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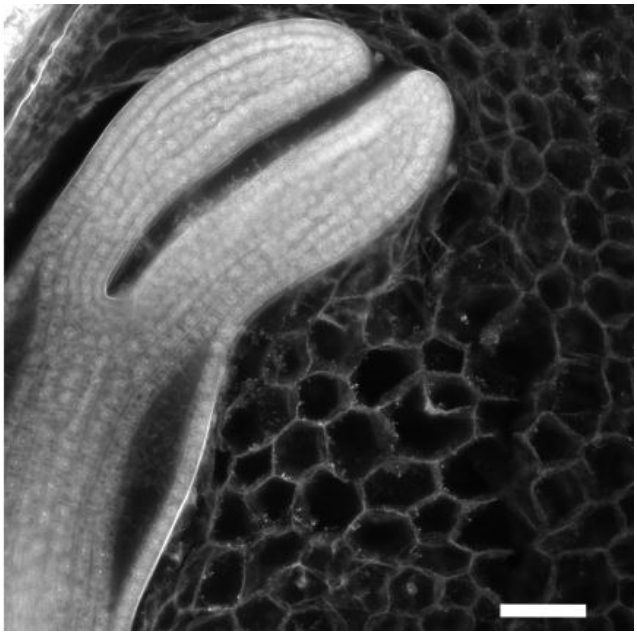

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Endosperm based postzygotic hybridization barriers in *Arabidopsis* and *Capsella*

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Abstract

In *Arabidopsis*, endosperm development is very important for viable seed development. Especially, endosperm cellularization is a crucial event for embryo survival and if it fails, can lead to embryo arrest, as observed in seeds derived from interploidy and interspecies hybridization. In case of interploidy crosses, increased ploidy level creates genome dosage imbalance in endosperm and in case of different species, a similar dosage imbalance basis is suspected, but remains to be broadly established. In this study, we investigated the viability of hybrid seeds between two outbreeding species, *Arabidopsis lyrata* and *A. arenosa,* in relation with the development of their endosperm*. A. lyrata* × *A. arenosa* (Female × Male) hybrid seeds were shrivelled, dark brown and inviable, with delayed endosperm cellularization. In contrast, *A. arenosa* × *A. lyrata* produced tiny inviable seeds with precocious endosperm cellularization. We also investigated if similar mechanisms could exist between different populations of the same species *A. lyrata*. In case of intraspecies crosses, *A. lyrata* Austrian population, when used as seed plant, produced tiny seeds and when used as pollen donor, produced shrivelled dark brown aborted seeds. However, no differences in endosperm cellularization were identified. According to our results, we propose that the hybridization barrier observed between *A. lyrata* and *A. arenosa* and between *A. lyrata* populations is a consequence of different levels of parental conflict experienced by the mating partners. Finally, the genetic analysis of *A. arenosa* and *A. lyrata* hybridization barrier suggests that cross direction-dependent multiple loci are responsible for the non-reciprocal hybrid seed defects we observed.

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Abbreviations

DAP	Day after pollination
EBN	Endosperm balance number
WISO	Week inbreeder strong outbreeder
FIS–PRC2	Fertilization independent seeds- Polycomb Repressive Complex2
TAR1	Tryptophan Aminotransferase related1
NaClO	Sodium hypochlorite
RH	Relative humidity
EtOH	Ethanol
DNA	Deoxyribonucleic acid
GFP	Green fluorescent protein
MYA	Million years ago

1 Introduction

Seed development is a vital process to ensure sexual reproduction. In *Arabidopsis thaliana* and other diploid angiosperm species, it is generated by the double fertilization, where the pollen tube contains two sperm cells, one fertilizing the egg cell in the ovule to generate the embryo and the other one fertilizing the homodiploid central cell to generate the triploid endosperm (Berger, 1999; Brown *et al.* 1999; Becraft *et al.*, 2001). The endosperm serves as a nourishing tissue for the embryo. Among angiosperms, the endosperm can persist until the seed is mature, such as in grasses, or be consumed by the embryo during seed development, such as in *Arabidopsis* (Olsen *et al.*, 1999; Brown *et al.* 1999 ; Sabelli & Larkins, 2009). Generally, the development of the embryo and the endosperm occurs inside the ovary. Before fertilization the ovary is covered by inner and outer integuments but after fertilization, those integuments differentiated into the seed coat (Haughn & Chaudhury, 2005).

1.1 Endosperm Development

In most angiosperms including Arabidopsis, the endosperm follows a syncytial mode of development. It mainly consists in four phases: syncytial, cellularization, differentiation and cell death (Berger, 2003). Before first zygotic division, the nucleus of the fertilized central cell divides without cytokinesis (Christensen *et al.*1997). During the syncytial phase, the endosperm enlarges rapidly, contains the central vacuole and nuclei keep on dividing without cytokinesis (Olsen, 2004) (Fig. 1a).

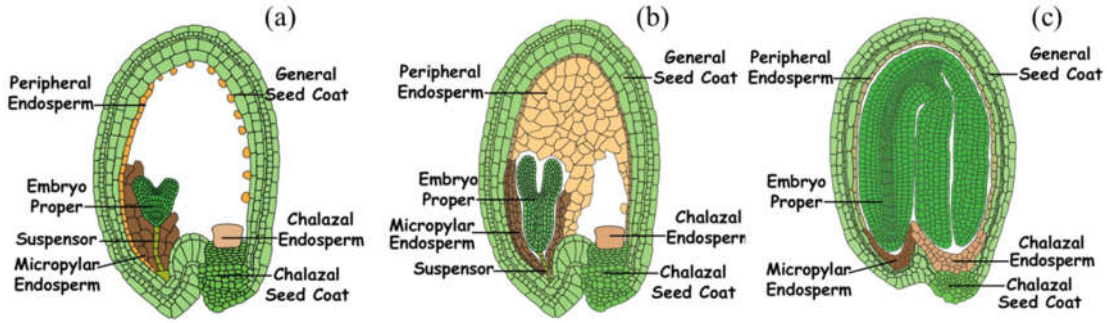

Figure 1: Developmental stages of *Arabidopsis* seed. (a) Syncytial endosperm before cellularization, (b) Cellularized endosperm, (c) Mature seed. (Adapted from Belmonte *et al.*, 2013)

In *Arabidopsis* endosperm, during 16-nuclei stage, the syncytium starts to differentiate into three distinct domains: the micropylar, peripheral and chalazal domains (Berger, 2003). Compartmentalization into different domains is an important event for seed development (Hehenberger *et al.*, 2012). The large central vacuole forces cytoplasm to the peripheral domain while micropylar and chalazal syncytial cytoplasm maintains connections with seed parent vascular tissues (Brown *et al.*, 2003). After the 8th cycle of mitotic nuclei division, cellularization starts in the micropylar endosperm (Charlton *et al.*, 1995; Brown *et al.*1999; Brown & Lemmon, 2001). Endosperm, cellularization occurs via development of radial microtubule systems (RMS) and alveolation. RMS form on nuclear surfaces, consequently

establishing cytoplasmic phragmoplasts in nuclear cytoplasmic domain that mediate alveolar cell wall formation around nuclei (Brown *et al.* 1999). The whole endosperm except nuclei at chalazal pole is cellularized when the embryo reaches at torpedo stage (Berger, 1999; Olsen, 2001) (Fig 1b).

During early stages of embryo development, the suspensor connects the embryo proper to surrounding maternal tissues, serves as a channel for nutrients and growth regulators essential for embryonic development (Kawashima & Goldberg, 2010). The suspensor degenerates during cellularization stage and the embryo starts uptaking nutrients directly from the endosperm. Before cellularization, sucrose moves into the seed via the phloem and continues its movement through integuments until it reaches at the central endosperm vacuole (Lafon-Placette & Köhler, 2014). In endosperm vacuole, the sucrose converts into hexoses and serves as the main storage for hexoses during seed development (Morley-Smith *et al.*, 2008). With progressing endosperm cellularization, the large central vacuole shrinks until it gets completely replaced by the endosperm cells. Therefore in response to cellularization, hexose starts to convert to sucrose (Hehenberger *et al.*, 2012). At the same time, the sucrose transporters expressed in the embryo epidermis and endosperm cells around the embryo allows direct sucrose transport from the endosperm to the embryo (Lafon-Placette & Köhler, 2014).

The endosperm cellularization is essential for embryo development. The *fertilization independent seeds2* (*fis2*) mutant shows failure of endosperm cellularization and in *Arabidopsis thaliana* seeds abort at eight day after pollination (DAP) (Chaudhury *et al.*, 1997) (Fig. 2a,b).

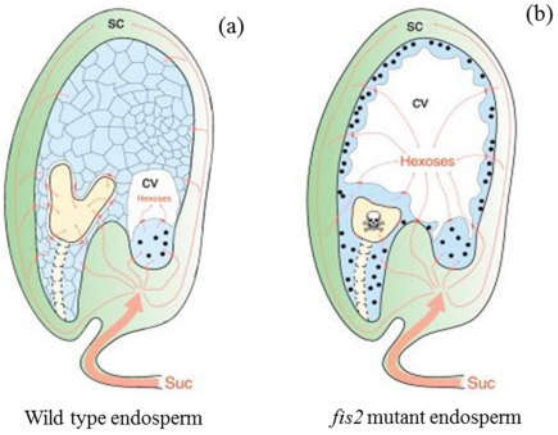

Figure 2: Endosperm cellularization is essential for seed development. (a) Endosperm of wild type *Arabidopsis thaliana*, (b) Endosperm of *fis2* mutant. Endosperm of *fis2* mutant fails to cellularize, as a result embryo arrest at late heart stage (Adapted from Lafon-Placette & Köhler, 2014)

The *endosperm defective1* mutant also causes failure of endosperm cellularization and embryo arrest at late heart stage (Pignocchi *et al.*, 2009). All together, these mutation analyses reveal that endosperm cellularization is crucial for embryo survival and if it fails, it leads to embryo arrest (Hehenberger *et al.*, 2012). The embryo cannot use the resources directly from the central

vacuole, both because sugars are stored as hexoses in the central vacuole, a form not useable by the embryo, and because the central vacuole forms a physical barrier. Therefore, it is likely that, in case of cellularization failure, the embryo arrest is due to disturbed sucrose supply to the embryo (Hehenberger *et al.*, 2012).

