PHARMACOLOGICALLY INDUCED MEIOSIS APOMEIOSIS

INTERCONVERSIONS IN BOECHERA,

ARABIDOPSIS AND VIGNA

by

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ABSTRACT
Pharmacologically Induced Meiosis Apomeiosis Interconversions in Boechera, Arabidopsis and Vigna
by
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In flowering plants, apomixis is asexual reproduction by seed without meiosis or fertilization. Differing from sexual embryos, apomictic embryos do not contain a paternal contribution, thus, they are genetically identical to the maternal plant. This attribute provides a potential method of propagation in agriculture, especially in hybrid seed production to fix hybrid vigor over generations. Due to its rarity in major crops and to a lack of knowledge concerning its molecular regulation, harness this wonderful trait of apomixis for crop improvement remains a major challenge. In this study, I used differential interference contrast (DIC) microscopy to elucidate apomixis mechanisms in four wild Boechera taxa: two accessions of B. exilis, B. Inlahenaensis and B. yellowstonensis. All of these showed major attributes of sexual reproduction. In fact, except for possibly a few obligate sexual species, the taxa of Boechera are facultative, meaning that both sexual and apomictic reproduction coexist.

To further investigate molecular pathways of the apomixis/sex switch, we
performed pharmacological treatments to impose stress on three apomictic *Boechera* taxa: *B. cf. gunnisoniana*, *B. lignifera*, and *B. retrofracta x stricta*. The results indicated short-term stress (H$_2$O$_2$) as well as long-term stress (drought, sugar starvation and BRZ) induce shifts from apomeiosis to meiosis. The most effective timing of treatment application for inducing the shift was the 2-IV stage (Meiosis I) which is essential for sexual reproduction. On the other side, we performed pharmacological treatments to induce apomixis by disturbing the sexual status of three sexual plants: *B. stricta*, *Arabidopsis* and cowpea. All treatments, glucose, epiBL, DTBA and 5-azaC, were effective in inducing apomictic reproduction, especially 5-azaC, which suggests that disturbance of the epigenetic program responsible for sex allows apomixis to proceed in plants.

Overall, our experiments supported the hypothesis that apomixis and sex are polyphenisms of each other, i.e., that they coexist in plant genomes and can switch to one another by imposing or eliminating stress.

(168 pages)
Pharmacologically Induced Meiosis Apomeiosis Interconversions in *Boechera, Arabidopsis* and *Vigna*

Lei Gao

Apomixis is a clonal propagation method that produces offspring identical to the mother plant. With this feature, superior traits could be maintained over generations. However, our knowledge about apomixis is limited. In this study, we analyzed several apomictic *Boechera* embryologically to learn the details of apomixis. Meanwhile, we designed chemical treatments to successfully induce sex in apomictic plants and apomixis in sexual plants. Our experiments suggest that sex and apomixis coexist in plants and that sexual and apomictic reproduction are switchable by treating with specific chemicals.
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## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>57</td>
</tr>
<tr>
<td>5</td>
<td>62</td>
</tr>
<tr>
<td>6</td>
<td>106</td>
</tr>
<tr>
<td>7</td>
<td>120</td>
</tr>
</tbody>
</table>

1. Accession numbers and collection information for *Boechera* species.
2. Frequencies of sexual dyads, sexual tetrads and aposporous initials (AI) observed in appropriately-staged ovules of three *Boechera* taxa.
3. Average pistil length by germline development stage for pistils of four *Boechera* taxa.
4. Accession and herbarium numbers and other collection information for *Boechera* taxa evaluated herein.
5. Average pistil length by germline development stage for pistils of three *Boechera* taxa.
6. Average pistil length by ovule development stage for *B. stricta*, *Arabidopsis* and cowpea.
7. Numbers of cowpea ovules developing either sexually (dyads, tetrads, embryo sacs from tetrads) or by Antennaria type diplospory as affected by glucose as sole carbohydrate, ii) epiBL (epibrassinolide), iii) DTBA, (S-2-aminobutane-1,4-dithiol hydrochloride), iv) epiBL plus DTBA, or v) 5-azaC (5-azacytidine).
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Putative parentage of sexual diploid and apomictic interspecific hybrid <em>Boechera</em> studied herein</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>Microsporogenesis and megasporogenesis in the Utah <em>B. exilis</em> (a-d), <em>B. yellowstonensis</em> (e-h), <em>B. innahaensis</em> (i-l) and the Nevada <em>B. exilis</em> (m-n)</td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td>Reproduction in <em>B. cf. gunnisoniana</em> (a-d), <em>B. retrofracta x stricta</em> (e-h), <em>B. lignifera</em> (i, j), and <em>B. stricta</em> (k)</td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>Meiosis or apomeiosis stages of microsporogenesis associated with ovule developmental stages in <em>B. cf. gunnisoniana</em> (a, b), <em>B. retrofracta x stricta</em> (c, d), <em>B. lignifera</em> (e, f) and <em>B. stricta</em> (g, h)</td>
<td>45</td>
</tr>
<tr>
<td>5</td>
<td>Frequencies of <em>B. retrofracta x stricta</em> ovules that were undergoing sexual tetrad, sexual or diplosporous dyad, or aposporous embryo sac (AES) formation at the end of pistil culture as affected by <em>i</em>) decreasing culture media Ψ (from right to left) and <em>ii</em>) ovule stage at pistil culture initiation (1-I, 2-II, 2-IV)</td>
<td>65</td>
</tr>
<tr>
<td>6</td>
<td>Frequencies of <em>B. cf. gunnisoniana</em> ovules that were undergoing sexual tetrad formation at the end of pistil culture as affected by <em>i</em>) ovule stage at culture initiation (2-II, 2-III, 2-IV) and <em>ii</em>) decreasing culture media Ψ (from right to left)</td>
<td>66</td>
</tr>
<tr>
<td>7</td>
<td>Ovules of <em>B. gunnisoniana</em> (a-d), <em>B. lignifera</em> (e-h) and <em>B. retrofracta x stricta</em> (i-l) from the drought stress experiments</td>
<td>67</td>
</tr>
<tr>
<td>8</td>
<td>Frequencies of <em>B. retrofracta x stricta</em> ovules that were undergoing sexual tetrad, sexual or diplosporous dyad, or aposporous embryo sac (AES) formation at the end of the pistil culture treatment as affected by removal of sucrose from the culture medium</td>
<td>68</td>
</tr>
<tr>
<td>9</td>
<td>Frequencies of <em>B. cf. gunnisoniana</em> ovules that were undergoing sexual tetrad, sexual or diplosporous dyad, or aposporous embryo sac (AES) formation at the end of pistil culture as affected by <em>i</em>) removal of sucrose from culture media and <em>ii</em>) ovule stage at pistil culture initiation (2-III or 2-IV)</td>
<td>69</td>
</tr>
<tr>
<td>10</td>
<td>Ovules of <em>B. cf. gunnisoniana</em> (a-d) and <em>B. retrofracta x stricta</em> (e-h) from the sucrose starvation experiments</td>
<td>70</td>
</tr>
</tbody>
</table>
11 Frequencies of *B. retrofracta x stricta* ovules that were undergoing sexual tetrad, sexual or diplosporous dyad, or aposporous embryo sac formation at the end of the pistil culture treatment as affected by *i*) increasing concentrations of H$_2$O$_2$ in the pistil preculture treatment and *ii*) ovule stage at pistil culture initiation (1-I, 2-III and 2-IV) .........................................................71

12 Frequencies of *B. lignifera* ovules that were undergoing sexual tetrad, sexual or diplosporous dyad, or aposporous embryo sac formation at the end of the pistil culture treatment as affected by *i*) increasing concentrations of H$_2$O$_2$ in the pistil preculture treatment and *ii*) ovule stage at pistil culture initiation (2-III and 2-IV) .........................................................72

13 Frequencies of *B. cf. gunnisoniana* ovules that were undergoing sexual tetrad, sexual or diplosporous dyad or aposporous embryo sac (AES) formation at the end of pistil culture as affected by *i*) increasing H$_2$O$_2$ concentrations in the pistil preculture treatment solution and *ii*) ovule stage at pistil culture initiation (2-III and 2-IV) .........................................................73

14 Ovules of *B. cf. gunnisoniana* (a-d), *B. lignifera* (e-h) and *B. retrofracta x stricta* (i-l) treated with H$_2$O$_2$ ........................................................................................................74

15 Frequencies of *B. cf. gunnisoniana* ovules that were undergoing sexual tetrad, sexual or diplosporous dyad, or aposporous embryo sac formation at the end of pistil culture as affected by BRZ concentration ............................................75

16 Ovules of *B. cf. gunnisoniana* treated with BRZ .................................................76

17 Molecular pathway model for apomixis-to-sex switching in *Boechera* with locations in the pathway (1-4) that were tested herein ........................................78

18 Molecular pathway model for sex/apomixis switching in plants (5-8)............98

19 Frequencies of triploid *B. cf. gunnisoniana* ovules that were initiating Taraxacum-type diplospory, Polygonum-type sexual development, Antennaria-type diplospory, or Hieracium-type apospory at the end of pistil culture treatment as affected by a 5 min pre-culture soak in solutions containing 5-azacytidine (5-azaC) ........................................................................107

20 Ovules from pistils of *B. gunnisoniana* treated at the pre-MMC stage with 5-azaC as a pretreatment solution and then cultured on 5-azaC-containing tissue culture media for 48 h ................................................................................109

21 Frequencies of *B. stricta* ovules (dyads and tetrads) that were undergoing
dyad formation (sexual or diplosporous) at the end of pistil treatment as
affected by i) 3 h in vivo floral dip in solutions containing epibrassinolide
(epiBL) or ii) culturing of pistils in epiBL-containing media with in vitro
culture of pistils initiated when pistils were in the 1-I or the 2-III to 2-IV
stages.

22 Ovules of *B. stricta* treated with epiBL in vivo (a-c) and in vitro (d-f) ........111

23 Frequencies of *Arabidopsis* ovules undergoing tetrad, dyad, putative
aposporous embryo sac (PAES), and Antennaria type diplosporous
embryo sac (AntES) formation at the end of pistil culture as affected by i)
absence of 5-azacytidine (5-azaC) in the pretreatment solution and the
tissue culture medium (none), ii) 5-azaC in the pretreatment only (before),
or iii) 5-azaC in the pretreatment and culture medium (before and during). 115

24 Ovules of *Arabidopsis* treated with epiBL, DTBA, epiBL plus DTBA and
5-azaC in sucrose or glucose medium .................................................................116

25 Frequencies of *Arabidopsis* ovules undergoing tetrad, dyad, aposporous
embryo sac (PAES), and Antennaria type diplosporous embryo sac
(AntES) formation at the end of pistil culture as affected by epiBL,
epibrassinolide; DTBA, (S)-2-aminobutane-1,4-dithiol hydrochloride; BL
plus DTBA; or 5-azaC, 5-azacytidine .................................................................117

26 Frequencies of *B. stricta* ovules that were undergoing dyad formation at the
end of pistil culture as affected by i) energy source (glucose or sucrose)
and ii) five treatments ..........................................................................................118

27 Ovules of *B. stricta* treated with epiBL, DTBA, epiBL plus DTBA and 5-
azaC in sucrose or glucose medium ................................................................119

28 Ovules of cowpea treated with epiBL, DTBA, epiBL plus DTBA and 5-
azaC in either sucrose or glucose-containing medium .................................121

29 Parthenogenetic embryos and unreduced pollen microspores in *Arabidopsis*
pistils and anthers cultured in vitro and treated with DTBA and epiBL,
respectively ........................................................................................................122
CHAPTER 1

LITERATURE REVIEW

In eukaryote kingdoms, sexual reproduction is considered as an evolutionarily superior mode compared to asexual reproduction because of heterosis (Marshall & Brown, 1981). But many eukaryotes still reproduce asexually through their germlines (apomixis). In angiosperms (flowering plants), apomixis is defined as asexual reproduction by seed without meiosis or fertilization (Bicknell & Koltunow, 2004). Sexual embryos result from the union of male and female gametes often producing genetically varied offspring, which have advantages under natural selection. In contrast, apomictic embryos form without paternal contribution. Thus, progeny genetically identical to the mother plant are produced (Grossniklaus et al., 2001). Apomixis provides the opportunity to produce F₂ seeds that are genetically identical to F₁ hybrid seeds. In fact, any high-quality trait transferred into an apomictic plant, either by interspecific breeding or genetic engineering, will not be lost by sexual processes of genetic segregation (Moffat, 2001). Unfortunately, apomixis is rarely found in major crops although it has arisen seemingly independently in over 400 plant species of more than 40 families (Carman, 1997). The motivation of using apomixis to fix hybrid vigor in major crops has driven scientists to explore this potential ‘Holy Grail’ of agriculture nearly continuously for the past 70 years. However, even with intensive efforts, the mechanisms of apomixis are still poorly understood.
Types of apomixis in angiosperms

In general, there are two modes of apomixis that occur in plants: gametophytic and sporophytic (Asker & Jerling, 1992). In gametophytic apomixis, an unreduced (2n) gametophyte (embryo sac) forms from the megaspore mother cell (MMC) (diplospory) or from a neighboring somatic nucellar cell (apospory). Apomeiosis is the term used for processes of diplospory and apospory in which 2n embryo sacs with their 2n eggs form. Once the unreduced egg has formed, parthenogenesis occurs wherein the embryo forms from the unreduced egg without fertilization. In contrast, fertilization of the central cell (fusion product of two unreduced nuclei in the embryo sacs of most apomictic plants) may or may not be required for endosperm formation, i.e., endosperm formation in apomictic plants is either autonomous or pseudogamous, respectively. In sporophytic apomixis, one or more somatic cells from the nucellus or an adjacent integument initiate embryogenesis. The adventitious embryo uses for its growth and development nutrients from a sexually derived endosperm. This is referred to as adventitious embryony, and it is commonly observed in Citrus and Orchis (Koltunow, 1993).

Diplospory is divided into the following types based on different behaviors of the MMC: i) Taraxacum type, chromosomes in the MMC replicate, meiosis I fails (1st division restitution), meiosis II, which is mitotic-like, produces a dyad of genetically identical 2n megaspores, and the surviving megaspore undergoes three endomitoses to form an unreduced 8-nucleate embryo sac, ii) Ixeris type, chromosomes in the MMC
replicate, meiosis I fails (1st division restitution), karyokinesis of meiosis II (mitotic-like) occurs but cytokinesis does not, which produces a 2-nucleate megaspore, and the two nuclei of the single megaspore undergo two endomitoses to form an unreduced 8-nucleate mature embryo sac, iii) Antennaria type, the MMC develops directly into an unreduced 8-nucleate mature embryo sac, iv) Eragrostis type, the MMC develops directly into an unreduced 4-nucleate mature embryo sac avoiding meiosis completely, v) *Allium* type, two complete replications of chromosomes (instead of one replication) occur prior to meiosis, chromosome pairing does not occur, recombination occurs within the four replicated chromatids of each individual chromosome, with no genetic consequence, all chromosomes (each consisting of four chromatids) align independently on the metaphase I plate (rather than as pairs), chromatids separate during meiosis I such that each daughter cell contains a complete set of replicated chromosomes (two chromatids), meiosis II produces four spores each with two complete sets of chromosomes (unreduced). This mechanism is rare in angiosperms, except in *Allium odorum*, but is the most common form of apomixis in ferns (Döpp-Manton scheme of agamospory), where it is commonly followed by adventitious embryo formation from a somatic cell of the unreduced gametophyte (Mogie, 1992). Interestingly, 1st division restitution apomeiosis is common in angiosperms (Taraxacum type) and it is the most common form of apomeiosis in animals; and Allium type apomeiosis, which is rare in plants, is common in animals (Suomalainen et al, 1987).
In apospory, $2n$ embryo sacs arise from somatic cells of the nucellus while the MMC or its meiotic products degenerate. More than one cell in the nucellus may initiate aposporous development, but usually only one matures into an unreduced embryo sac. There are two main types of apospory: i) the Hieracium type, where the aposporous initial cell (AI) goes through three mitoses to form an unreduced 8-nucleate mature embryo sac, and ii) the Panicum type, where the AI goes through two mitoses to form an unreduced mature 4-nucleate embryo sac.

In both diplospory and apospory, fertilization of the central cell is usually necessary for endosperm to form (Khush, 1994), but autonomous endosperm formation (endosperm formation without fertilization) is also observed in some genera, especially in the Asteraceae (Carman, 1997). With autonomous endosperm formation, female plants become completely independent of the male gender with regard to seed formation.

**Boechera (Brassicaceae) as a model genus for studying apomixis**

Apomixis is widely distributed in all five eukaryote kingdoms, i.e., in Protista, Fungi, Animalia, Chromista and Plantae (Carman et al. 2011, Hojsgaard et al. 2014). Of the more than 400 species of angiosperms that reproduce apomictically, about 300 of them occur in Poaceae, Asteraceae or Rosaceae (Carman, 1997). Recently, *Boechera* (Brassicaceae) has been brought to the spotlight as a model genus for ecological and evolutionary genomics studies (Rushworth, 2011), as well as for the study of apomixis.
(Alexander et al., 2013). *Boechera* contains 110 species distributed mainly in North America, Greenland and the Far East of Russia (Al-Shehbaz and Windham, 2010). The relatively concentrated habitats and tolerance of inbreeding make the gene pool of *Boechera* relatively stable. Among the species, 71 are sexual and 38 are apomictic (Al-Shehbaz and Windham, 2006, 2007a, 2007b), including both diplospory and apospory (Carman et al., 2015). Naturally occurring diploid apomicts allow for the study of gene expression between sexuals and apomicts without the confounding effects of polyploidy (Sharbel et al., 2010).

In 1951, Tyge W. Bőcher observed and documented stress-induced shifts in *Boechera* from apomeiosis to meiosis in male meiocytes, and he suggested and presented limited evidence that the same shift may be occurring in female meiocytes as well. Recently Mateo de Arias (2015), from our lab, demonstrated that drought and heat stress do cause a shift from apomeiosis to meiosis in female meiocytes (MMC) when plants are stressed throughout the flower-development season. RNASEq studies conducted using RNA extracted from Dr. Mateo de Arias’ immature pistils taken from drought-stressed and well-watered sexual and apomictic *Boechera* show that this stress-induced switching includes global epigenetics-based changes in gene expression (Schilling, 2016). Gene ontology analyses of these gene expression differences in our lab indicate that oxidative stress induces meiosis to occur instead of apomeiosis in apomictic *Boechera*. Apomictic plants in general appear to maximize their fecundity through early flowering and seed set (Asker and Jerling, 1992). These attributes may make apomicts more competitive than
their related sexuals and partially explain their more aggressive establishment of previously glaciated areas (Carman et al., 2016).

*Boechera* is closely related to the model plant *Arabidopsis thaliana*, which provides tremendous tools for studying *Boechera*. In 2013, *Boechera stricta* (sexual diploid) was fully sequenced by the Joint Genome Institute ([http://phytozome.jgi.doe.gov/](http://phytozome.jgi.doe.gov/)). This sequence provided the scaffold for alignment our RNASEq reads (Schilling, 2016).

**Methods used to study apomixis**

*Embryological analyses* – Angiosperm embryology is the study of germline development in flowers. Thus, it includes a study of ovule and ovary formation and their development into seeds and fruit, respectively. Types of apomixis, as described above, and more thoroughly in Nogler (1984) and Asker & Jerling (1992), are studied cytologically in our laboratory using differential interference contrast (DIC) microscopy of cleared pistils (Crane and Carman, 1987).

