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The effect of maternal epigenetics on hybridization barriers

Diploma thesis

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Declaration

I wrote this final thesis independently and listed all the information sources and literature used. This work, or a substantial part, has not been submitted for the award of another or the same academic degree.

In Prague, 8. 8. 2024 ……

Acknowledgments

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Abstract

Hybridization barriers are reproductive barriers that contribute to plant speciation. Understanding the genetic and epigenetic mechanisms of both pre- and post-zygotic hybridization barriers may improve our understanding of evolutionary biology but also may bear applications for breeding purposes.

Among all the hybridization barriers contributing to plant reproductive isolation, pollen rejection, and hybrid seed lethality have received particular attention. Pollen rejection is a prezygotic barrier in which incompatible pollen-pistil interactions prevent a seed between two species from being formed. If a hybrid seed is formed, it might not be viable, leading to a postzygotic barrier. The failure of endosperm development is the major cause of inviability in interspecific hybrid seeds. Epigenetics is well known to play a role in this process. More precisely, the epigenome of the pollen donor seems to have an important impact on hybrid seed viability. However, the role of the maternal epigenome is less well understood. Regarding the implication of epigenetic mechanisms in pollen rejection, very scarce evidence is currently available.

Here, I assessed the role of maternal epigenetics in these pre- and post-zygotic hybridization barriers between two species: *Arabidopsis thaliana* and *Arabidopsis arenosa*. For this purpose, I used *A. thaliana* epigenetically recombinant inbreed lines (epiRILs) that are genetically similar but epigenetically variable and crossed them with *A. arenosa* as a pollen donor. I measured seed set as a proxy for pollen rejection and seed abortion rate as a proxy for hybrid seed viability. There was significant variation between epiRILs in the pre-and post-zygotic barriers, suggesting an effect of maternal epigenetics in both hybridization barriers. To further evaluate this hypothesis, I mapped epigenetic quantitative trait loci (epiQTL) to find the epialleles involved in pollen rejection and hybrid seed viability. We did not find any epiloci associated with these traits, potentially due to technical limitations. Overall, maternal epigenetics likely play a role in the two studied hybridization barriers, with a likely complex epigenetic structure involving several small effect epiloci controlling these barriers.

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1. Introduction

1.1. Diversity of angiosperms

Angiosperms comprise the majority of plant species (Stebbins, 1974). Flowering plants show a large diversity of life forms, vegetative and reproductive traits, and habitats. While this diversity is apparent, its delimitation into species and its biological relevance have been discussed throughout the 20th century, such as Mayr and Dobzhansky, and are still under debate (Nosil, P., 2012).

Species can be explained via several concepts: morphological, evolutionary, phylogenetic, ecological, biological, and more (De Queiroz, 2007). The biological species concept (BSC) defines species as ”groups of interbreeding natural populations reproductively isolated from other such groups.” (Mayr, 1996) (Briggs & Walters, 2016). This definition has the particularity to offer a mechanistic focus on *how* species emerge and are maintained as discrete units of life (Coyne & Orr, 2004). In this thesis, I aim to understand the mechanisms of reproductive isolation and use the BSC.

a) Reproductive isolation

Two main types of reproductive barriers prevent hybridization and thus contribute to genetically and phenotypically distinct groups (**Figure 1**), acting either before or after fertilization: pre- and post-zygotic barriers. In plants, prezygotic barriers are numerous, ranging from geographical isolation, ecological speciation, and pollinator preference to pollen-pistil interactions (L. Wang & Filatov, 2023). For the sake of simplicity, in this introduction, I will describe in more detail the barriers that act after the pollen is deposited on the stigma, i.e., postpollination pre- and postzygotic barriers, since these barriers are the ones I studied.

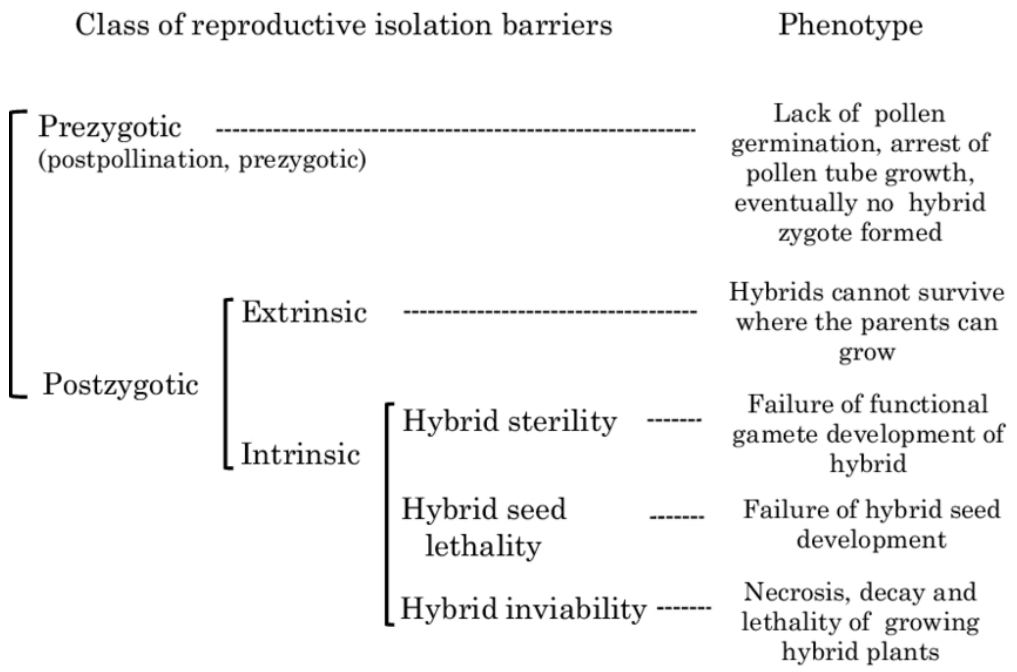

Figure 1 *scheme of general pre- and post-zygotic barriers (Mino et al., 2022)*

i) Pre-zygotic barriers

Postpollination pre-zygotic barriers are essential for preventing hybridization, and they are thought to be more effective than post-zygotic barriers in reducing gene flow as they act first in the reproductive sequence (Kirkpatrick & Ravigné, 2002; Widmer et al., 2009). They act after the pollen is deposited on the stigma until it reaches the ovule (fertilization). These include pollen-stigma, pollen-style, and pollen-ovary interactions. The self-incompatibility (SI) system, which prevents self-fertilization and has independently evolved in different plant families (Takayama & Isogai, 2005), seems also to play a vital role in interspecific reproductive barriers by rejecting heterospecific pollen at the stigma or style level (Kitashiba & Nasrallah, 2014; Pease et al., 2016). Additionally, pollen-pistil interactions consist of a molecular complex system that depends on the structure of stigma and size, hydration of pollen, pollen coat, specific proteins, and protein-protein interactions (PPI)(Cheung et al., 2022). These mechanisms have been shown to play a role in interspecific isolation via the failure of pollen tube guidance or targeting of the ovule (Haghighatnia et al., 2023). Nevertheless, many shadow zones remain on the molecular components of these barriers, and further studies are required.

ii) Post-zygotic barriers

Post-zygotic barriers are divided into two: extrinsic and intrinsic barriers (Coughlan & Matute, 2020). Extrinsic barriers involve an interplay with the environment, such as hybrids, even with typical development, showing reduced growth in parental habitats. Intrinsic barriers manifest independently of the environment, such as hybrid inviability or sterility. This thesis focused on intrinsic hybrid inviability, particularly hybrid seed inviability.

In angiosperms, after fertilization, two sperm cells flow in the female gametophyte; one fuses with the egg cell, and the other fuses with the central cell to form the diploid zygote and the triploid endosperm (Butel & Köhler, 2024). Endosperm is essential as a nourishing tissue for the growth and support of the embryo. Endosperm development is crucial for embryo development and having viable, healthy offspring. In *Arabidopsis thaliana* and most angiosperms, endosperm develops in nuclear type, first undergoing mitotic division of nuclei without cytokinesis (syncytial phase). Endosperm cellularizes at the end of the eighth syncytial mitosis cycle (kinesis happens; (Lafon-Placette & Köhler, 2014; Li & Berger, 2012). Endosperm cellularization in *A. thaliana* starts around four days after pollination (DAP), while the embryo is at the heart stage and finishes at approximately six DAP, while the embryo at the torpedo stage (Boisnard-Lorig et al., 2001; Xu et al., 2023). The time synchronization of endosperm cellularization and embryo development is essential for embryo survival and the formation of viable seeds. In hybrid seeds with nuclear endosperm, inviability occurs due to early or late endosperm-cellularization (**Figure** 2). More generally, endosperm development failure is the leading cause of hybrid seed lethality, named explicitly as an endosperm-based hybridization barrier, in diverse species groups having nuclear endosperm such as *Arabidopsis* (Lafon-Placette & Köhler, 2016), rice (Ishikawa et al., 2011), and *Capsella* (Rebernig et al., 2015) but also in cellular development species groups such as *Mimulus* (Flores-Vergara et al., 2020; Oneal et al., 2016), tomato (Florez-Rueda et al., 2016; Roth et al., 2019), and potato (Cornejo et al., 2012). This hybridization barrier can either act between different ploidy levels of a species, i.e., interploidy, or between different species, i.e., interspecific hybridizations. Both types of hybridization lead to strikingly similar developmental defects of the resulting hybrid seeds. The contribution of maternal and paternal genomes to the endosperm is two to one (2m:1p), and this dosage of parental contributions is necessary for successful development (Lafon-Placette & Köhler, 2016). Furthermore, observations based on imbalanced contributions of parental genomes led to the Endosperm Balance Number (EBN) concept in Solanum species. This

concept explains the fact that crossing species of the same ploidy leads to hybrid seed defects similar to interploidy hybridization by suggesting that some species have a higher "effective ploidy" despite having the same number of chromosomes (Johnston et al., 1980; Johnston & Hanneman, 1982). For example, in *Arabidopsis*, interspecific hybridization between ♀ diploid *A. thaliana* (selfer) and ♂ diploid *A. arenosa* (outcrosser) leads to similar defects than interploidy hybridization with a pollen donor of higher ploidy (Burkart-Waco et al., 2015; Scott et al., 1998), suggesting that *A. arenosa* has a higher EBN, or higher "effective ploidy" than *A. thaliana*. To this day, the molecular causes leading to a higher EBN/effective ploidy in one species compared to the other remain largely unresolved.

