the report of 1 particle μm^{-2} as the density of *CESA* complexes which is lower than reported of the density of cellulose microfibrils in single wall when imaged by

atomic force and electron microscopy (Xiao *et al*., 2016; Zhang *et al*., 2016, 2017). Although, confocal microscopy is still an accessible way to image these proteins in living plants as they move in the cell, they do not offer a super resolution of individual molecule. These shortcomings raise the possibility that there may be further factors that influence the motility, patterning, or the behaviour of both *CESA*6 and *PMR4* together with their corresponding polymers in the plant cells, that are yet to be completely unravelled by confocal imaging. Therefore, understanding the nanoscale behaviours of these synthases would not only help to decipher the dynamics of cellulose-callose polymer interaction but also provide additional clarity on the density, patterning, and relationships of the glucan polymers with the protein trajectories.

Super-resolution microscopy has massively facilitated studies in cell and molecular biology including plants (George *et al*., 2018; Schubert, 2017). A type of super-resolution microscopy called direct stochastic optical reconstruction microscopy (dSTORM) (Rust *et al*., 2006; Jensen and Crossman, 2014), improves upon the high resolution of conventional confocal microscopy by about four-fold, differentiating objects spaced as little as 20 nm apart for STORM versus ~100 nm for confocal microscopy. An advantage of STORM imaging over atomic force and electron microscopy that can also reveal ultrastructural details of fluorescently tagged proteins in plant tissues, is the ability to capture time-resolved photon localization sequence capturing in living cells (Huang *et al.,* 2008). dSTORM uses photo-switchable probes which can be switched on and off by emission control thereby, transitioning between emitting and dark state (Rust *et al*., 2006). When these photo-switchable probes get activated by a low intensity but adequate light, a random probe gets triggered and independent single molecules are detected and localized with high precision. Following numerous cycles of "on-and-off", many of the probes can be localized and a super-resolution image can be established from the localizations recorded from many individual emitters with each having a distinct set of coordinates in the lateral image plane (Hosy *et al.,* 2015).

Using the dSTORM, approximately 20 to 40 nm resolution, multicolour, three-dimensional imaging has been achieved in mammalian cells (Rust *et al*., 2006; Huang *et al.*, 2008, 2010). Many super-resolution images are still limited in their applicability techniques and therefore limited in use, especially in plant cells due to the complexity of the cell walls. However, recent developments in live-cell imaging indicate that adaptive optics may be used to partly compensate for the complexity of plant cell walls (Betzig, 2015). For instance, adaptive optics

in dSTORM enables the visualization of glucan polymers (Schneider *et al.,* 2016). Dong *et al.,* (2015) visualized cortical microtubules in fixed root cells that were immunolabeled with antibodies conjugated to the photo-switchable fluorophore AlexaFluor 647 at 50 nm resolution. Also, numerous subcellular structures have been resolved with super-resolution using STORM, including clathrin-coated vesicles, microtubules, and actin (Huang *et al.*, 2009; Huang *et al.*, 2010; George, *et al.* 2018). Imaging with STORM requires thousands of cycles that need prolonged time to complete. Hence, a trade-off between spatial and temporal resolution with STORM cannot be avoided (George, *et al.* 2018). Localization microscopy such as dSTORM, improves speed of image acquisition and is compatible with conventional fluorophores (Lemmer *et al*., 2009; Ries *et al*., 2012; Platonova *et al*., 2015). Hence, in this study, it was possible to use fluorescently tagged cellulose and callose synthase, for STORM imaging during biotic stress.

In this work, we successfully used dSTORM for imaging single molecule in living cells of *Arabidopsis thaliana*. STORM was used for tracking the movements of molecules using the photo-switchable fluorescent proteins with a super-resolution image of approximately 50 nanometers. The STORM imaging successfully captured the deposition, movement, pattern, and quantified speed of fluorescently tagged cellulose synthase, callose synthase and aniline-blue stained callose polymer in the living leaf tissue simultaneously upon powdery mildew transfection. The quantity and quality of information obtained as image data from the dSTORM was improved and analyzed by using tracking algorithms such as ThunderSTORM and TrackMate plugins in ImageJ software. The STORM technique and image data analysis described in this work can be modified and used to image proteins that are localized to other cell membranes in plants of different species.

4.3 Materials and Methods

4.3.1 Plant materials, transformation, and growth

The transgenic lines used in this work were generated and provided by Dr. C. Voigt (BASF Innovation Centre, Belgium). The constitutive cauliflower mosaic virus promoter (35S) was used to control the fusion of PMR4 to GFP to create 35S::PMR4-GFP transgenic strains (Ellinger *et al,* 2013). tdTomato (red fluorescent protein) were used as fluorescent tags for *CESA*6 (unpublished). A BASTA selection cassette was included to select for positive construct presence in the expression vectors. The presence of this herbicide-resistance cassette

allowed identification of transformed seedlings from untransformed seedlings. All constructs were confirmed by restriction digestion with XhoI (for *PMR4*) and SfiI (for *CESA*6) and sequencing. The transformation of *A. thaliana* with the generated plant expression vector and selection of successfully transformed plants, followed an established protocol (Ellinger *et al*., 2013). In summary, plasmids containing 35S::*PMR4*-GFP and 35S::tdTomato-*CESA*6 were transformed into agrobacterium strains, GV3101by electroporation, and successful clones were tested with colony PCR. To transform *Arabidopsis thaliana*, floral tissues were dipped into a solution containing *Agrobacterium tumefaciens* GV3101, 5% sucrose and 500ml per liter of surfactant Silwet L-77 as described by Clough and Bent, (1998). Plants were further grown for four weeks until siliques were brown and dry. The dried seeds were harvested, kept in microfuge tubes, and stored at room temperature till further use.

BASTA resistant *Arabidopsis* transformants were selected in soil. Seeds from primary *Arabidopsis thaliana* homozygous T3 transformants (transformed to express *35S::PMR4-GFP* and *35S::tdTomato-CESA6*) were grown and the seven-day old seedlings were sprayed with BASTA solution (Harrison *et al.*, 2006). Non-transformed plants showed yellowing of cotyledons after 3 days. Seeds were harvested from seed pods of transformed plants after 8 to 10 weeks and dried. Seeds from transformed plants were grown as described by (Duncombe *et al*., 2020). Seeds were sown into compost (M3 soil + silica sand: 3:1) in flat containers placed in plastic trays. The trays were then covered with a tight-fitting clear plastic dome to retain humidity and were then transferred to the growth chamber with a 16-hour light/dark cycle at 21°C (light) and 17°C (dark), 150 mol/m2/s1 fluorescent lighting, and 70% humidity. The dome remained on the trays until seedlings started to emerge and green cotyledons becomes evident. Courgettes (*Cucurbita pepo*) plants used for cultivating and maintaining the powdery mildew, *Podosphaera xanthii* for plant pathogenic transfection, were grown in M3 compost and sand (3:1) with a 16-hour light/dark cycle at 21°C (light) and 17°C (dark) under 150µmolm^{-2}s^{-1} fluorescent illumination and at 70% humidity, in the plant growth chamber.

