TESTING EFFECTIVE PLOIDY IN RNA-directed DNA-METHYLATION IN THE GENUS *Capsella*

By

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Abstract

In plants, hybridization is an important breeding technique; however, reproductive barriers can hinder viable hybrid seed production. Interploidy and interspecific crosses are common hybridization conflicts caused by improper endosperm development, leading to embryo arrest and seed abortion. This endosperm hybridization barrier is mediated by effective ploidy as it considers both the absolute ploidy (n) and the genomic strength, determined by the mating system. The RNA-directed DNA Methylation (RdDM) pathway plays an important role in plant reproduction and seed development. The loss of RdDM reduces interploidy seed lethality in diploid by tetraploid crosses in *Arabidopsis thaliana*. However, this has not been studied in *Capsella* interspecific crosses. To test the effective ploidy of RdDM mutants in *Capsella*, I performed intraspecific and interspecific crosses with *Capsella grandiflora* and *Capsella rubella*, where either one or both of the parents were RdDM mutants. I observed that loss of RdDM in *C. grandiflora* WT *x C. rubella* WT resulted in slightly increased normal seed production. However, there was no change in normal seed production when *C. rubella* RdDM mutant was crossed with *C. grandiflora.* This suggests that loss of RdDM plays a greater role in seed production in *C. grandiflora* than *C. rubella*, therefore, has a greater impact on the effective ploidy of *C. grandiflora*.

Introduction

Hybridization Barriers

Proper endosperm development is critical for normal seed development. In angiosperms, double fertilization occurs when one haploid sperm fertilizes the egg to form a zygote and the other fertilizes the two haploid polar nuclei in the central cell to form the endosperm (Nawaschin, 1898). The endosperm is a triploid tissue that is responsible for providing nutrients from the maternal parent to the developing embryos (Sarkar and Coe, 1971; Lopes and Larkins, 1993). The endosperm consists of two maternal genomes for every one paternal genome (2m:1p) (Nishiyama and Inomata, 1966). This ratio is critical for conserving normal seed development.

Hybridization is an important breeding technique for improving crop quality, yield, and tolerance. However, reproductive barriers can hinder successful hybridization and often result in high proportions of seed abortion. Interploidy and interspecific crosses disrupt the endosperm development ratio, resulting in similar seed abnormalities (Haig and Westoby, 1991; Rebernig et al., 2015; Lafon-Placette et al., 2018). These crosses can result in high proportions of seed lethality because the endosperm fails to develop properly (Scott et al., 1998). However, unlike in interploidy crosses, the ploidy levels remain balanced in interspecific crosses. This suggests there is an underlying mechanism that contributes to reproductive barriers. Understanding the causes and mechanisms of these reproductive barriers is crucial for improving modern crop breeding strategies.

Interploidy crosses can result in distinctive seed and endosperm phenotypes. Interploidy hybridization occurs between individuals of different ploidy levels, often resulting in seed abnormality or lethality. In the Scott *et al*. (1998) study, the effects of interploidy hybridization on seed development were demonstrated in *Arabidopsis thaliana*. They observed balanced crosses (6x6, 4x4, and 2x2, maternal parent listed first) successfully produced high proportions of normal seed. However, the success of the unbalanced crosses depended on the degree of difference in ploidy level between the parents. The 4x2 crosses resulted in a high proportion of viable seeds, while the 6x2 embryos were arrested, resulting in inviable seeds. The reciprocal crosses presented a similar pattern. This suggests that the success of viable seed production from interploidy crosses decreases the greater the ploidy difference between the parents. Furthermore, the reciprocal unbalanced interploidy crosses demonstrated different seed phenotypes depending on which parent had the greater ploidy level. Maternal excess occurs when the maternal parent has a greater ploidy level than the paternal parent. While paternal excess occurs when the paternal parent has the greater ploidy level. They found maternal excess seeds were significantly smaller than normal seeds, while paternal excess seeds were significantly larger than normal seeds. In the study by Scott et al. (1998), they demonstrated that reciprocal interploidy crosses in *A. thaliana* resulted in opposing seed and endosperm phenotypes.

Interspecific and interploidy crosses can result in similar seed and endosperm developmental phenotypes. Interspecific crosses occur when two different species are crossed,

however, the ploidy level remains balanced. Rebernig *et al*. (2015) demonstrated seed and endosperm development in interspecific crosses between *Capsella rubella* and *Capsella grandiflora.* They observed a higher proportion of seed abortion in *C. rubella* x *C. grandiflora* than *C. grandiflora x C. rubella,* 100% and 40%, respectively. Additionally, endosperm cellularization was delayed in *C. rubella* x *C. grandiflora* crosses, which can be further characterized by the large, flat aborted seeds. This phenotype is indicative of paternal excess, while in the reciprocal cross exhibits maternal excess. In this case, endosperm cellularization occurred precociously, causing the seed coat to improperly expand. As a result, the seeds were small and shriveled. These interspecific seed and endosperm phenotypes are similar to those from interploidy crosses, suggesting a common mechanistic basis.