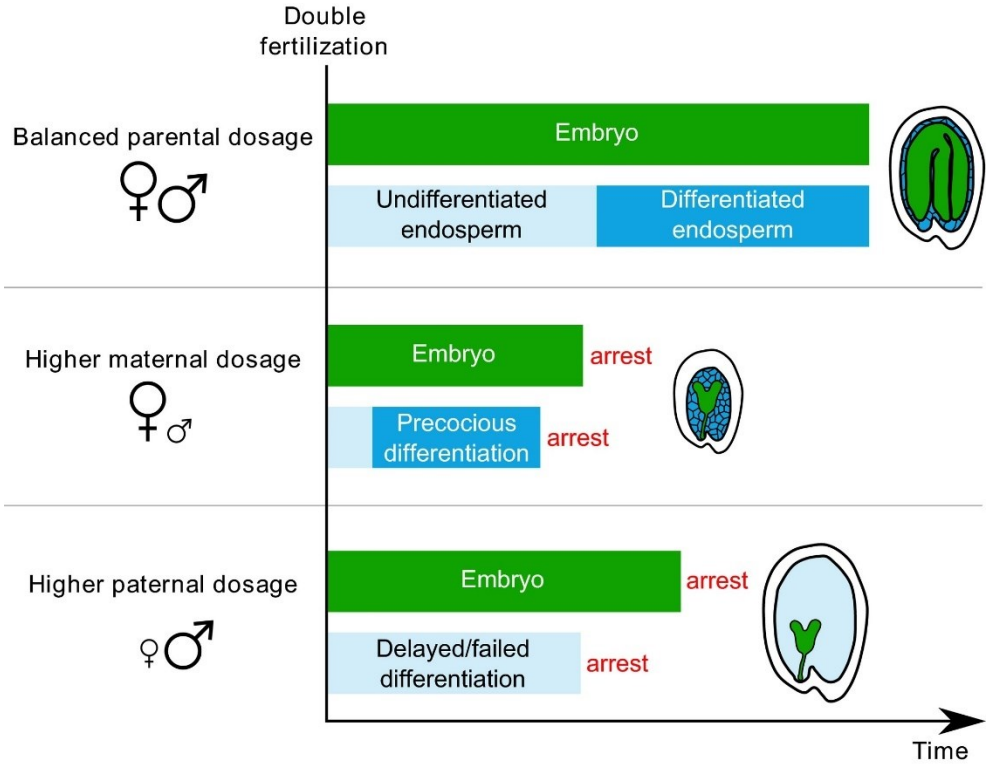

Figure 2 *Illustration of how parental dosage imbalance causes nuclear and cellular endosperm failure and ends with embryo arrest (Lafon-Placette & Köhler, 2016)*

1.2. Epigenome effect on hybrid seed lethality

Beyond genes, other mechanisms affect hybrid seed lethality. Various studies showed that epigenetic modifications are essential in endosperm-based hybridization barriers (Butel et al., 2023; Lafon-Placette & Köhler, 2016; Schatlowski et al., 2014).

a) Epigenetics: What is it?

Epigenetics is a 'stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence.' (Berger et al., 2009). What is altered is, instead, chromatin, which is composed of nucleosomes, ~147 bp of DNA wrapped around an octamer of histone proteins (Andrews & Luger, 2011). The chromatin can be either transcriptionally active (euchromatin) or transcriptionally prohibited (heterochromatin). The state of the chromatin is determined by a set of epigenetic modifications and their interactions. In plants, epigenetic modifications are ongoing during all life stages, including histone modification and DNA methylation (Bender, 2002). DNA methylation primarily affects cytosines in plants in specific sequence contexts: CG, CHG, and CHH, where H is A, T, or C (Maeji & Nishimura, 2018). Histones can undergo several post-translational modifications, methylation (Me), acetylation (Ac), phosphorylation (P), and ubiquitination (Ub), which can affect gene transcription (Bannister & Kouzarides, 2011). Methylation is considered transcriptionally either repressive (H3K9me2 or H3K27me3) or active (H3K4me3 or H3K36me3)(Maeji & Nishimura, 2018), depending on the positions. In addition, small RNA (sRNA) molecules, especially small-interfering RNAs (siRNAs), control epigenetic silencing and are responsible for inducing chromatin silencing in a pathway called RNA-dependent DNA methylation (Slotkin et al., 2009). The tight regulation of epigenetic modifications is essential to plant stress response and development, including the proper formation of gametes and seeds (Calarco et al., 2012; Köhler & Kradolfer, 2011).

b) Epigenetics: Mechanisms in post-pollination pre-zygotic barriers

Mechanisms in post-pollination pre-zygotic barriers, such as self-incompatibility or pollen pistil interactions, have a genetic background. Several genes and kinases, such as S-RNase, cytosine-rich proteins, and pollen coat protein B, are involved in these mechanisms (L. Wang & Filatov, 2023). However, whether and if postpollination prezygotic barriers are controlled via epigenetic modifications is still unknown. Self-incompatibility mechanisms, which contribute to these barriers, involve epigenetic silencing (Batista et al., 2024), suggesting a potential role of epigenetics in post-pollination of pre-zygotic barriers. However, very little evidence links epigenetics and post-pollination pre-zygotic barriers so far. In this master project, I assessed this link.

c) Epigenetics: Mechanisms in endosperm development

Epigenetic control mechanisms are essential for producing functional gametes for sexual reproduction (Bourc'his & Voinnet, 2010). Before fertilization, male and female gametophytes undergo epigenetic regulations through histone reprogramming and DNA demethylation (Calarco et al., 2012; Martinez & Köhler, 2017; Pillot et al., 2010). This reprogramming is believed to reinforce transposable element (TE) silencing and thus prevent the transmission of new TE insertions to the next generation (Calarco et al., 2012; Ibarra et al., 2012).

In the male gametophyte, the vegetative cell (VC) undergoes DNA demethylation by the DNA glycosylase *DEMETER* (*DME*), and this results in the reactivation of TEs in *Arabidopsis*, rice, and maize species (Martinez & Köhler, 2017; Nobuta et al., 2007). This is enhanced by the absence of DECREASE OF DNA METHYLATION1 (DDM1) in the VC, a chromatin remodeling complex involved in the maintenance of heterochromatin (Schoft et al., 2011). The reactivation of TEs leads to the production of TE-derived 21nt siRNAs in the VC, which migrate and accumulate in the sperm cells (SCs) (Martínez et al., 2016). The mechanism is likely to ensure transgenerational genome stability of SCs by re-activating TEs in VCs, ending with siRNAs needed for TE silencing in SCs. In the female gametophyte, similar to the VC, the central cell (CC) undergoes epigenetic reprogramming (Pillot et al., 2010). More precisely, DME is expressed in the central cell, and demethylates in particular TEs (Ibarra et al., 2012). This DNA demethylation allows the Polycomb repressive complex 2 (PRC2) to access chromatin and silence genes, particularly those flanked by TEs (Godwin & Farrona, 2022). The PRC2 complex in the female gametophyte comprises *FERTILIZATION INDEPENDENT SEED 2 (FIS2), MEDEA (MEA), FERTILIZATION INDEPENDENT ENDOSPERM,* and *MULTICOPY SUPPRESSOR OF IRA1,* and silences its targets via the deposition of H3K27me3 (Makarevich et al., 2006). Finally, unlike the male gametophyte, the intercellular movement of sRNA from the companion cell to the gamete is not well characterized in the female gametophyte (Martinez & Köhler, 2017).

The reprogramming before fertilization, which is asymmetric between the central cell genome, which is demethylated, and the sperm cell genome, which is not, sets the stage for parent-of-origin gene expression, i.e., genomic imprinting, to happen in the endosperm (after fertilization). Genomic imprinting is essential to endosperm development. For example, the PRC2 complex silences maternal alleles of paternally expressed genes (PEGs) genes (Moreno-Romero et al., 2016). PEGs

include the auxin biosynthesis genes *YUC10* and *TAR1* (Cheng et al., 2007; Stepanova et al., 2008). Auxin is needed to initiate central cell division and maintain endosperm development (Figueiredo et al., 2015), and the paternal expression (and repression of the maternal allele) ensures that endosperm development is initiated only with the presence of the paternal genome, i.e., it is dependent on the fertilization event. Both paternal and maternal genomes are subsequently required for sustained endosperm development (Figueiredo et al., 2015), showing the complementarity of paternal and maternal genomes due to the specific set of genes each expresses.

d) Epigenetics: Mechanisms in interspecific hybrid seed lethality

In interspecific hybridization, unbalanced genomic contribution between parents leads hybrid seeds to lethality ((Rebernig et al., 2015); see "Post-zygotic barriers" section). The molecular mechanisms underlying this imbalance in interspecific hybrid seeds are poorly understood. Nevertheless, evidence suggests that it is a consequence of abnormal imprinting of maternally and paternally expressed genes (MEGs and PEGs) (Hornslien et al., 2019). Indeed, several works have shown that the imprinted patterns are disrupted, with the loss of imprinting, or the shift from paternal to maternal expression, in Arabidopsis and Solanum hybrid seeds (Florez-Rueda et al., 2021; Kirkbride et al., 2015). One reason for this abnormality might be the disrupted FIS-PRC2 expression (Kirkbride et al., 2015), which is a major regulator of genomic imprinting (Hennig & Derkacheva, 2009). Another potential cause may be maternal DNA methylation being disrupted by Methyltransferase 1 (*MET1*), resulting in DNA demethylation of maternal genomes and, as a probable consequence of activated TEs, leading to the upregulation of PEGs for maintaining gene expressions (Kirkbride et al., 2015).