4.3.2 Plant transfection with powdery mildew

A new powdery mildew strain, *Podosphaera xanthii* Shef1 which was identified and isolated from a Courgette plant (*Cucurbita pepo*) hybrid variety ‘Eight Ball’ plant in Sheffield, S10, Crookes (53°23'13"N 1°30'24"W) was provided by Dr. C. Voigt (BASF Innovation Centre, Belgium). *Podosphaera xanthii* Shef1 was maintained on courgette plants which were planted in pots containing M3 compost soil and housed in a growth chamber with a 16:8 light:dark

cycle (21°C, 60% constant humidity, 160 $molm^{-2}s^{-1}$ light intensity). After 4-5 weeks of courgette plants' germination, Shef1 spores were moved from the infected courgette leaves by carefully tapping the infected leaves over the uninfected courgette plants, as described by Urbanietz and Dunemann (2005). After 3 weeks of growth, the transformed *Arabidopsis* plants were inoculated with powdery mildew. Conidia from infected courgette leaves were sprayed onto the uninfected plants using an air duster and a cardboard inoculation tower that matched the size of the plants' tray. Spraying was paused intermittently to allow the spores to settle. Spraying was stopped when the spore density of the inoculation reached 40 conidia/mm^2 which was measured with a haemocytometer. At 6 hours post inoculation (6hpi), transfected leaves were harvested for imaging analysis.

4.3.3 dSTORM imaging of leaf tissues

The imaging of enzymes and callose were performed using dSTORM, following the description of van de Linde *et al*. (2011). Rosette leaves from transfected transgenic plants were stained with 0.01% aniline-blue (Hong *et al.*, 2001) for callose detection. Samples were imaged at room temperature by direct STORM on an inverted N-STORM microscope (Nikon STORM Microscopy, Wolfson Light Microscopy Facility, University of Sheffield) in highly inclined illumination mode using 100x NA oil immersion objective. STORM imaging was conducted with a high signal-to-background ratio using an inverted microscope (Zeiss) equipped with a total internal reflection fluorescence (TIRF) module (van de Ven *et al.*, 2011). A solid-state laser operating at different wavelengths was used to excite aniline blue at 408 nm wavelength and was focused on the back focal plane of the oil-immersion objective (Nikon, 100X Oil, N.A. 1.49). Fluorophore blinking of tdTomato and GFP were excited and activated with 488 and 561 nm lasers, respectively. Emission light collected by this objective then was focussed onto an EMCCD chip (Andor iXon+, Sheffield, UK) enabling a final pixel size of ~50 nm. The camera integration time was set to 30 ms, with images acquired at a frame rate of 33 Hz. Approximately, 6800 images (30ms per frame) were obtained, with a laser excitation intensity of 1–5 kW cm^{-2} for a total acquisition time of 3–4min for a single dSTORM image. The resulting image data of the emission of fluorescence (provided by Dr. C. Voigt) was calculated using ImageJ tools (W.S. Rasband, National Institutes of Health; http://imagej.nih.gov/ij/).

It is important to note that when studying proteins in fixed cells the super resolution imaging technique typically relies on a reducing environment, such as a mercaptoethylamine buffer.

However, caution is advised when studying proteins in living cells (as in this study), as these buffers can be harmful to the cell's viability. In the case of live cell dSTORM, a different approach as afore described is used, taking advantage of the suitable reducing conditions naturally present within living cells. All living cells contain reducing agents like glutathione (GSH), which enables the live-cell dSTORM technique (van de Linde et al., 2011). This method has been successfully applied to investigate the distribution and dynamics of proteins in living cells (Wombacher *et al*., 2010, Klein *et al*., 2011). Pertaining to the "blinking" phenomenon in super-resolution microscopy, it is important to clarify that in living cells, fluorophores do not require light-induced oxidation to achieve the "on-state" for imaging. Living cells already have a natural "redox cocktail" comprising thiol (GSH) and oxygen at appropriate concentrations, which is sufficient for activating the fluorophores. Hence, in the experiment, all fluorophores are initially transferred to a nonfluorescent "OFF" state by irradiation with laser light of an appropriate wavelength and intensity. Subsequently, they are excited and activated to the "ON" state using laser lights in the range of 488 to 561 nm (van de Linde *et al*., 2011).

4.3.4 Single-molecule localization data and statistical analysis

The lists of molecular localization were extracted from dSTORM to single-molecule data analysis software available at https://github.com/inatamara/Grafeo-dSTORM-analysis and processed, prior to data curation in R package. The image data generated from STORM contained approximately 330,000 data points from 6800 data frames for thirty timepoints (30ms per frame for 3.4 minutes). These data points represent photon distribution of molecules within that frame which is denoted by the position (x, y and z coordinates) of each fluorophore representing the fluorescence signal. For the analysis of the generated image data of callose deposits and fluorescent tagged proteins trafficking from the live plant leaf tissues, an automated image analysis with ThunderSTORM (Martin *et al*., 2014) and TrackMate plugins (Tinevez *et al.*, 2017) Fiji ImageJ software (W.S. Rasband, National Institutes of Health; http://imagej.nih.gov/ij/) was used. Raw data were cleaned, structured, and enriched into a desired format using R-package tidyverse, prior to analysis in imageJ. Analysis involved three datasets of tdTomato signal (Cellulose synthase – *CESA*, red channel), GFP signal (Callose synthase – *PMR4*, green channel) and aniline blue (ABF) signal (Callose polymer, blue channel). Statistical test was performed using a Mann–Whitney test, analysis of variance

(ANOVA). Variation between two normal distribution was determined using Kolmogorov-Smirnov Tests. The two sample Kolmogorov-Smirnov test (Grover, 1977) is a nonparametric test that compares the cumulative distributions of two data sets.

4.4 Results

To generate plants for imaging *PMR4* and *CESA*6, constructs in which GFP is fused to the C-terminal of *A. thaliana PMR4* coding sequences, under the control of the constitutive cauliflower mosaic virus promoter (35S) was generated. Likewise, 35S::tdTomato-*CESA*6 transgenic lines were generated. Four weeks after transformation, plants were inoculated with powdery mildew and selected for imaging. Qualitatively and quantitatively, these two lines were distinguishable and here I present results for the *35S::PMR4-GFP* and 35S::tdTomato-*CESA*6. Imaging protein particle movements in leaf tissue of *A. thaliana*, revealed varying patterns of particles in different regions within and between the *CESA*6 and *PMR4* trajectories, and great variation in the speed of the particles.