Effective Ploidy/Endosperm Balance Number

The similar seed and endosperm developmental phenotypes between interspecific and interploidy crosses suggest an underlying mechanism contributing to the reproductive barrier between the parents in addition to the absolute ploidy. Johnston *et al*. (1980) hypothesized that the Endosperm Balance Number (EBN) assigns each species a value in the endosperm based on an individual's genomic strength, which may be different for species of the same ploidy. The EBN dictates the effective ploidy on the endosperm, which will impact the way the endosperm develops. Divergent mating systems have been proposed to contribute to genomic strength and therefore, the effective ploidy. The WISO (Weak Inbreeder, Strong Outbreeder) hypothesis proposes that parental strength/conflict is proportional to the extent of outbreeding in an individual (Brandvain and Haig, 2005; Brandvain and Haig, 2018). As embryos develop and receive nutrients from the maternal parent, the more unrelated the embryos, the greater competition for maternal resources (Queller, 1983). Thus, outbreeders (naturally fertilized by multiple individuals) are more likely to have greater genomic strength compared to inbreeders (self-fertilized) because outbreeding embryos are more unrelated. Therefore, it is expected that *C. grandiflora*, an outbreeding species, to have higher genomic strength and effective ploidy than *C. rubella*, an inbreeding species.

RNA-directed DNA Methylation (RdDM) pathway

The RNA-directed DNA Methylation pathway (RdDM) is an important epigenetic pathway that triggers *de novo* DNA methylation (Matzke and Mosher, 2014). RdDM facilitates DNA methylation using 24-nucleotide (nt) small interfering (siRNAs), which are produced by RNA Polymerase IV, RNA-DEPENDENT RNA POLYMERASE2 (RDR2), and DICER-LIKE3 (Singh et al., 2019; Loffer et al., 2022). These 24-nt siRNAs are abundant in reproductive tissues, specifically in ovule and endosperm tissues (Chow and Mosher, 2023). This suggests that RdDM plays an important role in plant reproduction, particularly in seed development. The role of RdDM in seed development is dependent on the breeding system (Dew-Budd et al., 2024). Outbreeding species are more dependent on RdDM for successful seed development than inbreeding species. In Dew-Budd *et al*. (2024), the loss of RdDM in the outbreeding *C.*

grandiflora resulted in 97 – 99% reduction in seed set, while the inbreeding *C. rubella* resulted in a 50% reduction in normal seed production. A similar pattern has been observed in the inbreeding *A. thaliana* and outbreeding *Brassica rapa.* Loss of RdDM in *A. thaliana* did not result in overt seed phenotypes, while loss of RdDM in *B. rapa* resulted in severe seed phenotypes (Grover et al., 2018). Together, this suggests that the role of RdDM is greater in outbreeding species than in inbreeding species.

RdDM might have a role in balancing parental genomes in the endosperm. Erdmann *et al*. (2017) and Satyaki and Gehring (2019) demonstrated in *A. thaliana,* loss of RdDM to reduce interploidy seed abortion. Loss of *nrpd1* in tetraploid males crossed with diploid wild type mothers reduced seed abortion by 57%. This suggests that RdDM may mediate the parental genomes in the endosperm in interploidy crosses. Considering interploidy and interspecific crosses result in similar endosperm and seed phenotypes, RdDM may mediate the parental genomes in interspecific crosses. I propose that the loss of RdDM could alleviate seed lethality in interspecific crosses between *C. grandiflora* and *C. rubella.* Particularly, I hypothesize that the loss of RdDM in *C. grandiflora* could reduce the effective ploidy enough to reduce the interspecific hybridization barrier. To test this hypothesis, I performed intraspecific and interspecific crosses with *C. grandiflora* and *C. rubella* where either one or both parents are *rdr2* mutants. Here, I demonstrate that loss of RdDM in *C. grandiflora* reduces interspecific seed lethality when crossed by *C. rubella*. While the loss of RdDM in *C. rubella* did not change interspecific seed lethality when crossed by *C. grandiflora.* This supports the hypothesis that RdDM is required for proper seed development in outbreeding species, and therefore plays a greater role on the effective ploidy.

Results

Control intraspecific and interspecific crosses

To assess the effect of RdDM on the effective ploidy in *C. rubella* and *C. grandiflora,* control intraspecific and interspecific wild-type (WT) crosses were performed. The control *C. grandiflora* WT and *C. rubella* WT intraspecific crosses produced plump and healthy normal seeds (Fig. 1). These crosses resulted in 93% and 99% normal seeds, respectively (Fig. 2A). The average number of normal seeds per silicle was 9.4 and 11.4, respectively (Fig. 3A). Additionally, both produced high rates of successful germination, 90% and 96%, respectively (Fig. 4). The interspecific crosses resulted in significantly high proportions of seed abortion and distinctly different seed phenotypes. The maternal excess cross (*C. grandiflora* WT x *C. rubella* WT) produced small and shriveled seeds, while the reciprocal paternal excess cross resulted in large, dark, and flat seeds (Fig. 1). *C. grandiflora* WT x *C. rubella* WT resulted in 99% seed abortion and the reciprocal cross resulted in 91% ($P < 0.01$) (Fig. 2A). Both interspecific crosses resulted in significant decreases in the average number of normal seeds per silicle when compared to intraspecific controls ($P < 0.01$) (Fig. 3A). Additionally, both interspecific crosses

resulted in high proportions of unsuccessful germination. *C. rubella* WT x *C. grandiflora* WT resulted in 87% unsuccessful germination and the reciprocal cross resulted in 96% (Fig. 4). These results confirm the effective ploidy between the two species and as expected, *C. grandiflora* exhibits a greater effective ploidy than *C. rubella*.