On the other hand, studies on the loss of DNA methylation of paternal epigenome rescued the hybrid seed inviability (Huc et al., 2022; Jiang et al., 2017). This suggests that the paternal epigenome is important for hybrid seed lethality. Specifically, interspecific hybrid seed lethality in Capsella can be bypassed by using a DNA methylation inhibitor, 5-azacytidine, on the pollen donor, and here, they showed a stable transgenerational loss of DNA methylation and paternal imprinting (Huc et al., 2022). Additionally to the effect of paternal epigenome, the viability of *A. thaliana* × *A. arenosa* hybrid seeds, as well as, the expression levels of *A. arenosa* PEGs in these seeds, was dependent on the maternal genotype (Kirkbride et al., 2015; Burkart-Waco et al., 2015).

However, it remains unclear whether the maternal genotype's effect on hybrid seed viability is due to the maternal genome or epigenome. This is the central question of this thesis.

1.3. Methodological approach: A deeper look into methods

I used some methods introduced in the following sections to understand how epigenetics affects hybridization barriers.

a) The epiRILs

The epigenetic recombinant inbred lines (epiRILs) are *A. thaliana* lines (Col) that are genetically similar but epigenetically variable (Johannes et al., 2009). The purpose of creating epiRILs is to maximize the DNA methylation variation and minimize the genetic variation, reducing the effect of DNA sequence polymorphism on the epialleles, allowing to disentangle the genetic and epigenetic basis of a phenotypic trait (Catoni & Cortijo, 2018). The epiRILs were formed via two genetically similar parental lines, one wild type (Col-wt) and the other with a mutation at the *DDM1* gene. *DDM1* encodes an ATPase chromatin remodeler mainly functioning in maintaining DNA methylation and silencing transposable elements (TEs) (Jeddeloh et al., 1999; Johannes et al., 2009; Kakutani et al., 1995; Lippman et al., 2004; Vongs et al., 1993). Consequently, the *ddm1-2* mutation decreases DNA methylation in all contexts (CG, CHG, and CHH). After the cross between Col-wt and *ddm1-2* to obtain F1 plants, the authors performed backcrosses to Col-wt to more easily select out the *ddm1-2* mutation and only keep the progeny carrying two functional alleles of *DDM1*. If the *ddm1-2* mutation remains, this would add genetic variation in subsequent generations, which would be a confounding factor in epigenetic variation. After the second generation, produced individuals selfed for six to eight generations to obtain fixed homozygous unmethylated/methylated lines, i.e., epiRLs (**Figure 3**).

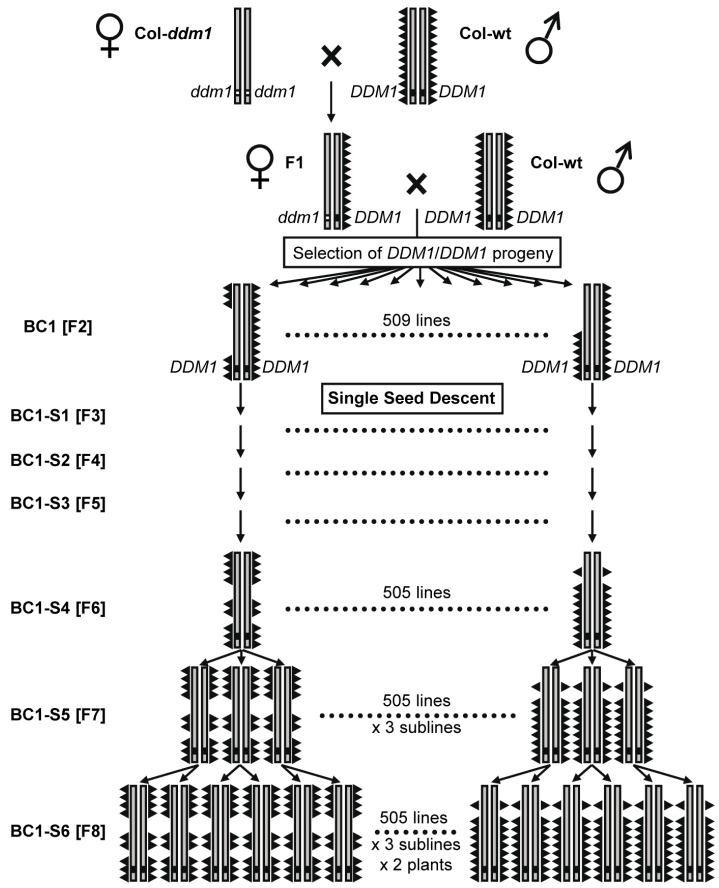

Figure 3 *Illustration of epiRILs construction. Obtained from (Johannes et al., 2009)*

In a subsequent study, the epiRILs were epigenetically characterized (Colomé-Tatché et al., 2012). Researchers conducted methylated DNA immunoprecipitation (MeDIP) followed by hybridization to a whole-genome DNA tiling array (MeDIP-chip) on 123 epiRILs and the two parental lines to characterize the methylome of the epiRILs. Furthermore, they used a three-state Hidden Markov Model (HMM) to classify tiling array signals into three underlying DNA methylation states (unmethylated (U), intermediate methylation (I), or methylated (M)). Researchers confirmed this method by doing whole-genome bisulfite sequencing (WGBS) on six epiRILs. Additionally, the researchers conducted a probe-level comparison of the HMM calls between the *ddm1* and WT parents to define parental DMRs. These DMRs were used as physical epigenetic markers to detect the frequency and distribution of recombination events along chromosomes (**Figure 4A**). These 126 meiotically stable epigenetic markers were the basis for constructing an epigenomic map for subsequent use to perform epiQTL analyses (**Figure 4B**).

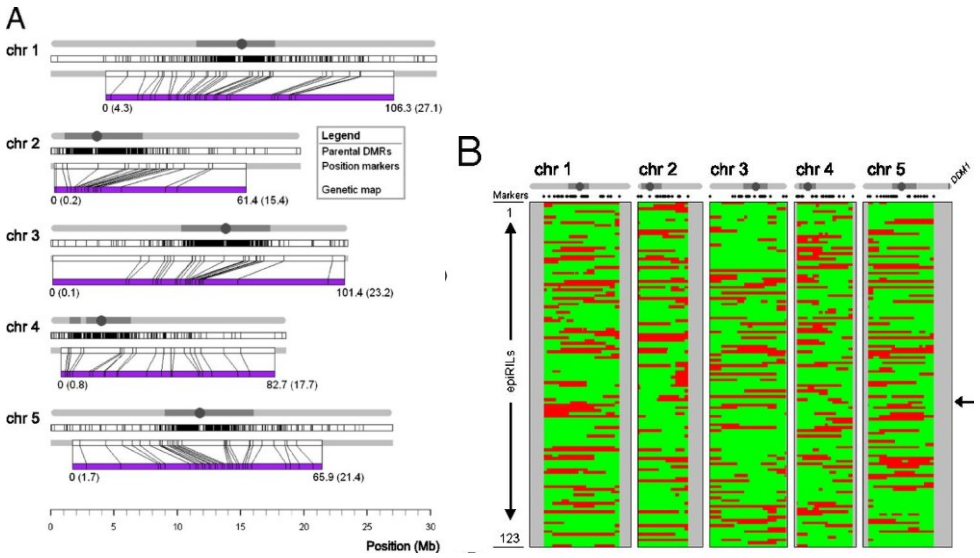

Figure 4 *Recombinant map construction. (A) Genome-wide distribution of the parental DMRs (Top) and the 126 DMRs (i.e., markers; Middle) retained for the construction of the recombination map (purple, Bottom) for each of the five Arabidopsis chromosomes. (B) Inference of inherited WT (green) and ddm1 (red) haplotypes along the genome (x-axis) as inferred from the recombination map for each of the 123 epiRILs (y-axis). Chromosome extremities not covered by the genetic map are indicated in gray. A schematic representation of each chromosome is plotted above the map, with the physical location of the DDM1 gene shown at the end of chromosome 5. Obtained from (Colomé-Tatché et al., 2012)*

b) The epiQTL mapping

The epigenetic quantitative trait loci (epiQTL) analysis is a statistical tool that links phenotypic data and epigenomic data to explain the epigenetic basis of variation in complex traits. The epiQTL can find possible phenotypic differences principally due to a few loci with more significant effects or many loci with each having minor effects (Miles, C. & Wayne, M., 2008). Methods apply in two ways: single and multiple QTL mapping. Single QTL analysis posits the occurrence of a single QTL and considers each position, one at a time, as the putative loci of that QTL (Broman & Sen, 2009). Four models can be used without covariate: standard interval mapping, Haley-Knott regression, extended Haley-Knott regression, and multiple imputation. I initially used standard interval mapping to find possible QTL in this study. However, I needed covariates, such as genetic and environmental factors, to reach a precise result of epiQTL mapping. Covariates are independent variables that can affect the outcome of epiQTL mapping. Additionally, applying covariates reduces residual phenotypic variation and increases the power to detect QTL. In contrast to Single-QTL, Multiple QTL Mapping (MQM) provides a broader approach for mapping QTL by scanning multiple genotypes modeled with their estimated probabilities (Arends et al., 2010). In general, the significance of a QTL is measured using a logarithm of odds (LOD) score that represents the

difference in likelihood between the alternative hypothesis (presence of a QTL) and the null hypothesis (absence of a QTL) (Shi, 2020).

In this thesis, I used the epiRILs and epiQTL mapping to search for an epigenetic basis of pre- and post-zygotic barriers between *Arabidopsis arenosa* and *A. thaliana*. The use of epiRILs for epiQTL mapping has led to successfully identifying epialleles contributing to phenotypic traits such as flowering time and root length (Colomé-Tatché et al., 2012) and more recently, autonomous seed formation (Pankaj et al., 2024).

2. Objectives

Explaining the effect of maternal epigenome in pre- and post-zygotic hybridization barriers by using epiQTL mapping.