4.4.1 Cellulose and callose synthases: qualitative observations.

Three-week-old, transformed *Arabidopsis thaliana* plants were inoculated with a new powdery mildew strain, *Podosphaera xanthii* Shef1 (unpublished). For imaging, I used dSTORM imaging with an inverted microscope (Zeiss) equipped with a TIRF module reducing background noise (van de Ven *et al*., 2011). Aniline blue stain allowed visualization of callose. Few hours after inoculation, powdery mildew induced both single and clusters of callose spots in the 35S::*PMR4*-GFP and 35S::tdTomato-*CESA*6 leaves tissues. The patterning and quantified motility of *PMR4* spots were substantially different from that of *CESA*6. The appearance of bright red tdTomato flourescent tagged *CESA*6 track vary widely within the leaf cells. This study would have benefited from cell membranes stained and visualized for analysis of the distance or postion of the proteins in relation to plasma membrane. Without this information I was not able to precisely describe the relative angle of these protein localization. Nevertheless, a uniform thin, linear shape of *CESA*6 tracks were observed at the middle region, and regions closer to the edges adopt more rounded shapes (Figure 4.1A). The *CESA*6 patterns displayed in various regions within the tissue was different from that of the *PMR4* tracks patterns, which display similar molecule patterns in neighbouring regions. Green fluorescent tagged *PMR4* are constantly more uniformly shaped, close to a round shape or more densely ball-shaped clustered and were similar to the aniline blue stained callose polymers. Larger

PMR4 tracks toward the central region of the leaf tissue 2D image displayed some clustering patterns.

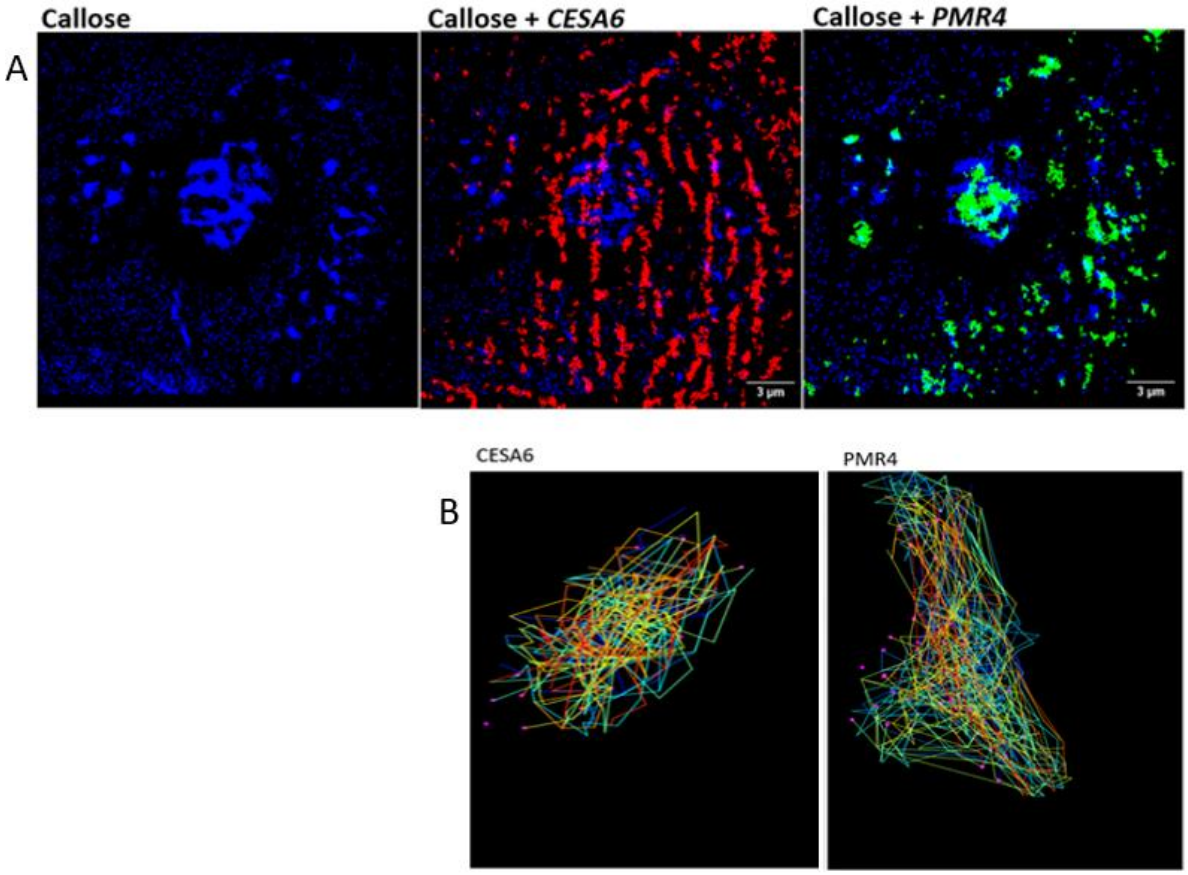

Figure 4.1 Localization of *PMR4*-GFP and tdTomato-*CESA*6 in the leaf tissue, after inoculation with powdery mildew. Three-week-old transgenic lines were inoculated with the powdery mildew *Podosphaera xanthii* Shef1. All tests were performed with rosette leaves. Images were taken by Direct stochastic optical reconstruction microscopy (dSTORM). Green colour denotes GFP-emitted fluorescence (*PMR4*), red colour: tdTomato-emitted fluorescence (*CESA*6), and blue colour: aniline blue-stained callose. Visualization and localization of callose deposition, tdTomato-*CESA*6 and *PMR4*-GFP 6 hours post inoculation in maximum intensity 2D reconstruction stacked images (A: left, centre and right columns). 3D reconstruction of fluorophore localization at the same site with the view of distribution within the cytosol and possibly to the cell membrane (B: left and right columns). Scale bars = 3 µm.

In single frames and projections, the *PMR4* puncta visually appeared qualitatively similar to those of callose depostion but different from *CESA*6. Concatenating the two dimensional images stacks into a time-lapse 4D image with single particle tracking allowed the visualization

of the overal networks and distribution of the proteins (Figure 4. 1 B). Callose synthase (*PMR4*) is apparently more widely distributed in the leaf cells in comparison to cellulose synthases. In the 2D image of the time-lapse data (Figure 4. 1 A) obtained from cells expressing *CESA*6 and the aniline blue stained callose, *CESA*6 particles tends to move along the sides of callose rather than directly on top of them as observed for the interaction between callose and *PMR4* where *PMR4* colocalizes with callose.

4.4.2 Motility of cellulose and callose synthase: quantitative observations.

To quantify motility of the fluorescently tagged molecules, I performed single-particle tracking analysis using ThunderSTORM (Martin *et al*., 2014) and TrackMate plugins (Tinevez *et al*., 2017) in Fiji ImageJ software. The dynamics of the proteins and their interactions with callose were evaluated over a time period of 1 to 3 minutes. The ImageJ plugin Trackmate provided a method to automatically segment puncta 3D image and track them over a period of time (tracks the puncta through subsequent frames automatically). In each photon localization sequence, particles were tracked for a minimum of 70 seconds and were used to determine the average speed. In Figure 4. 2, individual bar denotes the average speed of particles in a given photon localization sequence, with the middle bar of a sequence group (the bar having error bars) denoting the mean speed averaged over the photon localization sequences in the group. The variability within each photon localization sequence group (between diferent cells, often on diferent plants) is notable (Figure 4. 2).

