Consequences of the mating system shift on the evolution of sexual traits in flowering plants

Důsledky změn způsobu reprodukce na evoluci pohlavních znaků krytosemenných rostlin

by

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In memory of my beloved grandmother, "Helima zer"

Sevgili büyükannem Halime İltaş'ın anısına

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Declaration

I hereby declare that this thesis has been composed by myself using the mentioned references and that it has not been submitted elsewhere, in whole or in part, to obtain the same or other academic degree.

Prohlašuji, že jsem tuto práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 06. 11. 2024 Ömer Iltaş

Author contribution statement

I hereby declare that I have contributed to all the papers included in this thesis. My specific contributions to each paper are detailed in the table below.

Contribution	CS1	CS2	CS3	CS4	CS5
Conceptualization	✓	✓	–	–	–
Sample/data collection and preparation	✓	✓	–	–	✓
Experiment	✓	–	–	✓	–
Data analysis and interpretation	✓	✓	✓	✓	–
Writing – original draft	✓	✓	✓	✓	–
Manuscript review/editing	✓	✓	✓	✓	✓
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Abstract

The diversity of reproductive strategies among angiosperms is considered as a key feature behind their success as one of the most species-rich group of land plants. Among them, the transition from outcrossing to selfing appears as one of the significant reproductive strategies altering the evolution of key sexual traits, underlying genes and speciation processes. Although the impact of transition to selfing on reproductive traits is well documented, its consequences on sex-specific selection at the trait and gene level remain unexplored. In this thesis, by combining developmental and comparative -omics approaches, I aimed at investigating the consequences of mating system shift on the evolution of male and female traits, underlying molecular mechanisms, and subsequent impact of this shift on establishing reproductive barriers in flowering plants. I focused on species that have undergone transitions to selfing across the plant model genera *Arabidopsis* and *Capsella*, with transition times ranging from 10k - 600k years old. In case studies 1 and 2 (CS1 and CS2), I investigated how the evolution of sexual traits and underlying molecular mechanism is impacted by the transition to selfing under the theoretical frame of sexual selection. Together with experimental approaches, I used transcriptomic data to identify the molecular mechanism underlying the evolution of the male and female gametophytic function in recently diverged sister lineages. I found that outcrossers, which experience strong sexual selection in nature, exhibited higher pollen performance and stricter female choice compared to selfers (CS1), consistent with predictions from modelling study (CS2). Additionally, I identified candidate genes involved in vesicle transport and cytoskeleton organization associated with pollen trait divergence, as well as genes related to auxin and stress responses that may explain differences in female choice through molecular crosstalk at the post-pollination stage (CS1). These results suggest that the transition to selfing can rapidly alter the intensity of sexual selection between outcrossing and selfing populations, driving the evolution of sexual traits and their associated gene expression patterns. In CS3, by conducting comparative proteomics in independent selfing lineages, I found that the pollen coat morphology evolved similarly in species exhibiting the selfing syndrome, such as *Arabidopsis thaliana* and *Capsella rubella* (CS3). Moreover, I identified differentially expressed pollen coat proteins that were significantly enriched for pathogen response functions in outcrossing species compared to their selfing counterparts across all three independent systems. These results reveal that both morphological and protein-level changes in the pollen coat have occurred similarly across independent transitions to selfing, demonstrating rapid convergent evolution of pollen coat functions related to defence and pathogen response, an aspect that was previously unknown. Finally, by performing reciprocal crosses and contributing to population genomic analyses between outcrossing and selfing *A. lyrata* lineages, I investigated whether the recent transition to selfing impacts hybrid seed lethality and selection on parent-specific imprinted genes (CS4 and CS5). I found that parental genomes in hybrid seeds interact negatively, leading to extensive hybrid seed lethality associated with endosperm cellularization disturbances (CS4). However, the work I contributed to did not detect differences of selection on imprinted genes between selfing and outcrossing populations of *A. lyrata*, suggesting that the transition to selfing has not significantly affected the evolution of these genes (CS5). These results indicate that, despite a relatively short divergence time, the transition to selfing can give rise to strong postzygotic barriers, causing hybrid seed inviability between two intraspecific *A. lyrata* lineages. Overall, my PhD thesis brings a novel empirical contribution to our understanding of how mating system shifts impact the evolution of sexual traits and their underlying molecular and genetic elements, and how they drive the emergence of reproductive barriers and ultimately speciation.

Abstrakt

Diverzita reprodukčních strategií mezi krytosemennými rostlinami je považována za klíčovou vlastnost, která stojí za jejich úspěchem jako jedné z nejvíce druhově bohatých skupin suchozemských rostlin. Mezi těmito strategiemi je přechod od cizosprašnosti k samosprašnosti jednou z významných reprodukčních strategií, která ovlivňuje evoluci klíčových pohlavních znaků, souvisejících genů a procesů speciace. Ačkoli je dopad přechodu k samosprašnosti na reprodukční znaky dobře zdokumentován, jeho důsledky pro pohlavně specifický výběr znaků a genů zůstávají neprozkoumány. V této disertační práci jsem kombinací vývojových a komparativních -omických přístupů zkoumal důsledky změny rozmnožovacího systému na evoluci samčích a samičích znaků a za nimi stojících molekulárních mechanismů a následný dopad této změny na vznik reprodukčních bariér u krytosemenných rostlin. Zaměřil jsem se na druhy, které přešly k samosprašnosti v rámci modelových rodů *Arabidopsis* a *Capsella* v období před 10 tisíci až 600 tisíci lety. V případových studiích 1 a 2 (CS1 a CS2) jsem zkoumal, jak je evoluce pohlavních znaků a za nimi stojících molekulárních mechanismů ovlivněna přechodem k samosprašnosti v kontextu pohlavního výběru. Spolu s experimentálními přístupy jsem použil transkriptomická data k identifikaci molekulárních mechanismů, které stojí za evolucí funkce samčího a samičího gametofytu u nedávno rozdělených sesterských linií. Zjistil jsem, že cizosprašné druhy, které jsou v přírodě pod silným pohlavním výběrem, mají vyšší úspěšnost pylu a větší selektivitu ze strany samic ve srovnání se samosprašnými druhy (CS1), což je v souladu s predikcemi modelové studie (CS2). Navíc jsem identifikoval kandidátní geny zapojené do vezikulárního transportu váčků a organizace cytoskeletu, které jsou spojeny s divergencí pylových znaků, stejně jako geny související s auxinem a stresovými reakcemi, které mohou vysvětlovat rozdíly v samičím výběru prostřednictvím molekulárních interakcí ve fázi po opylení (CS1). Tyto výsledky naznačují, že přechod k samosprašnosti může rapidně změnit intenzitu pohlavního výběru mezi cizosprašnými a samosprašnými populacemi, což vede k evoluci pohlavních znaků a s nimi souvisejících vzorců genové exprese. V CS3 jsem pomocí komparativní proteomiky u nezávislých samosprašných linií zjistil, že morfologie pylového obalu se vyvíjela podobně u druhů, které vykazují syndrom samosprašnosti, jako jsou *Arabidopsis thaliana* a *Capsella rubella* (CS3). Navíc jsem identifikoval odlišně exprimované proteiny pylového obalu, které byly významně nabohaceny na funkce spojené s odpovědí na patogeny u cizosprašných druhů ve srovnání s jejich samosprašnými protějšky ve všech třech nezávislých systémech. Tyto výsledky ukazují, že jak morfologické, tak proteinové změny v pylovém obalu proběhly podobně napříč nezávislými přechody k samosprašnosti, což dokazuje rychlou konvergentní evoluci funkcí pylového obalu související s obranou a odpovědí na patogeny, což nebylo dosud popsáno. Nakonec jsem pomocí reciprokých křížení a příspěvkem k populačním genomickým analýzám mezi cizosprašnými a samosprašnými liniemi *A. lyrata* zkoumal, zda recentní přechod k samosprašnosti ovlivňuje letalitu hybridních semen a selekci na imprinting genů (CS4 a CS5). Zjistil jsem, že rodičovské genomy v hybridních semenech negativně interagují, což vede k rozsáhlé letalitě hybridních semen spojené s narušením buněčné organizace endospermu (CS4). Nicméně práce, na níž jsem se podílel, neodhalila rozdíly v selekci na imprintované geny mezi samosprašnými a cizosprašnými populacemi *A. lyrata*, což naznačuje, že přechod k samosprašnosti významně neovlivnil evoluci těchto genů (CS5). Tyto výsledky naznačují, že navzdory relativně krátké době divergence může přechod k samosprašnosti vést ke vzniku silných postzygotických bariér, které způsobují neschopnost hybridních semen z intraspecifických linií *A.*

lyrata přežít. Celkově moje disertační práce přináší nový empirický příspěvek k našemu chápání toho, jak změny v reprodukčním systému ovlivňují evoluci pohlavních znaků a za nimi stojících molekulárních a genetických mechanismů, a jak řídí vznik reprodukčních bariér a nakonec i speciaci.

Part I – General chapters

1. The transition from outcrossing to selfing, one of many mating strategies in flowering plants

Angiosperms (flowering plants) so far appears as one of the most species rich groups of land plants. This exceptional dominancy of angiosperms has also been an element among Darwin's notes as the "abominable mystery" (Francis & Seward, 1903). A key to this mystery lies in the extraordinary diversity of mating patterns and sexual systems to promote reproductive success among angiosperms. Their mode of reproduction (from asexual like apomixis or vegetative clonal propagation to sexual reproduction), allocation of resources between male and female functions (from hermaphrodites with bisexual flowers to unisexual individuals), and mating strategies (from outcrossing to selfing) can vary significantly (Barrett, 2002, 2010). This diversity of reproductive strategies has long captured the attention of evolutionary biologists and remains a core interest in understanding process of genome, phenotype and population evolution. Among these strategies, research on mating systems shift, particularly the transition from outcrossing to selfing, has flourished in recent years due to its potential impact on altering population structure, selective processes, genome evolution, reproductive traits evolution, and speciation processes (Cutter, 2019; Tsuchimatsu & Fujii, 2022; Wright et al., 2008). Outcrossing, or cross-pollination, appears as one of the earlier ancestral and predominant mating strategies in flowering plants (angiosperms), with estimates ranging from ~40–65% (Barrett, 2002; Igic et al., 2008). Despite this high frequency, transitions to self-fertilization from ancestral outcrossing species have frequently occurred within lineages (Igic et al., 2008; Stebbins, 1974). The shift from outcrossing to selfing is typically viewed as a derived condition and has independently occurred multiple times in different lineages. Recently approximately 20% of flowering plants known to use selfing as mating strategy (Barrett, 2002; Stebbins, 1974).

This widespread evolutionary shift often has important consequences for plant reproduction at both the phenotypic and genotypic levels over time (Tsuchimatsu & Fujii, 2022; Wright et al., 2013). Self-fertilization is as an assurance strategy to overcome mating limitations when the number of compatible mates is scarce due to low population densities (Kennedy & Elle, 2008). Initially, such a transition can be advantageous because of reproductive assurance under the pollinator scarcity and transmission advantage of allele to the offspring (Darwin, 1877; Kennedy & Elle, 2008). Despite the short-term benefits, predominant selfing is often considered as an evolutionary dead end (Stebbins, 1957). This can be explained through the challenges and trade-off selfing populations experience during the long-term evolution. Transition to selfing decreases the genetic diversity within populations because individuals are mating with themselves (Cutter, 2019). Consequently, it leads to increased homozygosity among selfing population and reduces the effectiveness of recombination, thereby elevating linkage disequilibrium (Nordborg, 1997; Wright et al., 2013). Moreover, due to reduced number of random gamete samplings, selfing leads to reduction in effective population size (Barrett et al., 2014; Wright et al., 2008). This immediate reduction in effective population size mitigates the beneficial effects of purging (Glémin, 2007) and render selection less efficient in selfers, particularly against weakly deleterious mutations (Wright et al., 2013). Consequently, the transition to selfing reduces the efficacy of selection and leads to accumulation of deleterious mutation (Glémin, 2007). This assumption has been evidenced in literature by showing the higher accumulation of deleterious

mutations in pollen genes in predominantly selfing *Arabidopsis thaliana* (Harrison et al., 2019), and as well as in *Eichhornia paniculata* (Arunkumar et al., 2015). Despite the profound genomic consequences, in most cases, transition to selfing is accompanied by similar drastic changes in the morphology and function of floral traits (known as 'selfing syndrome' (Sicard & Lenhard, 2011a). These traits include a reduction in floral size, loss of odor or nectar production, limited pollen production, reduced pollen-to-ovule ratios, and reduced herkogamy (Sicard & Lenhard, 2011a; Tsuchimatsu & Fujii, 2022). These changes appear to be similar among independent selfing lineages, suggesting the convergent evolution of the selfing syndrome traits across different taxa following the transition to selfing (Tsuchimatsu & Fujii, 2022; Woźniak et al., 2020). Although impact of transition to selfing on reproductive traits is well documented, its consequences on sex-specific of male and female function remained to be elucidated. Moreover, the impact of mating system on the evolution of sexual traits important for sexual selection remains incomplete due to the confounding factor arise from selfing syndrome effect.

2. Mating system shift and male and female gametophyte evolution

Multiple mating is prevalent in flowering plants (Pannell & Labouche, 2013), with several pollen donors competing to fertilize ovules. This probably influences both the mechanisms and strength of the selective forces driving genome and sexual traits evolution, through sexual selection. Sexual selection in plants is commonly accepted on theoretical ground, but studies focusing on this question, especially at the molecular and genetic levels, are scarce (Moore & Pannell, 2011). As explained above, transition from outcrossing to selfing, is well-known to alter population genetic structure and selective processes (Cutter, 2019; Tsuchimatsu & Fujii, 2022), and likely to influence how sexual selection works as well as its intensity. Indeed, it has been shown that outcrossing behaviour in plants is associated with higher pollen competition ability compared to selfing (Mazer et al., 2018a), suggesting divergent sexual selection between outcrossing and selfing populations. Therefore, differences arising from mating system shift is likely to provide an opportunity to study components of sexual selection comparatively. Besides sexual selection, outcrossing also includes other selective forces on sexual traits and genes, such as natural selection for pollen grain survival, driving sex-specific adaptations that enhance resilience to environmental stresses and pathogens, ensuring reproductive success under challenging conditions.

2.1. Sexual selection

The origins of sexual selection theory trace back to Charles Darwin and his groundbreaking voyage to the Galapagos Islands in September 1835. During his trip, Darwin observed the prevalence of conspicuous male secondary sexual traits, which seemed to contradict his theory of natural selection due to the detrimental effects these traits could have on survival. However, he proposed that these flamboyant traits might confer a reproductive advantage by increasing mating opportunities, potentially offsetting their negative impact on survival. This idea was later expanded into the concept of sexual selection, explaining how exaggerated sexual traits positively impact an individual's mating success (Darwin, 1871). Despite its contentious definition history, sexual selection can be succinctly defined as the non-random success in competition for access to gametes for fertilization (Alonzo & Servedio, 2019a; Shuker & Kvarnemo, 2021).

Sexual selection theory encompasses and elucidates two selective forces:1/ intra-sexual selection, which involves competition among members of one sex, typically males, for the chance to fertilize the gametes of the other sex; and 2/ intersexual selection, which involves the selection of outperformed mates by members of the opposite sex, predominantly females. In the past century, this concept has been broadened, mostly in relation to animals, and much experimental evidence has been published to explain prevalence of sexual selection associated with intra-sexual selection (Emlen et al., 2005; Hagelin, 2002) and intersexual selection (Birkhead, 1998; Eberhard, 1996). Darwin's (1871) exclusive focus on sexual selection in animals excluded plants from the scope of his theory, delaying the recognition of sexual selection in plants until the late 1970s (Janzen, 1977; Queller, 1983; Willson, 1979).

In theory, the operation of sexual selection is widely believed to be rooted in the evolution of differential gamete investment, or anisogamy between sexes, where usually male gametes are produced in much higher number than female ones (formulated as 'Bateman's principle' (Bateman, 1948)). According to this principle, male reproductive success is limited by the number of mating events, while female reproductive success is limited by resource availability. When applied to plants, it is now well known that the male and female functions evolve separately with their own tissue, genes, and selective interests (pollen: Da Costa-Nunes & Grossniklaus, 2004, gynoecium: Kivivirta et al., 2020, even in the case of hermaphroditism. Moreover, differential gamete investment, and anisogamy between pollen and ovule is also persistent in plants, and often skewed towards pollen (Gong & Huang, 2014). Therefore, male reproductive success depends on ovule availability, leading to competition among conspecific pollen grains for fertilization, in line with Bateman's principles of female choice.

Nowadays, the operation of sexual selection in plants has become widely accepted, and many researchers have invested considerable effort in studying it. There is now substantial evidence showing that sexual selection operates in plants, both before and after pollination, in ways similar to those observed in the animal kingdom (Lankinen & Green, 2015). Because it provides the opportunity for males to interact with each other in accessing pollinator bodies (Cocucci et al., 2014), the pre-pollination phase can be considered equivalent to pre-mating sexual selection in animals. Sexual selection can also operate after (-post) pollination, when pollen from different donors arrived on recipient stigma and competes to access ovules (intra-sexual selection) under female selective pressures (intersexual selection), which is equivalent to post-copulatory selection in animals (Moore & Pannell, 2011; Queller, 1983; Tonnabel et al., 2021). However, despite the possible role of sexual selection in the pre-pollination phase, most of the studies on sexual selection in plants focus on post-pollination process.

The transition from outcrossing to selfing is likely to influence how sexual selection works. Indeed, the number of genetically distinct mating partners is usually high in outcrossing species. In contrast, selfing species tend to evolve over time to produce identical pollen and female gametophytes within the same plant, potentially eliminating competition among conspecific pollen grains for fertilization, as well as female choice. This striking differences between outcrossing and selfing species set a solid ground for divergent selection pressure between selfers and outcrossers and to test the evolution of reproductive traits impacted by sexual selection. However, reduction or loss of floral traits arising with transition to selfing, so-called the "selfing syndrome", are not always directly connected with sexual

selection (Sicard & Lenhard, 2011a), which brings a confounding factor when studying the effect of sexual selection on sexual traits evolution. Moreover, such evidence remained to be scarce, and mainly tested on the same biological system, *Clarkia* species (Kerwin & Smith-Huerta, 2000; Smith-Huerta, 1996). Therefore, our understanding of the consequences of the mating system shifts on traits under intra- and intersexual selection is still relatively limited. In the next chapter, I will introduce the components of sexual selection that operate at post-pollination stages and provide examples of pollen and pistil traits that are likely subject to both intra- and intersexual selection, particularly in plant genera that show divergence between outcrossing and selfing species. As the molecular basis of sexual selection in plants is largely unresolved, I also describe in the next section the molecular mechanisms underlying pollen and pistil development, potential candidates under sexual selection.

2.1.1. Intrasexual selection

During pollination, stigmas often receive more pollen than available ovules, and the pollen grains are often from genetically distinct donors (Pannell & Labouche, 2013), allowing the possibility of mating with multiple males within a single reproductive cycle. The number of distinct mating partners and the asymmetry between number of ovules and pollen grains often restricts reproduction for males and intensifies competition for fertilization (Lankinen & Skogsmyr, 2002). The siring success of a given pollen grain is likely underlain by traits that provide an advantage over other pollen grains under intrasexual selection (Lankinen & Skogsmyr, 2002; Marshall et al., 1996a; Mazer et al., 2018b). These traits, known as pollen performance traits, include pollen germination rate (Austerlitz et al., 2012), pollen tube growth (Lankinen & Skogsmyr, 2002; Pasonen et al., 1999; Snow & Spira, 1991; Tonnabel et al., 2022), and the inhibition of competitor pollen grains (Marshall et al., 1996a; Swanson et al., 2016). The comparison of sister lineages with different mating systems provides great opportunity to test the effect of intrasexual selection on the evolution of pollen performance traits. Consistently with the expectation that sexual selection is relaxed in selfing lineages, several studies have shown evidence that pollen tubes of outcrossing species tend to grow faster than those of selfing species (Hove & Mazer, 2013; Mazer et al., 2018b; Smith-Huerta, 1996; Taylor & Williams, 2012). The increased tube growth of outcrossing pollen is consistent with strong positive selection acting on pollen genes in outcrossing species such as *Capsella grandiflora* (Arunkumar et al., 2013; Gutiérrez-Valencia et al., 2022), while deleterious mutations appear to accumulate in pollen genes for the selfing species *A. thaliana* (Harrison et al., 2019). The growth of pollen tube growth is mostly heterotrophic and extensively depends on intrinsic pathways, such as vesicle transport, Ca^{2+} signalling and cytoskeleton movement, cell wall modification, but also on extrinsic factors, i.e. nutrients and signalling peptides secreted by female tissues (Cascallares et al., 2020; Scholz et al., 2020). Intrinsic factors may provide an advantage in male-male competition at earlier stages, while extrinsic factors may offer an advantage when interacting with the chemical composition of the pistil through female choice mechanisms (reviewed in Tonnabel et al., 2021). The development of pollen germination and pollen tube growth is governed by the expression of specific genes, and knowledge of these genes has been extensively documented through molecular studies (Guan et al., 2013; Krichevsky et al., 2007). Although these studies provide valuable insights into the molecular basis of pollen germination and pollen tube growth, it remains unknown which of these molecular mechanisms are influenced by sexual selection.

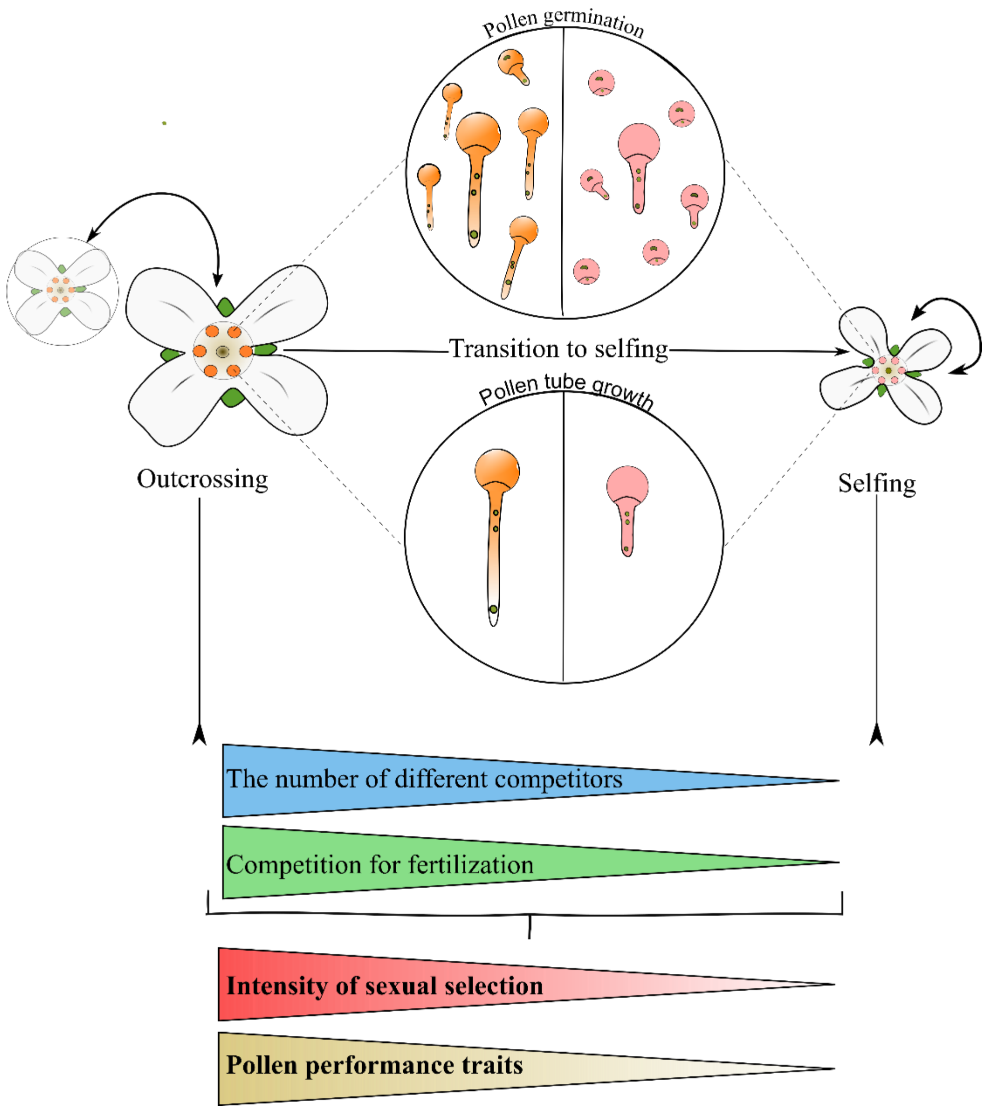

Figure 2: Mating system shift and pollen performance traits evolution. The transition to selfing reduces the number of different mating partners and the competition for fertilization, which in turn weaken the intensity of sexual selection on traits related to male-male competition (intrasexual). Traits important for male-male competition include pollen germination and pollen tube growth. Color ramps indicate a decrease in the given term.

2.1.2. Intersexual selection

Female choice (intersexual selection) in postpollination phase can exert a role in two ways, either by indirect sorting via pistil traits (such as delayed stigma receptivity (Galen et al., 1986; Lankinen & Madjidian, 2011a), style length (Lankinen & Skogsmyr, 2001; Ramesha et al., 2011; Ruane, 2009), size of the stigmatic surface (Tonnabel et al., 2022), or directly by the molecular crosstalk between pollen and stylar tissues (Dresselhaus & Franklin-Tong, 2013; Pereira et al., 2021). Following pollen germination, the style, a narrow tube structure connecting stigma and ovary, guides the pollen tube towards to the ovules, and likely serves as an important pistil trait for females to select pollen grains with faster pollen tube growth (Mazer et al., 2010a; Ramesha et al., 2011). This may happen indirectly,

with a longer distance allowing clearer differences of tube growth speed between pollen grains (Lankinen & Skogsmyr, 2001; Ramesha et al., 2011). The correlation between style length and pollen tube attrition rate (the number of pollen tubes enter the style but fail to reach ovary) has been shown in previous studies (Smith-Huerta, 1997). Moreover, studies showed that the styles of outcrossing species are significantly longer than those of related selfing species, providing evidence, yet indirect, of style length being under sexual selection (Ramesha et al., 2011; Runions & Geber, 2000). Style development in flowers is regulated by multiple phytohormones (auxin, cytokinin, and brassinosteroids) and transcription factors, which dictate the initial patterning of the gynoecium and transition it from bilateral to radial symmetry. For example, the *NGATHA* (NGA) transcription factors positively regulate the expression of three angiosperm-specific genes (SSS; *STIGMA AND STYLE STYLIST 1–3*), which in turn repress cell elongation and proliferation of the style in *Arabidopsis* (W. Li et al., 2020). In primrose, style length is controlled by the gene *CYP734A50*, which is involved in the spatial accumulation of brassinosteroids and regulates style length (Huu et al., 2016).

In addition, as explained above, pollen tube growth in the style is highly dependent on extrinsic factors, such as signalling peptides or nutrients secreted from the stylar tissue (Higashiyama & Takeuchi, 2015; Higashiyama & Yang, 2017). Through this molecular interaction, females can exert selective power during fertilization by promoting or inhibiting certain pollen grains. The research focused on the molecular mechanisms underlying the interactions between pollen tube growth and pistil tissue following pollination has revealed that the pollen tube communicates with the stylar tissue during its journey toward the ovules, a process known as pre-ovular guidance (Chae et al., 2007a; Dresselhaus & Franklin-Tong, 2013; Mollet et al., 2000). These pollen-pistil interactions may allow the female gametophyte to exert selection on specific pollen grains (Pannell & Labouche, 2013). Similar aspect of this proposal is well documented in animals, showing the female choice of specific sperm traits (namely cryptic female choice; (Eberhard, 1996). However, although the molecular mechanisms involved in pollen-pistil interactions are well documented, it remains to be determined whether these mechanisms play a role in female choice of conspecific mating partners in natural populations.

In general, the genes under sexual selection in plants are poorly understood. The development of male and female traits relies on ubiquitous cellular and molecular mechanisms (pollen; (Wang et al., 2010); style; (Zúñiga-Mayo et al., 2019). Therefore, genes involved in such a process are likely to have a pleiotropic effect. In this context, mutations occurring in the coding region of a gene are likely to have deleterious pleiotropic effect and therefore to remain out of scope for sexual selection. In contrast, pleiotropy might be alleviated by mutations occurring in cis-regulatory elements, which can restrict the mutational effect to cell type-specific gene expression, thereby reducing the pleiotropy. In line with this concept, Sicard et al. (2016) demonstrated that a mutation in a tissue-specific regulatory element of a gene controlling the level of STERILE APETALA (SAP) protein led to a reduction in petal size in an organ-specific manner following the transition to selfing in *Capsella rubella*. If a mutation in the cis-regulatory elements of a gene responsible for sexually selected traits enhances reproductive success, then sexual selection may promote its spread. As a result, sexual selection may have larger room to act on tissue-specific gene expression levels rather than on the coding sequence of these genes. However, this assumption remains to be tested.

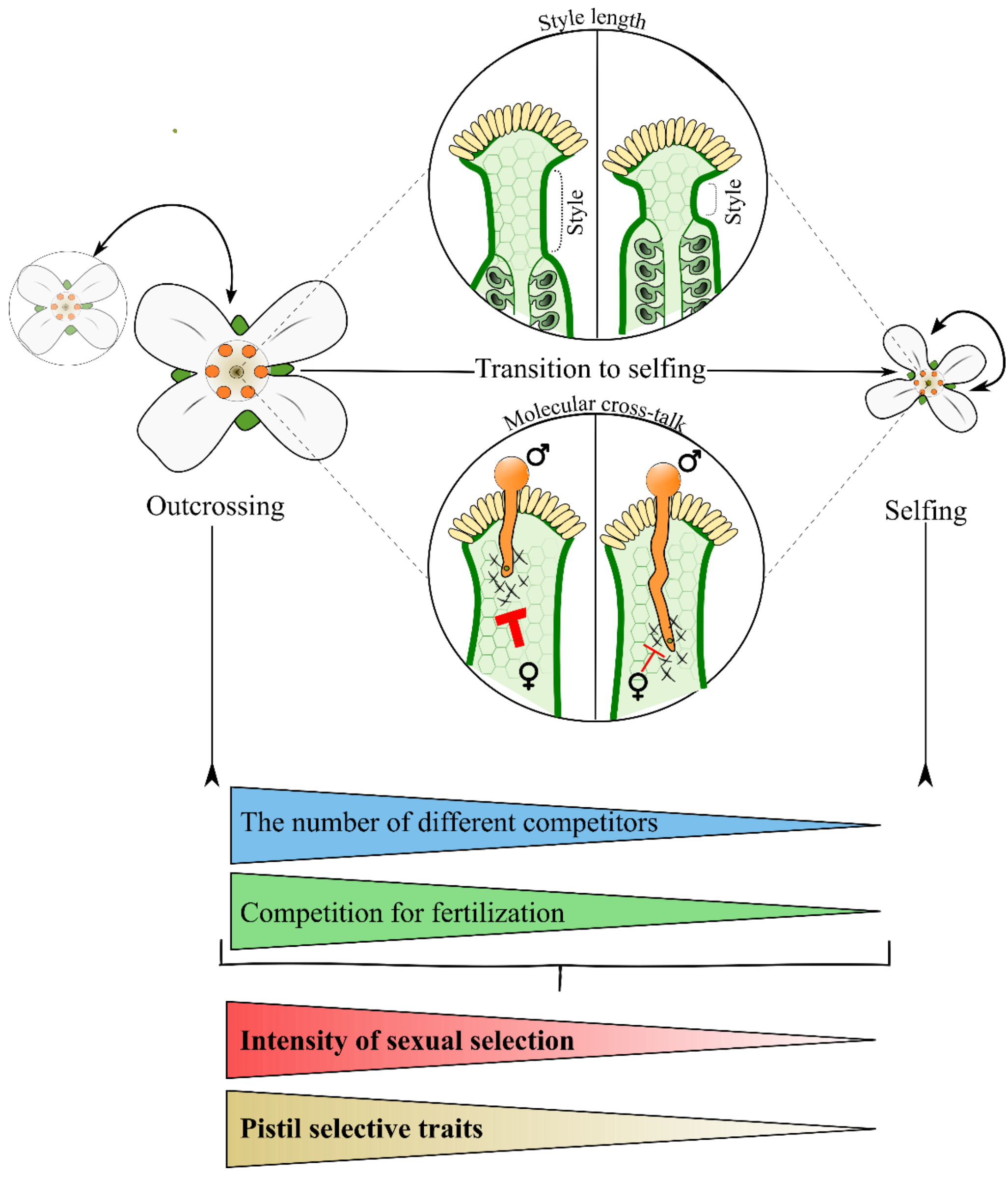

Figure 2: Mating system shift and pistil selective traits evolution. The transition to selfing reduces the number of different mating partners and the competition for fertilization, which in turn weaken the intensity of inter-sexual selection on traits related to female mate choice (intersexual). Traits important for female mate choice includes style length and molecular crosstalk sorting abilities that which can hinder pollen tube growth. Red block bars in the molecular crosstalk part represent the strength of female influence on pollen tube growth from outcrosser female tissue. The size of the block bar correlated with the strength of female influence, the bigger the stronger. Color ramps indicate a decrease in the given term.

2.2 Male gametophyte-specific selection

Besides sexual selection, other selective forces act on the male and female gametophytes. On the male side, which is the focus of this work, the journey of pollen grains from the donor plant to the recipient stigma includes several stresses and challenges. These challenges include water loss, UV radiation, herbivores, and pathogens, as well as adhesion to and visibility by pollinators, and the preservation of proteins necessary for pollen-pistil interactions (Pacini & Hesse, 2005; Rivest & Forrest, 2020). A major structure coping with these challenges is the pollen coat (Pacini & Hesse, 2005). It is derived from the tapetum and is the outermost layer of pollen grains. It consists of secretory elements and a complex mixture of proteins, lipids, and pigments (Rejón, et al., 2016). In outcrossing taxa, the role of the pollen coat in the survival of pollen grains and their successful transfer to the recipient stigma is likely to be crucial. Reproduction is assured in selfing species, thus pollinator-assisted pollen transfer is no longer needed (Kalisz et al., 2004). Also, as the duration and distance of the travel between pollen and stigma is strongly reduced in selfing species, protection against UV, dehydration or pathogens is likely less needed than in outcrossers. Therefore, the importance of the pollen coat may be reduced in selfing compared to outcrossing lineages. Although the impact of mating system shifts on reproductive traits has been extensively studied, it remains to be determined whether the transition to selfing affects pollen coat morphology and molecular composition.

3. Mating system shift and reproductive barrier

Speciation is the evolutionary process in which reproductive isolation leads populations of a single species to diverge into distinct species (Dobzhansky, 1937; Mayr, 1982). Reproductive isolation is fundamental to the speciation process and can arise in two forms: pre-zygotic isolation, which occurs before fertilization, and post-zygotic isolation, which occurs after fertilization (Wang & Filatov, 2023; Widmer et al., 2009). Postzygotic barriers often result in the formation of hybrids with reduced viability or fertility, which are typically associated with genetic incompatibilities (Schluter, 2001). In plants reproductive barriers are sometimes asymmetric (highly depend on the direction of cross; (Lowry et al., 2008). This is the case for example of hybrid seed lethality (development failure of hybrid seeds), which displays different defects and viability rates depending on the cross directions (Garner et al., 2016; Lafon-Placette et al., 2017). This asymmetry may arise from incompatibilities between genes with parent-of-origin specific expression, namely imprinted genes, which may evolve under parental conflict over resource allocation to the progeny (Rodrigues & Zilberman, 2015). According to this idea, the maternal parent is the main contributor of resources to the progeny, and the maternal parent is equally genetically related to each of its offspring. In this context, maternal interests are likely to favor equal resource allocation among all offspring (Brandvain & Haig, 2005; Smith & Fretwell, 1974). In contrast, paternal contributors provide no resources to the offspring and thus their inclusive fitness may be maximized if they can drive more maternal resources to their own offspring to the detriment of others, leading to an evolutionary conflict between paternal and maternal interests (Haig & Westoby, 1989). The endosperm, a biparental tissue nourishing the embryo, appears as a crucial place for antagonistic interaction between the maternal and paternal genome for resource

allocation. These parent-specific selective interests are thought to drive the evolution of genomic imprinting, which depends on parent-specific gene expression in the endosperm (Haig, 2004).

Parental conflict, driven by competition among different pollen donors for maternal resources, is expected to be stronger in outcrossing species, where seeds are often sired by multiple fathers (Queller, 1983). In predominantly selfing species, however, most of the seeds are sired by genetically identical paternal and maternal genomes, reducing this conflict. Consequently, the degree of parental conflict is closely correlated with the level of outcrossing within a species. The differences in the intensity of parental conflict between outcrossing and selfing species have been conceptualized as the "Weak Inbreeder/Strong Outbreeder" hypothesis (Brandvain & Haig, 2005; Lafon-Placette & Köhler, 2016). According to this hypothesis, in outcrossing species, paternal and maternal genomes are selected to have an increased influence on endosperm development and resource accumulation in an arms race scenario. Conversely, in selfing species, this arms race does not happen as parental conflict is relaxed. As a consequence, when a cross occurs between an outcrossing and a selfing species, the outcrossing parental genome is expected to "overpower" the selfing one in terms of influence on resource allocation over the respective progeny. The outcome of this imbalanced interaction in the endosperm can lead to development defects such as abnormal seed size and ultimately may result in the death of the hybrid seed (Garner et al., 2016; Lafon-Placette et al., 2018; Willi, 2013), contributing to reproductive isolation between the two species. However, this hypothesis has mostly been tested either in systems where species are well-separated (Brandvain & Haig, 2005; Rebernig et al., 2015), or in closely related intraspecific populations (Raunsgard et al., 2018; Willi, 2013). It remains unclear whether the transition to selfing affects hybrid seed inviability between recently diverged lineages.

II. Aims of my PhD thesis

In my PhD thesis, I aimed to investigate 1) the consequences of mating system shift on the evolution of sexual traits, underlying genes, proteins, and 2) subsequent impact of this shift on establishing post-zygotic reproductive barriers in flowering plants. I used the transition from outcrossing to selfing as a tool to leverage divergent selection pressures. Within this framework, I followed an integrative approach that linked evolutionary processes with developmental and molecular mechanisms, using a range of methods from wet-lab experiments and microscopy to transcriptomics and proteomics.

The specific objectives of my PhD thesis was as following:

1. **Impact of mating system shift on sexually selected traits and genes**
    - 1.1. What is the consequence of a recent transition from outcrossing to selfing on pollen and pistil traits important for sexual selection? (**CS1**)
    - 1.2. What are the genes behind these traits and their divergence in outcrossers vs. selfers? (**CS1**)
    - 1.3. Is coevolution between male and female traits under sexual selection possible in plants? How can a shift in the mating system provide a basis for testing the coevolution of male and female traits? (**CS2**)

2. **Consequences of mating system shift on pollen morphology and protein profiles**
   2.1. Does the transition to selfing impact the evolution of pollen morphology and proteins in independent lineages? (**CS3**)
   2.2. What is the evolutionary timeline for pollen morphology and protein evolution across independent transitions to selfing? (**CS3**)

3. **Impact of mating system shift on hybrid seed lethality and genomic imprinting**
   3.1. What is the consequence of a recent transition to selfing on hybrid seed lethality? (**CS4**)
   3.2. Do parent-specific imprinted genes show footprint of selection in outcrossing versus selfing *A. lyrata*? (**CS5**)

III. Comments on material and methods

The primary focus of my PhD project is to demonstrate how transitions from outcrossing to selfing influence the evolution of sexual traits, including their underlying genes and proteins, and effect on intraspecific hybridization barriers. I used natural populations of outcrossing and selfing Brassicaceae species, particularly *Arabidopsis* and *Capsella*, and tested for parallel sexual evolution in comparative systems. I adopted an integrative approach to connect evolutionary processes with developmental and molecular mechanisms. To achieve this, I employed a variety of methods, including modelling, wet-lab experiments, microscopy, transcriptomics, and proteomics.

3.1. Model systems

In my PhD project, I used *Arabidopsis lyrata* as a main model system in each of my case studies (**CS1, CS3, CS4 and CS5**). *A. lyrata* is a perennial diploid plant species distributed across Eastern Asia, Europe, and Northwestern America, with populations varying in habitat preference, mating system, and environmental adaptation. European populations (*A. lyrata* ssp. *petraea*) typically grow in large populations on open habitats such as lakeshores, gypsum rock outcrops, scree slopes, cliffs, and boulders (Jonsell et al., 1995; O'Kane & Al-Shehbaz, 1997), and in semi-shaded areas (Hemp A, 1996). Northwestern American populations (*A. lyrata* subsp. *lyrata*) are found on sand dunes or rocky outcrops with some natural disturbances (Heblack et al., 2024).

There are several important reasons why I mainly used *A. lyrata* as a model system for my PhD project. First, it offers significant genetic diversity within two closely related recognized subspecies: *A. lyrata* subsp. *petraea* in Eurasia and *A. lyrata* ssp. *lyrata* in North America (Figure 3B, Ross-Ibarra et al., 2008; Schmickl et al., 2010). The North American populations are believed to have arisen from a colonization event from Europe around 35,000 years ago, accompanied by a strong bottleneck (Ross-Ibarra et al., 2008). As a result, North American populations are genetically less diverse than their European counterparts (Schmickl et al., 2010). Second, *A. lyrata* exhibits divergent mating systems within the same species, having both selfing and outcrossing populations. While European populations are exclusively outcrossing, North American populations are predominantly self-incompatible.

However, after the colonization event, predominantly selfing populations with a loss of self-incompatibility system emerged multiple times in the Great Lakes region. Third, North American selfing populations show no signature of reduction in floral traits typically associated with the selfing syndrome (Carleial et al., 2017). Because the breakdown of self-incompatibility in North American populations is a recent event, occurring approximately 10,000 years ago (Foxe et al., 2010a; Willi & Määttänen, 2010), the selfing syndrome is not apparent yet. This recent transition to selfing allowed me to study the effect of sexual selection on sexual trait evolution without confounding factors arising from selfing syndrome. Finally, *A. lyrata* provides a powerful model for investigating the genetic basis of evolutionary changes. It genome has already been assembled (Hu et al., 2011), and genome annotation is well characterized using RNAseq data (Rawat et al., 2015).

In CS3, I used additional systems where the transition to selfing represented by different age of transition (intermediate and old) (Figure 3B). Using an array of outcrossing and selfing species with different ages of selfing transitions allow me to have a range of divergence between lineages to assess how fast male gametophyte specific selection can evolve and how well male-specific trait and underlying protein is conserved/divergent in closely related systems, which is virtually unknown. In this context, I used multiple arrays of the model systems that consisting of recent, intermediate and older independent selfing lineages with different ages of selfing transition from *Arabidopsis* and *Capsella* genera (**CS3,** Figure 3). The Brassicaceae family represents an excellent system to study the effects of the transition to self-fertilization on population genomic variation, molecular evolution, and sexual trait evolution (Vekemans et al., 2014). For intermediate transition to selfing, I used contrasting mating system from *Capsella* genus; outcrossing *Capsella grandiflora* versus selfing *Capsella rubella*. *Capsella grandiflora* is a diploid outcrossing and only outcrossing (self-incompatible) species in the *Capsella* genus species distributed across Albania, western Greece, and Northern Italy (Neuffer et al., 2014). *Capsella rubella* is a diploid self-compatible (SC) and the breakdown of self-incompatibility from its outcrossing ancestor and subsequent split from its sister outcrossing species *C. grandiflora* occurred approximately 100,000–200,000 years ago (Guo et al., 2009). For old transition to selfing case, I used contrasting mating system set of species from *Arabidopsis* genus, including outcrossing *Arabidopsis arenosa* and selfing *Arabidopsis thaliana*. *A.thaliana* is an annual obligate self-compatible species to native to Europe, Asia, and Africa. It is a highly self-compatible plant that is separated from the other *Arabidopsis* species by 6 My (Novikova et al., 2016) . It has been reported that the loss of self-incompatibility in *Arabidopsis thaliana* occurred 600, 000 years ago (Strütt et al., 2023)(Figure 3B). Due to a high rate of selfing, *A. thaliana* flowers display a strong reduction in flower phenotypes as predicted by selfing syndrome. *Arabidopsis arenosa* is an obligate outcrossing species with an annual, biennial, or short-lived perennial life cycle that widely occurs throughout Europe (Al-Shehbaz & O’Kane, 2002).

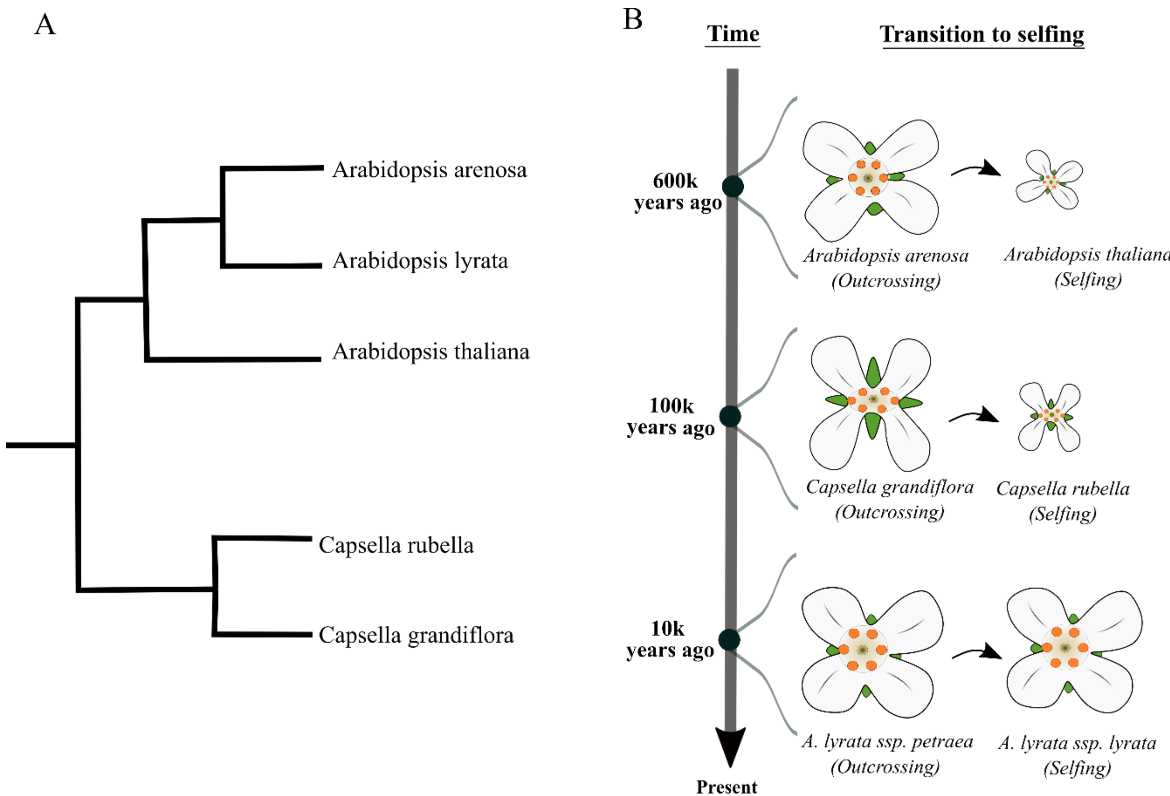

Figure 3. Illustration of model systems used in my PhD project. A) Schematic drawings of phylogenetic relationships among *Arabidopsis* and *Capsella* species used. B) Evolutionary timescale of transition to selfing in *Arabidopsis thaliana, Capsella rubella* and *A.lyrata ssp. lyrata.* Size of the flowers correlate with characteristic flower phenotypes associated with the selfing syndrome. All of the species used in this PhD project are diploid.

3.2. Developmental approaches to detect variation in sexual traits

Evolutionary developmental (Evo-Devo) research has gained significant interest in recent years as scientists seek to understand how developmental changes within individuals over a single generation are connected to evolutionary changes over multiple generations. Microscopy techniques have been a crucial part of Evo-Devo research, and advancements in these techniques have allowed scientists to uncover deeper evolutionary insights on trait development by examining how differences in growth rate and cell-type proliferation give rise to features in organisms. In my PhD project, I performed *in vitro/vivo* experiments to detect fine details of sexual traits development using various microscopy techniques, followed by measuring the variation in these traits between selfing and outcrossing populations in each of my case studies.

Previous studies considering the consequence of the mating system shift on the evolution of pollen performance traits, often take a one-sided approach to study their evolution under sexual selection.

Most studies have remained at the prediction level (Lankinen and Green, 2015; Cutter, 2019), often focusing on the same biological system, primarily *Clarkia* species (Mazer et al., 2018) and evaluating pollen performance using either *in vitro* or *in vivo* methods separately. Therefore, it remains unclear to what extent the observed male performance traits are shaped by interactions with female tissues versus intrinsic abilities alone, whether these traits vary between selfing and outcrossing populations of the same species, and how quickly these traits can evolve. To address this gap, I first tested my hypothesis through mathematical predictions to evaluate how quickly pollen performance traits could evolve to reach their optimal levels in outcrossers, potentially driving rapid phenotypic divergence between outcrosser and selfer (**CS2**). Then, I used a combination of *in vitro* (nonexistence of female influence) and *in vivo* (existence of female influence) pollen germination experiments. Through *in vitro* pollen germination assays, I investigated the inherent abilities of pollen donors under the different pollen concentrations and different time points to account for impact of pollen concentration (chemical interaction among pollen grains) on pollen performance abilities. Through *in vivo* (reciprocal crosses) pollen germination, I investigated the how pollen intrinsic abilities influenced by the existence of female tissues, which resemble the natural conditions (**CS1**). In this context, I brought comprehensive experimental evidence on disentangle the intrinsic pollen performance abilities, from its interaction with other pollens, and as well its interaction with the female partner at different time points. Such comprehensive approach of combining *in vitro* and *in vivo* pollen developmental using microscopy rarely used to understand the evolution of pollen performance traits impacted by intra-inter sexual selection, and this has been comprehensively addressed in my PhD project for the first time.