*Single seed flow cytometry (SSFC)* – Apomixis in angiosperms involves three main processes: i) apomeiosis, embryo sac formation in the absence of meiosis and includes formation of an unreduced egg, ii) parthenogenesis, the development of an embryo independent of fertilization, either diplosporously or aposporously, and iii) endosperm formation with or without fertilization by sperm (Bicknell & Koltunow, 2004). The cellular maternal to paternal genome ratio contributions (M: P) in embryos and in
endosperm of seed are hard to determine cytologically, i.e., by mitotic chromosome squashes, but these ratios can be determined by flow cytometry, which measures the amounts of DNA (C values) in cells of embryos and endosperm (Matzk et al., 2000). The C value depends on whether the egg (becomes the embryo) and central cell (becomes the endosperm) was reduced or unreduced and whether these cells are fertilized by reduced or unreduced sperm or not fertilized at all (completely autonomous). For example, sexual reproduction almost always leads to a 2C:3C embryo to endosperm genome ratio in angiosperms where the embryo is formed by a reduced egg (n) fertilized by a reduced sperm (n) and the endosperm is formed by the 2n fusion product of the two 1n polar bodies of the embryo sac (central cell) being fertilized by the second reduced 1n sperm. In apomictic reproduction, the embryo is formed from an unreduced egg (2n) without fertilization, and the endosperm is formed by the two unreduced polar bodies (2n) fusing together to produce a 4C central cell with or without fertilization by a reduced or unreduced sperm (n). Thus, instead of the 2C:3C ratio seen in sexual seeds, we usually see in Taraxacum-type diplosporous apomicts a 2C:4C, 2C:5C or 2C:6C ratio (Scott et al., 2004). In Boechera, pseudogamously-derived endosperm (central cell fertilized by sperm) is more common than autonomous endosperm (without fertilization) (Aliyu et al., 2010), and both reduced and unreduced pollen commonly participate in the formation of endosperm during apomictic seed production.

We perform SSFC in our lab to test the ploidy level of the embryo and endosperm of individual Boechera seeds. The number of embryo nuclei is much larger than the number
of endosperm nuclei in *Boechera* seed. In many cases, there are too few endosperm nuclei to get an accurate endosperm peak, especially with older seeds (Mateo de Arias, 2015).

*Gene expression profiling* – Although apomixis has been observed and documented morphologically by embryological studies, the genetic mechanism of apomixis is still poorly understood. In order to understand global gene expression levels between sexual and apomictic plants during a specific development stage (early germline development), gene expression profiling, i.e. microarray and next-generation sequencing, has been done in the Carman lab and in other labs. In *Poa pratensis*, the cDNA-AFLP method of mRNA profiling was performed between sexual and apomictic genotypes during flowering (Albertini et al., 2004), and 179 out of 2248 transcript fragments were differentially expressed. Three candidate genes (*PpRAB1*, *PpARM* and *PpAPK*) involving signal transduction were selected for future study. In *Pennisetum squamulatum*, two transcriptomes from ovules of *P. squamulatum* and its backcross line were compared at the stage of apospory initiation by 454 sequencing (Zeng et al., 2011), and 49 transcripts were verified to occur in the Apospory-Specific Genomic Region (ASGR). Most of them were related to reproductive tissue. A large-scale EST/cDNA expression analysis was conducted between sexual and apomictic *Eragrostis curvula*, including across-ploidy levels (Cervigni et al., 2008), and 8864 unigenes were obtained, which fell into six major categories: catalytic activity, nucleotide binding, protein metabolism, binding, transport and energy pathways. Another differential display expression was investigated at the
meiotic stage between sexual and apomictic tetraploid *Paspalum notatum* (Laspina et al., 2008), and 65 out of 94 highly differentially expressed sequences were selected as candidate unigenes. Among them, eight were related to an extracellular receptor kinase (ERK) signal transduction cascade, and others were involved in a variety of central cellular processes like cell-cycle control, protein turnover, intercellular signaling, transposon activity, transcriptional regulation and endoplasmic reticulum-mediated biosynthesis. Sharbel et al. (2009, 2010) compared gene expression between diploid sexual and apomictic *Boechera* across four development stages by 454 sequencing. Only 20 ovules per sample were dissected for testing, and 543 differentially expressed genes were identified. A global down-regulation of gene expression was reported in the apomict compared to the sexual at the MMC stage. Silveira et al. (2012) developed EST libraries made from ovaries of sexual and apomictic *Brachiaria brizantha*, and the macroarray analysis showed 11 sequences to be differentially expressed. *BbrizSti1* showed similarity to a gene encoding a stress-induced protein and *BbrizHelic* showed similarity to a gene encoding a helicase. Both were expressed in the early stage of apomictic ovary development suggesting that stress-related genes are important for apomixis. These several studies have revealed that signaling transduction and stress response play an important role in apomixis genetics. In a digital gene expression tag profiling experiment involving *Zea mays* mutants (Singh et al., 2011), AGO104, which is related to AGO9 (ARGONAUTE9) in *Arabidopsis thaliana*, was identified and shown to modify chromatin through DNA methylation. The *ago104* mutants showed a meiosis failure like
diplosporous apomixis. Thus, we could assume that epigenetic regulation also plays an important role regarding apomixis.

In our lab, we performed gene differential expression analysis by microarray between several sexual and apomictic species of *Boechera* at two different stages: i) meiosis/apomeiosis and ii) embryo sac formation/parthenogenesis. Genes from ovules of *B. formosa* (sexual), *B. lignifera* (apomictic) and *B. microphylla* (apomictic) were compared across stages, and additional comparisons were made using pistils of *B. stricta* (sexual) and *B. lignifera* (apomictic). Overall, 5305 genes were differentially expressed between sexual and apomictic plants, and a global up-regulation of genes in sexual plants (or down-regulated in apomictic plants) in both ovules and pistils was observed. Further, gene ontology (GO) was analyzed by GOEAST (Zheng & Wang, 2008) and showed 1123 enriched GO categories including response to stress, oxidoreductase activity, signal transducer activity and gene silencing by miRNA. Our results were consistent with recent studies from other labs, which indicated that genes related to stress response, signaling transduction and epigenetic regulation have significant effects on apomixis (manuscripts in preparation).

*Plant Tissue Culture* – Plant tissue culture is used to grow plant tissues or cells under aseptic and controlled environment conditions (El-Missiry et al., 2012). This technique was established in 1939 by Philip R. White, and it developed rapidly with the use of plant nutrient solution, e.g., Murashige and Skoog (1962) MS medium. Tissue culture is used widely and intensively for both commercial and scientific studies (Gautam et al., 2014).
Because the whole system is under a controlled environment, people investigate a single factor that interacts with the cultured tissue without the interference of other variables. Many experiments were conducted by means of tissue culture to study plant responses to environmental stimuli and stress physiology (Cassells and Curry, 2001; Gaspar et al., 2002; Sivritepe et al., 2008; Cui et al., 2010; Shehab et al., 2010). As we consider apomixis to be regulated by stress signals, tissue culture could be used to study apomixis. Adventitious embryogenesis was induced in *Vitis vinifera* L. (Mullins et al., 1976), *Malus púmila* (James et al., 1984), *Paspalum dilatatum* (Akashi & Adachi, 1992), and many others (Carman, 1990). Quarin et al. (2001) regenerated a *Paspalum notatum* tetraploid plant that expressed apomixis by treating a tissue culture of a diploid sexual plant with colchicine. A series of stress treatments (colchicine, water deficit and tissue culture) of *Eragrostis curvula*, an apomictic perennial grass, caused a shift in reproduction from apomictic to sexual, which indicated that stress can temporarily activate sexuality in apomictic genotypes of this grass (Echenique et al., 2014; Rodrigo et al., 2015).

Based on experiments conducted in our lab (Mateo de Arias, 2015), it was shown that apomictic *Boechera* when stressed will revert to sexuality. In her studies, Mateo de Arias exposed apomictic and sexual plants to various drought and heat stresses continuously from the end of vernalization until seed set. Hence, we do not know at what stage stress causes reversion from apomeiosis to meiosis. A major objective of my study was to evaluate timing and strength of stress on reversion from apomeiosis to meiosis. In preparation for this, I conducted preliminary studies which I stressed cultured pistils of *B.*
*lignifera* by exposing them to \( \text{H}_2\text{O}_2 \), and an increased frequency of tetrads (sexual ovules) in the pistils showed that reactive oxygen species (ROS) could induce sexual reproduction in the apomictic species. My preliminary experiments also involved heat and other stresses and the application of 5-azacytidine to evaluate the putative role of epigenetic regulation (methylation) on the conversion from apomeiosis to meiosis.

*Environmental effects on apomixis facultativeness*

Obligate sexual plants are common in nature, but obligate apomictic plants are thought to be rare because most apomictic plants show some facultative sexuality. Perhaps all apomictic plants occasionally reproduce sexually (Asker and Jerling, 1992). Facultative apomixis is defined as both sexual and apomictic reproduction occurred simultaneously in the same plant. The degree of sexuality, or the ratio of sexual/apomictic seed during seed set, is not fixed. It can be altered by both genetic (Barcaccia et al., 2006; van Dijk, 2003) and environmental factors (Böcher, 1951; Barcaccia et al., 1997b; Mazzucato et al., 1997; Carman et al., 2011).

As discussed above, heat and drought induced a major switch to meiosis from apomeiosis in *Boechera*. However, SSFC showed that the sexual ovules were sterile, i.e., almost all of the seeds that formed were of apomictic origin (Mateo de Arias, 2015). This is explained by the triploid or otherwise wide-hybrid origin of the apomicts. Balanced gamete formation by meiosis in triploids and species hybrids can be extremely rare.
Plant hormone effects on apomixis

Brassinosteroids (BRs) are defined as the sixth plant hormone that contribute to cell elongation and division (Fridman & Savaldi-Goldstein, 2013; Gudesblat & Russinova, 2011; Bajguz, 2007), vascular differentiation (Zhang et al., 2014), reproductive determination (Gerashchenkov & Rozhnova, 2013; Hartwig et al., 2011) and regulation of gene expression (Hou et al., 2017; Wang et al., 2014; Guo et al., 2013). A well-studied BR signaling model in *Arabidopsis* indicated that BR activates transcription factors such as Brassinazole Resistant 1 (BZR1), which subsequently regulates the expression of more than one thousand downstream genes that lead to plant growth (Wang et al., 2012).

Interestingly, an important BR receptor, SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3), which is one of five SERK genes in *Arabidopsis*, is reported to be involved in forming of aposporous initial cells (AI) (Podio et al., 2014; Albertini et al., 2005). In our RNASeq data, 85 enriched GO categories involve responses to BR stimuli and were up regulated in apomicts, which suggests that BR may be involved in plant stress defense possibly to eliminate stress. In agricultural practice, exogenous BR have been applied to assist plants in dealing with stress (Kagale et al., 2007; Li et al., 2013; Xia et al., 2009). In our view, the elimination of stress responses may switch sex to apomixis. For example, Rojek et al., 2015 reported exogenous steroid hormones (including BR) stimulate central cell divisions and fertilization-independent (autonomous) endosperm development in *Arabidopsis*, and Kitani (1994) reported
haploid parthenogenesis by applying BR to the stigma of emasculated flower buds of

*Arabidopsis*.

Apomixis once harnessed for crop improvement could initiate another green revolution by permanently fixing hybrid vigor in high-yielding hybrid plants. In our studies, apomixis in *Boechera* was investigated by microarray and by RNA-Seq to screen for candidate genes and to further verify them. I hope that my tissue culture experiments, as described herein, will advance our understanding of the genetics and epigenetics of apomixis and bring the goal of apomictic crops several steps closer.

**OBJECTIVES AND SIGNIFICANCE**

My research involved studying wild species of plants that have not been domesticated, many of which have not been characterized for mode of reproduction. Hence, I first determined mode of reproduction for several plants that I participated in collecting (Chapter 2). In Dr. Carman’s lab, we have used *Arabidopsis* microarray technology to identify genes up and down regulated in pistils and ovules of apomictic and sexual *Boechera*. Recently, our lab obtained a much more complete transcriptome profiling of pistils from sexual and apomictic plants using RNA-Seq (Schilling, 2016). I have used plant tissue culture, of immature cultured inflorescences, to test timing and strength of in-vitro-applied stress treatments and specific in-vitro-applied pharmacological treatments, which were designed to disrupt specific components of the stress-induced signal transduction pathway, on the apomeiosis to meiosis switch (Chapter
3). If a model *in vitro* system can be identified through these studies, which consistently regulates the apomixis to sex switch, it might be possible to reverse the process and induce apomixis in sexual plants (Chapter 4). These studies were designed based on our previous gene expression studies (reviewed above) and on our previous stress experimentation (reviewed above). Collectively, these studies led us to develop a model for the molecular and biochemical regulation of the apomixis sex switch (Chapter 3, Fig. 3.13; Chapter 4, Fig. 4.1), and my research was designed to test various aspects of this model (Chapters 3-4).

My specific objectives were as follows:

- Identify and study the embryology of several wild species of *Boechera* and document the degree of facultativeness in each species; establish a general staging system for these species where pistil length, a sporophytic parameter, is correlated with germline development stage (Chapter 2)
- Determine the timing and strength of chemically-induced stresses necessary to induce an apomeiosis to meiosis switch; determine if a putative stress-defense buffering system exists in apomictic *Boechera*, which buffers against low levels of stress but induces a reversion to sex when a stress threshold is exceeded (Chapter 3)
- Determine whether epigenetic regulation is extensively involved in the apomeiosis to meiosis conversion or *vice versa* (as suggested by our RNA-Seq data) and evaluate whether extensive epigenome modifications are an
elementary or fundamental component of the sex apomixis switch in

*Boechera* (Chapter 4)

The ultimate goal of my apomixis research, and many apomixis research scientists around the world, is to engineer a switchable version of this ‘holy grail of agriculture’ into major crops. Apomixis, especially a switchable version, would allow breeders to improve crops sexually and then perpetuate their superior-yielding hybrids clonally through apomixis-produced seed. Such a system would greatly reduce hybrid seed production costs, and this would enable crops generally grown today as inbred varieties, including wheat and most rice, to be converted to superior-yielding hybrids.

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CHAPTER 2

SEXUAL REPRODUCTION AND GAMETOPHYTIC APOMIXIS IN SEVERAL

BOECHERA TAXA

ABSTRACT

• **Background and Aims** Boechera (Brassicaceae) is an ideal genus to study apomixis due to the existence of diploid apomicts in this genus. The embryology of reproductive mode, the degree of facultativeness and the germline developmental stage are important for studying apomixis. The aims of this study, were to identify the reproductive mode of several wild Boechera species and document their level of facultativeness.

• **Methods** Pistil length was used as a sporophytic parameter to correlate with germline developmental stages in several sexual and apomictic Boechera taxa. Pistils were cleared and studied using differential interference contrast (DIC) microscope.

• **Key Results** High frequency meiosis and Polygonum type embryo sac formation was observed in pistils of two *B. exilis* accessions, *B. immahaensis* and *B. yellowstonensis*. Low frequency Taraxacum type diplospory and Hieracium type apospory were also observed. Additionally, correlations between pistil length and megasporogenesis and microsporogenesis for *B. cf. gunnisoniana*, *B. lignifera*, *B. retrofracta x stricta* and *B. stricta* were obtained.
• Conclusions Except for possibly a few obligate sexual species, the taxa of Boechera are facultative, meaning they to some degree are both sexual and apomictic. The coexistence of different types of apomixis as observed herein suggest that the responsible mechanisms that regulate each type of apomixis may be closely related. The complexity of speciation and diversity of reproduction make Boechera a unique model for studying the mechanisms of apomixis.

Key words: apomixis; diplospory; apospory; Boechera; facultative apomixis
INTRODUCTION

*Boechera* is a genus of Brassicaceae. It contains 110 species, which are distributed mainly in North America, Greenland and the Far East of Russia. Most members of the genus are perennial plants that have pubescent leaves, stellate trichomes, narrow curving fruits, and small white to purple flowers in elongated racemes (Al-Shehbaz and Windham, 2010). In North America, *Boechera* grows in environments ranging from deserts to moist alpine meadows of the Western United States (Rushworth et al., 2011). Although most *Boechera* species are self-pollinating, their relatively close geographic distribution permits hybridization between two or even three species (Al-Shehbaz and Windham, 2010).

High levels of cross hybridization makes this genus taxonomically complex (Fig. 2.1). Both polyploidy and apomixis are very common in this genus, the latter of which provides an escape from interspecific hybrid sterility. Among *Boechera* species, 71 are thought to be sexual and 38 are thought to be apomictic (Alexander et al., 2013, Kantama et al., 2007). Both diplospory (unreduced embryo sac formation from an ameiotic megasporocyte) and apospory (meiocyte degeneration accompanied by unreduced embryo sac formation from an adjacent nucellar cell) have been reported in *Boechera* (Carman, 2007, Carman et al., 2015). Diploid (2n = 2x = 14) and triploid (2n = 3x = 21) apomicts as well as diploid sexuals are found in *Boechera*. These provide good opportunities for researchers to study apomixis without the confounding effects of
polyploidy (Sharbel et al., 2010). Besides, *Boechera* is closely related to the model plant *Arabidopsis thaliana* (Brassicaceae) \(2n = 2x = 10\). *Arabidopsis*, *Arabis* and *Boechera* diverged 19-25 million years ago. Nevertheless, sequence similarities are close enough to permit usage of many *Arabidopsis*-based genomics tools with *Boechera* (Koch et al., 2001, Mitchell-Olds, 2001, Schranz et al., 2005).

Because many apomictic *Boechera* are diploid, many researchers use *Boechera* as a model to study apomixis. Many of these researchers have suggested that hybridization may cause apomixis in *Boechera* (Roy, 1995, Beck et al., 2012, Dobes et al).
al., 2004a, Dobes et al., 2004b, Dobes et al., 2007, Kiefer et al., 2009b, Kiefer et al.,
2009a, Kiefer and Koch, 2012). However, hybrids have been artificially produced among
several sexual Boechera collected from the Western United States that did not express
apomixis, which suggests that hybridization alone, at least when performed artificially, is
not an efficient way of producing apomicts (Schranz et al., 2005). Boechera stricta
(diploid sexual), B. retrofracta (diploid apomict), B. polyantha (diploid apomict or
facultative apomict) and B. pendulocarpa (diploid sexual or facultative apomict) and their
naturally-occurring interspecific hybrids were studied, and high levels of heterozygosity
were observed in triploid apomicts, but heterozygosity was not high in diploid apomicts,
which suggests that intraspecific, not interspecific, hybridization is the cause of apomixis
in the diploid apomicts (Lovell et al., 2013).

Due to the comprehensive hybridization patterns of the 110 plus Boechera species
in North America, the taxonomic complexity of Boechera is extreme. Except for obligate
sexual species, the taxa are facultative, meaning they to some degree are both sexual and
apomictic. The ratio of sexual to apomictic embryo sac development in the ovules of each
pistil is not constant. Abiotic stress, such as drought or heat, can induce megaspore
mother cells (MMC) in ovules of apomictic plants to undergo meiosis and produce
genetically-reduced (sexual) embryo sacs (Carman et al., 2011). Stress-induced shifts in
Boechera from apomeiosis to meiosis in male meiocytes (Bocher, 1951). In our lab,
drought and heat-stressed B. lignifera and B. cf. gunnisoniana (triploid apomict involving
diploid apomictic B. cf. gunnisoniana) shifted apomeiosis to meiosis in MMC (Mateo de
Arias, 2015). The frequency of sexual to apomictic embryo sac formation is different in each facultative Boechera species, and it likely depends to some extent on the environmental conditions during which the pistils were collected. In apomixis research aimed at understanding signals that induce sex or apomixis, documentation of the degree of facultativeness of the experimental material under specific environmental conditions is very important.