1.2 Abnormal endosperm development in interploidy and interspecies hybridization: a matter of genome dosage imbalance

A normal endosperm development requires two maternal and one paternal genome (2m:1p). In most cases, the disruption of this 2m:1p ratio by interploidy crosses show endosperm defect (Müntzing, 1933; Lin, 1984; Haig & Westoby, 1991; Birchler, 1993). Typically, increased maternal genome correlates with precocious endosperm cellularization and smaller seed size; on the other hand excess paternal genome correlates with delayed or no endosperm cellularization, bigger and mostly aborted seeds (Scott *et al.*, 1998; Bushell *et al.*, 2003). Moreover, studies on interploidy intra-cultivar rice seeds had also similar phenotypes. Endosperm cellularization occurred early in autotetraploid (4x) × diploid (2x) rice hybrids whereas reciprocal cross showed delayed cellularization (Sekine et al., 2013). These endosperm defects are thought to be the cause for interploidy hybrid seed lethality. Indeed, reciprocal interploidy (2x and 4x) crosses of *Lycopersicon pimpinellifoliumm* produce mostly non-functional aborted seeds (Cooper & Brink, 1945). *A. thaliana* reciprocal interploidy crosses also inhibit normal endosperm development and produce aborted seeds (Scott *et al.*, 1998). Similarly, interploidy maize hybrids exhibit defective kernel with aborted embryo (Bauer, 2006).

Besides, crosses between species of same ploidy level but interspecies populations also demonstrate hybridization barriers similar to interploidy crosses. Postzygotic interspecific hybridization barrier was observed in some genus like *Primula, Nierembergia, Cyphomandra* and *Vigna* (Valentine & Woodell, 1963; Pringle & Murray, 1991; Ngampongsai, 1997; Soto *et al.*, 2012). For instance, reciprocal *P. reris* and *P. elatioi* crosses produced abnormal seeds with opposite phenotypes. *P. reris* × *P. elatioi* produced smaller seeds with thicker testa and *P. elatioi* × *P. reris* produced normal sizes seeds with thinner testa also both seeds were fail to germinate (Valentine & Woodell, 1963). Furthermore crosses of *N. linariaefolia* × *N. ericoides* showed abnormal zygote formation with aborted seed (Soto *et al.*, 2012). But opposite cross was produced normal seeds. Fruit crop of *Cyphomandra betacea* (Cav.) Sendt. also showed hybrid incompatibility while crossed with nine other species. Fruit set was very poor among these hybrids and none of them produced viable seeds (Pringle & Murray, 1991).

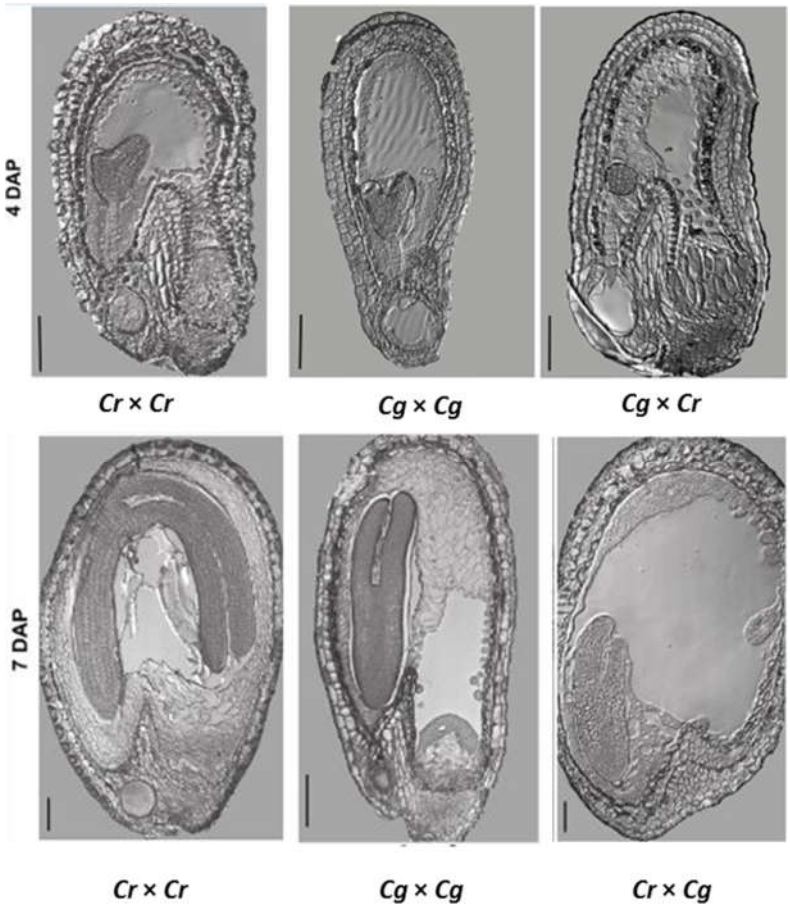

Figure 3: Precocious and delayed endosperm cellularization in interspecies *Capsella rubella* and *Capsella grandiflora* hybrid endosperm. Cr= *C. rubella*; Cg= *C. grandiflora*. At 4DAP Cg × Cr endosperm cellularized precociously but both parental endosperms did not cellularized. On the other hand, at 7DAP in Cr × Cg endosperm cellularization delayed and parental endosperm cellularized normally. (Adapted from Rebernig *et al.*, 2015)

In addition, recent studies revealed reason behind hybridization barriers in closely related *Capsella* species. Crosses between *Capsella rubella* × *Capsella grandiflora* provided delayed cellularization with mostly aborted, blackish, shrivelled seeds while *C. grandiflora* × *C. rubella* had precocious cellularization with small tiny seeds. Seed germination results also indicated that *C. rubella* × *C. grandiflora* hybrids were failed to germinate while *C. grandiflora* × *C. rubella* were germinated partially (Rebernig *et al.*, 2015)(Fig.3). These precocious and delayed endosperm cellularizations in interspecies hybrids are very much similar with interploidy hybrid endosperm cellularization results. Interestingly, in case of interploidy crosses hybridization barriers can be rescued by increasing ploidy of one species (Johnston & Hanneman, 1982). Likewise in *C. rubella* × *C. grandiflora* hybrids are also rescued by increasing ploidy of *C. rubella* (Lafon-Placette *et al.*, unpublished data). Therefore these interploidy and interspecies hybridization barriers mainly followed quantitative basis and a matter of dosage imbalance, rather than something qualitative basis.

Genome dosage imbalance in endosperm of interploidy crosses (the disruption of the 2m: 1p ratio) results in post-zygotic hybridization barriers. But an hypothesis called endosperm balance number (EBN) hypothesis could explain basis for interspecific hybridization barriers (Johnston & Hanneman, 1982). According to this hypothesis each species has specific EBN number and for viable seed production mating species required 2m: 1p ratio of EBN in endosperm. Imbalance of this EBN ratio resulted hybridization barriers which can be easily overcome by increasing or decreasing EBN number through changing ploidy number of one species (Jansky, 2006). EBN hypothesis was first explained for crosses between *Solanum* species (Johnston & Hanneman, 1982). These authors used *Solanum chalconase* as a standard potato species with EBN 2 and EBN for other *Solanum* species was measured according to failure or success of crosses to *Solanum chalconase.* This EBN hypothesis primarily proposed for *Solanum* later on was also used for some other species like *Lycopersicon* (tomato) *Trifolium* (clover) (Parrott & Smith, 1986; Ehlenfeldt & Hanneman). Nevertheless, the reason why different species have different EBNs is largely unknown.

1.3 Parental conflict theory and endosperm-based hybridization barriers

During angiosperm seed development, reserves nutrients are transported directly from maternal tissues to the endosperm and the embryo after double fertilization. In other words, the developing seed is carried by the maternal plant and the maternal plant transfers resources to its progenies.

Maternal resources are limited. Therefore, in case of multiple paternities, progenies from different male parents compete for maternal resources transferred to them. Thus, paternal loci from a given male responsible for driving more resources to its progeny and outcompeting the others will be positively selected with more resources available during embryo development; the progeny will be more fit (Haig & Westoby, 1989). Over time, males will be selected for this "selfish" behaviour. The selective interest for females is the opposite. A female able to counteract the competition between males, by allocating resources equally to all progenies, will be positively selected (more equally fit progenies instead of one very fit and the others with low fitness). Over time, this will lead to females being selected for a "repressive behaviour". This is known as the parental conflict theory (Haig & Westoby, 1989).

The influence of the male genome on maternal resource transfer is directly possible due to its contribution to the nourishing tissue, the endosperm. It is thus likely that this biparental tissue is the battlefield for parental conflict, with maternal and paternal genomes influencing endosperm development towards opposite directions: accumulation of resources for the paternal genome, restriction for the maternal one. This asymmetric influence suggests that parental genomes are not equivalent and it has been proposed that genomic imprinting, the parent-of-origin gene expression, is the molecular mechanism underlying this phenomenon (Moore, 2001).

In outbreeding species, the females can be fertilized by multiple males and the competition between males for maternal resources transferred to the developing progenies is high. As a consequence, a high level of parental conflict is maintained in such species, and both males and females are selected for having a strong and opposite influence on maternal resources

allocation to the progeny (i.e., on endosperm development). On the other hand, in inbreeding species, each plant is mainly fertilized by itself. Therefore, the selection pressure on both males and females over maternal resource allocation to the progeny is released. In other words, parents are not selected for their influence on endosperm development. If outbreeding and inbreeding species mate, the hybrid endosperm will consist of two different genomes: one from a parent selected for a strong influence on endosperm development (the outbreeder) and one from a parent not selected for this (the inbreeder). This can result on an imbalance of genome influences, or genome dosages, leading to endosperm development defects similar to the ones observed in interploidy seeds as explained above. This theory has been coined as the weak inbreeder/strong outbreeder hypothesis (WISO) (Brandvain & Haig, 2005). This hypothesis has been supported by recent experimental data (Rebernig et al., 2015; Lafon-Placette and Köhler, 2016).

1.4 Arabidopsis species to study inter and intraspecies hybridization barriers

According to the WISO hypothesis, it is difficult to predict the outcome of hybridization between two outbreeding species. In theory, two outbreeding species evolve under the same level of parental conflict and individuals are therefore selected for the same level of influence on endosperm development. No imbalance-related endosperm defects should be observed in hybrid seeds. Nevertheless, hybrid seed inviability was previously found between two outbreeding *Lycopersicon* species (Rick, 1963; Brandvain & Haig, 2005), contradicting theoretical expectations. Endosperm-based hybridization barriers could exist as well between outbreeding species and need further investigation.