Next, to better understand the evolutionary background of phenotypical variation in pollen coat morphology affected by the transition to selfing, I used transmission electron microscopy (TEM) to investigate differences in the internal structure of pollen grains (pollen coat) between selfing and outcrossing species from three independent model systems (**CS3**). The effects of mating system shift on floral morphology and sexual traits have been widely reported, but the impact of transition to selfing on pollen coat morphology remained to determined. In CS3, I addressed this issue by using extensive model systems with varying ages of transition to selfing, consist of three independent transitions to selfing in *Arabidopsis* and *Capsella* genera. This allowed me to bring the first large-scale experimental evidence to evaluate how fast shifts in mating systems can influence male gametophyte-specific selection, particularly their effect on pollen coat morphology (**CS3**). This design, which includes several independent comparative systems and the detailed visualization of pollen coat structure with underlying proteome profile, has not been done before and represents another novelty of my PhD project.

Finally, to assess the overall impact of the transition to selfing on hybridization barriers, I investigated the early evolution of reproductive isolation. Previous studies testing this hypothesis have often used model systems to assess the impact of EBN divergence on hybrid seeds between closely related species (interspecific), with the earliest reported case leading to EBN divergence and hybrid seed lethality around 100k years ago (Guo et al., 2009). However, it remains to be determined how quickly hybrid seed lethality and divergence in EBN can arise and whether these processes are observable within the same species (intraspecific). To test this hypothesis, I used a model system in which transition to selfing occurred recently (10k years ago) and performed manual reciprocal crosses between a selfing

and an outcrossing lineage within the same species of *A.lyrata* (**CS4**). I finally assessed hybrid seed viability and monitored hybrid endosperm development using confocal microscopy.

3.3. Molecular approaches to detect sexual genes and proteins

Natural variation in traits essential for pollen performance and preferred donor selection can arise from mutations or expression changes in genes linked to pollen tube growth and pollen-pistil interactions, providing a component for sexual selection to act. However, the molecular and genetic basis for traits under sexual selection in plants remains to be elucidated. Previous studies have primarily used population genomics approaches to estimate the molecular evolutionary rate of pollen-expressed genes focusing on either selfing or outcrossing species (Harrison et al., 2019; Arunkumar et al., 2013; Gutiérrez-Valencia et al., 2022) and do not pinpoint candidate genes that are likely to evolve under sexual selection. To address this gap, I employed a novel transcriptomics approach, and I generated species-specific and tissue-specific transcriptomes from both selfing and outcrossing populations of *Arabidopsis lyrata* (**CS1**). I generated RNAseq data of pollen (intra-sexual selection) and pollinated-pistil (inter-sexual selection) tissues and identified differentially expressed genes (DEGs) between selfng and outcrossing *A. lyrata*. The comprehensive transcriptomic approach I employed is novel in terms of advancing our understanding on genomics of sexual selection in plants, a field that remains largely unexplored compared to animals (Dean et al., 2015; Harrison et al., 2015; Veltsos et al., 2017). Moreover, by utilizing transcriptomics in natural populations, I was able to distinguish the selection pressures acting on pollen developmental genes, an aspect often overlooked in comparison to common lab-based knock-out approaches.

I addition to transcriptomics approach, I also used proteomics approach to understand impact of transition to selfing on evolution of pollen proteins under male gametophyte-specific selection (**CS3**). Floral traits impacted by transition to selfing is well-documented in literature (Sicard & Lenhard, 2011), but it remained to be determined whether shift in mating system affect the evolution of pollen coat, and underlying proteins under male gametophyte-specific interest. To address this knowledge gap, I used proteomics approach to detect footprints of male gametophyte-specific selection on pollen coat proteins in three independent selfing lineages. I generated the proteome profiles of pollen coat from selfing and outcrossing species within the *Arabidopsis* and *Capsella* genera (representing three independent transitions to selfing at different evolutionary timescale) and identified differentially expressed total pollen and pollen coat proteins between selfing and outcrossing species. Such a comprehensive approach including three independent transitions to selfing and detailed screening of pollen coat proteome profile in a comparative way has not done before, and it is one of the novelties of my PhD project.

The antagonistic interaction between maternal and paternal genomes over resource allocation is expected to occur in the endosperm, a biparental tissue that nourishes the embryo, through imprinted genes expressed in a parent-of-origin-dependent manner (Haig, 2004). Previous studies addressing the genomic bases of parent-specific imprinted genes have mostly focused on selfing species, in which parental conflict is not present. To address these knowledge gaps, I investigate footprint of selection on parent-specific imprinted genes in outcrossing versus selfing *A. lyrata* (CS5). I used pool-

sequencing data (Willi et al., 2018) and paternally (PEGs)/maternally (MEGs) gene expression data identified by (Klosinska et al., 2016). Then I used population genomics and various selection scans approaches to detect the footprint of selection on maternally and paternally expressed imprinted genes in outcrossing and selfing populations of *Arabidopsis lyrata* (**CS5**).

IV. Discussion of my Results

4.1. Mating system shifts drive the rapid evolution of male and female gametophyte

The evolution of male and female gametophytes is likely influenced by the strength of sexual selection, particularly evident during pollen–pistil interactions (Tonnabel et al., 2021). As explained in section 2.1, the intensity of sexual selection is expected to be weaker in selfing lineages compared to outcrossing ones, and likely to result in a reduction of pollen performance and pistil traits compared to their outcrossing counterparts (Mazer et al., 2018; Cutter, 2019). Although the impact of mating system shifts on male competition ability (Cutter, 2019; Lankinen & Karlsson Green, 2015; Mazer et al., 2010) and female choice (Cutter, 2019) has long captured the attention of plant biologists, empirical studies have been limited, primarily within the same species and how fast divergent selection pressure can shape the evolution of these traits remained to be elusive (Hove & Mazer, 2013; Mazer et al., 2018; Németh & Smith-Huerta, 2003). Moreover, interpretations of results about the impact of sexual selection on sexual trait evolution in divergent mating systems have been complicated by confounding factors arising from selfing syndrome that are independent of sexual selection. In my PhD project, I took advantage of closely related outcrosser and selfer populations from *Arabidopsis lyrata* subspecies and brought more stronger evidence on the role of sexual selection on shaping the pollen performance and female choice traits. Consistent with the expectation of decreased efficiency of sexual selection in selfers, I found outcrossing pollen exhibited a significant higher germination rate and longer pollen tube growth compared to the selfing pollen (**CS1**). This observation is also confirmed by mathematical predictions, where I showed that pollen performance traits could quickly evolve and lead to a fast phenotypic divergence between outcrossers and selfers (**CS2**). These findings highlight the impact of mating system shifts on the rapid evolution of pollen performance traits impacted by divergent sexual selection pressure that two lineages experience, independently of the typical 'selfing syndrome'. Nevertheless, it is known that a growing pollen tube communicates with the stylar tissue during its journey toward the ovules, meaning that pollen tube growth is not fully intrinsic male performances (Chae et al. 2007, Dresselhaus and Franklin-Tong 2013). To test this hypothesis, I combined *in vitro* results with *in vivo* (reciprocal crosses) pollen germination experiments and distinguished the intrinsic abilities of pollen from the impact of female tissues on pollen tube growth, a comprehensive strategy that has not been previously applied in this context (**CS1**). I showed that female outcrossers had a significant negative impact on pollen tube growth compared to female selfers, independent of the pollen donor used. These findings align with molecular evidence suggesting that female tissues provide nutrients for pollen tube growth (Johnson & Preuss, 2002; Pereira et al., 2021). This suggests female outcrossers may have evolved to limit their nutrient provisioning to select the most vigorous pollen grains, potentially acting as a substrate for intersexual selection. (Tonnabel et al., 2021). Further studies

focusing on controlled pollination experiments, followed by microdissection and metabolomic profiling of pollinated pistils, could reveal specific nutrients from female tissues that support pollen tube growth, allowing quantification of key elements/metabolites between outcrossing and selfing populations. Additionally, manipulating candidate elements using reverse genetics may clarify if nutrient allocation serves as a mechanism for intersexual selection. Last but not least, the observed differences may also reflect factors beyond sexual selection, such as lineage divergence, genetic drift, or ecological variation. The interaction between environmental conditions and sexual selection is crucial, as the ecological context can shape pollen performance, influencing how selection acts on these traits (Delph et al. 1997). Given that the two populations (one outcrossing and one selfing) differ ecologically, these factors might explain some of the variation I observed. Future research should incorporate more closely related outcrossing populations from North America or a wider range of independent lineages, such as the recently identified selfing *A. lyrata* population in Siberia (Kolesnikova et al. 2023), to strengthen the conclusions.

Beyond the evolution of male and female gametophytes under sexual selection, outcrossing species imposes additional selective pressures on sexual traits, i.e. sex-specific adaptations to enhance survival under various stresses and challenges during the reproductive process. While the importance of pollen coat protection against environmental stresses and pathogens is well documented (Pacini & Hesse, 2005), it remains to be determined whether the transition to selfing affects pollen coat morphology and how fast sex-specific interests may evolve, leading to divergence in pollen coat between outcrossing and selfing lineages. To test this hypothesis, I used comprehensive independent selfing and outcrossing species within the *Arabidopsis* and *Capsella* genera, representing three independent transitions to selfing at different evolutionary timescales, and compared pollen morphology in pairwise combinations of outcrossers vs. selfers. I found that the pollen coat area decreased concomitantly with the selfing transition in *A. thaliana* and *C. rubella* compared to their outcrosser counterparts *A.arenosa*, and *C.grandiflora* respectively (**CS3**). However, this trend was not observed in selfing *A. lyrata* (no appearance of selfing syndrome). In summary, these results suggest that male-specific traits can rapidly evolve after the transition to selfing in *C. rubella* (100k of years transition), with reduced pollen coat area potentially being another trait of the selfing syndrome, a novel finding proposed in my PhD thesis. However, changes in pollen coat morphology may be influenced by local environmental adaptations, not just mating system shifts, complicating interpretation. Indeed, we also found that similar pollen coat functions associated with pathogen response were recurrently observed in outcrosser-outcrosser comparisons, suggesting that factors other than the selfing transition may drive the evolution of pollen coat protein composition. Testing pollen from selfing and outcrossing populations under different stress conditions (e.g., UV, pathogens) could help clarify whether observed traits provide adaptive benefits specific to selfing or are responses to environmental pressures

4.2. Mating system shift cause rapid changes on the molecular basis of sexual traits

Mutation or expression variation of genes involved in pollen tube growth pathways and pollen-pistil interactions may lead to natural variation in traits important for pollen performance and the selection of preferred pollen donors, providing a substrate for sexual selection. Divergence in sexual traits between selfing and outcrossing lineages and is likely to leave a molecular signature on the underlying

traits in the genome. However, the impact of transition to selfing on shaping the molecular and genetic basis for traits under sexual selection in plants remains to be elucidated. To this aim, I investigated the sexual gene expression patterns in selfing and outcrossing populations of *Arabidopsis lyrata*. I generated RNA-seq data from pollen and pollinated pistil tissues and identified tissue-specific candidate genes showing divergent expression between selfing and outcrossing populations. Then, I showed the functional interpretation of these genes, which are involved in cytoskeleton and vesicle transport (**CS1**). The observed functions are known to be crucial for pollen tube growth, making these genes strong candidates for intra-sexual selection in plants. Additionally, to identify genes involved in pollen-pistil interactions, I demonstrated variation in pollinated pistil-specific and differentially expressed genes between selfers and outcrossers and identified 25 DEGs as potential candidates for female choice. I further showed that these candidate genes in pollinated pistils were significantly enriched in functions related to auxin synthesis, auxin response, and stress response (**CS1**). I propose that, these functions might serve as female choice mechanisms in plants by controlling pollen tubes through a pathway similar to the pathogen response. However, further research is needed to dissect this interaction in more detail. In conclusion, I revealed that strong intra-sexual selection in outcrossers drives higher gene expression in sexual tissues, associated with enhanced pollen performance and stricter female choice, compared to selfers. Furthermore, by employing a transcriptomic approach in sexual selection studies in plants, my PhD project paves the way for further research in the largely unexplored field of plant sexual selection genomics. Last but not least, as the annotation used for functional interpretation is largely based on mutant observations from wet-lab conditions, unforeseen candidates that did not show enriched functions may still be responsible for natural variation in pollen and pistil traits in the wild, presenting opportunities for future research. Functional validation of these candidate genes identified in pollen and pistil tissues may further clarify our understanding about their roles in pollen tube growth and female choice in plant reproduction. For functional validation, gene knockout and overexpression using CRISPR/Cas9 or RNA interference (RNAi) can elucidate gene-specific effects on pollen tube growth and female choice.

Similarly, the transition to selfing likely alters selection pressure on molecular components, such as protein, involved in male-specific sexual traits, as the need for pollen coat protection against pathogens is greatly reduced. However, the extent to which male-gametophyte selection drives protein evolution, and whether this process is impacted by mating system shift, remains to be tested. To address this, I generated proteome profiles of pollen from selfing and outcrossing species within the *Arabidopsis* and *Capsella* genera and quantified differentially expressed pollen coat proteins between the two mating systems across three independent model systems. I found that proteins enriched in pathogen response functions were significantly differentially expressed in outcrossing species compared to their selfing counterparts (**CS3**), and these results involving three independent selfing transitions suggest convergent evolution at work. These findings suggest that the transition to selfing can drive the rapid evolution of reproductive proteome profiles, particularly in male-specific traits. In this context, my PhD project is the first to address the divergence in pollen coat proteins and their convergent functions across independent selfing lineages.

4.3. Mating system shift promote early evolution of reproductive isolation

Transition from outcrossing to selfing can influence parent-specific interests on resource allocation to offspring, potentially resulting in post-zygotic hybridization barriers. To investigate the consequences of mating system shifts on post-zygotic hybridization barriers, I examined how differences in paternal and maternal genomes, particularly through endosperm balance, affected hybrid seed lethality between closely related outcrossing and selfing populations. Several studies have shown that crosses between selfing and outcrossing parents often result in hybrid seed lethality in closely related species (Rebernig et al., 2015; Lafon-Placette et al., 2018). However, it remains unclear at what pace the transition to selfing can lead to sufficient divergence in EBN to initiate reproductive barriers and whether this process is observable within the closely related lineages with lower genetic diversity. I hypothesized that reproductive isolation within closely related selfer and outcrosser populations could arise rapidly because selection acting on parent-specific endosperm dosage (i.e. the EBN) in selfers would gradually decrease after the transition to selfing. To address this gap, I investigated the early evolution of reproductive isolation in two closely related subspecies of *Arabidopsis lyrata*, where a recent transition to selfing occurred approximately 10,000 years ago (Foxe et al., 2010; Willi and Määttänen, 2010). I demonstrated that the shift in mating system has induced significant divergence in paternal genome between selfers and outcrossers (likely through EBN divergence), leading to negative interactions in hybrid seeds. This divergence results in extensive disturbances in endosperm cellularization and ultimately hybrid seed lethality. Previous studies partially studied the impact of mating system shifts on the evolution reproductive barriers, mostly using a well diverged sister lineages (Brandvain, and Haig 2005; Raunsgard et al., 2018; Rebernig et al., 2015; Lafon-Placette et al., 2018; Guo et al., 2009). By using a recently diverged sister lineages, I demonstrated that the divergence in the paternal genome between selfers and outcrossers, along with hybrid seed lethality, may play a crucial role in the early stages of speciation. Prior to this study, the shortest divergence time associated with hybrid seed lethality was observed between two closely related species, *Capsella rubella* (a selfer) and *Capsella grandiflora* (an outcrosser), which diverged approximately 100k years ago. Here in CS4, I provided evidence that hybrid seed inviability can evolve more rapidly (10k years after) following the transition to selfing and can leads to a strong postzygotic barrier between two closely related intraspecific lineages of *Arabidopsis lyrata*, may play a crucial role in the early stages of speciation. Consistently, a recent study has also shown that, hybrid seed lethality arises between populations of divergent mating systems in *Arabis alpina*, resulting in strong reproductive isolation between selfing and outcrossing populations (Petrén et al., 2023). However, it should be noted that, I used only one selfing and one outcrossing *A. lyrata* population to investigate the hybrid seed lethality. Therefore, future studies should include a broader range of *A. lyrata* populations across its geographic distribution, along with demographic analyses, to bring more complete understanding of EBN divergence and its role in hybridization barriers.

A mechanism on how sex-specific interest between paternal and maternal contributors give rise to dead offspring may explained through their regulation of gene expression in the endosperm depending on whether the gene is inherited from the father or mother (known as “genomic imprinting”; Haig, 1997; Wilkins, 2011). Previous studies have detected directional selection on imprinted genes involved in parent-specific functions, specifically showing that paternally expressed genes (PEGs) in selfing

species like *Capsella rubella* and *Arabidopsis thaliana* are under such selection (Hatorangan et al., 2016; Tuteja et al., 2019). Although these results offer valuable insights genomic bases of parent-specific imprinted genes, the authors only used selfing species in which parental conflict is not present, see the section 3. Therefore, literature is lacking comprehensive studies on the signatures of selection on imprinted genes, including comparing outcrossing and selfing lineages. Together with other members of the lab, I hypothesized that stronger selective pressure acting on the paternal and maternal genomes of outcrossers should leave a footprint of positive selection on maternally and paternally imprinted genes in the genome compared to selfing counterparts. To test this, A. Le Vève used genomic data from outcrossing and selfing populations of *A. lyrata* and searched for signatures of positive selection on imprinted genes, a manuscript I contributed to (**CS5**). However, we did not find any differences in the signal of selection between selfing and outcrossing populations (**CS5**). These results suggest that mating system shifts appeared to have limited effect on the evolution of imprinted genes. To address the limitation of detecting selection signals in imprinted genes, a more comprehensive comparison of selection signals in endosperm and imprinted genes across multiple outcrossing populations with varying levels of polymorphism is required. Additionally, focusing on the interaction between paternally and maternally expressed genes could provide a better insight into role of imprinted genes in endosperm development. Approaches like gene regulatory networks (GRNs) could further help to understand how these genes coordinate each other in the endosperm and underlying molecular mechanisms of genomic imprinting in plants.

V. Conclusion

The transition from outcrossing to selfing provides valuable insights into the ecological and genetic factors that shape the phenotypic evolution of sexual traits. In this thesis, I explored the evolutionary consequences of mating system shifts in independent selfing species and investigated how sexual traits and their underlying molecular mechanisms evolve in both male and female gametophytes under different evolutionary process (CS1-CS3), and whether, this shift can lead to the emergence of reproductive isolation between closely related lineages (CS4, CS5). Firstly, I demonstrated that the transition from outcrossing to selfing leads to rapid divergence in male and female gametophyte traits. I showed that outcrossers, experiencing stronger intra-sexual selection pressures, exhibit higher pollen germination rates and longer pollen tube growth compared to selfers (CS1 and CS2). Additionally, I found that outcrossing pistils exert stronger negative influence on pollen tube growth than selfing pistils, indicating the persistence of female choice mechanisms in outcrossers (CS1 and CS2). These findings highlight that the reduction in pollen performance traits and the less strict female influence on pollen tube growth rates (PTGR) are consistent with the expected impact of relaxed sexual selection in selfing species together with independent of confounding factor arising due to selfing syndrome.

Secondly, the molecular and genetic basis of traits under sexual selection remains poorly understood. To address this gap, I investigated the genetic basis for the divergence of sexual traits between selfing and outcrossing populations. Using RNA-seq data, I identified differentially expressed genes in pollen and pollinated pistil tissues, revealing tissue-specific genes associated with pollen tube growth and

pistil-mediated selection (CS1). My findings indicate that candidate pollen-specific genes in outcrossers involved in cytoskeleton organization and vesicle transport, which are key developmental components for pollen performance important for intrasexual selection. I also identified candidate genes in pollinated pistils that may play a role in female choice, particularly those related to auxin synthesis and response, which could regulate pollen tube growth through mechanisms similar to pathogen responses evolving under the pressure of intersexual selection. Altogether, these findings provide strong evidence for the role of sexual selection in shaping sexual gene expression in flowering plants. Furthermore, through proteomic analysis of pollen coat proteins, I uncovered convergent evolution in proteins associated with pathogen defense in three independent transitions to selfing, suggesting that selfers experience relaxed sex-specific selection on this trait (CS3). Beyond disentangling the effects of selfing syndrome from relaxed sexual and sex-specific selection, these findings offer insight into how rapidly underlying molecular mechanisms of male and female sexual traits can evolve following the recent transition to selfing in independent lineages (CS1 and CS3).

Thirdly, I demonstrated that mating system shifts contribute to the early evolution of reproductive isolation, particularly through the emergence of post-zygotic barriers. By examining hybrid seed lethality in crosses between selfing and outcrossing populations of *Arabidopsis lyrata*, I provided evidence that the transition to selfing leads to significant decrease in divergence in paternal genome contributions, resulting in hybrid seed inviability. This divergence, likely driven by differences in endosperm balance numbers (EBN), highlights the role of paternal and maternal genomic conflict in driving reproductive barriers. My findings suggest that reproductive isolation can evolve more rapidly than previously thought, occurring within 10,000 years after the transition to selfing (CS4).

Taken together, this thesis advances our understanding of how shifts in mating systems influence the evolution of male and female gametophyte traits, their underlying molecular mechanisms, and the development of post-zygotic hybridization barriers in flowering plants. By providing new insights into the evolutionary consequences of transitions to selfing, my findings lay the groundwork for future research on the genomics of sexual selection, sex-specific selection, and reproductive barriers in flowering plants. Additionally, the results present a system involving natural variation in male and female gametophyte traits can help uncover the molecular mechanisms driving the evolution of pollen and pistil traits in natural settings, an aspect often overlooked compared to lab-based systems. The broader implications of these findings extend beyond basic evolutionary biology, with potential applications in agriculture, conservation, and understanding plant reproductive dynamics in natural populations

VI. References

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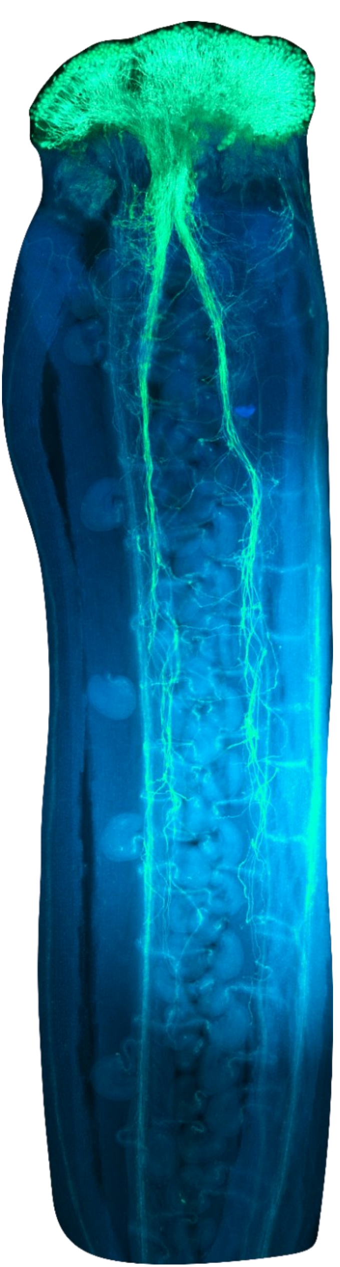
Part VII – Case Studies

Case study 1

Natural variation in sexual traits and gene expression between selfing and outcrossing *Arabidopsis lyrata* suggests sexual selection at work



Natural Variation in Sexual Traits and Gene Expression between Selfing and Outcrossing *Arabidopsis lyrata* Suggests Sexual Selection at Work

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Flowering plants show significant diversity in sexual strategies, profoundly impacting the evolution of sexual traits and associated genes. Sexual selection is one of the primary evolutionary forces driving sexual trait variation, particularly evident during pollen–pistil interactions, where pollen grains compete for fertilization and females select mating partners. Multiple mating may intensify competition among pollen donors for siring, while in contrast, selffertilization reduces sire–sire competition, relaxing the sexual selection pressure. Traits involved in male–male competition and female choice are well described, and molecular mechanisms underlying pollen development and pollen– pistil interactions have been extensively studied in the model species *Arabidopsis thaliana*. However, whether these molecular mechanisms are involved in sexual selection in nature remains unclear. To address this gap, we measured intrinsic pollen performance and its interaction with female choice and investigated the associated gene expression patterns in a selfing and an outcrossing population of *Arabidopsis lyrata*. We found that pollen germination and pollen tube growth were significantly higher in outcrossers than selfers, and this difference was accompanied by changes in the expression of genes involved in vesicle transport and cytoskeleton. Outcrosser mother plants showed a negative impact on pollen tube growth compared to selfer mother plants, together with a difference of expression for genes involved in auxin and stress response, suggesting a potential mechanism for female choice through molecular cross talk at the post-pollination stage. Our study provides insight into the impact of sexual selection on the evolution of sexual gene expression in plants.

Keywords: Natural variation • Pollen development • Pollen–pistil interactions • Selfing transition • Sexual gene expression • Sexual selection

Introduction

The evolution of sexual traits driven by sexual selection has been a long interest among biologists since Darwin's (1871) first proposal. Sexual selection theory explains the evolution and persistence of favorable sexual traits associated with nonrandom success in competition for access to gametes (Alonzo and Servedio 2019, Shuker et al. 2021). It includes two selective forces: male–male competition, or intrasexual selection, where individuals of the same sex vie for the opportunity to mate, and female choice, or intersexual selection, where females select mates based on specific male traits. In plants, after the contact between pollen and pistil tissue (similar to mating in animals), sexual selection may occur through both male–male competition and female choice (Tonnabel et al. 2021). While sexual selection is now well accepted in plants (Moore and Pannell 2011), the genes underlying this evolutionary process remain understudied.

Upon landing on the stigma, pollen germinates and extends the sperm-carrying tube through the stylar tissue to fertilize the ovules (Johnson et al. 2019, Pereira et al. 2021). As the number of pollen grains received often exceeds the number of available ovules, this leads to competition among pollen donors for siring success (Christopher et al. 2020). Pollen germination and pollen tube growth are two significant traits likely to evolve under intra-sexual selection (Snow and Spira 1991, Delph et al. 1998, Mazer et al. 2010, Hove and Mazer 2013, Tonnabel et al. 2022). This assumption is supported by evidence suggesting that pollen donors with higher siring success demonstrate elevated germination rates (Austerlitz et al. 2012) and faster pollen tube growth (Snow and Spira 1996, Pasonen et al. 1999, Skogsmyr and Lankinen 2002) compared to those with lower siring success. The initial growth of the pollen tube is autotrophic and relies on precise gene regulation mechanisms, including vesicle transport, cytoskeleton dynamics, cell wall modification

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and Ca^{2+} signaling, to maintain continuous growth (reviewed in Cascallares et al. 2020, Scholz et al. 2020, Weng and Wang 2022). However, whether these molecular mechanisms are involved in sexual selection in nature remains unclear.

In addition to its autotrophic growth, a growing pollen tube communicates with the stylar tissue during its journey toward the ovules, a process known as pre-ovular guidance (Marshall et al. 1996, Mollet et al. 2000, Chae et al. 2007, Dresselhaus and Franklin-Tong 2013). The presence of numerous genetically distinct pollen donors on the stigma potentially allows the female gametophyte to exert selective force on tube growth abilities (Pannell and Labouche 2013). The female choice of superior sperm traits is well documented in animals at postmating stage (namely cryptic female choice; Eberhard 1996) and represents similar aspects when considering the interaction between pollen and pistil tissue (Tonnabel et al. 2021). Female choice could influence mating outcomes directly by the molecular cross talk within the pistil or via the provisioning of elements required for pollen tube growth (Cheung et al. 1995, Park et al. 2000, Tonnabel et al. 2021). Nevertheless, while the molecular mechanisms involved in the interaction between the pollen tube and stylar tissues have been extensively studied, it remains to be investigated whether they contribute to female choice of conspecific mating partners in natural populations.

Several studies have found stronger footprints of positive and purifying selection on genes expressed in pollen than on sporophytic genes (Arunkumar et al. 2013, Gutiérrez-Valencia et al. 2022), which may be explained by sexual selection and/or haploid selection. Nevertheless, the developmental processes of pollen and pistil tissues are known to be highly conserved at the cellular and molecular levels (pollen: Wang et al. 2010; pistil: Zúñiga-Mayo et al. 2019). Therefore, genes involved in these processes are expected to exhibit pleiotropic effects (Rodríguez-Trelles et al. 2003, Signor and Nuzhdin 2018), potentially explaining the observed footprints of purifying selection on pollen genes (Arunkumar et al. 2013, Gutiérrez-Valencia et al. 2022). In contrast, positive selection is more likely to act on mutations with low pleiotropy, which may confine their detrimental effects (Connallon and Hall 2018). Pleiotropy might be alleviated by mutations occurring in cis-regulatory elements, which can restrict the mutational effect to cell type–specific gene expression. Indeed, most of the genetic variation underlying differences in gene expression is attributed to the effects on the activity of gene regulatory elements (Vuylsteke et al. 2005, Sicard et al. 2016). Therefore, sexual selection may favor the evolution of pollen performance and female choice traits through changes in gene regulatory elements. With transcriptome sequencing becoming more accessible, research on sexual selection is moving toward understanding the gene expression patterns evolving under selection (Wilkinson et al. 2015, Beaudry et al. 2020, Price et al. 2022, Tosto et al. 2023), a method well established in animals (Dean et al. 2015, Harrison et al. 2015, Veltsos et al. 2022), but yet to be tested in plants.

The diversity of reproductive strategies in plants provides valuable insights into how sexual selection operates in natural populations. For example, selfing species often mate with genetically identical partners, while outcrossing species primarily mate with genetically distinct partners. As a result, traits related to male–male competition and female choice may be relaxed in selfing lineages compared to their outcrossing counterparts (Skogsmyr and Lankinen 2002, Mazer et al. 2010, Wright et al. 2013, Cutter 2019). Consistently, several studies have shown that pollen tubes of outcrossing species tend to grow faster than those of selfing species (Smith-Huerta 1996, Taylor and Williams 2012, Hove and Mazer 2013, Mazer et al. 2018). Mating system shifts can also lead to significant genomic changes, potentially affecting how efficiently selection operates in selfing lineages. Due to higher levels of inbreeding, selfing species experience increased homozygosity, which reduces recombination efficiency and results in elevated linkage disequilibrium (Nordborg 1997, Wright et al. 2013). Although a shift in the mating system is known to play a profound role in the evolution of sexual traits, it may not be the sole driver. The evolution of sexual traits is likely to interact with local adaptation and is frequently influenced by multiple environmental factors acting as selective forces on genetic variation (Delph et al. 1997). For example, several studies have shown that environmental factors such as temperature and resource availability may also contribute to variation in pollen germination and pollen tube growth (Hedhly et al. 2004, Smith-Huerta et al. 2007, Liu et al. 2023a).

In this context, the transition to selfing may bear profound genomic consequences, including (but not restricted to) genes responsible for traits evolving under male–male competition and female choice. However, the transition to selfing leads to important long-term changes in sexual traits ('selfing syndrome') that are independent of sexual selection, bringing in a confounding factor when studying the impact of sexual selection on trait and gene evolution.

Arabidopsis lyrata appears as a relevant model system to test the effects of sexual selection on sexual traits and the evolution of gene expression without the confounding factor arising from the selfing syndrome. *Arabidopsis lyrata* is a perennial herbaceous plant with widespread distribution and made up of two recognized subspecies: *A. lyrata* ssp. *petraea* occurs in Eurasia, while *A. lyrata* ssp. *lyrata* occurs in North America (AlShehbaz and O'Kane 2002) (Ross-Ibarra et al. 2008, Schmickl et al. 2010). They vary in their habitat preferences, mating system and adaptation to their environment. European populations (*A. lyrata* ssp. *petraea*) are predominantly obligate outcrossers with a functional self-incompatibility system. They have a large population size and grow in open habitats including lakeshores, gypsum rock outcrops, scree slopes, cliffs and boulders (Jonsell et al. 1995, O'Kane and Al-Shehbaz 1997), as well as in semi-shaded places (Hemp 1996). It is assumed that the North American populations are the result of a colonization event from Europe

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that happened around 35,000 years ago (Ross-Ibarra et al. 2008). Colonization of the North American population was accompanied by a strong bottleneck (Ross-Ibarra et al. 2008, Schmickl et al. 2010), leading to genetically less diverse *A. lyrata* populations in North America (*A. lyrata* ssp. *lyrata*) compared with Europe (Schmickl et al. 2010). *Arabidopsis lyrata* ssp. *lyrata* occurs on sand dunes or rocky outcrops with some natural disturbance (Heblack et al. 2024). While most of the *A. lyrata* populations in North America (*A. lyrata* ssp. *lyrata*) are outcrossing, predominantly selfing populations with a loss of self-incompatibility have also been reported (Mable et al. 2005, Hoebe et al. 2009, Foxe et al. 2010). The breakdown of self-incompatibility occurred multiple times in populations located in the Great Lakes region and appears to be a recent event, occurring approximately 10,000 years ago (Foxe et al. 2010, Willi and Maatt¨ anen 2010˙). Due to this recent transition, North American selfing populations show no reduction in floral traits (aka 'selfing syndrome') compared to their ancestral outcrossing populations (Carleial et al. 2017). Moreover, the genome of *A. lyrata* has been already sequenced (Hu et al. 2011) and the annotation of the genome is well characterized using RNA-seq data (Rawat et al. 2015), making this species valuable to study consequences of the mating system shifts on the evolution of sexual traits and the underlying genes under sexual selection.

In this study, we used the two geographically isolated subspecies of *A. lyrata*, which differ in their mating systems, to test the following objectives: (i) whether pollen performance and pistil sorting ability upon pollination differ between them and (ii) whether the underlying genes responsible for pollen performance and female choice traits show variation in expression profiles between selfing *A. lyrata* ssp. *lyrata* and outcrossing *A. lyrata* ssp. *petraea* populations. For this purpose, we measured and compared pollen and female traits involved in intra- and intersexual selection between the selfing and outcrossing subspecies. In addition, we generated RNAseq data of pollen and pollinated-pistil tissues and identified differentially expressed genes (DEGs) between selfing and outcrossing *A. lyrata*. Our study shows variation in sexual traits and gene expression between selfing and outcrossing *A. lyrata* that is compatible with the effect of differences in the intensity of sexual selection.

Results

Concentration-dependent differences between outcrosser and selfer pollen performance

To test the phenotypic prevalence of gametophytic selection on pollen performance traits between the selfing *A. lyrata* ssp. *lyrata* and outcrossing *A. lyrata* ssp. *petraea*, we performed an in vitro liquid pollen germination assay and measured the pollen germination and pollen tube growth at an early (4 h) and late (16 h) time point (**Fig. 1A**). To experimentally simulate different levels of pollen competition for resources, we used a range of pollen concentration in the medium. At both time points, pollen concentration had a significant negative impact on germination (**Fig. 1B**, **Supplementary Fig. S4**, **Table 1**) and tube growth (**Fig. 1D**, **Supplementary Fig. S4**, **Table 1**). As a measure of pollen concentration, we tried using dilution factors or estimated pollen concentration values, and both approaches provided highly comparable results (**Fig. 1**).

At 4 h, both pollen germination and pollen tube growth were reduced with increasing pollen concentration in selfers, while the pollen performance in outcrossers was largely independent of pollen concentration, as reflected by the significant Pollen concentration × Mating system interaction in a linear mixedeffects (LME) model (**Fig. 1C**, **E**, **Table 1**). Consequently, the difference in pollen germination between outcrossers and selfers was most pronounced under high pollen concentrations and was least apparent under low pollen concentrations (**Fig. 1**). In addition, we observed a significantly faster pollen tube growth in outcrossers compared to selfers across the gradient of pollen concentration, as indicated by the significant effect of mating system in the LME model (**Fig. 1D**, **Table 1**).

At 16 h, pollen germination and tube growth were only affected by the pollen concentration, and no significant differences were detected between the selfer and the outcrosser (**Supplementary Fig. S4**, **Table 1**).

Outcrossing mother plants show strong interference on pollen tube growth

We then tested the impact of the female tissues on pollen tube growth, under the hypothesis that female selfers and outcrossers would show different effects. For this purpose, we performed reciprocal crosses and measured pollen tube length at an early (10 h) and late (16 h) time point (**Fig. 2A**). Independently of treatment combination and experimental design, pollen tube length was significantly positively associated with the length of the pistil (**Supplementary Fig. S5**). Selfers and outcrossers did not differ in the pistil length (**Supplementary Fig. S5**). To account for variation in the pistil length among individuals and its effect on pollen tube length, we used relative pollen tube length (a ratio between absolute pollen tube length and pistil length) in all downstream analyses.

At 10 h, pollen of outcrossers developed longer tubes than pollen of selfers, independently of the mating system of the maternal plant (i.e. significant effect of paternal mating system in an analysis of variance (ANOVA) model; **Fig. 2B**, **Table 1C**). Additionally, outcrosser maternal plants had a negative impact on pollen tube growth compared to selfer maternal plants, independently of the pollen donor

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(significant effect of the maternal mating system in ANOVA; Fig. 2B, Table 1C). At 16 h, only the mating system of the maternal parent had an effect on pollen tube length, and pollen donor contribution was no longer apparent (Fig. 2B, Table 1C). We were only able to account for the effects of seed family variation in both maternal and paternal parents by fitting an LME model to the larger, combined dataset (10 + 16 h). This additional analysis confirmed the significant role of the mating system, even after including seed family information as random effects in the statistical model. Specifically, we found a significant Maternal mating system × Paternal mating system interaction, with the outcrosser maternal × selfer paternal crosses showing significantly lower

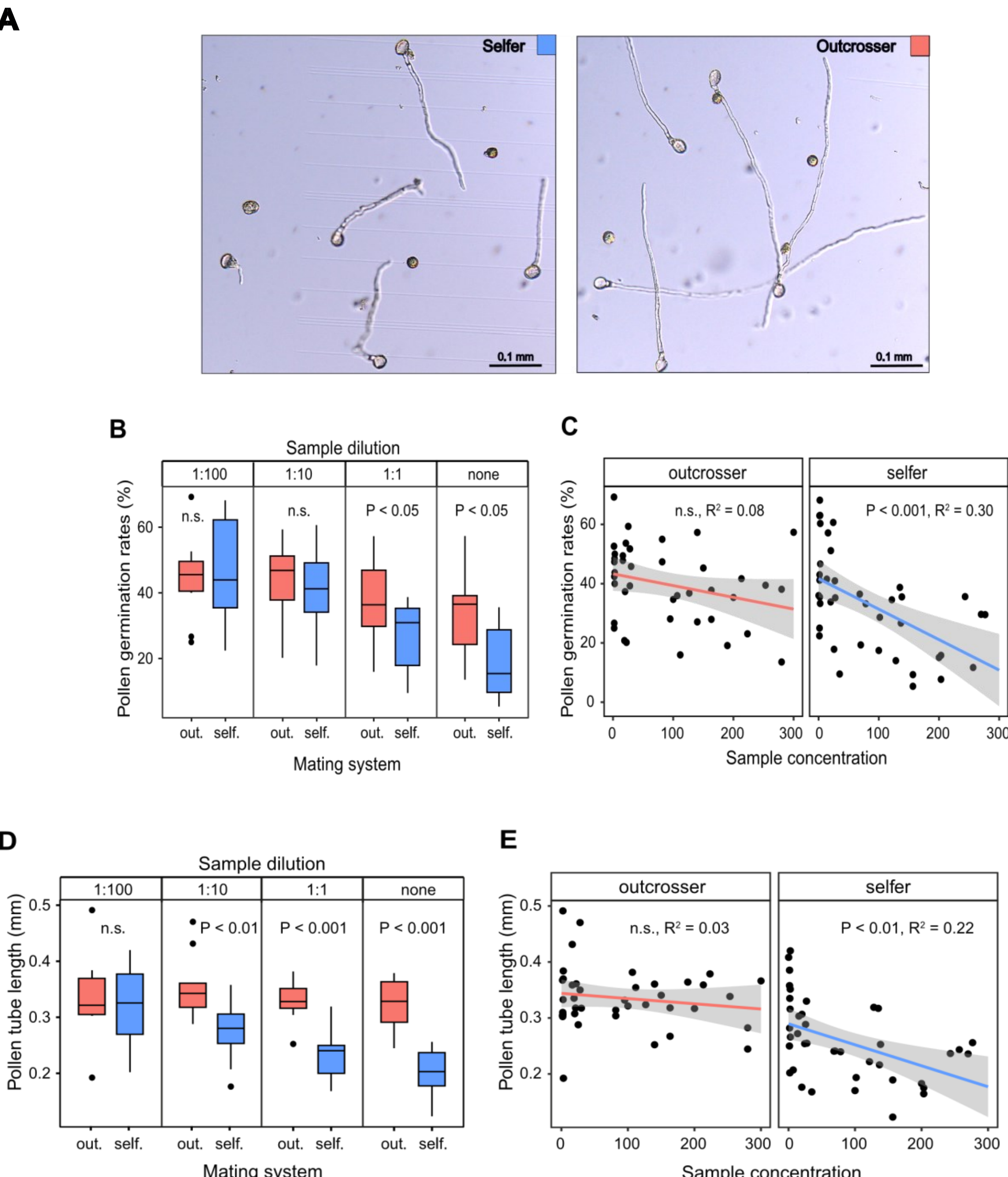

Fig. 1 Higher performance of pollen originating from outcrosser than selfer individuals of *A. lyrata*. (A) Microscopy pictures showing the differences between the selfer and outcrosser pollen tube growth. (B, C) Pollen germination rates (%) and (D, E) pollen tube growth (mm) were assessed under experimentally manipulated gradients of pollen concentration resulting from different levels of sample dilution (1:100, 1:10, 1:1, none) after 4 h. Aside from plotting both response variables against the predictor pollen concentration (used in statistical models; Table 1), in (B) and (D) we provide alternative visualizations of the same relationships using sample dilution categories. All scale bars are 0.1 mm.

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Table 1 Summary of linear models testing the effects of the parental mating system (selfer vs. outcrosser), experimentally manipulated pollen concentration (a proxy of resource limitation) and their interactions on pollen performance

	d.f.	*F*	*P*	d.f.	*F*	*P*
A. Pollen germination rate						
LME model	Time = 4 h			Time = 16 h		
Pollen concentration	1, 66.4	34.5	**<0.001**	1, 48.2	14.9	**<0.001**
Mating system	1, 15.2	0.5	0.492	1, 6.4	1.5	0.258
Pollen concentration × Mating system	1, 66.4	7	**0.01**	1, 48.2	0.8	0.372
B. Pollen tube growth in vitro						
LME model	Time = 4 h			Time = 16 h		
Pollen concentration	1, 65.8	18.6	**<0.001**	1, 46.4	12.9	**<0.001**
Mating system	1, 14.7	5.5	**0.034**	1, 7.6	0.03	0.869
Pollen concentration × Mating system	1, 65.8	8.1	**0.006**	1, 46.4	1.9	0.172
C. Pollen tube growth in vivo						
ANOVA	Time = 10 h			Time = 16 h		
Maternal mating system (MMS)	1, 38	7.7	**0.009**	1, 60	6.5	**0.013**
Paternal mating system (PMS)	1, 38	6.9	**0.012**	1, 60	0.4	0.54
MMS × PMS	1, 38	0.5	0.488	1, 60	3.9	0.054

Pollen germination rates and pollen tube growth were established both in vitro under controlled conditions (A, B) and in vivo as pollen tube growth in pistils subjected to manipulated pollinations (C). Variation among different seed families was used as a random factor in LME models (A, B). Significant predictors and interactions (α= 0.05)
are highlighted in bold.

pollen tube length than all other parental combinations (**Supplementary Table S5**).

Pollen DEGs between the selfer and the outcrosser involve cytoskeleton and vesicle transport

We searched for gene expression patterns associated with the difference in pollen traits observed between selfers and outcrossers (**Fig. 1**). To do so, we first identified genes preferentially expressed in pollen by performing a differential expression analysis (DEA) between pollen germinated in vitro and other tissues (leaves, roots, emasculated pistils and endosperm; see methods for the criteria we used; **Supplementary Fig. S2A**). A total of 3,055 DEGs were detected from this comparison and used as pollen-specific genes for further analysis. To validate biological functions of pollen-specific genes, we performed Gene Ontology (GO) enrichment analysis. 'Pollen tube growth', 'pollination', 'pollen tube development', 'multi-organism reproductive process' and 'cell tip growth' were some of the highly represented GO terms within the biological process (False Discovery Rate, FDR <0.05 and q < 0.05; **Supplementary Fig. S3**). Then, to find pollen genes differentially expressed between pollen from the outcrosser and the selfer subspecies, we performed a DEA between the outcrosser and selfer pollen. A total of 4,031 genes were identified as differentially expressed with 2,033 of them being downregulated and 1,998 being upregulated in outcrosser compared to selfer pollen. As many of these genes are likely to be expressed in other tissues than just pollen and thus to have a general function, we aimed at narrowing down the genes responsible for pollen performance differences between selfers and outcrossers. To do so, we overlapped the pollen-specific genes (3,055) with pollen DEGs between selfers and outcrossers (4,033 genes). By doing so, we identified 716 genes (**Fig. 3A**). These genes showed three significantly enriched GO terms (FDR <0.05 and q < 0.05; **Supplementary Table S6**), namely SNARE (soluble NSF attachment protein receptor) complex (a complex of proteins drives the fusion of vesicles with the target membrane), cytoskeleton and cytoskeletal parts (**Fig. 3B**).

Pollinated pistil DEGs show enriched functions in auxin and stress signaling

We finally searched for genes associated with the differential maternal plant response to pollen between selfers and outcrossers (**Fig. 2**). To characterize the transcriptome profile of pollen and pistil interaction, we first identified genes that are preferentially expressed in pollinated pistils (within-mating system pollination only). We performed a DEA between pollinated pistil and the tissues composing it (pollen, emasculated pistils and endosperm as markers for already formed seeds) and detected 208 genes as pollinated pistil–specific genes (see methods for criteria; **Supplementary Fig. S2B**). Among these pollinated pistil–specific genes, several enriched GO terms were found, including 'response to organic substance', 'response to chemical', 'defense response' and 'response to stimulus' (FDR <0.05 and q < 0.05; **Supplementary Fig. S3**).

Furthermore, we performed a DEA between the selfer- and outcrosser-pollinated pistils to identify the candidate

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genes associated with mating system–dependent maternal response to pollen. In total, 4,016 genes were detected, among which 1,876 were upregulated and 2,140 downregulated (see methods for the criteria). As many of these genes are likely expressed in other tissues and are thus not related to pistil–pollen interactions, we narrowed down the list of candidates by overlapping pollinated pistil–specific genes with DEGs obtained between the selfer- and outcrosser-pollinated pistils. A total of 25 genes were found (**Fig. 4A**). These genes showed significantly enriched GO terms such as 'response to stimulus', 'response to stress', 'response to auxin' and 'indole-3-acetic acid amido synthetase activity' (**Fig. 4B**). The gene list is available in **Table 2**.

A

out x out self x self self x out out x self

B

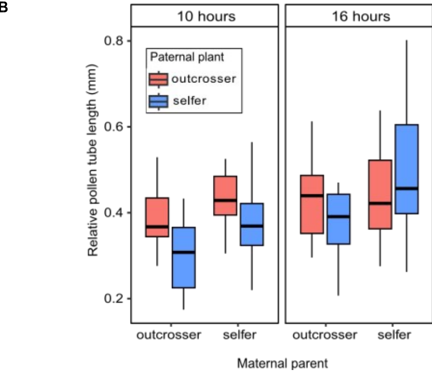

Fig. 2 Male performance in pistils in experimental crosses testing the effects of the mating system (selfer vs. outcrosser) of both the maternal and paternal parent. (A) Aniline blue-stained pollinated pistil among the control and reciprocal crosses from the selfer and the outcrosser *A. lyrata* 16 h after pollination (cross directions always noted as ♀ × ♂). Arrows indicate the final point of the length of the majority of pollen tubes, forming a bulk. (B) Relative tube length among the crosses performed between paternal and maternal plants. Results are shown separately for two experiments differing in time allowed for pollen germination and growth (10 vs. 16 h). All scale bars are 0.25 mm.

Discussion

The differences in sexual traits between selfing and outcrossing *A. lyrata* are consistent with the effect of sexual selection

In this study, we tested whether male performance and female choice differ between selfing and outcrossing *A. lyrata*. We assumed that these two traits would show a reduction in the selfing lineage compared to the outcrossing one due to relaxed sexual selection (Mazer et al. 2018, Cutter 2019). By combining in vitro and in vivo (reciprocal crosses) pollen germination experiments, we could separate the pollen's intrinsic abilities from the impact of female tissues on pollen tube growth.