RdDM Intraspecific Controls

Intraspecific crosses of RdDM mutants in *C. grandiflora* and *C. rubella* were performed to evaluate the effect of RdDM on seed productivity. Additionally, to test the parental role of RdDM, intraspecific crosses were conducted where only one parent was an RdDM mutant. I observed that loss of RdDM reduced seed productivity to a greater extent in *C. grandiflora* than *C. rubella*, consistent with Dew-Budd *et al.* (2024). The *C. grandiflora rdr2* intraspecific seeds were disfigured compared to WT (Fig. 1). These crosses resulted in 1% normal seed production and the remaining seeds were identified as either abnormal or aborted, 38% and 61%, respectively (Fig. 2B). Interestingly, *C. grandiflora rdr2* self-crosses resulted in 56% successful seed germination (Fig. 4). This suggests that while the *C. grandiflora rdr2* abnormal seeds look disfigured, they are viable enough to germinate. I observed *C. grandiflora* WT x *C. grandiflora rdr2* normal seeds were similar to WT (Fig. 1). The normal seed production was found to be no different than *C. grandiflora* WT as both sets of crosses resulted in 92% normal seed (Fig. 2B). Furthermore, the average number of normal seeds per silicle in *C. grandiflora* WT x *C. grandiflora rdr2* was no different than *C. grandiflora* WT (Fig. 3B). However, *C. grandiflora* WT x *C. grandiflora rdr2* resulted in a reduction in successful germination by 16% ($P < 0.01$) (Fig. 4). There was a slight increase in abnormal seed production in *C. grandiflora rdr2* x *C. grandiflora* WT compared to *C. grandiflora rdr2* x *C. grandiflora rdr2*, 55% and 38%, respectively ($P < 0.05$) (Fig. 2B). There was no difference in the average number of normal seeds per silicle between these *C. grandiflora rdr2* maternal intraspecific crosses (Fig. 3B). However, germination of *C. grandiflora rdr2* maternal intraspecific crosses resulted in a significant decrease in successful germination ($P < 0.01$) (Fig. 4). Together, these results suggest as long as the maternal genotype is capable of RdDM, seed production is conserved and the loss of paternal RdDM has no impact on healthy seed production in *C. grandiflora* intraspecific crosses.

Normal seed production in *C. rubella* was less impacted by loss of RdDM than in *C. grandiflora.* Additionally, there was no clear parental role as the loss of RdDM in both parents did not have an additive effect on seed production. While the *C. rubella rdr2* seeds were plump and similarly sized to *C. rubella* WT seeds (Fig. 1), there was a 26% reduction in normal seed (Fig. 2C). Successful germination was significantly reduced by 39% in C. rubella *rdr2* seeds compared to WT ($P < 0.01$) (Fig. 4). *C. rubella* WT x *C. rubella rdr2* resulted in a 29% reduction in normal seed compared to *C. rubella* WT ($P < 0.01$) (Fig. 2C). However, the average number of normal seeds per silicle was found to be no different than WT (Fig. 3C). Successful germination was significantly reduced in *C. rubella* WT x *C. rubella rdr2* by 30% compared to WT ($P < 0.01$) (Fig. 4). Both *C. rubella rdr2* x *C. rubella* WT and *C. rubella rdr2* x *C. rubella rdr2* resulted in a 26% reduction in normal seed compared to *C. rubella* WT (Fig. 2C). The average number of

normal seeds per silicle was observed to be the same between *C. rubella rdr2* x *C. rubella* WT and *C. rubella rdr2* x *C. rubella rdr2* (Fig. 3C). Additionally, these crosses resulted in a significant reduction in successful germination when compared to WT (Fig. 4). Together, these crosses suggest that loss of RdDM in both parents does not have an additive effect on seed production in *C. rubella* and RdDM is less important for normal seed development than *C. grandiflora*.

RdDM Interspecific Crosses

I hypothesized that the loss of RdDM would reduce interspecific seed abortion because the effective ploidy could be equalized between *C. grandiflora* and *C. rubella.* Thus, I conducted interspecific crosses between *C. grandiflora* and *C. rubella* where one or both parents was a RdDM mutant. I observed that the loss of RdDM had a greater effect on interspecific seed production in *C. grandiflora* than in *C. rubella*. For instance, *C. rubella* WT x *C. grandiflora rdr2* normal seeds were similar to WT and the aborted seeds maintained a paternal excess phenotype (Fig. 1). There was a significant increase in normal seed production in *C. rubella* WT x *C. grandiflora rdr2* by 13% compared to *C. rubella* WT x *C. grandiflora* WT ($P < 0.01$) (Fig. 2E). However, there was no difference in the average number of normal seeds per silicle between these crosses (Fig. 3E). Additionally, there was no difference in successful germination between *C. rubella* WT x *C. grandiflora rdr2* and *C. rubella* WT x *C. grandiflora* WT (Fig. 4). The reciprocal cross, *C. grandiflora rdr2* x *C. rubella* WT, seeds were small and shriveled, which implies maternal excess (Fig. 1). While there was no change in normal seed production, this cross demonstrated an increase in abnormal seed production when compared to the interspecific control ($P < 0.01$) (Fig. 2D). However, the percentage of successful germination was no different between *C. grandiflora* WT x *C. rubella* WT and *C. grandiflora rdr2* x *C. rubella* WT (Fig. 4). Together, these results suggest that the effective ploidy of *C. grandiflora rdr2* remains higher than *C. rubella* WT. Additionally, the loss of RdDM in *C. grandiflora* slightly alleviates interspecific seed abortion when *C. rubella* WT is the maternal parent.