Arabidopsis arenosa and *A. lyrata* are two outbreeding species. They are closely related to each other. Both are perennial and naturally exist as diploid and tetraploid species (Schmickl *et al.*, 2010; Schmickl *et al.*, 2012). Those species are also distributed in most parts of Europe (central Europe, Scandinavia) and *A. lyrata* is also present in North America (Fig. 4) (Koch & Matschinger, 2007). The process of divergence from *A. arenosa* to *A. thaliana* took place approximately 5 mya, and it is believed that *A. lyrata* and *A. arenosa* radiated around two mya (Koch *et al.*, 2000). Study of speciation process of Arabidopsis in Central Europe indicated that diploid of *A. arenosa* and *A. lyrata* are strongly genetically isolated from each other (Schmickl & Koch, 2011). This isolation suggests the existence of hybridization barriers between *A. arenosa* and *A. lyrata*. Especially, the viability of hybrid seeds between the two species could be impaired. Interestingly, reciprocal crosses performed between

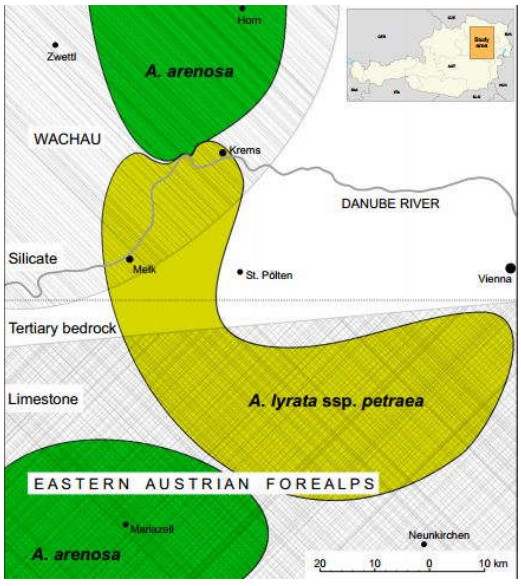

Figure 4: Non overlapping distribution pattern of *A. lyrata* and *A. arenosa* species. (Adapted from Schmickl & Koch, 2011)

A. arenosa and *A. lyrata* showed it is the case (Muir *et al.*, 2015). Hybrid seeds with *A. lyrata* mother germinated much less than the reciprocal hybrid (Muir *et al.*, 2015). Several seed based hybridization barrier mechanisms may be considered for this hybrid incompatibility, and whether endosperm defects are the cause for it remains to be addressed. *A. lyrata* also used as a model plant to study initial phases of speciation and to estimate divergence time among different populations (Leppälä *et al.*, 2007). Evolutionary lineage studies of *A. lyrata* point out that populations are migrated from central Europe to Scandinavia even till North America (Schmickl *et al.*, 2010). Migrated populations of *A. lyrata* have been adapted to several climatic conditions with diverse soil types (Turner *et al.*, 2008). Divergence results among European population also indicated that Scandinavian populations were originated from central European population with a significant loss of variation during colonization (Muller *et al.*, 2008). Scandinavian populations were diverged 39000 generations (one generation= two years) ago from central European population and it was not possible to estimate divergence among Scandinavian populations due to absence of fixed differences (Pyhäjärvi *et al.*, 2012). Distribution map of *A. lyrata* populations from different adapted regions also showed considerable morphological and genetic differentiation between central European and other populations (Schmickl *et al.*, 2010; Pyhäjärvi *et al.*, 2012). Genetic diversity results specified that central European Austrian population has highest nucleotide diversity and diversity in Scandinavian population was reduced by ca. 50% than central European populations (Ross-Ibarra *et al.*, 2008; Pyhäjärvi *et al.*, 2012) (Fig. 5). It is not known whether this divergence between *A. lyrata* populations is enough for reproductive barriers, such as endosperm based, to be established. Some postzygotic hybridization barriers were already observed between genetically diverged *Tigriopus californicus* populations (Willett & Berkowitz, 2007). An experiment between genetically diverged North American and European *A. lyrata* populations, similarly showed reduced male fertility in F_2 hybrids (Leppälä & Savolainen, 2011). But there was no clear indication about hybridization barriers among European populations. Therefore, to investigate intraspecies hybridization barriers we used four different European *A. lyrata* populations (Fig. 5). These four populations separated individually with distinct genetic variation and adapted to different environments (Pyhäjärvi *et al.*, 2012). Hence, to testify seed based postzygotic hybridization barriers we have studied endosperm development of intraspecies population hybrid seeds.

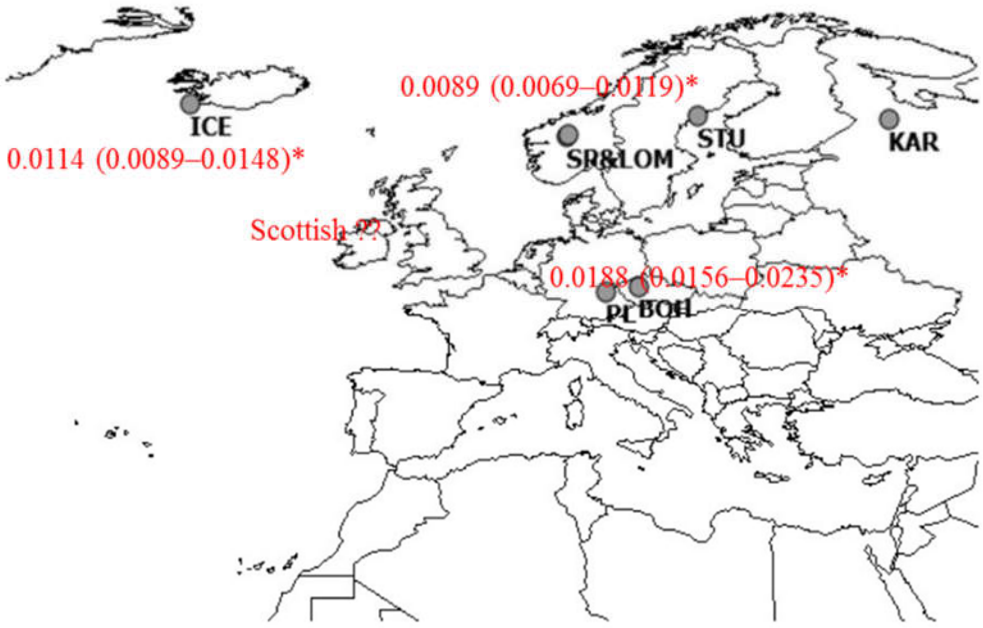

Figure 5: Distribution and genetic divergence map of *Arabidopsis lyrata* population. Bohemia (BOH) named as Austrian, LOM as Norwegian, ICE as Icelandic and another population from Scotland. Nucleotide diversity (*) is measured by Watterson's θ (Adapted from Pyhäjärvi *et al.*, 2012)

1.5 Auxin is essential for endosperm development and proliferation

Auxin plays a major role in endosperm development. Functional analysis of auxin biosynthesis and auxin signalling double mutants showed endosperm proliferation defects in maize and Arabidopsis endosperm (Bernardi *et al.*, 2012; Figueiredo *et al.*, 2015). In endosperm, availability of auxin mainly depends on biosynthesis pathways. There are several pathways for auxin biosynthesis that varies on different extent in different species (Vanneste & Friml, 2009). In endosperm, auxin biosynthesis depends on activity of FERTILIZATION INDEPENDENT SEED–Polycomb Repressive Complex2 proteins (FIS–PRC2). FIS–PRC2 represses auxin biosynthesis in the central cell before fertilization but after fertilization, the paternally expressed *YUCCA10* (*YUC* family) and *TRYPTOPHAN AMINOTRANSFERASE RELATED 1* (*TAR1*) auxin biosynthesis genes generate auxin production that leads to endosperm development (Fig. 6) (Hsieh *et al.*, 2011; Figueiredo *et al.*, 2015). In addition, higher level of auxin was observed in hybrid endosperm while *Arabidopsis thaliana* (2n) mother crossed with *Arabidopsis thaliana* (4n) father (Figueiredo *et al.* unpublished data). As *Capsella* interspecies hybrid endosperm showed similar results like interploidy crosses therefore we hypothesise that cross direction dependency of hybrid seed defects and paternally expressed auxin biosynthesis may share underlying mechanism.

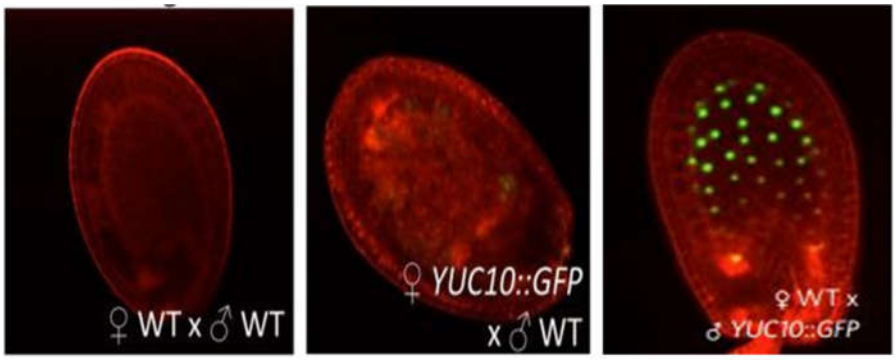

Figure 6: Parent specific auxin biosynthesis occurs in *Arabidopsis thaliana* endosperm. (Adapted from Figueiredo *et al.*, 2015)

Interspecies crosses of *C. rubella* × *C. grandiflora* show delayed cellularization (Rebernig *et al.*, 2015), and *C. rubella* × *C. orientalis* exhibits early cellularization with inviable hybrid seeds (Lafon-Placette et al., unpublished data). So, to test whether *C. grandiflora* express higher level of auxin in *C. rubella* × *C. grandiflora* hybrid endosperm and *C. orientalis* express less amount of auxin in *C. rubella* × *C. orientalis* hybrid endosperm, we constructed *GH3.3::GFP* reporter line in C. rubella.

1.6 Aim of this study

The objective of this study was to investigate factors impairing hybrid seed development. The research work was carried out in three main aspects:

I. Test whether hybrid seeds between *A. arenosa* and *A. lyrata* exhibit impaired viability and if so, determine the role of endosperm cellularization and the genetic basis behind it.
II. Investigate a potential endosperm-based postzygotic hybridization barrier between *A. lyrata* European populations.
III. Determine auxin levels in inviable *C. rubella* × *C. grandiflora* and *C. rubella* × *C. orientalis* interspecies hybrid seeds.