To determine whether *CESA*6 and *PMR4* particle movement demonstrated a bias based on callose deposition, I compared the speed of protein particle movement at the region where callose is present to the region when callose is absent. The average speed of the sequences for each enzyme with callose and enzymes without callose appeared similar. In other words, *CESA* particles do not speed up or slow down significantly in the region where callose is deposited, although, callose seems to slightly reduce the speed of *PMR4* particles. Overall, callose synthases appear to be slower than cellulose synthases (Figure 4. 2). To compare the enzymes more precisely, the tracked particles from each sequence were obtained for analysis of the overall distribution (Figure 4. 4 and 4. 6). A faster mean particle speed of 493.457±11.909nm/min (n = 1396 particles) of *CESA*6 compared to an average speed of 281.579±4.854nm/min (n = 1854 particles) for *PMR4* particles was observed. The distribution for *PMR4* appeared to be shifed to lower values across its speed range (Figure 4. 3). The

cellulose synthases seem to slightly slow down in the region where callose is present (Figure 4.3) but the trend is not significant. I tested the hypothesis that the effect of protein type on the speed (F=41.24; df=1, p=1e-07) is dependent on the presence or absence of callose. I found no evidencce to support the presence of an interaction between callose and the enzymes.

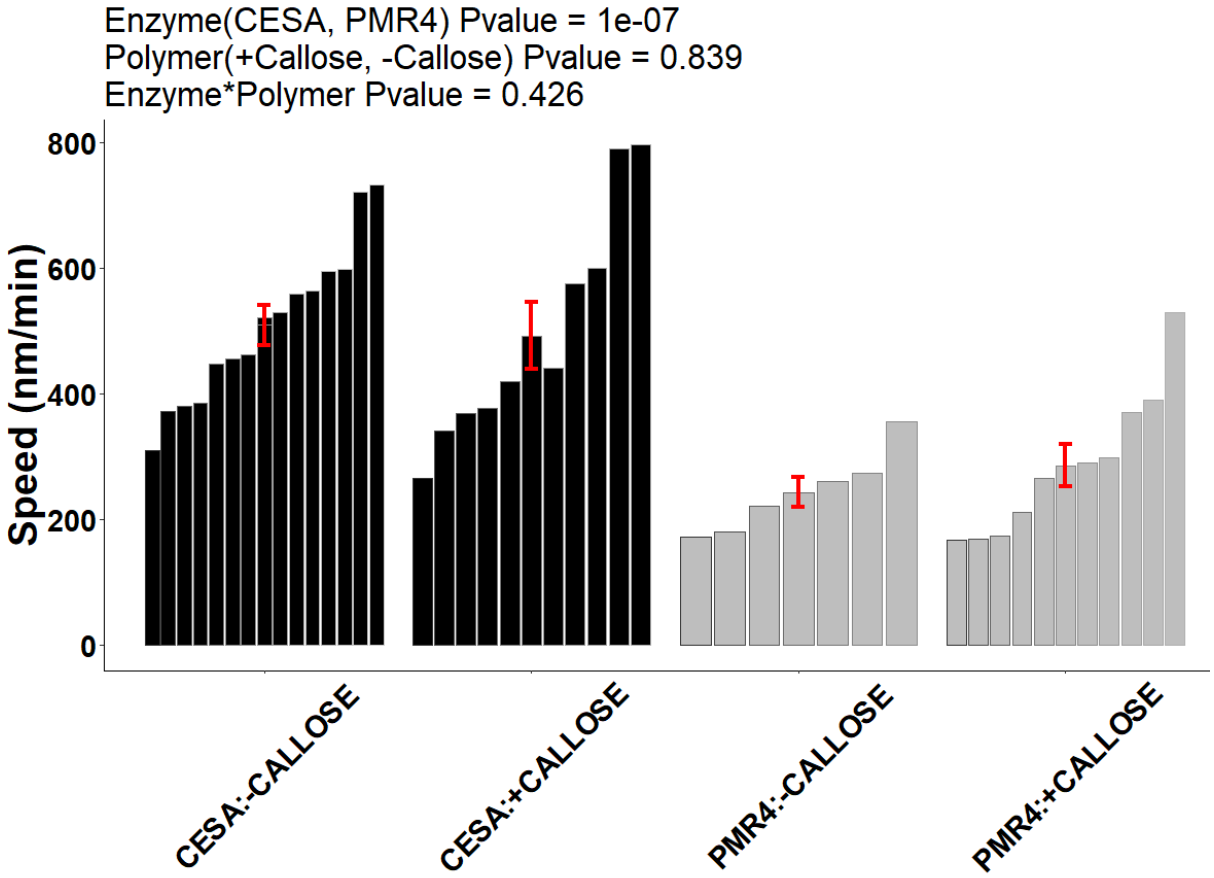

Figure 4.2 Quantifcation of *CESA*6 and *PMR4* motility. Lightly shaded bars are callose synthases with or without callose and darker bars are cellulose synthases with or without callose. For each group, bars denote the mean speed for tracked particles in a photon localization sequence. Bars with errorbars plot mean±SE of the average speeds displayed. Bars are sorted in an ascending order for the purpose of clarity. Count of tracked particle in each sequence ranges from 23 to 91 (*CESA*+); 33 to 142 (*CESA*-); 44 to 541 (*PMR4*+) and 38 TO 409 (*PMR4*-). Statistical test: ANOVA.