Through in vitro pollen germination assays, we assessed the inherent abilities of pollen donors independently of female gametophyte interaction. Our findings revealed significantly higher pollen germination and pollen tube length in the outcrosser compared to their selfing counterparts, particularly at the early time point (4 h). These in vitro results mirror the in vivo findings from controlled crosses, showing that outcrosser pollen had longer tubes than the selfer one at the early time point (10 h). To date, despite theoretical predictions on the impact of mating system shifts on male competition ability (Mazer et al. 2010, Lankinen and Karlsson Green 2015, Cutter 2019), empirical studies have been limited, primarily focusing on Clarkia species (Németh and Smith-Huerta 2003, Hove and Mazer 2013, Mazer et al. 2018). Especially, how fast a transition to selfing can lead to divergence in pollen performance traits between sister lineages remains largely unexplored. It is known that the divergence between the Eurasian and North American lineages occurred around 35,000 years ago (Ross-Ibarra et al. 2008). The breakdown of self-incompatibility in North American populations appears to be a more recent event, occurring approximately 10,000 years ago (Foxe et al. 2010), making *A. lyrata* a valuable model system for studying recent transitions to selfing. Our study shows that pollen performance can rapidly decline following the transition to selfing. This decline seems to have appeared faster than and independently from the 'selfing syndrome', which is not visible in the selfing *A. lyrata* lineage we used (Carleial et al. 2017). The difference in pollen performance between selfing and outcrossing *A. lyrata* was the most pronounced in higher pollen concentration in the liquid medium. Higher pollen concentration is likely to be associated with stronger competition for nutrients present in the medium and may to a certain extent mimic pollen–pollen competition for the nutrients provided by female tissues (Erbar 2003). In this context, outcrosser pollen may perform better in more competitive environments than selfer pollen, which may be explained by differences in the intensity of sexual selection between the two lineages (Cutter 2019).

By performing reciprocal crosses, we could identify a divergent role of female selfers and outcrossers on pollen

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tube growth. It is important to note that our experimental design did not allow us to test the impact of individual maternal plants on pollen tube growth at each time point separately, which could be important. Larger scale studies should be considered, even though conducting microscopy work at a larger scale would be tedious and time-consuming. Nevertheless, our findings suggest that female outcrossers have a significant and negative impact on pollen tube growth compared to female selfers, independently of the pollen donor. Variation in pollen tube length due to maternal genotype interference may stem from molecular interactions between the growing pollen tube and stylar tissue at post-pollination stage, potentially serving as a substrate for sexual selection (Tonnabel et al. 2021). As female tissues provide nutrients for pollen tubes to grow (Johnson and Preuss 2002, Chae and Lord 2011, Pereira et al. 2021), female outcrossers may have evolved to restrict their provisioning to select for the most vigorous pollen grains.

With this in mind, we are aware that our comparison involved only one outcrossing population and one selfing from two geographically isolated subspecies: *A. lyrata* ssp. *petraea* (outcrossing) occurs in Eurasia, while *A. lyrata* ssp. *lyrata* (selfing) occurs in North America (Al-Shehbaz and O'Kane 2002),

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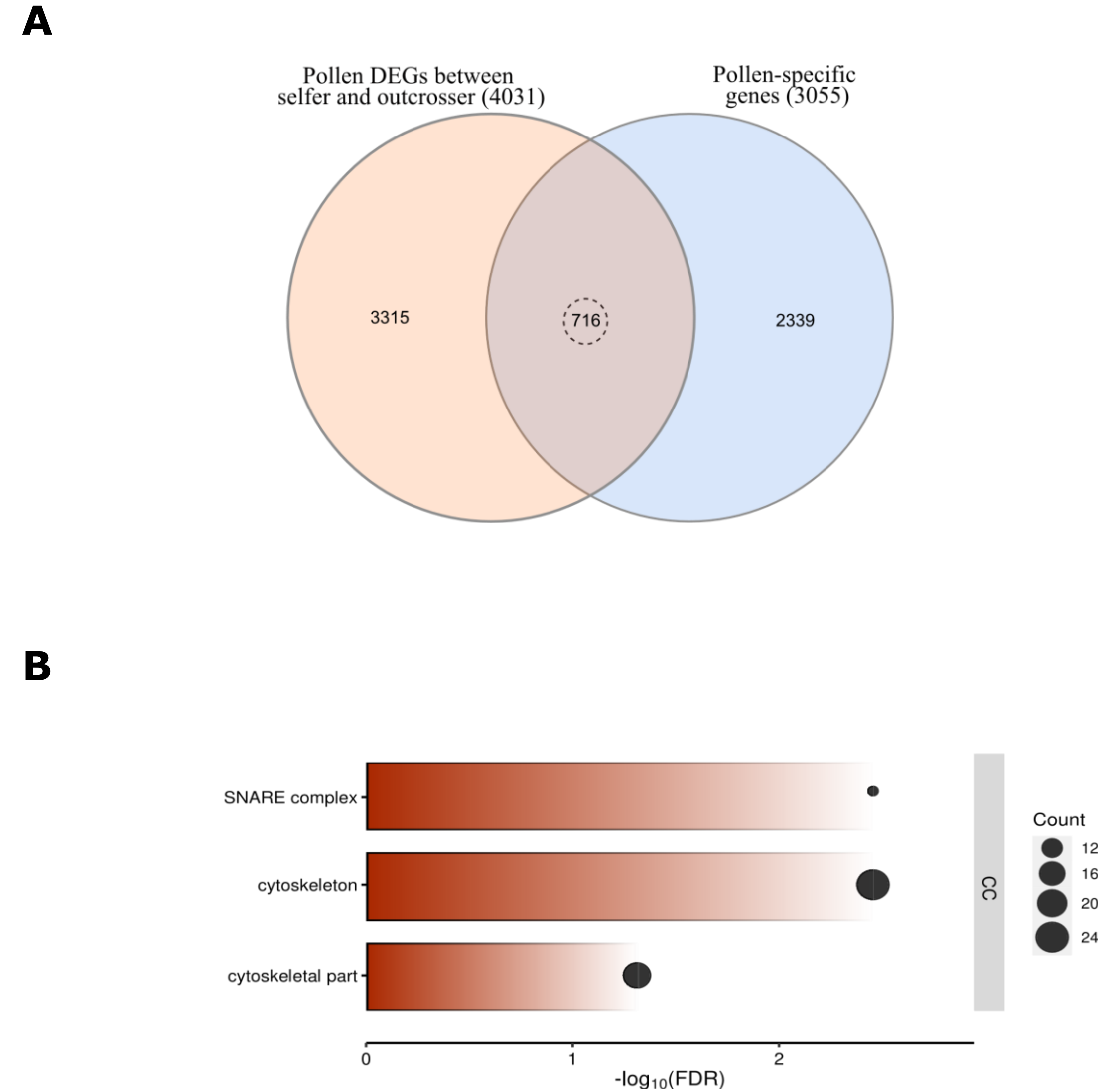

Fig. 3 Candidate pollen-specific genes together showing divergent expression between selfing and outcrossing plants with their biological functions. (A) Venn diagram showing the number of candidate genes under the sexual selection in pollen (716 DEGs) and (B) GO term enrichment analysis of those candidate genes in pollen within the biological process (FDR <0.05 and $q < 0.05$). The size of the black bubble refers to the number of genes enriched in each category.

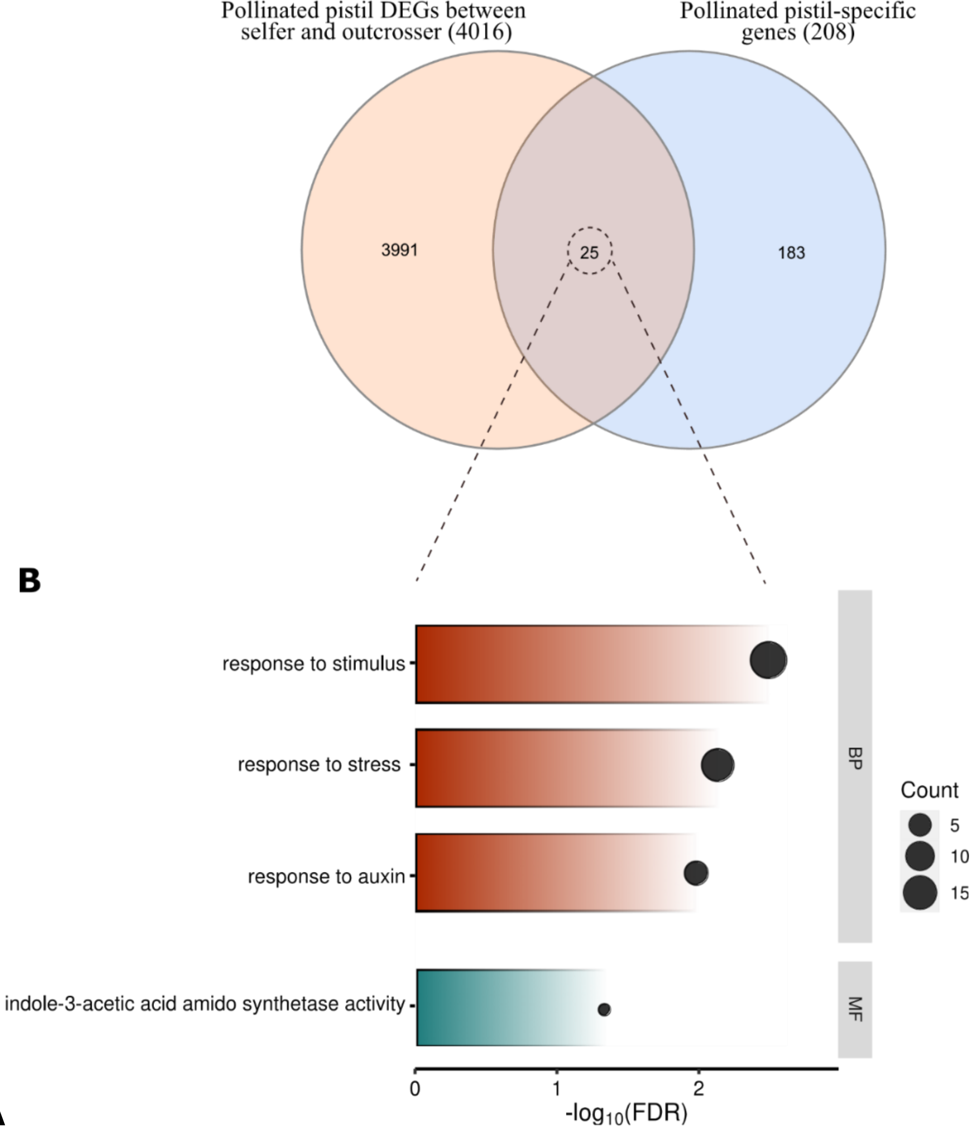

Fig. 4. Candidate pollinated pistil–specific genes together showing divergent expression between selfing and outcrossing plants with their biological functions. (A) Venn diagram shows the number of pollinated pistil candidate genes (25 DEGs) for the sexual selection and (B) GO term enrichment analysis of those candidate genes in pollinated pistil within the biological process and molecular function (FDR <0.05 and q < 0.05). The size of the black bubble refers to the number of genes enriched in each category.

and observed differences could be due to other factors such as lineage divergence, genetic drift or other selective processes (like ecological differences) rather than sexual selection. The interaction between ecological factors and sexual selection is noteworthy, as the environmentally plastic nature of pollen
performance is likely to impact the efficacy of selection acting on it (Delph et al. 1997) and could explain our results, as the two populations we used show ecological differences. A further study should include North American outcrossers closely related to and geographically located near the selfers (Li

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Table 2 Detailed list of 25 pollinated pistil–specific candidate genes showing divergent expression between selfing and outcrossing plants and their description and expression values

Arabidopsis lyrata gene name	*Arabidopsis thaliana* ortholog	Description	log2FoldChange	P_{adj}	Regulation
AL1G66590	AT1G56650	Production of anthocyanin pigment 1	2.27738456	4.01E-05	Up
AL1G34180	AT1G21130	*O*-methyltransferase family protein	5.771687311	4.09E-05	Up
AL4G27410	AT2G32030	Acyl-CoA *N*-acyltransferases superfamily protein	3.124196917	4.57E-05	Up
AL6G21160	AT5G10625	NA	2.389417277	0.000114949	Up
AL4G10820	AT2G21620	Adenine nucleotide alpha hydrolases–like superfamily protein	1.117985273	0.000248226	Up
AL6G47870	AT4G04330	Chaperonin-like RbcX protein	−2.529241661	0.000334042	Down
AL1G33580	AT1G20620	Catalase 3	−1.626009186	0.000400621	Down
AL3G52410	AT1G69730	Wall-associated kinase family protein	5.682140958	0.00053256	Up
AL5G44800	AT3G62150	*P*-glycoprotein 21	3.741307124	0.000636484	Up
AL5G14590	AT3G25930	Adenine nucleotide alpha hydrolases–like superfamily protein	2.106417629	0.000715791	Up
AL8G24230	AT5G50200	Nitrate transmembrane transporters	2.362197731	0.000916552	Up
AL6G50520	AT4G02520	Glutathione *S*-transferase PHI 2	2.535846662	0.00103574	Up
AL1G22630	AT1G11530	C-terminal cysteine residue is changed to a serine 1	1.585825356	0.001559205	Up
AL1G41660	AT1G28130	Auxin-responsive GH3 family protein	1.872423364	0.002825036	Up
AL2G27690	AT1G68760	Nudix hydrolase 1	−0.983314842	0.003276667	Down
AL8G28640	AT5G53730	Late embryogenesis abundant hydroxyproline-rich glycoprotein family	1.496028194	0.006214946	Up
AL7G21490	AT4G30650	Low-temperature and saltresponsive protein family	1.973107626	0.008353171	Up
AL4G27150	AT2G31865	Poly (ADP-ribose) glycohydrolase 2	2.8765681	0.012083748	Up
AL1G54460	AT1G47960	Cell wall/vacuolar inhibitor of fructosidase 1	−2.235527378	0.013239139	Down
AL8G14450	AT5G44990	Glutathione *S*-transferase family protein	2.993517576	0.016405063	Up
AL1G16470	AT1G06460	Alpha-crystallin domain 32.1	1.046839627	0.017572315	Up
AL3G51340	AT2G18150	Peroxidase superfamily protein	2.349665778	0.027822045	Up
AL3G36350	AT3G22160	VQ motif-containing protein	2.201899453	0.037081683	Up
AL4G13030	AT2G23170	Auxin-responsive GH3 family protein	1.996218442	0.040542136	Up
AL4G27560	AT2G32200	NA	1.4652235	0.049394014	Up

Descriptions of genes retrieved from the *A. lyrata* v2.1 annotation file (Rawat et al. 2015).

et al. 2023) and/or a broader range of independent lineages to build stronger conclusions, such as the recently discovered selfing *A. lyrata* in Siberia (Kolesnikova et al. 2023). Nevertheless, the higher pollen performance and more strict female influence on pollen tube growth we observed in the outcrossers are consistent with the impact of sexual selection. Independently of this conclusion, our findings provide a system involving natural variation in pollen and female traits that may prove useful to understand the molecular mechanisms underlying the evolution of these traits in nature.

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Mating system shift differences in pollen performance mirror differential expression of genes important for pollen development

To uncover the molecular mechanisms behind the pollen performance differences we observed between selfers and outcrossers, we compared their pollen gene expression profiles. We identified 716 genes being both pollen-specific and differentially expressed between selfer and outcrosser *A. lyrata*. Among these genes, significantly enriched functions were associated with the SNARE complex, cytoskeleton and cytoskeletal parts, reflecting biological functions important for pollen development.

Indeed, these processes are vital for efficient pollen tube growth during fertilization. Pollen tube growth is characterized by polar tip growth and is regulated by cytoplasmic streaming during the synthesis of a new cell wall at the tip region (LovyWheeler et al. 2007). Active streaming of cell wall materials, enzymes and signal molecules to the growing tip region, facilitated by secretory vesicles, is essential for pollen tube growth (Ruan et al. 2021, Weng and Wang 2022). The SNARE complex regulates intracellular membrane fusion events, facilitating the delivery of necessary cell wall materials for expansion and elongation, promoting pollen tube growth toward the ovule for fertilization (Guo and McCubbin 2012, Slane et al. 2017, Macgregor et al. 2023, Liu et al. 2023b). Several SNARE proteins are specifically expressed in pollen tissue, with some located at the pollen tube apex and behind the tip, as well as on the plasma membrane of pollen tubes (Enami et al. 2009, Ichikawa et al. 2014, Slane et al. 2017). It has been shown that knockout mutations in the SNARE complex cause severe defects in pollen tube growth within the style (Slane et al. 2017). Additionally, the essential role of the cytoskeleton and its components in organizing the trafficking of endo- and exocytotic vesicles during pollen tube growth is well understood and has been shown by numerous studies (Cole and Fowler 2006, Qin and Yang 2011, Xu and Huang 2020). As the genes involved in these processes were differentially expressed between selfing and outcrossing *A. lyrata*, this suggests that sexual selection may act on the expression of these genes. Previous population genomics studies estimated the molecular evolutionary rate of pollen-expressed genes in selfing (Harrison et al. 2019) and outcrossing (Arunkumar et al. 2013, Gutiérrez-Valencia et al. 2022) populations. While informative, these studies do not pinpoint specific traits and genes essential for intra-sexual selection. In our study, we used species-specific and pollen-specific transcriptomics and identified candidate genes involved in pollen tube growth in outcrosser *A. lyrata*, potentially evolving under strong intra-sexual selection pressure at the post-pollination stage. Finally, the enriched functions we found only included 56 genes attributed to these functions from *A. thaliana* annotation. As this annotation is largely based on the observation of mutants in lab conditions, the 661 remaining genes from our transcriptomic study may be unforeseen candidates responsible for natural variation in pollen traits in the wild, presenting opportunities for future research.

Female choice may operate as a defense response mechanism following pollination

Upon germination on the stigma, the pollen tube receives external signals from the stylar tissue, guiding its growth toward the ovule (pre-ovular guidance; reviewed in Cheung et al. 1995, Park et al. 2000, Higashiyama and Yang 2017). This finding has drawn attention to possible mechanisms for pistil traits to discriminate among preferable pollen donors, similar to cryptic female choice in animals (Tonnabel et al. 2021). However, studies focusing on the molecular mechanisms of pollen and pistil interaction often utilize knock-out approaches to identify genes, overlooking selection pressures in natural populations. In this context, we aimed at finding differential gene expression profiles involved in pollen–pistil interactions upon pollination from natural outcrossing and selfing populations. We found 25 genes both being pollinated pistil–specific and differentially expressed between outcrossers and selfers, representing potential candidates for female choice. Among these, enriched functions were related to auxin synthesis and response as well as stress response.

Multiple studies highlight the importance of auxin and its derivatives [indole-3-acetic acid amido (IAA)] in pollen germination and tube growth following pollination. For instance, Aloni et al. (2006) observed elevated auxin levels in the pollen tube tip and beneath the stigma, suggesting their role in regulating pollen germination and tube growth. Similarly, Chen and Zhao (2008) emphasized the significance of free IAA in stylar tissue during pollen tube growth after pollination. Moreover, they discovered elevated IAA levels in the apical and middle parts of the style, correlating with enhanced pollen tube growth. In this context, pistil tissue with differential IAA expression and auxin response in the stylar region may function as a mechanism for female choice, spatially regulating IAA production to allow pollen donors to display their performance. Another recent study by Chebli and Geitmann (2024) demonstrated the invasive growth of pollen tubes in the pistil, revealing that the enzyme pectate lyase–like (PLL), secreted by pollen tubes, digests pectin from the transmitting tissue of the pistil to facilitate its growth. This may explain why pollen–style interactions after penetration resemble a pathogen response at the molecular level (Elleman and Dickinson 1999, Dresselhaus and Márton 2009, Shi et al. 2017). The transcriptome of pollinated pistils in our study also revealed enriched functions associated with defense response. Although direct evidence of pistil counteraction against the invasive growth of the pollen tube is lacking, it is known that pistil secretes proteins that interact with the pollen tube (Cheung et al. 1995, Park et al. 2000). These pistil-secreted molecules may reduce pollen invasiveness by affecting PLL activity (Sanati Nezhad and Geitmann 2013). As pistil genes

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differentially expressed between selfers and outcrossers included enriched function in stress response in our study, we hypothesize that female choice, and the more restrictive influence of female outcrossers on pollen tube growth, may act through a similar pathway as a pathogen response against growing pollen tubes. Further studies may test this hypothesis.

In conclusion, we found that outcrossers, experiencing strong intra-sexual selection in nature, showed higher pollen performance and stricter female choice than selfers. We revealed candidate genes underlying this trait divergence with a transcriptomic approach, paving the way for further studies on the genomics of sexual selection in plants, a largely unexplored field.

Material and Methods

Plant material and cultivation conditions

In this study, we used individuals from two subspecies of *A. lyrata* that are known to differ in their mating system, one selfing and the other outcrossing. Seeds from the selfer population (*A. lyrata* ssp. *lyrata*) were collected from Point Pelee, ON, Canada (GPS coordinates: 41°55′40.0″N, 82°30′58.0″W), and seeds from the outcrosser population (*A. lyrata* ssp. *petrea*) were collected from Nová Ves near Oslavany, Czech Republic (GPS coordinates: 49°07′03.1″N, 16°18′21.4″E). Prior to germination, seeds were surface-sterilized using a sterilization solution [consisting of 5% NaClO, 0.01% (v/v) Triton X-100, and sterile water] and planted on agar plates containing MS-Salts, MES hydrate and 0.8% (w/v) plant agar with pH 5.8. Plates were then transferred to a growth chamber with the following conditions: a 12-h day at 23°C and a 12-h night at 13°C, and cultivated for 2 weeks. After that, seedlings were transplanted into small pots filled with garden substrate and grown for 8 weeks under short-day conditions (8 h of light per day at 4°C) to induce flowering. Finally, young plants were repotted into 0.5-l pots, transferred to a growth chamber and cultivated under long-day conditions (a 16-h day at 21°C, a 8-h night at 15°C) until they were used in experiments.

In vitro pollen germination and pollen tube growth assay

To test the intrinsic pollen performance abilities, we performed an in vitro liquid pollen germination assay using pollen from selfer and outcrosser subspecies of *A. lyrata*. The assay was performed following an original protocol adapted from Xiyan (2011) with some modifications outlined later. For all experiments, for feasibility reasons, we prioritized the number of mother plants over the number of replicates per mother plant. This way, we could catch a larger amount of genetic diversity to have a representative sampling of each population, with the drawback that the low number of replicates per mother plant did not allow us to test the effect of mother plants.

Initially, we performed a pilot experiment to determine the timeline of pollen tube growth, as well as to assess the impact of different conditions on pollen germination. Time was indeed the most relevant difference for this experiment and other distinctions were rather technical, such as volume in which germination took place and dilution. We began by using 2-ml tubes for pollen incubation and allowing the pollen to germinate for 16 h at room temperature (RT) to ensure sufficient time for germination. For this pilot experiment, we used four mother plants (with two to four offspring per mother as individual replicates) for both the selfer and the outcrosser.

Subsequently, we decided to refine the assay conditions by reducing the incubation time to 4 h at 25°C to examine the early response of intrinsic pollen performance abilities. Moreover, we transitioned to using 15-ml Falcon tubes to ensure ample oxygen availability for pollen tube respiration compared to the limited oxygen supply in the 2-ml tubes. For this experiment, we used four mother plants (with one to two offspring per mother as individual replicates) for both the selfer and the outcrosser.

To start the pollen germination assay, 15 freshly opened flowers from the apical inflorescence were collected in 2-ml tubes, and chilled at RT for 30 min, with the tube cap remaining open. Subsequently, 1 ml of freshly prepared germination medium (containing 5 mM MES-Tris adjusted to pH 5.8, 1 mM KCl, 0.8 mM $MgSO_4$, 1.5 mM boric acid, 10 mM $CaCl_2$, 5% w/v sucrose and 15% w/v PEG4000) was added to the tubes and vortexed at maximal speed for 1 min. Then, pollen grains were concentrated by centrifugation at 6,000 rpm for 5 min at RT. The supernatant and flower residues were removed, and the pollen pellets were resuspended in 1 ml of germination medium by vortexing. The concentration of pollen grains was measured using 10 µl of pollen suspension placed on a hemocytometer counting chamber under a light microscope. Subsequently, pollen suspensions were diluted in serial batches (including no dilution, 1/2, 1/10 and 1/100) in 15-ml falcon tubes for the 4-h experiment and 2-ml tubes for the 16-h pilot experiment to test pollen performance under resource limitation. Following that, pollen suspensions were incubated at 25°C for 4 h or at RT for 16 h with 250 rpm agitation. Finally, 10 µl of the germinated pollen suspension was placed on a hemocytometer counting chamber and photographed by using differential interference contrast microscopy with an

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Olympus BX51 (Olympus Corp., Tokyo, Japan) equipped with Canon EOS 700D camera (Canon Inc., Tokyo, Japan).

Pollen germination and tube length were analyzed using the ImageJ program (https://imagej.nih.gov/ij/) in a double-blind manner. Pollen grains were considered germinated when a pollen tube emerged, and its length was equal to or greater than the pollen grain diameter. The germination rates (%) were calculated by dividing the number of germinated pollen grains by the total number of pollen grains per sample. The length of the pollen tube (µm) was measured using the segmented line option in the ImageJ program, and the mean pollen tube length was determined as the average length of 30 germinated pollen grains. Detailed measurements can be found in **Supplementary Table S1**.

In vivo pollen tube growth assay

To assess the female action on pollen tube growth, we performed control and reciprocal crosses between selfers and outcrossers of *A. lyrata*. For this experiment, we again used two different time points to observe pollen tube growth at 10 and 16 h after the pollination. Each time point was done in a separate experiment using a different set of maternal and paternal individuals to perform manual crosses (see **Supplementary Table S2** for details). For each designated cross and time point, we performed 10–15 manual pollinations for each cross-type (♀ × ♂; selfer × selfer, outcrosser × outcrosser, outcrosser × selfer and selfer × outcrosser). Between two to four different maternal plants and two to four different paternal plants were randomly combined for each type of cross (**Supplementary Table S2**). For outcrosser crosses, we first did a test cross to see whether plants were compatible and could produce fruits to avoid crossing plants with the same self-incompatibility allele that would result in no pollen tube growth. For all crosses, flowers were emasculated before anthesis, and pistils were hand-pollinated 2 d after emasculation. Pollinated pistils were collected after 10 and 16 h of pollination and stored in a fixation solution (containing acetic acid/EtOH, 1:3 ratio) for 1 d at RT. After fixation, the fixed pistils were rehydrated through a series of ethanol dilutions using 70%, 50% and 30% ethanol series, respectively, and washed with ddH_2O. Pistils were softened overnight at RT using 8M NaOH and subsequently stained with a decolorized aniline blue solution (0.1% w/v aniline blue in 108 mM K_3PO_4, pH 11) for 3 h in the dark. Stained pistils were observed and pictured under UV light conditions using a fluorescence Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) equipped with a Nikon Digital Sight 10 camera (Nikon Instruments Inc., Melville, NY, USA). Finally, images were analyzed using the ImageJ program (https://imagej.nih.gov/ ij/). The length of the majority of pollen tubes, forming a bulk, was measured as a mean value for pollen tube length. All measurements were conducted in a double-blind manner.

Statistical analyses of pollen tube growth

LME models were used to compare the differences in pollen germination and pollen tube growth between selfer and outcrosser individuals. Separate models were fitted for different designs of experiments, differing in time at which the male performance was assessed and using different sets of *A. lyrata* individuals (recruited from mostly non-overlapping suites of seed families). The mating system of an individual (selfer and outcrosser), in case of manipulated crosses for the maternal and paternal parent separately, pollen concentration (in vitro experiments only) and their interactions were used as fixed effects in the models. Seed family (nested within the mating system) was used as a random effect in the models. LME models of the Gaussian family were fitted with the restricted maximum likelihood method as implemented in the R package 'lme4' ver. 1.134 (Bates et al. 2015). The response variable Pollen tube growth in vitro was log-transformed for the 16-h dataset to meet the model assumptions. Statistical significance was inferred in a type III analysis of variance with Satterthwaite's method to estimate the denominator degrees of freedom using the R package 'lmerTest' (Kuznetsova et al. 2017).

When analyzing the dataset of in vivo pollen performance in manipulated crosses, incorporating the seed family identity for both maternal and paternal parents as random effects resulted in too complex LME models (singular fit) given the number of observations available within each of the two experimental designs (time = 10 h, 16 h). Therefore, we instead used two-way ANOVA models to analyze the two designs separately. In addition, we applied an LME model to the combined dataset of both designs, with maternal and paternal seed families as random effects, to check the consistency of the main results after including random effects in the statistical model.

Simple linear models (*t*-test, least-squares regression) were applied to illustrate differences between compared groups and the strength of relationships plotted in graphs. Unless stated otherwise, statistical data analysis was done with R ver. 4.3.1 (R Core Team 2023).

RNA extraction, library preparation and sequencing

We isolated total RNA from sexual and non-sexual tissues from both outcrossers and selfers of *A. lyrata* (see **Supplementary Table S3** for details). For each tissue, we collected material from one individual per mother plant from four different mother plants for both the selfer and outcrosser

subspecies (2 subspecies × 4 mother plants × 1 individual = 8 samples sequenced per tissue in total). In total, we sequenced 40 samples (2 subspecies × 5 tissues × 4 mother plants × 1 individual) for the transcriptomic analysis.

Non-sexual tissues included leaves and roots, and sexual tissues consisted of pollen, emasculated unpollinated pistils, and pollinated pistils. For emasculated pistils, we first emasculated the flowers before anthesis, then collected 10 pistils 2 d after emasculation and immediately froze them in liquid nitrogen until the day of RNA extraction. For pollinated pistils, we first emasculated the flowers before anthesis. Two days after emasculation, we then performed 10 manual control crosses (selfer × selfer and outcrosser × outcrosser) and collected pollinated pistils 6 h after pollination by immediately freezing them in liquid nitrogen until the day of RNA extraction. To extract RNA from germinated pollen, a total of 60–100 freshly opened flowers were collected from a single offspring of four different mother plants for both selfing and outcrossing populations (eight plants in total). A single plant cannot produce this amount of flowers simultaneously, and therefore we collected flowers >3 d to ensure having 60–100 flowers per plant. Pollen was extracted from the flowers and germinated in a liquid medium for 6 h (using the same protocol as for the in vitro pollen germination analyses). The germinated pollen was subsequently centrifuged, and pollen pellets were stored using 100 µl RNAlater™ Stabilization Solution (Thermo Fisher Scientific, Waltham, MA, USA) until the RNA extraction.

For the non-sexual tissues, approximately 60 mg of leaf and root tissue was harvested and immediately frozen in liquid nitrogen until the day of RNA extraction. Just as for the other tissues, we collected material from one individual per mother plant and four mother plants per subspecies (2 subspecies × 1 individual × 4 mother plants = 8 samples per tissue). Total RNA was extracted using the protocol from MagMAX™ Plant RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) for all the tissues. The quality of the total RNA extracted from these tissues was quantified by Qubit (Thermo-Fisher Qubit 2.0 Fluorometer), and RNA concentration exceeded 72 ng/µl for all the samples. Library preparation and mRNA enrichment of the extracted RNA were prepared at the SEQme company in Dobříš, Czech Republic, using the QuantSeq 3′ mRNASeq Library Prep kit (Lexogen, Vienna, Austria). Finally, single-end libraries were sequenced at 100-bp read length using an Illumina NovaSeq6000 SP (**Supplementary Table S3**).

Endosperm RNAseq data used in this study were included to filter out seedoriginated RNA from the pollinated pistils, as early seeds may have already been formed. Also, we included endosperm data to widen the range of tissue identities as comparison points to find tissue-specific genes. Due to the difficulty of isolating endosperm/early seed tissues, we obtained RNAseq data from a previous study conducted in *A. lyrata* (Klosinska et al. 2016) and downloaded from the Sequence Read Archive (GEO: GSE76076). The RNAseq data of five outcrosser endosperm samples were included in the data processing starting from the trimming step. While these data were only from outcrossers, we do not expect tissue-specificity expression patterns to significantly vary between the two subspecies. To make sure this is the case, we identified pollen-specific genes separately in selfers and outcrossers and verified that most of the genes show a similar pattern in both subspecies (**Supplementary Fig. S7**).

Read trimming, mapping and counting

The quality of the raw RNA-seq reads was checked using FastQC ver. 0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Before alignment, over-represented TruSeq adapters were trimmed using CUTADAPT ver. 3.5 (Martin 2011). Subsequently, we used trimmomatic ver. 0.39 (Bolger et al. 2014) to remove adapters and low-quality data with the following parameters: ILLUMINACLIP:TruSeq3-SE.fa:2:30:10, LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15 and MINLEN:36. The trimmed reads were then mapped to the *A. lyrata* genome ver. 2.1 (Rawat et al. 2015) using the STAR alignment tool ver. 2.7.0a (Dobin et al. 2013) with option –outFilterMultimapNmax 1. This option retained only uniquely mapped reads in the output BAM file for subsequent analysis. Uniquely aligned reads were counted using the HTSeq count tool ver. 0.11.1 (Anders et al. 2015) with default parameters. *Arabidopsis lyrata* ver. 2.1 genome annotation (Rawat et al. 2015) was used for counting uniquely mapped reads.

Differential gene expression and GO enrichment analysis

Before conducting the DEA, data normalization was performed based on sequencing depth and composition to assess sample quality according to their expression profiles. For this purpose, the vst normalization (variance stabilizing transformation) function from the DESeq2 package ver. 1.34.0 was used (Love et al. 2014). In order to see the profile of gene expression of each sample, normalized read counts among each replicate were visualized using principal component analysis (PCA) with the 'plotPCA' DESeq2 function, and a cluster dendrogram using the 'hclust' DESeq2 function in R (**Supplementary Fig. S1**).

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DEA was performed using the DESeq2 tool ver. 1.34.0 in R (Love et al. 2014). To identify tissue-specific genes, we performed a DEA against other tissues.

We considered genes as tissue-specific if they were upregulated (log_2FoldChange ≥ 1 and adjusted $P < 0.05$) in all tissue pairwise comparisons. We chose a log_2FoldChange threshold of 1 (i.e. a 2-fold change) to be sure of enough expression level differences between tissues for genes we call 'tissue-specific'. For DEA between selfers and outcrossers, we used a different fold change threshold, $|log_2FoldChange| \geq 0.5$ (1.5-fold change) with adjusted $P < 0.05$ (**Supplementary Table S4**). We used this threshold to focus on small differences in gene expression between genetically closely related sister lineages. It is likely that small differences in gene expression can hold significant biological importance, especially when the impact of these differences is magnified through their influence on numerous genes (Huang et al. 2010). To compare the gene sets identified by DEA, Venn diagrams (Heberle et al. 2015) were generated for both tissue-specific and mating type–specific pairwise comparisons of the DEGs (**Supplementary Fig. S2**).

Biological roles of the obtained list of genes were identified with GO enrichment analysis (**Supplementary Fig. S3**). The GO enrichment analysis was performed using the PlantRegMap online tool (Tian et al. 2019). To improve the GO annotation of our genes, we decided to use *Arabidopsis thaliana* homologs of *A. lyrata* genes for enrichment analysis. We retrieved *A. thaliana* homologs from the *A. lyrata* ver. 2.1 annotation (Rawat et al. 2015). We used the list of these homologs as a background gene list to reduce the bias due to different genomes and gene content between the two species. By default, topGO and Fisher's exact tests were applied to find the significantly overrepresented GO terms. Enriched GO terms corrected by parameters from the Benjamini and Hochberg method for multiple comparisons (FDR <0.05, $q < 0.05$) were considered as significant output. The q-value in the output file is defined as a natural FDR (positive FDR) analog to the P-value. The significance level of q-values ($q < 0.05$) was selected and transformed to negative fold changes (−log10) to present results in a bar chart. The bar charts were generated using the SRplot online platform (Tang et al. 2023).

Supplementary Data

Supplementary data are available at *PCP* online.

Data Availability

The RNA-Seq data used in this study have been submitted to the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/geo/) under submission number PRJNA1067428.

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Author Contributions

C.L.P. and Ö.İ. conceptualized the study. Ö.İ. performed the experiments. Ö.İ. and M.C. analyzed the data. C.L.P., Ö.İ. and

M.C. wrote and edited the manuscript.ˇ

Disclosures

The authors have no conflicts of interest to declare.

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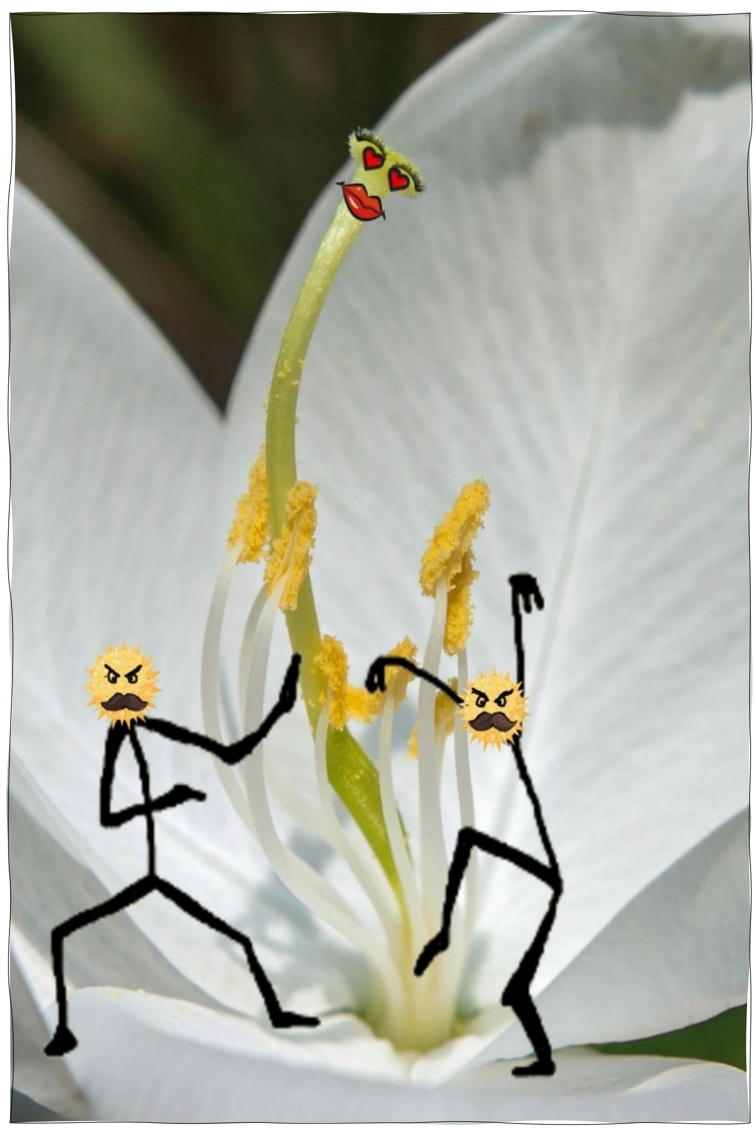
Case study 2

The coevolution between pollen tube growth and style length: a molecular love story



Runaway selection and the coevolution between pollen tube growth and style length: a molecular love story

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Abstract

Sexual selection in plants is not a controversy anymore, and yet, to which extent sexual selection theory can be broadened beyond the animal kingdom and applied to plants remain unclear. For example, runaway selection, leading to the coevolution of male traits and female preference, remains untested in plants. In this article, focusing on pollen tube growth as an example of trait involved male performance, and style length as an example of trait involved in female choice, we evaluate the plausibility of runaway selection in plants. We not only review the available literature, but also use mathematical modelling to predict the potential coevolution between pollen tube growth and style length. We find that the coevolution between these two traits is likely to happen and is influenced by plant-specific reproductive strategies such as mating systems. We then propose potential molecular and evolutionary pathways responsible for gene-gene coevolution caused by runaway selection in plants and review genomic approaches to detect this coevolution.

KEYWORDS

Coevolution; Development and Evolution; Plant Mating Systems; Sexual Selection; Genomics; Pollen-pistil interactions

1. INTRODUCTION

'...The sight of a feather in a peacock's tail, whenever I gaze at it, makes me sick!' - Darwin

It has been more than 150 years since Darwin wrote this sentence in his theory of sexual selection to explain exaggerated ornaments in animals (C. R. Darwin, 1871). Because of the negative effects of these traits on survival ability, Darwin could not explain the ostentatious plumage (more visibility for predators) of peacocks by his natural selection theory. However, in the following years, Darwin realized that the evolution of exaggerated traits would compensate the negative effects on survival ability by increasing the number of mating events through female choice and proposed the concept of sexual selection (C. R. Darwin, 1871). According to the broadly accepted definition, sexual selection explains the evolution of sexual traits based on reproductive success caused by competition for mating (Alonzo & Servedio, 2019b). It comprises two main components that can be applied for both animals and plants. The first component is intra-sexual selection (most of the time male-male competition, even though female-female competition also exists (Alonzo & Servedio, 2019b) and basically explains competition among conspecific members of one sex for the opportunity to mate with individuals of the other sex. The second component, known as inter-sexual selection, relies on non-random selection of mate (female ability to bias paternity) or at least fitness advantage provided by mating with partners that have higher performance in the intra-sexual competition (Willson, 1979). Indeed, the advantage of biasing paternity for the female may include an improved general fitness of the progeny ("good genes" selection; Byers and Waits, 2006) or an improved reproductive success of the male progeny due to the heritability of male competition abilities, indirectly increasing the female fitness ("sexy son" hypothesis; Weatherhead & Robertson, 1979). The benefits of sexually selected male traits might then lead to a "runaway process" wherein female choice for the preferred trait and male traits evolve synergistically (detailed under the coevolution section) (Fisher, 1930). In the past century, these concepts have been developed and broadened mostly in relation to animals (due to the noticeable visibility of visual cues and mating behaviours), while it relatively received less attention in plants (Lankinen & Green, 2015; Moore & Pannell, 2011; Tonnabel et al., 2021). However, in the late 1970's the idea of sexual selection was extended into a new era with an innovative proposal from Janzen (Janzen, 1977), by putting attention on the possibility of "mate preference" in plants.

Sexual selection in plants can take place at several reproductive stages. Such as, before pollination (pre-pollination), when insect-pollinated plants compete for pollinator service, via floral traits or floral architectures (Cocucci et al., 2014; Delph, 2006; Delph & Ashman, 2006; Minnaar et al., 2019), or when wind-pollinated plants are selected for increased pollen production (Schoen & Stewart, 1986) and their dispersal ability (Tonnabel et al., 2019) to enhance the fertilization capability for a limited number of ovules. Sexual selection can also occur after mating (post-pollination stage), when pollen grains from different individuals compete to access ovules under female selectivity (reviewed in Tonnabel *et al.*, 2021). Before pollination, because plants usually produce male gametes in a much higher extent compared to female gametes, it is assumed that the male reproductive success is overall more limited by mating events than the female function (Bateman, 1948), and thus, pollinator attraction exert a stronger selective pressure on the male function of hermaphroditic plants (also known as the "Fleurs-du-Male" hypothesis; Queller, 1983) (Cocucci *et al.*, 2014; Minnaar *et al.*, 2019; Paterno *et al.*, 2020). However, it should be noted that in case of pollen limitation, pollinator attraction is likely to be under sexual selection through the female function (Ashman & Morgan, 2004; Hansen & Totland, 2006) After pollination, the potential for intra- and inter-sexual selection is important, as pollen grains deposited on the stigma and will have a direct contact with the pistil (the female function) through germinating and growing pollen tubes to access the ovules (somehow similar to sperm competition in animals; Bernasconi et al., 2004; Gage & Morrow, 2003; Parker, 1970). At this stage, several pollen performance traits have been proposed to be under intra-sexual selection (male-male competition): pollen germination rate (Austerlitz et al., 2011), pollen tube growth (Lankinen et al., 2017; Pasonen et al., 1999; Skogsmyr & Lankinen, 2000; Tonnabel et al., 2022) or chemical interference between pollen grains where pollen grains inhibit the growth and germination of one another (Marshall et al., 1996b; Pasonen & Käpylä, 1998; R. J. Swanson et al., 2016). The female ability to bias paternity in plants occurs during the post-pollination process when maternal tissues may discriminate the pollen donors based on particular traits or genotypes (Marshall, 1991; Marshall & Ellstrand, 1988). In this context, several traits has been proposed (but importantly, not formally evidenced yet) to be involved in paternity bias (inter-sexual selection), such as delayed stigma receptivity (Galen et al., 1986; Lankinen & Madjidian, 2011), style length (Lankinen & Skogsmyr, 2001); meta-analyis by Ramesha *et al.*, 2011), size of the stigmatic surface (Tonnabel et al., 2022) and pollen tube guiding via chemical interactions (Bhattacharya and Baldwin, 2012; reviewed in Tonnabel *et al.*, 2021). During the journey of the pollen tube through the pistil, style length is likely to be an important trait for female selectivity, as longer styles may increase the time and space window for 1/ differences in pollen tube growth performance to manifest themselves and 2/ chemical selection of pollen tubes by females or pollen-pollen interactions (Herrero

& Hormaza, 1996; Ramesha et al., 2011; Tonnabel et al., 2021).

Pollen tube growth and style length have been one of the most expected candidate traits involved in postmating male-male competition and female choice respectively in plants (Pasonen et al., 1999; Ramesha et al., 2011; Skogsmyr & Lankinen, 2000). If longer styles select for pollen grains with faster tube growth and faster pollen tube growth brings a reproductive advantage, longer style will then be indirectly selected for. These two traits will be preferentially transmitted to progeny and may become genetically correlated. In other words, pollen tube growth and style length may coevolve under a runaway selection scenario (Fisher, 1930) and consequently, the underlying genes may coevolve too. This hypothesis largely remains to be tested, and in this review, we will evaluate the potential for pollen tube growth and style length coevolution, the evolvability of the molecular mechanisms underlying pollen tube growth and style length, and the promises of research focused on male-female gene-gene coevolution in this context.

2. POLLEN TUBE GROWTH AND STYLE LENGTH: POTENTIAL CANDIDATE TRAITS FOR MALE-FEMALE COEVOLUTION

In the following section, we review the literature by focusing on pollen tube growth (as an indicator trait for male performance) and style length (as an indicator trait for female choice) as potential candidate traits for models of runaway or good-genes. We then, evaluate the possibility of pollen tube-style length coevolution under a runaway selection scenario.

2.1 Pollen tube growth

After deposition on the stigma, the pollen:ovule ratio is often skewed towards pollen (data extracted from Gong and Huang, 2014, 2014; Figure 1a). Therefore, male reproduction is limited by the number of available ovules, and this creates male-male competition between conspecific pollen grains. To be successful in this siring race, pollen grains need to have specific competitive traits such as fastest-growing pollen tubes to achieve fertilisation in the first place (Delph et al., 1998; Lankinen & Skogsmyr, 2002; Mazer et al., 2010a; Snow et al., 2000; Snow & Spira, 1991). A previous study in *Hibiscus moscheutos* have shown that a higher number of seeds were non-randomly sired by a pollen donor with a faster-growing pollen tube compared to pollen of lower performance (Snow & Spira, 1991). The positive correlation between pollen tube growth rate and siring success has been also shown by other studies on different species (see the references; Delph *et al.*, 1998; Lankinen and Skogsmyr, 2002; Mazer *et al.*, 2010; McCallum and Chang, 2016; Snow *et al.*, 2000).

Another set of evidence for sexual selection acting on pollen tube growth comes from the comparison of sister lineages with different mating systems. It has been proposed that while sexual selection is strong in outcrossing lineages, the transition to selfing may lead to relaxed sexual selection (Cutter, 2019). The reasons for this phenomenon may be multiple: firstly, due to increased homozygosity and decreased effective population size and effective recombination, selection in general is proposed to be less efficient in selfing lineages (Burgarella & Glémin, 2017). Moreover, while in outcrossing lineages, pollen grains from multiple donors can be deposited on the stigma and compete for mating, this is rarely the case in selfers. Thus, because the genotype of all pollen grains and the female on the stigma is nearly identical, traits related to intra or intersexual selection are unlikely to provide any reproductive advantage to the male or female function (i.e., relaxed sexual selection; Mazer *et al.*, 2010). Consistently with the expectation that sexual selection is relaxed in selfing lineages, evidence has shown that outcrossing lineages tend to have faster pollen tube growth compared to their selfing relatives (Diaz & Macnair, 1999; Hove & Mazer, 2013; İltaş et al., 2024; Mazer et al., 2018b; Smith-Huerta, 1996; Taylor & Williams, 2012). Nevertheless, such evidence remains relatively scarce, and these studies focus on the same biological system, *Clarkia* species (Kerwin & Smith-Huerta, 2000; Smith-Huerta, 1996). However, the "selfing syndrome" (set of floral traits accompanying recurrently the transition to selfing) includes the reduction of female organs and pollen production (Duncan & Rausher, 2013; Snell & Aarssen, 2005) and thus, many sexual changes linked with selfing are not necessarily related to sexual selection. Using divergent mating systems to infer the impact of sexual selection may thus have some drawbacks. One way to alleviate this issue may be to study recent transitions to selfing, where the selfing syndrome did not have time to evolve yet or comparing the impact of recent vs older transition to selfing.

Overall, faster pollen tube growth as a trait increasing male reproductive success under intrasexual competition is a relatively well accepted phenomenon.