2 Materials and methods

2.1 Plant materials

Seeds were surface sterilized with sterilizing solution (5% NaClO + 0.01% (v/v) Tween20) for 10 minutes and washed four times with sterile water. Sterile seeds were plated in 1% MS Medium and kept in 4° C cold dark room for vernalisation. After six weeks, plates were transferred in growth chamber (22°C, 16h light /8h dark and 40% RH). Ten days old seedlings were transferred into soil and grown at 22°C during light and 18°C during dark, 16h light /8h dark and 70% humidity conditions. All F_1 hybrids and parents used for backcrossing were grown at 26°C, 16h light /8h dark conditions. For interspecies crosses, six *A. lyrata* and four *A. arenosa* plants were used. Two to six F_1 hybrid plants were used per cross type for backcrossing. For intra population crosses four to six plants were used for each population. All flower buds were emasculated one day before anthesis and pistils were hand pollinated two days after emasculation in the morning under normal light stereoscope. All siliques were harvested according to mentioned time points.

2.2 Germination analysis

Thirty days after harvesting, all parental and hybrid seeds were sterilized according to above explained seed surface sterilizing protocols and plated on MS media without sucrose. All plates were kept at 4°C for six weeks to break seed dormancy. After that plates were transferred to light chamber for germination. Seeds with ruptured seed coat and protruding radicles were counted as germinated and others as ungerminated seeds. Seed germination rates were calculated dividing germinated seeds by total number of seeds.

2.3 Embryo rescue

A. lyrata × *A. arenosa* hybrid seeds were used for embryo rescue at 27 DAP by *in vitro* cultivation. The siliques were incubated in 70% ethanol for short time and then the embryos were isolated by dissection using hypodermic needles. Rescued embryos were placed immediately on MS media containing 2% sucrose. All plates were covered with tissue and incubated in a light chamber. All surviving seedlings were transferred to soil and kept in growth chamber for normal growth and development.

2.4 Seed size, weight and abortion rate analysis

Dry seeds were taken on white plastic dishes and cleaned from all siliques debris. Pictures were taken using a Lecia Z16apo microscope. Abortion rate and seed size were analysed using ImageJ software. Aborted seeds and plump seeds were counted by “cell count” function. For seed size measurement images were converted into red colour by “colour threshold” function and seed size was measured by “Analyse particles”. For actual size measurement, a picture of ruler paper was measured and then microscopic seed size was converted to the actual seed size. Seed weight of all the seeds were weighed by Ohaus GA 110 balance and weight of each sample was divided by number of seeds to get individual seed weight.

2.5 Clearing of seeds for nuclei counting

Five crosses were done for each time point and from each crosses three siliques were harvested. Siliques were opened on one side just after harvesting, placed in Fixing solution (Ethanol (EtOH): Acetic acid 9:1) and stored over night at 4°C. Siliques were washed with 90% EtOH for 10 min and then 70% EtOH for 10 min. They were then stored at 4°C in 70% EtOH.

EtOH was removed from stored samples via addition of clearing solution (66.7 % Chloralhydrate 25 % H2O and 8.3% Glycerol) into all samples. Siliques were incubated for 48 hours. After incubation, pods were removed and only seeds were mounted on slide with clearing solution. From three slides five seeds were selected for nuclei counting according to average embryo growth condition. Pictures of cleared sample were taken using Lecia DMI4000B microscope under DIC light with 20X zoom settings.

2.6 Feulgen staining of Seeds

Five crosses were done for each time point and from each crosses three siliques were harvested. Harvested siliques were opened from one side by needles and were incubated in fixing solution (EtOH:Acetic acid 3: 1). After 24 hours of incubation, the fixing solution was replaced by 70% EtOH and the siliques were stored at 4°C.

Seeds were prepared using Feulgen staining method described by (Braselton *et al.* 1996). Three to five siliques for each time point were taken to prepare slides. Seeds were mounted in LR resin and pictures of all samples were taken using ZESIS LSM 710 NLO multiphoton microscope with an excitation wavelength of 770 nm and emission from 518 nm and onwards.

2.7 Cloning and gene expression

Destination vector was constructed previously in our lab (Figueiredo et al., unpublished data). *Agrobacterium* GV3101 strain was used as competent cell for transformation as described in (Main *et al.*, 1995). Then, Agrobacterium cells were spread on LB plates with Rifampicin and Spectinomycin antibiotics (selection markers) (LB plates provided by Cecilia Wärdig). Then these antibiotic plates were incubated at 28°C for 48h. After that, colony PCR amplification and gel electrophoresis were performed, in order to identify positive transformants. One positive colony was harvested and put in 300ml of liquid LB with Rifampicin (100µg/ml) and Spectinomycin (100µg/ml). *Agrobacterium* was then grown at 28°C at 200 rpm for 16 hours in the dark. After spinning down the culture (4000rpm, 16minutes), the pellet was resuspended in transformation medium (10% sucrose, ½ MS salts and 0.05% Silwet L-77 per liter). Unopen flower buds of *Capsella rubella* were used for transformation. Buds were dipped in transformation medium for 20 sec. Plants were placed horizontally in trays, covered with plastic foil and kept in dark for overnight. Next morning plastic foil was removed and plants were put back into the growth chamber. Same procedure was done 7 days later. Three weeks after transformation, all seeds were harvested and left to rest for 1 month. Seeds were then sown on soil and BASTA was applied 3 times per week from when plants reached 2 leaves stage.

3 Results

3.1 Abnormal seed phenotypes in hybrid seeds

In order to test whether a postzygotic hybridization barrier prevents the formation of viable hybrid seeds between *Arabidopsis arenosa* and *Arabidopsis lyrata*, reciprocal crosses were performed between the two species. Parental crosses produced normal seeds as shown in (Fig. 1 a,b) whereas *A. lyrata* × *A. arenosa* (Female × Male) hybrid seeds were shrivelled dark brown seeds (Fig. 1c). On the other hand, the reciprocal cross produced plump seeds (Fig. 1d). Hybrid seeds from *A. lyrata* × *A. arenosa* cross- resulted in 10% viable seeds while *A. arenosa* × *A. lyrata* resulted in 0% germination despite a high number of non-collapsed seeds(Fig.1e).

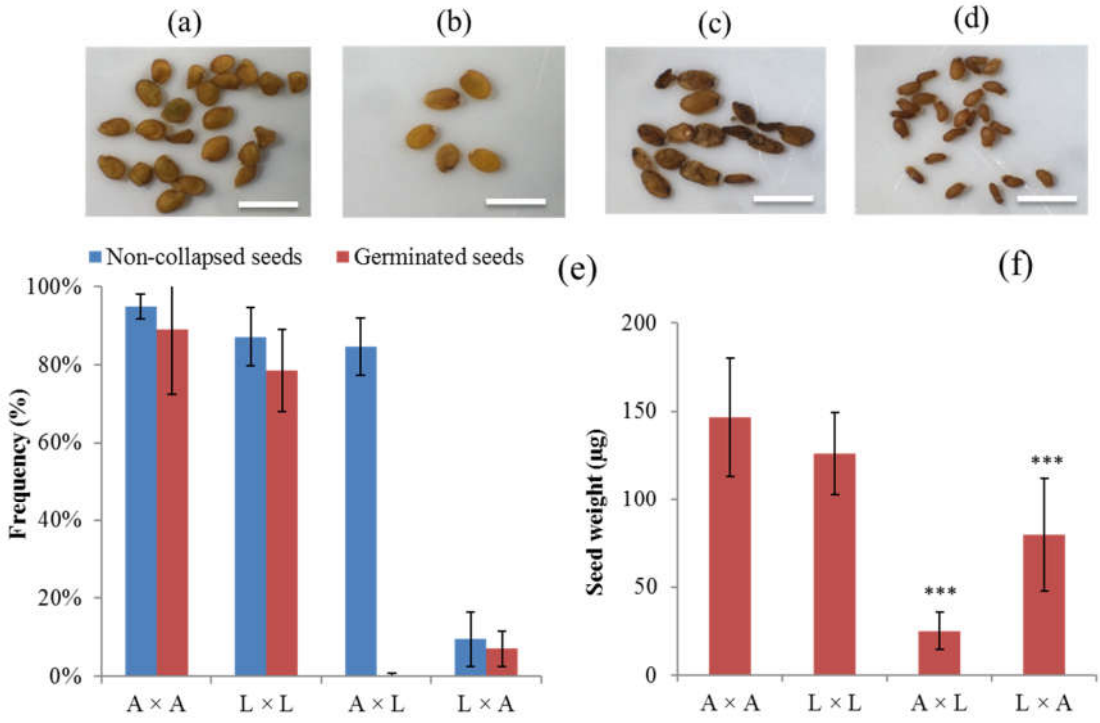

Figure 1: Parent-of-origin phenotypic defects leads to unviability of hybrid seeds between *Arabidopsis arenosa* and *Arabidopsis lyrata.* Pictures of (a) *A. arenosa* seeds;(b) *A. lyrata*; (c) *A. lyrata* × *A. arenosa* (Female × Male) hybrid seeds and *(*d) *A. arenosa* × *A. lyrata*. The scale bars represent 1mm. (e) seed viability is expressed as the rate of non-collapsed seeds and germination (%) for parental species and hybrids. (f) Average seed weight (µg) of parents and hybrids. A= *A. arenosa,* L= *A. lyrata*. (***) indicates significant reduction of seed weight as compare to mid parent seed weight. Significance was determined by *t* test analysis (P≤ 0.001). Error bar indicates standard deviation.

Seed size measurement of *A. arenosa* × *A. lyrata* also showed significant reduction of hybrid seed size as compared to parent seeds (Fig. S1). Moreover, reciprocal hybrids also resulted in significant reduction of seed weight as compared to mid parent seeds weight ($P \leq 0.001$) (Fig.1f).

Therefore, reciprocal hybrids between *A. arenosa* and *A. lyrata* are in majority inviable, despite the cross-direction dependent aspect of the hybrid seeds. This acts as postzygotic hybridization barrier between these two species.