3. Materials and Methods

3.1. Plant material

a) The epiRILs and epigenotype data

The epiRILs seeds were purchased from Versailles Arabidopsis Stock Centre, INRA, France (https://publiclines.versailles.inrae.fr/catalogue/epiril). There were 123 epiRILs, including five Col-wt controls. The epiRILs were derived from two closely related parents of the exact accession (Columbia, Col); one is the wild-type *DDM1* allele (Col-wt), and the other one is the *ddm1-2* mutant allele (Col-*ddm1*) (Johannes et al., 2009)The epiRILs were produced using a knocked-out mutation in the DDM1 gene of A. thaliana. The F2 population was produced by crossing between Col-wt and ddm1 parents. The F2 population was fixed in homozygous form by self-pollination for six generations (**Figure 3**)(Catoni & Cortijo, 2018).

Epigenotypic data of epiRILs was obtained from the study of Colomé-Tatché et al., 2012, by conducting a Methylated DNA immunoprecipitation (MeDIP) with a custom NimbleGen tiling array. For each array, they classified the probe signals into three methylation states: methylated (M), intermediate (I), or unmethylated (U). The parental origin of each epiRIL DMR was defined using an HMM-based inference method. Overall, epiRILs were epigenotyped by 126 markers of differential DNA methylation. These markers show if there is a methylation on specific markers at

specific loci. Epigenotypes were labeled 'A' for *ddm1-2* mutant alleles and 'B' for wild-type *DDM1* alleles (Col-wt).

b) Pollen donor

Diploid *A. arenosa* Western Carpathian (WC) plants were used as pollen donors (Kolář et al., 2016). Seeds of this lineage, GC1_3, GC1_10, and GC1_17, were collected from (48.82208N 19.02565E, 48.83034N 19.01859E, and 48.83522N 19.00842E) Harmanec, Slovakia. Additionally, *A. arenosa* WC plant AA084 seeds from (49.162N, 20.15419444E) Velická Dolina, Slovakia, were used. OI77-1 (GC1_3 as the mother plant and the parental plant as wild seeds) was used as a pollen donor during pollination experiments. GC1_10 and 17 plants were also crossed in the lab (GC1_17 as maternal and GC1_10 as paternal plants) to produce the donor plant. Siblings of this cross were used as main pollen donors for pollination experiments (**Table 1**). However, the genetic effect of pollen donors on epigenetic variation among the epiRILs was important; three different mother plants from the WC population were used as pollen donors (**Table 1**) and specified as pollen effect.

Table 1 *Information of the parental species*

Pollen donor	**Species**	**Lineage**	**Ploidy**	**Location**	**Location**	**Maternal plant**	**Paternal plant**
AA084 2/2663	*A. arenosa*	W. Carpathian	2X	Velická dolina (SK)	From field	wild seeds	wild seeds
AA084 4/2662	*A. arenosa*	W. Carpathian	2X	Velická dolina (SK)	From field	wild seeds	wild seeds
OI77-1	*A. arenosa*	W. Carpathian	2X	Harmanec (SK)	From field	GC1_3	wild seeds
ANO1-2	*A. arenosa*	W. Carpathian	2X	Harmanec (SK)	Lab cross	GC1_17	GC1_10
ANO1-1	*A. arenosa*	W. Carpathian	2X	Harmanec (SK)	Lab cross	GC1_17	GC1_10
ANO1-20	*A. arenosa*	W. Carpathian	2X	Harmanec (SK)	Lab cross	GC1_17	GC1_10
ANO1-17	*A. arenosa*	W. Carpathian	2X	Harmanec (SK)	Lab cross	GC1_17	GC1_10
ANO1-36	*A. arenosa*	W. Carpathian	2X	Harmanec (SK)	Lab cross	GC1_17	GC1_10

3.2. Experimental design, germination, and growth conditions

a) Growth conditions

To run the experiments, 123 epiRILs were divided into 11 batches, and these differences were specified as a batch effect. Approximately 20 epiRILs were planted in each batch, and a wt-control

was used in every second batch (**Figure 5A, Table 2**). For *A. arenosa* plants, four batches were grown at different times. Twenty seeds for each epiRIL and 10 for each *A. arenosa* plant were used. Seeds were treated by heat/freeze protocol (phytotron rules, https://www.natur.cuni.cz/biology/botany/working-information/walk-in-chamber). Seeds were put in tubes at 37°C for two days and then -18°C for two days, according to heat/freeze protocol. Sterilized *A. arenosa* seeds were treated with 1 ml of sterilizing solution (5% NaClO, 0.01% (v/v) Triton X-100, and sterile water) and mixed by inverting for 10 minutes. The seeds were rinsed with double-distilled water, and then the water was removed. This process was repeated two times. Seeds were then sowed onto 9x15 mm agar plates (containing 1× MS-Salts, MES hydrate, and 0.8% (w/v) plant agar; pH 5.8) with a head-trimmed 200 ml tip (**Figure 6A**). The sowing process was performed in a sterile laminar flow hood. After labeling, plates were sealed with paper tape (3M Micropore tapes). Plates were put in a growth chamber with 21/18 °C day/night settings and 16 hours of light per day for three weeks. After three weeks, *A. arenosa* seedlings were sent to the vernalization chamber into small pots and were grown for eight weeks in short-day conditions, 8 hours of light per day, at four °C to induce flowering (**Figure 5B**). Germinated epiRILs were directly transferred into medium-sized pots (9x9x10 cm) (no vernalization) with a design of eight individuals per line and four plants per pot (**Figure 5A**). After vernalization, *A. arenosa* individuals were sowed into medium-sized pots. The soil was sterilized according to phytotron rules by autoclaving. Pots were transferred into phytotron growth chambers (PSI growth units) with 21/18°C day/night, 16 hours of light per day with 40% cool white and far-red settings. One or two batches were kept in the phytotron under the same conditions except for 20/15°C day/night temperatures. This difference is specified as the temperature effect. Additionally, in batches four and five, epiRILs were cut from the main stem and watered with fertilizer, which was specified as a cutting effect. Moreover, seedlings were watered two times per week.

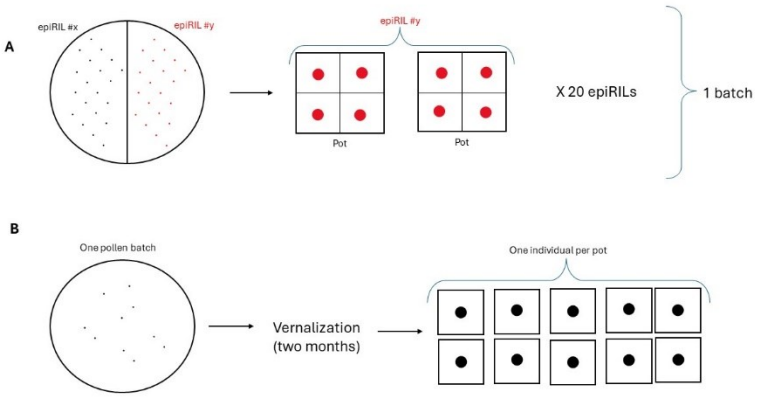

Figure 5 *Experimental design of epiRILs and pollen donors.* ***A*** *is the design of epiRILs. Pots were represented by squares, and individual epiRIL seeds by black/red dots.* ***B*** *is the design of pollen donors. Again, squares represent pots, and black dots represent each A. arenosa individual.*

Table 2 *Planting order of pollen and epiRILs batches with the addition of WT control in specified batch.*

Batch name	Batch number	Wild-type control
pollen batch	1	
	2	
epiRILs batch	1	+ WT control
	2	
	3	+ WT control
	4	
pollen batch	3	
epiRILs batch	5	+ WT control
	6	+ WT control
	7	+ WT control
	8	+ WT control
pollen batch	3	
epiRILs batch	9	+ WT control
	10	
	11	

b) Hybridization experiments

When epiRILs started flowering, approximately after three weeks in a phytotron, flowers were emasculated before the anthesis, working under a stereoscope (Olympus SZ51). For each epiRIL, 15-20 manual emasculations were done using 3-8 individuals of an epiRIL. Two days after

emasculation, stigmas were hand pollinated. Mature *A. arenosa* stamens were used as pollen donors. Filaments were taken by a tweezer (P-lab, size 5) and brushed onto the stigma of emasculated epiRILs (**Figure 6B**).

3.2. Seed collection and screening

a) Seed collection

After pollination, when mature siliques became yellowish, +/- 20 days, individual siliques were collected in different seed bags. Seed samples were kept in paper bags until they were dry at room temperature in the dark. Hybrid seeds were photographed by arranging them on white paper using stereo microscopy (Leica M205 with Fusion Optics) with default settings (**Figure 6C**).

b) Phenotypic analyses/measure of phenotypic traits

Normal and inviable seeds were visually observed by using ImageJ (Schneider et al., 2012). Normal or viable seeds have a light-brown color and regular oval shape. Inviable seeds are dark brown/green, shriveled, and irregularly shaped (**Figure 6D**).

To calculate the effect of post-zygotic hybridization barriers, the viability rate was calculated by the number of viable seeds divided by the total number of seeds. To estimate the effect of pre-zygotic hybridization barriers, the seed set ratio is calculated by the number of seeds divided by the number of siliques. Standard deviation and average values of epiRILs were calculated for both traits. For each epiRIL, a minimum of 70 seeds and three replicates were set for reliable and verifiable results. Each seed bag was considered a replicate, and in each bag, had one to six siliques per epiRIL. Furthermore, the lines that had not reached the decided number of siliques and seeds were repeated.

c) Statistical analysis

First, for the seed set and viability rate independently, I researched if the distribution of the phenotypes was significantly different from normal distribution by Shapiro's test using R version 4.2.2 (www.r-project.org). If the phenotype distribution differed significantly (p-value<0.05), the phenotype was transformed into log10. Then, I estimate the correlation between the two phenotypes by Pearson's test.