2.2 *Style length*

In flowering plants, the style is a narrow stalk that connects the stigma and the ovary (Figure 5B). It has a radially symmetric form surrounded by chlorenchyma tissue and a distinctive epidermis. The style plays a crucial role during the fertilisation process as it serves as a path for the growth of compatible pollen tubes. Similar to many floral traits, the style displays prominent phenotypic variation at morphological and genetic level within and between plant species (Gotelli et al., 2017; Hao et al., 2020; Huu et al., 2016; Ramesha et al., 2011). Its length can range from virtually null (e.g. *Tulipa* L.) to a few centimetres and its structure can be

open (with few or no cells in the central region) or close (tightly packed with cells throughout) (Gotelli et al., 2017). For example, in lily, the style is very long and has an open structure, whereas, in *Arabidopsis* gynoecium, it is short (a couple hundred micrometres) and closed.

This huge variation in style length within and between taxa has created several inferences about its possible role in the selection of mating partners (Lankinen & Skogsmyr, 2001; Mazer et al., 2016; Mulcahy, 1979; Ramesha et al., 2011). One hypothesis is that a longer style increases pollen transfer onto the stigma by improving the access to pollinator bodies (Lee et al., 2008). Thereby, with high pollen deposition onto the stigmatic surface, competition between each pollen grain to access ovules would be intense during germination and tube growth (Erbar, 2003; Mulcahy, 1979; Stephenson et al., 2003). A longer style in this circumstance would expand the pool of mate choice with high number pollen deposition, allowing the selection of the best mating partner. Another proposed hypothesis is that with a longer style, the distance between the stigma and ovules, across which growing pollen tubes compete, increases, giving a larger window for differences in pollen performance to manifest themselves: a longer distance for pollen grains to compete means larger differences in pollen tube lengths at the end of the "race" (Mazer et al., 2010a; Ramesha et al., 2011). Thus, both hypotheses suggest that a longer style length may provide a way for females to select high-quality pollen grains, and this is likely to be advantageous for females ("good genes" or "sexy son" selection; see previous section). Therefore, under sexual selection, females are expected to evolve towards a longer style.

Evidence in this direction, even though indirect, comes from the comparison of outcrossing and selfing sister lineages, which is one approach to measure the impact of sexual selection. As mentioned above, it is expected that sexual selection is high in outcrossers and relaxed in selfers. Consistently, previous studies have recurrently found that the style of outcrossing species is significantly longer than those of related selfing species (Diaz & Macnair, 1999; Georgiady & Lord, 2002; Ramesha et al., 2011; Runions & Geber, 2000). In addition to the selfer-outcrosser contrast, gynodioecy is another relevant system to assess the impact of sexual selection on style length evolution. Gynodioecy is a reproductive mode enabling the co-existence of female and hermaphrodite individuals within a given species (C. R. Darwin, 1877; Shykoff et al., 2003). It represents an opportunity to study female-specific interests related to sexual selection: it is assumed that in hermaphrodite species, neither the male nor the female function can reach their fitness optimum due to physical constraints, where sexual organs may be size-limited due to other organs (e.g. coexistence of anthers and gynoecium). Another constraint is resources, as individuals investing in both male and female functions (e.g. pollen or ovule production) cannot provide as much in one particular function as a specialised individual can (Delph & Ashman, 2006). This evolutionary trade-off gets solved with the emergence of separated sexes, such as in gynodioecy and dioecy in general. In gynodioecy, we can thus expect that the female traits in female individuals may be optimal for the female function. In a frame of sexual selection, we may expect that style length would be longer in females compared to hermaphrodites, allowing a more extensive mate choice. Indeed, this trend has been reported in many model systems including non-exhaustively *Maytenus obtusifolia*, *Plantago maritima*, *Stellaria graminea*, *Silene acaulis* or *Sidalcea hirtipes* (Benevides et al., 2013; Canelles et al., 2018; Dinnétz, 1997; Kučera et al., 2021; Schultz, 2012). However, this trend is not absolute, as no difference or even the opposite, a shorter style in females, was also found in some instances (Delph & Ashman, 2006; Jang et al., 2015; Rodríguez-Riaño & Dafni, 2007). Importantly however, it is crucial not to ignore that females in gynodioecious systems do not need to invest energy in the formation of male organs and gametes (Charlesworth & Charlesworth, 1978). This means that they invest more resources in female organs, and the resulting qualitative and quantitative shifts in traits between females and hermaphrodites such as length of style (see references above) but also higher seed sets and overall offspring fitness (Miller et al., 2015; Oak et al., 2018; Shykoff et al., 2003; Spigler & Ashman, 2012) are not necessarily the result of sexual selection and female choice *per se* but rather reflect differences in resource allocation between sexual forms. Nevertheless, we believe that female choice could be explored in gynodioecious systems by comparing the stringency of female choice in hermaphrodite vs female individuals of the same population, for example.

2.3 Coevolution between pollen tube growth and style length: a runaway selection scenario?

Runaway selection is a process by which male traits evolve under directional and recurrent selection by females, explaining exaggerated male attributes in birds for example (Fisher, 1930). In a runaway selection scenario, male trait and female preference are not only genetically correlated in a natural population, but they also coevolve (Laura M. Travers, 2017): a male trait is selected under a certain female preference and thus spreads in the population, and this establishes selection on stricter female preference to evolve, leading to an evolutionary feedback loop. This may well apply to pollen tube growth and style length: a longer style may select for faster pollen tube growth; as the alleles responsible for more performant pollen increase in frequency in the population, an even longer style may select for the most performant among the newly selected

pool of males and may thus provide a selective advantage to females, etc. In other words, we expect pollen tube growth and style length to coevolve. While this remains to be demonstrated, these two traits are at minimum genetically correlated, as for example, the same outcrossing species have both faster pollen tube growth and longer style compared to their selfing relatives in *Mimulus* or *Clarkia* genera (Diaz & Macnair, 1999; Mazer et al., 2018b; Runions & Geber, 2000). Unravelling the genomic architecture of these two traits and screening genomes may help to detect coevolution between underlying genes. Gene-gene coevolution is a current challenge in population genomics approaches (Märkle et al., 2021), which we further explore in the last section of this article.

3. MATHEMATICAL MODELLING OF COEVOLUTION BETWEEN POLLEN TUBE GROWTH AND STYLE LENGTH

To further evaluate the plausibility of a pollen tube growth/style length coevolution, we carried out stochastic individual centred modelling (Supporting Information Methods S1) where pollen grains of different performance (tube growth velocity) are selected according to style length (the longer the style, the less stochasticity in fertilisation success) and tested whether this simple criterion was enough to drive an evolutionary response towards faster pollen tube growth and longer style length. In animals, female choice (in our model via style length) is assumed to be costly (Pomiankowski, 1987). For this reason, we also assessed the effect of the cost of style length on style length and pollen tube growth evolution. To do so, we assumed that the cost of style length has consequences on the number of ovules (longer style = less ovules) due to resource limitations number is limited by its cost. We then tested the effect of different parameters on this coevolution: the extent of the cost of female choice, the mating system (selfer vs outcrosser), the ratio between the number of deposited pollens on the stigma and the number of available ovules and the diversity of different pollen donors by pollination. Finally, we assessed whether "good genes" selection (fitness of the progeny correlated with pollen tube growth or style length) can enhance the coevolution between pollen tube growth and style length.

It is worth mentioning that a model has previously studied the coevolution of pollen tube growth and style length in plants (Lankinen & Skogsmyr, 2001). This previous model showed that, theoretically, the two traits could co-evolve under the theory of selection for "good genes". But important theoretical differences distinguish our two models. First, in our model and contrary to the other one (Lankinen & Skogsmyr, 2001), pollen tube growth is not a trait that depends on the viability of individuals, although it can be a proxy. In other words, we considered that pollen tube growth can be selected without selection for the "good genes" (intra- and intersexual selection for mating alone). Secondly, we considered the possibility that the length of the style could also be a proxy for the survival capacity of an individual, which was not the case in the previous model. In addition, our model analysed new factors that may influence the co-evolution between pollen tube growth and style length, such as the mating system or the number of pollen donors per pollination, (which is clearly distinguished from the ratio between the number of pollen grains deposited on the style and the number of available ovules). Finally, from a conceptual point of view, our model being stochastic, and not deterministic, it allowed us to evaluate the dynamics of the two traits but also of each gene over time, for different sets of parameters. This allowed us to make predictions about expected phenotypic and genetic variations on population. Because stochastic models create virtual populations in each simulation, these models also allow us to estimate power of detection for phenotypic and genotypic signals of these coevolution on samples conceivable in an experimental procedure. On the other hand, stochastic models limit the amplitude of the parameters studied.

In more detail, our model simulates the allelic variation and phenotypic value of two genes over the generations, named V and L, determining respectively the velocity of the pollen tube and the length of the style (arbitrary units). We have also voluntarily added one neutral gene, *Nt,* which have, at the start of each simulation, the same characteristics as V and L, but have no effect on the individuals. At the start of each simulation, each diploid hermaphrodite individual has two randomly chosen alleles out of the ten possible ones of V (V_x, V_y) and of L (L_x, L_y). We then allowed a mutation rate to happen for both genes at every generation. Our model thus allowed us to estimate the variation in style length and pollen tube velocity as a function of the different parameters mentioned above after 1000 generations, but also to estimate the effects on the polymorphism of V and L genes.

3.1 The cost of female choice strongly hinders the synergistic coevolution between style length and pollen tube growth

We tested the effect of cost of style length (c) on the evolution of pollen tube growth and style length. The value of c means that for every arbitrary unit of style length, the plant produces a number of ovule minus c. We observed that the cost of style length on the number of ovules impacted the evolution of pollen tube growth and style size (Figure 2A and B). Indeed, if this cost was high (c=1), the size of style was null after 1000 generations (Table S2), and the average of velocity of pollen tube growth was highly decreased compared to

the case with c = 0.1 (Table S2). However, if this cost was null, the size of style and the velocity of pollen tube growth were highly increased (Table S2). Moreover, in case of low or no cost, the initial optimum is reached at 20 generations, whereas this optimum was never reached if the cost is high (Table S2). While this suggests that pollen tube growth and style length indeed coevolve, it also shows that even with a minimal cost, style length cannot evolve towards higher values, impacting the evolution of pollen tube growth as well.

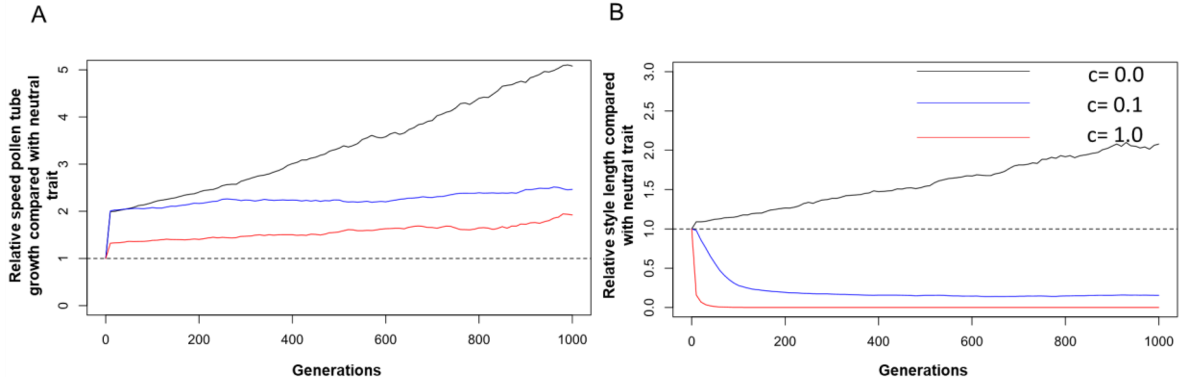


FIGURE 2. Relative value of the velocity of pollen tube growth (A) and style length (B) compared with equivalent neutral traits during 1000 generations for different levels of cost associated with style length. Simulations for allogamous crosses with r=5, s=0.3, D=5, P_L=0 and P_v=0. Black : no cost c=0, Blue: low cost c=0.1, Red : high cost c=1.

3.2 Runaway selection does happen between style length and pollen tube growth under pollen-pollen competition.

As an absence of cost of female choice seems unlikely according to the literature, for all other simulations, we fixed the cost c at 0.1 value. We then tested whether the intensity of pollen competition could affect the evolution of both pollen tube growth and style length, as expected if they coevolve. To do so, we used as proxies for male competition a) the ratio r between number of pollen deposited and number of ovules with r=1, 2, 5, 10 or 100 and b) the number D of pollen donors per pollination event with D = 1, 2, 5 or 10. We looked at pollen tube growth and style length evolution across 1000 generations under these parameters (see Supporting Information Methods S1 for more details). Regardless of the value of r, the average pollen tube growth in the population quickly reached the initial optimum (Figure1B; 20 generations; Supporting Information Table S2), except for the less biased pollen/ovule ratios (for a ratio r=1, the necessary time was 120 generations; Table S2). At the end of the 1000 generations, the averages velocity of pollen tube growth and the style length (Figure 1.C and Table S2) were similar whatever the ratio considered. Thus, the ratio of the number of pollens deposited on the style to the number of available ovules appeared to have no important effect on pollen performance evolution on the long term. Moreover, for the less biased ratio, the style size appeared to decrease more quickly than for the other ratio, suggesting a synergistic coevolution between pollen and style antagonizing the decrease in style length caused by its fitness cost. Similarly, we observed that, regardless of the number of pollen donors, the average velocity of pollen tube growth in the population quickly reached the initial optimum (Figure S3A; 20 generations; Table S2), except for a number of donors D = 1 (30 generations; Table S2). At the end of the 1000 generations, the selection on the velocity of pollen tube growth and on the style length compared to neutral trait (Figure S3.A and Table S2) were similar whatever the number of donor considered.

In conclusion, in our model where style length influences the efficacy of selection of pollen tube growth by male-male competition, all phenomena that modify selection on style length modify indirectly selection on pollen, suggesting a scenario consistent with runaway selection and a coevolution. Interestingly, except for early generations, the intensity of male-male competition did not significantly impact the selection on pollen performance, which quickly reached an optimum. This suggests a reduced role of the intensity of intrasexual competition on pollen performance evolution, and this is consistent with a meta-analysis showing that this ratio does not impact the degree of non-random mating (Ruane, 2009).

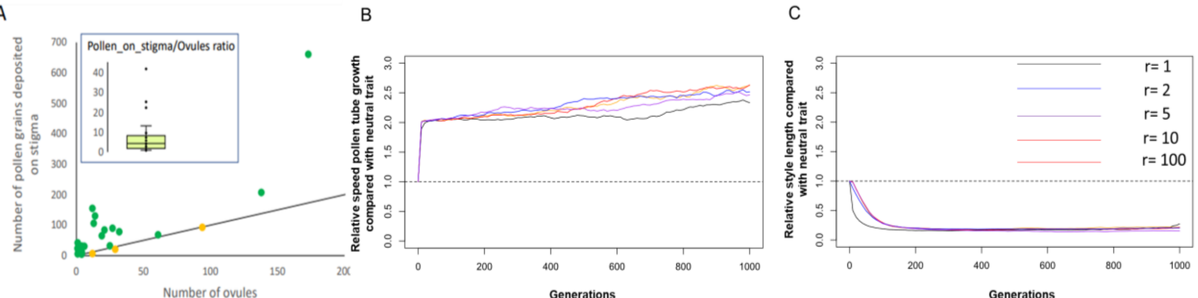


FIGURE 1. Pollen-pollen competition for mating is ubiquitous and influences pollen tube growth and style length evolution. (a) Relationship between number of pollen grains deposited on the stigma and the number of ovules (adapted from Gong & Huang, 2014). Each dot represents one species. The black line shows a 1:1 ratio between pollen grains and ovule number, meaning virtually no competition for mating between pollen grains. Green dots show species with more pollen deposited than available ovules, and orange dots show the opposite trend. The insert shows the ratio between deposited pollen and ovule number from the same data. (b) and (c) Relative value of the velocity of pollen tube growth (b) and style length (c) compared with equivalent neutral trait during 1000 generations for different ratios between number of pollens on pistil and number of ovules available. Simulations for allogamous crosses with s=0.3, D=5, c=0.1, P_L=0 and P_v=0. Black: r=1, Blue: r=2, Purple: r=5, Orange: r=10, Red: r=100.

3.3 Pollen-style coevolution is enhanced under "good genes" selection

Pollen tube growth velocity, relying on metabolic mechanisms (see next chapter), may be correlated to general growth capacities and therefore may be a proxy for the fitness of the progeny. Females selecting for faster pollen tubes may therefore select for higher fitness progeny, providing an advantage to females, a scenario known as "good genes" sexual selection (Byers & Waits, 2006). A consequence would be that pollen tube growth and style length coevolve faster under this scenario. To test this hypothesis, we compared stochastic simulations in which the velocity of pollen tube growth and the probability of survival of offspring were correlated or not (P_V= 0.1, Figure 3). We observed that if the velocity of pollen tube growth and the probability of survival of offspring were correlated, both the style length and the speed of pollen tube growth increased compared to the absence of correlation (Figure 3). The increase in style length was however limited, likely due to the fitness cost imposed on this trait. Thus, the mean velocity of pollen tube growth is higher than the initial optimum in this case, easily explained by quick selection of rare advantageous mutant (Table S2). We interpret these results as a more efficient selection both on pollen tube growth and style length, as more performant pollen will have more fit progeny, and females selecting for these males (via style length) will be positively selected as their progeny will also be fitter, and in turn the evolution of longer style length will increase the selection for faster pollen tubes, etc. In other words, under "good genes" selection, the coevolution between pollen tube growth and style length is enhanced. Interestingly, this coevolution was also enhanced when style length was a proxy for the progeny fitness, suggesting a possible "good genes" selection on the female side (P_L= 0.1, Figure 3). Moreover, the style length increased in this case, despite its cost.

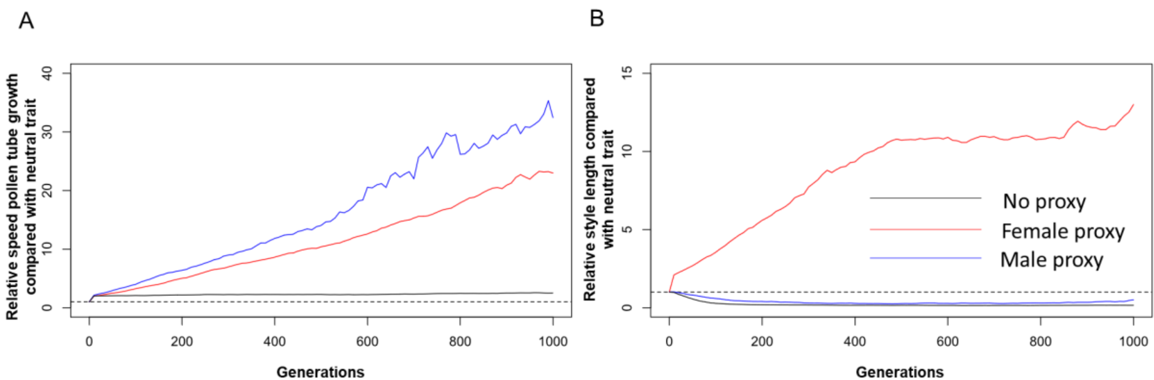


FIGURE 3. Relative value of the velocity of pollen tube growth (A) and style length (B) compared with equivalent neutral traits during 100 generations if the velocity of pollen tube growth or the style length are proxy of fitness. Simulations for allogamous crosses with s=0.3, r=5, D=5, c=0.1. Red : P_v=0.1; Black: P_L=0 and P_v=0; Blue: P_L=0.1

3.4 Sexual selection and consequent pollen-style coevolution are relaxed in selfers

Pollen-pollen competition is expected to decrease in the selfers (Cutter, 2019). This should decrease the intensity of selection for the velocity of pollen tube growth and style length. To test this hypothesis, we compared stochastic simulations for allogamous plants with simulations obtained for autogamous plants (Figure 4).

We observed that, as an expected result of selection, the value of pollen tube growth increased strongly for the allogamous plants (Figure 4A, red lines). At the 1000th generation, at the opposite of the allogamous populations, the mean pollen tube growth did not reach the initial optimal value in the autogamous populations (Figure 4A; Supporting Information Table S2). Moreover, the equilibrium for the mean relative style length was obtained more quickly (<=40 generation; Figure 4B; Table S2) in the autogamous population compared to the allogamous one. This is likely explained by stronger selection on pollen tube growth induced by male-male competition in the allogamous scenario, increasing style length as a coevolutionary response, and counteracting the selection against style length related to its fitness cost.

Overall, the fact that pollen tube growth evolved to be lower in selfers compared to outcrossers, and the quick fixation of short styles are consistent with relaxed male-male competition and consequently relaxed selective pressure on style length (relaxed coevolution).

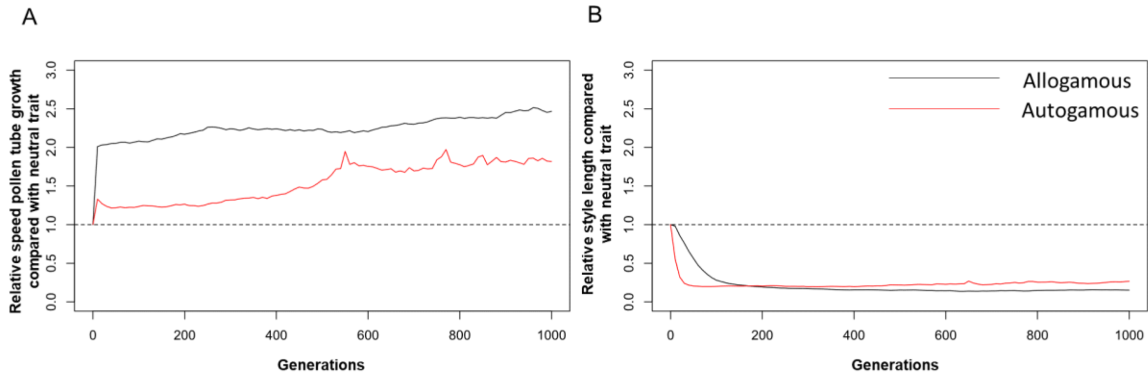


FIGURE 4. Relative value of the velocity of pollen tube growth (A) and style length (B) compared with equivalent neutral traits during 1000 generations with mating type. Simulations for crosses with s=0.3, r=5, D=5, c=0.1, P_L=0 and P_v=0. Red : autogamous, Black: allogamous.

4. UNDERLYING GENES: WHICH EVOLVABILITY AND POTENTIAL FOR GENE-GENE COEVOLUTION?

In this section, we first review the literature about the molecular mechanisms underlying male competitive traits through their effect on pollen tube growth and female choice through their effect on style length. We then evaluate whether and how sexual selection may act on these mechanisms, and how to detect this using modern approaches.

4.1 Molecular mechanisms underlying pollen tube growth

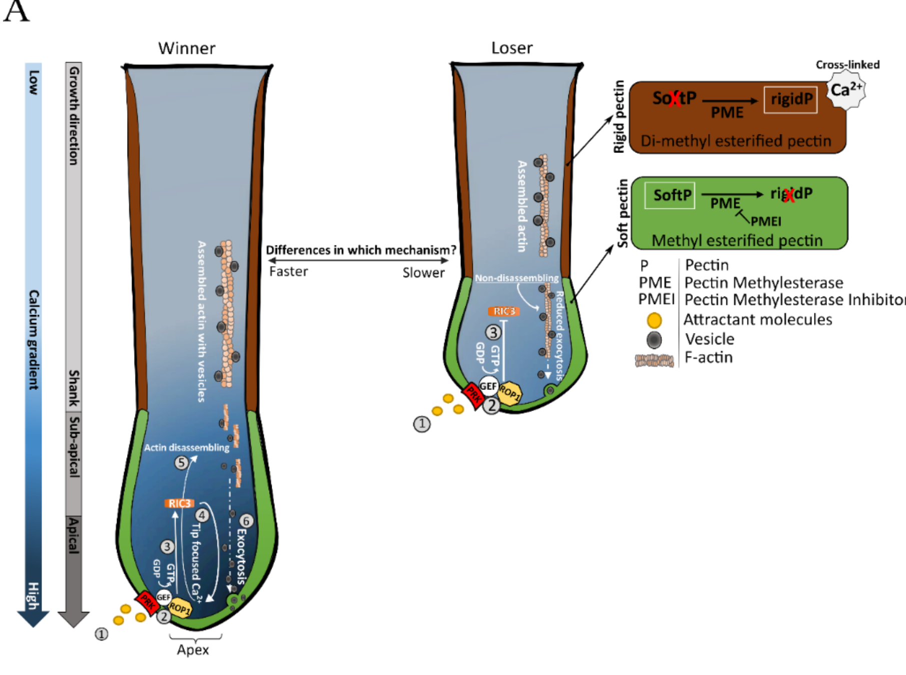
Pollen tubes function as a vehicle to carry the male gamete cells from the pollen grain to the female gametophyte during double fertilisation. To fulfil this task, following pollen deposition, the pollen grains adhere to the surface of the stigma and receive water and signals from the papillary cell to trigger hydration and germination (reviewed in Hiscock & Allen, 2008). After germination, the compatible pollen grains extrude a tube from germ pores and elongate it through the extracellular space in the transmitting tract of the pistil. Depending on the species, the growth rate of pollen tubes can reach up to tens and even hundreds of micrometers per minute (e.g. 12-18 µm/min in lily and 168 µm/min in maize) (Barnabas, & Fridvalszky, 1984; Messerli & Robinson, 1997).

The pollen tube shows polar tip growth and is controlled by cytoplasmic streaming during synthesis of the new cell wall at the tip region (De Win et al., 1999; Lovy-Wheeler et al., 2007). In particular, stiffness on the side (shank region) and extensibility of the cell wall at the tip play a significant role in the polar tip growth of the pollen tube (reviewed in Guo & Yang, 2020). It has been shown that the degree of pectin esterification plays a crucial role in determining the cell wall properties (Voragen et al., 2009; Wu et al., 2018). During the tube elongation, Golgi-secreted soft methyl-esterified pectin is loaded into Golgi-derived vesicles and transported to the tip region via actin filaments (Harholt et al., 2010; Jiang et al., 2005; Figure 5a). Vesicles are then excreted by exocytosis from the cytosol to the plasma membrane

to maintain a soft expandable zone at the tip (Geitmann et al., 1995; Li et al., 1994). Following the tip elongation, a major part of pectin is gradually de-esterified by the activation of pectin methylesterase (PME) (Bosch & Hepler, 2006; L. Jiang et al., 2005). Demethyl esterified pectin is then cross-linked with calcium ions, increasing its rigidity and contributing to the mechanical stability and integrity of the pollen tube at the shank region (Catoire et al., 1998; Harholt et al., 2010; Steinhorst & Kudla, 2013; Zheng et al., 2019).

Once the pollen tube arrives in the style, the female tissues start to provide nutrients and signals to guide growing pollen tubes toward the ovary (preovular guidance). This direction is guided by attractant molecules secreted from stylar Transmitting Tissue Epidermis (TTE) (Mizuta & Higashiyama, 2018; Pereira et al., 2021). For example, small cysteine-rich peptides (CRPs) and transmitting tissue-specific arabinogalactan proteins (TTS) secreted by the stylar transmitting tract have been shown to play an important role in pollen–pistil communication (Cheung et al., 1995). These include Stigma/style Cysteine-rich Adhesin (SCA) peptides in Lily (Chae et al., 2007b; Mollet et al., 2000), and LTP5 (a SCA-like LTP) peptides in *Arabidopsis* (Chae et al., 2010). Attractant molecules secreted from stylar TTE has been proposed to bind initially at the tip region of pollen tubes and are then travel through the cytoplasm of the tube cell by endocytotic pathway (Kim et al., 2006). It is likely that attractant molecules captured by the receptors at the tip region of pollen tube are the signal molecules that will be involved in maintaining the reorientation and cell polarity of the pollen tube during the paternity journey (Chae et al., 2009; Kim et al., 2021). Membrane-localized pollen receptor-like kinase (PRK), one of the receptors, is located at the tip of the growing pollen tube (Chang et al., 2013; Takeuchi & Higashiyama, 2016; Yu et al., 2018). This receptor perceives signals from the extracellular matrix of stylar tissue (attractant molecules) and activates the tip-localized ROP GTPases (Berken et al., 2005; Gu et al., 2006), via guanine exchange factors (GEF). ROP GTPases play an important role during the coordination of pollen tube growth by promoting both tip localized F-actin assembly and disassembly via the coordination of Ca^{2+} influx channels (Figure 5A). The influx of Ca^{2+} ions from the external medium into the cytosolic matrix of tube cell plays a crucial role in pollen tube tip growth, and is achieved by plasma membrane-localized Ca^{2+} channels (Chang et al., 2007; Frietsch et al., 2007; Michard et al., 2011). Hereat, the intracellular concentration of Ca2+ shows a tip-focused gradient with a higher concentration at the tip apex and a decrease along the axis of the pollen tube (reviewed in Hafidh et al., 2016). Ca^{2+} is notably involved in F-actin disassembly, and Ca^{2+} accumulation at the tip of the pollen tube ensures the disassembly of tip localized F-actin (H. J. Wang et al., 2008; Yokota et al., 2005). As mentioned above, actin plays a key role during the intracellular movement of secretory vesicles, transporting molecules such as pectin along the axially aligned actin throughout the elongated tube. Disassembled actin filaments together with cytoplasmic streaming transport leads to the release of secretory vesicles to the tube apex and the exocytosis of molecules required for apical tube growth (Qu et al., 2017).

In summary, pollen tube growth depends on intrinsic pathways, such as vesicle transport and cytoskeleton, cell wall modification, and Ca^{2+} signaling, but also on extrinsic factors, i.e. signaling peptides secreted by female tissues (Figure 5A). The former pathways may underlie intrinsic male traits involved in male-male competition while the latter may be responsible for female choice through variation in chemical composition of the pistil (reviewed in Tonnabel et al., 2021). Mutation or expression variation of genes involved in the pollen tube growth intrinsic pathways may lead to natural variation in pollen performance and thus be a substrate for sexual selection.



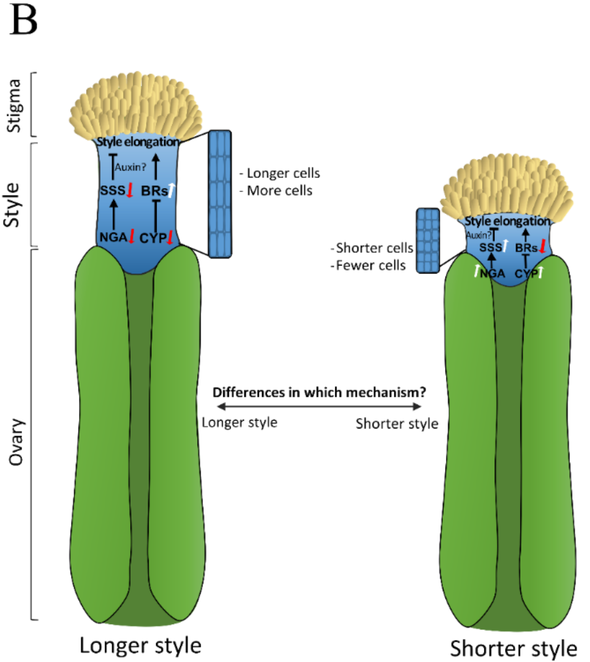


FIGURE 5. Molecular mechanisms underlying pollen tube growth and style length, and candidates under sexual selection. (A) Molecular mechanisms of pollen tube growth. The calcium gradient scale at the left represents the calcium distribution in the pollen tube cytoplasm, calcium concentration gradually increases from the shank zone through the apical zone. The second gray scale represents the different growth zones of the pollen tube. Brown box at the right describes the formation of demethyl esterified pectin (a rigid form of pectin) after cross-linked with calcium ions at the shank zone. The green box at the right describes the formation of Golgi-secreted methyl esterified pectin (a soft form of pectin) at the sub-apical and apical zone. Winner pollen tube; (1) Perception of pollen tube attractants secreted by stylar tissue by the tip-localized pollen-specific receptor-like kinase PRK. (2) PRK-induced guanine exchange factor (GEF) activates the tip-localized ROP GTPases1 (ROP1). (3) Activated ROP1 positively regulates the CRIB-motif proteins 3 (RIC3) and (4) increases the tip-focused calcium level by coordinating the influx of Ca2+ ions. (5) High concentration of calcium at the tip region leads to disassembling of tip localized F-actins and releases the essential Golgi-secreted vesicles from it is actin cage. (6) Released vesicles streamed towards to apical zone and exocytosis through the plasma membrane (PM) of the growing pollen tube apex. Loser; (1) Perception of pollen tube attractant cysteine-rich peptide (LURE) by the tip-localized pollen-specific receptor-like kinase PRK. (2) PRK-induced guanine exchange factor (GEF) activates the tip-localized ROP GTPases1 (ROP1). (B) Molecular mechanisms of style length. Two possible molecular pathways controlling style length

are shown, one (left) evidenced in *Arabidopsis* and the second one (right) in primrose. In *Arabidopsis*, NGATHA (NGA) transcription factor positively regulates the expression of three angiosperm-specific genes (SSS; STIGMA AND STYLE STYLIST genes 1–3), and these three genes repress cell elongation and proliferation of the style. We propose than natural variation in style length may be underlain by variation in expression of these genes (low expression level of genes is shown by red arrows and high expression by white arrows). In primrose, brassinosteroids (BRs) are degraded by CYP734A50 gene (CYP), only present in the "short style" morph, leading to reduced elongation of the style. CYP734A50 is absent in the "long style" morph, where BRs can accumulate and leads to the elongation of the style. We here propose that natural variation in style length out of the primroses taxon, may be underlain by expression variation of the CYP gene, rather than a presence/absence.

4.2 Molecular mechanisms underlying style length

The gynoecium, which includes the stigma, style and ovary, is a complex organ that is essential for plant sexual reproduction. Gynoecium development encompasses several stages which require different tissue formation with specific identities. Style is one of these specific tissues, surrounded by chlorenchyma tissue, and a distinctive epidermis, unifying the ovary and stigma (Figure 5B). As it serves as a tunnel for pollen tube growth, its length plays a crucial role in pollen tube competency and guidance towards the ovules (Okuda et al., 2009, 2013).

The molecular pathway through gynoecium development and style differentiation events has been mostly characterized in the model plant *Arabidopsis thaliana*. In this species, near the time of ovary closure (known as floral stage 9-11; Smyth et al., 1990), style formation initiates with the vertical extension of tissues located at the top of the ovary. The formation of these newly synthesized tissues is achieved by a combination of cell division and cell elongation process that are regulated by complex interactions between transcription factors (TF) activity and hormone dynamics (Girin et al., 2011; Larsson et al., 2014; Magnus Eklund et al., 2010; Moubayidin & Østergaard, 2014; Reyes-Olalde et al., 2017).

Auxin is one of the phytohormones that are intricately involved in the gynoecium patterning by specifying the tissue orientation along the apical/basal axis (Deb et al., 2018; Zúñiga-Mayo et al., 2019). Proper auxin distribution at the top of the gynoecium primordia is mediated by auxin polar transport and it is a crucial step for style specification and development (Larsson et al., 2014; Moubayidin & Østergaard, 2014). Auxin transport notably ensures the transition from bilateral to radial symmetry that is required for radial style initiation (Moubayidin, 2015). The next stage includes style differentiation and elongation. At this stage, a network of transcription factors controls style development (Simonini & Østergaard, 2019). Among these transcription factor, the *NGATHA* (*NGA*) genes are more specifically involved in style formation and style length (Alvarez et al., 2009, 2009; Kuusk et al., 2002; Li et al., 2020). This control is achieved via the activation of three angiosperm-specific genes (*SSS*; *STIGMA AND STYLE STYLIST 1–3* genes) by *NGA* that are mainly involved in gynoecium development (*SSS1* and *SSS3* are predominantly expressed in the style) in *Arabidopsis* (Li et al., 2020). The silencing of these triple genes resulted in gynoecium with longer styles and vice versa, smaller styles when they are overexpressed, suggesting that *NGA*, via the activation of *SSS* genes, represses style elongation, and this is done by controlling cell proliferation and elongation (Li et al., 2020; Figure 5b). Interestingly, pollen tubes grow slower on a style overexpressing *SSS* genes, suggesting a potential role of *SSS* genes in female selection of mating partners beyond the control of style length (Li et al., 2020).

In addition to the role of auxin, other phytohormones have been involved in style development, such as cytokinins and brassinosteroids (Reyes-Olalde et al., 2017; Zúñiga-Mayo et al., 2019). Especially, brassinosteroids have been involved in heterostyly in Primula species, which is the coexistence of "short style" and "long style" morphs thought to have evolved to prevent self-fertilization (Huu et al., 2016; Vogler et al., 2014). In these species, the reduction of style length in short-styled plants is controlled by the activation of *CYP734A50* gene, a gene only found in the "short style" morph haplotype (Huu et al., 2016). This gene degrades brassinosteroids, which limits cell expansion and style elongation. *CYP734A50* mutants also have longer style, confirming the negative impact of this gene on style elongation (Huu et al., 2016).

In conclusion, while the pathways leading to style elongation are far from being fully understood, it appears that style length is underlain by different molecular bases depending on the taxon, suggesting a case of convergent evolution.

4.3 Which molecular candidates under sexual selection and male-female coevolution?

The genes behind sexual selection in plants are largely unknown. A lot of efforts have focused on the molecular interactions between pollen tube and female tissues (Tonnabel et al., 2021), but while these mechanisms bear potential to be under sexual selection, they may simply be involved in general male-female recognition during sexual reproduction. To us, realistic candidate genes to be under sexual selection should have all the

following properties: 1/ have a demonstrated function in a trait involved in intrasexual competition (such as pollen germination or tube growth) and/or intersexual selection (such as stigma width or style length); 2/ show evolvability, by expression or sequence differences between systems with divergent sexual selection (selfing vs outcrossing lineage, for example); 3/ show genetic signatures of selection and/or coevolution in natural populations. Generally, a reduction in polymorphism in genes under sexual selection is expected as a result of positive or purifying selection (Walsh & Charlesworth, 1992). Both positive and purifying selection are expected to be more effective than in sporophytic genes, due to the exposure of all new mutations, even recessive ones, in genes expressed at haploid stages (Charlesworth & Charlesworth, 1992; Gerstein & Otto, 2009; Haldane, 1933). If a gene has an impact on pollen performance or female choice, the reduction in polymorphism should be accentuated. With these features altogether only, a gene may qualify as "sexual selection gene".

While this means that virtually no sexual selection gene has been identified to date in plants, in the next section, we will evaluate in more detail the candidate mechanisms that may be involved in the evolution of pollen tube growth and style length under sexual selection.

Mutations on genes affecting a wide range of traits are likely to be maladaptive, as they may have pleiotropic side effects. Consequently, selective responses are thought to happen via mutations on low-pleiotropy genes (Connallon & Hall, 2018). In the case of sexual selection, both pollen tube growth and style length rely on cellular and molecular processes that are general (vesicle transport, calcium signaling, phytohormone signaling; see above). Therefore, mutations on the coding sequence of such genes are likely to have deleterious pleiotropic effects, and are unlikely candidates as sexual selection genes. Pleiotropy might nevertheless be alleviated by mutations affecting tissue-specific regulatory elements controlling the expression of related genes: the mutational effect may therefore occur in a restricted space (tissue) and time (developmental) window, i.e. pleiotropy would be low. This view is supported by the mutations responsible for petal size reduction as a result of the transition to selfing ("selfing syndrome") in *Capsella rubella*, affecting the regulatory element of general growth regulator genes rather than their coding sequence (Sicard et al., 2016; Woźniak et al., 2019), leading to gene expression differences restricted to petal organs. When it comes to the traits of interest in this review (pollen tube growth and style length), indirect evidence suggests indeed that sexual selection may act on expression levels in a given sexual organ rather than on the protein sequence: the transcriptome of developing flower buds, where floral organs are being formed, showed significant expression differences between selfing and outcrossing *Collinsia* Nutt. species for gene functions enriched in auxin signaling notably, which is likely to cause organ size differences, potentially including style length (Frazee et al., 2021). More direct evidence is nevertheless required to test whether sexual selection act on regulatory elements, and thus on sexual gene expression patterns, influencing style length and pollen performance differences in natural populations.

The assumption of pleiotropic effects of mutations in sexual genes, and therefore reduced potential for evolvability, is also partially contradicted by the finding that pollen-specific genes are under stronger purifying selection, but also show higher rate of adaptive substitutions, compared to sporophytic genes in *Capsella grandiflora* (Arunkumar et al., 2013). Similar observations were found in *Arabis alpina* (Gutiérrez-Valencia et al., 2022), and this study also found that the efficacy of purifying selection increased with outcrossing specifically for genes expressed in pollen, consistent with sexual selection acting on these genes. These two studies however considered together all pollen-specific genes (Arunkumar et al., 2013) or even simply “expressed in pollen” (and thus potentially also in other tissues; Gutiérrez-Valencia et al., 2022), and therefore the impact of sexual selection specifically on genes involved in pollen tube growth remains unknown.

Beyond the action of sexual selection on gene (regulatory) sequences, a remaining question is whether gene-gene coevolution can underlie male-female coevolution in plants, and whether it can be detected with current tools. Cases of gene-gene coevolution have been reported in the case of a physical interaction between proteins, for example between *ZP3* and *ZP3R* human genes involved in gamete recognition (Rohlfs et al., 2010). In the case of pollen tube growth/style length coevolution, we expect the gene-gene coevolution not to be driven by direct molecular interactions but instead to be caused by trait-trait coevolution (runaway selection), i.e. to occur between genes involved in different molecular pathways. In this context, the coevolution may happen at the gene sequence level or instead, the coevolution may target regulatory elements leading to a coevolution of expression levels between certain pollen and style genes, involved in pollen tube growth and style length respectively (Figure 6).

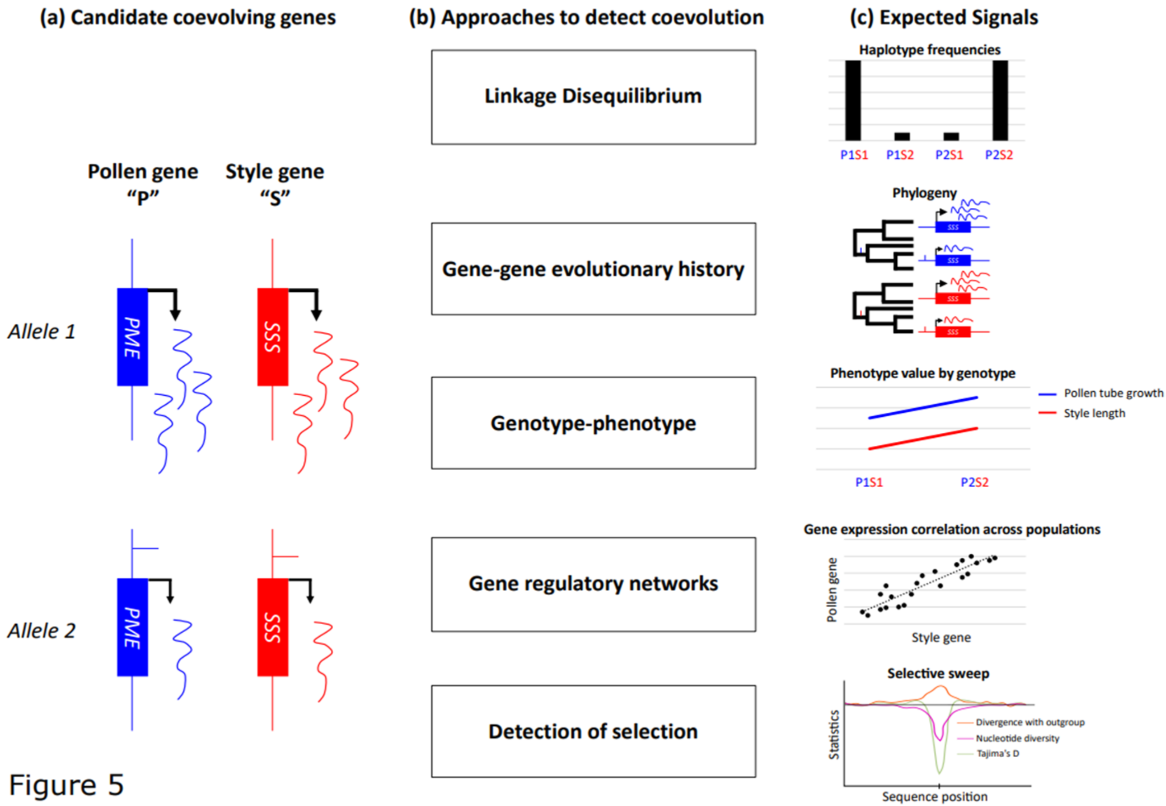


FIGURE 6. Approaches and expected signals of coevolution between pollen tube and style genes. (a) As an example, a pectin methylesterase encoding gene (*PME*) for pollen tube growth and *STIGMA AND STYLE STYLIST* (*SSS*) for style length are shown as genes potentially coevolving. Two alleles are shown, allele 1 leading to slower pollen tube growth and shorter style length (higher expression of *PME* and *SSS*, both being negative regulators of each trait respectively) and allele 2 leading to faster pollen tube growth and longer style (mutation in the promoter leading to lower expression). (b) Sets of approaches required to detect coevolution between these genes. (c) Expected signals for each of these approaches. P1/P2: Allele 1/2 of the *PME* gene; S1/S2: Alleles 1/2 of the *SSS* gene

While detecting gene-gene coevolution remains challenging, the combination of functional and population genomics may allow the identification of genes underlying runaway selection in plants. The search for coevolving loci under sexual selection can be based on several criteria: 1/ this coevolution should be beneficial for the male and female function, i.e. these loci should show signs of positive selection; 2/ under this assumption, selection should favor certain combinations of alleles at the coevolving loci, so these loci should show signs of genetic linkage even though physically distant (Rohlfs et al., 2010); 3/ similarly, coevolving loci should show a common evolutionary history that does not necessarily follow the evolutionary history of the populations/lineages/species studied (Galtier & Dutheil, 2007). The approaches to be used to test these expectations may depend on whether the coevolution happens between regulatory elements (coevolution of expression levels) or between coding sequences.

Regarding screening for selection footprints, positive selection is expected to reduce local genetic diversity at the selected loci, and therefore Tajima's D has been used to identify such a footprint (Oleksyk et al., 2010). The advantage of this approach is that it can work on non-coding sequences, however, reduced local genetic diversity is also a sign of purifying selection and therefore, other approaches such as Fst outlier screens may be necessary (Oleksyk et al., 2010). The footprints of positive selection could be evaluated as departures from neutral expectations of genetic diversity at a particular genomic region. For example, the Hudson–Kreitman–Aguadé (HKA) test (Hudson et al., 1987) uses a chi-square statistic to assess whether genomic regions have higher density of polymorphic sites when compared with a putative neutral genomic background, whereas Tajima's D (Tajima, 1989) measures the distortion of allele frequencies from the neutral site frequency spectrum under a model with constant population size. However, these approaches are extremely sensitive to confounding effects of nonequilibrium demography and population structure (see Fijarczyk & Babik, 2015 for review about detection of balancing selection). We recommend the use of more robust methods that are less sensitive to demographic

parameters, such as the summary statistics (Bitarello et al., 2018; Siewert & Voight, 2017) and model-based approaches (Cheng & DeGiorgio, 2019, 2020; DeGiorgio et al., 2014). For example, the method developed by Cheng and Degiorgio (2020) was found to be robust in detecting purifying selection on genes involved in gamete functionality but also in other important functions in humans. Finally, using estimation of the ratio of nonsynonymous to synonymous substitutions (ω-ratio), study have investigated the evolutionary dynamics of genes assumed to be involved in sexual selection as to determine if the proteins are adaptively coevolving (N. L. Clark & Aquadro, 2010). These methods, aimed at screening for footprints of selection, can support the search for coevolving genes in combination with other methods mentioned above, as under sexual selection, we expect a selective sweep to happen for each gene of the coevolving pair.

The detection of gene-gene coevolution may be done by finding genetic linkage between potentially coevolving genes with linkage disequilibrium (LD), which measures the probability of two alleles being inherited together. While a high LD can be explained by physical linkage between two loci (reduced probability of recombination between them), it is used to detect epistatic selection between loci that are physically unlinked (Boyrie et al., 2021). For example, composite LD coupled with genotype association were used to identify the coevolution of *ZP3* and *ZP3R* human genes involved in gamete recognition (Rohlfs et al., 2010). However, there are a number of significant weaknesses in measuring the linkage disequilibrium between two sites. The first is that these measurements are very sensitive to allelic frequencies, so much that it is generally recommended in good practice not to consider frequencies below 5 or 10%. Even if the standard measures D′ (Lewontin, 1964) or r^2 (Hill & Robertson, 1968) are intended to be less sensitive to allelic frequencies, it is not feasible to estimate the linkage disequilibrium between a site and a fixed site. Furthermore, even if we consider that co-evolving sites are not fixed, the measurement of linkage disequilibrium is sensitive to a large number of parameters, such as demographic parameters or recombination rates. Thus, a thorough knowledge of recombination rates and demographic history is necessary before any estimation can be made. All these limitations, and many others, are clearly detailed in the review by Gaut & Long, 2003. In the case of coevolving coding sequences, LD may be used as it has the advantage of identifying potential coevolution SNP-by-SNP, allowing the identification of fine-scale coevolving alleles (Rohlfs et al., 2010).

More holistic approaches are based on reconstructing the evolutionary history of candidate coevolving genes, assuming that their evolutionary history should be common if they coevolve and also likely diverges from the evolutionary history of the rest of the genome (Figure 6; Galtier & Dutheil, 2007). Most of these methods consist of comparisons of the phylogenies, sometimes completed by functional data about the genes, to finally detect co-occurring changes in a phylogeny. One weakness of these methods, however, is that they typically focus on pairs of genes, while coevolution could occur between groups of more sites. Moreover, the detection of these co-occurring changes in the phylogenies between genes are sensitive to probabilistic modelling used.