3.2 Endosperm proliferation is not correlated to hybrid seed inviability

It was previously shown that endosperm proliferation was impaired in interploidy hybrid seeds (Scott *et al*., 1998; Sekine *et al.* rice 2013). Therefore, we tested whether the altered hybrid seed viability was due to changes in the rate of endosperm syncytial nuclear division. To challenge this hypothesis, the number of endosperm nuclei was counted in three early time points (5Days After Pollination, DAP; 7DAP and 9DAP) which were used to check the variation of endosperm nuclei in both parental and reciprocal hybrid seeds. At 5 DAP, in seeds of *A. arenosa* × *A. lyrata* cross, the number of nuclei was similar as compared to the maternal *A. arenosa* seeds, but at 7 DAP, it was lower as compared to both parent seeds (Fig. 2a). This was associated with a low endosperm proliferation from 5 to 7 DAP (Fig. 2b). At 9 DAP, no endosperm nuclei was visible anymore. In case of *A. lyrata* × *A. arenosa* hybrid seeds, the endosperm proliferation was similar to both parents between 5 and 9 DAP, despite a higher number of nuclei as compared to *A. lyrata* control seeds (Fig. 2a,b).

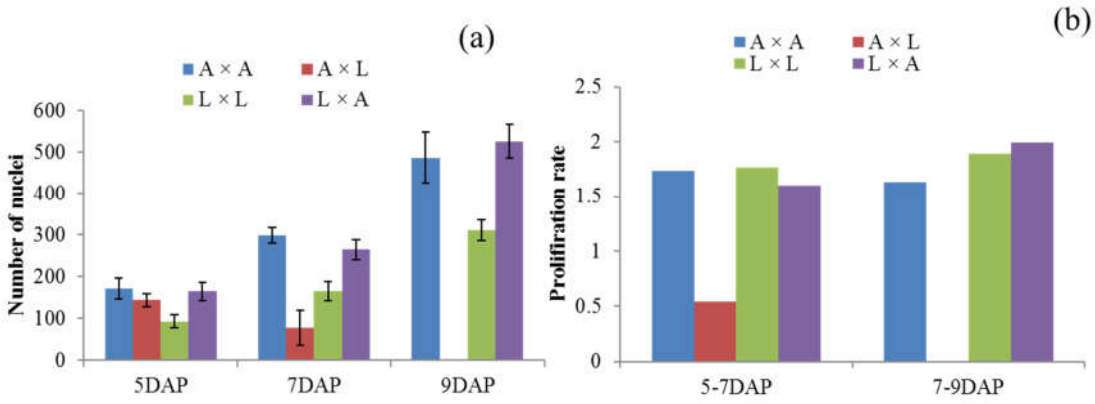

Figure 2: Endosperm proliferation rate is only affected in *A. arenosa* × *A. lyrata* hybrid seeds. (a) Number of endosperm nuclei after 5, 7 and 9 DAP. (b) Endosperm proliferation rate between 5-7DAP and 7-9DAP. Endosperm proliferation rate was counted through dividing 7DAP nuclei by 5DAP nuclei and 9DAP nuclei dividing by 7DAP nuclei. A= *A. arenosa,* L= *A. lyrata*. Indigo colour bar represents *A. arenosa* × *A. arenosa*, dark red for *A. arenosa* × *A. lyrata*, green for *A. lyrata* × *A. lyrata* violet for *A. lyrata* × *A. arenosa*. Error bar indicates standard deviation.

In conclusion, while endosperm proliferation was reduced in *A. arenosa* × *A. lyrata* seeds, no defect could be found in the reciprocal hybrid. Both cross directions give nearly full inviability and therefore, endosperm proliferation defects could not be correlated to hybrid seed survival,

making it unlikely that endosperm proliferation problems are the cause for this hybridization barrier.

3.3 Precocious and delayed endosperm cellularization occurs in hybrid seeds

It has been proposed that endosperm cellularization is crucial for embryo proper development and if it fails, it leads to embryo arrest (ref cited in the introduction). We therefore considered that, impaired endosperm cellularization would impact on embryo development which leads to hybrid seed lethality.

Endosperm cellularization was investigated at an early (9 DAP) and late (15 DAP) time point. In *A. arenosa* at 9DAP, endosperm not started to cellularize at micropylar end and embryo reached up to heart stage. Consequently, at 15DAP very few parts of cellularized endosperm were left and the embryo developed completely. On the other hand, in *A. lyrata* at 9DAP, endosperm didn't start to cellularize and the embryo reached globular stage while at 15DAP, the endosperm was completely cellularized and embryo cotyledons started to bend (Fig. 3). Meanwhile, hybrid seeds of *A. arenosa* × *A. lyrata* cross showed complete endosperm cellularization at 9DAP and in contrast hybrid seeds of *A. lyrata* × *A. arenosa cross* showed no cellularization at 9DAP or not even at 15DAP (Fig. 3). In hybrid seeds of *A. arenosa* × *A. lyrata* cross, the embryo developed completely at 15DAP but occupied less space as compared to parental seeds while, in this time point, hybrid embryo of *A. lyrata* × *A. arenosa* developed up to heart stage (Fig. 3).

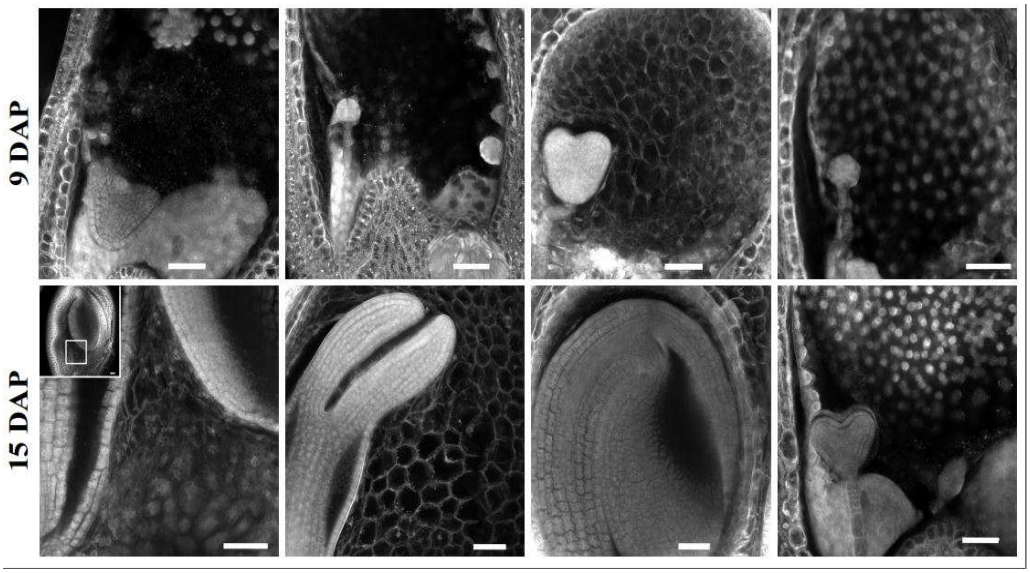

Figure 3: Abnormal endosperm cellularization of *A. arenosa* and *A. lyrata* hybrid seeds. Feulgen-stained parental and reciprocal hybrid seeds. (a) *A. arenosa* × *A. arenosa*, (b) *A. lyrata* × *A. lyrata*, (c) *A. arenosa* × *A. lyrata* and (d) *A. lyrata* × *A. arenosa* . A= *A. arenosa*, L= *A. lyrata*. Top row indicates 9 DAP and bottom row indicates 15 DAP seeds. The scale bars represent 50µm.

Endosperm cellularization analysis of both parental and hybrid seeds showed differential cellularization time points in reciprocal hybrid seeds as compared to parental seeds. To conclude, *A. arenosa* × *A. lyrata* cross showed precocious endosperm cellularization and *A. lyrata* × *A. arenosa* cross showed delayed endosperm cellularization. These results suggest that these endosperm cellularization defects are responsible for hybrid seed inviability because embryo from *A. lyrata* × *A. arenosa* hybrids was rescued to grow in-vitro condition at 27DAP which produced viable hybrid plants (Fig. S2).

3.4 Involvement of multiple loci for hybridization barriers in reciprocal crosses of *A. arenosa* and *A. lyrata*

To understand the genetic interaction between *A. arenosa* and *A. lyrata* genomes leading to hybrid seed inviability, it is very essential to know how many parental loci are involved in the hybridization barrier. The number of parental loci involved in reciprocal crosses of *A. arenosa* and *A. lyrata* can be achieved via analysing seed phenotype of F_1 backcross seeds. In *A. lyrata* × *A. arenosa* cross, seed abortion rate was scored while for *A. arenosa* × *A. lyrata*, seed size (small seeds) was scored. And then, the proportion of the phenotype following F_1 backcrosses were allowed to know how many loci were involved on the side of the parent replaced by F_1, according to Mendelian segregation rules (Table S1). For example, the theoretical involvement of one locus would produce 50% impaired seeds in F_1 backcrosses, involvement of two loci would produce 25% and three loci would produce 12.5% impaired seeds.

In *A. lyrata* × *A. arenosa* cross direction, F_1 hybrids were used both as pollen donor and pollen receiver to reveal the involvement of *A. arenosa* paternal loci and *A. lyrata* maternal loci respectively. *A. lyrata* × F_1 backcross gave 15% of aborted seeds, which is not significantly (Chi-square test analysis, $P > 0.05$) different from 12.5% abortion for having the involvement of three *A. arenosa* loci for hybrid incompatibility (Fig. 4a). On the other hand, F_1× *A. arenosa* backcross showed approximately 50% of impaired seeds, which was not significantly (Chi-square test analysis, $P > 0.05$) different to the theoretical expectation of 50% suggesting one maternal *A. lyrata* loci responsible for the hybridization barrier (Fig. 4b).

For the reciprocal *A. arenosa* × *A. lyrata* cross, very tiny seeds were scored since *A. arenosa* × *A. lyrata* hybridization produces tiny seeds. When *A. lyrata* was replaced by F_1, 16% of seeds were of similar size as compared to *A. arenosa* × *A. lyrata* hybrid seeds (Fig. 4c). This was not significantly (Chi-square test analysis, $P > 0.05$) different with theoretical 12.5% for having three paternal loci of *A. lyrata* involved in the hybridization barriers. Subsequently, in F_1 × *A. lyrata* backcross, the same pattern was observed: 14% had a reduced size, which is not significantly different (Chi-square test analysis, $P > 0.05$) with theoretical expectation of 12.5% for three maternal loci involved in the hybrid seed phenotype.