Through embryological analyses, it is possible to document the stage of ovule development in which meiosis or apomeiosis, embryo sac formation, and embryo and endosperm formation occur, and these stages can be correlated with pistil length (Schneitz et al., 1995). My research includes efforts to induce sexual development in ovules of apomictic plants and apomictic development in ovules of sexual plants (Chapters 3, 4). Hence, it was important to document when apomeiosis occurs in apomicts and when meiosis occurs in sexuals. In the present chapter, I provide embryological details of reproduction for putatively sexual collections of B. immahaensis and B. yellowstonensis, which are related to apomictic B. microphylla (Carman, 2007; Mateo de Arias, 2015), for putatively sexual B. exilis and B. stricta, and for putatively apomictic B. cf. gunnisoniana, B. lignifera, and B. retrofracta x stricta.
MATERIALS AND METHODS

Plant materials

Plant materials included putatively sexual *B. yellowstonensis*, *B. imnahaensis*, and two accessions of *B. exilis*, which were collected from the western United States as live specimens (Table 2.1). Voucher specimens were prepared and deposited in the Intermountain Herbarium, Utah State University. Seeds of these plants were germinated on moist germination paper in petri dishes at 26 °C for 1-2 weeks and planted in pots containing Sunshine Mix #1 potting soil (Sun Gro Horticulture Canada Ltd, Vancouver, BC). At least 36 plants of each species were grown for 3-4 weeks at 24 °C (8-10 leaves) until they were strong enough for vernalization. Seedlings were vernalized at 4 °C for 10-12 weeks with minimal lighting (8/16 day/night photoperiod) from soft-white fluorescent bulbs. The vernalized plants were then transferred to a walk-in growth chamber set for 22/16 °C day/night temperatures with soft-white fluorescent lighting (16/8 day/night photoperiod) supplemented by light from 1000 W high-pressure sodium-vapor lamps. The plants were watered regularly with a dilute solution (250 mg L⁻¹) of Peters Professional 20:20:20 fertilizer (Scotts, Maryville, Ohio).
**Embryological analyses**

Clusters of floral buds at the pre-anthesis stage were fixed in formalin acetic acid alcohol (FAA) for 48 h. The buds were then cleared in 2:1 benzyl benzoate to dibutyl phthalate (BBDP) (Crane and Carman, 1987) as follows: 70 % EtOH, 30 min; 95 % EtOH, 4 h; 2:1 95% EtOH to BBDP, 2 h; 1:2 95% EtOH to BBDP, 4 h; 100 % BBDP, 4 h;
and 100% BBDP overnight. Pistils were then dissected from the floral buds. Pistil lengths, measured from the base of the pedicel to the top of the stigma (± 0.05 mm), were then obtained using a dissection microscope, and the pistils were mounted on slides with a minimal amount of BBDP clearing solution. The developmental stages of each ovule in each pistil was studied using a BX53 microscope (Olympus, Center Valley, PA, USA) equipped with differential interference contrast (DIC) optics. Photographs of ovule development were taken using a MicroFire 599809 camera (Olympus). Ovules were scored for developmental stage, e.g., MMC, meiotic or diplosporous dyad, sexual tetrad, aposporous initial (AI), 2, 4 and 8-nucleate embryo sac (ES2, 4, 8), mature embryo sac (MES), and aposporous embryo sac (AES). For each pistil, the pistil length and the developmental stage of the majority of ovules in the pistil was recorded as the pistil stage. Pistil length was determined by measuring the distance from the base of the pedicel to the top of the stigma.

RESULTS AND DISCUSSION

Sexual Boechera

High frequency sexual megasporogenesis (female meiosis) and megagametophyte (embryo sac) formation was observed in pistils of two B. exilis accessions, B. imnahaensis and B. yellowstonensis (Table 2.2, Fig. 2.2). In each MMC of these sexual ovules, pairs of replicated homologous chromosomes separated during meiosis I
(reduction division) to produce dyads of megaspores, and the sister chromatids of each dyad member separated during meiosis II (equational division) to produce tetrads of genetically-reduced \((n)\) spores. The three spores closest to the micropyle degenerated.

The chalazal spore \((n)\) survived and underwent three endomitoses to form a genetically-reduced \((1n)\) 8-nucleate Polygonum-type embryo sac. Because of the small amount of endosperm in mature Boechera seeds, the detection of embryo-to-endosperm genome ratios by single seed flow cytometry was only rarely successful in our lab (Mateo de Arias, 2015). However, observations of consistent meiotic reductions on both the male and female sides suggest that the embryo-to-endosperm genome ratio was primarily 2C:3C (Fig. 2.2a, e, i, m). Collectively, these observations suggest that the two \(B. \ exilis\) accessions, \(B. \ yellowstonensis\) and the \(B. \ innahaensis\) studied herein (Table 2.1) are

<table>
<thead>
<tr>
<th>Species</th>
<th>Scored no.</th>
<th>Dyads</th>
<th>Tetrads</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td>(B. \ yellowstonensis)</td>
<td>208</td>
<td>5</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td>(B. \ innahaensis)</td>
<td>210</td>
<td>5</td>
<td>92</td>
<td>3</td>
</tr>
<tr>
<td>(B. \ exilis) (Nevada)</td>
<td>148</td>
<td>7</td>
<td>91</td>
<td>2</td>
</tr>
<tr>
<td>(B. \ exilis) (Utah)</td>
<td>39</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
sexual. Nevertheless, I occasionally observed aposporous initials, which suggests that low frequency apospory might occur (Table 2.2, Fig. 2.2), but aposporous embryo sacs (2-nucleate or greater with pronounced vacuolation) were not observed in these taxa.
Diplospory, apospory and sexual reproduction occurring together in the same plant is rare. It has been observed rarely in *B. microphylla*, interspecific hybrids of diploid sexual *Tripsacum*, interspecific hybrids of diploid sexual *Antennaria*, intraspecific hybrids of diploid sexual *Sorghum* and in a few other taxa (Carman, 2007).

The type of diplospory commonly observed in *Boechera* is the Taraxacum type (Fig 2.2c, g, k) wherein the reduction division (meiosis I) fails, a restitution nucleus forms, and meiosis II (mitotic like) occurs resulting in a dyad of unreduced (2n) spores. The micropylar spore degenerates, but the chalazal spore undergoes three mitoses to form an unreduced (2n) 8-nucleate embryo sac. In *Boechera*, Taraxacum type diplospory occurs in *B. holboellii* (Bocher, 1951), *B. cf. gunnisoniana* (Taskin et al., 2004), *B. divaricarpa* (Schranz et al., 2006), *B. retrofracta* (Sharbel et al., 2010), *B. lignifera* (Windham et al., 2015), *B. exilis x retrofracta*, *B. lignifera*, *B. retrofracta x stricta* (Mateo de Arias, 2015), and *B. duchesnensis* and interspecific triploid hybrids involving *B. formosa*, *B. lincolnensis* and *B. stricta* (Carman et al., 2015).

In *B. exilis*, *B. yellowstonensis* and *B. imnahaensis* (Fig. 2.2d, h, l), the type of apospory that occasionally initiates, but seldom if ever advances to seed formation, is likely the Hieracium type, which has previously been observed in *B. microphylla* (Carman, 2007, Mateo de Arias, 2015). This form occurs in many genera of the Asteraceae, Poaceae and Rosaceae (Carman, 1997). Though I did not observe evidence of these aposporous initials advancing into embryo sacs in the taxa I studied, other studies in our lab suggest that apospory in *Boechera* may be widespread (Carman et al., 2015).
The mechanism of facultative apomixis, i.e., why development can switch between sex and apomixis, is still poorly understood. It has been suggested that apomixis in eukaryotes is an ancient alternative to sex and has been retained in many eukaryotic taxa where epigenetic or metabolic disturbances, such as wide hybridization and polyploidy, occasionally induce its expression (Carman et al., 2011, Hojsgaard et al., 2014).

According to the Boechera microsatellite website (Li et al., 2017), many B. microphylla accessions found in Utah, including those sampled by Carman (2007) and Mateo de Arias (2015), are tri-parental diploid hybrids involving B. imnahaensis, B. yellowstonensis and B. pendulina. That B. imnahaensis and B. yellowstonensis are sexual (Table 2.2) provides additional rationale for their species status and for separating them from the highly apomictic tri-parental hybrids that occupy more southern habitats. If B. microphylla is retained as representing an apomictic species of interspecific hybrid origin, then B. yellowstonensis and B. imnahaensis are appropriate names for the two sexual accessions studied herein. Alternatively, these two taxa might be synonymized as different subspecies of one or the other. Additional taxonomic evaluations are required to determine how best to treat them.

Recently, a hybrid between B. exilis and B. retrofracta was found to be highly diplosporous (Mateo de Arias, 2015). My studies indicate that the parents of this hybrid are sexual (Table 2.2). This is another example of hybridity-induced apomixis, and it is consistent with the hypothesis that a capacity for apomixis may reside silently in
angiosperms that is epigenetically triggered by hybridization (Hojsgaard et al., 2014, Carman et al., 2011).

**Timeline of male and female gametophyte development**

To describe the temporal details of ovule development, the staging system of *Arabidopsis thaliana* (Schneitz et al., 1995), a close relative of *Boechera*, was used for three apomictic *Boechera*, *B. cf. gunnisoniana*, *B. lignifera*, and *B. retrofracta x stricta*, and one sexual *Boechera*, *B. stricta*. This ovule development timeline can be divided into four stages. Stage 1 is the early stage of ovule development or pre-MMC stage. It begins with active mitotic divisions that result in ovules protruding from the ovary placenta, and it ends with the mitotic division in the ovule nucellus that generates the MMC. Stage 2 covers megasporogenesis, from the early MMC stage to tetrad formation. This stage is also called the meiosis stage. Stage 3 is the embryo sac development stage. It starts with the onset of degeneration of the three non-surviving spores and the expansion of the surviving spore in preparation for embryo sac formation (functional megaspore stage). The functional megaspore stage ends with the first of three endomitotic divisions to produce the 2-nucleate embryo sac, which acquires prominent vacuoles. The second endomitotic division then occurs to produce the 4-nucleate embryo sac, and the third endomitotic division occurs to produce the 8-nucleate embryo sac. Embryo sac maturation involves cellularization and differentiation of the eight embryo sac nuclei into
the egg apparatus (egg and two synergids), the central cell (two polar nuclei that fuse) and the antipodals (three cells that are generally located along the sides or at the chalazal end of the embryo sac. Stage 4 is the syngamy stage where double fertilization occurs. This stage encompasses early endosperm formation and early embryony.

Each stage is further divided into several shorter stages (marked by Roman numerals). For example, 2-IV and 2-V represent meiosis I and meiosis II, respectively. These are the best stages for identifying reproductive mode when diplospory is expected. In my study, I adjusted the *Arabidopsis* staging system to accommodate *Boechera*. Because I was interested in both apomeiosis (Stage 2) and parthenogenesis (Stage 4), I chose the following stages for extensive evaluations: i) 1-II, pre-MMC stage, ii) 2-IV and 2-V, meiosis stage, iii) 3-II and 3-III, 2-nucleate embryo sac stage (with vacuole), and iv) 4-VI and later, parthenogenesis stage.

In previous studies, bud and pistil lengths have been found to be highly correlated with stage of ovule development (Kandeler and Hugel, 1974, Hugel, 1976, Peterson et al., 1992, Erickson and Markhart, 2002, Taskin et al., 2004) (Salinas-Gamboa et al., 2016). Hence, I used pistil length as a sporophytic parameter to identify pistil lengths that are correlated with specific stages of megasporogenesis in several *Boechera* (Table 2.3).

The developmental stage of ovules within pistils was variable. Usually, ovules at the pedicel end were more mature than those at the stigma end. For example, at the stigma end, ovules might tend to be at the late MMC stage, but at the pedicel end, they might tend to have already completed meiosis to form a tetrad of genetically reduced
spores or apomeiosis to form a Taraxacum-type dyad of unreduced spores. Therefore, I used the germline stage for ovules observed in the middle of the pistil to represent the whole pistil.

From Table 2.3, apomeiosis (stage 2-IV) occurred in the three apomicts at a shorter pistil-length stage than meiosis did in the sexual *B. stricta*. This might simply reflect the fact that *B. stricta* contains more ovules in its pistils than the apomicts do. But it might also reflect a precocity of development in apomicts (the germline of apomicts developing faster than that of sexuals relative to the sporophytic tissues of the ovule and pistil) as noted in many other apomicts (Carman et al., 2011). This precocity of germline development may be related to a precocity of flowering as observed in many apomicts compared to their close sexual relatives (Carman et al., 2011).

**Table 2.3.** Average pistil length by germline development stage for pistils of three *Boechera* taxa. Lengths are an average of 3-8 pistils per stage by species combination.

<table>
<thead>
<tr>
<th>Species</th>
<th>Average pistil length (mm) by stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-II</td>
</tr>
<tr>
<td><em>B. cf. gunnisoniana</em></td>
<td>1.1</td>
</tr>
<tr>
<td><em>B. lignifera</em></td>
<td>0.9</td>
</tr>
<tr>
<td><em>B. retrofracta x stricta</em></td>
<td>1.2</td>
</tr>
<tr>
<td><em>B. stricta</em></td>
<td>1.5</td>
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</tbody>
</table>
Our *B. cf. gunnisoniana* is an obligate triploid apomict (Mateo de Arias, 2015) with Taraxacum type apomeiosis (Fig. 2.3a). However, we also observed in this line “triad” and “tetrad” formation during meiosis (Fig. 2.3b-d), and even higher numbers of megaspores, e.g., five and six, have been observed for this line (Mateo de Arias, 2015).
Likewise, pentads and hexads of male microspores form occasionally in other triploid *Boechera* (Bocher, 1951). The imbalanced distribution of chromosomes in triploid *Boechera* likely cause odd numbers of spores to form, and most of these spores likely abort due to grossly unbalanced chromosome complements.

Previous studies in our lab found that our *B. retrofracta x stricta* accession is a facultative apomict with 55 % Taraxacum type apomictic embryo sac formation (Mateo de Arias, 2015). My studies also found 55 % Taraxacum-type diplospory for this line, but I also documented 30 % Hieracium-type apospory and 3% Antennaria-type diplospory (Fig. 2.3e, f, h). The hybrid nature of this plant may be responsible for its complexity of reproduction. Three major types of apomixis being observed in one species is very rare, and it suggests that the mechanisms that regulate each type may be more closely related than previously thought. Antennaria-type diplospory involves the direct formation of an embryo sac from the MMC. A distinct characteristic of this type of apomixis is the early formation of a large vacuole in the MMC (Fig. 2.3h). This type of diplospory avoids both meiosis I and meiosis II. Thus, it is considered that the sexual pathway is completely shut down. In comparison, Taraxacum type apomixis bypasses meiosis I, and in this case, it is considered that the sexual pathway is only partially shut down. The observation of these different types of apomixis have provided us with new ideas for inducing partial or full apomixis in sexual plants by metabolic, genetic and epigenetic modifications.

I also correlated the timing of meiosis in microspore mother cells with pistil length (Fig. 2.4). Meiosis in these cells occurred early, during the 1-I to 2-I stages, which
is much earlier than female meiosis. Pollen grains had matured by the mature embryo sac stage. Triploid *B. cf. gunnisoniana* produced unreduced pollen at the 2-I stage (Fig. 2.4a, b), which suggests that the embryo to endosperm genome ratio would be 3C: 9C. The pollen of diploid *B. retrofracta x stricta* was also unreduced (1-II stage, Fig. 2.4c, d), which suggests that the embryo to endosperm genome ratio would be 2C: 6C. As expected, pollen in the diploid sexual *B. stricta* was reduced (Fig. 2.4g, h) with microsporogenesis occurring at the 1-I stage. Here, the embryo to endosperm genome ratio is expected to be 2C: 3C. Interestingly, *B. lignifera* produced both reduced and
unreduced pollen (1-II stage, Fig. 2.4e, f) at the same time, suggesting that the embryo to endosperm genome ratio could be 2C: 5C (reduced) or 2C: 6C (unreduced). Thus, both reduced and unreduced egg and sperm formation appears to be facultative in *B. lignifera*.

The complexity of speciation among *Boechera* has been considered “legendary” by plant taxonomists (Rollins, 1941). We now know that this complexity is due to high-frequency interspecific hybridization with resulting sexually-sterile hybrids being rescued from their sterility by apomixis. This is especially the case with *Boechera* where apomixis occurs readily in diploid interspecific as well as intraspecific (inter-racial) hybrids. Adding to this complexity is the frequent occurrence of polyploidy, where diploid apomicts pick up an additional genome by *B. III* hybridization. As documented herein, this “legendary” complexity is additionally accentuated by the occurrence of different types of gametophytic apomixis, e.g., Taraxacum and Antennaria types of diplospory and the Hieracium type of apospory, and the ready occurrence of apomeiosis occurring in both the male and female sides. Collectively, this complexity makes *Boechera* a unique model among angiosperms for studying and elucidating the mechanisms of apomixis.

LITERATURE CITED


CHAPTER 3
FROM APOMEIOSIS TO MEIOSIS: INDUCING MEIOSIS IN APOMIC TIC BOECHERA

ABSTRACT

• **Background and Aims** Apomixis is asexual reproduction by seed without meiosis or fertilization. Previous studies in our lab showed long term stresses induce meiosis in apomictic plants. In this study, I tested how late the stress can be applied and how strong the stress must be to shift apomeiosis to meiosis in apomictic *Boechera*. Stresses include peroxide, water deficit, sugar deprivation, and suppression of endogenous brassinosteroid function.

• **Methods** Preapomeiotic pistils of apomictic *B. cf. gunnisoniana*, *B. lignifera*, and *B. retrofracta x stricta* were excised and exposed to stress *in vitro*. Stressed included exposure to H<sub>2</sub>O<sub>2</sub>, culture on media containing polyethylene glycol (PEG), culture on media lacking a sugar source, or culture on media containing brassinozole (BRZ), a competitive inhibitor of brassinosteroid). When pistils reached the mid-apomeiosis stage (tetrad stage in sexual plants), they were fixed, cleared and studied using differential interference contrast (DIC) microscopy.

• **Key Results** For all three species, the 2-IV stage (meiosis I in sexual plants) was the most effective pistil stage for inducing meiosis (tetrad formation) in apomictic *Boechera*. Significantly higher frequencies of meiosis (conversion from apomeiosis to
meiosis) were achieved with 50 $\mu$mol L$^{-1}$ H$_2$O$_2$ for *B. lignifera* and 100 $\mu$mol L$^{-1}$ H$_2$O$_2$ for *B. retrofracta x stricta* and *B. cf. gunnisoniana*. For *B. retrofracta x stricta*, 20 g L$^{-1}$ PEG 6000 induced high frequencies (40-100 %) of apospory with degenerating tetrads, but effects of sugar deprivation for this taxon were not significant. For *B. cf. gunnisoniana*, 30 g L$^{-1}$ PEG 6000 induced high frequency (55 %) tetrad formation with rare occurrences of apospory being observed, and effects of sugar deprivation were also significant with ca. 60 % ovules producing tetrads. Likewise, applying BRZ to *B. cf. gunnisoniana* showed a significant shift from apomeiosis to meiosis.

- **Conclusions** In addition to long term stress, which was previously documented in our lab, short term stress also induces shifts from apomeiosis to meiosis, and additionally high frequency apospory was also induced in some cases. Molecular pathways associated with ROS, osmotic potential, cellular energetic status and TOR-BR appear to be involved in apomeiosis to meiosis switching. Such stresses may lead to DNA lesions, which are often repaired during Meiosis I (2-IV stage) of sexual reproduction.

**Key words:** apomixis; *Boechera*; ROS; PEG 6000; molecular pathway components
INTRODUCTION

Sexual reproduction is prevalent in most eukaryotes and considered as an evolutionarily superior mode compared to asexual reproduction because of heterosis (Marshall & Brown, 1981). Apomixis, defined herein as asexual seed formation, is relatively rare but still occurs in many angiosperm families (Carman, 1997; Hojsgaard, 2014). It is generally assumed that sex is an ancient pathway and that apomixis has evolved independently multiple times (Van Dijk & Vijverberg, 2005). However, recent genetic analyses of most studied apomictic species, such as Taraxacum (Van Dijk et al., 1999), Erigeron (Noyes and Rieseberg, 2000), Poa (Albertini et al., 2001), Hieracium (Catanach et al., 2006) and Hypericum (Schallau et al., 2010), indicate that apomeiosis, parthenogenesis, and fertilization-independent endosperm formation are controlled by independent loci (Hand & Koltunow, 2014). This suggests that if apomixis is derived as a result of mutations of sexual processes, then three independent loci must have evolved simultaneously for apomixis to arise. Independent mutations of these processes by themselves are deleterious, and the probability that all three occurred simultaneously seems to be null, especially with apomixis supposedly evolving independently in many angiospermous families. Koltunow et al. (2012) noted that sex and apomixis coexist in most if not all angiospermous apomicts (facultative apomixis). Based on their studies, they suggest that sexual reproduction is the default mode in facultatively apomictic
Hieracium subgenus Pilosella. From their evidence they suggested that apomixis occurs due to alterations to the spatial and temporal regulation of sexual reproduction processes.