Also, as the coevolution between regulatory elements may lead to the coevolution of expression levels between genes, elucidating gene regulatory networks may allow, indirectly, to find coevolving genes as their expression may be correlated (Figure 6; Fraser et al., 2004). In plants, an example of a well-characterised coevolution process between regulatory elements and genes implicated on pollen competition mediated by style has been empirically described in *Arabidopsis halleri* and *Arabidopsis lyrata*, between the microRNAs responsible for pollen expression of sporophytic incompatibility alleles (*S*-alleles) and these alleles themselves (Durand et al., 2014). This study explained, by a network based on the expression pattern of each microRNAs linked to each *S*-allele, the pollen expression of each allele in many combinations. To establish this type of network, it is necessary to have expression data for these regulatory elements, which can be of different genetic types (sRNA, microRNA, transcription factors…), the expression data for potentially regulated genes, the genotypes of each of them in the individuals analysed, and this for a large number of replicas, and tools for predicting interactions between the regulatory elements and the regulated elements.

At last, screening genomes for footprints of coevolution may be only a way to identify candidate genes, and ultimately, these coevolving candidate genes require to be functionally validated by searching genetic correlations with male and female function by QTL/GWAS mapping. Typically, this method is based on the combined analysis of molecular information and a quantitative trait in segregating progeny, in order to statistically test the link between genetic variation and phenotypic variation. However, an important limitation of the QTL approach is that it requires many crosses over a large number of generations in order to identify the genomic regions linked to a trait as precisely as possible. The GWAS method allows more easily the analysis of polymorphism at the population level. However, it is sensitive to population structure, and the genes detected remain dependent on the test models used (Tam et al., 2019). In addition, both methods remain limited for the detection of genes implicated on complex traits. Finally, functional methods, such as CRISPR knockout mutations or genetically engineered plants for example, could be used to validate candidate

genes involved in specific phenotypes and interactions between candidate genes (Park et al., 2019) but these methods are tedious and can only test a limited number of candidate genes in coevolution.

5. CONCLUSIONS

In this review, we propose, and show *in silico*, that the coevolution between male and female traits under sexual selection is possible in plants. While the molecular mechanisms involved in these different traits start to be elucidated, the genes and functions under sexual selection in plants remain virtually unknown. Finally, while genomic tools to detect gene-gene coevolution exist, they have not been used to detect male-female gene coevolution in plants yet, probably because sexual selection itself has been largely disregarded in plants. This phenomenon has nevertheless gained interest in the recent years, which opens a whole population and functional genomics field to explore.

AUTHOR CONTRIBUTION

OI and CLP planned and conceptualized the article. OI and MS performed the literature survey. ALV performed the mathematical modelling. OI, ALV, MS and CLP wrote the manuscript. OI and ALV contributed equally.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY

The data that supports the findings of this study are either part of the public literature or available in the supplementary material of this article.

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SUPPORTING INFORMATION

The following Supporting Information is available for this article:

Method S1 Mathematical Modeling

FIGURE S1 Description of the relationship between genotypes and phenotypes.

FIGURE S2 Description of the steps in the stochastic model.

FIGURE S3 Variation of velocity of pollen tube growth (A) and style length (B) compared with equivalent neutral trait during 100 generations for different numbers of pollen donors.

TABLE S1 Summary of the simulations tested.

TABLE S2 Summary of results obtained in the stochastic model for each tested condition.

TABLE S3 Summary of genomic effects of selfing obtained in the stochastic model.

Method S1

Mathematical modelling

a. *Hypothesis tested*

We supposed that the velocity of pollen tube growth and style length in hermaphrodite plants could coevolved if intersexual selection depending on style length (*s*) is implicated on pollen tube growth selection. Exactly, we supposed that more the intensity of intersexual selection by female is correlated with the style length, more the female with longer style will be able to discriminate and choose better pollens and generate male offspring with higher pollen tube velocity. We suppose also that this coevolution depending on others selection pressures implicated on the selection on the style length or on the selection on the velocity of pollen tube growth. Exactly, we supposed that the style length selection is limited by the cost on ovule production (*c*), because this cost creates competition between females on the number of offspring produced (Female intrasexual selection) linked to style length. On the other hand, we supposed that this coevolution depends also on the selection on pollen tube velocity. Exactly, we supposed that the pollen tube growth selection is limited by the factors that limits the male intrasexual selection, as the mating type, the ratio between the number of pollens deposited on stigma and the number of ovule

available (r) or the number of pollen donors by deposition (D). Finally, we supposed that the selection on the two traits is also amplified if these traits are positively correlated with survival (P_L and P_V; "good gene" theory). We supposed that the effects of these different factors modified the observable phenotypic and genotypic variation in population. To test these hypotheses, we simulated variation of velocity of pollen tube growth and style length in hermaphrodite plants using individual centred stochastic simulations, depending on the type of crosses (strictly allogamous or autogamous), the ratio between pollens deposited on pistil and number of ovules available (r=1; 2 ; 5; 10 ; 100 pollens for one ovule), the number of pollen donors by deposition (D=1; 2; 5; 10), and the cost on produced ovules determined by style length (c= 0; 0.1; 1). Each simulation was performed with 100 independent replicates. Moreover, each simulation was compared with a simulation for allogamous cross with r=5, D=5, c=0.1, s=0.3 P_L=0 and P_V=0. We summarised all the simulations tested in Table S1.

b. Definition of genotypes and phenotypes analysed

In the models, we simulated variation of different genes that defined the style size (L) and the velocity of pollen tube growth (V). The two genes were genetically independent. At the initial state, all alleles for the two genes were associated with a value randomly chosen between 0 and 10. The style length of each diploid individual (L') was determined by addition of two codominant alleles (L_x, L_y). However, the velocity of pollen tube growth was determined in haploid pollen by the value of the allele transmitted, randomly chosen between the two alleles in individual (V_x, V_y).

The number of ovules produced by individuals (n_o) depending on the style length and its cost (c), following the equation [a]: $n_o = 10 - (c * L')$. Moreover, the proportion of pollens grains selected to fertilise ovules (S) depending on the style length and the intensity of intersexual selection (s), following the equation [b]: $S = 1 / (L' \times s)$. Finally, the viability of each seed (p) depending on style length and on addition of the two codominant alleles for the pollen tube growth (V'), following the equation [c]: $p = 1 + (L' * P_L) + (V' * P_v)$. P_L and P_v represented the correlations between the viability of individuals and the style length or the velocity of pollen tube growth, respectively.

To estimate the variation of each gene by selection, not drift, we compared the variation of each gene with the variation of neutral gene Nt that present the same initial parameters but did not implicate on the studied phenotypes and survival. The genotypes of each individual were recorded every 10 generations. The relationship between the genotypes and the different phenotypes are summarised in the supplementary Figure 1.

c. Populations definition

We simulated a population of 1000 diploid individuals during 1000 non overlapping generations. The population size was chosen high enough so that no neutral genes were fixed at the end by genetic drift. At the initial state, the two alleles for the L, V, Nt genes were independently and randomly chosen between the available alleles (see above) for each individual independently.

d. Life cycle

The life cycle in our simulations had four steps (Supporting Information Figure S2):

Gametogenesis: The individuals produced n_o ovules (following the equation [a]) and infinity of pollen. Each gamete presents one allele of L, V and Nt genes, randomly chosen between the two alleles present in individuals.

Syngamy: a cluster of chosen pollen was deposited on each individual (n_p). These pollen were randomly chosen between the self-pollen or pollen of D donor, randomly chosen between the other plants if the plants were autogamous or allogamous respectively. The size of this cluster depended on the ratio r multiplied by 10, the maximum of available ovules produced by individual. The cluster of pollen inside n_p that could fecund available ovules (n_s) was composed of the pollen with faster pollen tube. The size of this cluster was n_p multiplied by the proportion of pollen grains selected to fertilise ovules (S; following the equation [b]). Then, we randomly chose pollen inside n_s that fecunded each ovule of each individual (n). The seeds produced were conserved in a cluster (N_0).

The gametogenesis and syngamy steps are reiterated for all individuals in the population (N) before the next step.

Viability selection: we assumed that the survival trait p of a seed depended on the style size and on the two alleles of V gene following the equation [c].

To form the next generation of 1000 individuals (N), we computed p for all the seeds in N_0 (maximum of 10000 if each individual produced 10 seeds) and chose the 1000 seeds with higher survival trait p. If p was equal for all individuals (for example if P_V and P_L were null), we randomly chose 1000 seeds in N_0.

Mutagenesis: mutations on alleles associated with the speed of the pollen tube growth or the style size occurred after the viability selection of the *N* seeds and before the gametogenesis of each generation (Supporting Information Figure S2). the mutations occurred depending on a mutation rate $\boldsymbol{\mu}$ of 10^{e-6} for each allele of *L*, *V*, *Nt* genes. If an allele of *V*, *L* or *Nt* is mutated (V_x', L_x', Nt_x'), the new value associated with this allele was randomly chosen in a distribution of values between the ancient value (V_x, L_x, Nt_x) more or less 3.

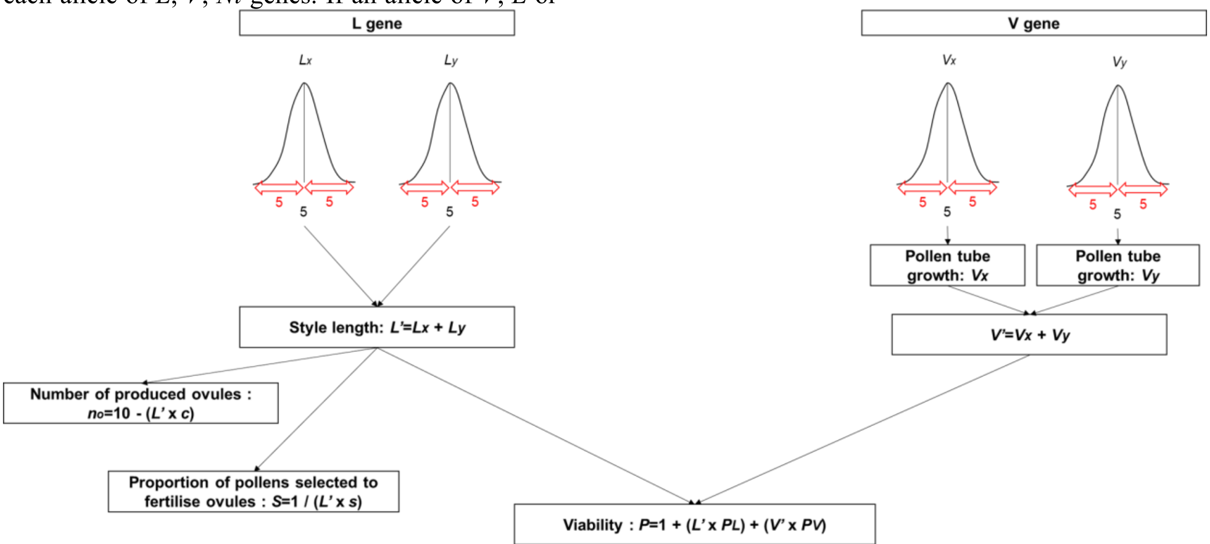


FIGURE. S1 Description of the relationship between genotypes and phenotypes. The L and V genes (top) are associated with different traits (arrows). The two alleles of the L gene (L_x,L_y) are associated with style length (L') whereas each V allele (V_x,V_y) is first associated with the pollen tube growth in haploid state and then in seed survival in diploid state (V'). Finally, L' and V' are associated with different other traits: L' is associated with the number of produced ovules by mother plant (n_o), the proportion of pollen selected based on pollen tube growth velocity (S) and the viability of seed (P), whereas V' is associated only with P. We signified each equation that linked each phenotype. c: cost on ovule production associated with L', s: coefficient of selection by style length, P_L: coefficient of correlation between P and L', P_V: coefficient of correlation between P and V'.

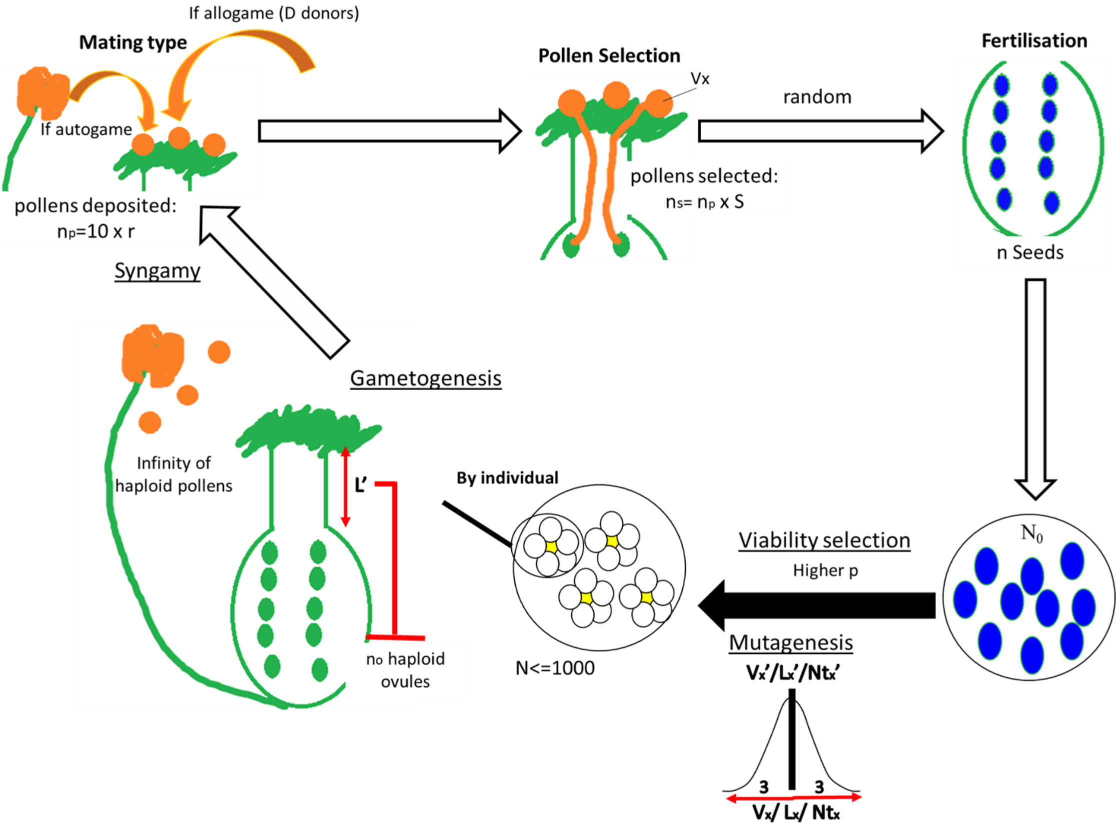


FIGURE. S2 Description of the steps in the stochastic model. For each generation, the seeds in the population depended on the seeds produced in each individual, depending on the steps on top (white arrows). The main steps of the simulations are underlined. The style length (L') of each individual was the sum of the two values linked to each allele (Lx',Ly'), and determined the number of ovules produced (n_o). The velocity of pollen tube growth (V) of each pollen of each individual was the values linked to each allele (V_x,V_y). The seeds conserved in the final population for the next generation were those with higher survival probability (p). After the constitution of the next generation, the alleles that determine the size of the pistil (L_x, L_y), the speed of the pollen tube growth (V_x, V_y) and the neutral gene (Nt_x, Nt_y) were defined for each individual, depending of a mutation rate μ of 10^{e-6}. The new values of mutated alleles were randomly chosen in values distributed between the ancient values inherited more or less 3. If the alleles were not mutated, the values associated with the alleles were the ancient values inherited. N: population size, n_p: cluster of the pollen deposited on each style, D: number of pollen donors if the population is allogamous, r: ratio between the number of pollens deposited and the maximum number of ovule, n_s: cluster of selected pollen deposited on style to fecund ovule, s: coefficient of selection by style length, n: cluster of seeds produced by each individual, N_0: cluster of seeds produced by all individuals.

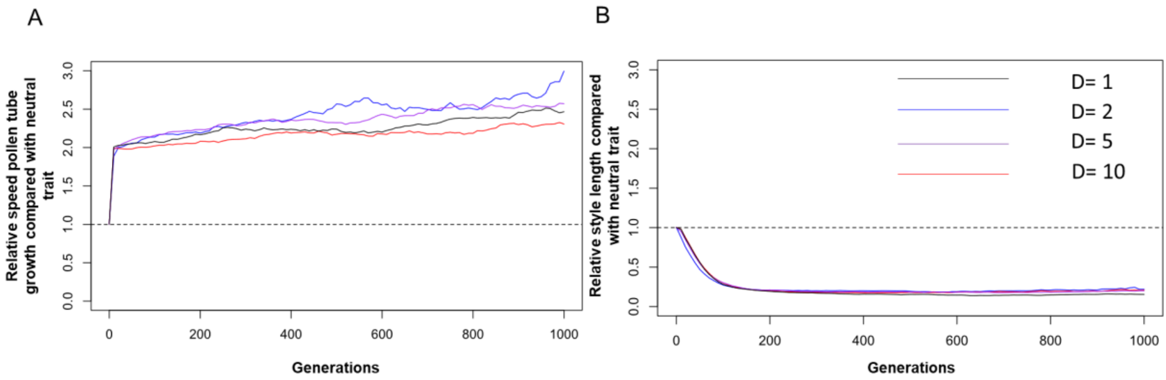


FIGURE. S3 Variation of velocity of pollen tube growth (A) and style length (B) relatively to equivalent neutral trait during 1000 generations for different numbers of pollen donors. Simulations for allogamous crosses with s=0.3, c=0.1, PL=0 and Pv=0. Black: D=1, Blue: D=2, Purple: D=5, red: D=10.

TABLE S1 Summary of the simulations tested.

Simulation	c	Mating type	D	r	s	PL	PV	Process impacted
Control	0.1	Outcrosser	5	5	0.3	0	0	
No female intrasexual cost	0	Outcrosser	5	5	0.3	0	0	Female intrasexual selection
High female intrasexual cost	1	Outcrosser	5	5	0.3	0	0	
Mating type	0.1	Selfer	5	5	0.3	0	0	Mating type
Mating type without female intrasexual cost	0	Selfer	5	5	0.3	0	0	
Mating type with V is a proxy of survival trait	0.1	Selfer	5	5	0.3	0	0.1	
Mating type with L is a proxy of survival trait	0.1	Selfer	5	5	0.3	0.1	0	
No diversity of pollen donors	0.1	Outcrosser	1	5	0.3	0	0	Male competition
low diversity of pollen donors	0.1	Outcrosser	2	5	0.3	0	0	
High diversity of pollen donors	0.1	Outcrosser	10	5	0.3	0	0	
Lower number of pollens deposited by ovules	0.1	Outcrosser	10	1	0.3	0	0	
Low number of pollens deposited by ovules	0.1	Outcrosser	10	2	0.3	0	0	
High number of pollens deposited by ovules	0.1	Outcrosser	10	10	0.3	0	0	
Higher number of pollens deposited by ovules	0.1	Outcrosser	10	100	0.3	0	0	
No selection by female	0.1	Outcrosser	5	5	0.01	0	0	Intersexual selection
High selection by female	0.1	Outcrosser	5	5	1	0	0	
Intermediate selection by female	0.1	Outcrosser	5	5	0.5	0	0	
L is a proxy of survival trait	0.1	Outcrosser	5	5	0.2	0.1	0	Good genes selection

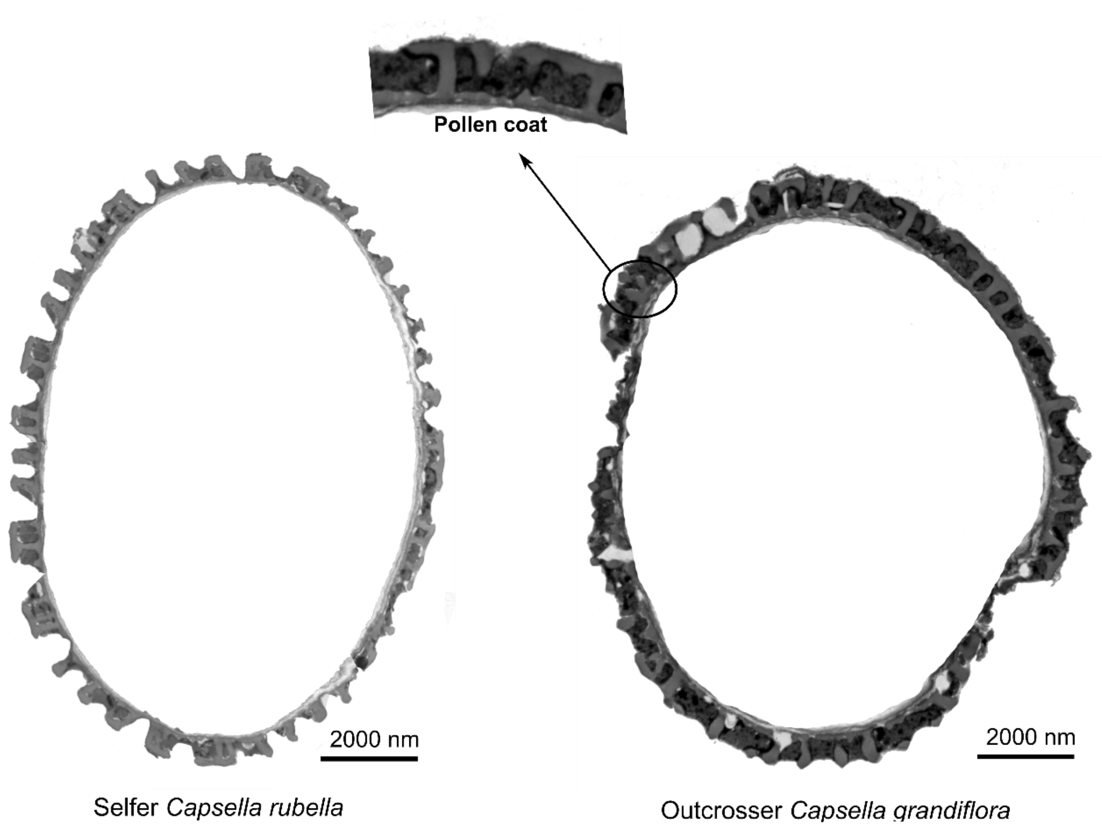
V is a proxy of survival trait	0.1	Outcrosser	5	5	0.2	0	0.1

TABLE S2: Summary of results obtained in the stochastic model for each tested condition.

Simulation	Generation with mean Vx=>10	Mean Vx at the 1000th generation	Mean Lx at the 1000th generation
Control	20	10.03	0.80
Ratio 1	120	10.04	0.72
Ratio 2	20	10.09	0.69
Ratio 10	20	10.04	0.71
Ratio 100	20	10.04	0.76
Donor 1	30	10.58	0.69
Donor 2	20	10.74	0.79
Donor 10	20	10.05	0.68
Cost 0.0	20	22.67	7.23
Cost 1.0	>1000	6.77	<0.01
S 0.01	>1000	4.89	0.73
S 0.1	>1000	8.51	0.70
S 0.9	10	15.81	0.91
Autogamous	>1000	5.72	0.96
PV 0.1	10	96.13	0.94
PL 0.1	20	84.01	42.7

Case study 3

Pollen coat proteins involved in pathogen response show evidence of convergent evolution in independent transitions to selfing in *Arabidopsis* and *Capsella* species



Pollen coat proteins involved in pathogen response show evidence of convergent evolution in independent transitions to selfing in *Arabidopsis* and *Capsella* species

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Abstract

Convergent evolution, the independent evolution of similar traits in different species under similar environmental pressures, is a widespread phenomenon observed across many taxa. In plants, one striking example of this is the repeated evolution of similar floral phenotypes in species that have transitioned from outcrossing to self-fertilization. This shift is often accompanied by the emergence of the 'selfing syndrome,' characterized by similar changes in floral traits like reduced flower size, loss of scent, and altered reproductive strategies. However, potentially overlooked phenotypic changes concern the pollen coat, which plays a role in different aspects of outcrossing strategy. To investigate this, we first compared pollen morphology between selfing and outcrossing species within the *Arabidopsis* and *Capsella* genus, representing three independent transitions to selfing at different evolutionary timescales. We then generated the proteome profiles of pollen from these pairs and identified differentially expressed total pollen and pollen coat proteins between selfing and outcrossing species. Our results showed that the pollen coat area diminished with the age of the selfing transition, showing a significant loss in selfing species *A. thaliana* and *Capsella rubella*, but not in selfing *A. lyrata* (recent transition, no selfing syndrome). Moreover, differentially expressed pollen coat proteins were shared between the three selfer-outcrosser contrasts more than expected by chance and were related to pathogen response. However, these functions were recurrently found in outcrosser-outcrosser comparisons too, suggesting that other drivers than the selfing transition may explain the evolution of pollen coat protein composition. Our findings point towards signals of convergent evolution of the pollen coat morphology and protein content following the transition to selfing. They also suggest that pathogen response drives the fast evolution of pollen coat protein composition independently of the mating system.

Introduction

Sexual reproduction is the predominant reproductive strategy in most land plants, with around 80% of land plants relying on this strategy. Plant taxa display enormous variation in terms of sexual reproduction, and this variation can have important consequences on selective processes, genome evolution, and reproductive traits evolution (Barrett, 2010; Sicard & Lenhard, 2011). The shift from outcrossing to self-fertilization is one of the most significant and widespread evolutionary transitions in plant reproduction and provides an excellent opportunity to study the evolutionary fate of floral traits. There is extensive evidence showing that the transition from the ancestral outcrossing state to selfing occurred recurrently and independently across angiosperms evolution (Igic et al., 2006; Sicard & Lenhard, 2011; Stebbins, 2013) . Initially, such a transition can be advantageous because of reproductive assurance under pollinator scarcity and transmission advantage of allele to the offspring (Kennedy & Elle, 2008). However, in the long term, selfing is considered as an evolutionary dead end because of genomic challenges populations experience, such as increased homozygosity, reduced effective population size, and weakened efficiency of selection (Barrett et al., 2014; Cutter, 2019). At the trait level, transition to selfing is recurrently accompanied by characteristic changes in the morphology and function of floral traits in independent selfing lineages (known as 'selfing syndrome' (Sicard & Lenhard, 2011). In most cases, these changes are similar among independent taxa and leads to the vestigialization of floral traits involved in pollinator attraction, such as reduction in floral size, and number, loss of odor and nectar production, reduced pollen-to-ovule ratios, and reduced herkogamy (Sicard & Lenhard, 2011; Tsuchimatsu & Fujii, 2022).

The transition to selfing is likely to impact pollen traits as well, even though these have been less studied. Pollen serves as the vital vehicle for delivering male gametes, ensuring the continuity of plant life through fertilization (Anderson & Minnaar, 2020). The journey of pollen grain from the anther to the receptive stigma is critical, as it must preserve its functional integrity to successfully initiate fertilization upon arrival. However, in nature, this journey is fraught with numerous stresses and challenges, from environmental pressures to potential pathogens before pollination. To navigate these challenges, pollen must be protected from herbivores and pathogens, shield against UV radiation, and maintain the components necessary for attracting pollinators and interacting with the stigma (Pacini & Hesse, 2005; Rivest & Forrest, 2020). Pollen grains are equipped with specialized structures at their outermost layer, known as the pollen coat, which acts as a protective shield against these challenges they face

(Pacini & Hesse, 2005). It is rich in secretory elements and comprises a complex mixture of proteins, lipids, and pigments (Rejón et al., 2016). Considering these features, one would expect that pollen functions involved in survival of pollen grains and their successful transfer to the recipient stigma would be more important particularly in outcrossing species due to higher dependency on pollinator movement. In contrast, reproduction is assured in selfing species, thus pollinator-assisted pollen transfer is no longer needed, and duration of distance of the pollen movement is only restricted within the same flower (Kalisz et al., 2004). Therefore, the importance of the pollen coat may be reduced, along with the need for protection against environmental stresses, compared to outcrossing lineages. Although comparative studies on pollen coat variation between closely related species are limited, it has been shown that the transition from insect pollination to wind pollination leads to a corresponding decrease in pollen coat amount and density (Hesse, 1979). Moreover, few studies revealed a complex array of pollen coat proteins involved in defense against pathogens and herbivores, as well as in interactions with the pistil in model species like *Arabidopsis* and other plants (Gong et al., 2015; Rejón et al., 2016; Wang et al., 2023). However, it remains to be determined whether the transition to selfing affects pollen coat morphology and molecular composition.

The repeated evolution of similar floral phenotypes associated with the transition to selfing across independent lineages is a case of convergent evolution, a phenomenon that has garnered significant attention in the last decade. The evolution of repeated similar phenotypical traits, known as convergence, is explained by a similar response of independent populations or species when faced with similar selective pressures (Stayton, 2015). Convergent evolution is widespread in nature, and has been widely observed among different taxa, like mammals, birds, insects and plants (Li et al., 2010; Woodhouse & Hufford, 2019; Woźniak et al., 2024; Zhang et al., 2020). At the genetic level, convergent evolution of similar traits can rely on mutations in the same gene (gene reuse) or different genes with similar functions (Stern, 2013). Recent studies revealed that the extent of gene reuse decreases with the divergence between the lineages showing convergent evolution, while convergence at the function level is more likely to happen between distant clades (Bohutínská et al., 2021; Bohutínská et al., 2021a; Birkeland et al.2020). Regarding the convergent evolution of the selfing syndrome, several studies have revealed the underlying molecular mechanisms. For example, flower scent evolution in *Capsella* revealed that the transition from outcrossing to self-fertilization led to a reduction in fragrance, with certain compounds such as β-ocimene. The loss of β-ocimene in the different selfing species appeared to have been caused by mutations in different genes, one of them affecting the subcellular localization of the ortholog of TERPENE SYNTHASE 2 (Woźniak et al., 2024). Besides mutations affecting protein sequence, convergent changes may be quantitative and affect mRNA and/or protein accumulation in a particular tissue. Such changes are likely to be hotspots for convergent evolution, as they have fewer pleiotropic effects, altering gene expression without necessarily changing the structure of the gene product (Stern, 2013). However, to what extent convergence happens at the protein accumulation level in the case of the transition to selfing remains to be tested.

The Brassicaceae family, including the *Arabidopsis* and *Capsella* genera, provides an excellent system for studying the effects of transition to self-fertilization on repeated evolution of sexual traits. It exhibits a wide diversity of independent selfing transitions across different species, which are known to have occurred at various evolutionary timescales, ranging from recent, intermediate, and older. For instance, in *Arabidopsis lyrata*, the transition to selfing is a relatively recent event and is restricted to a few populations in North America and Siberia (Foxe et al., 2010; Kolesnikova et al., 2023), while the rest of the species is an obligate outcrosser (Schierup 1998; Koch et al. 1999; Nasrallah et al. 2000). More specifically, in *A. lyrata* subsp. *lyrata*, multiple breakdowns of self-incompatibility have been reported in the Great Lakes region about 10k years ago (Foxe et al., 2010; Willi & Määttänen, 2010). Despite this recent shift to selfing, these populations do not yet exhibit the characteristic reproductive trait associated with the selfing syndrome (Carleial et al., 2017). Selfing *C. rubella* is one of the most studied model systems for mating system shift, and already exhibits strong reductions in floral traits caused by selfing syndrome, with its developmental and genetic bases well documented (Sicard & Lenhard, 2011). The

breakdown of self-incompatibility from its outcrossing ancestor and subsequent split from its sister outcrossing species *C. grandiflora* occurred approximately 100,000–200,000 years ago (Guo et al., 2009), making it a good model to study the effects of an intermediate timescale for transition to selfing. *Arabidopsis thaliana* is the most intensively studied model species for the evolution of selfing. It is an annual, self-compatible plant native to Europe, Asia, and Africa, and has been separated from other *Arabidopsis* species for approximately 6 million years (Novikova et al., 2016). Recent studies suggest that the loss of self-incompatibility in *A. thaliana* occurred around 600k years ago (Strütt et al., 2023). *A. thaliana* exhibits a clear selfing syndrome. In contrast, its relative *Arabidopsis arenosa* is an obligate outcrossing species with an annual, biennial, or short-lived perennial life cycle, widely distributed across Europe (Al-Shehbaz & O'Kane, 2002).

In this study, using selfing and outcrossing diploid Brassicaceae lineages with recent, intermediate, and older selfing transition events, we aimed to address the following objectives: 1) to determine whether the transition to selfing similarly impacts the evolution of pollen coat morphology in independent lineages, 2) to assess whether these convergent changes in pollen coat morphology are accompanied by convergent changes in pollen coat protein composition, and 3) to investigate at what pace changes in pollen morphology and underlying proteins arise after the transition to selfing. To do so, we first compared pollen grain morphology between selfers and their closely related outcrossing lineage, representing three independent transitions to selfing in *Arabidopsis* and *Capsella* genera. We then generated protein profiles of pollen from these pairs and identified differentially expressed total pollen and pollen coat proteins between selfing and outcrossing species.

Material and Methods

Plant material and growth conditions

To compare the pollen coat structure and pollen cytoplasm from selfer and outcrosser species, 100 plants from each selfer species, including *A. lyrata ssp. lyrata (1 population)*, *Arabidopsis thaliana* (2 populations), and *Capsella rubella (1 population)* were cultivated. For outcrosser species, we selected 25 plants each from *Arabidopsis arenosa* (2 populations), *Arabidopsis lyrata ssp. petrea* (1 population), and *Capsella grandiflora* (1 population) (Supp. Table S1). Seeds of these species were germinated on agar plates containing MS salts, MES hydrate, and 0.8% (w/v) plant agar, adjusted to a pH of 5.8. Then plates were then placed in a growth chamber set to a 12-hour photoperiod at 23°C and a 12-hour dark period at 13°C and cultivated for two weeks. Following germination, seedlings were transferred to small pots filled with garden substrate. For species requiring vernalization, such as *A.arenosa* and *A.lyrata*, seedlings were initially grown under the short-day conditions (8 hours of light per day at 4°C) for eight weeks to induce flowering. While these species being under vernalization process, other species that did not require vernalization were grown simultaneously to perform experiments in batch. These species included *Arabidopsis thaliana*, *Capsella rubella*, and *Capsella grandiflora*. They were germinated under cycle of 12 hours of light at 23°C and 12 hours of dark at 13°C for three weeks before being moved to standard conditions.*A. arenosaA. lyrata*. Finally, all seedlings were then repotted into 0.5-liter pots and transferred to a growth chamber to grow under long-day conditions (16 hours of light at 21°C and 8 hours of dark at 15°C), where they were maintained until ready for experimental use.

Pollen collection

An adaptation of the device described by Brousseau and McCormick (2004) was built to harvest pollen from individual flowers: one 200ul pipette tip was coupled with a 1000ul pipette tip, incorporating a 50un mesh in between to collect unwanted plant tissue and debris; a 5un mesh was attached to the wider section of the 1000ul tip, where the pollen would be collected, and this inserted in a flexible pvc tube. All the structure was sealed with tape and the tube was connected to a mini-Diaphragm vacuum pump. To remove the pollen from the 5un mesh from the pipette tip setup, the filter mesh was removed, placed in weighted Eppendorf tubes (to know the final pollen quantity) and pelleted by high-speed centrifugation for about 15.

Samples preparation for the TEM microscopy

For TEM observation, the collected pollen pellets were fixed by adding to the tubes 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) in a ratio of 1:100 for 24 hs. After this time, the pellet was washed with a washing buffer solution (WB, 0.1M cacodylate with 1.5% glucose). Subsequently, the sample was concentrated in agarose as follows: equal volume of 4% low melting agarose in 0,1 M cacodyl buffer, in

liquid in 37°C was added so sample suspension, carefully resuspended and transferred at 4 °C for 20 minutes. The samples were rinsed in the same buffer and post-fixed in 2% osmium tetroxide for 2 hours. After washing, pollen samples were dehydrated through an ascending ethanol and acetone series and embedded in Araldite - Poly/Bed ® 812 mixture. Thin ultra sections (70-90 nm) were cut on a Reichert-Jung Ultracut E ultramicrotome and stained using uranyl acetate and lead citrate. Sections were examined and photographed using JEOL JEM-1011 electron microscope. Fine structure measurements were performed using a Veleta camera and iTEM 5.1 software (Olympus Soft Imaging Solution GmbH). For visualization of conspicuous membranes of mitochondria and ER has been used this variety of postfixation: 2% OsO4 + 0,8% K4Fe (CN6) in buffer (0,5ml 4%OsO4 +0,5ml PBS+ 0,008g K4Fe (CN)6). Pictures were processed using GIMP 2.10 to obtain a clear differentiation between the pollen coat and the remaining structures from the pollen grain. Finally, images were analyzed using the ImageJ program (https://imagej.nih.gov/ij/). The pollen coat was determined as the outermost layer of the pollen wall filling between the sexine spaces. Total area was measured from cytoplasm area starting from the intine layer of pollen wall. Relative area of pollen grains was measured by dividing the area of pollen coat by the total pollen area per sample. All measurements were conducted in a double-blind manner. Significance between pairwise outcrossing vs. selfing comparisons (Are. vs. Ath., Cgr. vs. Crb., and AlyO. vs. AlyS.) was determined using the Wilcoxon test, with a p-value of < 0.05.

Protein Digestion

Pollen grains were washed by hexane. Hexane fraction was dried and resuspended in 100 mM TEAB (Triethylammonium bicarbonate) and same volume of dichlormethane was added. After shaking, samples were centrifuged and TEAB fraction was further reduced and alkylated by 40mM chloroacetamide, 10mM TCEP (Tris(2-carboxyethyl) phosphine) for 5 min at 95°C. Core of pollen grains left after extraction to hexane were mixed with 100 mM TEAB containing 2 % SDC (sodium deoxycholate) and abrasive glass beads and homogenized using FastPrep bead beating grinder. After homogenization samples were centrifuged and supernatants were further reduced and alkylated by 40mM chloroacetamide, 10mM TCEP (Tris(2-carboxyethyl) phosphine) for 5 min at 95°C. Samples were further processed using SP3 beads (Hughes et al.) on the Thermo KingFisher Flex Automated Extraction & Purification System in a 96 well plate. Briefly, 65 µl of sample was added to 65 µl of 100 % ethanol and mixed with the SP3 beads. After binding, the beads were washed three times with 80% ethanol. After washing, samples were digested in 50 mM TEAB at 40 °C with 1 µg of trypsin for two hours, then another 1 µg of trypsin was added and digested overnight. After digestion samples were acidified with TFA to 1% final concentration and peptides were desalted using in-house made stage tips packed with C18 disks (Empore) according to Rappsilber et al.

nLC-MS 2 Analysis

Nano Reversed phase column (Ion Opticks, Aurora Ultimate TS 25×75 C18 UHPLC column) was used for LC/MS analysis. Mobile phase buffer A was composed of water and 0.1% formic acid. Mobile phase B was composed of acetonitrile and 0.1% formic acid. Samples were loaded onto the trap column (C18 PepMap100, 5 µm particle size, 300 µm x 5 mm, Thermo Scientific) for 4 min at 18 µl/min loading buffer was composed of water, 2% acetonitrile and 0.1% trifluoroacetic acid. Peptides were eluted with Mobile phase B gradient from 4% to 35% B in 15 min. Eluting peptide cations were converted to gas-phase ions by electrospray ionization and analyzed on a Thermo Orbitrap Ascend (Thermo Scientific) by data dependent aproach. Survey scans of peptide precursors from 350 to 1400 m/z were performed in orbitrap at 120K resolution (at 200 m/z) with a 100 % ion count target. Tandem MS was performed by isolation at 1,6 Da with the quadrupole, HCD fragmentation with normalized collision energy of 30 % and 10 ms activation time. Fragmentation spectra were acquired in ion trap with scan rate set to Rapid. The MS2 ion count target was set to 150 % and the max injection time was 75 ms. Only those precursors with charge state 2–6 were sampled for MS2. The dynamic exclusion duration was set to 30 s with a 10ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. Cycle time was se to 1.5 s.

Protein quantification and data analysis

All data were analyzed and quantified with the MaxQuant software (version 2.4.13.0) (Cox, Mann 2008). The false discovery rate (FDR) was set to 1% for both proteins and peptides and we specified a minimum peptide length of seven amino acids. The

Andromeda search engine was used for the MS/MS spectra search. Samples from each species were searched separately against the organism specific database downloaded from Uniprot in April 2024 (*Arabidopsis arenosa*, containing 22 979 entries, *Arabidopsis lyrata*, containing 32 097 entries, *Arabidopsis thaliana*, containing 27 449 entries. *Capsella grandiflora* and *Capsella rubella* were searched together, databases contained 1 865 and 26 721 entries respectively). Enzyme specificity was set as C-terminal to Arg and Lys, also allowing cleavage at proline bonds and a maximum of two missed cleavages. Carbamidomethylation of cysteine was selected as fixed modification and N- terminal protein acetylation and methionine oxidation as variable modifications. The "match between runs" feature of MaxQuant was used to transfer identifications to other LC-MS/MS runs based on their masses and retention time (maximum deviation 0.7 min) and this was also used in quantification experiments. Data analysis was performed using Perseus 1.6.15.0 software (Tyanova et al.)

Bioinformatic analysis

All proteomics analyses were conducted using Perseus 1.6.15.0 software (Tyanova et al., 2016). For each dataset, reverse hits and potential contaminants were filtered out, and intensity values were converted to binary logarithms. To be able to compare protein profile of different species with each other we used *A.thaliana* homologues for each species to identify differentially expressed proteins. We used BLAST 2.14.1+ (Camacho et al., 2009) with default parameters to link results from proteomic analysis with specific genes in current genome annotation of different species. Specifically, we performed protein blast of *A. thaliana*, *A. lyrata*, *A. arenosa*, and *C. rubella* UniProt IDs on respective genome annotations (Lamesch et al., 2011; Rawat et al., 2015; Slotte et al., 2013). Identified proteins from *A. lyrata*, *A. arenosa*, and *C. rubella* were then reciprocally blasted on *A. thaliana* to get additional information from homologous genes. Further, to identify differentially expressed proteins for each pairwise comparison, a two-sample t-test ($p < 0.01$) was used for statistical comparisons, with the significance level determined by a permutation-based FDR (< 0.05). To compare the protein sets identified by DEA, Venn diagrams (Hulsen, 2022) were generated for each pairwise comparison. Finally, biological roles of the obtained differentially expressed proteins from pairwise comparisons were identified with Gene Ontology (GO) enrichment analysis. The GO enrichment analysis was performed using the PlantRegMap online tool (Tian et al. 2019). Enriched GO terms, corrected for multiple comparisons using the Benjamini and Hochberg method (FDR < 0.05), were considered significant. Q-values ($q < 0.05$) were selected and transformed into negative fold changes (−log10) for presentation in bar charts, which were generated using the SRplot online platform (Tang et al., 2023).

Results

A decrease in pollen coat area accompanies the selfing syndrome

To test whether the transition to selfing affects pollen coat area, we compared pollen coat area between selfing (SC) lineages and their outcrossing (SI) close relatives. These comparisons included outcrossing *Arabidopsis arenosa* (SI) with selfing *Arabidopsis thaliana* (SC), outcrossing *Capsella grandiflora* (SI) with selfing *Capsella rubella* (SC), and outcrossing *Arabidopsis lyrata* ssp. *petraea* (SI) with selfing *Arabidopsis lyrata* ssp. *lyrata* (SC).

The pollen coat area was significantly larger in the outcrossing species *A. arenosa* and *C. grandiflora* compared to their selfing counterparts *A. thaliana* and *C. rubella* (p-value: 0.00013 and p-value: 0.07, respectively; Figure 1A). In contrast, the pollen coat area of the selfing *A. lyrata* population was not significantly different from that of its outcrossing counterpart (p-value: 0.406). Differences in pollen coat area might be simply due to differences in pollen grain size (larger grain size = larger perimeter = larger surface of pollen coat). To correct for this bias, we calculate the ratio between pollen coat area and total pollen area (Figure S2A), hereafter "relative pollen coat area". Outcrossing *A. arenosa* and *C. grandiflora* still showed significantly larger relative pollen coat area compared to selfing *A. thaliana* and *C. rubella* (p-value: 2.215e-11 and p-value: 0.0022, respectively, Figure S2B). In contrast, the outcrossing *A. lyrata* had a significantly lower relative pollen coat area than its selfing counterpart (p-value: 0.038).

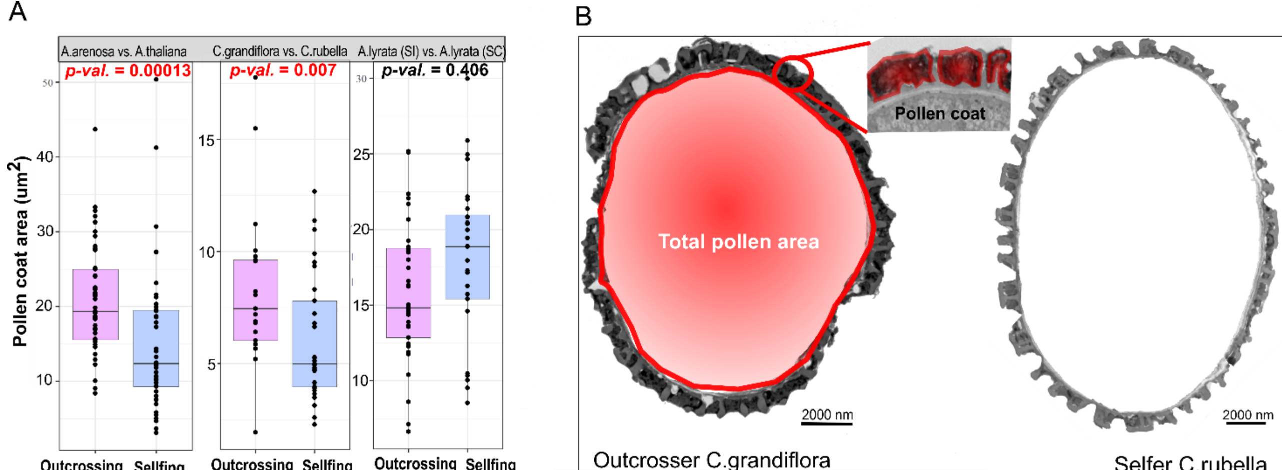


Figure 1: Pollen coat area differences between selfer and outcrosser pollen in three species comparisons: *A. lyrata* (SI) vs. *A. lyrata* (SC), *C. grandiflora* (SI) vs. *C. rubella* (SC), and *A. arenosa* (SI) vs. *A. thaliana* (SC). A) The pollen coat area, differences in the selfer-outcrosser comparisons of the studied species. B) Transmission electron micrsocopy pictures of outcrosser *C. grandiflora* and selfer *C. rubella* pollen grains. The red areas in the *C. grandiflora* pollen grains indicate the pollen coat and total pollen region included in the measurement for each comparison. Significance was determined using the Wilcoxon test, with a p-value of < 0.05 indicating significant differences between comparisons.

Differentially expressed pollen coat proteins between selfers and outcrossers are significantly enriched in functions related to pathogen and defense response

To test whether pollen coat proteins show divergence in expression levels across the three independent selfer-outcrosser contrasts, we characterized the protein profiles of pollen coat from the six lineages and identified proteins differentially expressed in outcrossers compared to selfers separately for each of the three contrasts. We first focused on identifying the total pollen coat proteins that are expressed in outcrossing and selfing *A. lyrata.* A total of 393 pollen coat proteins were detected at least in either selfing or outcrossing *A. lyrata*. To gain insights into the functional characteristics of the pollen coat proteomes, we conducted gene ontology (GO) enrichment analyses. “Modification of morphology or physiology of other organism," "disruption of cells of other organism," "killing of cells of other organism," "cell killing," and "defense response to other fungus" were some of the highly represented GO terms within the biological process (FDR <0.05, and q-value < 0.05, Supplementary Fig.1A). Of these 393 total pollen coat proteins identifies, 56 proteins showed significant differential expression between the outcrosser vs. selfer, with 42 proteins being upregulated and 14 proteins downregulated in the pollen of outcrossing compared to selfing *A. lyrata* (Figure 2A, FDR < 0.05, q-value < 0.05). To gain insights into the biological functions of differentially expressed pollen coat proteins detected in outcrossing vs. selfing *A. lyrata* pollen, we then performed a gene ontology (GO) enrichment analysis. This analysis utilized the 42 upregulated and 14 downregulated pollen coat proteins as the target set, with all detected pollen coat proteins (393 in total) serving as the background reference to correct for prominent functions in the pollen coat. Ten biological processes were significantly enriched in GO terms (FDR < 0.05, q-value < 0.05, Figure 2A). These terms include "modification of morphology or physiology of other organism," "disruption of cells of other organism," "killing of cells of other organism," "cell killing," "regulation of biological quality," and various terms related to defense responses to fungus, biotic stimuli, other organisms, or external stimuli (see details in Figure 2A).

We used a similar approach to analyze the pollen coat proteins of outcrossing *C. grandiflora* vs. selfing *C. rubella*. We identified a total of 284 pollen coat proteins expressed in at least one of the two species. The biological function of these proteins was investigated with GO enrichment analysis, and highly represented GO terms within the biological process were similar to those observed in *A. lyrata* (FDR <0.05, and q-value < 0.05, see details in Supplementary Fig.1A). A total of 144 proteins were found to be significantly upregulated in *C. grandiflora* compared to *C. rubella* (Figure 2B). To further evaluate the biological functions of the 144

significantly upregulated proteins, a GO enrichment analysis was performed using whole *Capsella* pollen coat proteins as background. Similar to the results obtained for outcrossing *A. lyrata*, the proteins with higher expression in *C. grandiflora* were significantly enriched for GO terms such as "disruption of cells of other organism," "modification of morphology or physiology of other organism," "killing of cells of other organism," and "cell killing" (FDR < 0.05, q-value < 0.05, Figure 2B).