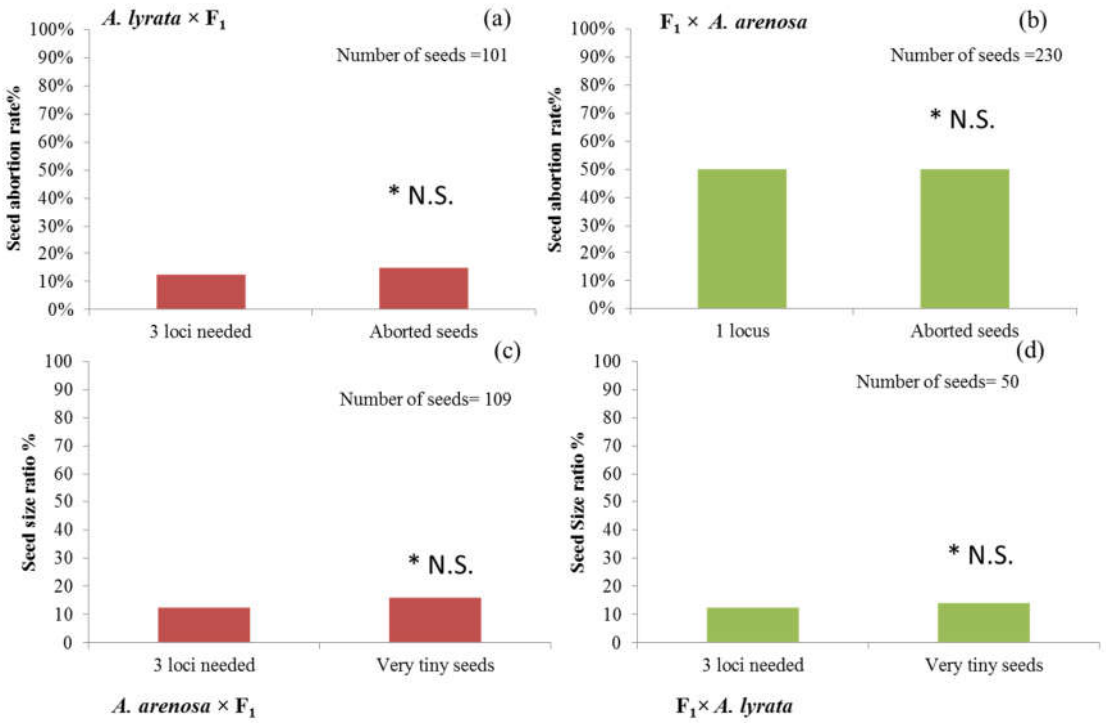

Figure 4: Multiple and variable number of loci involved in reciprocal *A. arenosa* and *A. lyrata* hybrid incompatibility. (a, b) F_1 backcross seed abortion rate to determine number of loci in *A. lyrata* × *A. arenosa* cross direction. (a) When *A. arenosa* father, three loci are responsible for incompatibility and (b) In case of *A. lyrata* mother, one locus responsible for incompatibility. (c, d) F_1 backcross seed size frequency to determine number of loci in *A. arenosa* × *A. lyrata* cross direction. Histogram bars represent three independent loci of both parents responsible for incompatibility. N.S.= Not significant. (*) indicates no significant differences from expected value. Significance was determined by chi square test analysis ($P \leq 0.05$).

Overall all these results revealed that a complex genetic network exist for the hybridization barrier between *A. arenosa* and *A. lyrata* hybrids. These results also suggest that the number of loci is different depending on the cross direction, consistent with the parent-of-origin nature of hybrid seed defects.

3.5 Intra-species hybrid incompatibility between four *A. lyrata* populations

To investigate *A. lyrata* intraspecies hybrid seed incompatibility, we used four populations of *A. lyrata* from four different countries: Austria, Iceland, Scotland and Norway. All the possible combinations of crosses were done previously (Rebernig et al., unpublished data; Figure 5). Collapsed seed rate, seed size and weight were measured to check hybrid incompatibilities among all hybrids.

Figure 5: Hybrid incompatibility of Austrian hybrid population while cross with other populations. Column indicates seed plant and row indicates pollen donor population. Seeds with red borders indicate intrapopulation control cross. Scale bar represents 1mm

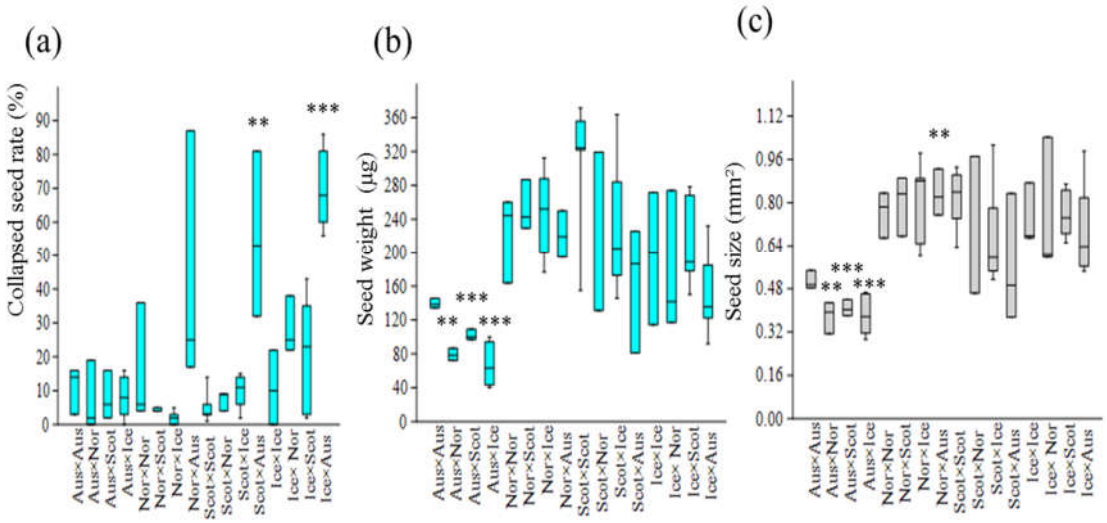

Figure 6: Abnormal seed development of Austrian hybrids in reciprocal cross directions. (a) Collapsed seed rate (%) of hybrids and parental populations, (b) Seed weight (µg) and (c) Seed size (mm²) of parents and hybrids. Error bar indicates Standard Error. (*) indicates significant reduction of seed collapsed rate, seed weight and seed size as compare to mid parent value. Significance is determined by *t* test analysis. **P ≤ 0.01 and **P ≤ 0.001.

Collapsed rate of hybrid seeds from Scottish × Austrian (mean 61%) and Icelandic × Austrian (mean 71%) increased significantly (*t* test analysis, P≤ 0.01) as compared to mid parent value (Fig. 6a). In case of Norwegian × Austrian cross, one replicate had 87% collapsed seeds while, other replicates had 36%, 25% and 17% respectively. Meanwhile, collapsed rate of all other hybrid seeds had less than 20%, which is not significant (*t* test analysis, P> 0.05) as compared to mid parent value.

The weight of Austrian × Norwegian (hybrid mean 79.09 µg, mid parent mean 181.12µg), Austrian × Scottish (hybrid mean 102 µg, mid parent mean 223µg), and Austrian × Icelandic (hybrid mean 65.73 µg, mid parent mean117.14µg) hybrid seeds were reduced significantly (*t* test analysis, P≤ 0.01) compared to the mid parent value (Fig. 6b). Consequently, seed sizes of those hybrids were also reduced significantly (*t* test analysis, P≤ 0.01) (Fig. 6c). At the same time, seed size of Norwegian × Austrian hybrids was increased significantly (*t* test analysis, P≤ 0.01) as compared to mid parent seed size. All the other types of interpopulation hybrid seeds did not exhibit any significant difference in size or weight as compared to the midparent value (*t* test analysis, P> 0.05).

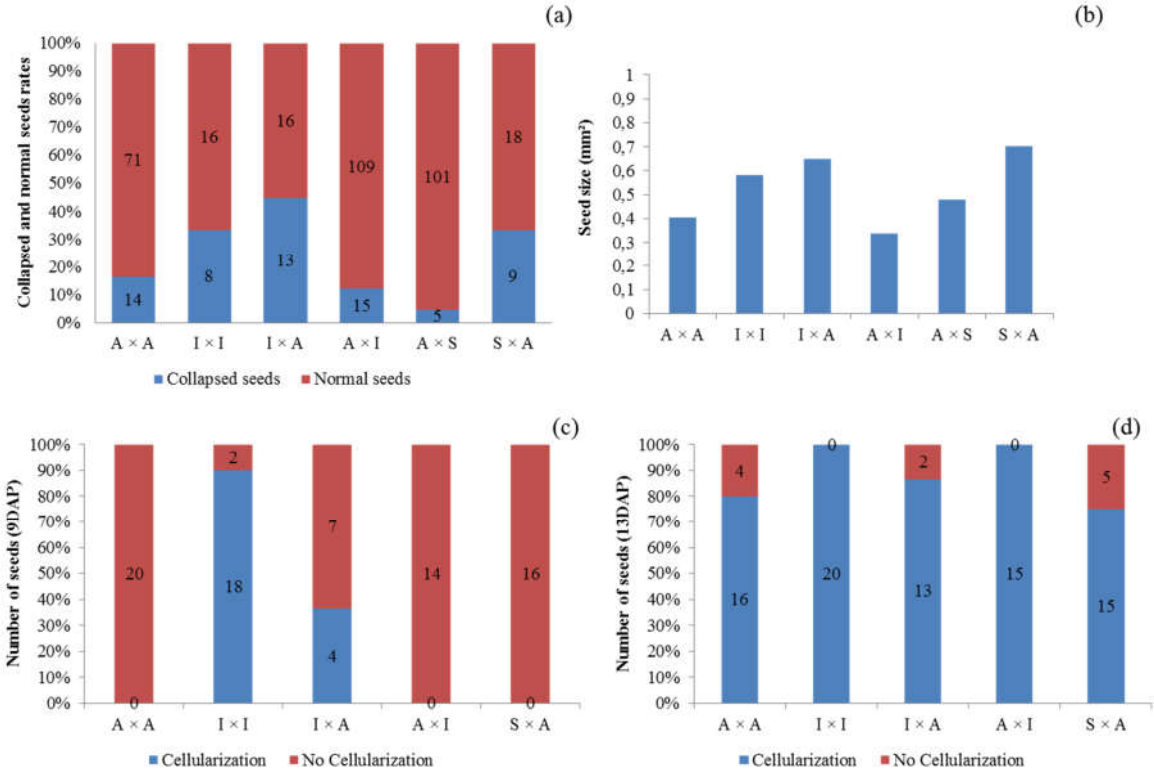

Figure 7: Lower number of collapsed seeds and normal cellularized endosperm are found among repeated intra species hybrids.