To test the significant effect of different factors on non-transformed seed set and viability rate, I performed Kruskal–Wallis tests and 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 FSA (Ogle, D. H., Wheeler, P. , & Dinno, A., 2020). I independently assessed the effect of epiRIL, batch, pollen donor, temperature, and cutting effects. I created box plots for each phenotype and each factor independently using the package 'ggplot2' (Wickham, 2016).

Then, for seed set and viability rate independently, I choose the more accurate model to explain each phenotype using the AIC in a stepwise algorithm implemented in the R package MASS (Venables & Ripley, 2002)The initial model tested was the phenotypes depending on all the factors with a significant effect detected previously by Krustal-Wallis's test. The final model chosen for each phenotype was the model with the lower AIC value. If two models were associated with the same AIC value, the simpler model (e.g., with fewer factors) was conserved (**Figure 6D**).

3.3. Mapping of epigenetic quantitative trait loci (epiQTLs)

An epiQTL mapping analysis was performed to research the association of epigenetic variation on phenotypic traits. Mapping epiQTL was done to find associations between epialleles and phenotypic traits (**Figure 6E**).

For epiQTL mapping, the data was filtered by several conditions. Depending on the batch and pollen effect, the outliers in each epiRIL were removed in both the viability rate and seed set. For the seed set, seeds less than ten were discarded, except for the ones still in the margin. Additionally, depending on other replicate values for each epiRIL, 5-10 seeds per silique were accepted.

a) Single QTL mapping

A single QTL mapping was performed using the 'scanone' function of the packages R/genetics (https://CRAN.R-project.org/package=genetics) and R/ qtl (Broman et al., 2003)Mapping was performed using default mode and standard interval mapping using maximum likelihood via the EM algorithm (https://rqtl.org/rqtltour2.pdf). The number of permutations and LOD score were selected as 1000 and 3, respectively. Mapping was done for both the viability rate and seed set.

b) Multiple QTL mapping

Second, multiple QTL mapping was done for both traits using the R/qtl package (Arends et al., 2010). Missing genotypic data were filled up by function 'maugment.' Cofactors were automatically assigned to the markers using the function 'mqmautocofactor.' Single trait permutations 'mqmpermutation' were calculated by a 5% threshold. For visualization, the 'geno.image' function was used.

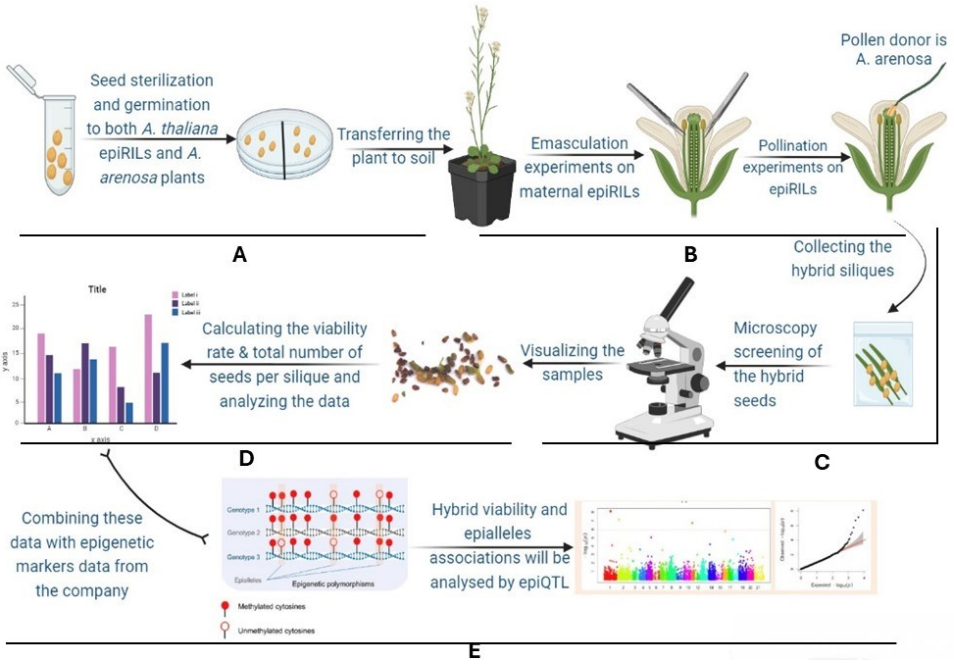

Figure 6 *Generalized scheme for applied methods (BioRender).* ***A*** *represents the steps from seed sterilization to plantation of epiRILs and A. arenosa plants.* ***B*** *represents pollination experiments.* ***C*** *represents the steps from seed collection to visualization.* ***D*** *represents the steps from measurements of phenotypic traits and statistical analysis.* ***E*** *represents the steps in epiQTL mapping.*

4. Results

4.1. There is a significant correlation between variables

To understand the impact of potential confounding factors on seed set and seed viability, I represented KW correlations of seed set and viability rate within other potential factors I were aware of (see Methods for a description of these factors). I calculated the correlations (Chi2), p-value, degree of freedom, and basic AIC test (**Table 3**). From **Table 3**, many variables were

significantly correlated to the seed set and viability rate (p-value<0.05). I also represented the results from **Table 3** in a correlation matrix in **Figure 7**, showing the variable correlations by color and radius differences.

Table 3 *Kruskal-Wallis significance test result with seed set and viability rate combinations. Values in bold indicate a significant effect (P < 0.05).*

First variable	Second variable	Chi-square	P value	Degree of freedom	AIC
Seed set	epiRIL names	244.4060941	**1.10548E-10**	119	1443.046026
Seed set	Plant donor	106.241134	**8.54703E-19**	9	1425.850698
Seed set	Date	212.3627296	**3.19897E-22**	49	1399.426735
Seed set	Batch effect	156.4548115	**1.74575E-28**	10	1384.647837
Seed set	Pollen effect	6.190031552	0.102722048	3	1495.96121
Seed set	Cutting effect	11.63399009	**0.000647575**	1	1481.174987
Seed set	Temperature effect	5.002788925	**0.025306509**	1	1492.090804
Viability rate	epiRIL names	181.7979666	**0.000186453**	119	1409.49786
Viability rate	Plant donor	90.6926812	**1.18208E-15**	9	1314.295329
Viability rate	Date	136.1338896	**7.55192E-11**	46	1332.926497
Viability rate	Batch effect	101.3951941	**2.86407E-17**	10	1304.794006
Viability rate	Pollen effect	29.34612177	**1.89406E-06**	3	1374.514487
Viability rate	Cutting effect	0.678040413	0.410262318	1	1400.719428
Viability rate	Temperature effect	2.683090398	0.101418739	1	1395.062762

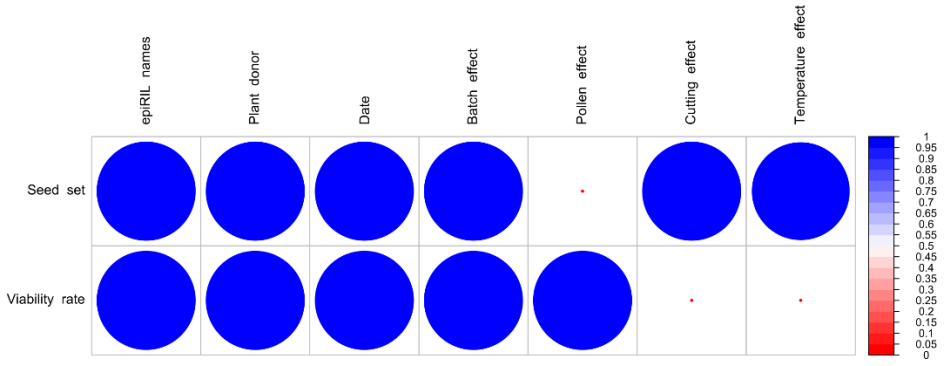

Figure 7 *Correlation plot of seed set and viability rate with epiRIL names, plant donor, date batch effect, pollen effect, cutting effect, and temperature effect. This correlation matrix was performed using the KW test from* ***Table 1****.*

I also wanted to see the relationship between seed set and viability rate, so I performed a Pearson test (**Figure 8**). This test showed a negatively significant correlation between the seed set and viability rate from a P value <0.05 and a coefficient value of -0.33.

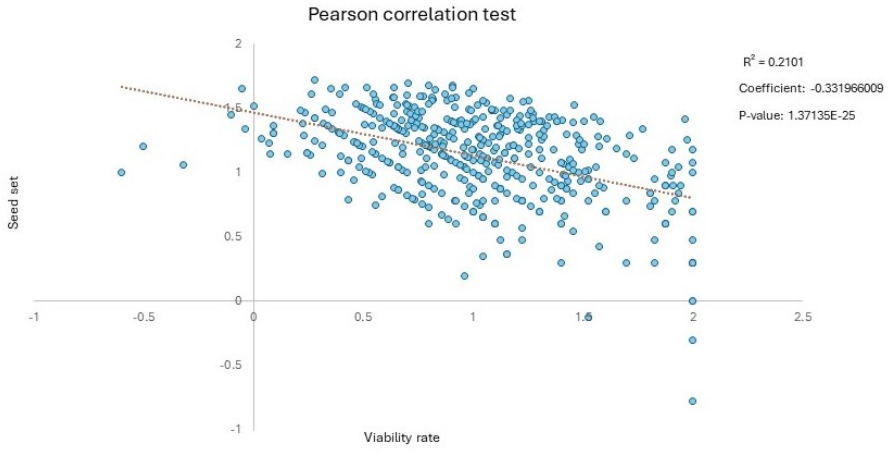

Figure 8 *The Pearson test results from the correlation between seed set and viability rate. The coefficient is -0.331966009, the P-value is 1.37135E-25, and the R^2 is 0.2101. Blue dots represent the intersection of viability rate and seed set values, and the orange dotted line represents the regression line.*

4.2. There is significant variation between epiRILs in pre- and post-zygotic barriers

The viability rate ranged from 0 to 87.5. The viability rate of *A. thaliana* × *A. arenosa* hybrid seeds significantly varied between epiRILs according to a KW test (**Figure 9A**; KW test; P<0.05). The seed set ranged from 0.75 to 53 seeds per silique. The seed set resulting from *A. thaliana* × *A. arenosa* crosses significantly varied between epiRILs according to a Kruskal-Wallis (KW) non-parametric test (**Figure 9B**; KW test; P<0.05).