Loss of RdDM in *C. rubella* did not change interspecific seed production. *C. grandiflora* WT x *C. rubella rdr2* exhibited similar maternal excess phenotypes as *C. grandiflora* WT x *C. rubella* WT*,* as the hybrid mutant seeds were small and shriveled (Fig. 1). Both *C. grandiflora* WT x *C. rubella rdr2* and *C. grandiflora* WT x *C. rubella* WT resulted in high proportion of seed abortion, 96% and 99%, respectively, with no statistical difference (Fig. 2D). Additionally, there was no difference in the average number of normal seeds per silicle between these crosses (Fig. 3D). There was no difference in successful germination between *C. grandiflora* WT x *C. rubella rdr2* and *C. grandiflora* WT x *C. rubella* WT (Fig. 4). The reciprocal cross, *C. rubella rdr2* x *C. grandiflora* WT*,* exhibited paternal excess seed phenotype as the seeds were large and flat (Fig. 1). Additionally, *C. rubella rdr2* x *C. grandiflora* WT resulted in no significant differences in normal seed production compared to the control interspecific cross (Fig. 2E). There was no difference in the average number of normal seeds produced per silicle between *C. rubella rdr2* x *C. grandiflora* WT and *C. rubella rdr2* x *C. grandiflora rdr2* (Fig. 3E). Successful germination

of *C. rubella rdr2* x *C. grandiflora* WT was found to be no different than *C. rubella* WT x *C. grandiflora* WT (Fig. 4). The expectation is that loss of RdDM in *C. rubella* will exacerbate the difference in effective ploidy. However, if RdDM only has a small role in effective ploidy in *C. rubella,* the impact on interspecific seed production would also be minimal.

Discussion

I aimed to evaluate the effective ploidy of RdDM mutants in interspecific crosses in the genus *Capsella*. I demonstrated that loss of RdDM in *C. grandiflora* increases normal seed production when *C. rubella* WT is the maternal parent, while loss of RdDM in *C. rubella* does not affect interspecific seed normal production. These results support the hypothesis that RdDM is more important for seed development in the outbreeding *C. grandiflora* than the inbreeding *C. rubella.* The expectation is that RdDM would have the greatest impact on the effective ploidy in *C. grandiflora* than in *C. rubella*. Therefore, loss of RdDM in *C. grandiflora* would have the greatest impact on interspecific seed abortion than in *C. rubella*.

The control intraspecific crosses established a clear effective ploidy relationship. I observed high rates of seed abortion in both *C. rubella* WT x *C. grandiflora* WT and the reciprocal cross. Rebernig *et al*. (2015) reported only a 40% abortion rate in *C. grandiflora* x *C. rubella*. Additionally, I observed a 4% germination rate, while Rebernig *et al.* (2015) observed a 60% germination rate. The discrepancy in seed abortion and germination might be due to differences in environment, plant accession, and seed classifications. However, both sets of data depict clear parental excess seed phenotypes. The *C. rubella* WT x *C. grandiflora* WT seeds were flat, large, and darkly discolored. While the reciprocal cross resulted in small and shriveled seeds, consistent with maternal excess phenotypes. These distinctive seed phenotypes confirm that *C. grandiflora,* the outbreeding species, has a greater parental strength and effective ploidy than the inbreeding *C. rubella.*

RdDM is required for seed development in *C. grandiflora* and plays a maternal role. Dew-Budd *et al*. (2024) observed 97% to 99% fewer healthy seeds in *C. grandiflora rdr2*, while I observed a 60% seed abortion rate. In addition to environmental differences, this discrepancy could be because in Dew-Budd *et al*. (2024) healthy fruits were classified at 7 days after pollination, while our mature fruits were classified upon dehiscence. Dew-Budd *et al*. (2024) reported an indistinguishable proportion of normal seeds per fruit in heterozygous *C. grandiflora* mothers crossed by wild-type or homozygous mutant pollen. This suggests that as long as the maternal sporophyte is capable of RdDM, seed production is conserved. I observed no difference in normal seed production when comparing *C. grandiflora* WT x *C. grandiflora rdr2* to WT as both crosses resulted in 92% normal seed (Fig. 2B). The reciprocal cross resulted in no normal seeds. This further emphasizes the importance of the maternal genotype to be capable of RdDM for viable seed production in *C. grandiflora*.

C. rubella does not require RdDM for normal seed production. I observed higher levels of normal seed production in *C. rubella* WT compared to Dew-Budd *et al*. (2024) who observed high levels of seed inviability independent of RdDM genotype. The *C. rubella* WT and *C. rubella rdr2* crosses were allowed to self-fertilize (no manual emasculation or pollination) and produced 99% and 74% normal seeds, respectively (Fig. 2C). While the *C. rubella WT* x *C. rubella rdr2* and the reciprocal cross were done manually. These crosses resulted in 70% and 74% normal seed production, respectively. I observed great variation in the average number of normal seeds per silicle in the manual crosses compared to the selfed crosses (Fig. 3C). This implies that manual pollination reduces successful seed production. The loss of RdDM in both parents in *C. rubella* intraspecific crosses did not have an additive effect on normal seed production. The following crosses all produced similar proportions of normal seed: *C. rubella rdr2* x *C. rubella rdr2*, *C. rubella rdr2* x *C. rubella* WT, and *C. rubella* WT x *C. rubella rdr2* (Fig. 2C). This suggests that loss of RdDM does not have an additive effect on seed production in *C. rubella.* Overall, these *C. rubella* RdDM crosses indicate RdDM is less important for seed production, which further supports the hypothesis that RdDM is less important in inbreeding species.