Finally, we identified pollen coat proteins that were differentially expressed between the outcrosser *A. arenosa* and the selfer *A. thaliana*. A total of 721 pollen coat proteins were detected in at least one of the two species."Disruption of cells of other organism," "killing of cells of other organism," and "cell killing," were some of the significantly represented GO terms within the biological process (FDR < 0.05, q-value < 0.05 see other terms in Supplementary Fig.1A). Among these, 237 proteins were found to be significantly differentially expressed, with 137 being upregulated and 100 downregulated in *A. arenosa* compared to *A. thaliana* (Figure 2C). GO enrichment analysis of the 237 differentially expressed proteins (including both upregulated and downregulated) using whole *A.arenosa* and *A.thaliana* pollen coat proteins as background revealed significant enrichment for 10 GO terms in the biological process category. These terms included "disruption of cells of other organism," "killing of cells of other organism," "cell killing," "modification of morphology or physiology of other organism," "multi-organism process," and various terms related to defense responses to fungi, biotic stimuli, other organisms, or external biotic stimuli (see other related terms in Figure 2C). Furthermore, two GO terms were significantly enriched in the cellular component category, specifically "extracellular region" and "lipid particle" (Figure 2C, FDR < 0.05, q-value < 0.05).

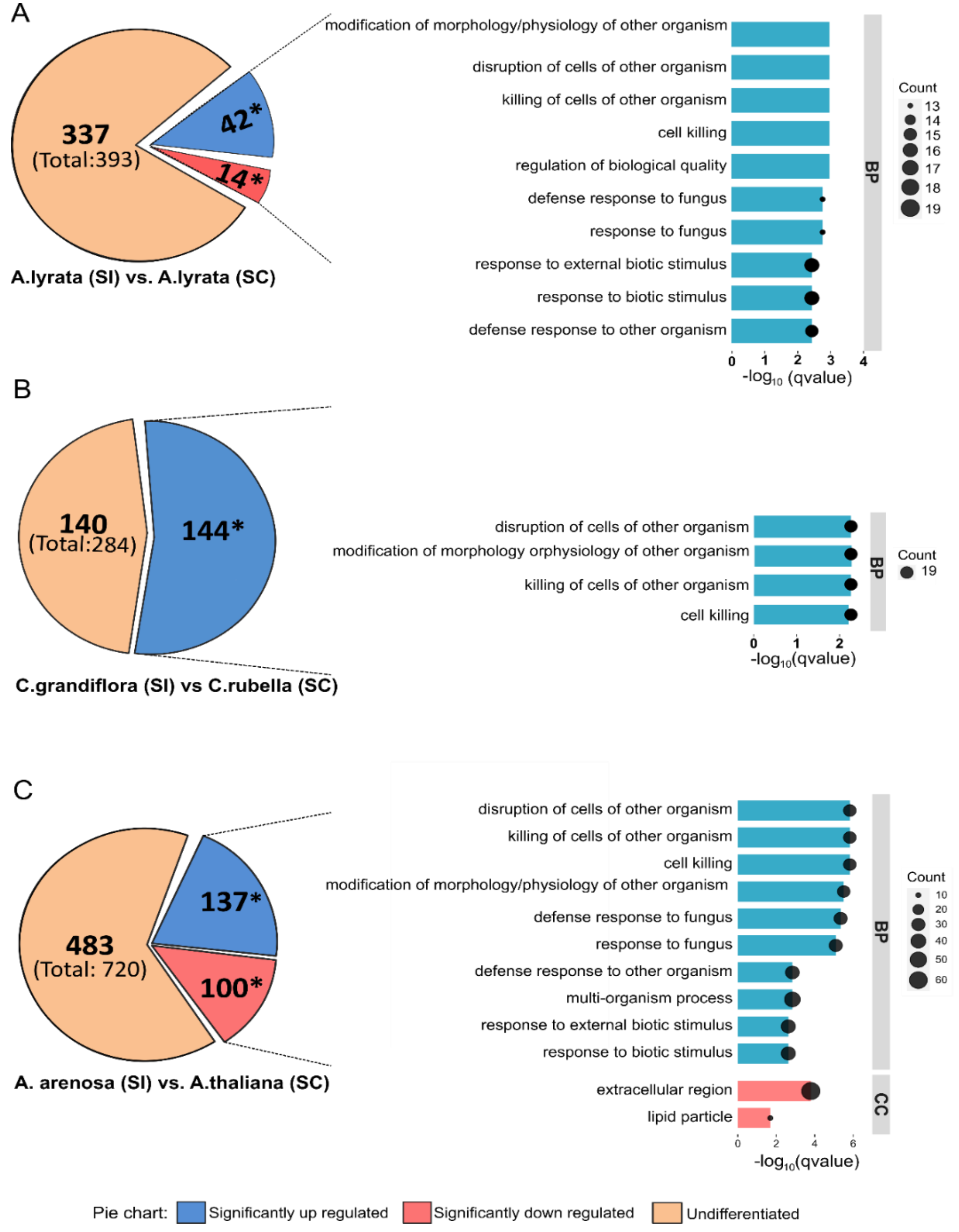


Figure 2: Differential expression of pollen coat proteins and their respective enriched Gene Ontology (GO) terms between selfer and outcrosser pollen in three comparisons: *A. lyrata* (SI) vs. *A. lyrata* (SC), *C. grandiflora* (SI) vs. *C. rubella* (SC), and *A. arenosa* (SI) vs. *A. thaliana* (SC). A) The pie chart shows the number of up, down, and total pollen coat protein between selfer and outcrosser *A.lyrata*, and Gene Ontology (GO) term enrichment analysis of those significantly up and down regulated proteins candidate in pollen coat . B) The number of up, down, and total pollen coat proteins between outcrosser *C.grandiflora* and selfer *C.rubella* with significantly enriched GO terms of those up and down regulated proteins candidates. C) The number of up, down,and total pollen coat protein between outcrosser *A.arenosa* and selfer *A.thaliana* with significantly enriched GO terms of those up and down regulated proteins candidates. The asterisk in the pie charts indicates significantly different pollen coat proteins between selfer and outcrosser species (FDR < 0.05 and p-value < 0.01). The GO term enrichment analysis focuses on biological process (BP) and cellular component (CC) categories, with significance criteria of FDR < 0.05 and q-value < 0.05. The size of each black bubble corresponds to the number of genes enriched in each category.

Differentially expressed proteins from total pollen tend to be located outside the cytoplasm in selfer-outcrosser comparisons

We then investigated the rest of the pollen proteome (excluding the coat during the extraction protocol, see Methods section) and tested whether it also showed signatures associated with the transition to selfing in the three independent comparison pairs. First, the number of detected proteins for each selfer-outcrosser contrast was 1369 proteins in *A. lyrata*, 1543 proteins in *C. grandiflora*-*C. rubella*, and 2085 proteins in *A. arenosa*-*A. thaliana* (Figure 3A). Similar enriched GO terms in the biological process category were found across the three groups, including 'response to metal ions,' 'organonitrogen compound metabolic process,' 'response to cadmium ion,' 'translation,' and 'peptide biosynthetic process' (FDR < 0.05, q-value < 0.05; see other terms in Supplementary Fig. 1B). Of the 1369 total pollen proteins identified between outcrossing and selfing *A. lyrata*, 132 showed significant differential expression, with 81 upregulated and 51 downregulated proteins (Figure 3A, FDR < 0.05, q-value < 0.05). In the comparison between outcrossers *C. grandiflora* and selfers *C. rubella*, 38 proteins were significantly upregulated, while 38 showed significantly lower expression in *C. grandiflora* compared to *C. rubella*. Lastly, among the 202 total pollen proteins identified in *A. arenosa* and *A. thaliana*, 126 were significantly upregulated and 76 were downregulated in *A. arenosa* compared to *A. thaliana* (Figure 3A).

To further explore the biological functions of differentially expressed pollen proteins between outcrossing and selfing species across three independent comparison groups, we performed a gene ontology (GO) enrichment analysis. Significantly up- and downregulated proteins were included in the analysis, using a background list of whole pollen proteins from species involved in each respective pairwise comparison to correct for overrepresented functions in pollen. Among the 132 differentially expressed proteins in the *A. lyrata* (SI) vs. *A. lyrata* (SC) comparison, four GO terms were significantly enriched (FDR < 0.05, q-value < 0.05, Figure 3A). These included "anchored component of membrane" and "anchored component of plasma membrane" for cellular component, and "hydrolyzing O-glycosyl compounds, hydrolase activity" and "acting on glycosyl bond" for molecular function (Figure 3B). For the 202 differentially expressed proteins in the *A. arenosa* (SI) vs. *A. thaliana* (SC) comparison, significant enrichment of GO terms was observed in cellular component functions such as “cell periphery,” “vacuole,” “symplast,” “cell junction,” “plasmodesma,” “extracellular region,” and “apoplast” (Figure 3B, FDR < 0.05, q-value < 0.05). No significantly enriched GO term was found for the comparison between *C. rubella* and *C. grandiflora*.

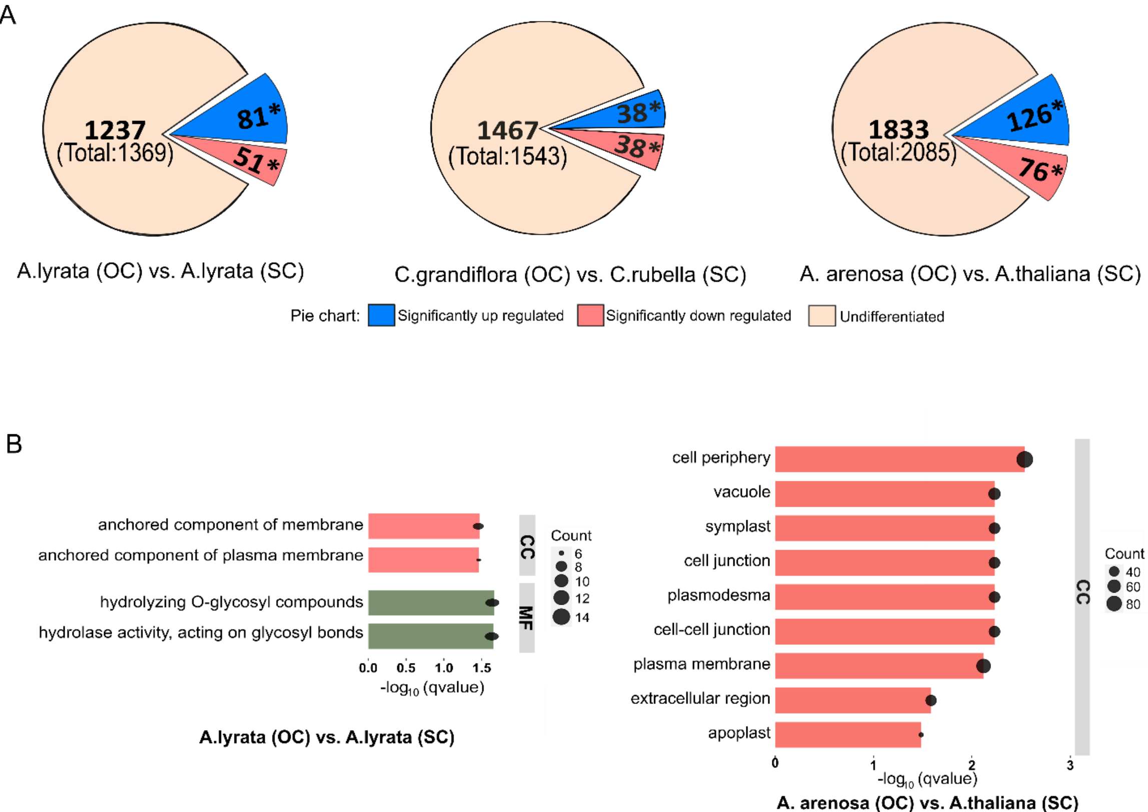


Figure 3: Differential expression of total pollen proteins and enriched Gene Ontology (GO) terms between selfer and outcrosser pollen in three comparisons: *A. lyrata* (SI) vs. *A. lyrata* (SC), *C. grandiflora* (SI) vs. *C. rubella* (SC), and *A. arenosa* (SI) vs. *A. thaliana* (SC). A) Pie charts categorize the number of upregulated, downregulated, and total pollen proteins in these species comparisons. B) GO term enrichment analysis highlights the significantly upregulated and downregulated protein candidates in total pollen within cellular component (CC) and molecular function (MF) categories (FDR < 0.05 and q-value < 0.05). The size of each black bubble corresponds to the number of genes enriched in each category. An asterisk in the pie chart indicates significantly different total pollen proteins between selfers and outcrossers, with significance determined by FDR < 0.05 and p-value < 0.01.

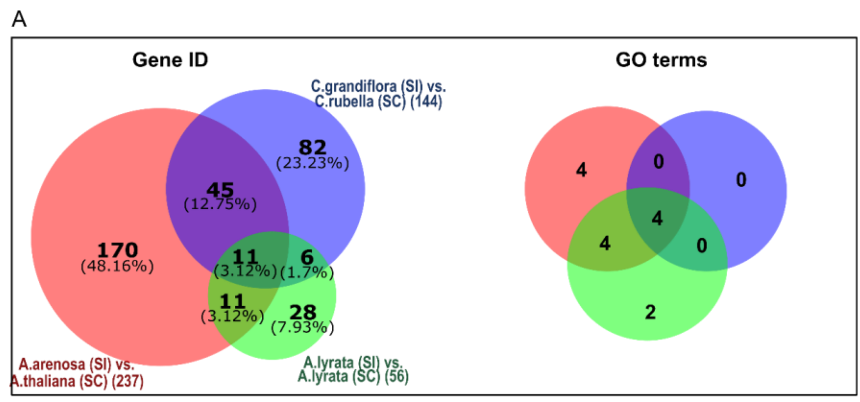
Pollen coat proteins show signals of convergent evolution across the three transitions to selfing

To investigate whether pollen coat and total pollen proteins expression repeatedly diverges among independent selfer-outcrosser systems, we compared the differentially expressed proteins identified in the three comparisons: *A. arenosa* (SI) vs. *A. thaliana* (SC), *A. lyrata* (SI) vs. *A. lyrata* (SC), and *C. grandiflora* (SI) vs. *C. rubella* (SC). As a reminder, a total of 237 significantly differentially expressed pollen coat proteins were identified in the comparison between *A. arenosa* (SI) and *A. thaliana* (SC), 56 in the comparison between *A. lyrata* (SI) and *A. lyrata* (SC), and 144 in the comparison between *C. grandiflora* (SI) and *C. rubella* (SC). Among these, 11 proteins (3.12%) were found to overlap across all three independent groups (Figure 4A). These proteins were members of plant invertase/pectin methylesterase inhibitor superfamily protein, SCR-like, defensin-like (DEFL) family protein, cysteine-rich, glycine-rich protein zinc finger (C2H2 type) family protein and oleosin family protein families known to be important for pollen hydration, pollen germination, pollen tube elongation and signaling process (Arrey-Salas et al., 2021; Takeuchi and Tetsuya, 2012; Rejón et al., 2016; Ge et al, 2011). The gene list is available in Figure 4C. The most abundant overlap was observed between the *A. arenosa* (SI) vs. *A. thaliana* (SC) and *C. grandiflora* (SI) vs. *C. rubella* (SC) comparisons despite their phylogenetic distance, with 56 proteins (half of *C. grandiflora*-*C. rubella* differentially expressed proteins). Finally, to test for a convergence at the function level rather than gene reuse between the three transitions to selfing, we overlapped a total of 12 significantly enriched GO terms for *A. arenosa* (SI) vs. *A. thaliana* (SC), 10 for *A. lyrata* (SI) vs. *A. lyrata* (SC), and 4 for *C. grandiflora* (SI) vs. *C. rubella* (SC). The overlap of GO terms among the three groups was

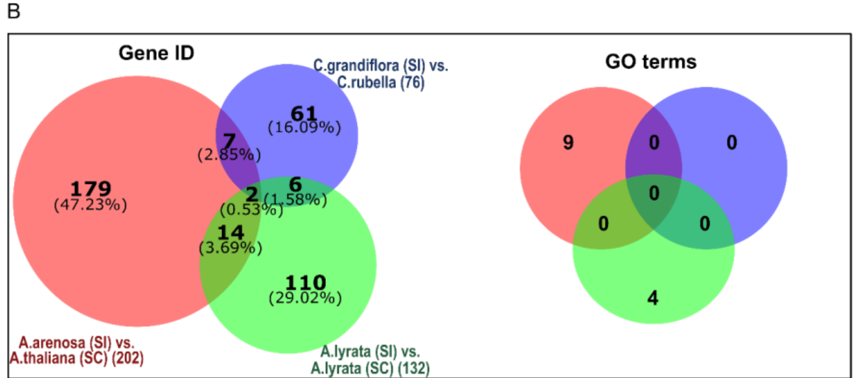
greater than the overlap observed from genes, with 4 common GO terms shared across the three comparisons being all enriched GO terms for *Capsella*, half for *A. lyrata* and one third for the *A. arenosa*-*A. thaliana* contrast (Figure 4A). These 4 GO terms were all related to pathogen response.

We then also tested whether the total pollen proteome shows signals of convergent evolution between the three transitions to selfing. A total of 202 significantly differentially expressed total pollen proteins were identified in the comparison between *A. arenosa* (SI) and *A. thaliana* (SC), 132 in the comparison between *A. lyrata* (SI) and *A. lyrata* (SC), and 76 in the comparison between *C. grandiflora* (SI) and *C. rubella* (SC). The number of shared proteins among the three groups was relatively low, with only 2 proteins (0.53%) overlapping across all comparisons (Figure 4B). These shared proteins were members of the neutral/alkaline non-lysosomal ceramidase and beta-glucosidase families (Figure 4C). Additionally, the overlap of significantly enriched GO terms for total pollen proteins was null.

A



B



C

Overlapped pollen coat proteins (11)

GENE NAME	Gene description	A.arenosa vs. A.thaliana		C.grandiflora vs. C.rubella		A.lyrata(SI) vs. A.lyrata (SC)	
		Fold change	*Log T-test p-value*	*Fold change*	*Log T-test p-value*	*Fold change*	*Log T-test p-value*
AT1G54620 [x]	Plant invertase/pectin methylesterase inhibitor superfamily protein	10.124	34.776	29.16	5.3760	103.86	8.2561
AT1G65113	SCR-like 2	-8.940	17.069	54.11	5.3267	5.24	4.0622
AT2G22941	Defensin-like (DEFL) family protein	-7.909	14.836	10.25	2.2324	-78.65	5.2841
AT2G27145	low-molecular-weight cysteine-rich 9	-3.728	7.6631	665.74	9.6078	36.74	11.0322
AT2G27670 [x]	Domain of unknown function DUF220	5.916	16.888	9.09	2.9352	-11.53	3.7958
AT3G22050 [x]	Domain of unknown function (DUF26)	-2.955	2.3245	6.11	2.4506	23.44	6.7173
AT3G53080 [x]	D-galactoside/L-rhamnose binding SUEL lectin protein	-11.33	22.6550	24.43	4.1197	5.08	3.9663
AT4G14785 [x]	SCR-like 23	-8.100	14.801	24.16	4.7295	-5.19	4.1741
AT5G07560 [x]	glycine-rich protein 20	13.77	43.3355	9.18	2.6331	11.69	4.3938
AT5G16470 [x]	zinc finger (C2H2 type) family protein	-5.469	9.43596	4.29	2.0701	5.42	4.0202
AT5G61610 [x]	Oleosin family protein	-6.210	18.7496	2.90	2.1443	-3.33	2.4018

Overlapped total pollen proteins (2)

GENE NAME	Gene description	A.arenosa vs. A.thaliana Fold change	A.arenosa vs. A.thaliana Log T-test p-value	C.grandiflora vs. C.rubella Fold change	C.grandiflora vs. C.rubella Log T-test p-value	A.lyrata(SI) vs. A.lyrata (SC) Fold change	A.lyrata(SI) vs. A.lyrata (SC) Log T-test p-value
AT2G38010	Neutral/alkaline non-lysosomal ceramidase	-1.957	9.4512	-4.71	4.4339	4.46	4.1041
AT5G44640	beta glucosidase 13	-3.716	4.808	3.09	2.9317	-2.39	2.6276

Figure 4: Venn diagrams illustrating the overlap of pollen coat and total pollen proteins across three independent selfer-outcrosser systems, accompanied by a detailed description of these overlapping proteins. A) Venn diagram illustrating the overlapping genes and GO terms of significant pollen coat proteins (FDR < 0.05 and q-value < 0.05) among the comparisons of *Arabidopsis lyrata* (self-incompatible, SI) vs. *A. lyrata* (SC), *C. grandiflora* (SI) vs. *C. rubella* (SC), and *A. arenosa* (SI) vs. *A. thaliana* (SC). B) Venn diagram showing the overlapping genes and GO terms of significant (FDR < 0.05 and q-value < 0.05) total pollen proteins among the same sets of species comparisons. C) Detailed description and statistical analysis (False Discovery Rate, FDR < 0.05, and p-value < 0.01) of the overlapping pollen coat (11) and total pollen (2) proteins identified across these three selfer-outcrosser systems. Gene descriptions were obtained from the *A. lyrata* v2.1 annotation file (Rawat et al., 2015). The colors used in GO terms Venn diagrams are consistent with gene ID diagram. Multiplication signs on gene names are from overlapped pollen coat proteins unique to the transition to selfing (8 proteins) from Figure 5C.

To test whether the convergent evolution of pollen coat protein composition was due to the selfing transition, we performed outcrosser-outcrosser comparisons and overlapped the differentially expressed proteins between the three outcrossing species *A. arenosa*, *A. lyrata* (SI), and *C. grandiflora*. We identified 209 significantly differentially expressed pollen coat proteins in the comparison between *A. arenosa* (SI) and *A. lyrata* (SI), 180 in the comparison between *C. grandiflora* (SI) and *A. arenosa*, and 97 in the comparison between *C. grandiflora* (SI) and *A. lyrata* (SI). Of these, 19 proteins overlapped across the three comparisons (Figure 5A). Then, to examine convergence at the functional level rather than gene reuse across the three outcrosser-outcrosser comparisons, we compared the significantly enriched GO terms derived from each comparison. We found 10 enriched GO terms for the *A. arenosa* (SI) vs. *A. lyrata* (SI) comparison, 4 for *C. grandiflora* (SI) vs. *A. arenosa*, and 8 for *C. grandiflora* (SI) vs. *A. lyrata* (SI). Four GO terms were shared across all three comparisons, representing all enriched terms in *C. grandiflora* vs. *A. arenosa*, half of those in *C. grandiflora* vs. *A. lyrata*, and one-third of those in *A. arenosa* vs. *A. lyrata* (Figure 5A). These four GO terms were all related to pathogen response.

In addition, we checked whether these proteins were similar to those showing convergent expression evolution following the three transitions to selfing (Figure 5B). This comparison revealed an overlap of three proteins (*AT1G65113*, *AT2G22941* and *AT2G27145*) with 16 and 8 proteins unique to the outcrosser-outcrosser and selfer-outcrosser contrasts, respectively. The description of proteins unique to the transition to selfing (8 proteins) are marked with a star sign in Figure 4C.

Finally, we looked at signatures of mating system on the expression profile of all pollen coat proteins for each species. A PCA plot of the first two principal components, explaining 41.5% and 11.3% of the variation, respectively, indicated that samples did not group according to their phylogenetic relationships (Figure 5C). In contrast, the second component of total pollen protein expression patterns showed a gradient matching the phylogenetic distance between species (Figure S3).

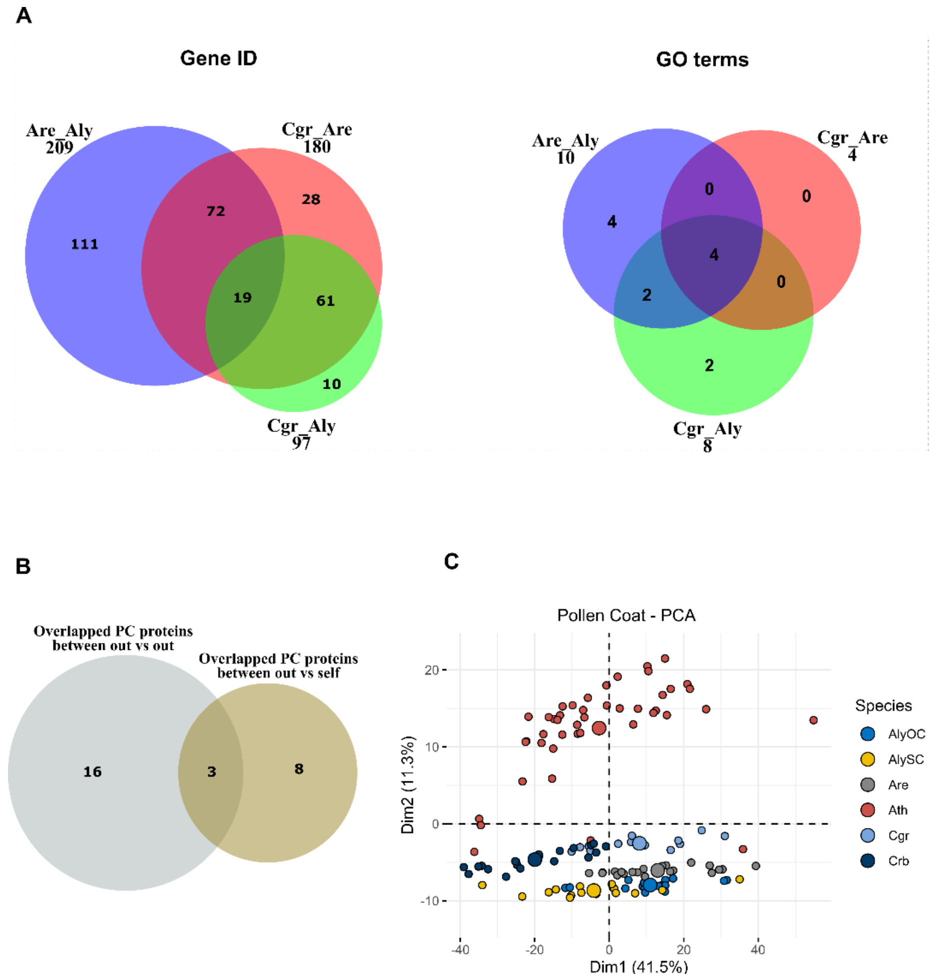


Figure 5: Convergent evolution of pollen coat protein expression following independent transitions to selfing in *A. lyrata, C.rubella and A.thaliana*. A) Venn diagram illustrating overlapping significant pollen coat proteins and GO terms between outcrossing species (FDR < 0.05 and q-value < 0.05). B) Overlapped pollen coat proteins (FDR < 0.05 and q-value < 0.05) from outcrosser vs. outcrossser and outcrosser vs. selfer pollen coat proteins. C) Principal Component Analysis (PCA) showing variability in pollen coat protein expression across all species.

Discussion

Reduction in pollen coat area in selfing *A.thaliana* and *C.rubella* species aligns with characteristic of selfing syndrome

Vestigialization of ancestral outcrossing floral traits following the transition to selfing has largely documented in literature (Sicard and Lenhard, 20111; Tsuchimatsu and Fujii, 2020). The loss of traits in selfing species often includes traits important for pollinator attraction associated with pre-pollination processes which are no longer needed due to reproductive assurance. Besides the independence from pollinators, other changes accompany the transition to selfing, such as the re-allocation of resources to male and female gametes (reduced pollen-ovule ratio) (Barrett 2003), or the enhancement of the efficiency of self-pollination (Torang et al., 2016; Shimizu and Tsuchimatsu, 2015). Although certain traits are well known to evolve with the selfing syndrome, it remains to be elucidated whether additional traits are associated with it. The pollen coat structure plays a vital role in pollinator mediated sexual reproduction and thus we hypothesize that the pollen coat becomes less crucial in selfing species, leading to reduced resource allocation to its development compared to their outcrossing sister lineages. Our findings showed that independent transitions to selfing in *A. thaliana* and *C. rubella*, both of which display a strong selfing syndrome, resulted in a significant reduction in pollen coat area, suggesting convergent evolution of the pollen coat (Figure 1).

Most animal-pollinated angiosperms, particularly those relying on insect pollination or similar strategies, are known to have pollen coats. Although comparative studies on pollen coat variation between closely related species are limited, it has been shown that the transition from insect pollination to wind pollination leads to a corresponding decrease in pollen coat amount and density (Hesse, 1979). The transition from outcrossing to selfing may resemble similar effects, as pollinator dependence gradually decreases in selfing species. In this context, a reduction in pollen coat area in independently evolved selfing species exhibiting strong selfing syndromes, in our study *A. thaliana* and *Capsella rubella*, could represent pollen coat as another trait associated with the selfing syndrome.

An intriguing result from our study is the observation that selfing *A. lyrata* did not show the same trend. This finding may reflect the relatively recent breakdown of self-incompatibility in *A. lyrata* populations (Foxe et al. 2010), with no evidence of selfing syndrome yet (Carleial et al. 2017). Consistently, the number of differentially expressed pollen coat proteins between *A. lyrata* selfer and outcrosser was substantially lower than for the two other selfer-outcrosser comparisons (Figure 2A).

Pollen coat protein composition evolves quickly, in contrast with the proteome of rest of the pollen

The evolution of reproductive proteins is likely driven by strong positive selection, influenced by sexual selection pressures such as cryptic female choice (Eberhard, 1996) and sperm competition (Clark, 2002). Beyond sexual selection, before pollination, male gametophytes face challenges, such as water loss and defense against pathogens, which are critical for survival of pollen grains upon present on anther (Rivest & Forrest, 2020). These pressures may contribute to the rapid evolution of reproductive proteins, as selection favors advantageous modifications that improve pollen grain survival. This rapid evolution may happen at the protein sequence level, or protein expression level. Here, we tested for the latter. In all the comparisons we performed between species (independently of the mating system), we generally found a higher proportion of differentially expressed proteins between lineages in the pollen coat compared to the rest of the pollen (Figures 2 and 3). In addition, the PCAs showed a clear phylogenetic signal on the total pollen proteome, while this signal was less clear for the pollen coat proteome. This suggests that the pollen coat proteome evolves faster than the proteome of the rest of the pollen. The core pollen proteome may be genetically constrained, likely due to its involvement in some of the most basic and general reproductive processes (Hafidh et al., 2016; Scholz et al., 2020). In contrast, the pollen coat may be more susceptible to evolutionary changes due to the constantly changing ecological demands, as it is directly involved in the interactions with the ambient environment (Rivest and Forrest, 2019). Consistently, while there was no clear convergence for the total pollen proteome in all the comparisons we performed between lineages, similar proteins and functions recurrently showed differential expression in the pollen coat, and these were related to pathogen response. This suggests that the fast evolution of the pollen coat proteome is due to strong positive selection and similar selective pressure, i.e. pathogen threat. This is consistent with the challenges faced by pollen to survive until its deposition on the stigma (Rivest & Forrest, 2020). Nevertheless, it is important to note that the proteins we identified as recurrently differential may be annotated as pathogen response due to features such as cysteine-rich, pectine methylesterase activity, or defensin-like (Figure 4C), all being connected with pathogen defense (Lionetti et al., 2012; Wormit and Usadel, 2018; Khan et al., 2019; Wang et al., 2020). However, this type of protein features is also well known to be important for pollen germination and tube growth (Takeuchi and Tetsuya, 2012; Rejón et al., 2016; Ge et al, 2011). In fact, the pollen tube growth across the transmission tract resembles a pathogen response at the transcriptome level (Elleman and Dickinson 1999, Dresselhaus and Márton 2009; İltaş et al., 2024). It could thus well be that the similar selective pressures leading to the fast pollen coat proteome divergence between lineages would be sexual selection rather than pathogen response. Future works should aim at disentangling the two.

Independent transitions to selfing in the three different species are accompanied by some signals of convergent changes in pollen coat proteins

As discussed above, the selfing syndrome may be accompanied by changes in pollen traits such as pollen coat morphology. These changes may display features of convergent evolution, as selfing emerged independently in separate lineages and similar selective pressures are at play. Namely, for the pollen coat, protection from environmental stresses may be less needed in case of selfing, and we might expect pollen coat convergence across independently evolved selfing lineages as they adapt to reduced need for pathogen protection. Consistent with convergent changes in pollen morphology, we found that the pollen coat proteome showed convergent changes

between outcrossing and selfing lineages, targeting functions associated with pathogen defense (Figure 4). This convergent change likely reflects reduced selection pressure on pollen coat proteins for pathogen defense in independent selfing lineages, where pollen no longer needs to withstand varied environmental factors.

The convergent patterns we observe bring up the question of whether such changes reflect gene reuse (meaning the same genes are recruited repeatedly across lineages) or functional convergence (where different genes evolve similar functional outcomes in response to selfing) (Miller and Matute, 2016; Bohutínská and Peichel, 2024). We found that gene reuse between the three independent transition to selfing was reduced yet non neglectable (11 proteins), belonging to protein families essential for pollen hydration, germination, tube growth, and signaling (Rejón et al., 2016; Ge et al., 2011). Despite the low gene reuse, we found a higher convergence at the function level. Previous studies have shown that gene reuse decreases with increasing divergence between lineages exhibiting convergent evolution. In contrast, functional-level convergence is more likely to occur between distant clades or across the full divergence scale and has been frequently observed in studies using divergent species (Birkeland et al., 2020; Whiting et al., 2021; Bohutínská et al., 2021), which is the case in our study, where we used species from two different genera (*Arabidopsis* and *Capsella*).

With this in mind, it is important to discuss critically the possibility that some of the observed proteome convergence is not necessarily related to the selfing transition. Indeed, our results revealed pathogen defense functions commonly enriched in all outcrosser-outcrosser proteome comparisons too. This result suggests that broader ecological factors may drive the evolution of the pollen coat proteome rather than the response to selfing. Although our study found convergent changes in pollen coat proteins expression specific to the selfing transition, care should thus be taken in directly ascribing all convergence with selfing transitions per se.

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Author Contributions

C.L.P. and L.S. conceptualized the study. Ö.I., and L.S., performed the experiments. Ö.I., M.K., P.T. and K.H., analyzed the data. Ö.I and C.L.P. wrote the manuscript with contributions from all authors

Disclosures

The authors have no conflicts of interest to declare.

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Supplementary figures

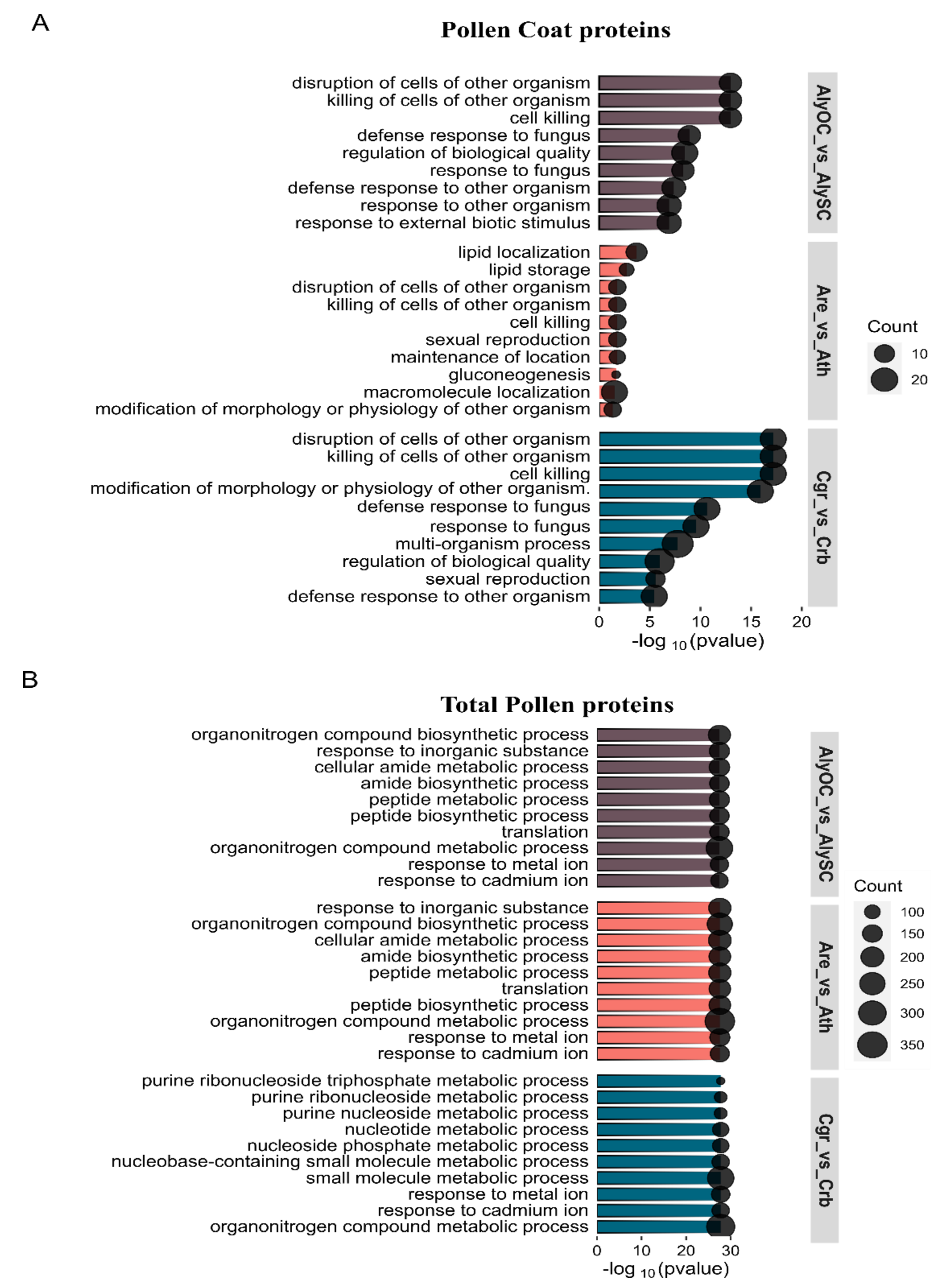

Figure S1. Gene enrichment analysis of all pollen coat proteins (A), and all total pollen proteins (B) identified in comparisons between outcrosser and selfer species. Each bar represents significantly enriched terms (FDR < 0.05 and q < 0.05) within a specific species comparison (e.g., *A. lyrata* outcrosser vs. *A. lyrata* selfer, *A. arenosa* vs. *A. thaliana*, and *C. grandiflora* vs. *C. rubella*). The count of enriched terms is indicated by bubble size, with larger bubbles representing higher counts. Species-specific reference genomes were used as the background list for each respective enrichment analysis.

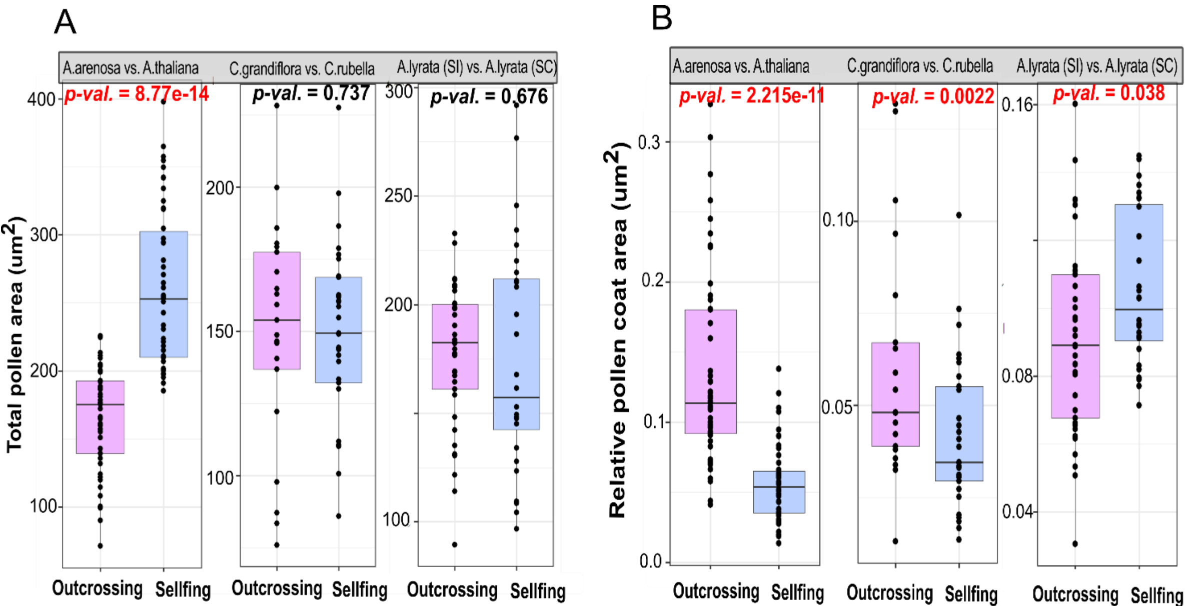

Figure S2: Total and relative pollen coat area differences between selfer and outcrosser pollen in three species comparisons: *A. lyrata* (SI) vs. *A. lyrata* (SC), *C. grandiflora* (SI) vs. *C. rubella* (SC), and *A. arenosa* (SI) vs. *A. thaliana* (SC). A) Total pollen coat area differences in the selfer-outcrosser comparisons of the studied species. B) The relative area differences in the selfer-outcrosser comparisons of the studied species. Significance was determined using the Wilcoxon test, with a p-value of < 0.05 indicating significant differences between comparisons.

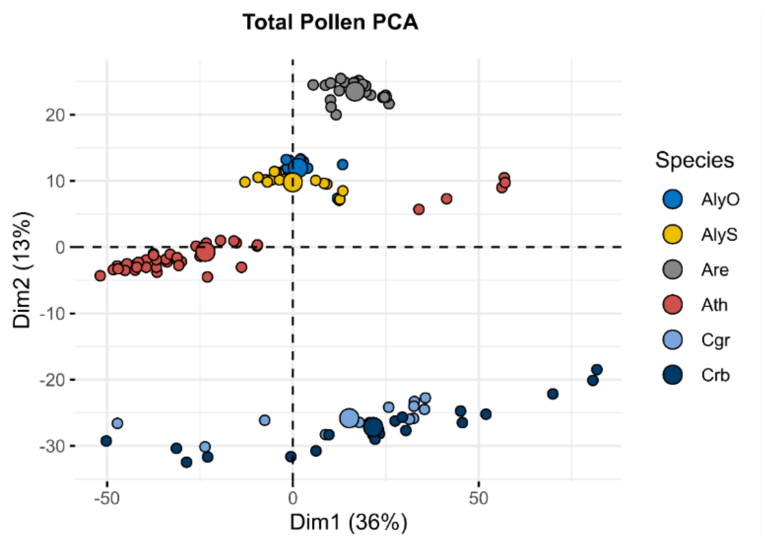

Figure S3: Total pollen protein expression patterns of species. Principal Component Analysis (PCA) showing variability in total pollen proteins expression across species.

Supplementary Tables

Table S1. Population index of Brassicacea lineages used in this study

Species	*Mating system*	*Lineage*	*Ploidy*	*Location*	*GPS coordinates*	*Origin*
A. arenosa	Outcrossing	Western Carpathian	2x	Harmanec, Slovakia	48.82825N 19.01688E	wild seeds
A. arenosa	Outcrossing	Pannonian	2x	Harmanec, Slovakia	48.83448N 19.01348E	wild seeds
A. lyrata sps. lyrata	Selfing		2x	Point Pelee, ON, Canada	41.5540.0N 82.30580W	
A. lyrata sps. petrea	Outcrossing		2x	Nová Ves near Oslavany, Czech Republic	49.07031N, 16.18214E	
C. rubella	Selfing		2x			
C. grandiflora	Outcrossing		2x			
A. thaliana	Selfing		2x			
A. thaliana	Selfing		2x			

Case study 4

Early evolution of reproductive isolation: A case of weak inbreeder/strong outbreeder leads to an intraspecific hybridization barrier in *Arabidopsis lyrata*

ORIGINAL ARTICLE

Early evolution of reproductive isolation: A case of weak inbreeder/strong outbreeder leads to an intraspecific hybridization barrier in *Arabidopsis lyrata*

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Reproductive strategies play a major role in plant speciation. Notably, transitions from outcrossing to selfing may lead to relaxed sexual selection and parental conflict. Shifts in mating systems can affect maternal and paternal interests, and thus parent–specific influence on endosperm development, leading to reproductive isolation: if selfing and outcrossing species hybridize, the resulting seeds may not be viable due to endosperm failure. Nevertheless, it remains unclear how the switch in mating systems can impact reproductive isolation between recently diverged lineages, that is, during the process of speciation. We investigated this question using *Arabidopsis lyrata*, which recently transitioned to selfing (10,000 years ago) in certain North American populations, where European populations remain outcrossing. We performed reciprocal crosses between selfers and outcrossers, and measured seed viability and endosperm development. We show that parental genomes in the hybrid seed negatively interact, as predicted by parental conflict. This leads to extensive hybrid seed lethality associated with endosperm cellularization disturbance. Our results suggest that this is primarily driven by divergent evolution of the paternal genome between selfers and outcrossers. In addition, we observed other hybrid seed defects, suggesting that sex-specific interests are not the only processes contributing to postzygotic reproductive isolation.

KEY WORDS: Hybrid seed lethality, endosperm balance number, *Arabidopsis lyrata*, gray zone of speciation, mating system divergence.

The evolutionary interests of female and male parents do not always align with regard to the production of high fitness offspring (Parker and Partridge 1998). When sexes differ in their contribution to offspring provisioning, sex-specific selective interests are likely to diverge and may even be in conflict. Selection through maternal interests will favor allocating resources equally among offspring, as maternal parents are equally related to each embryo (Smith and Fretwell 1974). In contrast, when multiple mating is the rule, each paternal contributor competes for maternal resources allocated to its offspring. Therefore, promoting more resource allocation to their respective progeny may bring a selective advantage to paternal contributors (Trivers 1974; Rice 1982). Thus, the apparent collaboration between parental genomes may instead be a fragile equilibrium between the antagonistic

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influences of maternal (resource limitation) and paternal (re- source drive) genomes. This conflict between maternal and pa- ternal interests is well known as "parental conflict" (Haig and Westoby 1989). In angiosperms, seeds arise as the product of double fertilization, whereby most of the seed, that is, the embryo and its nourishing tissue, the endosperm, inherit both the mater- nal and paternal genomes (Baroux et al. 2002). The interaction of the two parental genomes seems robust and concerted as it usu- ally leads to viable seeds. Nevertheless, females are the only re- source providers for the embryo, while the paternal contribution to the endosperm may allow its influence in maternal resource allocation to the developing offspring, laying the groundwork for parental conflict to occur.

Conflict among parental interests in resource allocation to offspring is predicted to be observed most strongly in the en- dosperm, as this tissue nourishes the embryo and is contributed to by both maternal and paternal genomes (Haig and Westoby 1989). These two contributions are quantified under the theoreti- cal concept of the Endosperm Balance Number (EBN) (Johnston and Hanneman 1980, 1982). The EBN concept proposes that the endosperm relies on the equilibrium between paternal and ma- ternal molecular factors (determining the EBN) to be functional. If the EBN of one lineage is higher than the EBN of another, the hybrid seed between the two will show failure of endosperm development that will cause embryo defects and result in seed lethality (Johnston and Hanneman 1980, 1982; Roth et al. 2018). Most angiosperms have a nuclear endosperm, and, in this case, hybrid endosperm defects encompass parent-of-origin disturbance in the timing of endosperm cellularization, a crucial developmental transition for embryo survival (Hehenberger et al. 2012). When the pollen donor has a higher EBN than the maternal plant, endosperm cellularization is delayed, whereas in the reciprocal cross it occurs precociously (Lafon-Placette et al. 2017). Changing the ploidy of one parental species can restore hybrid seed viability and endosperm development, supporting the idea that this hybridization barrier is underlain by a quantitative imbalance between paternal and maternal EBN factors (Johnston and Hanneman 1982; Lafon-Placette et al. 2017). Imprinted genes (genes expressed from either the maternally or paternally inherited allele) are strong candidates for the enigmatic "EBN parental factors" (Garner et al. 2016). Indeed, imprinted genes are implicated in hybrid seed lethality in multiple angiosperms, providing a potential molecular basis linking EBN and speciation (Wolff et al. 2015; Florez-Rueda et al. 2016; Lafon-Placette et al. 2018).

Parental conflict may act as a selective pressure on these parental factors (potentially imprinted genes) to increase their role and serve antagonistic parental interests over endosperm re- source accumulation (Brandvain and Haig 2005; Lafon-Placette et al. 2018). This conflict is dependent on the degree to which a species is outcrossing, as parental conflict is driven by sire–sire competition for maternal resources. Indeed, across angiosperms, many lineages have shifted from an outcrossing mating system to various degrees of selfing (Wright et al. 2013). In a self-pollinating species, every offspring has the same father and, further, the father and mother are the same individual, which together lead to the absence of conflict. In contrast, outcrossed offspring generally have multiple different fathers, setting the stage for intense conflict (Queller 1984). Consequently, the stronger selective pressure for parental influence in outcrossing compared to selfing species can explain why outcrossers are mostly found to have a higher EBN than their selfer counterparts, a phenomenon coined as "Weak Inbreeder/Strong Outbreeder," later referred to as "WISO" (Brandvain and Haig 2005; Lafon-Placette and Köhler 2016). It is interesting to note that a higher EBN in outcrossers compared to selfers may well be explained by another parent-specific selective pressure. Namely, EBN may be a trait related to male–male competition for maternal resources and mate choice by females, that is, sexual selection (Burley and Willson 1984; Lafon Placette 2020). Indeed, a repressive influence on endosperm by the maternal genome may act as selection of paternal contributors able to counteract the maternal influence ("good gene-like" selection; Lafon Placette 2020).