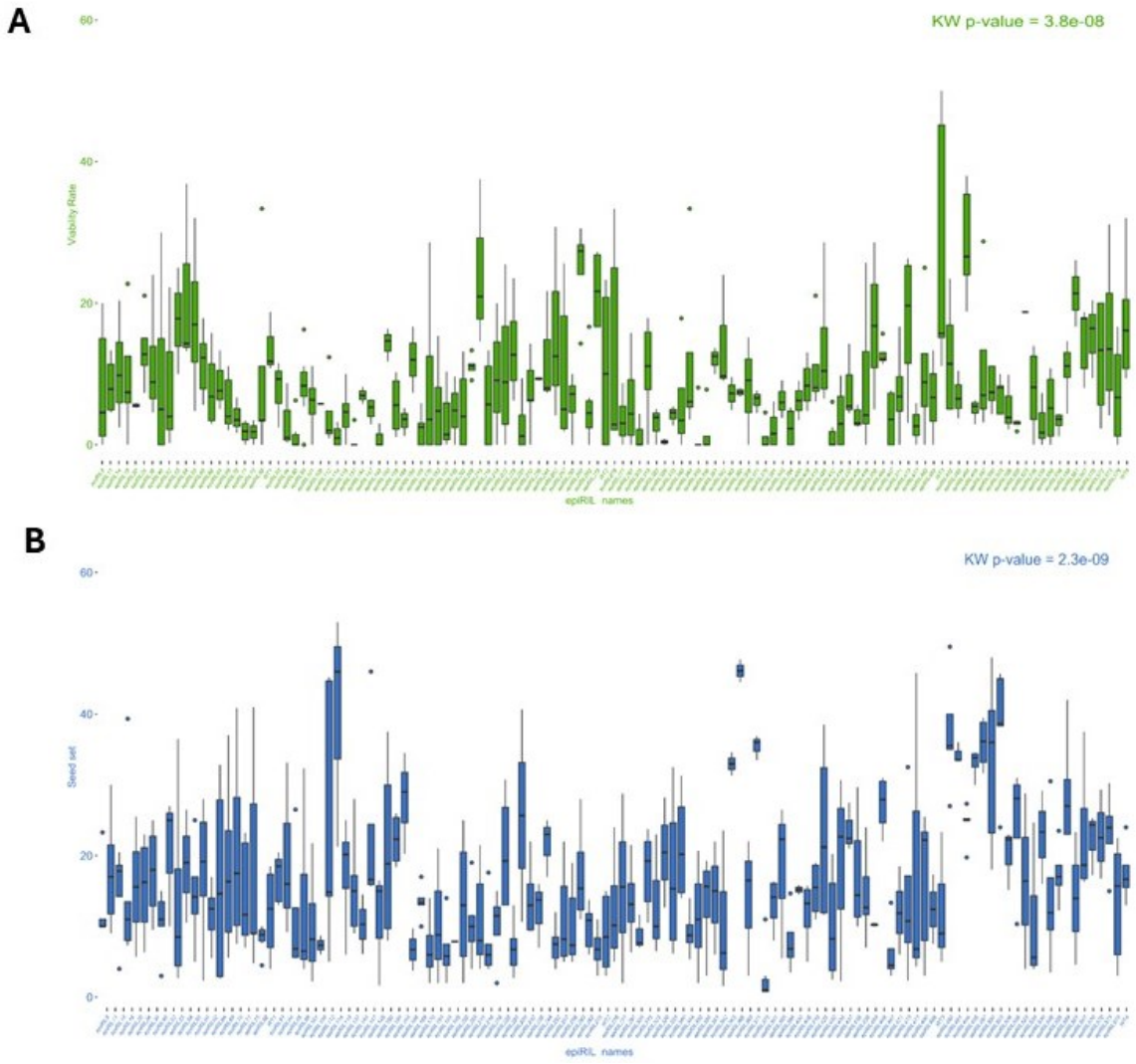

Figure 9 *Phenotypic traits among epiRILs by Kruskal-Wallis test. The epiRILs are represented on the x-axis.* ***A*** *(Green plot) The viability rates are on the y-axis (KW p value= 3.8x10-*[8]*).* ***B*** *(Blue plot) The seed set values are on the y-axis (KW p value= 2.3x10-*[9]*). In both* ***Figure****s, the epiRILs are sorted according to the chronological order in which they were crossed.*

4.3. AIC test reveals which variables affect viability rate and seed set

I performed the step AIC test to assess the variables further and decide whether they impact the seed set and viability rate (**Tables 4 and 5**). I searched the model to explain the viability rate and seed set by step AIC. I considered the most accurate model to have a lower AIC value. After varying combinations of variables, I found the most accurate model for the seed set (**Table 4**). The lowest AIC value showed a strong effect depending on epiRILs, plant donor, and batch effect. I found the

most accurate model for viability rate (**Table 5**). This model also shows a robust effect depending on epiRILs, date, batch effect, pollen effect, and temperature effect.

Table 4 *The stepAIC model result for the logarithm of the seed set. The lines highlighted in red represent an effect on the model and the lowest AIC value (-302.76).*

	Seed_set ~ epiRIL_names + Plant_donor + Date + Batch_effect + Pollen_effect + Cutting_effect + Temperature_effect				
Effect (+/-)	**Step:**	**AIC=**	-297.1		
		Df	**Sum of Sq**	**RSS**	**AIC**
-	**Date**	43	28.204	207.02	-299.33
-	**Plant_donor**	6	3.165	181.99	-299.06
-	**Pollen_effect**	1	0.081	178.9	-298.84
	<none>			178.82	-297.1
-	**Temperature_effect**	1	1.305	180.12	-294.94
-	**Batch_effect**	5	4.636	183.46	-292.46
-	**epiRIL_names**	116	128.274	307.09	-219.78
	Seed_set ~ epiRIL_names + Plant_donor + Batch_effect + Pollen_effect + Temperature_effect				
	Step:	**AIC=**	-299.33		
		Df	**Sum of Sq**	**RSS**	**AIC**
-	**Pollen_effect**	1	0.015	207.04	-301.29
-	**Temperature_effect**	1	0.199	207.22	-300.78
	<none>			207.02	-299.33
+	**Date**	**43**	**28.204**	**178.82**	**-297.1**
-	**Plant_donor**	7	6.606	213.63	-295.36
-	**Batch_effect**	9	17.336	224.36	-271.33
-	**epiRIL_names**	119	135.521	342.54	-249.29
	Seed_set ~ epiRIL_names + Plant_donor + Batch_effect + Temperature_effect				
	Step:	**AIC=**	-301.29		
		Df	**Sum of Sq**	**RSS**	**AIC**
-	**Temperature_effect**	1	0.191	207.23	-302.76
	<none>			207.04	-301.29
+	**Pollen_effect**	**1**	**0.015**	**207.02**	**-299.33**
+	**Date**	**43**	**28.138**	**178.9**	**-298.84**
-	**Plant_donor**	9	12.422	219.46	-285.96
-	**Batch_effect**	10	18.482	225.52	-272.38
-	**epiRIL_names**	119	135.701	342.74	-250.96
	Seed_set ~ epiRIL_names + Plant_donor + Batch_effect				

	Step:	**AIC=**	-302.76		
		Df	**Sum of Sq**	**RSS**	**AIC**
	<none>			207.23	-302.76
+	**Temperature_effect**	**1**	**0.191**	**207.04**	**-301.29**
+	**Pollen_effect**	**1**	**0.007**	**207.22**	**-300.78**
+	**Date**	**43**	**27.084**	**180.15**	**-296.87**
-	**Plant_donor**	9	12.231	219.46	-287.96
-	**Batch_effect**	10	18.29	225.52	-274.38
-	**epiRIL_names**	119	138.051	345.28	-248.74

Table 5 *The stepAIC model is for the logarithm of the viability rate. The lines highlighted in red represent an effect on the model and the lowest AIC value (-80.06).*

	Viability_rate ~ epiRIL_names + Plant_donor + Date + Batch_effect + Pollen_effect + Temperature_effect				
Effect (+/-)	**Step:**	**AIC=**	-73.58		
		Df	**Sum of Sq**	**RSS**	**AIC**
-	**Plant_donor**	5	1.396	185.97	-80.063
	<none>			184.58	-73.576
-	**Pollen_effect**	1	0.944	185.52	-73.199
-	**Batch_effect**	5	5.476	190.05	-69.95
-	**Temperature_effect**	1	3.3	187.88	-67.318
-	**Date**	38	44.309	228.88	-49.312
-	**epiRIL_names**	115	145.78	330.36	-32.311
	Viability_rate ~ epiRIL_names + Date + Batch_effect + Pollen_effect + Temperature_effect				
Effect (+/-)	**Step:**	**AIC=**	-80.06		
		Df	**Sum of Sq**	**RSS**	**AIC**
	<none>			185.97	-80.063
-	**Temperature_effect**	1	3.346	189.32	-73.754
+	**Plant_donor**	5	1.396	184.58	-73.576
-	**Pollen_effect**	3	5.552	191.52	-72.355
-	**epiRIL_names**	115	149.066	335.04	-35.753
-	**Batch_effect**	6	32.286	218.26	-17.465
-	**Date**	40	68.499	254.47	-13.929

4.4. The epiQTL mapping

To see if there are any quantitative trait loci matching the seed set and viability rate phenotypes, I performed epiQTL mapping. I searched for single and multiple epiQTL mapping to obtain one or several loci. I used filtered data (details in the methods section).

a) Single epiQTL mapping

The same epigenotype data were used for the seed set and viability rate previously obtained (**Figure 10;** (Colomé-Tatché et al., 2012)**.** No missing values were found (**Figure 11A**), and the epigenomic map is represented for each chromosome, with the distance between epigenetic markers shown in cM (**Figure 11B**). Furthermore, the distribution of both seed set, and viability rate phenotypes was represented (**Figures 11C and D**).