The loss of RdDM in *C. grandiflora* had the greatest effect on interspecific normal seed production when *C. rubella* WT is the maternal parent. I observed *C. rubella* WT x *C. grandiflora rdr2* increased normal seed production by 13% compared to *C. rubella* WT x *C. grandiflora* WT ($P < 0.01$) (Fig. 2E). However, the majority of the seeds were classified as aborted and were consistent with the paternal excess phenotype. This suggests that *C. grandiflora rdr2* has a greater effective ploidy than *C. rubella* WT. This is further supported by the reciprocal cross. The majority of the *C. grandiflora rdr2* x *C. rubella* WT aborted seeds were small and shriveled. This implies that *C. grandiflora rdr2* x *C. rubella* WT results in maternal excess, further indicating *C. grandiflora rdr2* has a higher effective ploidy than *C. rubella* WT. Rather than observing a change in normal seed production, there was an increase in abnormal seed production between *C. grandiflora rdr2* x *C. rubella* WT and *C. grandiflora* WT x *C. rubella* WT (Fig. 2D). Together, these crosses suggest that loss of RdDM in *C. grandiflora* has the greatest effect on interspecific normal seed production when *C. grandiflora* is the paternal parent. There is only a slight effect on abnormal interspecific seed production when *C. grandiflora* is the maternal parent, but no impact on normal seed production. The expectation is that loss of RdDM would reduce the effective ploidy of *C. grandiflora*, which could potentially alleviate interspecific seed abortion. While I observed an increase in normal seed production in *C. rubella* WT x *C. grandiflora rdr2*, the majority of the seeds exhibited paternal excess. This indicates that while the effective ploidy of *C. grandiflora rdr2* is reduced compared to WT, it is still greater than *C. rubella* WT.

The loss of RdDM in *C. rubella* did not impact interspecific normal seed production. I observed no difference in seed abortion between *C. grandiflora* WT x *C. rubella rdr2* and *C. grandiflora* WT x *C. rubella* WT (Fig. 2D). Additionally, no differences were observed in seed

abortion between *C. rubella rdr2* x *C. grandiflora* WT and *C. rubella rdr2* x *C. grandiflora rdr2* (Fig. 2E). These crosses suggest that the loss of RdDM in *C. rubella* does not affect normal seed production because the effective ploidy of *C. rubella rdr2* remains weaker than *C. grandiflora* WT and *rdr2.* The expectation is that loss of RdDM in *C. rubella* to exacerbate the difference in effective ploidy because RdDM positively contributes towards effective ploidy. The loss of RdDM should reduce the effective ploidy relative to WT. However, if RdDM only has a small role in *C. rubella*, then the difference in effective ploidy between *C. rubella* WT and *C. rubella rdr2* would be minimal. Therefore, it is unsurprising that loss of RdDM in *C. rubella* does not impact interspecific seed abortion.

Across all crosses, loss of RdDM in *C. rubella* and *C. grandiflora* intraspecific crosses resulted in significant decreases in successful germination (Fig. 4). However, there was no significant difference in successful germination in interspecific RdDM crosses. This suggests that germination success is impacted by loss of RdDM, but interspecific seed germination is independent of the RdDM genotype.

To further investigate the impact of RdDM on effective ploidy in the genus *Capsella,* I plan to first increase the statistical accuracy by redoing the *C. rubella* WT and *C. rubella rdr2* crosses through manual emasculation and fertilization. In addition, the crosses that have fewer silicle measurements such as *C. rubella rdr2* x *C. grandiflora rdr2* and the reciprocal. This would improve the statistical accuracy of the results and provide a better understanding of the impact of RdDM on effective ploidy. Secondly, by expanding these crosses with other RdDM genes such as *NRPE1*, we can better understand the role of RdDM on effective ploidy. To complement seed phenotypic data, seed clearing and Feulgen staining would expand insight on embryo and endosperm development in RdDM intraspecific and interspecific crosses. To better understand the molecular mechanisms, Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) can be performed to identify common genes that are deregulated in the endosperm in RdDM mutant interspecific and intraspecific crosses in the genus *Capsella.*

Methodology

Plant material and growing conditions

Capsella grandiflora accession 83.17 and *C. rubella* accession Monte Gargano were grown in growth chambers at 22 °C under 16h:8h light:dark conditions. *C. rubella* required vernalization for 3 weeks at 4 °C to induce flowering. When *C. rubella* was the maternal parent, flowers were emasculated 24 hours before anthesis and pollinated after 2 days. *C. rubella* wild-type and mutant self-crosses were allowed to self and collected at random. Both species' silicles will shatter when seeds are mature. Therefore, silicles were harvested just before shattering when the dried ovary valves began to detach from the septum.

Genotyping

Prior to genotyping, the *RDR2* genes were transformed in *C. grandiflora* and *C. rubella* using *Agrobacterium*-mediated floral dip and standard floral dip methods, respectively (Dew-Budd et al., 2019). The genotype of individuals was confirmed before use in crosses through DNA sequencing or gel electrophoresis. Leaf tissue was harvested and flashed frozen in liquid nitrogen and then ground to powder using 2.3 mm diameter silica beads in a tissue mill. DNA was extracted using cetyltrimethylammonium bromide (CTAB) buffer. 500 ul of CTAB buffer was added to each ground tissue sample and incubated for 30 minutes at 65 °C. Then 500 ul of 24:1 chloroform:isoamyl alcohol was mixed thoroughly with the sample. Then the samples were centrifuged at maximum speed for 5 minutes. 400 ul of upper clear supernatant was collected into a new tube. Then 280 ul of 100% isopropanol was added. These samples incubated at -20 °C overnight. The samples were centrifuged at maximum speed for 7 minutes in order to form a DNA pellet. The isopropanol was removed, and the DNA pellets were washed with 200 ul of 70% ethanol. All ethanol evaporated before resuspending the pellet in 30 ul nuclease-free water. Following DNA extraction, the RdDM gene *RDR2* was identified through polymerase chain reactions (PCR). Both species used the same primers for *RDR2*: CGCR.RDR.F (5' - TCCGTCGAGATCACCACCA - 3') and CGCR.RDR.R (5' - TATGTCCCTTCTGCATTTCAAATTCG - 3'). The PCR conditions were as follows: initial denaturation at 95 °C for 3 minutes, denaturation cycle at 95 °C for 30 seconds, annealing cycle at 55 °C for 30 seconds, extension cycle at 72 °C for 30 seconds. Repeat steps denaturation cycle through the extension cycle 39 times. Final extension at 72°C for 5 minutes and then 4 °C holding stage. *C. grandiflora* WT has an expected band size of 889 bp and *C. grandiflora rdr2* has an 18 bp deletion. Due to the small bp difference, the post-PCR product was purified with ExoSAP-IT and sent for DNA sequencing. The sequencing chromatograms results were mapped to a genomic reference of *C. grandiflora rdr2* in Geneious Prime. *C. rubella* WT has an expected band size of 887 bp and *C. rubella rdr2* has a 44 bp insertion which can be visualized on a 3% agarose gel while under UV light.