The above reports are based on the hypothesis that apomixis is derived from sex by mutation. However, in nature, mutation is not required when sex switches to apomixis or vice versa as occurs in cyclically apomictic organisms where the organism is usually apomictic in favorable growth conditions but becomes sexual when stressed (Suomalainen et al., 1987). For example, aphids rapidly reproduce genetically identical offspring in spring and summer by apomixis, but they reproduce sexually in the fall, producing eggs that tolerate cold stress and freezing in the winter. To address this question, Carman et al. (2011) hypothesized that apomixis/sex are polyphenisms, they evolved simultaneously during eukaryogenesis, shared the same genes, but regulated these genes epigenetically by environmental signals instead of mutation. If this is correct, and if the latent potentialities of both sex and apomixis still exist in typically sexual angiosperms, then sex or apomixis can be triggered by signals that cause epigenetic modifications.

In our lab, Mateo de Arias (2015) stressed B. lignifera and B. gunnisoniana with drought and heat and achieved major shifts from apomeiosis to meiosis in megaspore mother cells (MMC). In RNA-seq data obtained from immature ovules taken from her stressed and well-watered (control) plants, the well-watered apomicts were producing and metabolizing sugars (highly energetic) and glutathione transferases were up-regulated, which detoxify reactive oxygen species (ROS). When these plants were drought-stressed,
photosynthesis and ROS detoxification declined, growth ceased, gene expression patterns specific to epigenome reprogramming and sex were up-regulated, and sex ensued. Gene ontology (GO) patterns in apomictic plants under stressed conditions were similar to those in sexual plants under well-watered conditions (Mateo de Arias et al., in preparation).

In the Mateo de Arias (2015) experiments, stressed plants were stressed from the end of vernalization until seed maturation, which included the entire reproductive phase. Her research did not address the question as to when the stress had its effect in switching reproductive programming from apomixis to sex. Herein I address this question. Specifically, my research addressed the question of how late before female apomeiosis can stress be applied and still switch the program to sexual meiosis. My research also addressed the question as to how strong the stress signal must be to induce the switch from apomixis to sex? To answer these questions, as discussed in more detail in Chapter 1, I exposed immature pistils (ovaries) of *B. cf. gunnisoniana*, *B. lignifera*, and *B. retrofracta x stricta*, cultured in vitro, to the following pharmacological treatments: *i*) H$_2$O$_2$, to simulate ROS stress, *ii*) polyethylene glycol (PEG), to simulate osmotic stress, *iii*) sucrose starvation, to lower the energetic status of the tissues, and *iv*) brassinazole (BRZ), an inhibitor of brassinosteroid synthesis, to disrupt early high-energy-dependent brassinosteroid signaling, which induces rapid growth (Hao et al. 2016), and which we hypothesize may be involved in inducing apomictic rather than sexual reproduction under favorable growth conditions. If this is correct, we should be able to chemically induce sex
in apomictic plants without mutation. The objective of this study was to determine the most effective types, timings and concentrations of stress for inducing sexual meiosis pharmacologically in apomictic plants, which normally undergo apomeiosis.

MATERIALS AND METHODS

Plant materials

Plants of *B. gunnisoniana*, *B. lignifera*, and *B. retrofracta x stricta* were collected from the western United States as live specimens, seeds were harvested upon maturation, and voucher specimens were prepared and deposited in the Intermountain Herbarium, Utah State University (Table 3.1). Seeds were germinated on moist germination paper in petri dishes at 26 °C for 1-2 weeks and planted in pots containing Sunshine Mix #1 potting soil (Sun Gro Horticulture Canada Ltd, Vancouver, BC). Plants were grown in a walk-in growth chamber set at 22/16 °C day/night temperatures with lighting (16/8

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Accession &amp; Voucher no.</th>
<th>Location</th>
<th>GPS</th>
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</thead>
<tbody>
<tr>
<td><em>B. cf. gunnisoniana</em> <em>(3x)</em></td>
<td>CO11005 UTC00275883</td>
<td>CO, Gunnison Co., hillside east of Highway 50</td>
<td>38.5274 -106.8144</td>
</tr>
<tr>
<td><em>B. lignifera</em></td>
<td>WY05001 UTC00276008</td>
<td>WY, Sweetwater Co., South of Green River</td>
<td>41.5517 -109.5253</td>
</tr>
<tr>
<td><em>B. retrofracta x stricta</em></td>
<td>CO11010 UTC00276007</td>
<td>CO, Rio Blanco Co., Cow Creek Access Road</td>
<td>39.7029 -107.9986</td>
</tr>
</tbody>
</table>
d/night photoperiod) supplied by incandescent lights, soft white florescent bulbs, and 1000 W high-pressure sodium-vapor lamps, which provided a minimum photosynthetic photon flux of 400 μmol m⁻² s⁻¹ at the canopy surface. The plants were watered regularly with a dilute solution (250 mg/L) of Peters Professional 20:20:20 fertilizer (Scotts, Maryville, Ohio). After 4-6 weeks (8-10 leaf stage), the seedlings were vernalized at 4 °C for 10-12 weeks with minimal lighting (8/16 day/night photoperiod) from soft white fluorescent bulbs. The vernalized plants were then transferred to the growth chamber and incubated as above.

**Embryology**

Floral buds at the pre-anthesis stage were fixed in formalin acetic acid alcohol (FAA) for 48 h and cleared using mixtures of benzyl benzoate:dibutyl phthalate (2:1) (BBDP) and 95% EtOH. The following procedures were performed as described by Crane and Carman (1987) with minor modifications: i) 70% EtOH for 30 min, ii) 95% EtOH for 4 h, iii) 95% EtOH:BBDP (2:1) for 2 h, iv) 95% EtOH:BBDP (1:2) for 4 h, v) BBDP for 4 h, BBDP again for overnight. Plant materials should be infiltrated by clearing solution completely for each step. Hence, with larger clumps of buds, times for infiltration were extended.

Pistil lengths were measured using a dissection scope before they were mounted on slides. The developmental stage of each ovule in each pistil was then studied using a
BX53 microscope (Olympus, Center Valley, PA, USA) equipped with differential interference contrast (DIC) optics. The germline development staging notation of Schneitz et al. (1995), which was developed for *Arabidopsis*, was used. Staging in this system is as follows: 1-I, pre-MMC ovule primordium initiation; 2-II, inner integument and MMC enlargement; 2-III, outer integument initiation with MMC enlarged; 2-IV, beginning of meiosis; 2-V, meiosis complete. Ovules were further scored based on presence of megaspore mother cells (MMC), dyads, tetrads, aposporous initials (AI, nucellar cell as large as the developing meiocyte but without one or more large vacuoles), 2, 4, or 8-nucleate embryo sacs (ES2, ES4, and ES8) and aposporous embryo sacs (AES stage, large actively growing nucellar cells with one or more large vacuoles and one of more nuclei). For each pistil, the pistil length and the developmental stage of the majority of ovules was recorded. Pistil length was determined by measuring the distance from the base of the pedicel to the top of the stigma. Photomicrographs of ovule development were taken using a MicroFire 599809 camera (Olympus, Center Valley, PA, USA).

*Pharmacological treatments of pistils and in vitro culture*

To simulate drought stress, pistils were excised, randomly assigned to a control medium or a medium that imposed a specific level of drought (using a random number table), and cultured. The basal medium consisted of MS (Murashige and Skoog, 1962) salts and vitamins (Caisson Labs, Logan, UT, USA), pH 5.7, that contained 20 g L\(^{-1}\)
sucrose (Sigma-Aldrich, St. Louis, MO, USA) and 2.7 g L\(^{-1}\) Phytogel (Sigma-Aldrich, St. Louis, MO, USA). This basal medium served as the control, and additional aliquots of basal media were supplemented with 20, 30, 40 or 60 g L\(^{-1}\) PEG 6000 (Sigma-Aldrich, St. Louis, MO, USA), which produced estimated media water potentials (\(\Psi\)) of -0.5 MPa for the basal medium and -0.55, -0.56, -0.57 and -0.61 MPa for the PEG amended media, respectively. These estimates were determined as follows. The \(\Psi\) (osmotic plus matric) of MS medium with 30 g L\(^{-1}\) sucrose and supplemented with 2.0 g L\(^{-1}\) Gellan gum (equivalent to Phytogel) is ca. -0.50 MPa (Buah et al. 1999). \(\Psi\) of solutions of pure water containing 20, 30, 40, and 60 g L\(^{-1}\) PEG 6000 are ca. -0.013, -0.022, -0.034, -0.065 MPa, respectively (from equations in Michel, 1983). \(\Psi\) of PEG solutions combined with solutions of other solutes often are not additive but synergistic, with a synergism factor often being as high as 7.5 % (Michel & Kaufmann, 1973; Michel, 1983). Based on this information, I estimated \(\Psi\) of my PEG-amended basal media by adding the \(\Psi\) of the various PEG concentrations, in pure water, to the \(\Psi\) of the basal medium and multiplying the sums by a 1.075 synergism factor.

The pistil culture technique described above was used to impose additional stresses as follows. To study the effects of sugar starvation, excised pistils were randomly assigned and cultured horizontally on basal medium with or without sucrose. For exposure to high levels of ROS, randomly assigned excised pistils were treated for 5 min in water that contained \(\text{H}_2\text{O}_2\) (Sigma-Aldrich, St. Louis, MO, USA) at 0 (water control), 50, 100, 250 and 500 mmol L\(^{-1}\). The pistils were then cultured horizontally on basal
medium. To simulate stress-induced repression of brassinosteroid synthesis, randomly assigned excised pistils were cultured on basal medium and basal medium supplemented with BRZ (Sigma-Aldrich, St. Louis, MO, USA), which was dissolved in minimal DMSO (Fisher Scientific, Asheville, NC, USA) and added immediately after autoclaving by filter sterilization (0.2 µm, BioExpress, Kaysville, UT, USA) to achieve a 1.0 µmol L⁻¹ concentration. After exposure to these pharmacological treatments (1-8 d), pistils were fixed, cleared and studied embryologically as above.

Statistical analyses

For statistical analyses, only ovules where mode of reproduction could be reasonably assessed were scored. Ovules were not scored if they were too young (MMC stage or earlier), too old (older 2-nucleate embryo sac stage or older), or their orientation through the microscope prevented complete sagittal views of the dyad, tetrad, 1-nucleate sexual or apomictic embryo sac, or early 2-nucleate sexual or apomictic embryo sac. The resulting cross-tabulation data were analyzed using Pearson chi-square tests for independence of table rows and columns (SYSTAT, 2004).
RESULTS

Pistil length stages and germline development

Different species of Boechera contain from ca. 40-200 ovules per pistil (Al-Shehbaz and Windham, 2010). Hence, stages of germline development in ovules of pistils vary by species and pistil length. To expose ovules to chemical treatments at specific germline stages, it was necessary to characterize, for each species, the relationship between pistil length and germline stage, which was defined as the germline stage for the majority of ovules in pistils of given length. The average germline stage by pistil length is shown in Table 3.2 for B. cf. gunnisoniana, B. lignifera and B. retrofracta x stricta.

<table>
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<tr>
<th>Species</th>
<th>Average pistil length (mm) by stage</th>
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<tr>
<td></td>
<td>1-I</td>
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<tr>
<td>B. cf. gunnisoniana</td>
<td>1.5</td>
</tr>
<tr>
<td>B. lignifera</td>
<td>1.0</td>
</tr>
<tr>
<td>B. retrofracta x stricta</td>
<td>1.1</td>
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Multiple stresses induce shifts from apomeiosis to meiosis in MMC and from programmed
cell death to embryo sac formation in the MMC-adjacent sporogenous nucellar cells

Previously in our lab, apomicts of triploid B. cf. gunnisoniana and diploid B. lignifera were exposed from vernalization through flowering to drought or drought plus heat. These long-term treatments caused a reprogramming of most MMC so that they underwent meiosis instead of apomeiosis. Non-stressed control plants exhibited 87-98 % apomictic dyad formation, while heat and water stressed plants exhibited 75-80 % sexual tetrad formation (Mateo de Arias, 2015). Such polyphenic shifts in reproductive mode were well demonstrated, but many questions remained unsolved. Specifically, does the stress-induced genomic, epigenomic and/or biochemical reprogramming required for shifts from apomictic to sexual reproductive development occur gradually during flower development, i.e., requiring long exposures to stress, or can such shifts be induced by short exposures to stress, e.g., as late as immediately prior to the onset of apomeiosis in a late-stage MMC? Herein I used multiple stress treatments to determine how temporally close to the onset of apomeiosis in MMC a stress could be applied and still achieve a pronounced effect. In the previous study (Mateo de Arias, 2015), the effects of drought and heat were synergistic. In my study, I asked if drought or related stresses, applied individually and at specific stages of ovule development, can induce high-frequency shifts from apomeiosis to meiosis.
To study the effects of short-term drought on converting apomictic development to sexual development, pistils of apomictic *B. retrofracta x stricta*, *B. cf. gunnisoniana* and *B. lignifera* were excised and cultured at pre-apomeiotic stages of ovule development. Pistils exposed to treatments in vitro were harvested when they had grown to a length that corresponded with those of greenhouse grown plants that contained ovules in the 2-V stage (mature sexual tetrad or mature diplosporous dyad stage). For pistils grown on basal medium (MS with sucrose), growth averaged 0.2 mm d⁻¹.

From 40-100 % of ovules in *B. retrofracta x stricta* pistils were producing aposporous embryo sacs (includes a degenerating sexual tetrad) at the end of culture, and this high frequency of apospory coupled with sexual megaspore degeneration was most pronounced (80-100 % of ovules) in pistils that had been cultured at the earliest stage of ovule development regardless of Ψ treatment (1-I, these had remained in culture for the longest period of time). A sharp increase in apospory and sexual tetrad formation was observed in response to the least severe drop in Ψ (-0.55 MPa treatment), but only in the most mature pistils (Fig. 3.1, ovule stage 2-IV). Pearson Chi-square tests for independence were highly significant (P < 0.001) for i) the main effect that compared pistil age at culture initiation versus reproductive mode at the end of pistil culture and ii) the main effect of Ψ treatment versus reproductive mode at the end of pistil culture.

*Boechera lignifera* pistils were much smaller at the culture initiation stages used for this experiment than those of the two other taxa (Table 3.2), and most of their ovules
aborted. Consequently, sufficient data from *B. lignifera* pistils could not be obtained for statistical analyses.

Triploid *B. cf. gunnisoniana* is highly diplosporous (Mateo de Arias, 2015), but when pistils were exposed to low Ψ, the frequency of sexual meiosis increased to 55% (Fig. 3.2). Note that this was not accompanied by a corresponding increase in apospory, as occurred with *B. retrofracta x stricta*. As a triploid, it is expected that meiosis-produced megaspores will be inviable due to genetic imbalances that negatively affect gametophyte formation and gamete viability. Hence, megaspore inviability by itself (due to segregational genetic imbalance) does not explain the positive correlation between sexual...
tetrad formation and apospory in *B. retrofracta* x *stricta* and the lack of such a correlation in triploid *B. gunnisoniana*. Pearson Chi-square tests for independence were significant (P < 0.003) for pistil age at culture initiation versus reproductive mode and highly significant (P < 0.001) for Ψ versus reproductive mode. Aposporous, diplosporous and sexual embryo sac formation occurred in all three species, though apospory was rare in *B. cf. gunnisoniana* (Fig. 3.3).

Since drought can drain carbohydrates from tissues (Zhou et al. 2017), I tested whether changes in sugar signaling (Sheen, 2014), induced by sucrose starvation,
constitutes part of the signal transduction pathway responsible for stress-induced shifts from apomeiosis to meiosis (Mateo de Arias, 2015; Fig. 3.2). Pistils were cultured on basal medium with and without sucrose. Preliminary experiments indicated that sucrose starvation causes ovule death when pistils are cultured prior to 2-III. Hence, 2-III and 2-IV staged pistils were used. The small B. lignifera pistils (Table 2) were too sensitive to sucrose starvation, so data for this species was not obtained. When the experiment was
conducted using stage 2-IV pistils (late MMC stage) of aposporous *B. retrofracta x stricta*, no differences were induced by sucrose starvation in frequencies of aposporous, diplosporous or sexual development (Fig. 3.4; the Pearson Chi-square test for independence based on presence or absence of sucrose versus mode of development was not significant, P < 0.05). However, when the experiment was conducted with the highly diplosporous *B. cf. gunnisoniana*, sucrose starvation significantly increased frequencies of sexual tetrad formation at both stages of pistil development (Fig. 3.5). In this case of severe carbohydrate starvation (pistil detached from its photoassimilate supply and not provided with a substitute sugar source), positive correlations were observed between a

Fig. 3.4. Frequencies of *B. retrofracta x stricta* ovules that were undergoing sexual tetrad, sexual or diplosporous dyad, or aposporous embryo sac (AES) formation at the end of the pistil culture treatment as affected by removal of sucrose from the culture medium. Pistils were cultured at the 2-IV stage. Numbers of correctly staged ovules (containing a 1-2 nucleate AES and/or a dyad or tetrad) for the 20.0 and 0.0 g L\(^{-1}\) sucrose treatments were 61 and 90, respectively.
decreasing apomeiosis to meiosis ratio and the occurrence of apospory. At both culture
initiation stages, diplosporous dyad frequencies decreased sharply (Fig. 3.5). Pearson
Chi-square tests of independence were highly significant (P < 0.001) for pistil age versus
reproductive mode and for presence or absence of sucrose versus reproductive mode.

Photomicrographs of sexual, diplosporous and aposporous development from these sugar
starvation experiments are shown in Fig. 3.6.

TARGET OF RAPAMYCIN (TOR) is a major regulator of cell energetics that is
highly-conserved among eukaryotes. This serine/threonine protein kinase activates or
inactivates enzymes by its serine-threonine phosphorylation function (Xiong et al., 2013; Couso et al., 2016). As a regulator of bioenergetics, TOR is inactivated by ROS (Zhang et al. 2016), and its inactivation suppresses growth. Furthermore, high levels of ROS accumulate in plant cells in response to low glucose levels (Couee et al., 2006; Sarre et al., 2012; Keunen et al., 2013; Sheen, 2014). However, glucose/TOR signaling in high ROS low glucose environments is selective, with cell cycle regulation being specifically targeted (Xiong et al., 2013). Interestingly, this is consistent with the somewhat surprising results that stress-induced switching from apomeiosis to meiosis can occur very late in MMC development, possibly after apomeiotic prophase has initiated (Figs. 3.1, 2). Since

FIG. 3.6. Ovules of B. cf. gunnisoniana (a-d) and B. retrofracta x stricta (e-h) from the sucrose starvation experiments. a, e: unreduced dyads, white arrows point to surviving megaspores, black arrows point to degenerating megaspores; b, f: sexually-reduced tetrads in pistils exposed to sucrose starvation, white arrows point to surviving megaspores, black arrows point to degenerating megaspores; c, g: aposporous embryo sacs (AES) in pistils exposed to sucrose starvation, white arrows point to AES nuclei, black arrows point to degenerating megaspores; d, h: degenerating megaspores; v, vacuoles; scale bar: 20 μm.
major cell cycle alterations are central to apomixis/sex switching, I asked whether ROS/TOR signaling functions downstream from stress (drought or sugar starvation) perception? To address this question, I pretreated for 5 min appropriately staged pistils in solutions containing H$_2$O$_2$, cultured the pistils under otherwise favorable $\Psi$ and sucrose conditions, and observed, one to a few days later, whether apomeiosis to meiosis shifts had been induced. H$_2$O$_2$ is a relatively long-lived ROS that permeates whole cells and cell organelles (Horandl & Hadacek, 2013). A ROS-induced reduction in apomeiosis to

![Graph](image-url)

**Fig. 3.7.** Frequencies of *B. retrofracta x stricta* ovules that were undergoing sexual tetrad, sexual or diplosporous dyad, or aposporous embryo sac formation at the end of the pistil culture treatment as affected by i) increasing concentrations of H$_2$O$_2$ in the pistil preculture treatment and ii) ovule stage at pistil culture initiation (1-I, 2-III and 2-IV). Numbers of correctly staged ovules (containing a 1-2 nucleate aposporous embryo sac and/or a dyad or tetrad) at the 12 H$_2$O$_2$ concentration by pistil stage treatments averaged 77 and ranged from 6-138.
meiosis ratios would be consistent with TOR being a member of the causal signal
transduction (switch mechanism) pathway.