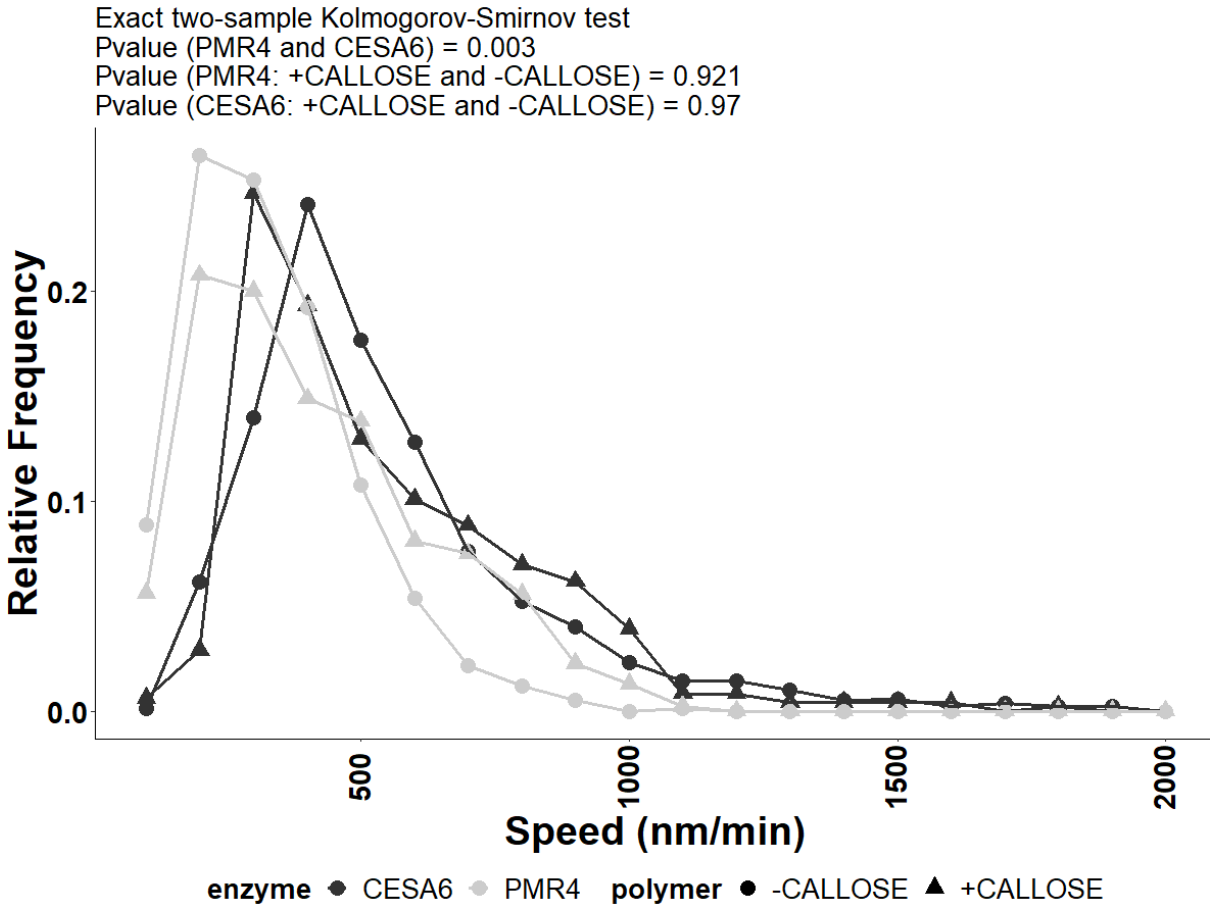

Figure 4.3 Frequency distribution for *CESA*6 and *PMR4* speeds with or without callose. Data are gathered from the individual sequence displayed in Figure 4.3. The average speed for *PMR4* is 281.579±4.854nm/min (mean±SE, n=1854 particles) and for *CESA*6 is 493.457±11.909nm/min (n=1396 particles).

Finally, the distribution of particles along different z positions of the stack images, gives an indication of the accumulation of proteins and polymer at different location within the challenged leaf tissue. Confirming by visual inspection, the distribution of callose synthases (with or without callose) along the Z-axis are distinguishable from those of the cellulose synthases with callose synthases having a wider distribution (Figure 4.4). Also, *CESA*6 particles appear to accumulate at a particular region within the cytosol and presumably not outside the cell membrane. In addition, there seems to be a notable effect of callose on the distribution of callose synthases. Presence of callose increased the wide distribution of *PMR4* by approximately 50%, an effect that was not apparent for the distribution of cellulose synthases. Taken together my results shows that *CESA*6 motility in the transfected leaf tissues is relatively fast but less distributed in comparison to *PMR4* and *PMR4* further spreads widely in the presence of callose.

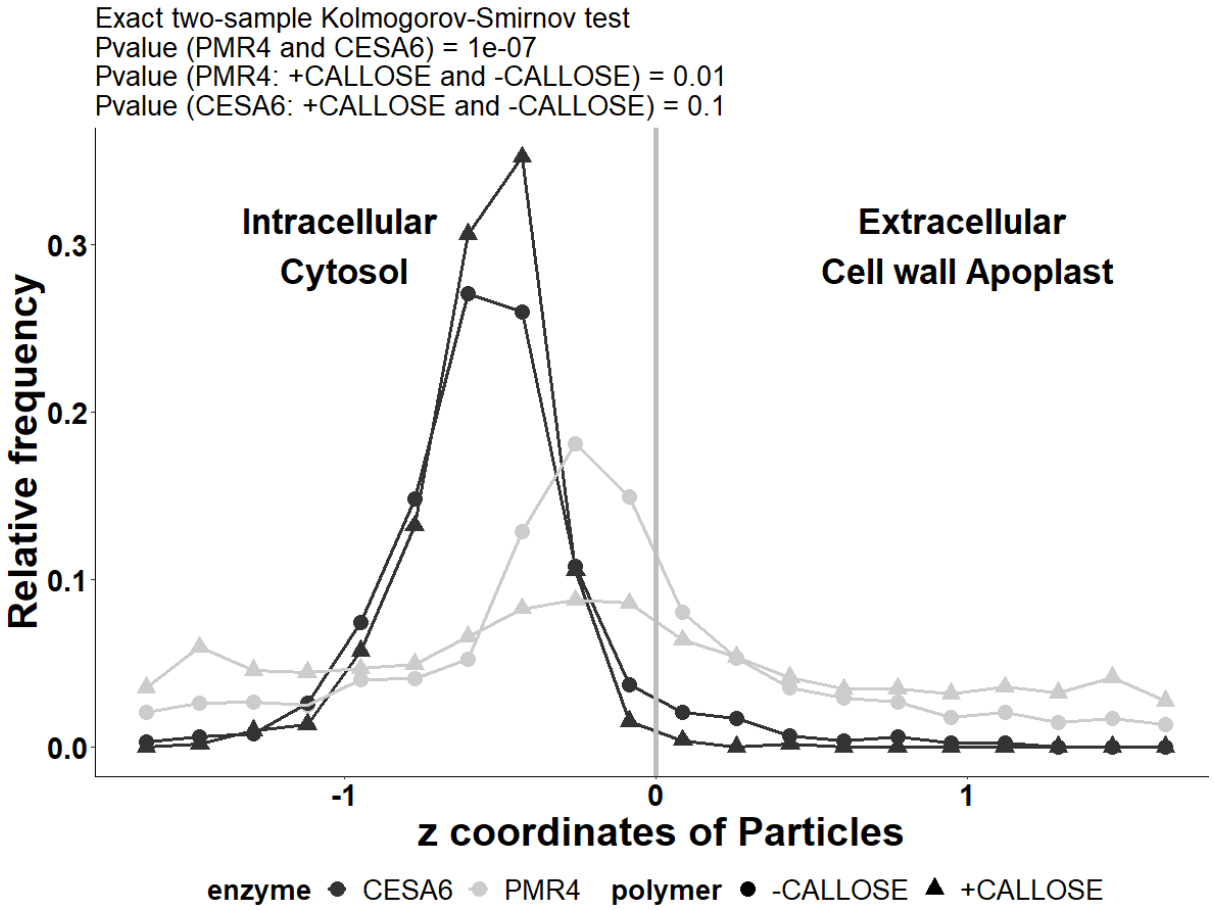

Figure 4.4 Frequency distribution of *CESA*6 and *PMR4* particles (with or without callose) over different z coordinates. V-line denotes the cell membrane