Seed Phenotype Classifications

Seeds were classified as normal if they were tan in color, plump in size, and regularly shaped. Abnormal seeds were those that were plump, but irregularly shaped and/or discolored. Aborted seeds were either shriveled and very small or dark, large, and flat. Images of seeds were taken with AmScope Dissecting Scope. In Chi-square tests, when normal and abnormal seed counts resulted in an expected value below 5, the categories were combined.

Germination Assay

All seeds were surface sterilized by soaking in sterile water for 30 minutes with agitation, then stratified in 10% Preservation for Plant Tissue Culture Media (PPM) with agitation for 5 days in 4 °C. After stratification seeds were plated on MS media containing 1% sucrose. Plates were grown at 22 °C under 16h:8h light:dark conditions. After 7 days, seeds were categorized as either germinated (cotyledons emerged), germinated (radicle emerged) or ungerminated (radicle

did not emerge). In Chi-square tests, when the radicle emerged category had few counts, radicle and ungerminated counts were combined and defined as unsuccessful germination.

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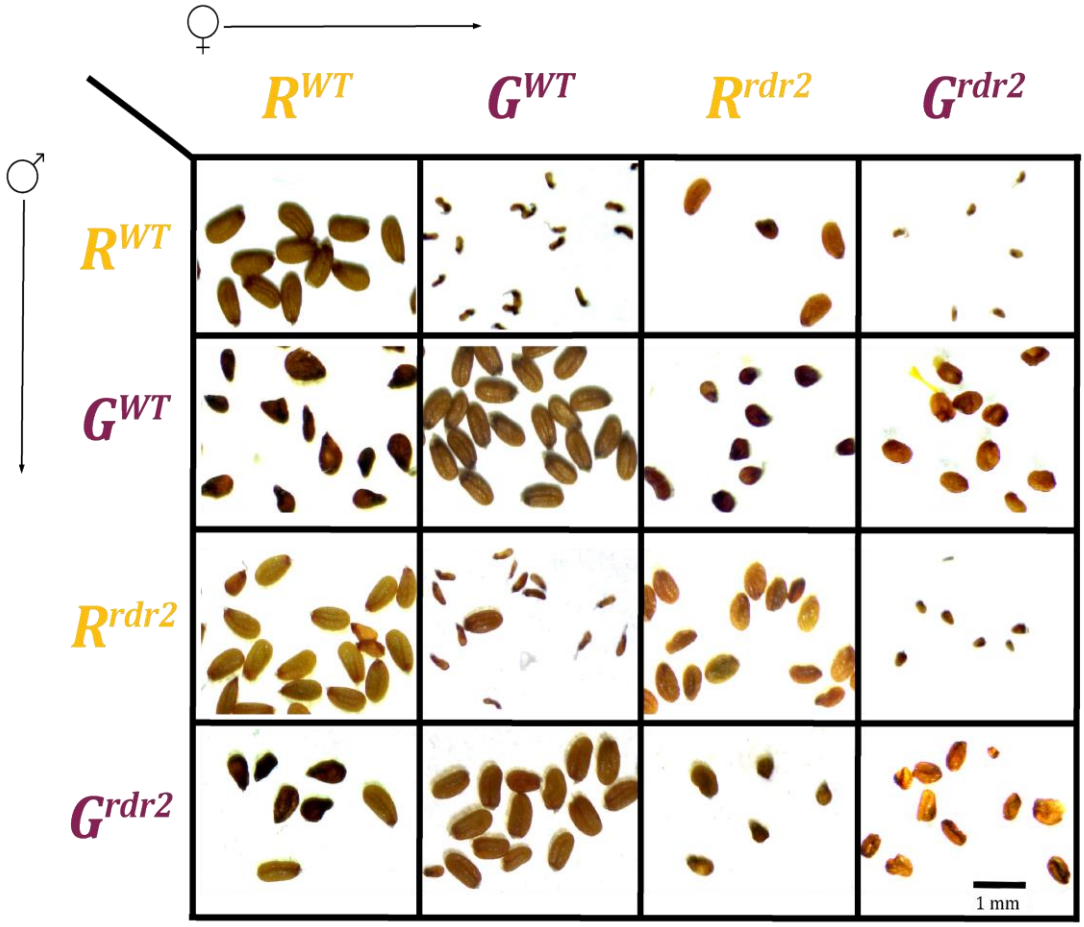

Figure 1: Seed phenotypes of WT and RdDM mutant intraspecific and interspecific crosses in *C. grandiflora* and *C. rubella.* Maternal parents represent the columns and paternal parent represent the rows.