The 50 and/or 100 mmol L\(^{-1}\) \(\text{H}_2\text{O}_2\) treatments effectively switched apomeiosis to
meiosis in apomictic \textit{B. retrofracta x stricta} (Fig. 3.7), \textit{B. lignifera} (Fig. 3.8) and \textit{B. cf.}
gunnisoniana (Fig. 3.9). In these cases, the switch from diplospory (producing dyads of
unreduced megaspores) to sex (producing tetrads of reduced megaspores) was mirrored
by adventitious embryo sac formation from evolutionarily-sporogenous nucellar cells
(apospory). In most cases of apospory, a sexual tetrad had also formed, but all four

![Graph](image)

\textbf{Fig. 3.8.} Frequencies of \textit{B. cf. lignifera} ovules that were undergoing sexual tetrad,
sexual or diplosporous dyad, or aposporous embryo sac formation at the end of the
pistil culture treatment as affected by \textit{i}) increasing concentrations of \textit{H}_2\text{O}_2 in the
pistil preculture treatment and \textit{ii}) ovule stage at pistil culture initiation (2-III and 2-
IV). Numbers of correctly staged ovules (containing a 1-2 nucleate aposporous
embryo sac and/or a dyad or tetrad) at the 10 \textit{H}_2\text{O}_2 concentration by pistil stage
treatments averaged 70 and ranged from 29-168.
megaspores were degenerating. The replacement of surviving megaspores of tetrads by aposporous embryo sacs was most apparent in the aposporous-leaning *B. retrofracta x stricta* (Fig. 3.7), but it also occurred to a fairly high degree (30 % of ovules) in *B. gunnisoniana* (Fig. 3.9), in which apospory is only rarely observed in nature (Chapter 2). Apparently, the decision of nucellar cells to undergo apoptosis and yield their protoplast nutrients to the developing female gametophyte, is redox sensitive. In the three *Boechera* species tested, the nucellus, regardless of genetics, displayed four inherent developmental potentialities: i) apomeiosis (Taraxacum-type diplospory), ii) meiosis, iii) apoptosis, with
nutrients becoming available to the growing gametophyte, and \( iv \) aposporous female gametophyte (embryo sac) formation. Interestingly, the switch from apoptosis to apospory was redox activated. Photomicrographs of these four developmental fates are shown for the \( \text{H}_2\text{O}_2 \) treatment experiments in Fig. 3.10.

The most effective timing in the 2-IV stage for inducing meiosis with \( \text{H}_2\text{O}_2 \) treatments differed depending on the species. For \( B. \text{ cf. gunnisoniana} \), the best timing occurred when pistils were 2.1 mm long, which is near the end of the 2-IV stage. For \( B. \)
lignifera, the best timing was when pistils were 1.5 mm long, which was near the middle of 2-IV stage. For B. retrofracta x stricta, the best time was when the pistils were 2.0 mm long, which is at the beginning of the 2-IV stage.

The observed rapid shift from apomeiosis to meiosis in late-stage MMC, as affected by H$_2$O$_2$ treatments (Fig. 3.7, 8, 9), is consistent with the activity status of TOR being a key regulator of the apomixis/sex switch. By a mechanism not yet elucidated (Wang et al., 2002; Zhang et al., 2016), TOR stimulates the production of brassinosteroids (BR), which are plant steroids that regulate growth (He et al., 2005) including many growth and development functions of ovules (Huang et al., 2013). By dephosphorylation (Zhang et
al., 2016), BR activates BRASSINAZOLE-RESISTANT 1 (BZR1), which is a major BR-regulated transcription factor that targets ca. 2000 genes (Oh et al., 2012). Thus, to further evaluate whether TOR is involved with apomeiosis to meiosis switching, I treated sets of *B. cf. gunnisoniana* pistils with the BR synthesis inhibitor brassinozole (BRZ). Pistils were collected during the 2-III and 2-IV stages. Pistils in the 2-III stage did not grow sufficiently to collect data. However, for pistils cultured at the 2-IV stage, BR synthesis

![Fig. 3.12](image_url) - Ovules of *B. cf. gunnisoniana* treated with BRZ. **a:** unreduced dyad, white arrows point to nuclei of surviving megaspore, black arrow points to degenerating megaspore; **b:** meiotically-reduced tetrad, white arrows point to surviving megaspore, black arrows point to degenerating megaspore; **c:** aposporous embryo sac (AES), white arrow points to the AES nucleus, black arrows point to degenerating megaspores of a degenerating tetrad; **d:** two AES in one ovule, white arrows point to the AES nuclei; v, vacuoles; scale bar: 20 μm.
inhibition caused a significant shift (P < 0.05) from apomeiosis to meiosis and from apoptosis to apospory (Fig. 11, 12). This is strong evidence that the TOR/BR/BZR1 pathway is a component of the apomixis sex switch in Boechera.

DISCUSSION

The goal of this study was to elucidate fundamental molecular components of the stress-activated apomixis-to-sex switch in Boechera apomicts (Mateo de Arias, 2015). To accomplish this, I conducted experiments that tested four molecular pathway components (Fig. 3.13). In plants, drought induces a holistic and lasting stress response that includes not only ROS formation but also morphological, physiological and biochemical changes such as the constriction of stomata, reduced respiration, and disruption of pigment formation associated with photosynthesis (Anjum et al., 2011). These additional factors may explain the “sensitivity” of B. lignifera to the PEG 6000-induced drought stress. Also, the drought stress was a continuous stress, unlike the H₂O₂ stress, which was applied for 5 minutes only.

Drought stress is one of the most common abiotic stresses that affects plant growth (Hamayun et al., 2010). Many studies report that water deficit seriously harms the physiology of the plant especially during flowering (Zhu et al., 2005; Daneshian & Jonobi, 2001; Barnabás et al., 2008; Jiang & Huang, 2000; Rampino et al., 2006; Anjum et al., 2011). ROS formation, as an early response to water stress, acts as a secondary
messenger to trigger subsequent defense reactions in plants (Hörandl & Hadacek, 2013; Cruz de Carvalho, 2008; Anjum et al., 2011).

The oxidative damages caused by ROS may trigger meiosis in some species with the consequent DNA recombination repairing the lesions. Under favorable summertime conditions, *Daphnia pulex* (water flea) produces apomictic diploid eggs by a 1st division restitution, as in diplosporous *Boechera*, and this is followed by parthenogenesis.

However, under the environmental stresses associated with the onset of winter, they produce haploid resting eggs by meiosis that are fertilized and acquire cold resistance to
tolerate winter conditions (Stollewerk, 2010). Likewise, in our lab, Mateo de Arias (2015) stressed *B. lignifera* and triploid *B. cf. gunnisoniana* with drought and heat and observed a similar shift from apomeiosis to meiosis.

Like drought stress, sucrose starvation was also continuous and very strong, perhaps even stronger than the drought stress because the only sources of carbohydrates available to the cultured pistils were the reserves that may have been stored in pistil cell vacuoles at the time of culture. In this respect, sucrose starvation may have taken a little longer to have an effect at culture initiation, but it might also explain why pistils younger than the 2-III stage died after a few days of culture. It is necessary to add exogenous sugar to the medium for all kinds of plant tissue culture (Iraqi & Tremblay, 2001). Sucrose is a disaccharide of glucose and fructose and has been used as the most suitable source of carbon and energy for plant tissue culture (George, 1993). It is generally accepted that moderate amounts of exogenous sucrose in plant tissue cultures will increase growth, but high sucrose levels reduce photosynthetic activity in vitro (Fuentes et al., 2005; Schäfer et al., 1992; Furbank et al., 1997; Van Huylensbroeck & Debergh, 1996). On the contrary, deprivation of sucrose from plant tissue triggers decreased mitochondrial respiration (Journet et al., 1986), which agrees with our RNA-seq data that bioenergetics-related genes were down-regulated in the pistils of sexual plants relative to pistils of apomictic plants (in preparation). That sucrose starvation in *Arabidopsis* induces epigenetic modifications is evident from the results of Nicolai et al. (2006) who showed an up-regulation of chromatin regulation genes such as HD1 histone deacetylase and histone H4.
This suggests that the switch from apomeiosis to meiosis in response to sugar starvation or other stresses may involve restructuring of the epigenome, and this might occur through TOR-involved sucrose signaling (Zhang et al., 2016).

ROS are responsible for oxidative stress, which can damage DNA, proteins, and lipids (Møller et al., 2007). For the DNA double helix, the damages include strand breaks, sister chromatid exchange, DNA–DNA and DNA–protein cross-linking, and base modifications (Friedberg et al., 2006). In order to control the damage caused by oxidative stress, eukaryotes have evolved highly efficient antioxidant systems that attempt to maintain ROS homeostasis (Hadacek et al., 2011). For damages not prevented by ROS detoxification, Bernstein (1998) proposed that crossing over during meiosis evolved to repair oxidative double strand DNA damage. Nedelcu et al. (2004) induced sex in an apomictic green alga by heat stress. In our lab, Mateo de Arias (2015) stressed B. lignifera and B. cf. gunnisoniana with drought and heat and obtained similar results.

BR signaling leads to the dephosphorylation and accumulation of the transcription factor BZR1. Similarly, sugar-TOR signaling leads to the accumulation of BZR1 by preventing its catabolism (Zhang et al., 2016; Fig. 3.13). Shutting off endogenous BR biosynthesis by BRZ makes plants vulnerable to stress, and this may have caused the switch from apomeiosis to meiosis. In general, all the stresses imposed in the current study were capable of switching apomeiosis to meiosis, and higher levels of stress lead to growth cessation and degeneration. For the short-term (5 min) H$_2$O$_2$ application, the best timing to induced tetrads was at the 2-IV stage. That responses to H$_2$O$_2$ were not as
strong in pistils exposed at an earlier stage of development may reflect the pistils’ ability
to detoxify the H$_2$O$_2$ prior to apomeiosis onset. But it might also indicate that the timing
for apomeiosis to meiosis switching is specific to the immediate pre-apomeiosis stage.
Further research is needed to clarify these possibilities, e.g., providing short term stress to
young pistils followed by ideal growth conditions. For the longer-term stresses, such as
drought, sucrose deficit and BRZ treatment, the timing during which apomeiosis to
meiosis conversion could be induced was extended from stage 2-IV back to stage 2-III.
However, this does not prove that an earlier stress can induce the switch, i.e., pistils
exposed to early stress were also exposed to the later stress because of the experimental
design.

*B. cf. gunnisoniana*, as an obligate triploid apomict, tolerated stronger stresses than
the diploid apomicts. *B. lignifera* was the most sensitive to stress injury, but also
produced the smallest pistils, which may explain this sensitivity. *B. retrofracta x stricta*
was prone to produce AES more so than tetrads under mild stress. The mechanism of
these phenomenon is not fully understood and will require further investigation.

Of all three species, stage 2-IV was the best for inducing meiosis. In sexual plants,
2-IV is the meiosis I stage where crossing over happens in prophase I and homologous
chromosomes segregate to form reduced dyads. In Taraxacum type diplospory, meiosis I
fails and a meiosis II-like (mitotic-like) division occurs, which produces a dyad of
unreduced spores. The oxidative damage that causes DNA double strand breaks can be
repaired by various mechanisms such as DNA recombination or polyploidy, in this case,
effective recombination events that happen during meiosis may be the removal of oxidative damages (Hörandl & Hadacek, 2013). The most effective \( \text{H}_2\text{O}_2 \) concentration also varied by species. For \( B. \text{lignifera} \), 50 mM \( \text{H}_2\text{O}_2 \) during 2-IV and 2-III stages were best with higher concentrations causing growth cessation or even degeneration. For \( B. \text{cf. gunnisoniana} \) and \( B. \text{retrofracta x stricta} \), 100 mM, 250 mM and 500 mM \( \text{H}_2\text{O}_2 \) were effective for inducing tetrads. The high concentrations for these two taxa did not cause degeneration, which suggests that these two species might tolerate even stronger oxidative stress. \( B. \text{lignifera} \) was the most sensitive to oxidative stress, which agrees with the previous study that showed that \( B. \text{lignifera} \) is 84 \% facultative and \( B. \text{cf. gunnisoniana} \) is nearly an obligate (>95\%) apomict (Mateo de Arias, 2015). For \( B. \text{retrofracta x stricta} \), a lot of apospory (40 – 60 \%) was induced along with tetrads. In aposporous apomicts, aposporous embryo sacs produced unreduced embryos mitotically while the sexual tetrads in young ovules degenerate. In \( \text{Boechera} \), apospory might assure reproduction under low-stress conditions. This result also suggests that there may be an oxidative buffing system in the plant that helps plants stabilize the oxidative level thus preventing oxidative damage.

Ovules are thought to be evolutionary elaborations of progymnosperm (fern-like) sporangia with nucellar cells of ovules corresponding to sporangial cells (Herr, 1995). In ferns and other lower plants sporangial cells undergo either meiosis to form haploid spores and gametophytes or diplosporous-like apomeiosis to form unreduced spores and gametophytes as in many ferns (e.g., the Braithwaite and Dopp-Manton schemes; Mogie,
In these respects, it is interesting that the nucellar cells of all three Boechera taxa studied herein are capable of four distinct developmental fates, meiosis, diplosporous apomeiosis, apoptosis, and apospory, depending on the oxidative status of the pistil (Fig. 3.13). This observation is consistent with the hypothesis that sex and apomixis are ancient developmental polyphenisms of each other with environmental signals dictating the phenism to be expressed (Carman et al., 2011; Hojsgaard et al., 2014). This hypothesis contrasts sharply with the 20th century notion that apomixis is polyphyletic and requires specific genetic mutations each time it evolves from sexual ancestors. In contrast, if the polyphenism hypothesis is correct, it should be possible to physiologically or pharmacologically induce the expression of apomixis in other angiosperms that currently are thought to be completely sexual. This is the topic of the next chapter.

LITERATURE CITED


Mateo de Arias, M. 2015. Effects of Plant Stress on Facultative Apomixis in *Boechera* (Brassicaceae). PhD Dissertation, Utah State University, Logan, UT.


CHAPTER 4
FROM MEIOSIS TO APOMEIOSIS: INDUCING
APOMEIOSIS IN SEXUAL PLANTS

ABSTRACT

• Background and Aims Having been studied for more than 100 years, the mechanism of apomixis is still poorly understood. It is widely accepted that apomixis is derived from mutations of sex, however, in our lab we hypothesize that apomixis and sex are polyphenisms of each other, they share the same genes and capacities for each coexist perhaps in most eukaryote genomes. In this study, I tested our hypothesis by treating germline and germline associated tissues of several sexual plant species with chemicals designed to prevent meiosis-inducing signals from occurring and to provide metabolically favorable conditions, which, according to our hypothesis should induce apomeiosis.

• Methods Factorial experiments were performed using in vitro cultured pre-meiotic pistils of sexual Boechera stricta (Brassicaceae), sexual Arabidopsis thaliana (Brassicaceae) and sexual Vigna unguiculata (cowpea, Fabaceae). Pistils were treated with the brassinosteroid epibrassinolide (epiBL), the antioxidant (S)-2-aminobutane-1,4-dithiol hydrochloride (DTBA), epiBL plus DTBA, or 5-azacytidine (5-azaC) in sucrose or glucose amended media. Pistils were fixed during the mid-meiosis stage or
early embryogenesis and studied using differential interference contrast (DIC) microscopy of cleared pistil.

- **Key Results** A 30% conversion from meiotic (sexual) tetrad formation to putative diplosporous (apomictic) dyad formation was observed in *B. stricta* treated *in vivo* or *in vitro* with epiBL. However, these experiments did not permit determinations of subsequent diplosporous embryo sac formation frequencies, which may have been rare. All treatments, including glucose instead of sucrose, significantly increased dyad frequencies in *B. stricta* and *Arabidopsis*. Hieracium type aposporous apomeiosis and Antennaria type diplosporous apomeiosis were also observed in several treatments, especially in *Arabidopsis*. For cowpea, 24 of 34 ovules showed Antennaria type diplosporous apomeiosis on 5-azaC, epiBL, DTBA or glucose amended mediums. In *B. cf. gunnisoniana*, 10% Antennaria type diplosporous apomeiosis was induced by 500 μmol L⁻¹ 5-azaC.

- **Conclusions** epiBL can induce high frequency dyad formation (putative Taraxacum-type diplosporous apomeiosis) in *B. stricta*, but epiBL might not be sufficient to induce subsequent unreduced gametophyte (embryo sac) formation. Inducing apomeiosis in sexual plants supports our hypothesis that apomixis and sex are polyphenisms of each other with capacities that coexist at least in the genomes of the plants tested herein.

**Key words:** apomixis; *Boechera; Arabidopsis*; cowpea; polyphenisms; epiBL; 5-azaC
INTRODUCTION

Since apomixis was first described in 1841, in *Alchornea ilicifolia* (Euphorbiaceae) (Asker & Jerling, 1992), this phenomenon has been observed in hundreds of plant species from more than 40 families. Efforts to understand the mechanisms of apomixis have been ongoing since its discovery, and many theories have been put forward. In 1912, Ostenfeld proposed that hybridization caused apospory (formation of the embryo sac from a nucellar cell) in *Hieracium*. Soon afterwards, it was hypothesized that hybridity as well as genetic background and polyploidy are important prerequisites for apomixis (Gustafsson, 1946). More recently, genetic studies have shown that mutations of meiosis-specific genes can disturb meiosis and turn it into mitosis. Ravi et al. (2008) showed that mutation of the *Arabidopsis* gene DYAD/SWITCH1 (SWI1), a regulator of meiotic chromosome organization, induces a 1st division restitution with some meiocytes maturing into sexually-functional unreduced spores and gametophytes. d'Erfurth et al. (2009) generated a triple mutation in *Arabidopsis, osd1/Atrec8/Atspo11-1*, which omitted the 2nd meiotic division, eliminated recombination, and modified chromatid segregation, respectively. This resulted in an apomeiotic-like phenotype. Additionally, gene loci have been identified that regulate parthenogenesis and autonomous endosperm formation. For instance, the *PsASGR-BABY BOOM-like* (PsASGR-BBML) induces parthenogenesis in *Pennisetum squamulatum* (Conner et al., 2015), and a mutation of *CUL4*, a ubiquitin ligase gene regulated by the Polycomb Repressive Complex 2 (PRC2), induces
autonomous endosperm formation in *Arabidopsis* (Dumbliauskas et al., 2011). Additionally, a large number of DNA methylation targeting genes have been found to be associated with autonomous endosperm formation in *Arabidopsis* (Schmidt et al., 2013). These discoveries have left many thinking that apomixis involves genetic mutations of genes responsible for sexual reproduction (Hand & Koltunow, 2014), but others wonder if apomixis is a polyphenism of sex wherein the same genes encode both modes of reproduction but different environment-induced epigenetic gene regulation processes cause the difference (Carman et al., 2011; Hojsgaard et al., 2014).

In favor of a genetic basis for apomixis, Koltunow et al. (2011) proposed that sexual reproduction is the default mode in facultatively-apomictic *Hieracium* subgenus *Pilosella* (capable of sex and apomixis) and that apomixis is caused by genetic mutations, such as *LOA* (*LOSS OF APOMEIOSIS*), which could deregulate the sexual pathway. So far, this view of apomixis regulation, that apomixis is derived from mutations affecting the sex pathway, is widely accepted. The implication is that it should be possible to create an artificial apomixis in crops by mutating specific genes.

The mutation-based theories, however, struggle to explain the frequently observed alternations from sex to apomixis in facultatively apomictic plants, where both modes of reproduction occur at the same time in different seeds of the same plant, or in cyclically apomictic animals and plants, where different modes of reproduction are nearly or completely favored based on cyclical changing of seasons during the year (Suomalainen et al., 1987). In both facultative and cyclical apomixis, the mode of reproduction chosen
for expression is affected by specific environmental signals. Generally, eukaryotic
apomictic, such as aphids, yeasts, diatoms, green algae, and others reproduce
apomictically in metabolically favorable environments but sexually when exposed to
stress. Often, sex is responsible for producing overwintering resting eggs or spores, a
phase of the life cycle that is essential for many of these organisms to exist in more
temperate climates (Suomalainen et al., 1987; Bilinski et al., 1989).

If sex and apomixis co-evolved, perhaps as early as eukaryogenesis, then capacities
for both modes of reproduction may still be deeply imbedded in the genomes of many
eukaryote, particularly in those groups of eukaryotes where both sex and apomixis are
still common. Many examples of environmental signaling regulating polyphenisms
epigenetically have now been studied (Jones & Sung, 2014; Sanchez et al., 2015; Berry
& Dean, 2015; Baulcombe & Dean, 2014). Why most plants are sexual while apomixis is
rare remains an interesting question. We suggest that species hybridization and/or
polyploidization can disturb the balance of epigenetic factors that might otherwise
maintain a single mode of reproduction, e.g., the sexual pathway. If this balance is
disturbed by interspecific hybridization, and if the interspecies hybrid is sexually sterile,
and if apomixis (as a polyphenism of sex) is at all expressed, then apomixis might
become established in the hybrid lineage.