4.5 Discussion

It is well established that cellulose, which is made by *CESA* complexes, directly interacts with callose synthesized by callose synthase complexes within the area of forming callosic papillae, during attack by powdery mildew (Eggert, 2014). However, the mechanism of these interactions at the nanoscale in living cells has remained poorly understood. In this study, I imaged and quantified the motility and pattern of cellulose and callose synthase during the invasion of powdery mildew. Super-resolution imaging of callose, cellulose and callose synthases using a total internal reflection fluorescence (TIRF) microscopy in combination with dSTORM, a rapidly growing localization microscopy technique, allowed detailed visualization of the polymer and the fluorescently tagged proteins in the leaf tissues of *A. thaliana* to a lateral resolution of less than 50 nm, which indicated that proteins and corresponding polymers can accumulate during a microbial attack. Similar imaging of cellulose was possible for visualizing

cellulose fibrils in epidermal leaf cells with the use of cellulose-specific dye Pontamine FaIt (Liesche *et al.*, 2013) as well as successful super-resoution imaging of *CESA* complexes using Structured Illumination Microscopy (SIM) (Sydney *et al*., 2022). Here, it was possible to image callose and the synthases in the living cells of *A. thaliana* leaves and illuminate sub-critical areas, with the use of adaptive optics incorporated in the dSTORM, despite the overall challenge of plant cells; thickness (>250 nm) of the cell walls (Betzig, 2015). Consequently, there were abundant fluorophore spots available for tracking which enabled a reliable quantification of the speed.

The linear pattern observed for *CESA*6 tracks in this work, may be partly reflecting the pattern of the trans-Golgi network (TGN) and small vesicle compartments termed microtubule-associated cellulose synthase compartments which embeds and trafficks the *CESA* complexes from the site of assemblage to the plasma membrane (Elizabeth *et al*., 2009; Gutierrez *et al*., 2009). Li *et al*. (2015) reported that the patterning of *CESA* tracks may be influenced by cortical microtubule organization because *CESA* spots were localized closely with microtubules. Also, *CESA* particle trajectories are shown to be dominantly influenced by cortical microtubules (Chan and Coen, 2020). The frequency distribution of *CESA*6 along z-axis indicates the spread of synthases within the cytosol and towards the outer cell wall apoplast. The wider spread of *PMR4* along the z-axis suggests a travel of callose synthase into the outer cell wall, presumably along the forming callose polymers and towards the site of microbial attack as well as expansion into the cytosol for guarding internal molecules. My findings support previous reports on callose as the most abundant chemical constituent in defence-related cell wall thickenings in plants called papillae (Sherwood and Vance, 1976; Aist 1976; Nishimura 2008; Stone and Clarke, 1992), at sites of microbial attack.

Interestingly, callose synthase becomes even more widely spread towards the outer plasma membrane in the region where callose is present (Figure 4.4). The *CESA*s and *PMR4* complexes utilize UDP glucose as substrate, and their catalytic activity may drive their motility (Morgan *et al*., 2013). Glucan chains crystallize into cellulose microfibrils that are intertwined with other cell wall polymers, thereby, making any further cellulose synthesis to move the *CESA* forward to the outside of the cell membrane. Here, as for cellulose synthases (Morgan *et al*., 2013), callose synthase (*PMR4*) pushes forward to the extracellular cell wall apoplast at sites where callose is deposited, thus suggesting a similar mechanism to cellulose biosynthesis and

CESA motility. Also, it is reported that the presence of glucans such as cellulose is a great determinant of the speed of the *CESA* complexes (Morgan *et al*., 2013, Fabiana and Bela, 2007). However, this was not the case with callose synthases in my study; the speed of callose does not seem to have any effect on the speed of either the *PMR4* or that of *CESA*6 (Figure 4. 2). However, the frequency distribution of *PMR4* speed in region where callose is present shifts to slightly lower values but this shift does not seem to be significant (Figure 4.3).

I observed that *CESA*6 particles in the presence or absence of callose had a mean speed significantly greater than *PMR4* particles mean speed (Figure 4. 2). The mean speed (493.457±11.909nm/min) measured for *CESA*6 motility in the leaf cells of *A. thaliana* during microbial attack is higher than usually reported for this protein. In past studies, the speeds of *CESA*s in plant tissues have been either quantified or estimated to be approximately 100–350 nm/min depending on the type of plant material used (Gu *et al*., 2006; Desprez *et al*., 2007; Diotallevi and Mulder, 2007; Persson, 2007; Young *et al*., 2015). *CESA*6 speed value (493.457±11.909nm/min) was significantly higher in this study than previously reported (350 nm/min at the maximum) and may indicate the inducible effect of microbial inoculation, with *CESA*6 possibly migrating quickly to the site of microbial attack to strengthen the cell wall against microbial penetration. To the best of my knowledge, there is no study yet on the speed quantification of *PMR4*. Nevertheless, this study shows a lower speed of *PMR4* compared to *CESA*6. Despite the lower speed, *PMR4* is still more widely distributed than *CESA*6 (Figure 4. 1 B and Figure 4.4), widely spreading possibly towards the membrane apoplast at sites of microbial attack. Callose synthase complexes are assembled in the endoplasmic reticulum and transported through the trans-Golgi network (TGN) to the plasma membrane as suggested in tobacco pollen tubes (Cai *et al*., 2011; Drakakaki *et al*., 2012). Vesicle trafficking of the callose synthases complexes may also translocate callose synthases localized in the cell membrane to sites of attempted microbial penetrations where callosic papillae is deposited for cell wall reinforcement (Nielsen *et al*., 2012; Ellinger *et al*., 2013). Bohlenius *et al*. (2010) reported that callose synthases complexes trafficking was being mediated by multivesicular bodies in barley and Cai *et al*. (2011) suggested the involvement of actin filament in such trafficking. Hence, further investigation is needed to fully understand the mechanism of callose synthases trans-localization to sites of attempted pathogen invasion.

Overall, my results show the impact that super-resolution microscopy can have to decipher molecular mechanisms of callose and cellulose synthases. Importantly, my study elucidates the interaction and dynamics of callose and cellulose synthase complexes at pathogen interaction sites, leading to the discovery of yet unknown migration mechanisms of callose synthase complexes and associated callose polymer synthesis in the apoplast. Super resolution imaging offers a novel and an interesting way to approach studies on protein interaction in plant cells and will provide further mechanistic insights into the interactions among cellulose synthases, callose synthases and their corresponding polymer during a microbial attack. Such insights may present new opportunities for studying regulatory mechanisms of papillae formation that are involved in disease resistance and supports novel techniques for molecular breeding to increase pathogen penetration resistance in food crop species which are important, given the demand of growing human populations.