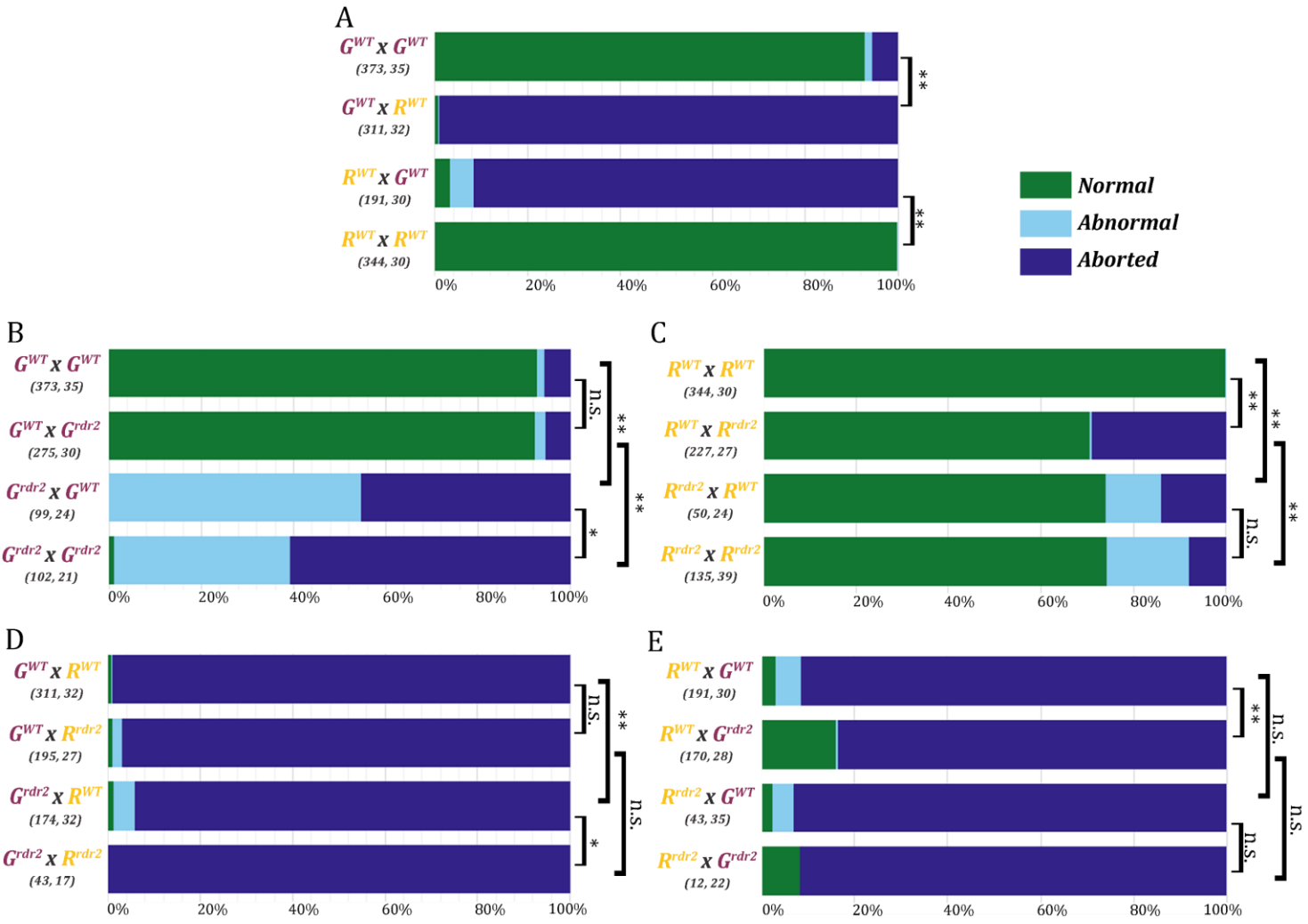

Figure 2. Seed production in *Capsella* RdDM interspecific and intraspecific crosses. Percentage of normal seed (dark green), abnormal (light blue), and purple (aborted). **A)** Wild-type interspecific and intraspecific *C. grandiflora* and *C. rubella* seed production. **B)** Intraspecific *C. grandiflora* RdDM seed production. **C)** Intraspecific *C. rubella* RdDM seed production. **D)** Interspecific RdDM seed production where *C. grandiflora* is the maternal parent. **E)** Interspecific RdDM seed production where *C. rubella* is the maternal parent. Notation under cross names = (number of seeds counted, number of silicles harvested). ** $P < 0.01$, * $P < 0.05$, n.s. = not significant.