(a) Collapsed and normal seed rate (%) of parental and hybrid seeds. Blue bar indicates collapsed seeds and dark red bar indicates normal seeds. Numbers on bars indicate number of seeds. (b) Seed size (mm²) of parental and hybrid seeds. (c,d) Endosperm cellularization rate (%) of parental and hybrid seeds. (c) 9DAP, (d) 13DAP seeds. Blue bar indicates cellularization

and dark red bar indicates no cellularized endosperm. A= Austrian, I=Icelandic, S= Scottish *A. lyrata* species. Scale bar 50µm.

In order to confirm these results, interpopulation crosses involving the Austrian plants were repeated again except Norwegian population. New Icelandic × Austrian cross produced 45% collapsed seeds (Fig. 7a) and Scottish × Austrian cross also produced 33% collapsed seeds (Fig. 7a). This is lower than the values obtained with previous crosses (Fig. 6a), but is still higher than the midparent value. More replicates are needed to be performed to know if this increase is significant. Seed size measurement of parental and hybrid seeds also indicated that hybrid seed size of Austrian × Icelandic cross was reduced as compare to mid parent value (Fig. 7b), but replicates will determine whether these seeds are significantly smaller. As we could not produce any parental Scottish seed, therefore it was not possible to make any conclusion about Austrian × Scottish hybrid seed size.

Afterwards, we tested whether an increased abortion rate or a smaller size could be associated with delayed or precocious endosperm cellularization in *A. lyrata* interpopulation hybrid seeds. For this purpose, endosperm cellularization rate (the proportion of seeds showing endosperm cellularization) was measured at 9 and 13 DAP (Fig. 7c,d and Fig. S3). Austrian parental endosperm was not cellularized at 9DAP. However, in Icelandic seeds, both embryo and endosperm development were faster, and most endosperms were already cellularized at 9DAP (Fig. 7c,d). Icelandic × Austrian hybrid showed 37% cellularized endosperm at 9DAP which is close to mid-parent value (45%) whereas in the reciprocal cross, no hybrid seed exhibited cellularized endosperm, similarly to the Austrian parent (Fig. 7c,d). At 13 DAP, all parental and hybrid seeds showed approximately 100% cellularized endosperm, at the same time Scottish × Austrian hybrid seeds also showed 75% cellularized endosperm (Fig. 7c,d).

In summary, cross direction dependent phenotypic defects were observed between Austrian and other *A. lyrata* populations, similarly to what was observed between *A. lyrata* and *A. arenosa*. Nevertheless, these results need confirmation and with the current results, it is not possible to propose that the interpopulation hybrid seed defects are related to endosperm cellularization problems.

3.6 Increased level of auxin in paternal excess phenotypes

Auxin is very essential for endosperm development, especially for endosperm proliferation defects (Figueiredo *et al.*, 2015). In addition, the endosperm of 2x *A. thaliana* × 4x *A. thaliana* hybrid seeds showed higher level of auxin expression as compared to the diploid control (Figueiredo *et al.*, unpublished data). *C. rubella* × *C. grandiflora* and *C. rubella* × *C. orientalis* hybrid seeds showed the same phenotypes as compared to 2x *A. thaliana* × 4x *A. thaliana* and 4x *A. thaliana* × 2x *A. thaliana* hybrid seeds respectively (Lafon-Placette *et al*., unpublished data). Therefore, we tested whether higher level of auxin in hybrid endosperm of *C. rubella* × *C. grandiflora* and lower level of auxin in *C. rubella* × *C. orientalis* hybrid endosperm could be observed.

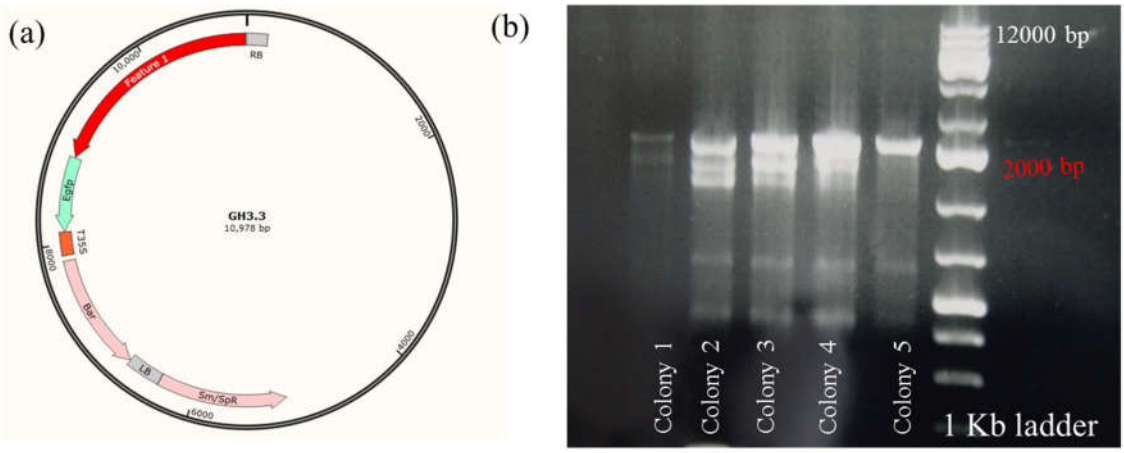

Figure 8: Transformation of *GH3.3::GFP* reporter line in C. rubella. (a) Construction of destination vector. Red colour indicates promoter region of GH3.3, light green colour indicates GFP reporter, pink colour bar for BASTA and Sm for Spectinomycine antibiotics. (b) Colony PCR amplicons of *GH3.3* promoter that confirms positive transformants of *Agrobacterium* colonies with our construction. (Expected size of amplicon 2118bp)

To test this hypothesis, we constructed auxin expression *GH3.3::GFP* reporter line in *C. rubella* (Fig. 8a). *Agrobacterium* transformation was done properly and appropriate colonies were confirmed by PCR reaction (Fig. 8b). *C. rubella* transformant seeds were grown in soils and BASTA antibiotic was applied on the leaf surface of 4 days old seedlings. BASTA antibiotic resistance sequence was inserted in the vector construction and after BASTA application we were expecting desired reporter line. Unfortunately, we did not get any positive reporter line in *C. rubella* after BASTA selection

4 Discussion

4.1 Endosperm based postzygotic hybridization barriers in interspecies of *A. arenosa* and *A. lyrata*

Abnormal seed development is a form of postzygotic hybridization barriers between several related species (Pringle & Murray, 1991; Ngampongsai, 1997; Ishikawa *et al.*, 2011; Rebernig *et al.*, 2015). Cross direction dependent seed phenotypes are also found in reciprocal hybrids of *C. rubella* and *C. grandiflora* (Rebernig *et al.*, 2015). In our experiment, *A. lyrata* × *A. arenosa* produced shrivelled dark brown seeds and the reciprocal cross produced small plump seeds. Seeds from both hybrids also failed to germinate. Therefore, our seed phenotype results support that there is a strong seed based hybridization barriers between *A. arenosa* and *A. lyrata*.

During syncytial endosperm development, abnormal pattern of endosperm proliferation were found in interploidy maize, *Oryza* and *Arabidopsis* hybrids (Scott *et al.*, 1998; Bushell *et al.*, 2003; Leblanc *et al.*, 2002; Sekine *et al.*, 2013). However, in *Capsella* and *Oryza* interspecies hybrid seeds did not show abnormal endosperm proliferation (Rebernig *et al.*, 2015; Ishikawa *et al.*, 2011). In reciprocal cross of *A. arenosa* and *A. lyrata*, we found that endosperm proliferation rate could not be correlated to hybrid seed survival. Therefore, this suggests that while interploidy and interspecies hybridization barriers share similar mechanisms, impaired endosperm proliferation is not one of them.

Interploidy *Arabidopsis* and rice endosperm of excess maternal genome showed early cellularization and excess paternal genome showed no cellularization (Scott *et al.*, 1998; Sekine *et al.*, 2013). Interspecies *C. rubella* × *C. grandiflora* also showed no cellularization and *C. grandiflora* × *C. rubella* showed early cellularization (Rebernig *et al.*, 2015). In this study, *A. arenosa* × *A. lyrata* endosperm cellularized early while in *A. lyrata* × *A. arenosa* seeds, endosperm failed to cellularize. The aborted seed phenotypes suggest that delayed endosperm cellularization could limit transfer of nutrients from endosperm to embryo, as (Morley-Smith *et al.*, 2008). These results suggest that endosperm cellularization defects are a widespread cause of interploidy and interspecies hybridization barriers.

In case of interploidy crosses, increased ploidy creates a genome dosage imbalance in endosperm. In case of interspecies cross, such as *C. grandiflora* and *C. rubella*, the former is an outbreeding species while the latter is an inbreeder, explaining according to the WISO hypothesis why, even though both species have the same ploidy level, *C. grandiflora* behaves as a higher genome dosage species (Brandvain & Haig, 2005; Rebernig *et al.*, 2015). In contrast, in our experiment both species are outbreeders and diploid species. Nevertheless, *A. arenosa* behaves like a higher genome dosage species; in other words, *A. arenosa* genome contributes in a higher amount to endosperm development (or has a higher EBN) comparing to *A. lyrata* genome (Johnston & Hanneman, 1982). The fact that both species are outbreeders and diploid but have different EBN numbers also could also be explain by the parental conflict hypothesis (Haig & Westoby, 1989). *A. arenosa* and *A. lyrata* have different genetic diversity (Koch & Matschinger, 2007). We suggest that higher genetic diversity of *A. arenosa* leads to

higher competition between males for maternal resource transfer and higher interest divergence between males and females, i.e. leads to a higher level of parental conflict, as compared to *A. lyrata*. Therefore, this leads to a higher selection pressure for a strong parental influence on endosperm development, or higher EBN, in *A. arenosa* as compared to *A. lyrata*. Finally, when these two species mate, a differential EBN generates dosage imbalance in the endosperm and the seed lethality frequently observed among hybrids.