4.6 Conclusions

Even though I could identify the differences in the speed and distribution between the synthases, I would like to also highlight some of the shortcomings of my approach when studying the behaviour of protein motility. In this work, I have expressed tagged *CESA* and *PMR4* sequences from *A. thaliana* in a wild-type background, hence, it has not been experimentally confirmed that the imaged constructs are functional. In other words, no test was carried out to confirm the functionality of the constructs, although this functionality is supported by prior studies (Ellinger *et al*., 2013). In addition, in *A. thaliana*, the pattern of accumulation or motility of imaged tagged synthases expressed in a wild-type background should have been compared with those of tagged genes expressed in the corresponding mutant background. Future work in this area of research should consider the aforementined short comings and make informed decisions on research methodology. In addition, the variability in the patterning of the protein particles among the different regions of the leaf tissue, might have an impact on the observed variability in protein speed among the different regions and further analysis, quantifying the patterning and speed of particles from each region would be informative.

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Chapter 5:

General Discussion

5.1 Overview

I investigated floral scent diversity, both constitutive and response to biotic stress in plant as well as plant defence responses at a cellular level to understand the effect of plant defence responses on floral signals and the underlying mechanism of plant defence. Firstly, I assessed the evolution of both induced and constitutive floral scent at two different levels of biological organizations. Across 20 closely related selfing and outcrossing wild potato species, I show that independent mating system shifts have driven a parallel pattern of scent vestigialization. Examining intraspecific variation in *Arabidopsis lyrata*, I also found remarkable shifts in floral VOC emissions, consistent with mating system transitions in 14 populations of *A. lyrata*, with higher magnitude VOC emissions shifting toward lower VOCs in the selfing plants. There was strong evidence of phylogenetic signal in the patterns among species for all floral scent compound classes and no phylogenetic signal was observed for inducibility, suggesting that inducibility is an evolutionarily labile trait subject to divergent selection, and highlighting the importance of accounting for evolutionary history when studying the drivers of VOC variation. Populations across the range of the *A. lyrata* complex diverge along the same axis, as wild potato species but over a more recent timescale. Overall, these results document a predictable pattern of floral scent evolution where variation expressed within a plant species may lead to specific evolutionary shifts at a macroscale. Finally, in my work on callose, I found that modification and strengthening of the plant cell wall for improved pathogen resistance could be a major focus of current research to improve resistance in crops to attacking plants

pathogens. I observed the interaction and dynamics of single callose and cellulose synthase complexes at pathogen interaction sites, leading to the discovery of yet unknown transport mechanism of callose synthase complexes and associated callose polymer synthesis in the apoplast. The results may help to identify new molecular targets for optimising pathogen-induced callose biosynthesis and deposition to increase resistance to pathogens in plants with direct focus on crops.

5.2 Total amount of emission and diversity of floral VOCs

Notably, total amount of VOC emissions and the emissions of main compound classes of floral VOCs (for example, green leaf volatiles, benzenoids, and terpenoids) were different among and within species. Our study is in concordance with other studies that found, species floral scent variation among species (Knudsen *et al*., 2006; Filella *et al*., 2013). However, I discovered that total floral VOC emission rates across species in a genus can vary predictably depending on the mating system which is often mediated by changes in ecology such as plant–pollinator interactions and abundance of pollinators. This indicates that consistent differences in the environment, shaped in part by mating systems may drive parallel (or convergent) selection on important biochemical pathways within and among species. Numerous factors might be involved in such selection. For example, floral VOC production, plant physiology and physiochemical properties of individual compounds can be positively affected by warmer temperatures (Sagae *et al*., 2008; Hu *et al*., 2013). Differences in water availability seasonally may also influence emission of floral VOCs (Campbell *et al*., 2019). Ultimately, differences in patterns of plant–pollinator interactions, pollination services, and plant reproductive systems can affect VOC biosynthesis. In the intraspecific floral scent variation, consistency with mating system shifts appeared to be contingent on the age of mating system transitions. Burkle and Runyon (2016) and Glenny *et al*. (2018) demonstrated that floral scent variation among species responded to experimental modifications of environmental conditions. In my studies, the among- and within- species floral scent variations were consistent, based on mating systems, despite my study species and populations occurring across a wide range environmental variation, including season, climate, timing of evolutionary transitions and environmental changes. In addition, a specific VOC compound class (benzenoids) known to be a significant set of attractants for plant-pollinator interactions, among the investigated compound classes was strongly associated with the age of mating shifts within species. This suggests plant-

pollinator interactions could have a great influence on mating system transitions that in turn could affect the production of floral VOCs.

5.3 Intraspecific and interspecific variation in floral scent

My range-wide analysis of *A. lyrata* are partially consistent with those of Petrén *et al*. (2021) which showed that *Arabis alpina* (Brassicaceae) floral scent emission rates are lower in selfing compared with outcrossing populations. However, the study did not consider the age of mating system transitions or population relatedness and was limited to looking at a single evolutionary shift to selfing. Accounting for population history, our study suggests that the predicted age of the mating system transition greatly impact the magnitude of floral scent variation. Younger selfing populations exhibited an enrichment in aromatic compounds known to be pollinator attractants; while other ecological factors are likely to have played additional roles in this pattern, my hypothesis of age-dependent selection was most consistent with the phenotypic data. On the other hand, the difference in total VOC emission between self-incompatible and self-compatible wild potato species is in concordance with the work of Sas *et al*. (2016) who demonstrated a reduced scent emission (most notably in benzaldehyde) in the selfing species *Capsella rubella* compared with the outcrossing *Capsella grandiflora*. With more thorough sampling and a phylogenetic framework in this study, I was able to deduce the variations in floral scent evolution, demonstrating with greater certainty that ancestral wild potato species which are outcrossing, produced significant amounts of VOCs. The initial diversification of closely related wild potatoes appears to have been mostly unaffected by these VOC emissions, and subsequent evolution of VOC variation coincided with transition to a selfing mating system. These findings support the hypothesis that Solanaceae originated more as outcrossing than the majority of its recently evolved selfing species, as well as the assertion that the transition from outcrossing to selfing occurred frequently within the group (Igic *et al*., 2006; Barrett, 2010; Goldberg *et al*., 2010).