Signal transduction pathways that may regulate apomixis/sex switching have been
deduced in our lab based on microarray and RNA-seq data (Fig. 4.1). For example, 203
genes associated with glycolysis and related bioenergetics pathways were up-regulated in
the immature pistils of well-watered apomictic species compared with the pistils of well-watered sexual species. Likewise, 26 glutathione transferase activity related genes were up-regulated in the pistils of well-watered versus stressed apomicts, 171 DNA methylation related genes were up-regulated in pistils of stressed compared to well-watered apomicts, and 85 brassinosteroid (BR) response genes were up-regulated in stressed compared to well-watered apomicts (Carman et al., in preparation). These pathways have now been well-studied in our lab (Mateo de Arias, 2015; Chapter 3), and we have designed treatments to induce apomixis pharmacologically in sexual plants provided they have retained in their genomes a capacity for it. In this Chapter, I report the results of such tests.

To test our proposed molecular pathways for inducing apomeiosis in sexual plants (Fig. 4.1), I conducted four sets of pharmacological experiments designed to \( i \) increase cellular bioenergetics, \( ii \) increase brassinosteroid (BR) levels, \( iii \) decrease reactive oxygen species levels (ROS), and \( iv \) decrease rates of DNA methylation (Fig. 4.1, black-circled numbers 5-8, respectively). These experiments were conducted using sexual *Boechera stricta* (A. Gray) A. Löve & D. Löve, *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh., Columbia 0), and cowpea (*Vigna unguiculata* (L.) Walp.). DNA methylation was also suppressed in a triploid apomict, *B. cf. gunnisoniana* (Rollins) W. A. Weber, which reproduces by Taraxacum-type (T-type) diplospory.
In T-type diplospory, the 1st meiotic division is restitutional (fails before recombination) and is followed by a functional 2nd meiotic (mitotic-like) division. If meiosis and syngamy are evolutionarily derived from a more primitive apomixis-like mode of reproduction, then suppressing methylation in T-type megaspore mother cells (MMC) of *B. cf. gunnisoniana* might revert them to Antennaria-type (A-type) diplospory. Here, meiosis in the MMC completely fails (no sign of meiosis initiating), and this is

![Diagram](image_url)

**Fig. 4.1.** Molecular pathway model for sex/apomixis switching in plants. White-circled numbers indicate locations in the pathway that were tested for apomixis-to-sex switching (Chapter 3). Black-circled numbers indicate locations in the pathway that were tested herein for sex-to-apomixis switching. According to our model, abundant energy supplies (light gray) induce apomixis by keeping TARGET OF RAPAMYCIN (TOR) active. This induces brassinosteroid (BR) synthesis, and BR activates many transcription factors including BRASSINAZOLE-RESISTANT 1 (BZR1). Under stress conditions (dark gray), sex is induced by reactive oxygen species (ROS). ROS suppress apomixis, by deactivating TOR, and they may also induce epigenome modifications specific to meiosis and syngamy. Experiments conducted at locations 5-8 involved: (5) enhancing cellular bioenergetics with exogenous applications of sucrose or glucose, (6) applying the BR epibrassinolide (epiBL) exogenously, (7) reducing cellular ROS levels by exogenous applications of the antioxidant (S)-2-aminobutane-1,4-dithiol hydrochloride (DTBA), and (8) preventing megaspore mother cell (MMC) DNA methylation by applying 5-azacytidine (5-azaC) exogenously to immature pistils at the pre-MMC stage. Molecular interactions represented among TOR, BR and BZR1 are after Zhang et al. (2016).
followed by female gametophyte (embryo sac) formation directly from the MMC. While efficiency improvements are needed, the conversions reported herein, from T-type to A-type diplospory, from meiosis to apomeiosis, from syngamy to parthenogenesis, and from fertilization-dependent to fertilization-independent (autonomous) endosperm formation, all support our hypothesis of sex being a derived polyphenism of more ancient apomixis-like reproductive mechanisms.

MATERIALS AND METHODS

Plant materials

Plants of *B. stricta* were collected from Duchesne County, UT as live specimens and grown in a growth chamber until seeds could be harvested. Vouchers (UTC00275884) were prepared and deposited in the Intermountain Herbarium, Utah State University, Logan, UT. Seeds of *B. cf. gunnisoniana* (Chapter 3) and *B. stricta* were germinated on moist germination paper in petri dishes at 26 °C for 1-2 weeks and planted in pots containing Sunshine Mix #1 potting soil (Sun Gro Horticulture Canada Ltd, Vancouver, BC). Plants were grown for 3-4 weeks at 24 °C (8-10 leaf stage) until they were strong enough for vernalization. *B. stricta* seedlings were vernalized at 4 °C for 10-12 weeks with minimal lighting (8/16 day/night photoperiod) from soft-white fluorescent bulbs. The vernalized *B. stricta* plants were then transferred to the growth chamber and grown using a 22/16 °C day/night temperature regime with lighting (16/8 day/night photoperiod)
supplied by incandescent, florescent and 1000 W high-pressure sodium-vapor lamps. The plants were watered regularly with a dilute solution (250 mg/L) of Peters Professional 20:20:20 fertilizer (Scotts, Maryville, Ohio). *Arabidopsis* seeds were germinated on moist germination paper in petri dishes at 26 °C for one week and planted as above. They were grown at 21 °C for one week with minimal lighting (8/16 day/night photoperiod) from soft-white fluorescent bulbs and then transferred to the growth chamber at 22/16 °C day/night temperatures with lighting and watering as indicated above for *B. stricta*. Cowpea (*Vigna unguiculata*) plants were obtained from the Plants, Soils and Climate Department teaching lab, Utah State University, and grown as above.

*Embryology*

Plant pistils were fixed in formalin acetic acid alcohol (FAA) for 48 h and mixtures of benzyl benzoate and dibutyl phthalate (Sigma-Aldrich, St. Louis, MO, USA), at a 2:1 v:v ratio (BBDP), and 95 % ethanol (EtOH) were used for clearing as described by Crane and Carman (1987) with minor modifications: i) 70 % EtOH for 30 min, ii) 95 % EtOH for 4 h, iii) 95 % EtOH:BBDP (2:1) for 2 h, iv) 95 % EtOH:BBDP (1:2) for 4 h, v) BBDP for 4 h, and BBDP overnight. For larger amounts of tissue, individual infiltration steps were extended. Pistils were measured lengthwise, from the base of the pedicel to the top of the stigma, under a dissection scope before they were mounted on slides. The developmental stages of each ovule in each pistil were studied using a BX53 microscope.
(Olympus, Center Valley, PA, USA) equipped with differential interference contrast (DIC) optics. Photomicrographs of ovule development were taken using an Olympus MicroFire 599809 camera. Ovules were scored depending on developmental stage, i.e., MMC, sexual or diplosporous dyad, sexual tetrad, aposporous initial (AI, nucellar cell as large as the meiocyte but with a single nucleus and no large vacuoles) 2, 4 or 8-nucleate embryo sac (ES2, 4, or 8), and aposporous embryo sac (AES, nucellar cell as large as the meiocyte with one or more nuclei and one or more large vacuoles). For each pistil, the pistil length and the developmental stage of the majority of ovules in the pistil was recorded. Average pistil lengths for each of the following ovule stages (Schneitz et al., 1995) were recorded: 1-II, ovule protrusions forming, pre-MMC; 2-IV, meiosis; 3-III, vacuole in immature embryo sac appears; 4-VI, nuclear divisions in endosperm well underway.

Pharmacological treatments of pistils in vivo and in vitro

Two procedures were used to expose pistils to pharmacological chemicals: i) floral dipping, where pistils remained on the plant, and ii) pistil culture, where pistils were excised and incubated on chemically amended media. In the latter case, pistils were randomly assigned to control and treatment media. The basal medium consisted of MS (Murashige and Skoog, 1962) salts with MS or B5 vitamins (Caisson Labs, Logan, UT,
USA), pH 5.75, that contained 20 g L\(^{-1}\) sucrose (Sigma-Aldrich) and 1.45 g L\(^{-1}\) Phytogel (Sigma-Aldrich).

Effects of the BR epibrassinolide (epiBL, Sigma-Aldrich, Cat No. E1641) on female meiosis in sexual \(B.\) stricta were tested by floral dipping and by pistil culture. For the floral dip experiment, epiBL was dissolved in 80 % EtOH, and intact racemes of sexual \(B.\) stricta, which contained multiple buds with pistils ranging from pre-meiosis to embryo sac maturity, were dipped in epiBL solution for 3 h. The following epiBL levels were tested: 0 (control), 0.1, 1.0 and 10.0 \(\mu\)mol L\(^{-1}\). Each treatment involved three racemes from different plants. Treated inflorescences were left on the plant for 24 h and were then fixed, cleared and studied as above. For in vitro exposure, pistils of \(B.\) stricta were excised at the MMC stage and cultured on basal medium amended with epiBL. For these experiments, epiBL was dissolved in 80 % EtOH and added by filter sterilization (0.2 \(\mu\)m filter, BioExpress, Kaysville, UT, USA) to basal media after autoclaving and before agar solidification. The following epiBL levels were tested: 0.0 (control), 0.1, 1.0, and 10 \(\mu\)mol L\(^{-1}\). Pistils were cultured on medium for 24 h (2.0-2.5 mm pistils) or 48 h (1.3-1.95 mm pistils), and were then fixed, cleared and studied as above.

The effects of incorporating 5-azacytidine (5-azaC, Sigma-Aldrich, Cat No. A2385) into pre-MMC ovules of the triploid T-type diplosporous \(B.\) cf. gunnisoniana were tested by pistil culture. Pistils of \(B.\) cf. gunnisoniana were excised at the pre-MMC stage (1.5-1.9 mm) and cultured on basal medium with B5 vitamins and amended with 5-azaC. 5-azaC was dissolved in water and added by filter sterilization as above. The following 5-
azaC levels were tested: 0.0 (control), 5, 50, and 500 μmol L⁻¹. Pistils were cultured on medium for 48 h and were then fixed, cleared and studied as above.

To evaluate effects of multiple pharmacological treatments, three pistil culture factorial experiments were designed and conducted. The first experiment was a 2 x 5 design that tested two basal medium energy sources, sucrose or glucose (Sigma-Aldrich), each at 20 g L⁻¹, against five basal media treatments, each of which included a 180 min pretreatment soak: i) basal medium, soaked in distilled water (control), ii) basal medium, soaked in 0.5 mmol L⁻¹ 5-azaC, iii) basal medium amended with 1.0 μmol L⁻¹ epiBL, soaked in 1.0 μmol L⁻¹ epiBL, iv) basal medium amended with 1.0 μmol L⁻¹ of the antioxidant (S)-2-aminobutane-1,4-dithiol hydrochloride (DTBA, Sigma-Aldrich, Cat No. 774405), soaked in 1.0 μmol L⁻¹ DTBA, and v) basal medium amended with 1.0 μmol L⁻¹ each of epiBL and DTBA, soaked in a solution containing 1.0 μmol L⁻¹ each of epiBL and DTBA. For *B. stricta*, pistils were excised at the 1-II stage (1.3-1.7 mm) to measure effects on apomeiosis and at the 2-II to 3-I (2.0 to 2.5 mm) stages to measure effects on parthenogenesis and autonomous endosperm formation. For *Arabidopsis*, pistils were excised at stages 1-II (0.5 mm) for apomeiosis, and 2-III (1.2 mm), for parthenogenesis and autonomous endosperm formation. Immediately after excision, *Arabidopsis* pistils were immersed for 60 min instead of 180 min (as above). Following their pretreatment soak, pistils were cultured horizontally on their respective chemical-amended MS media. The early-staged pistils were incubated for 5.5 d and 8 d for *B. stricta* and *Arabidopsis*, respectively. Pistils cultured at the later stages were incubated for
3.5 d and 3 d for *B. stricta* and *Arabidopsis*, respectively. For these taxa, each treatment contained at least four or at least 10 randomly-assigned pistils, respectively. Additional pistils of *B. stricta* ca. 1.5 mm long, and *Arabidopsis*, ca. 0.5 mm long, were pretreated in a 0.5 mmol L\(^{-1}\) 5-azaC solution for 1 h and transferred to one of the five media for 5.5 and 8 d for *B. stricta* and *Arabidopsis* pistils, respectively. After the designated incubation periods, pistils were fixed, cleared and studied as described above.

The second factorial experiment (2 x 6 design) was conducted using 2-II staged pistils of *Arabidopsis* and was conducted as in the first factorial, with sucrose and glucose at 20 g L\(^{-1}\), but with the following six chemical amendments: *i*) basal medium but with Gamborg’s B5 vitamins (Caisson), *ii*) basal medium (with MS vitamins), *iii*) 0.5 μmol L\(^{-1}\) epiBL, *iv*) 0.5 μmol L\(^{-1}\) DTBA, *v*) 0.5 μmol L\(^{-1}\) each of epiBL and DTBA, and *vi*) 0.5 mmol L\(^{-1}\) 5-azaC. Pistils (at least 10 per treatment) were immersed in treatment solutions for 1 h (as above) and then cultured on their respective media for 1.5 d. Additional pistils (each being ca. 0.5 mm long) were pretreated in a 0.5 mmol L\(^{-1}\) 5-azaC solution for 1 h and transferred to one of the six media for 4 d. Pistils were then fixed, cleared and studied as described above.

The third factorial (2 x 5 design) was conducted using 1-II (1.5 mm) staged cowpea pistils and was conducted as in the first two factorial experiments except for the following modifications. The basal MS medium contained Gamborg’s B5 vitamins (instead of MS vitamins), 0.5 μmol L\(^{-1}\) naphthalene acidic acid (NAA) and 5 μmol L\(^{-1}\) 6-benzylaminopurine (BAP) (MS media and hormones from Caisson). The two energy
sources were either 60 g L\(^{-1}\) sucrose or 30 g L\(^{-1}\) glucose, and the five media addenda were
\(i\) no further additions (control), \(ii\) 1.0 \(\mu\)mol L\(^{-1}\) epiBL, \(iii\) 1.0 \(\mu\)mol L\(^{-1}\) DTBA, \(iv\) 1.0 \(\mu\)mol L\(^{-1}\) of epiBL and DTBA, and \(v\) 0.1 mmol L\(^{-1}\) 5-azaC. Two pistils were incubated for 5.5 d on each of the media combinations, and an additional two pistils per combination were incubated for 8.5 d. The pistils were then fixed, cleared and studied as described above.

Statistical analyses

For statistical analyses, only ovules where mode of reproduction could be reasonably assessed were scored. Ovules were not scored if they were too young (MMC stage or earlier), too old (older 2-nucleate embryo sac stage or older), or their orientation through the microscope prevented complete sagittal views of the dyad, tetrad, 1-nucleate sexual or apomictic embryo sac, or early 2-nucleate sexual or apomictic embryo sac. The resulting cross-tabulation data were analyzed using Pearson chi-square tests for independence of table rows and columns (SYSTAT, 2004).

RESULTS

Pistil length stages and germline development

To expose ovules to chemicals at specific germline development stages, it was first necessary to characterize, for each species, the relationship between pistil length and
germline stage. The average germline stage by pistil length is shown in Table 4.1 for *B. stricta*, *Arabidopsis* and cowpea and was reported in Chapter 3 for *B. cf. gunnisoniana*.

### Table 4.1. Average pistil length by ovule development stage for *B. stricta*, *Arabidopsis* and cowpea. Length are an average of 1-6 pistils per stage by species combination.

<table>
<thead>
<tr>
<th>Species</th>
<th>Average pistil length (mm) by stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-II</td>
</tr>
<tr>
<td><em>B. stricta</em></td>
<td>1.5</td>
</tr>
<tr>
<td><em>Arabidopsis</em></td>
<td>0.5</td>
</tr>
<tr>
<td>Cowpea</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Suppressing MMC methylation in *B. cf. gunnisoniana* converts meiotically-disturbed *T*-type diplospory to meiotically-absent *A*-type diplospory

T-type diplospory, Hieracium-type apospory and sexual meiosis occur frequently in ovules of facultatively-apomictic *B. retrofracta x stricta*. Interestingly, this taxon also exhibits a low frequency (3 %) of A-type diplospory (Chapter 2). We hypothesized that
T-type diplospory represents a partial suppression of meiosis, while A-type diplospory represents complete meiotic suppression.

The observations that i) epigenome reprogramming is required for sexual reproduction (Kawashima & Berger, 2014; Grimanelli, 2012) and ii) inactivation of DNA methylation can induce apomixis-like phenotypes (Garcia-Aguilar et al., 2010; Galla et al., 2017) suggest that apomixis may be the default pathway with sex being the more

![Graph](image_url)

**Fig. 4.2.** Frequencies of triploid *B. cf. gunnisoniana* ovules that were initiating Taraxacum-type diplospory, Polygonum-type sexual development, Antennaria-type diplospory, or Hieracium-type apospory at the end of pistil culture treatment as affected by a 5 min pre-culture soak in solutions containing 5-azacytidine (5-azaC). Pistils were initiated in culture at the 1-I stage. Numbers of ovules per level of 5-azaC averaged 487 and ranged from 288-590.
derived add-on program. Thus, if methylation in sexual plants can be prevented, or if DNA can be demethylated, the apomictic pathway might surface as the default pathway. 5-azaC, an inhibitor of DNA-cytosine methyltransferases, produces upon DNA replication hypomethylated DNA (Santi et al., 1984; Christman, 2002). It is a chemical analogue of cytosine that is incorporated into DNA by competition with cytosine during DNA synthesis. In 5-azaC, the carbon-5 of the cytosine ring, which is essential to DNA methyltransferase function, is replaced by nitrogen, and this causes failure of methylation at each 5-azaC site (Stresemann & Lyko, 2008).

5-azaC was incorporated into archesporial nucellar cells of the T-type diplosporous Boechera cf. gunnisoniana (Mateo de Arias, 2015; Chapter 2) prior to the mitoses that produce MMC from archesporial cells. The highest concentration of 5-azaC (500 μmol L⁻¹) induced A-type diplospory (embryo sac forming directly from an a-meiotic MMC) in 10 % of the ovules (Figs. 4.2, 4.3).

Multiple pharmacological treatments convert meiosis to apomeiosis

The research reported in Chapter 3 proved that sexual processes can be induced in apomictic Boechera by introducing stresses at multiple steps along signal transduction pathways, which are commonly traversed as a result of plant stresses. Here, it is shown that the same steps as were modified in Chapter 3, to induce sex in apomictic Boechera, can be modified in the reverse direction (Fig. 4.1), to minimize sex-causing stress signals
and to induce the onset of apomixis in sexual *Boechera*, sexual *Arabidopsis*, and sexual cowpeas.

epiBL is the naturally occurring and most biologically active form of BR (Li et al., 2002). Applications of epiBL to pistils of sexual *B. stricta*, in vivo or in vitro, induced significantly higher (P < 0.001) frequencies of dyad formation than in the controls (Fig. 4.4). In vivo, the most effective treatment involved dipping flower buds of *B. stricta* for 3 h in a 10 μmol L⁻¹ solution of epiBL. This induced 31 % dyad formation, compared to 7 % for the controls. A similar dyad induction frequency (29 %) was obtained by culturing 1-I to 2-II staged *B. stricta* pistils on a 0.1 μmol L⁻¹ epiBL medium for 2 d (Fig. 4.4).
Under normal conditions, *B. stricta* is an obligate sexual plant (Mateo de Arias, 2015), and the sexual dyad stage, during meiosis, is very short. Often, but not always, it is difficult to catch the sexual dyad stage in pistil fixations because it is so brief (usually less than 5% of meiocyte-staged fixations). In contrast, the dyad stage of *Taraxacum*-type diplosporous apomixis (1st division restitution resulting in a dyad of unreduced megaspores) is a lengthy stage, like the tetrad stage of sexual plants. Hence, the dyad stage is readily observed in ovule fixations of diplosporous *Boechera*. Although ratios of observed dyads increased to 30% in the epiBL treatments (higher than expected for
sexual meiosis), unreduced embryo sacs forming from epiBL-induced dyads (with prominent vacuoles) were not observed. Such embryo sacs should have formed if the dyads were undergoing diplosporous development, i.e., the surviving megaspore (chalazal member) would have started to develop large vacuoles and would have begun consuming the degenerating dyad member and adjacent nucellar cells. Low frequencies of such observations (Fig. 4.5e) suggest that BR alone is insufficient to efficiently convert

Fig. 4.5 Ovules of *B. stricta* treated with epiBL in vivo (a-c) and in vitro (d-f). a, d: reduced tetrads, white arrows point to surviving megaspores, black arrows point to degenerating megaspores; b, e: unreduced dyads (typical of diplosporous apomeiosis in apomicts) in epiBL treated pistils, white arrows point to surviving megaspores, black arrows point to degenerating megaspores; c: sexual triad (failure of 2\textsuperscript{nd} meiotic division in the micropylar dyad member), alternatively, a diplosporous dyad with a parietal cell, black arrows point to degenerating megaspores, white arrow points to the putative surviving megaspore; f: aposporous embryo sac (AES) with degenerating tetrad, white arrow points to the AES nuclei, black arrows point to degenerating megaspores; v, vacuoles; scale bar: 20 μm.
meiosis to a persisting apomeiosis. Thus, our evidence indicating that Taraxacum-type apomictic dyad formation is inducible by BR treatments alone is minimal.