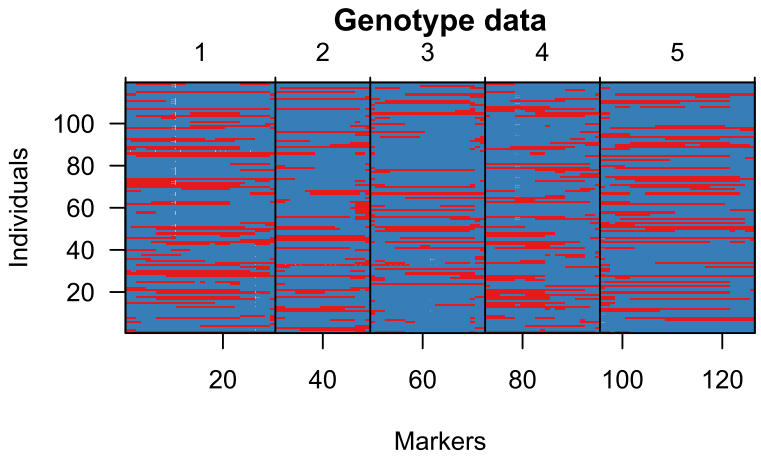

Figure 10 *Epigenotype data for both seed set and viability rate. Numbers 1-5 on the top are chromosome numbers. Individuals are the 123 epiRILs (y-axis). The epigenotypes are WT (methylated; blue) and ddm1 (unmethylated; red).*

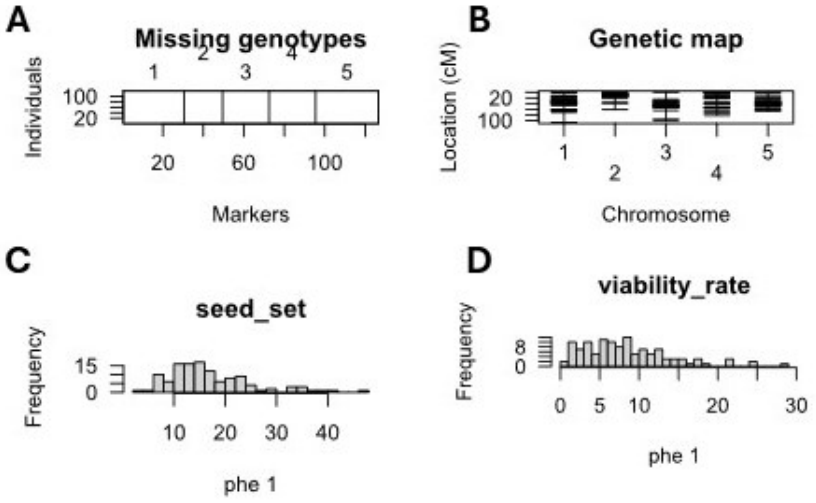

Figure 11 *Data information for seed set and viability rate.* ***A)*** *Graph representing the missing epigenotypes.* ***B)*** *Epigenetic map showing the methylation on chromosomes. Black lines are the markers, and they are located by cM distance.* ***C)*** *The frequency graph of phenotypic seed set data. The X-axis gives the values, and the y-axis represents the frequency.* ***D)*** *The frequency graph of phenotypic viability rate data. The X-axis gives the values, and the y-axis represents the frequency*

All the markers showed lower LOD scores for the traits than the significance threshold (obtained from 1000 permutations) for both the viability rate and seed set. In other words, I found no significant epiloci associated with viability rate and seed set using the single epiQTL mapping (**Figures 12A and B**).

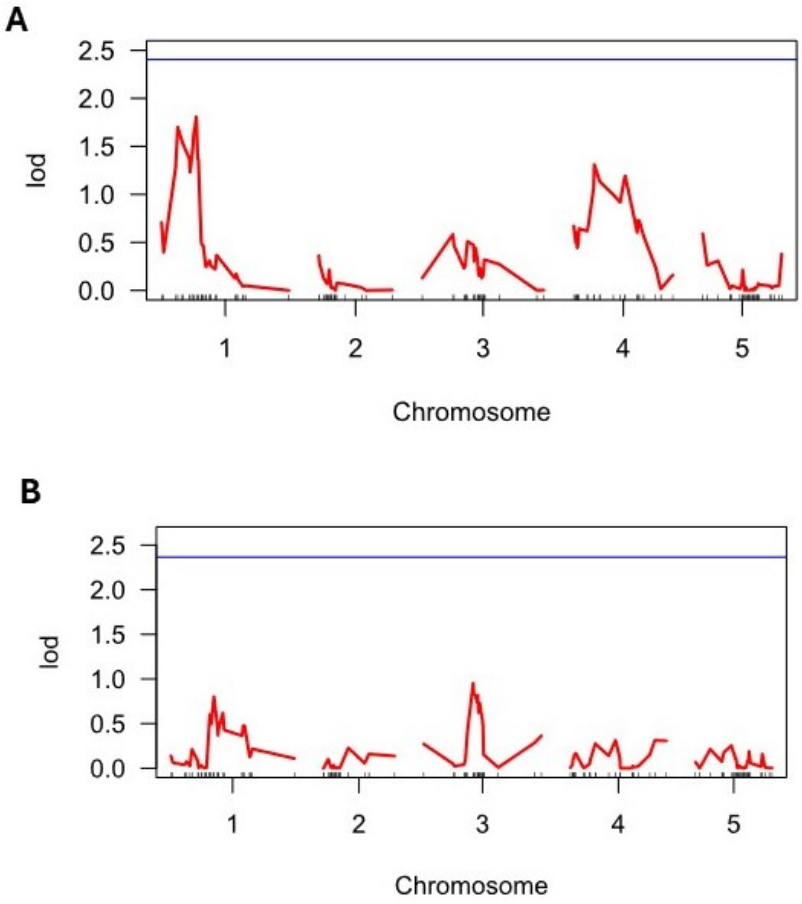

Figure 12 *The single QTL mapping of traits (1000 permutations). The blue horizontal line shows the threshold.* ***A)*** *The viability rate. The threshold is 2.4 for 5% LOD and 2.08 for 10% LOD.* ***B)*** *The seed set. The threshold is 2.53 for 5% LOD and 2.09 for 10% LOD.*

b) Multiple epiQTL mapping (MQM)

By MQM, I wanted to find several loci for our seed set and viability rate in case multiple loci would have encoded these traits. Similarly to the single mapping approach, the LOD score for all markers was under the significance threshold values for both the seed set and viability rate (**Figure 13**).

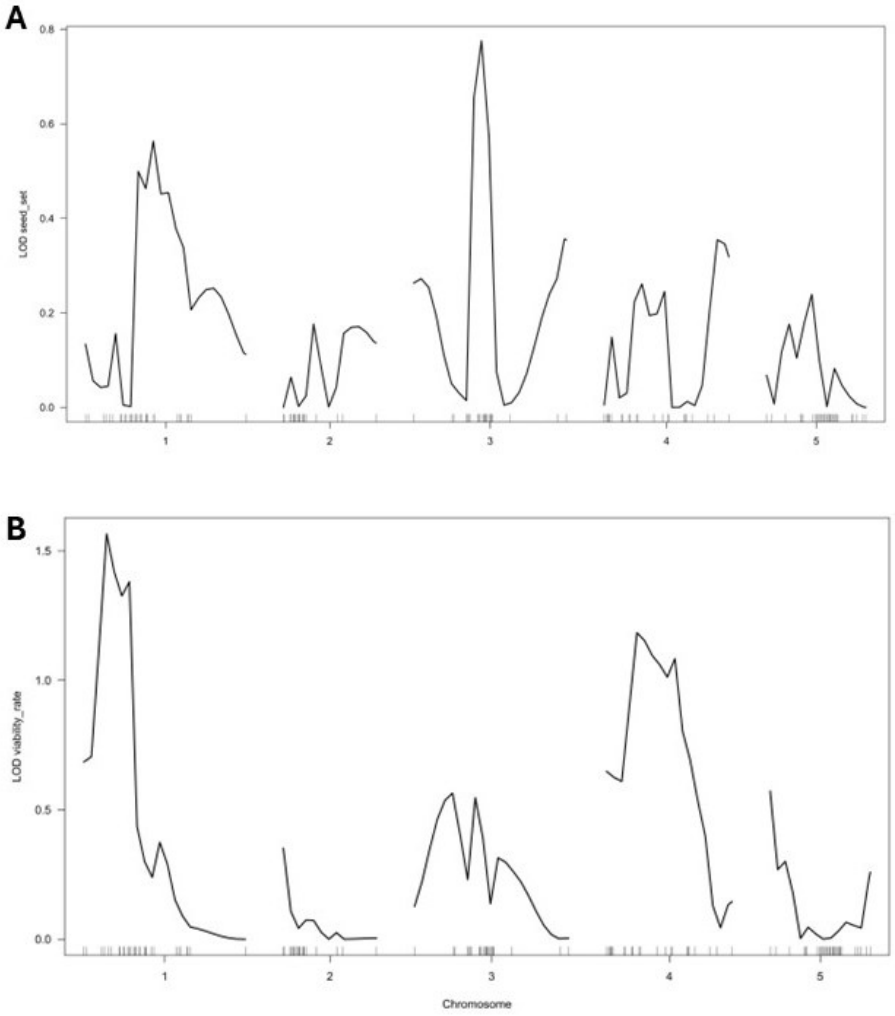

Figure 13 *Multiple QTL mapping (MQM) for both traits (number of permutations 1000).* ***A)*** *MQM for the seed set values. The threshold value is 2.3 for 5% and 1.97 for 10% of the LOD score.* ***B)*** *MQM for viability rate values. The threshold value is 2.39 for 5% and 1.94 for 10% of the LOD score.*

5. Discussion

The main objective of this thesis was to evaluate whether epigenetics, particularly the maternal epigenome, could be involved in two hybridization barriers: prezygotic postmating barrier, and hybrid seed lethality. The results to answer this question are discussed in the following sections.