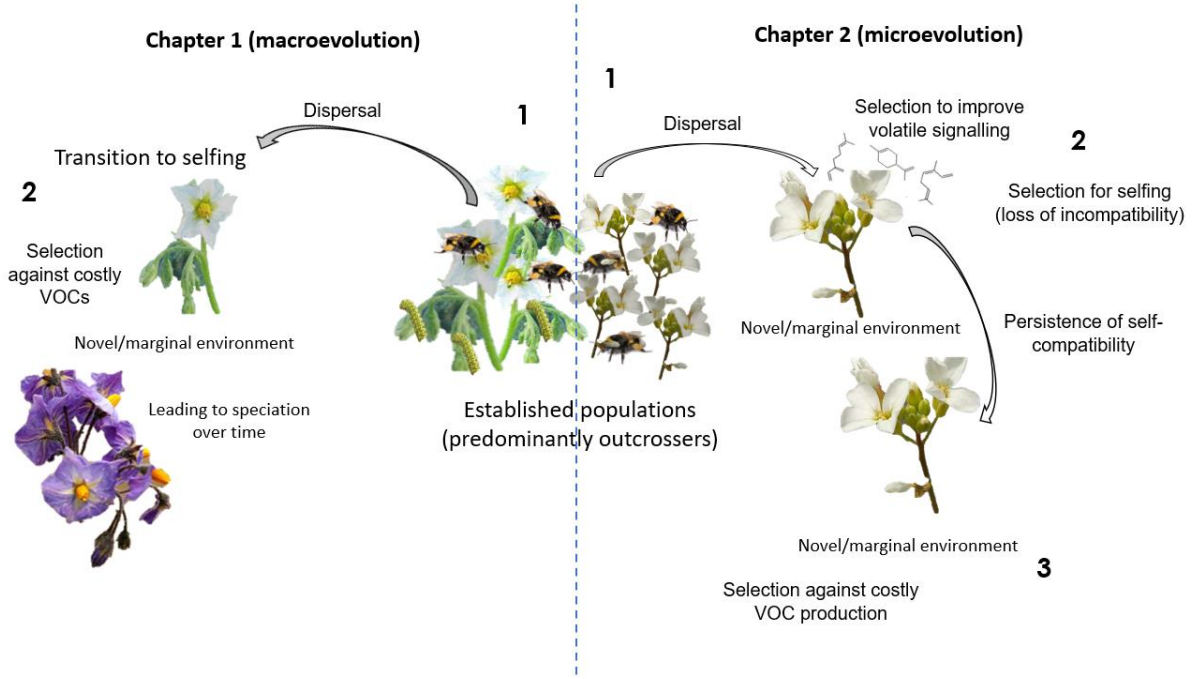

Figure 5.1 Schematics of the contribution this thesis has made to the understanding of interspecific and intraspecific floral scent variation in relation to mating system transitions usually mediated by pollinator-plant interactions in response to environmental changes. I propose a two-step process in the evolution of selfers at a microevolutionary scale prior to speciation over time (macroevolutionary scale). Younger selfing populations (microevolution, step 2) which are newly establishing a range, exhibit an enrichment in aromatic compounds known to be pollinator attractants. Over time, as self-fertilizing continues, there may be selection against increased VOC emission (microevolution, step 3). The microevolution patterns may predispose speciation (macroevolution, step 2).

Evidence of mating system related change in in floral scent emission in all 20 studied wild potato species indicates that it is common throughout the clade. In a single species (*A. lyrata*), I found that outcrossing species which are the ancestors produced significantly higher VOCs than selfers. In contrast, variation in floral scent emission with mating system shifts tended to be specific to the timing of transition to selfing (Figure 5.1). For instance, while VOC emissions were lower in old selfing populations (southern) than old outcrossers (southern), VOCs production was higher in young selfing populations (northern) than young outcrossing populations driven by selection on aromatic secondary metabolites at both genomic and phenotypic scales (Figure 2.1, Figure 5.2). These differences indicate that the covariance between floral traits and mating system shifts may vary with the timing of selfing and/or geographic location, and that floral scent variation can be associated with different traits such

as environmental changes affecting plant-pollinator or plant-herbivores interactions. However, among all the VOC compound classes studied (some data not shown), it is notable that green leaf volatiles which are involved in sustainable defence strategies of crops (Brilli *et al*., 2019) and benzenoids which are aromatic and may be more involved in pollinator attraction (Shi *et al*., 2019), were the most consistently variable floral scents because these are the two classes of floral scents that also vary among species. In other words, the magnitude and dynamics of variation within a species corresponded to that of among-species variation. That said, the *A. lyrata* complex displayed significant floral scent variation among population clades, and it is observed that selfing populations have accentuated the production of either the high emission of floral scents (in the north) or low production of VOCs (in the south). Our Hierarchical clustering tree (Figure 3.2) of the *A. lyrata* complex show considerable genetic population structure related to its geographic range and genetic differentiation related to mating systems traits. Hence, I suggest that the floral scent variation observed among populations may reflect a combination of adaptive differentiation in response to the environments faced by selfing populations, but also neutral and biogeographic processes (Levin, 2012).

Considering all our data sets, I suggest that the nature of the floral scent variation influenced by mating system shifts that are usually mediated by changing environments. Trait variation related to changing environment (especially changes in pollinator and herbivore abundance) differs from other variation related to developmental anomalies under extreme conditions, in its regularity, peculiarity and consistency. Among *A. lyrata* populations and among the investigated wild potatoes, floral scent variation with mating system shifts produced a predictable spectrum of VOC emissions that are exposed to selection due to the benefits of pollinator recruitment and the costs of VOC production. The repeatability and predictability of this variation suggest that the developmental factors guiding VOC production at each clade both within and among species can be easily modified to instigate evolutionary transitions among floral traits. and shifts to an "olfactory selfing syndrome". My results on scent vestigialization therefore also provide some of the strongest evidence to date that scent production (VOC biosynthesis) is physiologically costly, at least over longer evolutionary timescales. Overall, I demonstrate that floral scent emissions within a single species can vary from one another, and that this variation has high predictability. VOC emission varies consistently based on the mating systems and the variation seems to be related to the age of mating system evolution. I observed that the same floral VOCs classes that varies within species also vary among species. Our results suggest that the developmental transitions or trait

variations within a species may predispose that of among species and the among-species variation may potentially result from fixing alternate floral scent emission were in a variable ancestor.

5.4 Callose and cellulose synthase interactions for plant defence

In chapter 4, I demonstrated the need of using super-resolution microscopy to understand the molecular mechanisms of callose and cellulose synthase and the ensuing interactions between the two synthases and callose polymers. I made a significant contribution by describing the interaction of individual callose and cellulose synthase complexes at pathogen attack sites. These findings led to the identification of a previously unidentified interaction mechanisms for callose synthase complexes and the synthesis of the corresponding callose in the apoplast.

5.5 Conclusions

I have shown a clear parallelism in floral sent variation at two different levels of biological organizations. Despite finding a strong positive correspondence between the two levels assessed, many other factors can be possible. Further studies on a direct comparison of floral traits variation across multiple evolutionary scales may be required to establish a general understanding of how sub-specific and sub-individual variation may shape evolutionary trajectories. This thesis focused more on mating system related floral scent variation, but many other forms of seasonal structured variation happen in other plant organs (for example, leaf, fruits, pollinator behaviours). I am convinced that studies linking patterns of variation across various evolutionary scales in plants, with interspecific ecological interactions, and by integrating sub-individual variation (e.g., phenotypic plasticity) into evolutionary assessments will generate vital and novel insights into the drivers of adaptive variation.

5.6 References

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