Apospory is the formation of unreduced embryo sacs from one or more nucellar cells that reside adjacent to the MMC. Low frequency aposporous embryo sac (AES) formation occurred in ovules of the in vitro but not the in vivo epiBL-treated pistils (Fig. 4.5f). Also, AES formation was observed in a control treatment (without epiBL). For pistils cultured at the older stage (2-III to 2-IV), AES frequencies were 0 and 7 % for the 0.0 and 0.1 μmol L⁻¹ epiBL treatments, respectively. For pistils cultured at the younger stage (more time on the epiBL treatment), these frequencies were 0 and 7 %, respectively. Interestingly, several stress treatments reported in Chapter 3 either induced the onset or increased the frequencies of AES in Boechera apomicts that are generally diplosporous.

Since AES formation occurred in tissue cultured pistils without epiBL, it seems reasonable that this response was induced by the stress of removing pistils from the parent plant and culturing them on tissue culture media. It is also possible that the 7 % apospory (for pistils exposed to epiBL for a longer period of time) was caused by BR-induce expression of SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK), a gene that has been correlated with apospory in Paspalum (Podio et al., 2014).

Triads (Fig. 4.5c) were also observed in the BR treatments. During normal meioses, MMC do not divide to form triads, but the epiBL treatments may have stopped the 2nd meiotic division in the micropylar dyad member. Another possibility for “triads” is that the triad is a normal dyad with a parietal cell. Parietal cells are cell layers between the
epidermis of the nucellus and the megasporocyte (Rudall, 1997). They are derived from the archespore, which divides into a proximal MMC and a distal parietal cell (Davis, 1967), and the parietal cell may divide several times to form multiple layers. Parietal cells are common in the ovules of many plants (Nonomura et al., 2007; Canales et al., 2002; Yang & Sundaresan, 2000), including sexual and apomictic *Boechera* where they have been associated with diplosporous embryo sac formation (Bocher, 1951; Naumova et al., 2001). Due to the uncertainty of the origin of triads, they were not used to ascertain the occurrence of diplospory in the present study.

In the present study, pistils were treated with epiBL (Fig. 4.4) to induce BR-dependent pathways that might induce apomixis (Fig. 4.1) and indirectly reduce plant stress by upregulating ascorbate peroxidase, which reduces H$_2$O$_2$ (Belmonte et al., 2010). As H$_2$O$_2$ was used in Chapter 3 to increase ROS stress, thus inducing sexual meiosis in apomicts, here I asked whether ROS levels in ovules could be lowered by applications of antioxidants? If so, could this approach be more efficient than BR applications for reducing ROS levels and inducing apomixis? To answer this question, pistils were exposed to the antioxidant DTBA, a newly developed antioxidant that protects thiol groups in proteins from oxidation. Historically, dithiothreitol (DTT) was used, but DTBA is as much as 10-fold more effective as an antioxidant (Adamczyk et al., 2015; Reddy & Metanis, 2016; Suttirojpattana et al., 2016).

Glucose signaling is one of the most ancient and central signaling pathways in animals and plants and is involved in gene expression, primary and secondary
metabolism, and regulation of growth and development (Moore et al., 2003; Sheen, 2014; Cho et al., 2006; Smeekens et al., 2010). In *Arabidopsis*, rates of glycolysis and mitochondrial bioenergetics are sensed by TOR, which then control root meristem activation (Xiong et al., 2013). In Chapter 3, I induced meiosis in apomictic plants by depriving cultured pistils from sugars. The goal was to suppress TOR. Here, if sufficient sugar is supplied, TOR should be activated. Active TOR protects BZR1 and other important BR-regulated enzymes from degradation, and this leads to plant growth (Zhang et al., 2016).

To test the effects of sucrose, glucose, epiBL, DTBA and 5-azaC on apomixis induction, I designed factorial experiments for each of three sexual species, *B. stricta*, *Arabidopsis* and cowpea. The experiments were performed and ratios of apomeiosis to meiosis were documented. Two factorial experiments were conducted using immature *Arabidopsis* pistils. In the first experiment, numbers of scorable ovules were too low to perform Chi square analyses on individual chemical components, but they were high enough to compare the main effects (compiled across like treatments) of no 5-azaC exposure, 5-azaC in the pretreatment only, or 5-azaC in both the pretreatment and the tissue culture medium. In this experiment, 5-azaC significantly increased the frequency of enlarged, strongly-vacuolate nucellar cells (P < 0.05), which are referred to herein as
putative aposporous embryo sacs (PAES). In 6% of ovules, 5-azaC, in the pretreatment and tissue culture media, caused sexual meiosis to abort and be replaced with low-frequency A-type diplosporous embryo sac formation (Figs. 4.6, 4.7).

A second factorial experiment involving cultured Arabidopsis pistils was conducted based on the results of the first experiment. Here, larger numbers of pistils were cultured, and numbers of scorable ovules were sufficient to evaluate the effects of individual chemical supplements, and the differences among these were highly significant (P <
All of the treatments greatly increased dyad frequencies (putative T-type diplospory), and 5-azaC (as pretreatment and in the culture medium) induced A-type diplospory (AntES) in 26% of the ovules. Between AES formation, T-type diplospory and A-type diplospory, 90% of the 5-azaC-treated Arabidopsis ovules were putatively

Fig. 4.7. Ovules of Arabidopsis treated with epiBL, DTBA, epiBL plus DTBA and 5-azaC in sucrose or glucose medium. a: meiotically-reduced tetrad in the sucrose control medium, white arrow, surviving megaspore, black arrows, degenerating megaspores; b: 2-nucleate Taraxacum-type (T-type) diplosporous embryo sac (ES2) from unreduced dyad in the epiBL-amended treatment, white arrows, ES2 nuclei, black arrow, degenerating megaspore; c: aposporous embryo sac (AES) in the DTBA-amended treatment, white arrows, AES nucleus, black arrows, degenerating megaspores of a degenerating tetrad; d: unreduced T-type diplosporous dyad in the DTBA-amended treatment, white arrow, surviving megaspore, black arrow, degenerating megaspore; e: unreduced T-type diplosporous dyad in the 5-azaC-amended treatment, white arrow, surviving megaspore, black arrow, degenerating megaspore; f: AES in the 5-azaC-amended treatment, white arrows, the AES nuclei, black arrows, degenerating megaspores of a degenerating tetrad; g: Antennaria-type embryo sacs (AntES) in the 5-azaC-amended treatment, white arrows, AntES nuclei, v, vacuoles; scale bar, 20 μm.
initiating apo
mixis (Figs. 4.7, 4.8). Further research is needed to determine if these
apomixis onset events can persist to produce unreduced embryo sacs that contain
parthenogenetically competent unreduced eggs.

In the factorial experiment conducted using immature *B. stricta* pistils, all
treatments, including glucose instead of sucrose, increased dyad frequencies (putative T-
type diplospory; Fig. 4.9). Both the carbohydrate variable and the other components
variable were highly significant (P < 0.001) with regard to increasing dyad and reducing

**FIG. 4.8.** Frequencies of arabidopsis ovules undergoing tetrad, dyad, aposporous embryo sac (PAES), and Antennaria type diplosporous embryo sac (AntES) formation at the end of pistil culture as affected by epiBL, epibrassinolide; DTBA, (S)-2-aminobutane-1,4-dithiol hydrochloride; BL plus DTBA; or 5-azaC, 5-azacytidine. Numbers of correctly-staged ovules per treatment averaged 34 and ranged from 16-42.
tetrad frequencies. Additionally, three ovules from the sucrose plus epiBL treatment (epiBL in the pretreatment and in the medium) had formed AES. Also, two ovules, one from the sucrose-epiBL-epiBL treatment and one from the glucose-DTBA-DTBA treatment, had formed A-type diplosporous embryo sacs (Fig. 4.10).

The cowpea experiment was conducted as a preliminary experiment to determining if intentionally manipulating the biochemical pathways that switch apomixis to sex or vice versa in Boechera and Arabidopsis (both of the Brassicaceae family) would also work.
when applied to an important world crop from a different angiospermous family, the legume family, Fabaceae. Unfortunately, only 34 ovules from among the multiple treatments of this experiment were scorable, which was too few to evaluate chemical treatments individually for their effects on megasporogenesis and embryo sac development. However, of the 34 scorable ovules, 24 had initiated A-type diplospory
(Table 4.2; Fig. 4.11). Nine of these were from the 5-azaC treatment (pretreatment and in the culture medium), and 8 were from the DTBA treatment (in both the pretreatment and the culture medium).

Apomixis encompasses three major parts: apomeiosis, parthenogenesis and endosperm formation, which either requires fertilization of the embryo sac central cell (pseudogamous endosperm formation) or autonomous (fertilization independent) endosperm formation. The major goal of my research was to develop methods for inducing a natural form of apomixis in typically sexual plants. By doing so, we hypothesized that the resulting unreduced embryo sac might already be programmed for
parthenogenesis and possibly even autonomous endosperm formation, i.e., the embryo sac might not require additional experimental stimuli to conclude the apomictic program.

Our tissue culture experiments functioned well for the few days that was required for ovules to develop from the pre-MMC stage to the active meiocyte or apomeiocyte stage. But they failed to sustain normal ovary and ovule development for the lengths of time needed to mature the embryo sacs and test for parthenogenesis. The failure of long time
culturing might be because we couldn’t provide a suitable *in vitro* medium for pistils to grow healthily from meiosis stage to parthenogenesis (about 7 to 10 days). Another reason to explain the failure would be pseudogamy. In some apomictic plants, the endosperm formation still requires fertilization, and the development of the endosperm would be required for embryos to grow (Normark, 2014). In our study, pistils were cultured on medium before pistils or anthers were mature. Hence, there was no chance for pseudogamous endosperm formation, only autonomous endosperm formation, if it is possible. Interestingly, we still observed a few parthenogenetic embryos, on the female

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**FIG. 4.12.** Parthenogenetic embryos and unreduced pollen microspores in *Arabidopsis* pistils and anthers cultured *in vitro* and treated with DTBA and epiBL, respectively. a, b, c: parthenogenetic embryos (white arrows) induced by 0.1 μM DTBA on 2% glucose medium for 7 d; d, e: unreduced pollen dyads of microspores among reduced pollen tetrads of microspores (two microspores in focal plane for dyads versus three microspores in focal plane for tetrads) induced by 0.1 μM epiBL on 2% sucrose medium for 24 h; scale bars: 50 μm (a), 20 μm (b-e).
side, and a few unreduced pollen grains on the male side, but their frequencies were very low (Fig. 4.12).

DISCUSSION

Our results indicate that if we demethylate DNA before sexual MMC form, they will often develop apomictically into an unreduced female gametophyte. However, due to the general toxicity of 5-azaC (Christman, 2002), we were able to collect only a limited amount of data on 5-azaC treatments. In our study, most 5-azaC-treated ovules degenerated or were greatly suppressed in their growth. The situation was a little better when we reduced the 5-azaC concentration and the pretreatment exposure time to 1 h. Both DTBA (the antioxidant) and 5-azaC induced i) dyad formation, with some of them following the T-type of diplosporous embryo sac formation, ii) AES formation, and iii) the A-type of diplosporous embryo sac formation. On glucose medium with 5-azaC, the same patterns were observed.

In plants, BR are important growth regulators that contribute to cell elongation and division (Fridman & Savaldi-Goldstein, 2013; Gudesblat & Russinova, 2011; Bajguz, 2007), vascular differentiation (Zhang et al., 2014), reproductive determination (Gerashchenkov & Rozhnova, 2013; Hartwig et al., 2011) and regulation of gene expression (Hou et al., 2017; Wang et al., 2014; Guo et al., 2013). Recently, BR signaling model in Arabidopsis have been well studied (Wang et al., 2012). In the presence of BRs, BR binds to extracellular domains of the membrane receptor BRI1
(BRASSINOSTEROID INSENSITIVE 1) and BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1), which is also named SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3), which is related to the Arabidopsis AtSERK1 to AtSERK5 (Hecht et al., 2001). SERK genes play an important role in embryogenesis in angiosperms and have been implicated in many studies of apomixis (Podio et al., 2014; Talapatra et al., 2016; Santos & Aragão, 2009). Schmidt et al. (1997) reported somatic embryos formed (fertilization-independent formation of embryos) from cells expressing a SERK (DcSERK) gene in Daucus carota (carrot). Tucker et al. (2003) studied a SERK-Like gene in Hieracium (HpSERK-L) which is a homologue of AtSERK1 and DcSERK, was highly expressed during early ovule development until early seed development in both sexual and apomictic plants suggesting SERK genes might be a shared molecular element in sexual and apomictic reproduction. However, in Poa pratensis, SERK genes (PpSERK1 and PpSERK2) are highly expressed in the MMC of sexual plant but not in apomictic plant, also PpSERK were highly expressed in nucellar cells which suggesting that they could involve in forming of aposporous initials (AIs) (Albertini et al., 2005). Similarly, in Paspalum notatum, PnSERK2 is highly expressed in the MMC of sexual plants and in the nucellar cells of apomictic plants (Podio et al., 2014).

In the Arabidopsis model, the BRI1-BR-BAK1 complex induce BRI1 and SERK by phosphorylation (Zhang et al., 2014). The activated BRI1 phosphorylates two receptor-like cytoplasmic kinases: BSK1 (Brassinosteroid-Signaling Kinase 1) and CDG1 (Constitutive Differential Growth 1), which subsequently activate the BRI1-
SUPPRESSOR 1 (BSU1) phosphatase, a phosphatase that suppress the kinase activity of BIN2 (Brassinosteroid Insensitive 2) by dephosphorylation (Kim et al., 2011). With the suppression of BIN2, the inhibition of BZR1 (Brassinazole Resistant 1) and BZR2 (Brassinazole Resistant 2, also named as BES1, BRI1-EMS-SUPPRESSOR 1) is released. BZR1 and BZR2 would accumulate in the nucleus regulate the expression of more than one thousand downstream genes (Wang et al., 2012; Hou et al., 2017; Wang et al., 2014). In addition, activated BRI1 also phosphorylates BKI1 (BRI1 Kinase Inhibitor 1) to prevent the suppression of BZR1 and BZR2 (Wang et al., 2011).

As discussed in Chapter 3, BZR1 encodes a positive regulator of the BR signaling pathway that mediates both downstream BR responses and negative feedback regulation of BR biosynthesis (Wang et al., 2002). In short, BR released the inhibition of BZR1 would lead to plant growth, and as shown in this chapter, it could activate pathways leading to apomixis.

In addition to the role in plant growth, BRs also protect plants from a variety of environmental stresses including drought, changes of temperature, high salinity and heavy metal as well as the invasion of pathogen/pest (Divi et al., 2010, 2009; Bajguz & Hayat, 2009; Krishna, 2003). BRs have been studied to help plant to tolerant stress by employing the treatments with exogenous BRs (Kagale et al., 2007; Li et al., 2013; Xia et al., 2009).

In Chapter 3, I reported stress induced meiosis in apomictic Boechera. We hypothesized that sex (meiosis/syngamy) and apomixis (apomeiosis/parthenogenesis) are
polyphenisms that evolved simultaneously during eukaryogenesis, share the same genes, but express them differently by epigenetic regulation triggered by environmental signals. That is to say, apomeiosis and meiosis are possibly still embedded in the genomes of many plants. By eliminating stress signaling or blocking stress signaling pathways, apomeiosis and, to a limited extent, parthenogenesis were expressed.

One source of information leading to our view of apomixis evolution and regulation is the gene expression experiments previously conducted in our lab. From these data, involving sexual and apomictic Boechera and recent RNAseq data in stressed and nonstressed sexual and apomictic plants, we noticed DNA methylation related genes were up-regulated in sexual plants and in stressed apomicts wherein sex was induced. By following these leads, we were able to extend our intentional induction of sex in apomictic plants and apomixis in sexual plants by specific pharmacological treatments.

We hypothesized that by eliminating stress signals and methylation, we would be able to reveal apomixis from sex. So far in our study, we induced diplospory apomixis, apospory apomixis, Antennaria-type apomixis, parthenogenesis and unreduced pollen microspore formation in three different sexual plants. Although our experiments have need of a lot of improvements, our studies provide strong evidence for our hypotheses. I hope my studies will provide useful information for future investigations of apomixis.
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CHAPTER 5

SUMMARY

Apomixis in flowering plants is asexual reproduction through seeds, i.e., without meiosis or fertilization. This results in the clonal multiplication of the maternal plant. It provides a potential method of propagation in agriculture, especially in hybrid seed production, to fix hybrid vigor over generations. Although apomixis occurs naturally in more than 400 species, it is rarely found in major crops. The efforts to incorporate this trait into crops are challenging because of our limited knowledge of how apomixis evolved and how it is regulated. To understand the genetic mechanism of apomixis, our lab performed gene differential expression analyses using microarrays to compare floral tissues of sexual and apomictic plants and RNAseq to compare floral tissues of well-watered and stressed sexual and apomictic plants. Our data suggest that genes related to stress, signal transduction and epigenetics regulate whether apomixis or sex occurs. Also, sexual and apomictic reproduction pathways used the same sets of genes, which suggests that capacities for sex and apomixis coexist at least in the Boechera (Brassicaceae) genome. These findings are consistent with the concept that sex and apomixis are polyphenisms of each other, which possibly coevolved during eukaryogenesis (Carman et al., 2011; Hojsgaard et al. 2014). Accordingly, gene expression differences between apomictic and sexual plants might be triggered by the presence or absence of stress signaling. An objective of my research was to identify treatments that shift reproduction from apomixis to sex in Boechera and signal transduction pathways that regulate
apomixis/sex switching. I then performed pharmacological treatments to disturb these pathways. Similar treatments were performed by disturbing the epigenetic status of sexual *Boechera* before megaspore mother cells formed, and these studies indicated that removing the meiosis program epigenetically permitted apomixis to proceed, apparently as a default process.

*Boechera* (2*n* = 14, Brassicaceae) is an important genus for studying apomixis. 38 out of 110 species in *Boechera* are thought to be apomictic, and these reproduce either by diplospory and/or apospory (Chapter 2). Diploid apomicts and sexuals occur in *Boechera*, which we used to eliminate the confounding effects of polyploidy when comparing differential expression of genes between sex and apomixis. In addition, *Boechera* is closely related to *Arabidopsis thaliana* (2*n* = 10, Brassicaceae), which permit the usage of *Arabidopsis*-based genomics tools with *Boechera*. To investigate the embryology of *Boechera*, I identified and documented the reproduction mode of *B. yellowstonensis*, *B. innahaensis*, and two accessions of *B. exilis*. All of these species are sexual, although very low frequencies of possible diplospory and aposporous initials were observed in all three. It seems, from studying many *Boechera* taxa in our lab, that most *Boechera* retain a capacity for apomixis and are thus facultative to some extent. That naturally occurring interspecific and intraspecific hybridization between sexual *Boechera* species produces diploid apomicts (Beck et al., 2012) is also consistent with our hypothesis that capacities for apomixis and sex coexist in the plant genome, which are occasionally triggered by epigenetic disturbances such as hybridization and/or polyploidization.
To further investigate controls of the reproduction mode and embryology of *Boechera*, we used pistil length as a sporophytic parameter to identify the stages of meegasporogenesis and microsporogenesis in *B. cf. gunnisoniana*, *B. lignifera*, *B. retrofracta x stricta* and *B. stricta*. In general, the pistils of apomicts develop faster than their sexual relatives, which reflects a precocity of flowering as observed in many apomicts. Meiosis/apomeiosis occurred in microspore mother cells in all of these species much earlier than meiosis/apomeiosis occurred in megaspore mother cells.