The higher EBN in strictly outcrossing lineages compared to selfers often leads to the inviability of outcrosser × selfer re- ciprocal seeds, thus limiting hybridization and contributing to the ongoing divergence of such lineages (Brandvain and Haig 2005; Rebernig et al. 2015). Nevertheless, with limited diver- gence time (recently diverged lineages) or limited mating system divergence (e.g., mixed mating/partial selfing) between two lin- eages, the divergence of EBN may not be large enough to lead to seed lethality (Willi 2013; Raunsgard et al. 2018). With limited EBN divergence, crossing a pollen donor with a higher EBN (the more outcrossing lineage) compared to the maternal plant may yield larger seeds than normal, and the reciprocal cross (EBN ♀ > EBN ♂) may lead to smaller seeds (Willi 2013; Raunsgard et al. 2018). This is consistent with a stronger selective pres- sure in outcrossers compared to selfers on male progenitors to promote resource accumulation and for an antagonistic maternal influence (Brandvain and Haig 2005). Divergence in mating sys- tems associated with EBN differences were shown for cases of ei- ther well-separated species and extensive EBN divergence/hybrid seed lethality (Brandvain and Haig 2005; Rebernig et al. 2015) or cases of closely related intraspecific populations showing mild EBN divergence and hybrid seed size modulation with no impact on viability (Willi 2013; Raunsgard et al. 2018). Besides mating systems, it remains unclear whether other factors contribute to EBN evolution. Additional case studies of EBN divergence as- sociated with hybrid seed inviability, especially between recently diverged lineages, are therefore required to disentangle contribut- ing factors.

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Arabidopsis lyrata is a plant species that occurs in Eurasia as well as North America (Al-Shehbaz and O'Kane 2002; Schmickl et al. 2010). It is assumed to have colonized North America from ancestral European populations (Ross-Ibarra et al. 2008; Schmickl et al. 2010), and the divergence between the Eurasian and North American lineages has been dated to around 35,000 years ago (Ross-Ibarra et al. 2008). The colonization of North America was accompanied by a strong bottleneck, leading to genetically less diverse *A. lyrata* populations in North America compared with Europe (Schmickl et al. 2010). European populations of *A. lyrata* (*A. lyrata* subsp. *petraea*) have been found to be exclusively outcrossing (self-incompatible) (Schierup 1998; Koch et al. 1999; Nasrallah et al. 2000). On the other hand, North American populations of *A. lyrata* (*A. lyrata* subsp. *lyrata*) are still predominantly self-incompatible, but a loss of self-incompatibility occurred multiple times in populations located in the Great Lakes region (Mable et al. 2005; Hoebe et al. 2009; Foxe et al. 2010). The loss of self-incompatibility is associated with low outcrossing rates (Foxe et al. 2010). A previous study showed that North American *A. lyrata* outcrossers have a higher EBN than their selfer counterparts (Willi 2013). This affected hybrid seed size in a cross-direction-dependent manner, but the EBN divergence did not seem to be large enough to cause any loss of viability of the hybrid seeds. In this case, therefore, mating system shift does not seem to have led to substantial hybrid seed defects. Nevertheless, coupled with different demographic histories, such mating system shifts could lead to hybridization barriers between the North American selfers and other outcrossing populations across *A. lyrata*. Consistent with this idea, hybrid seeds between outcrossing Russian and North Amer- ican populations of *A. lyrata* showed parent-of-origin-specific reduction in germination rate, pointing to a divergence in EBN between outcrossing populations from North America and Eurasia (Hämälä et al. 2017). Nevertheless, factors other than EBN divergence, such as the random accumulation of incompatible alleles during genetic divergence, may well explain the reduced germination of hybrid seeds, which will require further investigation.

Here, we investigated the early evolution of reproductive isolation, potentially involving the combination of EBN divergence and other factors such as genetic divergence and demographic history in hybrid seed lethality between outcrossing and selfing populations of *A. lyrata*. We performed manual reciprocal crosses between North American selfing and Central European outcrossing populations of *A. lyrata*, measured hybrid seed viability, and monitored hybrid endosperm development. Our results suggest that changes in mating system accompanied with the successive colonization history of the species from Europe to North America appears to have induced a significant enough EBN divergence to cause extensive hybrid seed lethality within *A. lyrata*.

Materials and Methods

PLANT MATERIAL AND GROWTH CONDITIONS

In this study, both selfing and outcrossing populations of *A. lyrata* were used. Seeds were collected from Point Pelee, Canada (GPS coordinates 41°55'40.0"N 82°30'58.0"W) for the selfer *A. lyrata* subsp. *lyrata;* for the outcrosser *A. lyrata* subsp. *petraea*, seeds were collected from Nová Ves u Oslavan, Czech Republic (GPS coordinates 49°07'03.1"N 16°18'21.4"E).

In 2019, seeds were placed in paper bags and kept for two months at 4°C to break dormancy. After seed germination on damp soil, seedlings were transferred to pots filled with damp soil and were grown for six weeks in short-day conditions (8 h of light per day) and 4°C to induce flowering. Seedlings were then transferred to long-day conditions (16 h of light per day) and 21°C. Plants were reciprocally crossed within and between populations, and 6–14 manual pollinations (145–451 seeds) were performed for each type of cross (always noted as ♀ × ♂; selfer × selfer, outcrosser × outcrosser, outcrosser × selfer, selfer × outcrosser). Between two and six different maternal plants and four to six different pollen donors were used per type of cross in random combinations (see Supporting Information Table S1 for details).

For all crosses, designated female partners were emasculated before anthesis, and the pistils were hand-pollinated two days after emasculation (same procedure for selfing and outcrossing plants). Fully maturated siliques were harvested four to five weeks after pollination and stored at 4°C under dry and dark conditions.

SEED PHENOTYPING

Seeds were arranged on white paper and pictures were taken using a macroscope (Olympus SZX10 connected with a Zeiss Axiocam ERc5s camera system). Noncollapsed seeds were identified as brownish-yellow and plump, with variable size. Collapsed seeds were dark brown/black, shriveled and irregularly shaped. For measuring the seed size, images were converted to black and white using the "threshold" function on ImageJ (https://imagej.nih.gov/ij/) and size was measured using the "Analyze Particles" function.

SEED GERMINATION ASSAYS

After harvesting, seeds were kept at 4°C for one year. To perform germination assays, seeds were surface sterilized (with 0.5% NaClO and 0.01% (v/v) of Triton X-100) and plated on MS medium (containing: 1 x MS-Salts and 0.8% (w/v) Plant agar, at pH 5.8). Then, plates were transferred and kept for 2 weeks in a growth chamber (12 h at 23°C/day and 12 h at 13°C/night). Germination rates were assessed by counting seeds with a ruptured seed coat and protruding radicles (Supporting Information Fig. S1).

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ASSESSMENT OF ENDOSPERM CELLULARIZATION

Between three to four manual pollinations were performed for each type of cross. The same plants were used for control and hybrid crosses. To perform the crosses, flowers were emasculated before anthesis and pollinated one to two days after. Seeds were collected 13, 16, and 18 days after pollination (DAP) and placed into fixative solution (3:1 v: v ethanol:acetic acid). After overnight incubation at 4°C, the fixative solution was changed to 70% (v/v) ethanol and the samples were kept at −20°C until subsequent analysis.

Seeds were prepared for microscopy with Feulgen staining, as previously described (Braselton et al. 1996). The imaging of optical seed sections was performed using a multiphoton Zeiss LSM880 microscope with an excitation wavelength of 760 nm and emission from 518 nm and upwards. Approximately 15–20 seeds per cross per time point were imaged.

EMBRYO IN VITRO CULTURE

Mature dry seeds were surfaced sterilized as described above. Seed coat was then removed, and the embryo dissected out using fine needles. The embryos were then placed on plates containing MS media supplemented with 2% sucrose. Plates were put for 2 weeks in a growth chamber (12 h at 23°C/day and 12 h at 13°C/night), and the number of embryos with a grown radicle and green cotyledons were counted as germinated.

STATISTICAL ANALYSIS

We used generalized linear mixed models (GLMM) (Bolker et al. 2009) to test for the interacting effect of male and female mating systems (outcrossing vs. selfing) on size, abortion rate, and germination rate of the offspring seeds. Identities of maternal plants and pollen donors were included as random intercepts in the models to account for variation among individual plants. We used GLMM with a gamma distribution and a logarithmic link function to model nonnegative continuous values of seed sizes (McCullagh and Nelder 1989). Abortion rates, calculated as proportions of collapsed seeds to total seed numbers, were modeled using binomial distribution and a logit link function. Similarly, germination rates were quantified as proportions of germinated to total seeds used in germination assays. However, germination rates did not follow unit dispersion (approximately $\varphi = 2.03$), and thus we used beta-binomial distribution ($\theta = 7.23$) to model these over dispersed data (Bolker 2008). Likelihood ratio tests were employed to assess the significance of the model terms. Nested models with parameters estimated by maximum likelihood and restricted maximum likelihood were compared to test the fixed and random effects, respectively (Pinheiro and Bates 2000). When significant interaction terms were revealed, we performed post hoc tests with Tukey-adjusted probabilities to compare the estimated marginal means (Lenth 2016). Finally, we used beta regression (Ferrari and Cribari-Neto 2004) to assess the effect of mating system on endosperm cellularization rate across multiple stages of *A. lyrata* embryo development. Because cellularization rates are bounded between 0% and 100% and their variance is arguably not constant, we assumed that the response follows a beta probability distribution defined by mean and precision (inverse dispersion) parameters that naturally account for nonconstant variances (Cribari-Neto and Zeileis 2010). We fitted a three-way full factorial model with the main effects of female mating system, male mating system and embryo developmental stage, and all higher order terms (interactions). Prior to analysis, measurements from the first two developmental stages were excluded from the dataset as cellularization was recorded only seldom during these early stages. Again, likelihood ratio tests were employed to assess significance of the beta model terms.

The analyses were performed in R (R Core Team 2019) using the libraries betareg (Cribari-Neto and Zeileis 2010), emmeans (Lenth 2019), lmtest (Zeileis and Hothorn 2002), ggplot2 (Wickham 2016), and glmmTMB (Brooks et al. 2017).

Results

SEEDS FROM PATERNAL OUTCROSSERS ARE LARGER

To test for potential different EBN divergence between selfer and outcrosser *A. lyrata* populations, we performed reciprocal crosses between and within a population of selfers (SC) and a population of outcrossers (SI) (Fig. 1). The gamma GLMM revealed a significant influence of male mating system on the offspring seed size (Table 1). Plants with outcrossers used as pollen donors had significantly larger seeds than those with selfer pollen donors (0.40 ± 0.04 vs. 0.33 ± 0.03 mm^2) (Fig. 2a). Female mating system did not appear to play a significant role in seed size.

CROSSES BETWEEN SELFER AND OUTCROSSER POPULATIONS OF *A. LYRATA* LEAD TO SEED INVIABILITY

The binomial GLMM showed a significant interaction effect of female and male mating systems on the seed abortion rate (Table 1). When selfers were used as maternal plants and outcrossers as pollen donors, the percentage of collapsed seeds was significantly higher (57.1 ± 9.6%) than in any other com- bination of parental mating systems (Fig. 2b). In contrast, SC × SC crosses produced seeds with the lowest abortion rate (8.6 ± 3.2%). With germination assays, we then tested whether the morphological defects observed in hybrid seeds were associated with a loss of viability (Supporting Information Fig. S1). A significant interaction effect of female and male mating systems was

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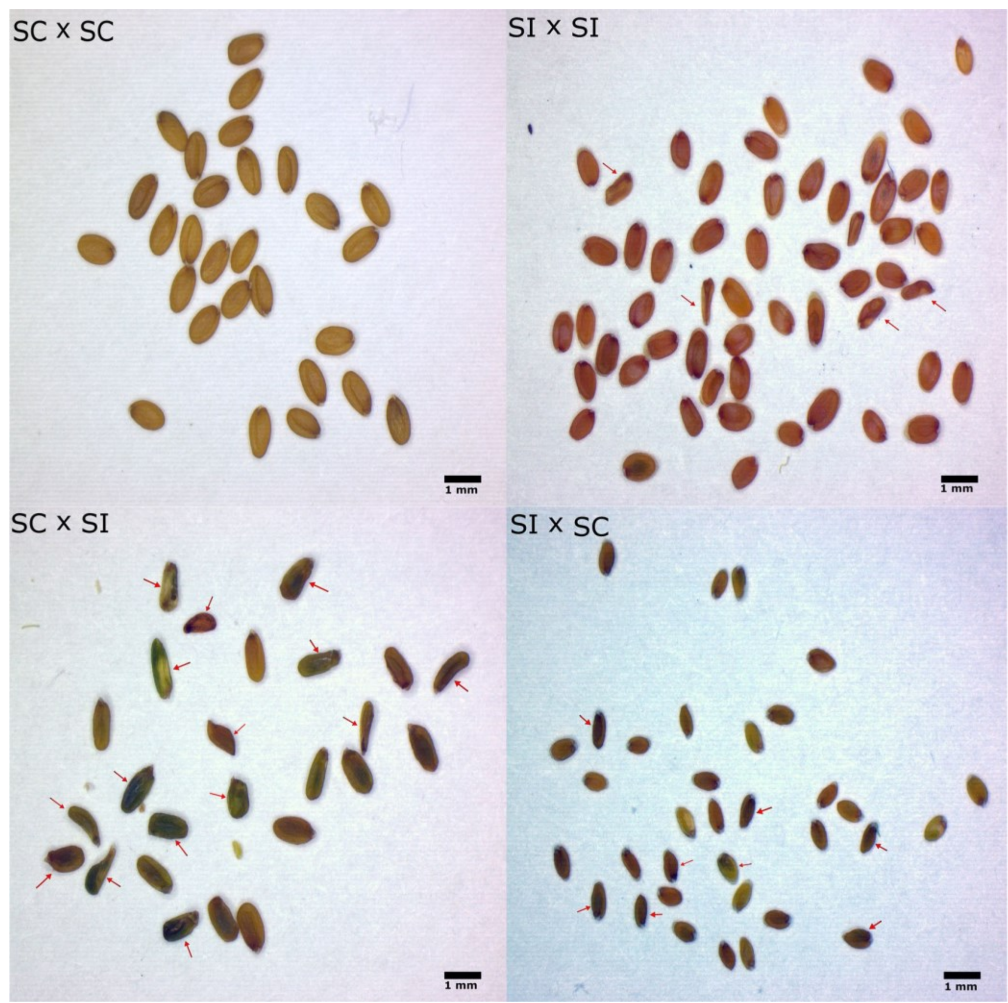

Figure 1. Phenotypic defects of *Arabidopsis lyrata* hybrid seeds. Pictures of seeds obtained from parental and reciprocal hybrid crosses between selfer (SC) and outcrosser (SI) populations of *A. lyrata*. By convention, crosses are always noted as ♀ × ♂. Arrows show seeds considered as aborted. Scale bars: 1 mm.

Tab le 1 . Summary of GLMMs testing for the fixed effects of female and male mating system (outcrossing vs. selfing) and their interaction on size, abortion rate, and germination rate of seeds for *Arabidopsis lyrata*.

	Seed size		Abortion rate		Germination rate	
Model terms	χ^2	P	χ^2	P	χ^2	P
Fixed effects (mating system)						
Female	0.06	0.8140	0.09	0.7688	**4.91**	**0.0267**
Male	**6.72**	**0.0096**	3.58	0.0585	0.41	0.5244
Female × male	0.04	0.8454	**24.30**	**<0.0001**	**33.30**	**<0.0001**
Random effects						
Maternal individual	**16.76**	**<0.0001**	2.17	0.1410	**6.13**	**0.0133**
Paternal individual	<0.01	0.9502	**11.96**	**0.0005**	<0.01	1.0000

Tests of the random effects (identity of maternal plants and pollen donors) are also displayed. Table shows likelihood ratio test statistics (χ^2) and associated probabilities (*p*). Results significant at α = 5% are highlighted in bold.

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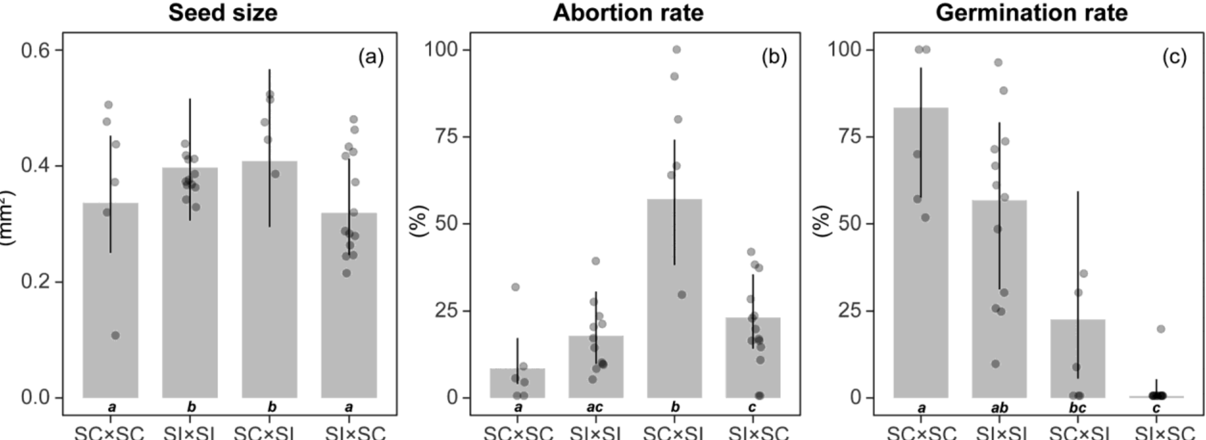

Figure 2. Hybrid seeds show impaired viability. Differences in seed size (a), seed abortion rate (b), and seed germination rate (c) among parental crosses (noted ♀ × ♂) of selfer (SC) and outcrosser (SI) populations of *Arabidopsis lyrata* ($n = 145$ seeds from six crosses for SC * SC; $n = 451$ seeds from 12 crosses for SI × SI, $n = 193$ seeds from seven crosses for SC × SI and $n = 268$ seeds from 14 crosses for SI × SC). GLMM-based estimates (gray bars) are displayed along with their 95% confidence intervals (error bars) and observed values (dots). Groups labeled with different lowercase letters are significantly different from each other ($\alpha = 5\%$).

also revealed in the beta-binomial GLMM of germination rates (Table 1). The seeds from selfer parents (SC × SC) had significantly higher germination rates (83.5 ± 9.2%) than those from reciprocal hybrid crosses (SC × SI: 22.7 ± 14.4%, SI × SC: 0.6 ± 0.7%) (Fig. 2c).

As SI × SC seeds were apparently viable (Figs. 1 and 2), we tested whether the low germination of SI × SC hybrid seeds could be explained by dormancy rather than inviability. Specifically, the seed coat may participate in establishing the seed dormancy. To test this hypothesis, embryos were dissected out of mature dry seeds and grown on MS medium complemented with sucrose (Supporting Information Fig. S2, see Material and Methods). In parental crosses, embryos germinated well and to similar levels as seeds (Supporting Information Table S2). SC × SI embryos also germinated to a large extent (six out of nine embryos germinated). However, despite any visible morphological defects, SI × SC embryos did not give rise to any seedlings, suggesting that seed coat-related dormancy was not responsible for the low

germination rate of SI × SC hybrid seeds.

HYBRID SEEDS SHOW CROSS-DIRECTION-DEPENDENT ENDOSPERM CELLULARIZATION DISTURBANCE

Hybrid seeds originating from parents with different EBNs usually show a disturbance in the timing of endosperm cellularization, an important developmental transition for embryo survival (Rebernig et al. 2015; Lafon-Placette et al. 2017). We tested whether the hybrid seeds between selfing and outcrossing *A. lyrata* showed such defects.

Across all time points considered (13, 16, and 18 days after pollination, referred to as "DAP" hereafter), the SC × SI cross showed delayed endosperm cellularization, as this transition was barely initiated, even at 18 DAP, while cellularization was almost completed in the parents at this time point (Fig. 3a). In contrast, in SI × SC seeds, most of the endosperm was cellularized at 16 DAP, whereas the embryo only reached heart stage (Fig. 3a).

To account for different development speeds in each type of cross, we used the embryo developmental stage (numbered from 1 to 6, Supporting Information Table S3) as a marker for the physiological age of the seed, and measured the progression of endosperm cellularization for each stage (Fig. 3b). Endosperm cellularization rate was significantly influenced by the mating system of parent plants and by the embryo developmental stage ($\chi^2 = 315.2$, df = 15, $P < 0.0001$). The influence of both male and female mating system significantly differed among developmental stages (Table 2).

The onset of cellularization (cellularization at stage 3) occurred significantly earlier in seeds of selfer pollen donors compared to those of outcrosser pollen donors (mean ± SE: 50.1 ± 5.5% vs. 13.2 ± 4.3%). However, as the endosperm development progressed, the difference gradually diminished and the seeds of selfer and outcrosser pollen donors eventually reached the same cellularization rate at stage 6 (significant male × stage interaction; Fig. 3b). In contrast, seeds of outcrosser maternal plants showed an earlier cellularization onset (stage 3) than the seeds of selfer maternal plants even though this effect was less pronounced (37.0 ±6.2% vs. 16.8 ±4.2%) than that of the pollen donor, and this difference was diminished at stage 6 (significant female × stage interaction).

Moreover, the effect of maternal plant mating system depended on the mating system of the pollen donor but this significant female × male interaction was independent from the

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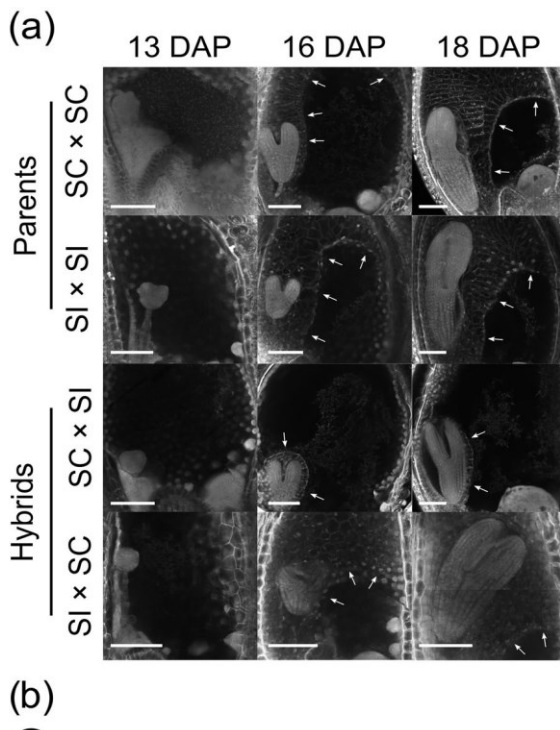

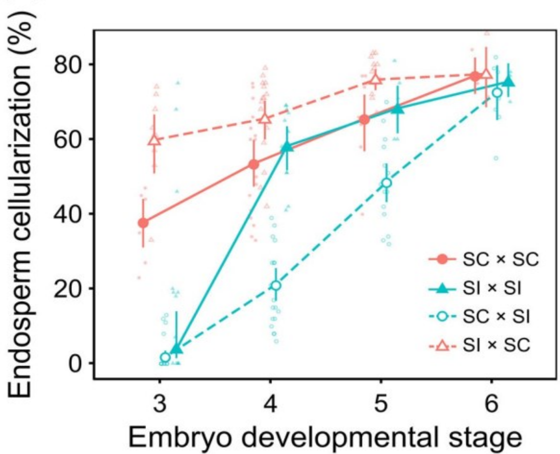

Figure 3. Hybrid seeds show reciprocal endosperm cellularization disturbance. (a) Examples of embryo and endosperm development in parental and hybrid seeds across the surveyed time points (13-18 DAP). Arrows indicate the border between cellular- ized and uncellularized endosperm. DAP: days after pollination. Scale bars: 100 µm; (b) differences in endosperm cellularization rate among parental crosses (noted ♀ × ♂) of selfer (SC) and outcrosser (SI) populations of *Arabidopsis lyrata* across four de- velopmental stages (see Supporting Information Table S3 for the description of embryo stages). Beta regression estimates (geom. shapes) are displayed along with their 95% confidence intervals (error bars) and observed values (dots). Stages 1–2 are not rep- resented on the figure as endosperm cellularization was not de- tected for most of the samples (see Supporting Information Table S1 for details).

Tab le 2 . Summary of beta regression model testing for the effect of female and male mating system (outcrossing vs. selfing), embryo developmental stage (stages 3-6), and their interactions on endosperm cellularization rate for *Arabidopsis lyrata*.

Model terms	χ^2	df	P
Female	**21.9**	**1**	**<0.0001**
Male	**60.1**	**1**	**<0.0001**
Stage	**165.5**	**3**	**<0.0001**
Female × stage	**9.4**	**3**	**0.0249**
Male × stage	**86.0**	**3**	**<0.0001**
Female × male	**6.8**	**1**	**0.0090**
Female × male × stage	5.7	3	0.1299

Table shows likelihood ratio test statistics (χ^2), degrees of freedom (df), and associated probabilities (*P*). Results significant at α = 5% are highlighted in bold.

developmental stage (nonsignificant three-way interaction). Notably, the onset of cellularization in SI × SC seeds occurred earlier than in parents, whereas the onset of cellularization was considerably delayed in crosses with SI pollen donors (Fig. 3b). In particular, SC × SI hybrid seeds showed consistently delayed cellularization along the developmental trajectory. Nevertheless, the SC × SI seeds which eventually reached later stages of embryo development also reached complete endosperm cellularization (Fig. 3b).

Discussion

In this study, we report substantial hybrid seed failure between two populations of *A. lyrata* despite their relatively recent divergence of around 35,000 years ago (Ross-Ibarra et al. 2008). One population, from Central Europe (Czech Republic), is outcrossing, whereas the other, from North America (USA, Great Lakes region), recently transitioned to selfing (10,000 years ago, Foxe et al. 2010). Hybrid seed defects, namely low viability and parent-of-origin-specific endosperm disturbance are compatible with the divergent evolution of parent-specific traits in response to divergence of mating systems between the two populations. We also uncovered additional failure of the hybrid embryo unrelated to parent-specific traits. This points toward the accumulation of intrinsic genetic incompatibility between the two populations independently of the incompatibility predicted by the parental conflict theory. This further incompatibility could be related to the genetic differentiation between Eurasian *A. lyrata* subsp. *petraea* and North American *A. lyrata* subsp. *lyrata* (Ross-Ibarra et al. 2008; Schmickl et al. 2010; Buckley et al. 2016). Given the rela- tively short evolutionary time scale, it is striking to observe diver- gence in parent-specific traits large enough to lead to substantial hybrid seed incompatibility.

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A CASE CONSISTENT WITH THE WEAK INBREEDER/STRONG OUTBREEDER THEORY

Brandvain and Haig (2005) proposed under the WISO hypothesis that outcrossing plant species may show stronger parental influence on endosperm (aka EBN) compared to selfing species, as the selective pressure on parental interests (i.e., promoting resources for paternal interests and antagonistic influence for maternal interests) is stronger in outcrossers than in selfers. According to this hypothesis, in the hybrid endosperm obtained from crosses between a selfing and an outcrossing species, the imbalance between EBN factors from the selfing and outcrossing parents is expected to have parent-of-origin specific deleterious effects on endosperm development and result in seed lethality (Brandvain and Haig 2005; Rebernig et al. 2015). Our results are consistent with this hypothesis: while SC × SI endosperm suffered from delayed cellularization, a typical consequence of the paternal contributor (SI) having a higher EBN compared to the maternal plant (Rebernig et al. 2015; Lafon-Placette et al. 2017, Lafon-Placette et al. 2018), the reciprocal was also consistent, with SI × SC seeds showing precocious endosperm cellularization. Our results are therefore consistent with the idea that divergent mating systems are associated with divergent selective pressure, such as parental conflict and/or sexual selection (Brandvain and Haig 2005; Lafon Placette 2020), leading to quantitative EBN differences between selfing and outcrossing lineages. Our results indicate that such divergence can occur rapidly since the two *A. lyrata* populations in this study diverged recently yet strong EBN divergence is already measurable. Prior to this study, the earliest reported divergence between a selfer and outcrosser causing EBN divergence and hybrid seed lethality was between *Capsella grandiflora* and *Capsella rubella* (Rebernig et al. 2015), which diverged around 50,000–100,000 years ago (Guo et al. 2009).

Interestingly, the effect of mating system divergence on EBN was more extreme on the paternal side. Indeed, with the outcrosser being the pollen donor, seeds were larger, independent of the mating system of the maternal parent. Similarly, the SI paternal genome delayed the onset of endosperm cellularization, independent of the mating system of the maternal genome (although) this effect was stronger with a selfing maternal plant). This suggests that over this short evolutionary time scale, the maternal genomes of selfers and outcrossers have not diverged for their EBN as much as the paternal genomes have. This implies that maternal and paternal EBN factors are not always in full balance at a given evolutionary time point, which is consistent with an arms race between the two parental genomes (Haig and Westoby 1989). Also, our results suggest that the paternal EBN factors evolve faster than the maternal ones, which is consistent with several studies reporting positive selection acting on imprinted paternally expressed genes (Hatorangan et al. 2016; Tuteja et al. 2019), while no footprints of selection on maternally expressed genes were found. The divergent evolution of seed size between the European outcrossers and North American selfers could also be explained by ecological factors (local adaptation; Metz et al. 2010). Nevertheless, we would expect a stronger maternal effect over seed size, as the maternal plant shares the same local en vironment as the progeny, while it is not necessarily the case for paternal contributors. However, we cannot rule out an important role of the paternal contribution in this process. In general, whether seed size evolution in response to ecological factors happens via parent-of-origin specific molecular mechanisms such as genomic imprinting is a fascinating perspective.

THE EVOLUTION OF SEED INVIABILITY DEPENDS ON FACTORS BESIDES EBN

Interestingly, both cross-directions suffered low germination rates, although only the SC × SI crosses were noticeably inviable. SI × SC seeds appeared viable, and their embryos were fully formed with no apparent morphological defect or arrest, suggesting that their precocious endosperm cellularization did not have a major impact on embryo development, similar to other studies (Rebernig et al. 2015). SI × SC seeds nevertheless mostly did not germinate, and the physiological reason remains unclear. We show that the low germination rate of SI × SC seeds cannot be explained by seed coat dormancy. As we germinated the seeds a relatively long time after they were obtained (a one-year gap), it is possible that the hybrid embryos exhibited reduced longevity, explaining the low germination rate. Consistently, the absence of germination even after embryo dissections suggests an incompatibility that manifests itself in the embryo. We cannot rule out strong physiological dormancy in SI × SC embryos. However, physiological dormancy appears as an unlikely scenario: the seeds were stored at 4°C during the year gap, which should have been long enough to break physiological dormancy. This suggests that postzygotic incompatibilities affect embryo lifespan, which could be related to the genetic divergence between Eurasian and North American *A. lyrata* (Ross-Ibarra et al. 2008; Schmickl et al. 2010; Buckley et al. 2016), independently of their mating system divergence.

It was previously shown that when North American outcrossing populations of *A. lyrata* are used as paternal donors with North American selfing *A. lyrata* as maternal plants, hybrid seed size was enlarged, supporting the WISO hypothesis (Willi 2013). Consistent with that study (Willi 2013), when we used European outcrossers as paternal donors, seed size was larger. Nevertheless, no loss of viability was found by Willi (2013), suggesting that the EBN difference between North American outcrossers and selfers is limited. Here, using an outcrossing population from Central Europe and the same selfing population as Willi (2013), we find a strong reduction in hybrid seed viabil- ity, suggesting a significantly higher EBN of Central European

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A. lyrata outcrossers than the North American ones. Although we did not test this directly, hybrid seeds between outcrossing Russian and North American populations of *A. lyrata* showed parent-of-origin-specific reduction in germination rate (Hämälä et al. 2017), suggesting a divergence in EBN between the two outcrossing populations. EBN divergence-related hybrid seed lethality has been reported between other outcrossing species (Lafon-Placette et al. 2017; Roth et al. 2018; Coughlan et al. 2020). This supports the idea that besides mating system divergence, additional factors may explain EBN divergence between lineages. The genetic divergence between Eurasian *A. lyrata*, to which the Russian population of Hämälä et al. (2017) belong, and North American *A. lyrata* (Ross-Ibarra et al. 2008; Schmickl et al. 2010) is likely one of them. Also, ongoing gene flow between self- ers and outcrossers in the Great Lakes region, a likely scenario because the selfers still outcross to a certain extent (Foxe et al. 2010), may slow down the emergence of incompatibilities be- tween the two lineages. Nevertheless, different EBNs imply a quantitative/hierarchical divergence rather than a qualitative one, unlike a "classic" case of BDM incompatibility. Instead, it has been suggested that even among outcrossing species, the intensity or efficacy of parental conflict differs as it does between selfing and outcrossing species, and this leads to a quantitative difference of EBN between these species. *Arabidopsis lyrata* went through a strong bottleneck during the colonization of North America, leading to reduced genetic diversity of the North American pop- ulations (Schmickl et al. 2010), and through additional bottlenecks during multiple transitions to selfing (Foxe et al. 2010; Willi et al. 2018). It was recently shown that genetic diversity of a given lineage positively correlates with its EBN (Coughlan et al. 2020). The authors of the study suggested that genetic diver- sity determines the effective number of paternal contributors in a population, which can affect the strength of parental conflict. Alternatively, we propose that this may be explained by the role of effective population size on selection efficiency (Chen et al. 2017), including the efficiency of parental conflict/sexual selec- tion as selective processes. In other words, the higher EBN in Central European *A. lyrata* outcrossers compared to the North American outcrossers may be explained by a reduced selection efficiency on EBN in the North Americans due to the bottlenecks they experienced. Nevertheless, our study included only one selfing and one outcrossing *A. lyrata* population. Thus, a more exhaustive study across the distribution range of *A. lyrata* in combination with demographic inference of the selected popula- tions is now required to reconstruct the EBN evolutionary history across the species and its potential consequence on intraspecific hybridization barriers.

In conclusion, we show that despite a relatively short divergence time, a strong postzygotic barrier affects hybrid seed viability between two intraspecific *A. lyrata* lineages. Hybrid seed inviability can therefore evolve rapidly, and in the case of *A. lyrata*, this rapid evolution may be driven by divergence in mating systems and EBN.

AUTHOR CONTRIBUTIONS

CLP, AC, and RS designed the research. ÖI performed the research and collected data. ÖI, MS, and CLP analyzed the data. ÖI, MS, CLP, AC, and RS interpreted the data. ÖI, MS, and CLP wrote the manuscript.

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DATA ARCHIVING

All data used in this article are published in it (gathered in Supporting Information Table S1).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Example of MS plates used for the germination assays for each type of cross.

Figure S2. Examples of embryo dissection.

Table S1. (Excel file). Details of the data used in this study.

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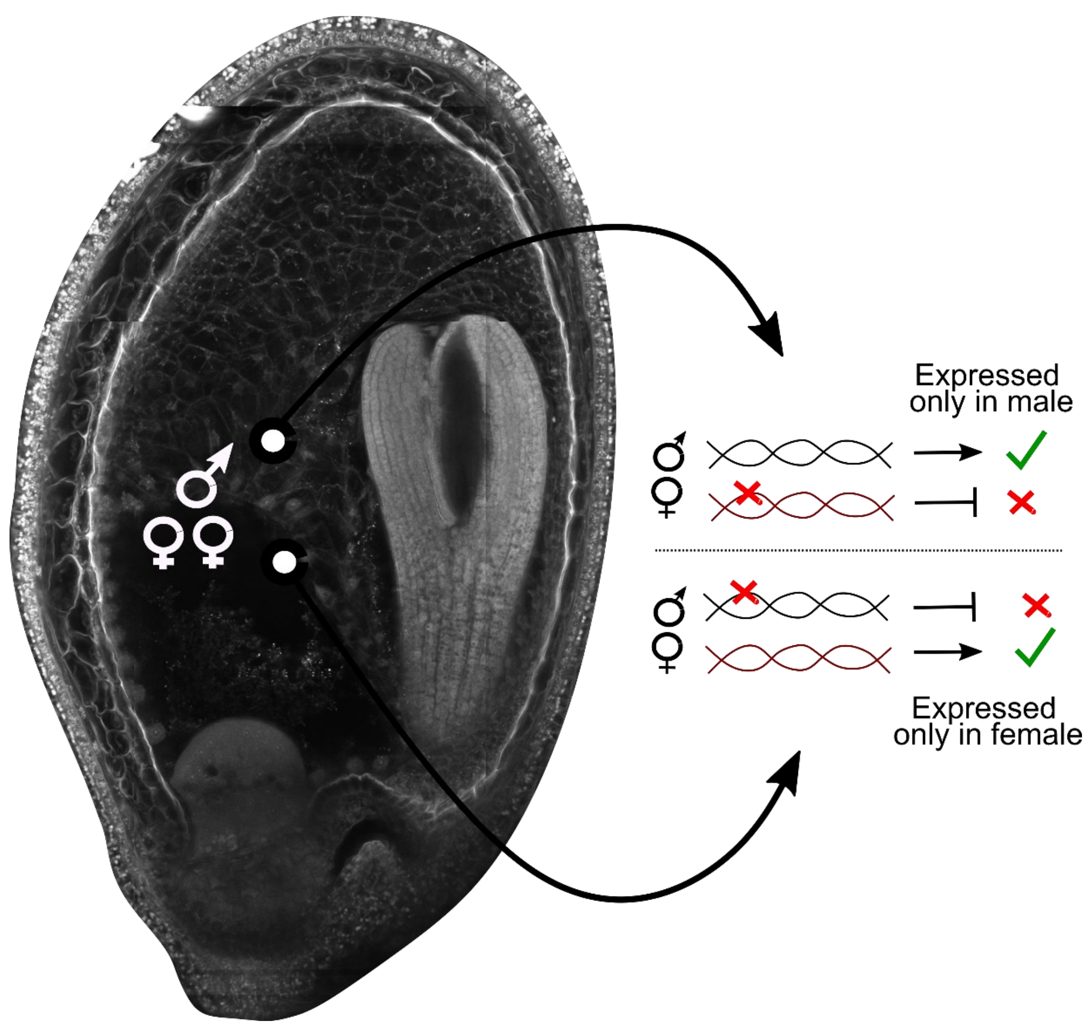
Case study 5

The limited effect of parental conflict on imprinted genes in
Arabidopsis lyrata



The effect of parental conflict on imprinted genes in *A. lyrata*

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Abstract

Genomic imprinting is a form of gene regulation resulting in the unequal expression of the two parental alleles. A common assumption is that the parental conflict over resource allocation to the offspring promotes directional selection on imprinted genes, but so far, most studies addressing this question have focused on autogamous species, whose parental conflict is expected to be low. Moreover, parental conflict is expected to promote antagonistic coevolution between imprinted genes, but this has never been tested so far. To address these knowledge gaps, we searched for signs of coevolution across the Brassicaceae family based on phylogenetic approaches. We then investigated and compared footprints of selection on imprinted genes in allogamous and autogamous populations of *Arabidopsis lyrata*. Overall, the analysis demonstrated that, first, non-imprinted genes presented the typical signals of balancing selection across the Brassicaceae and in allogamous populations of *A. lyrata*, but autogamy promoted directional selection on these genes. On the contrary, we did not find specific signals of selection in the allogamous populations of *A. lyrata* and a general effect of the mating system on imprinted genes, suggesting a limited role of parental conflict, even if the genes upregulated in endosperm appeared to be under negative selection across the Brassicaceae and that we found some signals of coevolution between imprinted genes.

Key words: genomic imprinting, parental conflict, positive Darwinian selection, endosperm, coevolution.

Introduction

Genomic imprinting is a form of epigenetic gene regulation in flowering plants and mammals in which alleles of genes are expressed in a parent-of-origin dependent manner (Haig,1997; Wilkins, 2011). In plants, imprinting is initiated by distinct epigenetic landscapes between male and female gametophytes (Batista and Köhler, 2020) that are stably inherited after fertilisation. The discovery of genomic imprinting has motivated a variety of theories attempting to explain its existence (Haig and Weston, 1989; Haig,1997; Spencer and Clark, 2014). The most frequently invoked theory is the parental conflict hypothesis (Haig, 2000). In species where the maternal parent directly provides growing progeny and has offspring by multiple males, the reproductive fitness of the mother is optimal when providing equal resources among all her offspring, while the father's fitness improves with the preferential allocation of maternal resources to his own offspring. The parental conflict theory posits that imprinting is selected to resolve this conflict over the allocation of resources to the embryo between the maternal and paternal genomes.

In plants, parental conflict is expected at the level of the endosperm, the embryo's nourishing tissue in the seed. Consistent with a conflict over resource allocation, many imprinted genes appear to be involved in resource accumulation and metabolism (Raissig et al., 2011). Moreover, growth-limiting this detection was based on the genome of the progeny of only three accessions from Greece. Similar signatures of positive selection on PEG were reported on the autogamous species *A. thaliana* (Tuteja et al., 2019). These observations raised the question of the generality of the implication of parental conflict on this positive selection because parental conflict is not expected in autogamous species. Thus, the signature of selection expected on imprinted genes and the implication of the parental conflict remains unclear in plants.

Another consequence of parental conflict expected at the molecular level is the coevolution of the pairs of reciprocally imprinted genes having antagonistic effects on offspring growth. This coevolution could be explained by direct interactions between the antagonist imprinted genes (Wilkins and Haig, 2001; Mills and Moore, 2004; Haig, 2014) or indirectly, by interactions with other genes implicated in parental conflict (Willi, 2013). Population genomic methods may be used to detect coevolving coding sequences. These methods, as genetic linkage for example, used the variations on allelic frequencies to estimate coevolution. The main advantage of these methods is the possibility of studying all polymorphic genes, whatever the distribution of this gene across species. This point is particularly important for imprinted genes that exhibited low conservation between *A. thaliana* and *C. rubella* (Hatorangan et al., 2016). genes are expected to be under maternal control, while growth-promoting genes should be under paternal control (Haig and Weston, 1989; Halg and Graham,1991; Moore and Haig, 1991). Consistent with this prediction, in *A. thaliana*, an increase in maternal genome dosage (cross between a tetraploid mother and a diploid father) produces small seeds, whereas a reciprocal cross (increased paternal dosage) produces larger seeds (Scott et al., 1998). However, the effects on seed size probably result from an interaction between cytoplasmic effects, dosage effects and genomic imprinting.

Such conflict should lead to positive selection on coding sequences of imprinted genes expressed from the maternally and paternally inherited genomes (Wilkins and Haig, 2001; Mills and Moore, 2004; Haig, 2014). According to this assumption, signatures of positive selection have been detected at the imprinted *MEDEA* gene in the allogamous plant *Arabidopsis lyrata* but not in the autogamous sister species *A. thaliana* (Spillane and al., 2007; Miyake and al., 2009). Evidence of positive selection has also been reported for some Paternally Expressed Genes (PEG), but not on Maternally Expressed Genes (MEG), of the autogamous Brassicaceae species, *Capsella rubella* (Hatorangan et al., 2016). This suggests that positive selection is not a general phenomenon expected on imprinting genes. However,

However, these methods require polymorphism across samples and could be biased by different factors such as genetic distance, demographic events or genetic structuration. More holistic approaches are based on reconstructing the evolutionary history of candidate coevolving genes, assuming that their evolutionary history should be common if they coevolve and also likely diverge from the evolutionary history of the rest of the genome (Dutheil and Galtier, 2007). Most of these methods consist of comparisons of the phylogenies, sometimes completed by functional data about the genes, to finally detect co-occurring changes in a phylogeny. However, to our knowledge, the signals of coevolution between imprinted genes, based on population genomics as on phylogenetics, were never investigated so far.

To determine the selection induced by the genomic imprinting and mating system in allogamous species, we analysed known imprinted genes of *A. lyrata* (Klosinska et al., 2016). This species is a hermaphroditic plant, close to the model species *A. thaliana*, which has cross-pollinating and selfing populations in North America (Ross-Ibarra et al., 2008). Using phylogenetic data across 23 species of *Brassicaceae* and genomic data from nine different allogamous and nine autogamous populations of *A. lyrata*, we searched: 1) the selective pressure acting on imprinted genes in allogamous populations, 2)

the implication of mating type on this selective pressure and 3) evidence of coevolution between imprinting genes and the other genes upregulated in the endosperm. Our findings suggest that parental conflict promotes diversification on non-imprinted genes, specifically in allogamous populations, but not specific selection on imprinted genes.

Materials and Methods

We expected that parental conflict promotes different genomic signatures of selection on the imprinted genes than in the other endosperm genes. To detect signals of selection on imprinted genes and the implication of the parental conflict on the selection, we developed an approach based on the comparison between the phylogenetic pattern and the variation of polymorphism of the 27 maternally expressed genes (MEG) and the 46 paternally expressed genes (PEG; Klosinska et al., 2016) clearly identified in the reference genome of *A. lyrata* (Hu et al., 2011) with the 2703 other genes upregulated in endosperm and a set of 100 randomly chosen ubiquitous genes.

Expression data

The RNAseq expression data for tissues, including leaf, root, pistil, and pollen, used in this study were acquired from populations of European and North American lineages of *A. lyrata* generated by Iltas et al. (2024). Additionally, endosperm RNAseq data from five outcrossed *A. lyrata* individuals were incorporated from a previous study by Klosinska et al. (2016). The endosperm data was integrated into the data processing pipeline as described by Iltas et al. (2024). Genes that showed significant upregulation (log2FC > 1 and adjusted P < 0.05) in all pairwise comparisons with other tissues were considered as endosperm-specific for further analysis. The RNAseq data for leaf, root, pistil, and pollen tissues can be found in the NCBI BioProject database under submission number PRJNA1067428. The endosperm RNAseq data is available under submission number GSE76076.

Genes clusters

We used the list of the 49 PEG, and 35 MEG found in *A. lyrata* (Klosinska et al., 2016). We filtered the genes with conserved coordinates in the reference genome of *A. lyrata* V1.0.23 (Hu et al., 2011) to finally study 27 MEG and 46 PEG. The previous studies in *C. rubella* (Hatorangan et al., 2016) and in *A. thaliana* (Tuteja et al., 2019) suggested different signatures of selection between the PEG and the MEG. Thus, we distinguished the two types of imprinted genes in our study.

To discriminate the signature of selection related to endosperm, considered under parental conflict, to the signature associated with other possible selection process, we distinguished the imprinted genes upregulated specifically in the endosperm ($14PEG_e/15MEG_e$) with the other imprinted genes not specifically upregulated in the endosperm ($32PEG_o/12MEG_o$) using differential expression analysis. The significance of the number of PEG and MEG upregulated in endosperm between all the PEG and MEG were tested by permutation tests based on 10,000 resamples of the genes with transcriptomic data. For all the following analyses, we compared these clusters of imprinted genes with the 2703 other genes upregulated in the endosperm (*e.g.* endosperm genes) and a set of 100 randomly chosen ubiquitous genes (*e.g.* control genes).

Based on the sequences on the reference genome of *A. lyrata* (Hu et al., 2011), we tested if the size of the gene, the GC content and the CDS content (%) on the gene were significantly different across the group of genes. To test for significant differences across the group of genes, we performed Kruskal–Wallis test as implemented in R version 4.3.2. Then for the traits with significant variations across groups of genes, we performed Dunn's test of multiple comparisons using rank sums, with two-sided P values adjusted using the Bonferroni method as implemented in the R package dunn.test (version 1.3.6; Dinno, 2024).

Read mapping and variant calling

We worked on natural accessions from *A. lyrata* of North America represented by 18 samples of 25 individuals from allogamous and autogamous populations (table S2), using available pool-sequencing data (Willi et al., 2018).

Raw reads from Willi et al (2018) datasets were mapped onto the complete *A. lyrata* reference genome V1.0.23 (Hu et al., 2011) using Bowtie2 v2.4.1 Langmead and Salzberg, 2012). File formats were then converted to BAM using samtools v1.3.1 (Li et al., 2009) and duplicated reads were removed with the MarkDuplicates program of picard-tools v1.119 (http://broadinstitute.github.io/picard). Biallelic SNPs in these regions were called using the Genome Analysis Toolkit v. 3.8 (GATK, *DePristo* et al., 2011) with the option GVCF and a quality score threshold of 60 using vcftool v0.1.15 (Danecek et al., 2011). We excluded sites with less than 15 reads aligned. Sites covered by at least 15 reads but containing no SNP were considered as monomorphic. The final number of sites in each sample set is summarised in table S2.

Effect of mating-type on global population polymorphism

We decomposed the signal of selection across the imprinted genes into two elementary statistics: the Tajima's D and the ratio of average polymorphism between the non-synonymous and synonymous sites π_{NS}/π_S. For the allogamous and autogamous populations, we compared the values obtained for the imprinted genes with the values obtained across the endosperm genes and the control genes. Because parental conflict is expected to promote positive directional selection on imprinted genes and balancing selection on non-imprinted genes in allogamous populations, we expected a general decrease of π_{NS}/π_S and of the Tajima's D on imprinted genes compared to endosperm genes in the allogamous populations. However, these differences of statistics between imprinted and non-imprinted genes were not expected for the autogamous populations. In fact, in the case of selfing, we expect that non-imprinted endosperm genes present signals of positive directional selection because of the absence of parental conflict.