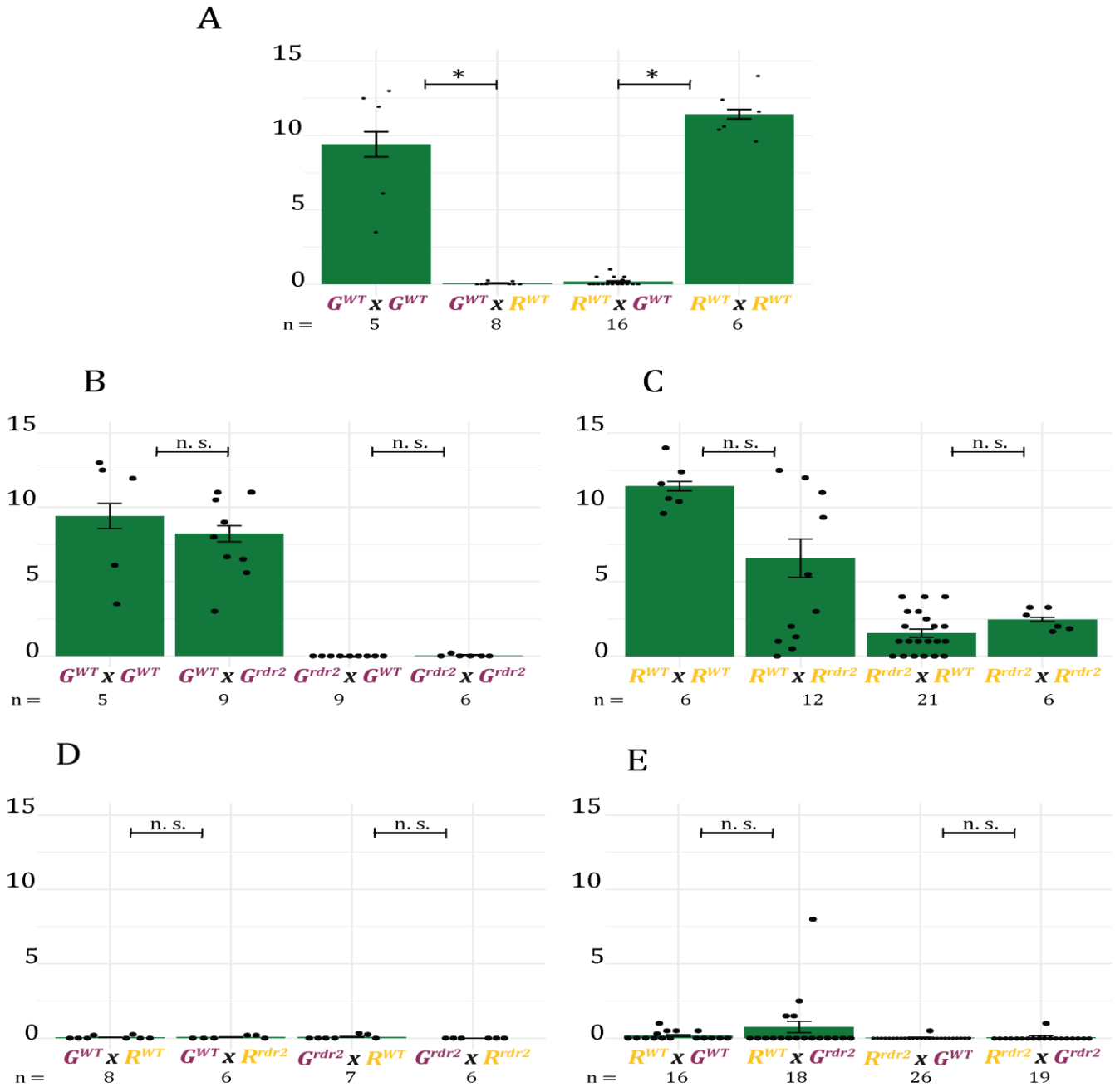

Figure 3. Average number of normal seeds produced per set of silicles in *Capsella* interspecific and intraspecific crosses. **A)** Wild-type interspecific and intraspecific *C. grandiflora* and *C. rubella* average normal seed production. **B)** Intraspecific *C. grandiflora* RdDM average normal seed production. **C)** Intraspecific *C. rubella* RdDM average normal seed production. **D)** Interspecific RdDM average normal seed production where *C. grandiflora* is the maternal parent. **E)** Interspecific RdDM average normal seed production where *C. rubella* is the maternal parent. Notation under cross names = number of measurements. Each measurement has 1-4 silicles. * $P < 0.01$, n.s. = not significant.

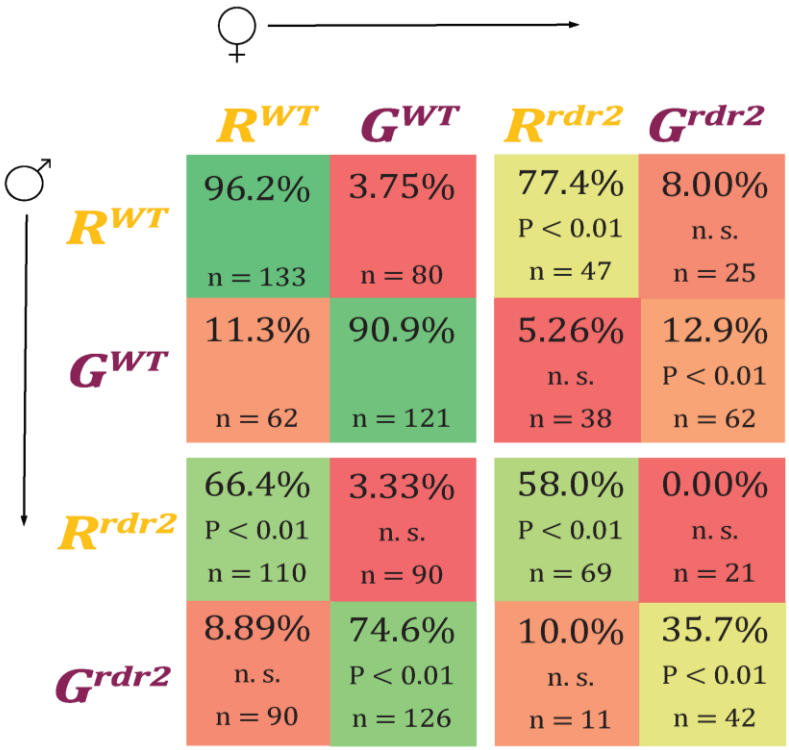

Figure 4. Cotyledon germination assay of RdDM mutant intraspecific and interspecific crosses in *C. grandiflora* and *C. rubella.* Maternal parents represent the columns and paternal parent represent the rows. The quadrants with statistical values were compared to the upper-left quadrant. n = number of total seeds used in assays. n.s = not significant.