Previously a complex genetic network was found for reciprocal crosses of *C. rubella* × *C. grandiflora* for endosperm based hybridization barriers (Rebernig *et al.*, 2015). Genetic analysis of reciprocal *A. arenosa* and *A. lyrata* crosses revealed as well involvement of multiple loci responsible for hybridization barriers. Our results support complex and cross-direction dependent genetic interactions for hybridization barriers between *A. arenosa* and *A. lyrata*.

4.2 Diverse genetic diversity might be responsible for postzygotic hybridization barriers in *A. lyrata* populations

To test hybridization barriers among four *A. lyrata* populations, hybrid seeds from all possible combinations were analysed. Seed weight and seed size was significantly reduced while Austrian population was mother while a significant number of dark shrivelled seeds was found in Scottish × Austrian and Icelandic × Austrian hybrid seeds. These phenotypes, suggest that the Austrian population behaves as a higher genome dosage population. Genetic diversity of three populations (except Scottish) was described previously, showing that the Austrian population exhibits the highest genetic diversity of all (Pyhäjärvi *et al.*, 2012). Due to higher genetic diversity of Austrian population, and the same way we proposed for *A. arenosa* and *A. lyrata*, we were expecting a higher EBN in the Austrians compared to the other populations. This would cause the hybrid seed defects we observed when crossed to other populations, as a consequence of genome dosage imbalance in the endosperm. To test this hypothesis, endosperm cellularization of all parental and hybrid seeds was also analysed. However, no major difference in endosperm cellularization rate could be found in any hybrid seeds. Nevertheless, this could be consistent with a lower rate of collapsed seeds found when the crosses were done a second time, as compared to the crosses previously performed (Rebernig et al., unpublished data). The second crosses were made in different growth conditions and it was shown that growing conditions such as temperature can influence seed development and especially endosperm cellularization(Chen *et al.*, 2016). Thus, variable results could link with plant growing conditions that effect on seed development.

4.3 Role of Auxin for hybridization barriers

Parent specific auxin biosynthesis was observed by *YUC10* transcriptional reporter line in *Arabidopsis thaliana* (Figueiredo *et al.*, 2015). Additionally, higher level of auxin was also observed in endosperm while *Arabidopsis thaliana* (2x) × *Arabidopsis thaliana* (4x) (Figueiredo *et al.*, unpublished data). Likewise, *C. rubella* × *C. grandiflora* and *C. rubella* × *C. orientalis* hybrid seeds also showed respectively paternal excess and maternal excess phenotypes (Rebernig *et al.*, 2015; Lafon-Placette *et al*., unpublished data). We were expecting to see differential level of auxin expression by constructing *GH3.3::GFP* reporter line in *C.*

rubella. We confirmed *Agrobacterium* transformation by colony PCR amplicons but we did not get any positive reporter line in *C. rubella* after BASTA antibiotics application on transformants *C. rubella* leaf surface. That may be our floral dipping method didn't work properly. In order to get positive reporter line, to avoid accession specific problem one can use another accession.

5 Conclusions

In case of interploidy and interspecies (initiated by different matting system species) hybrid endosperm, a postzygotic hybridization barriers were frequently found previously. The result presented in this study is the first indication of endosperm based hybridization barriers between two outbreeding species with same ploidy. In case of interspecies *A. arenosa* and *A. lyrata* reciprocal crosses, cross direction dependent opposite phenotypes were found in hybrid seeds. *A. arenosa* × *A. lyrata* produced tiny seeds with early cellularized endosperm and *A. lyrata* × *A. arenosa* produced aborted seeds with delayed cellularized endosperm. Due to presence of higher genetic diversity, we propose that *A. arenosa* shows higher parental conflict than *A. lyrata*. Our results suggest a complex genetic network for these hybridization barriers but the molecular basis for parental conflict is still unknown. Likewise, intraspecies hybrid seeds expressed the similar seed phenotypes as like as interspecies hybrid seeds.

Our works provide a good framework for further investigation to repeat intraspecies crosses to confirm hybridization barriers that could follow same genetic and molecular mechanism. Moreover, genomic imprinting is thought to be the molecular basis for parental conflict; therefore, analysis of imprinted genes could explain unsolved questions in future.

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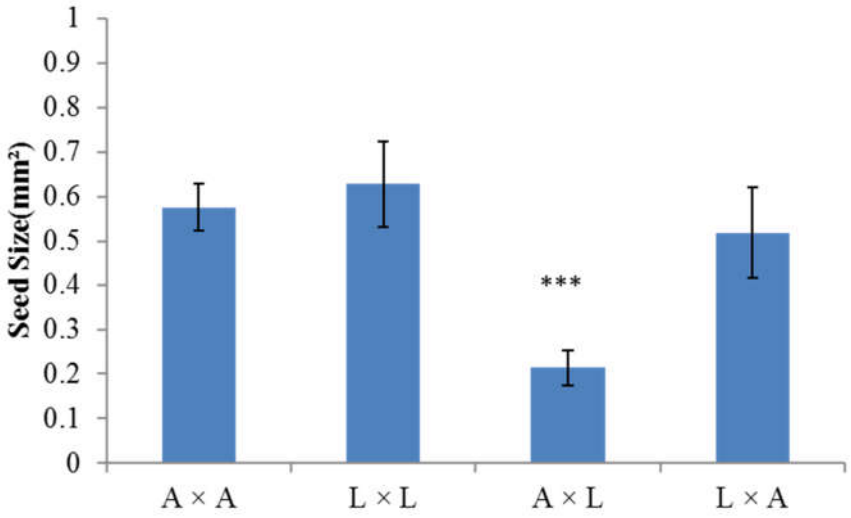
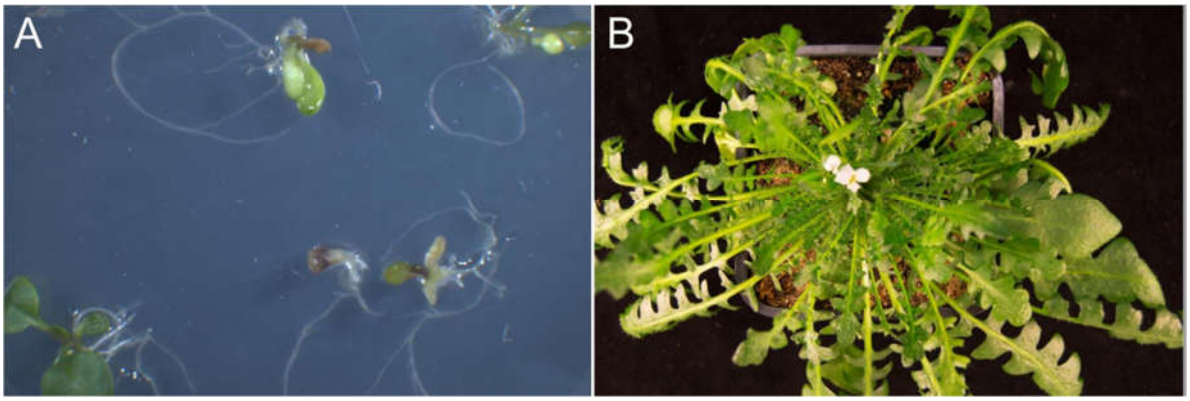
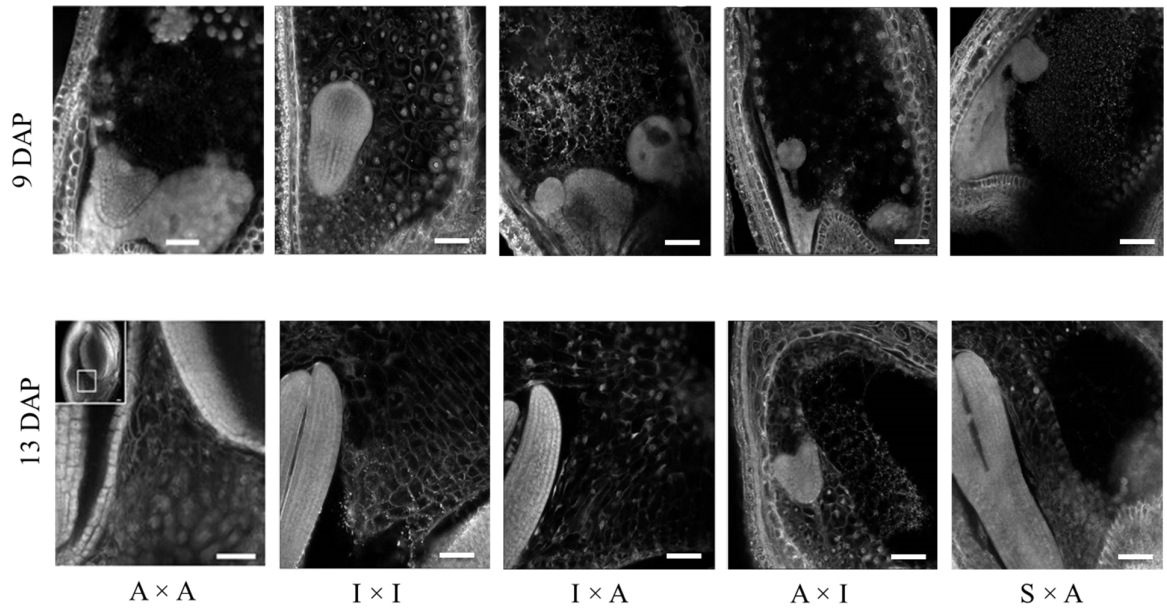
Supplementary Results



Supplementary Figure 1: **Seed size measurement of *A. arenosa* and *A. lyrata* parents and hybrids.** A= *A. arenosa,* L= *A. lyrata*. Significantly reduced seed size of *A. arenosa* × *A. lyrata* hybrid as compare to mid parent seed size is indicated by (***) and was determined by *t* test analysis (P≤ 0.001). Error bar indicates standard deviation.



Supplementary Figure 2: *A. lyrata* × *A. arenosa* hybrid seeds produced viable seedlings after using embryo rescue method. (A) Three weeks seedlings are in MS media, (B) Mature plant.



Supplementary Figure 3: Feulgen-stained parental and intra species endosperm. A= Austrian, I=Icelandic, S= Scottish *A. lyrata* species. Scale bar 50µm.

Supplementary Table 1: Distribution patterns of three incompatible loci that are responsible for hybrid incompatibility.

	Normal seeds(NS)	NS	NS	NS	NS	NS	NS	Impaired seeds
Loci Pattern	ABC	ABc	AbC	aBC	Abc	aBc	abC	abc
Theoritically expected %	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%