First, for each gene of each population, we estimated the π obtained for all positions of each gene using vcftools[37] with –site-pi option. If a position of the *A. lyrata* genome was covered but not polymorphic, the π was set to 0. Then, the 0-fold and 4-fold degenerate sites were identified and extracted from the reference genome and the gene annotation using the script NewAnnotateRef.py (Williamson et al., 2014). Because all mutations at 0-fold degenerate sites alter the sequence of the encoded protein, we assumed that these mutations are non-synonymous (NS). In contrast, mutations at the 4-fold degenerate sites never alter the encoded amino acid, so we assumed that these mutations are synonymous (S). For the sake of simplicity, we discarded mutations at 2- or 3-fold degenerate sites. Based on the π estimated for each site, we estimated, for each gene in each population, the mean π_{NS} and π_S, to finally estimate the π_{NS}/π_S. We removed the genes with a π_s to 0.

Secondly, for each gene of each population, we estimated the Tajima's D using vcftools (Danecek et al., 2011)[37] with --TajimaD option. We removed the genes without polymorphic sites.

To test for significant differences across the group of genes, for the allogamous and autogamous populations independently, we performed Kruskal–Wallis test as implemented in R version 4.3.2. Then for the statistics with significant variations across groups of genes, we performed Dunn's test of multiple comparisons using rank sums, with two-sided P values adjusted using the Bonferroni method as implemented in the R package dunn.test (version 1.3.6; Dinno, 2024).

Distribution of the genes across Brassicaceae

We researched if the imprinted genes present specific signals of selection compared to the endosperm and control genes in the phylogeny of 22 Brassicaceae. All the reference genomes were extracted from the Phytozome database (Goodstein et al., 2012; table S3). Thus, we extracted the sequences of the genes in the *A. lyrata* reference genome (Hu et al., 2011) using bedtools V2.30 (Quinlan et al., 2010). Then, we researched the best hit of these sequences with an e-value lower than 1^{e-5} on 22 other Brassicaceae by blast V2.10 (Altschul et al., 1990). If more of one hit were found for a gene in one specie, we filtered the best hit as the hit with: the higher length alignment, the higher % of identity, then the higher coverage on the gene of *A. lyrata* and the higher reciprocal coverage on the gene of the other species. Then we extracted the sequence of the best hit for each species of each gene.

Based on this data, to test for significant differences on the number of species with homologous genes across the group of genes, we performed Kruskal–Wallis test, as implemented in R version 4.3.2. Then, we performed Dunn's test of multiple comparisons using rank sums, with two-sided P values adjusted using the Bonferroni method as implemented in the R package dunn.test (version 1.3.6; Dinno, 2024).

Divergence of the imprinted genes across Brassicaceae

The rates of nonsynonymous mutations (D_{NS}) and of synonymous mutations (D_S) is assumed to follow the neutral evolutionary process and the ratio D_{NS}/D_S is therefore approximate to the selective pressure on the protein product of a gene. An increase of the D_{NS}/D_S in genes signifies positive selection, while a decrease indicates negative selection. To test if imprinted genes display accelerated evolutionary rates compared with endosperm genes, using the annotation of the 0-fold and 4-fold degenerate sites in *A. lyrata*, we estimated the mean evolutionary rates D_{NS}/D_S for each gene between the reference genome of *A. lyrata* and the species with an homologue found across the 22 other Brassicaceae. Only the genes found in at least 3 species and with a D_s higher than 0 were considered (table S4).

Then, we further compared the fixation index (FI) on imprinted genes with endosperm and control genes based on the ratio of the between-species divergence (D_{NS}/D_S) to the within-species polymorphism (π_{NS}/π_S) on the populations of allogamous and autogamous as used in Tuteja et al (2019).

Based on these data, to test for significant differences on the D_{NS}/D_S and FI across the group of

genes, we performed Kruskal–Wallis test, as implemented in R version 4.3.2. Then, we performed Dunn's test of multiple comparisons using rank sums, with two-sided P values adjusted using the Bonferroni method as implemented in the R package dunn.test (version 1.3.6; Dinno, 2024).

Topology of the genes across Brassicaceae

For each gene, we computed the gene, and all the best hits found in the different species in fasta files to do alignment with Muscle V3.8.155 (Edgar, 2004). Then, we build a phylogenetic tree following HKY85 model for each gene with phyml V3.3 (Guindon et al., 2010). Moreover, we concatenated the alignment of the 1055 endosperm genes found in the 22 others Brassicaceae in a fasta file to build a phylogenetic tree without branch length of the endosperm using phyml V3.3 (Guindon et al., 2010). This tree was used as a proxy of the topology of the endosperm. Then, we estimated the Robinson-Foulds distance between the topology of this tree with the topology of the tree of each gene obtained previously using the R package TreeDist. Then, to test for significant differences on the RF distance across the group of genes, we performed Kruskal–Wallis test, as implemented in R version 4.3.2.

Variation of the length of branches across Brassicaceae

We researched if the imprinted genes present a variation of the length of branches compared to the endosperm and control genes. For each gene, we estimated the length of branches for each species with the topology of the phylogenetic tree obtained with the 1055 endosperm genes using phyml V3.3 (Guindon et al., 2010). We extracted the length of branches of each gene with the R package 'ape' version 5.7-1. If the gene was not present in a species, the value was replaced by "NA". To test for significant differences on the length of branches across the group of genes, we performed Kruskal–Wallis test, as implemented in R version 4.3.2. Then, we performed Dunn's test of multiple comparisons using rank sums, with two-sided P values adjusted using the Bonferroni method as implemented in the R package dunn.test (version 1.3.6; Dinno, 2024).

Genes in coevolution

To detect coevolving coding sequences, we used three different methods based on the following assumptions: the coevolution could create 1) related polymorphic patterns between genes in populations, 2) related evolutionary rate across species and/or 3) similarity in the topologies across species (Dutheil and Galtier, 2007).

To detect coevolution based on polymorphism in population and on evolutionary rates across species, we estimated Pearson's linear correlations between each pair of imprinted and endosperm genes for the mean π and the length of branches on each species. Based on these matrices of correlations, we extracted the correlations between pairs of endosperm genes to define the thresholds of extreme values in the distributions. Then, all pairs of genes included imprinted genes with correlations lower than the value at 1% of the distributions or higher than the value at 99% of the distributions were considered as significant. We used these significant correlations of π and length of branches to build networks of coevolution.

To detect coevolution based on topology with other genes, first, we used the distribution of RF distance between endosperm genes and the global endosperm topology to estimate the value obtained in 95% of the distribution. Then, we removed all the imprinted and endosperm genes with lower RF distance than this value to remove genes with a topology explained by global topology. Finally, we compared the topology of each remaining imprinted gene and endosperm genes. All pairs of genes with a null RF distance were considered as significantly similar and used to build the last coevolution network.

For each network of coevolution, we compared the number of pairs of imprinted genes directly correlated with the number found between genes in 1000 random samples of 73 endosperm genes.

Identification of sites in coevolution

We used a model-based approach for detecting coevolving positions of each pair of genes with a coevolution signal. The pairs of imprinted genes studied were all the pairs directly associated within at least one network previously mentioned. To use this method, we concatenated each sequence of pairs of genes for each species. If one species was not found in the other gene, the sequence associated with this species was removed.

Coevolving positions within each pair of genes were predicted using the clustering method implemented in the CoMap package (version 1.5.2; Dutheil and Galtier, 2007). The built-in algorithm was then used to correct for multiple testing and estimate the false discovery rate (FDR). In this study, 1,000 simulations per tested pair were performed, and the groups of sites with an adjusted p value $< 1\%$ were considered coevolving. Simple correlation was used as a measure of coevolution. For each pair comparison, we extracted the coevolving groups of substitutions and classified them by size (between 2 and 10).

To test the significant excess of correlations, we compared the results obtained with the results for the same number of pair comparisons of imprinted genes not directly associated in any network, for each cluster of coevolving groups of substitutions of equivalent size by Krustal-Wallis tests. If the number of correlations is significantly higher than the number expected between two random genes by chance or by alignment errors, we expect a significant increase of these numbers in pairs of associated imprinted genes.

Finally, to test the significant excess of correlations in pairs of associated imprinted genes compared to other endosperm genes, we compared the results obtained with the results for the same number of pair comparisons of non-imprinted endosperm genes directly associated in, at least, one network, for each cluster of coevolving groups of substitutions of equivalent size by Krustal-Wallis tests.

Results

Expression patterns and characteristics of the genes studied

In order to disentangle the impact of parental conflict and tissue specificity on selective processes, the genes specifically upregulated in the endosperm of *A. lyrata* were identified based on transcriptomic data obtained for 19,425 genes from 5 tissues (leaf, root, pollen, pistil, endosperm). A total of 2732 genes were upregulated in the endosperm. By comparison with the list of imprinted genes, we found that among the 27 MEG and 46 PEG found in *A. lyrata* (Klosinska et al., 2016), 15 MEG and 14 PEG were specifically upregulated in the endosperm. These proportions of genes upregulated specifically in the endosperm between the MEG and PEG were significantly higher than those predicted by 10000 random resampling of all the genes studied (p value < 1^{e-04}). For the following analysis, PEG and MEG upregulated in the endosperm will be referred to as PEG_e and MEG_e. The other PEGs and MEGs have been named PEG_o and MEG_o. In these genes, we expected less effect of parental conflict because these genes could be under other selection processes in the other tissues. The 2703 non-imprinted genes upregulated in endosperm will be referred to as endosperm genes. A set of 100 randomly chosen not imprinted ubiquitous genes will be considered as control genes for genes not under parental conflict.

We compared the size of the gene, the coding sequences (CDS) content (%) and the GC content (%) of the imprinted genes in *A. lyrata* with the endosperm and the control genes by Krustal-Wallis tests because these statistics could modify the statistics used to detect selection in the rest of the analysis. Fortunately, only the size of the genes varied significantly across the group of genes (pvalue=1.63^{e-84}). The non-imprinted endosperm genes and the PEGo were significantly smaller than control genes following Dunn's tests after Bonferonni's corrections (table S5).

The PEG_e present phylogenetic characteristic of negative selection in Brassicaceae

We investigated the phylogenetic characteristics of the imprinted genes in *A. lyrata* and twenty-one other Brassicaceaes compared to other groups of genes. First, we compared the number of species with homologous genes across the group of genes (Fig 1A; table S6). This number varied significantly across groups (p value KW=2.97^{e-15}). In fact, this number increased significantly in endosperm genes compared to control and decreased in the PEG_e compared to endosperm. The numbers of species with homologous genes for the other PEG and MEG were intermediate to control and endosperm genes. However, the Robinson-Foulds (RF) distance based on comparison between the phylogenetic topology of each gene and the topology based on the endosperm genes found in all the Brassicaceae was not significantly different across the group of genes (p-value Krustal-Wallis = 0.6; Fig 1B). Thus, this topology was used to estimate the length of branch for each gene with homologous genes found in at least three species (including *A. lyrata*; table S4). The mean length of all branches found in the imprinted genes varied significantly across groups of genes (Fig 3C; p value KW< 2.2e-16; table S6). The mean length of all branches of all the groups of genes were significantly higher than control genes, but this value was significantly lower for the MEG_o and PEG_e compared to endosperm genes. Moreover, the branches of *A. lyrata* varied significantly across groups of genes (p value KW= 1.55^{e-115}; table S6). However, the mean length of branches found in the imprinted genes was not significantly different than in the endosperm genes (table S6).

Finally, we estimated the variations of the D_{NS}/D_S between *A. lyrata* and the other Brassicaceae species. We expect an increase of this ratio compared under positive directional selection, and the reverse under negative selection. Before analysis, the genes with homologous genes found in less than three other Brassicaceae and without divergent synonymous sites were removed (table S4). This ratio varied significantly across groups of genes (p-value Krustal-Wallis = 7.02^{e-05}; Fig 1D; table S6), with a significant decrease in the MEG_e and the PEG_e compared to endosperm genes.

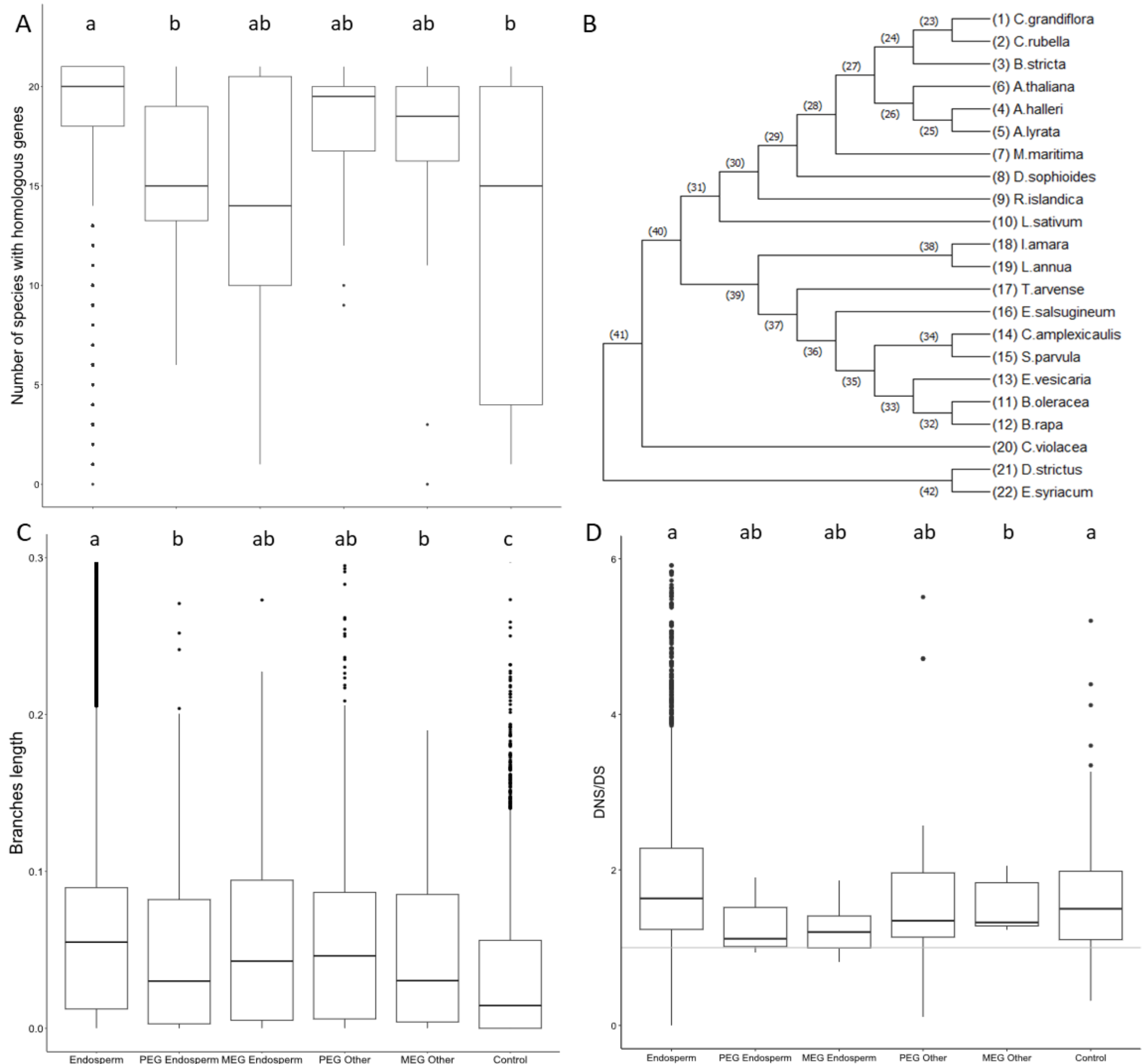

Figure 1: Phylogenetic characteristic of the imprinted genes compared to endosperm genes across the Brassicaceae *A: boxplot of the variations of the number of species with homologous genes by group of genes. B: phylogenetic tree obtained by HKY85 model[41] based on the 1055 endosperm genes found in all species after 1000 bootstrap. The percentage of trees in which the associated haplotypes clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. C: boxplot of the variations of the mean length branch by gene based on all the species. D: boxplot of the variations of the* D_{NS}/D_S *by gene. The light grey lines represent the threshold value for neutrality. The minuscule letters (a,b,c) signified the groups significantly different following Dunn's test (p-values < 0.05.).*

No signal of positive selection related to the mating system was detected on imprinted genes

The parental conflict on imprinted genes is expected to promote positive selection on these genes in allogamous populations compared to non-imprinted genes. In the populations, positive selection is expected to reduce polymorphism, specifically the non-synonymous polymorphism, by fast fixation of advantageous alleles. Conversely, in autogamous populations, with reduced parental conflict, we expected no such signatures on imprinted genes. To estimate the effect of mating type and imprinting on polymorphism in populations, we compared Tajima's D, the π_{NS}/π_S ratio and the fixation index (= $(D_{ns}/D_s)/(\pi_{NS}/\pi_S)$; FI) on the imprinted genes to the endosperm and control genes in the allogamous and autogamous populations.

In the case of directional selection, we expect a reduction of the Tajima's D, whereas we expect an increase of the Tajima's D in case of balancing selection. However, this method requires some polymorphic positions. Thus, the genes without polymorphic sites in all the populations were removed before analysis. The number of endosperms conserved increased significantly compared to control but the number of imprinted

genes were intermediate to control and endosperm genes, except the number of MEG_o that was significantly lower than endosperm genes (binomial tests; table S4). In the allogamous populations, the Tajima's D varied significantly across the groups (p-value Krustal-Wallis = 7.02^{e-05}), but the effect is explained only a significant decrease on PEG_e compared to MEG_o and is not related to variations with endosperm or control genes (Fig S1A; table S7). In the autogamous populations, the Tajima's D did not vary significantly across the groups (p-value Krustal-Wallis = 0.052; table S7). The Tajima's D decreased significantly on endosperm genes on autogamous populations, compared to allogamous populations, whereas the same statistics increased significantly on PEG_e (table S8). The Tajima's D on other groups did not vary with the mating system.

In the case of directional selection on imprinted genes, we expect a reduction of the π_{NS}/π_S. However, this method requires polymorphism on synonymous sites. Thus, the genes with a π_S to zero in all the populations were removed before analysis. The number of endosperms conserved increased significantly compared to control, but the number of imprinted genes were intermediate to control and endosperm genes, except the number of MEG_o that was significantly lower than endosperm genes (binomial tests; table S4). This ratio varied significantly across the groups of genes in allogamous and autogamous respectively (p-values Krustal-Wallis = 7.47^{e-04} and 7.48^{e-05} respectively). However, the tendencies observed were different: in the allogamous populations, the ratios on the PEG_e and the MEG_o increased significantly compared to the endosperm genes, and the ratio on control genes was similar than in endosperm genes (Fig 2A; table S7). In the autogamous populations, only the ratio on the PEG_e increased significantly compared to the endosperm genes, and the ratio on control genes was significantly higher than in endosperm genes (Fig 2B; table S7). The π_{NS}/π_S decreased significantly on endosperm genes on autogamous populations compared to allogamous populations (table S8). The π_{NS}/π_S on other groups did not vary with the mating system. Interestingly, the general polymorphism π increased significantly on MEG_o compared to endosperm genes, whatever the mating system of the populations considered (table S7).

We attempted to detect signals of selection by a variation of the FI as in Tuteja et al. (2019). We expected an increase of the FI in case of positive selection. However, this method requires divergent synonymous positions and a π_{NS}/π_S higher than 0. Thus, the genes that did not respect these criterias were removed before analysis. The number of endosperm and PEG_o genes conserved increased significantly compared to control but the number of MEG_e and PEG_e were intermediate to control and endosperm genes and the number of MEG_o was significantly lower than in the endosperm genes (binomial tests; table S4). The FI varied significantly across the groups of genes in allogamous and autogamous respectively (p-values Krustal-Wallis = 2.54^{e-07} and 4.16^{e-07} respectively). In the allogamous populations, according to the observations on π_{NS}/π_S, the FI on the PEG_e and the MEG_o decreased significantly compared to the endosperm genes, but also compared to control genes (Fig 2C; table S7). In the autogamous populations, the tendencies observed were the reverse of the observation on π_{NS}/π_S (Fig 2D; table S7). According to the observations on π_{NS}/π_S, the FI increased significantly on endosperm genes on autogamous populations compared to allogamous populations (table S8). The FI on other groups did not vary with the mating system.

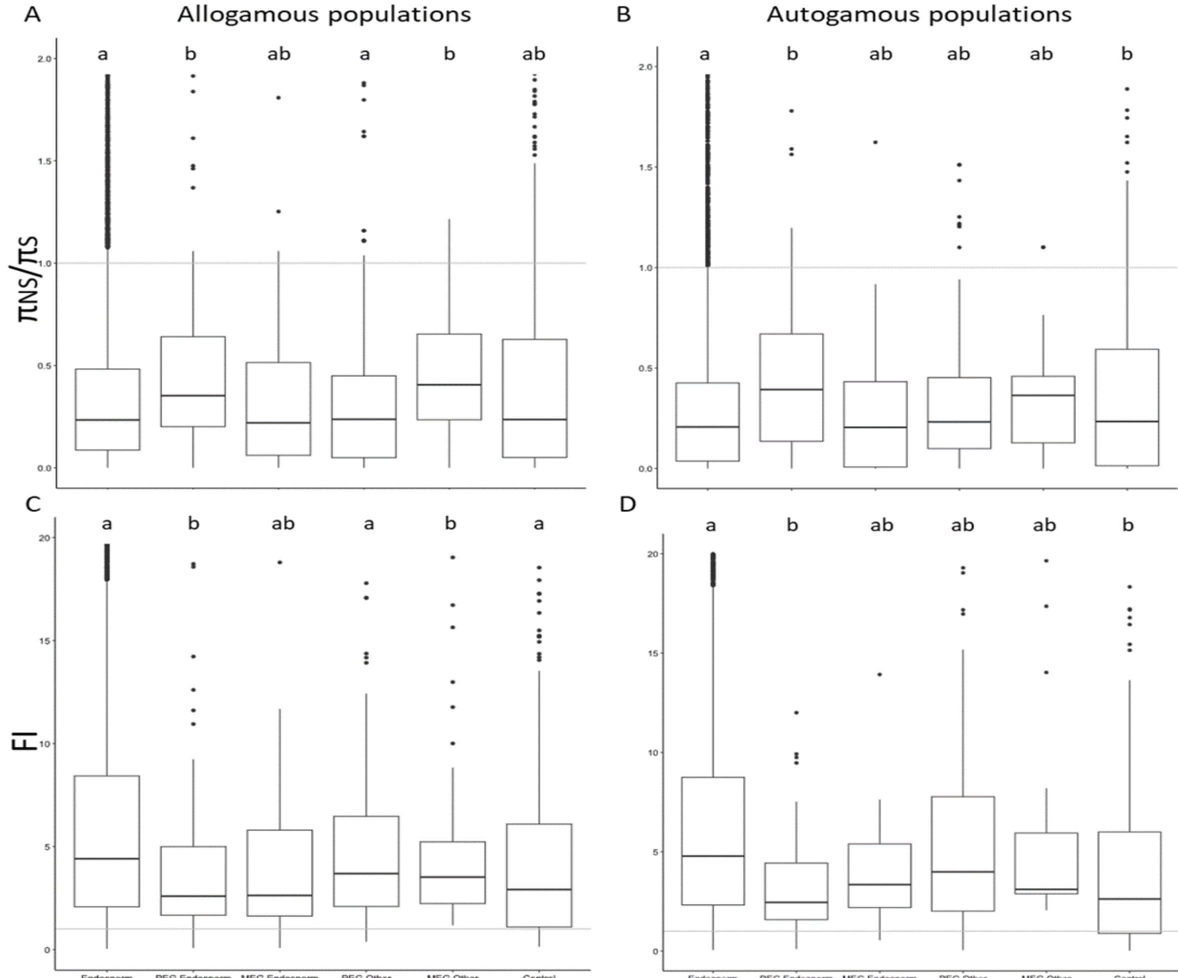

Figure 2: Variation of the polymorphism in the allogamous and autogamous populations in the imprinted genes compared to endosperm and control genes. *A and B: boxplot of the variations of the median π_{NS}/π_S by gene. C and D: boxplot of the variations of the median FI by gene. The light grey lines represent the threshold value for neutrality. A and C: allogamous populations. B and D: autogamous populations. The minuscule letters (a,b,c) signified the groups significantly different following Dunn's test (p-values < 0.05.).*

A limited number of imprinted genes presented intense signals of directional selection.

We researched the imprinted genes associated with strong signals of directional selection compared to endosperm genes by the research of imprinted genes with a lower mean length of branches across the Brassicaceae, and/or mean of Tajima's D in allogamous populations and/or mean π_{NS}/π_S in allogamous populations than 5% of these same values in the endosperm genes.

One MEG_o (*AL1G46480*), two MEG_e (*AL1G54190* and *AL2G25160*) and one PEG_o (*AL7G39320*) were associated with a lower mean length of branches across the Brassicaceae. No homologous gene in *A. thaliana* was found for the first and third genes (table S1). Based on homologous genes in *A. thaliana* (table S1), the other genes encode respectively an AGAMOUS-like protein expressed during proliferative endosperm development (Day et al., 2008) and implicated on female gametophyte, seed and early endosperm development (Bemer et al., 2010; Zhang et al., 2018), and a non-catalytic subunit common to Nuclear DNA-dependent RNA polymerases IV and V implicated on the RNA-Directed DNA Methylation pathway (Ma et al., 2015). The MEG_e *AL2G25160* was also associated with a lower mean Tajima's D in allogamous populations, as well as one other PEG_o (*AL4G10630*). The homologous gene in *A. thaliana* (table S1) encodes for chromatin remodelling protein implicated on resistance to oxidative stress tolerance of seed (Sujeeth et al., 2020) on seed germination (Dekkers et al., 2013) and on female gametophyte development (Wuest et al., 2010). On the contrary, no common gene was associated with a lower mean π_{NS}/π_S in allogamous populations. In fact, only one MEG_e (*AL7G33050*) and one PEG_e (*AL6G38210*) was associated with a reduced mean π_{NS}/π_S. Based on homologous genes in *A. thaliana*

(table S1), these genes encode respectively a Dof-type zinc finger domain-containing protein implicated on seed coat formation (Zou et al., 2012), and with an important role during proliferative endosperm development (Day et al., 2008), and a member of the Alfin1-like family of nuclear-localised plant homeodomain containing proteins implicated on the secondary cell wall synthesis (Taylor-Teeples et al., 2015) and in response to genotoxic stress (Chen et al., 2003).

Then, we researched the imprinted genes associated with strong signals of negative and positive selection in *A. lyrata* compared to endosperm genes by the research of imprinted genes with a lower and higher respectively mean D_{NS}/D_S than 5% of the endosperm genes. Two MEG_e previously mentioned, *AL1G54190* and *AL7G33050*, and associated respectively with a reduced mean length of branches and mean of π_{NS}/π_S, were also associated with a reduced D_{NS}/D_S, as well as two other PEG_o (*AL2G16860* and *AL4G11250*), encoding respectively a SIN3-like protein implicated on pollen germination and tube growth (Wang et al., 2008), on induction of flowering (Huang et al., 2019) and in stress response (Feng et al., 2021), and an another protein implicated on pollen germination and tube growth and on pathogen response (Wang et al., 2008; Ascencio-Ibáñez et al., 2008). On the contrary, no common gene was associated with a higher mean D_{NS}/D_S. In fact, only one MEG_o and one PEG_o (*AL5G18700* and *AL6G38200*) were associated with an increased mean D_{NS}/D_S, and these genes encode respectively a mini zinc finger protein implicated on regulation of floral meristem termination (Bollier et al., 2018), on seed and anther development (Meyer et al., 2012; Zhu et al., 2010) and a mitochondrial substrate carrier family protein implicated on nitrate, drought and high light response (Estavillo et al., 2011; Konishi et al., 2021)

Whatever the statistics and the group of imprinted genes considered, the number of genes with signals of directional selection was never significantly higher than 5% (p values binomial tests >= 0.17).

Clear trend of coevolution between imprinted genes in Brassicaceae

The last important expectation about imprinting genes is that parental conflict promotes coevolution between genes favouring paternal or maternal fitness in an arms race scenario. To test this assumption, different coevolution signals between PEG, MEG and the other endosperm genes were investigated. More precisely, we assessed whether some genes presented correlations based on 1) the π in the populations, 2) the length of branches or 3) Robinson-Foulds distance between pairs of topologies of the phylogenetic trees. First, the correlations of the π and the length of branches between each pair of PEG, MEG and endosperm genes were estimated by Pearson correlation. Then the values at 1 and 99% of each distribution obtained for the pairs of endosperm genes were used as limits for significant correlations with imprinted genes. These values were -0.66 and 3.20 for the polymorphism and -0.64 and 0.89 for the length of the branches.

Based on these values, for the polymorphism, 49 PEG and MEG were significantly correlated together, and with 145 common endosperm genes to 19 other PEG and MEG and with 1,865 other endosperm genes (Fig 3A; table S9). This complete network was associated with 75 biological processes, 5 molecular functions and 48 cellular compounds by gene ontology (GO, table S10). The number of direct correlations between PEG and MEG and the number of genes correlated were significantly higher than in 1000 random samplings of 73 endosperm genes (p-values= 6^{e-03} and $<1^{e-03}$ respectively). Three imprinted genes (*AL4G15650*, *AL3G18550* and *AL5G20240*) were correlated with more genes than 99% of the endosperm genes (table S9).

For the length of the branches, 36 PEG and MEG were significantly correlated together, and with 282 common endosperm genes with 34 other PEG and MEG and with 1,012 other endosperm genes (Fig 3B, table S11). Only 64 of these pairs of genes with significant correlations of length of branches presented also significant correlation of polymorphism, and these pairs of genes implicated always one imprinted and one endosperm gene. This complete network was associated with 10 biological processes, the catalytic activity and 26 cellular compounds by GO (table S10). The number of direct interactions and the number of imprinted genes directly linked were not significantly higher than in 1000 random samplings of 73 non imprinted endosperm genes (p-values = 0.08 and 0.051 respectively). However, the MEG_e *AL1G54190* was correlated with more genes than 99% of the endosperm genes (table S11).

Finally, we researched the pair of genes with a null RF distance. Moreover, to remove the pairs of genes with a signal of coevolution explained by the similarity with the global topology, we removed the pair of genes that implicated genes with a RF distance with the global topology of the endosperm genes (Fig 1B) lower than 95% of the distribution of the RF distance found in endosperm genes (=0.71). Based on these criterias, the topology of one PEG_e was significantly related to six endosperm genes. Three of them presented topologies significantly similar to one PEG_o. The topology of these imprinted genes was also similar with two other

endosperm genes (Fig 3C; table S12). Moreover, three endosperm genes presented significant similar topologies with two PEG_o, and one topology of these endosperm genes was also significantly similar to one other PEG_o. These two networks of genes were not associated with biological processes, molecular functions or cellular components by GO. However, the PEG_e (*AL6G33630*) presented a significant number of associations compared to 99% of non-imprinted endosperm genes (table S12).

Thus, within the 2,628 possible pairs of imprinted genes, 86 including 50 PEG/MEG (68%) were directly associated in one network based on π or the length of branches. To detect coevolution between specific mutations within these pairs of imprinted genes, we used the clustering method of the pipeline CoMap package (Dutheil and Galtier, 2007). Exactly, we researched groups compound by 2 to 10 sites in coevolution in each candidate pair of imprinted genes. To avoid false association, we compared the number of groups of each size found in the candidates with the number of groups with equivalent size for all the possible pairs of imprinted genes never associated in the previous networks and found in three or more species (Fig S1A). We found significant increases in the number of groups detected in candidates compared to control for the groups with at least eight sites in coevolution (table S13; Fig S1A). The groups of coevolving sites composed of less than eight sites were not considered.

For the 4 pairs of PEG/MEG directly associated by π (Fig 4A), we found 10 groups of at least eight correlated sites (table S13; Fig S1B for an example of one group of 9 coevolving sites between the MEG_o *AL2G40710* and the PEG_o *AL6G44530*). For the two PEGs directly associated by length of branches (Fig 4C), we found two groups of 10 correlated sites (table S13; Fig S1C for an example). Finally, for the 22 pair of PEG/MEG directly associated by topologies (Fig 4D), we found 74 groups of at least eight correlated sites for only twelve pairs of genes (55% of the candidates; table S13; Fig S1D for an example of one group of 9 correlated sites between the MEG_e *AL1G54190* and the PEG_o *AL1G64840*).

A Network based on π

B Network based on length of branches

C Networks based on topologies

Figure 3: Networks of the imprinted and endosperm genes based on A) polymorphism in population, B) length of branches and C) phylogenetic topologies across the Brassicaceae. *A) Network based on the Pearson's correlations of π found on genes in the populations. B) Network based on the Pearson's correlations of the length of branches for each gene estimated with the global topology. C) Networks based on the RF distance of topologies. Only the correlations that associate all the PEG/MEG of the networks together are represented. The correlations between PEG/MEG directly correlated and the endosperm genes are masked to simplify. The black and grey lines represent the negative and positive correlations respectively. Blue circles:* PEG_e*. Light blue circles:* PEG_o*. Red circles:* MEG_e*. Pink circles:* MEG_o*. White circles: endosperm genes required to correlate all the PEG/MEG.*

Discussion

Genomic imprinting is a form of epigenetic gene regulation in which alleles of genes are expressed in a parent-of-origin dependent manner (Haig, 1997; Wilkins, 2011). The most frequently invoked selective explanation for the evolution of imprinting is the parental conflict hypothesis (Haig, 2000). In species in which the maternal parent directly provides growing progeny and has offspring by multiple males, the mother's reproductive fitness is greater if the resources are equally distributed among all the offspring, while a father benefits from the concentration of maternal resources to his own offspring. This conflict is expected to lead imprinted genes to directional selection in allogamous populations and to promote the coevolution of imprinted genes having antagonistic effects on offspring growth (Wilkins and Haig, 2001; Mills and Moore, 2004). This coevolution could be explained by direct interactions between the antagonist imprinted genes (Wilkins and Haig, 2001; Mills and Moore, 2004) or indirectly by interactions with other genes implicated in parental conflict (Willi, 2013). Based on population genomic and phylogenetic approaches, we researched if imprinted genes are associated to specific signals of selection in allogamous populations of *A. lyrata*, if these signals are sensitive to mating systems and if these genes present signals of coevolution.

Phylogenetic signals of negative selection on imprinted genes upregulated in endosperm.

The parental conflict theory predicts that many genes under imprinting should affect growth (Haig, 2000). Thus, we supposed that, if the imprinted genes are implicated in parental conflict, these genes must be preferentially expressed on endosperm tissue. According to the parental conflict hypothesis, the imprinted genes were associated with a significant excess of genes specifically upregulated in the endosperm. However, over half of the imprinted genes were also upregulated in one or more other tissues. We supposed that this differential pattern of expression across imprinted genes could affect selection on these genes, because the genes not only upregulated on endosperm could be under different selection processes, not only related to parental conflict.

An important common point found in *C. rubella* and *A. thaliana* is the detection of rapid evolution in the imprinted genes compared to the rest of the genome (Hatorangan et al., 2016; Tuteja et al., 2019). Consistent with these studies, based on phylogenetic approaches, we found that the PEG_e and the MEG_o present a reduced length of branches, translating a fast evolutionary rate. However, contrary to previous studies, the reduction of the D_{NS}/D_S on these PEG_e and the MEG_e suggest more a process of negative selection than a process of positive selection. Interestingly, the PEG_o did not exhibit this acceleration of the evolutionary rate and the MEG_o were not associated with a reduction of D_{NS}/D_S. These observations tend to confirm that the PEG and MEG not specific to endosperm are under other processes of selection.

No specific signal of selection on imprinted genes in allogamous populations.

Parental conflict is expected to promote diversification of genes implicated on allocation ressources in allogamous populations because the alleles will be differentially selected in the two parents. However, in the case of imprinting, conflict should lead the imprinted genes to directional selection and promote the fixation or the loss of the advantageous or disadvantageous allele for the parent that expresses the genes (Wilkins and Haig, 2001; Mills and Moore, 2004). Contrary to our expectations, we did not detect a general signal of balancing on the endosperm genes in allogamous populations. This observation could be explained because the parental conflict does not apply in all genes expressed on endosperm but only in some of them.

For the imprinted genes, consistent with the study on *C. rubella* (Hatorangan et al., 2016*)*, we didn't find a homogenous signal of selection based on population genomics. In detail, we did not find a specific signal of selection on MEG_e and on PEG_o, whereas the PEG_e and MEG_o presented a decrease of the FI, according to negative selection previously mentionned, but also an increase of the π_{NS}/π_S, and an increase of the Tajima's D on MEG_o. These signals could translate a recent relaxation of selection. This relaxation of selection could be explained by the recent colonisation of *A. lyrata* of North America from Europe (Ross-Ibarra et al,. 2008; Otto et al., 2015; Willi et al., 2018), that could change the environmental pressures (biotic and abiotic) but also could decrease the efficiency of sexual selection by the general reduction of polymorphism and the increase of genetic load observed in these populations (Willi et al., 2018). To test this hypothesis, it will be required to compare the signal of selection in endosperm and imprinted genes in allogamous populations with different levels of polymorphism.

Moreover, it is important to emphasise that the list of imprinted genes used was from a previous study on *A. lyrata* lines (Klosinska et al., 2016) and not

directly from the populations studied. Indeed, we assumed that the imprinting was conserved across the species. However, the set of imprinted genes largely varies even within a species such as in *A. thaliana* (Gehring et al., 2011; Wolff et al., 2011; Hsieh et al., 2011; Pignatta et al., 2014). It is therefore possible that the "imprinted genes" studied are actually not imprinted in our populations, and thus are under different selective pressure than that expected if they were imprinted. A population-specific survey of genomic imprinting may solve this issue.

The autogamy promotes directional selection on non-imprinted endosperm genes.

Parental conflict is expected only in case of multiple mating (Haig, 2000). Thus, we supposed relaxation of selection on imprinted genes in the autogamous population. Consistent with this assumption, signatures of positive selection have been detected at the imprinted *MEDEA* locus in the allogamous plant *Arabidopsis lyrata* (Spillane et al., 2007) but not in the autogamous sister species *A. thaliana* (Miyake et al., 2009). However, positive selection on PEGs was reported on the autogamous species *A. thaliana* and *C. rubella* (Hatorangan et al., 2016; Tuteja et al., 2019). This observation could be explained if the absence of parental conflict does not promote relaxation but promotes directional selection for all genes implicated in resource allocation, including the imprinted genes. These opposite observations raise the question of the general effect of the mating system, and more specifically of the selfing rate, on the selection of imprinted genes in autogamous species or populations.

However, we did not detect singular and homogenous variations of polymorphism in imprinted genes compared to endosperm genes in autogamous populations. Exactly, we detected that only the Tajima's D for the MEG_o becomes equivalent to endosperm genes. Moreover, the autogamy increases of the Tajima's D only of the PEG_e, suggesting a relaxation of selection on these genes. All these observations suggest a limited effect of the mating system on imprinted genes.

An important argument to counteract our conclusion could be that the colonisation event of our populations is so recent (Ross-Ibarra et al., 2008; Foxe et al., 2010; Hough et al., 2013; Willi et al., 2018) that variation in selection process is not yet detectable. In addition, the transition to selfing is recent and partial (significant rates of outcrossing) in these populations, potentially limiting the impact of relaxed parental conflict on imprinted genes sequence evolution. However, in the autogamous populations, in non-imprinted endosperm genes, as expected, we observed an equivalent the Tajima's D, a decrease of the π_{NS}/π_S and an increase of the FI compared to control gene, suggesting a general directional positive selection on these genes. Moreover, the autogamy promoted a decrease of the π_{NS}/π_S and of the Tajima's D, and an increase of the FI on endosperm genes compared to allogamous populations, suggesting that the process of selection on these genes is sensitive to mating type, despite the recent transition to selfing.

Some imprinted genes with signals of directional selection sensitive to the mating system.

In the previous study on *C. rubella* (Tuteja et al., 2019), they compared the number of genes with signal of positive selection on imprinted genes with the number obtained of endosperm genes. Thus, we extracted the imprinted genes with a signal of directional selection based on endosperm genes and we compared with the number expected. Interestingly, the genes found were never associated with signals of directional selection on the autogamous populations. Between the eleven imprinted genes with signals of directional selection, four of them were associated with negative selection and two with positive selection. However, contrary to *C. rubella*, we did not detect an excess of this number in imprinted genes. Interestingly, one of these genes, the MEG_e that encodes an AGAMOUS-like protein implicated on female gametophyte, seed and endosperm development (Bemer et al., 2010; Zhang et al., 2018), presented also a polymorphism correlated with a significant number of genes, suggesting an important role on the evolution of endosperm genes.

Imprinted genes present a strong signal of coevolution on polymorphism.

Another expected consequence of parental conflict is the coevolution of the imprinted genes having antagonistic effects on offspring growth by direct or indirect interactions (Wilkins and Haig, 2001; Mills, and Moore, 2004). To detect coevolution between imprinted and endosperm genes, population genomic and phylogenetic methods were used (Dutheil and Galtier, 2007). As expected, the majority of the PEG/MEG were associated directly or indirectly by signals of coevolution and the number of direct associations based on polymorphism found was significantly higher than between endosperm genes. Together, these imprinted genes are involved in an important amount of biological functions. Further research may explore the functional significance of this coevolution signal. Moreover, we were able to find coevolving groups of sites for 18 pairs of the PEG/MEG in coevolution.

Conclusions

Here, we studied the phylogenetic and genomic signatures of selection on imprinted and endosperm genes in *A. lyrata*. By phylogenetic approaches, we found evidence for more efficient negative selection on imprinted genes expressed in endosperm genes. However, we did not detect this tendency in allogamous populations. Moreover, we detect only a limited effect of autogamy on selection on these genes. Thus, parental conflict appeared to have limited effect on evolution of imprinted genes. However, we detected that some imprinted genes, specifically one MEG, could have an importance on the evolution of genes expressed in endosperm and on parental conflict. In other PEG/MEG, we detected different and inhomogeneous signals of selection, suggesting that these genes are under other processes of selection.

Data availability

The RNAseq data for leaf, root, pistil, and pollen tissues can be found in the NCBI BioProject database under submission number PRJNA1067428. The endosperm RNAseq data is available under submission number GSE76076.

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Author contributions

O.I developed and performed the transcriptional analysis. A.L analysed and interpreted the data. AL and C.L-P wrote the manuscript. All authors edited the manuscript.

Declaration of interests

The authors declare no competing interests.

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Supplementary figures

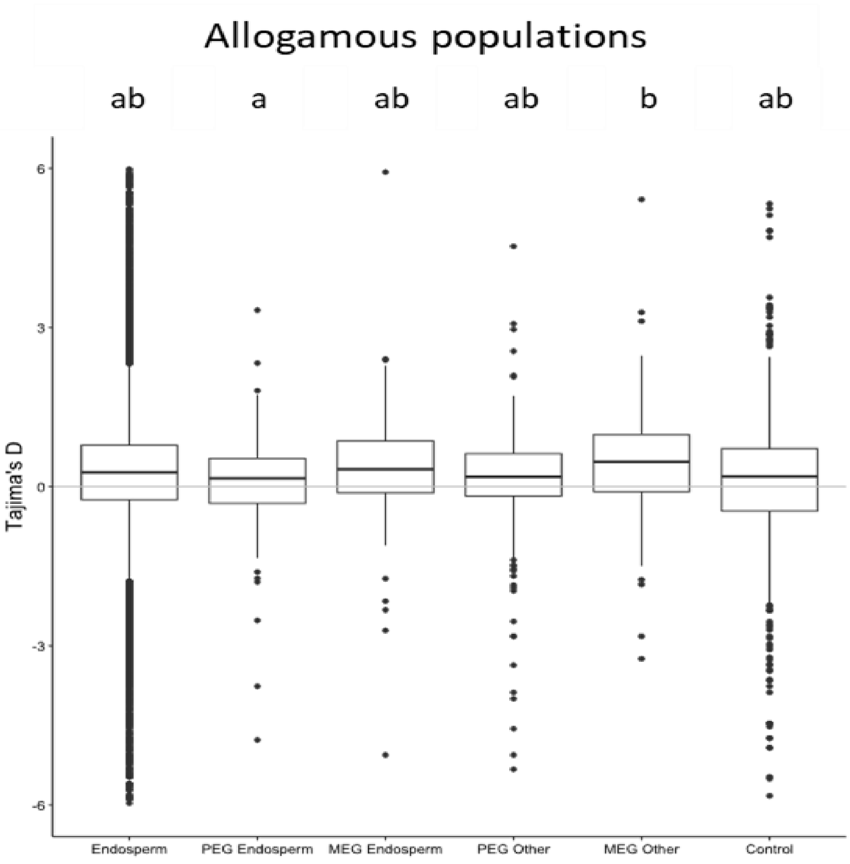

Figure S1: Variations of the Tajima's D in the allogamous populations in the imprinted genes compared to endosperm and control genes. *boxplot of the variations of the median Tajima's D by gene. The light grey line represents the threshold value for neutrality. The minuscule letters (a,b,c) signified the groups significantly different following Dunn's test (p-values < 0.0.).*

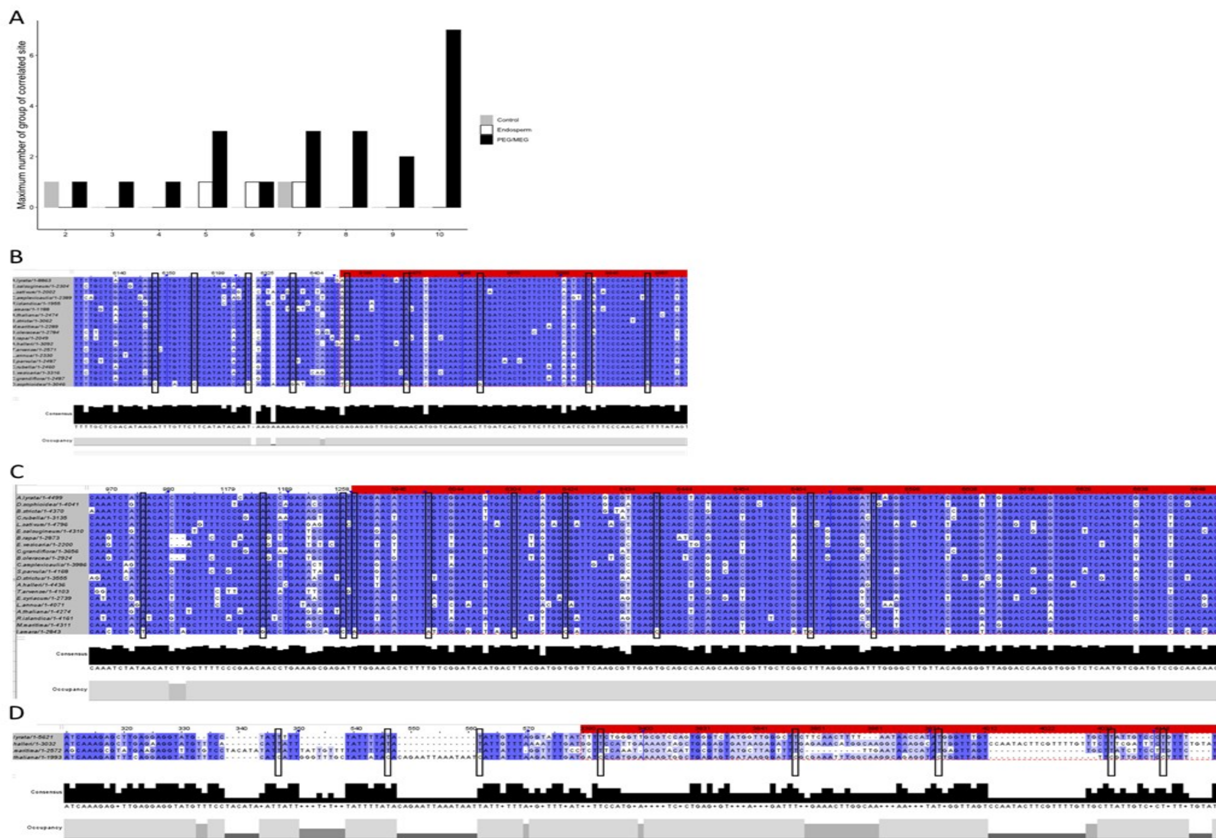

Figure S2: Correlated sites in imprinted genes directly associated in the networks in 22 Brassicaceaes. *A) Maximum number of groups with at least 2 correlated sites in each pair of genes. We distinguished the groups of correlated sites by the number of sites found in each group (between two and ten) and by type of pair of genes. We compared the real pair of imprinted genes directly associated in the networks (black bars) of the pair of imprinted genes never associated (control for false association; grey bars) and of the pair of non-imprinted endosperm genes directly associated in the networks (white bars). B) Example of alignment for one group of correlated sites for a pair of imprinted genes associated by polymorphism in populations. C) Example of alignment for one group of correlated sites for a pair of imprinted genes associated by length of branches. D) Example of alignment for one group of correlated sites for a pair of imprinted genes associated by topologies. The identity on each position is represented by a blue gradient. The arrows represent the hidden part of the alignment. The region on alignment for first and the second genes is delimited by the red scare (top). The correlated positions are represented by black scares. The consensus sequences and the occupancy are represented at the bottom.*