5.1. Maternal epigenetics affects pre-zygotic barriers

Previous studies on prezygotic barriers, precisely post-pollination prezygotic barriers, focused on the genetic elements underlying pollen-pistil interactions (Takayama & Isogai, 2005). However, the self-incompatibility (SI) system involves the regulation of pollen SI genes via epigenetic mechanisms, i.e. the production of sRNAs and the non-canonical silencing pathways or PTGS in *Arabidopsis halleri* (Batista et al., 2024). So far, it is unknown if epigenetic mechanisms further regulate pollen-pistil interactions. Thus, proposing a connection between maternal epigenetics and the post-pollination pre-zygotic barriers requires effort. I tried to find an answer to this issue by using the number of seeds per silique (seed set) as a quantitative proxy for pre-zygotic barriers (Widmer et al., 2009).

I found significant variation in *A. thaliana* epiRILs (♀) × *A. arenosa* (♂) seed set between epiRILs (**Figure 9B**), supported by an AIC test (**Table 4**). This suggests that the maternal epigenome has an impact on pre-zygotic post-mating barriers mediated by pollen-pistil interactions. Moreover, our result shows that the plant donor influences the seed set, suggesting a paternal contribution to this barrier as well (**AIC, table 4**).

Nevertheless, epiRILs with various seed set values could be a consequence of maternal fertility instead of the pre-zygotic barrier with *A. arenosa*. This means that differently methylated regions in epiRILs might cause the deregulation of genes in the ovules and in the flowers, which leads females to be more fertile or vice versa. Another possibility could be the ovule reservoir differences among epiRILs due to differently regulated genes in female organs. I could show this phenomenon by using self-cross control for each epiRIL, which would tell us how many ovules I have, or I could check the number of ovules per each epiRIL by dissecting pistils. Making both suggestions in hybrid crosses for the 123 lines is demanding, but it can be done for a long-time planned experimental design. In conclusion, our findings suggest that maternal epigenetics have a role in prezygotic barriers, which should be further evaluated. This role has not been described in the literature so far.

5.2. Maternal epigenetics affects post-zygotic barriers

Endosperm-based hybridizations, as a part of postzygotic barriers, result in hybrid seed lethality (Rebernig et al., 2015). In interspecific hybridization, the contribution of maternal and paternal genomes, normally 2m:1p, is disrupted in these hybrids (Lafon-Placette & Köhler, 2016). This imbalance is not linked to the number of chromosomes, as this can happen between species of the same chromosome numbers. Instead, it is an imbalance between their "effective ploidy" or EBN (Johnston et al., 1980 and 1982). In Arabidopsis interspecific hybridization, a high paternal EBN of *A. arenosa* over maternal *A. thaliana* causes a genomic imbalance, resulting in arrested seeds ((Kirkbride et al., 2015)**, Figure 2**). At the molecular level, the expression of genes like *FIS*, *FIE*, and *MEA* are disrupted by parentally imbalanced seeds (Kirkbride et al., 2015). In interspecific hybrids during endosperm development, dysfunction of FIS-PRC2 causes expression disruption of PEGs, causes disruption of endosperm, and leads to hybrid seed abortion (Burkart-Waco et al., 2013).

In this thesis, I aimed to find a mechanism to explain endosperm-based hybridization failure through maternal epigenetics. I found a significant variation in *A. thaliana* (epiRIL) × *A. arenosa* seed viability rate among epiRILs (**Figure 9A**), supported by an AIC test model (**Table 5**). These findings imply that the maternal epigenome is important in Arabidopsis interspecific hybridization (**Figure 9A and Table 5**) and post-zygotic barriers.

However, these results were limited by the genetic variability caused by plant donors. I considered this factor as part of a model (**AIC result, Table 5**). I used three different paternal populations (AA084, OI77-1, and ANO1) and several siblings from ANO1 (see details of donors in Table 1 in material and methods). This genetic diversity of the paternal genome might affect the variation of viability besides the maternal epigenetic effect from epiRILs (Burkart-Waco et al., 2013; Kirkbride et al., 2015). Even though I mostly use ANO1 full sibling individuals, I still likely had a similar donor issue due to genetic differences, but this time, the effect would be less than what I have. As *A. arenosa* is an obligate outcrosser, there is no perfect solution, as doing the crosses with one individual pollen donor would be impossible when I had 123 epiRILs. In addition, I divided epiRILs into 11 batches to separate the work into manageable bits. However, these batches influenced the viability of epiRILs (**AIC test, Table 5**). This influence can be explained by each batch's progress in improving the pollination experiments. Finally, I did not have time to support

viability rates by doing a germination assay on the hybrid seeds. Nevertheless, the viability rate was calculated visually from seed pictures, and studies show that the germination assay is consistent with phenotypic seed observations (Rebernig et al., 2015).

Besides all the limitations mentioned above, hybrid seed viability still varied among epiRILs, meaning epigenetics is important, and differently methylated regions might overcome post-zygotic barriers. Epi-mutagenesis, when applied to pollen donors, can rescue interspecific Capsella and Arabidopsis interploidy hybrid seeds (Huc et al., 2022); I added a new finding to this work by testing if the maternal epigenome can also affect interspecific hybrid seed viability. The way this may happen is unclear. The *ddm1* mutation is the source of methylation level variation in the epiRILs (Colomé-Tatché et al., 2012). The genome-wide variation in DNA methylation between the epiRILs might affect genes or transposons and consequently siRNAs levels in the maternal genome, and this could potentially change the regulation of maternally expressed genes and compensate the paternally expressed genes from *A. arenosa* genome. So far, this thesis's results have shown similarities with previous studies (Burkart-Waco et al., 2013; Walia et al., 2009), which can lead us to think that perhaps there are possible genes or gene pathways in the maternal epigenome that can bypass seed lethality caused by endosperm development.

On a side note, the temperature is also important in endosperm-based hybridization barriers, and even a four-degree decrease can result in increased viability of interspecific hybrid seeds (Bjerkan et al., 2020). This may be explained by the temperature-sensitivity of imprinted gene expression. For example, in rice, an imprinted *FIS* homolog gene, *OsFIE1*, is temperature-sensitive and functions in epigenetic regulation during hybrid seed development (Folsom et al., 2014). Due to technical problems in the growth chambers, I had two different temperature sets, 20/15°C to 21/18 °C day/night. In our results, I obtained an effect of temperature on hybrid seed viability rate (**AIC test, Table 5**). Even though this result is in line with previous studies, our experimental design was not made to study temperature effects and thus cannot fully support this conclusion. From this point on, an experimental design with temperature differences in hybridization with epiRILs could be an effective way to investigate how temperature affects maternal epigenetics and its impact on hybrid seed survival.

5.3. Understanding hybridization barriers with epiQTL mapping

The epiQTL mapping is a commonly used method to associate possible phenotypic traits with epigenotypes. So far, studies have focused on crop development (Gahlaut et al., 2020), TE mobilizations (Reinders et al., 2009), and structural phenotypes (e.g., plant height, main stem branching, flowering time, and leaf area (Kooke et al., 2015)) associated with an epigenetic basis. A recent study found one epiQTL associated with auxin-induced apomixis in epiRILs (Pankaj et al., 2024). These studies support the potential for a heritable impact of epigenetics on different traits, including sexual ones. However, no study has evaluated so far whether such an impact could also be observed on hybridization barriers. I evaluated this question in this thesis.

However, the pre-and postzygotic hybridization barriers I focused on were not associated with any epiloci (**Figures 12-13 A and B**). This could be because external factors induced variation unrelated to the epigenotypes, blurring the epigenetic signal and reducing the chance to find an epiQTL. I showed that for pre-zygotic barriers, plant donor and batch effects (**Table 4**), and for post-zygotic barriers, batch, pollen donor, and temperature effects had a significant impact (**Table 5**). In the future, these factors should be added as covariates in the epiQTL mapping analysis to obtain a more accurate result. Additionally, in general, QTL mapping requires a high number of samples (Miles, C. & Wayne, M., 2008), and the sample size (123 epiRILs) may have been less than needed. So, a possible solution would be to use a larger set of epiRILs. Unfortunately, the number of epigenotype epiRILs is currently restricted to 123.

It could also be that the epiQTL mapping for both hybridization barriers did not identify any epiloci because there is no epiloci influencing these hybridization barriers. This contradicts our finding that the epiRILs significantly impacted both barriers. One way to reconcile these results would be that the epiRILs effect is due to a large number of small-effect epiloci (perhaps transposons), which are harder to detect with an (epi)QTL approach compared to large-effect epiloci (S.-B. Wang et al., 2016). (Epi)GWAS might be an alternative, allowing more sensitive detection of small-effect (epi)loci (Tanić, 2020; S.-B. Wang et al., 2016).

6. Conclusion

In this thesis, I aimed to test whether epigenetics, particularly maternal epigenetics, plays a role in prezygotic (pollen rejection) and postzygotic (hybrid seed failure) barriers between *A. thaliana* and

A. arenosa species. For this purpose, I used epiRILs, showing epigenome diversity while having a similar genome, and tested whether maternal epigenetic diversity had an impact on the hybridization barrier between the two species. I found a significant impact of the maternal epigenome variation on both hybridization barriers. This thesis is the first work to investigate the role of epigenetics in prezygotic barriers. The involvement of epigenetics in hybrid seed failure has been evidenced but mostly focusing on the effect of the paternal epigenome on this reproductive barrier. This thesis provides a new piece of the puzzle by looking at the impact of the maternal epigenome.

Lastly, I searched for epiloci underlying the two hybridization barriers via epiQTL mapping. Due to possible technical difficulties, I did not find any epiloci associated with any of the two traits. Additional analyses may rule out the effect of technical issues on this result.

In conclusion, the maternal epigenome appears to impact both pollen rejection and hybrid seed failure between *A. thaliana* and *A. arenosa*, and future studies may reveal the molecular mechanisms behind this role.

7. References

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