Embryologically, we had some new findings. In triploid *B. cf. gunnisoniana*, we observed triad and tetrad formation during meiosis, but because of triploidy, the tetrad spores would rarely possess balanced sets of chromosomes, and previous flow cytometry experiments indicated that only the apomeiotically formed spores were viable (Mateo de Arias, 2015). In *B. retrofracta x stricta*, we observed 55 % Taraxacum-type diplosporous embryo sac formation along with 30 % Hieracium-type aposporous embryo sac formation and 3 % Antennaria-type diplosporous embryo sac formation. The coexistence of three major types of apomixis in one species is rare, and it suggested that the underlying causal mechanisms in each apomixis type may be similar. In *B. lignifera*, both reduced (tetrad) and unreduced (dyad) pollen were observed at the same time, which suggests a 2C:5C and a 2C:6C embryo to endosperm genome ratio, respectively. This was another evidence to explain facultativeness in *Boechera*.

According to our previous studies, long term stress causes the shift from apomeiosis to meiosis (Mateo de Arias, 2015). My research shows that short term stress
or the elimination of stress switches development from apomeiosis to meiosis or from meiosis to apomeiosis, respectively, and that these switches can be induced when treatments are applied within hours of when female apomeiosis or female meiosis would normally occur. To better elucidate these effects, I performed several pharmacological treatments designed to interrupt different components of stress signaling pathways. Using polyethylene glycol (PEG) as a supplement to tissue culture medium, drought stress was simulated, and this was imposed on appropriately stage immature pistils in vitro. For *B. retrofracta x stricta* (55% of MMC produce diplosporous embryo sacs under control conditions), low levels of PEG-6000 (20 g L\(^{-1}\)) caused nearly all MMC to switch to meiosis, but it also caused high frequency (40% - 100%) Hieracium-type apospory but with most of the associated tetrads (all four spores) undergoing degeneration. Thus, while this stress treatment caused MMC to undergo meiosis, it also caused the mode of apomixis to switch from diplospory, with no sexual meiosis, to apospory, with sexual meiosis followed by degeneration of all sexually derived spores. We suspected that stress might cause the shift in MMC cell fate, from apomeiosis to meiosis, but the shift in nucellar cell fate, from impending apoptosis to apospory, was surprising. For *B. cf. gunnisoniana*, which is normally highly diplosporous (90+%), 55% of ovules were induced to under meiosis when pistils were exposed to the 30 g L\(^{-1}\) PEG-6000 treatment, however apospory was also occasionally observed in these treatments. Ovules of *B. lignifera* pistils suffered severe degeneration under even the lowest PEG treatment, nevertheless, several aposporous and diplosporous ovules were also observed. These PEG
results are significant (P < 0.003) for both PEG concentration and pistil stage at culture initiation, and they are consistent with the long term stress results reported by Mateo de Arias (2015) in our lab.

The results of sugar starvation varied depending on species: no differences were observed for *B. retrofracta x stricta*, but up to 60 % (P < 0.001) of ovules produced tetrads (ovules switched from apomeiosis to meiosis) when sucrose was withheld from the tissue culture medium for *B. cf. gunnisoniana*. Pistil stage was also highly significant.

In order to elucidate effects of timing of exogenously applied reactive oxygen species (ROS) on apomeiosis to meiosis switching, excised pistils at varying stages of development were exposed to a short 5 min exposure to H\(_2\)O\(_2\) *in vitro*. 50 μmol L\(^{-1}\) H\(_2\)O\(_2\) worked effectively for *B. lignifera* (up to 36 % of ovules switched from apomeiosis to meiosis, to produce tetrads). 100 μmol L\(^{-1}\) H\(_2\)O\(_2\) significantly induced tetrad formation in *B. retrofracta x stricta* and *B. cf. gunnisoniana* (up to 70 % and 55 %, respectively). For all three species, the best pistil stage for inducing tetrads was 2-IV (meiosis I in sexual plants). Thus, the early apomeiosis stage was most sensitive to redox level switching from apomeiosis to meiosis. The reason why younger pistils failed to switch from an apomeiotic to a meiotic pathway when exposed to ROS remains to be determined. Possibly, the temporal window for switching reproductive development immediately proceeds apomeiosis (or meiosis), and pistils exposed to brief exposures at earlier stages were able to detoxify the ROS through endogenous antioxidant processes. If the DNA of apomictic ovules is damaged by ROS that are not detoxified by endogenous oxidative
buffer systems, then DNA repair mechanisms, such as recombination by crossing over, as happens during Meiosis I, may be initiated, and this may explain why sexual reproduction is induced.

We also treated pistils of *B. cf. gunnisoniana* with BRZ, a brassinosteroid (BR) synthesis inhibitor, and showed a significant shift \( P < 0.05 \) from apomeiosis to meiosis. This is strong evidence that the TARGET OF RAPAMYCIN (TOR)-BR-BZR1 molecular stress response pathway is involved in the apomeiosis/meiosis switch. In this respect, multiple stresses (long term and short term) were able to induce the shift from apomeiosis to meiosis, but many times apospory was also induced. For apomictic plants, the 2-IV stage was the most effective stage for shifting apomeiosis to meiosis.

We performed several pharmacological treatment experiments on three sexual plant species with the intention of switching sex to apomixis. Applications of epiBL (BR) to pistils of sexual *B. stricta*, *in vivo* or *in vitro*, induced highly significant \( P < 0.001 \) frequencies of dyad formation (30 %) compared to controls (≤5 %). The most effective concentrations of *in vivo* and *in vitro* epiBL treatments were 10 μmol L\(^{-1}\) and 0.1 μmol L\(^{-1}\), respectively. However, unreduced embryo sacs forming from epiBL-induced dyads were only rarely observed, which suggests that BR alone may be insufficient to efficiently convert meiosis to a persisting apomeiosis. Low frequencies of apospory (7 %) were also induced by the *in vitro* treatments, and we assume that *SERK* genes may play an important role in aposporous apomixis.
To better elucidate the effects of pharmacological treatments, I conducted factorial experiments wherein various levels of epiBL, DTBA, epiBL plus DTBA, and 5-azaC were applied using media amended with sucrose or glucose. These experiments were conducted using sexual *B. stricta*, sexual *Arabidopsis* and sexual cowpea. Two of the experiments did not produce enough data to perform statistical analysis for all tested chemicals, but we were still able to determine statistically (P < 0.05) that 5-azaC induces aposporous apomeiosis (30 % - 35 %) and a low frequency of Antennaria type diplosporous apomeiosis (6 %) in *Arabidopsis*. In cowpea, 5-azaC as well as epiBL, DTBA and glucose induced Antennaria type diplosporous apomeiosis (in 24 of 34 ovules). All treatments, including glucose instead of sucrose, significantly (P < 0.001) increased dyad frequencies (putative Taraxacum-type diplosporous apomeiosis) in *B. stricta* and *Arabidopsis*. Hieracium type aposporous apomeiosis and Antennaria type diplosporous apomeiosis were also induced by several of these treatments, especially in *Arabidopsis*.

We hypothesized that Taraxacum type diplospory occurs as a result of partial suppression of meiosis, while Heiracium type apospory and Antennaria type diplospory represent complete meiotic suppression, the former also usually comes along with abortion of a superimposed apoptosis program (associated with sexual expression) in a limited number of evolutionarily sporogenous nucellar cells. Accordingly, we observed Antennaria type diplospory (10 %) in typically diplosporous (Taraxacum type) *B. cf. gunnisoniana* pistils when treated with 500 μmol L⁻¹ 5-azaC *in vitro*. This result provided
us new thoughts on inducing partial or full apomixis in sexual plants by metabolic, genetic or epigenetic modifications. Although apomeiosis was induced in our experiments, inducing parthenogenetic embryo and autonomous endosperm formation are still challenges due to a putative pseudogamous endosperm formation requirement.

Overall, our experiments support the hypothesis that apomixis and sex are polyphenisms of each other. We induced meiosis in apomictic plants by pharmacological treatments and *vice versa*. I hope these few informative studies will provide useful information for the further investigation and development of an effective apomixis technology for crop improvement.

LITERATURE CITED


Mateo de Arias, M. 2015. Effects of Plant Stress on Facultative Apomixis in *Boechera* (Brassicaceae). PhD Dissertation, Utah State University, Logan, UT.
APPENDICES
Statistical analyses

\textit{B. retrofracta x stricta} pistils treated on PEG medium

Frequencies
\textsc{AGE$\$$(\text{rows})$$ \text{by MODE$\$$ (columns)}}

\begin{tabular}{lccc}
\hline
& Apospory & DYAD & TETRAD & Total \\
\hline
1-I & 143 & 11 & 145 & 299 \\
2-II & 251 & 138 & 262 & 651 \\
2-IV & 365 & 121 & 394 & 880 \\
Total & 759 & 270 & 801 & 1830 \\
\hline
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Frequencies
\textsc{PEG (rows)$$ \text{by MODE$\$$ (columns)}}

\begin{tabular}{lccc}
\hline
& Apospory & DYAD & TETRAD & Total \\
\hline
0 & 222 & 43 & 244 & 509 \\
20 & 277 & 38 & 294 & 609 \\
40 & 131 & 112 & 131 & 374 \\
60 & 129 & 77 & 132 & 338 \\
Total & 759 & 270 & 801 & 1830 \\
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\textit{B. cf. gunnisoniana} pistils treated on PEG medium

Frequencies
\textsc{PEG (rows)$$ \text{by MODE$\$$ (columns)}}
\textsc{AGE$\$ = 2-II}
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Frequencies

AG$E$ (rows) by MODE$ (columns)
Likelihood ratio Chi-square 11.899 2.000 0.003

**B. retrofracta x stricta** pistils treated on no sucrose medium

Frequencies
SUGAR (rows) by MODE$ (columns)

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**B. cf. gunnisoniana** pistils treated on no sucrose medium

Frequencies
AGE$ (rows) by MODE$ (columns)

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Frequencies
SUGAR (rows) by MODE$ (columns)

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### B. retrofracta \times stricta pistils treated with H\textsubscript{2}O\textsubscript{2}

Frequencies
STAGE\$ (rows) by MODE\$ (columns)

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### B. lignifera pistils treated with H\textsubscript{2}O\textsubscript{2}

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H\textsubscript{2}O\textsubscript{2} (rows) by MODE\$ (columns)

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Frequencies
H2O2 (rows) by MODE$ (columns)

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B. cf. gunnisoniana pistils treated with H2O2

Frequencies
AGE$ (rows) by MODE$ (columns)

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<th>Tetrad</th>
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<td>2-III</td>
<td>66</td>
<td>467</td>
<td>143</td>
</tr>
<tr>
<td>2-IV</td>
<td>309</td>
<td>965</td>
<td>598</td>
</tr>
<tr>
<td>Total</td>
<td>375</td>
<td>1432</td>
<td>741</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test statistic</th>
<th>Value</th>
<th>df</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-square</td>
<td>62.397</td>
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</tr>
<tr>
<td>Likelihood ratio Chi-square</td>
<td>63.991</td>
<td>2.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Cochran’s Linear Trend</td>
<td>1.949</td>
<td>1.000</td>
<td>0.163</td>
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</table>
Frequencies
H2O2 (rows) by MODE$ (columns)

<table>
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<tr>
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<th>Tetrad</th>
<th>Total</th>
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<tbody>
<tr>
<td>0</td>
<td>41</td>
<td>450</td>
<td>72</td>
<td>563</td>
</tr>
<tr>
<td>50</td>
<td>51</td>
<td>282</td>
<td>86</td>
<td>419</td>
</tr>
<tr>
<td>100</td>
<td>117</td>
<td>241</td>
<td>212</td>
<td>570</td>
</tr>
<tr>
<td>250</td>
<td>78</td>
<td>162</td>
<td>125</td>
<td>365</td>
</tr>
<tr>
<td>500</td>
<td>88</td>
<td>297</td>
<td>246</td>
<td>631</td>
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<tr>
<td>Total</td>
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<td>1432</td>
<td>741</td>
<td>2548</td>
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<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-square</td>
<td>250.435</td>
<td>8.000</td>
<td>0.000</td>
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<tr>
<td>Likelihood ratio Chi-square</td>
<td>260.286</td>
<td>8.000</td>
<td>0.000</td>
</tr>
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</table>

B. cf. gunnisoniana pistils treated on BRZ medium

Frequencies
BRZ (rows) by MODE$ (columns)

<table>
<thead>
<tr>
<th></th>
<th>Apospory</th>
<th>Dyad</th>
<th>Tetrad</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>69</td>
<td>15</td>
<td>94</td>
</tr>
<tr>
<td>1</td>
<td>22</td>
<td>80</td>
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</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>149</td>
<td>54</td>
<td>235</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Test statistic</th>
<th>Value</th>
<th>df</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-square</td>
<td>6.853</td>
<td>2.000</td>
<td>0.033</td>
</tr>
<tr>
<td>Likelihood ratio Chi-square</td>
<td>7.010</td>
<td>2.000</td>
<td>0.030</td>
</tr>
<tr>
<td>Cochran’s Linear Trend</td>
<td>0.717</td>
<td>1.000</td>
<td>0.397</td>
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</table>

B. cf. gunnisoniana pistils treated on 5-azaC medium

Frequencies
AZA (rows) by MODE$ (columns)

<table>
<thead>
<tr>
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<th>tet</th>
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</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>265</td>
<td>19</td>
<td>288</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>444</td>
<td>19</td>
<td>485</td>
</tr>
<tr>
<td>50</td>
<td>43</td>
<td>533</td>
<td>9</td>
<td>585</td>
</tr>
<tr>
<td>500</td>
<td>59</td>
<td>522</td>
<td>9</td>
<td>590</td>
</tr>
<tr>
<td>Total</td>
<td>128</td>
<td>1764</td>
<td>56</td>
<td>1948</td>
</tr>
<tr>
<td>-------</td>
<td>-----</td>
<td>------</td>
<td>----</td>
<td>------</td>
</tr>
<tr>
<td>Test statistic</td>
<td>Value</td>
<td>df</td>
<td>Prob</td>
<td></td>
</tr>
<tr>
<td>Pearson Chi-square</td>
<td>49.435</td>
<td>6.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Likelihood ratio Chi-square</td>
<td>51.548</td>
<td>6.000</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

*B. stricta* pistils treated with epiBL *in vitro*

Frequencies
BR (rows) by MODE$ (columns)

<table>
<thead>
<tr>
<th>dyad</th>
<th>tetrad</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>66</td>
</tr>
<tr>
<td>0.1</td>
<td>33</td>
<td>107</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>173</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test statistic</th>
<th>Value</th>
<th>df</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-square</td>
<td>8.716</td>
<td>1.000</td>
<td>0.003</td>
</tr>
<tr>
<td>Likelihood ratio Chi-square</td>
<td>9.912</td>
<td>1.000</td>
<td>0.002</td>
</tr>
<tr>
<td>McNemar Symmetry Chi-square</td>
<td>11.000</td>
<td>1.000</td>
<td>0.001</td>
</tr>
<tr>
<td>Yates corrected Chi-square</td>
<td>7.633</td>
<td>1.000</td>
<td>0.006</td>
</tr>
<tr>
<td>Fisher exact test (two-tail)</td>
<td>0.002</td>
<td></td>
<td></td>
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</tbody>
</table>

Frequencies
STAGE (rows) by MODE$ (columns)

<table>
<thead>
<tr>
<th>dyad</th>
<th>tetrad</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>1-I to 2-II</td>
<td>25</td>
<td>84</td>
</tr>
<tr>
<td>2-III to 2-IV</td>
<td>13</td>
<td>89</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>173</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test statistic</th>
<th>Value</th>
<th>df</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-square</td>
<td>3.706</td>
<td>1.000</td>
<td>0.054</td>
</tr>
<tr>
<td>Likelihood ratio Chi-square</td>
<td>3.767</td>
<td>1.000</td>
<td>0.052</td>
</tr>
<tr>
<td>McNemar Symmetry Chi-square</td>
<td>51.969</td>
<td>1.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Yates corrected Chi-square</td>
<td>3.048</td>
<td>1.000</td>
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<tr>
<td>Fisher exact test (two-tail)</td>
<td>0.072</td>
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</table>

*B. stricta* pistils treated with epiBL *in vivo*
Frequencies
BL (rows) by MODE$ (columns)

<table>
<thead>
<tr>
<th></th>
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<th>tetrad</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>56</td>
<td>60</td>
</tr>
<tr>
<td>0.1</td>
<td>8</td>
<td>51</td>
<td>59</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>17</td>
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<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>174</td>
<td>204</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test statistic</th>
<th>Value</th>
<th>df</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-square</td>
<td>17.759</td>
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<tr>
<td>Likelihood ratio Chi-square</td>
<td>17.357</td>
<td>3.000</td>
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</tbody>
</table>

*Arabidopsis* pistils pre-treated or treated on 5-azaC medium (factorial 1)

Frequencies
AZA$ (rows) by MODE$ (columns)

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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<td>9</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>pre</td>
<td>12</td>
<td>17</td>
<td>21</td>
<td>50</td>
</tr>
<tr>
<td>prepost</td>
<td>25</td>
<td>9</td>
<td>54</td>
<td>88</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>35</td>
<td>92</td>
<td>167</td>
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</table>

<table>
<thead>
<tr>
<th>Test statistic</th>
<th>Value</th>
<th>df</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson Chi-square</td>
<td>15.519</td>
<td>4.000</td>
<td>0.004</td>
</tr>
<tr>
<td>Likelihood ratio Chi-square</td>
<td>16.511</td>
<td>4.000</td>
<td>0.002</td>
</tr>
<tr>
<td>McNemar Symmetry Chi-square</td>
<td>6.752</td>
<td>3.000</td>
<td>0.080</td>
</tr>
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</table>

*Arabidopsis* pistils treated on factorial 2 medium

Frequencies
TRT$ (rows) by MODE$ (columns)

<table>
<thead>
<tr>
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<th>TET</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR</td>
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<td>19</td>
<td>23</td>
<td>46</td>
</tr>
<tr>
<td>BRDT</td>
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<td>3</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>Control</td>
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<td>1</td>
<td>39</td>
<td>47</td>
</tr>
<tr>
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<td>dt</td>
<td>aza</td>
<td>total</td>
<td></td>
</tr>
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<td>-----</td>
<td>------</td>
<td>------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>13</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>17</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>29</td>
<td>53</td>
<td>107</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>value</th>
<th>df</th>
<th>prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>pearson chi-square</td>
<td>32.929</td>
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<tr>
<td>likelihood ratio chi-square</td>
<td>40.137</td>
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</tbody>
</table>

**B. stricta pistils treated on factorial 3 medium**

Frequencies
SUGAR$ (rows) by MODE$ (columns)

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<tr>
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<tbody>
<tr>
<td>glucose</td>
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<td>114</td>
<td>152</td>
</tr>
<tr>
<td>sucrose</td>
<td>26</td>
<td>138</td>
<td>164</td>
</tr>
<tr>
<td>total</td>
<td>64</td>
<td>252</td>
<td>316</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>value</th>
<th>df</th>
<th>prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>pearson chi-square</td>
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<td>0.043</td>
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<tr>
<td>likelihood ratio chi-square</td>
<td>4.097</td>
<td>1.000</td>
<td>0.043</td>
</tr>
<tr>
<td>McNemar symmetry chi-square</td>
<td>55.314</td>
<td>1.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Yates corrected chi-square</td>
<td>3.539</td>
<td>1.000</td>
<td>0.060</td>
</tr>
<tr>
<td>Fisher exact test (two-tail)</td>
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<td>0.050</td>
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Frequencies
TRT$ (rows) by MODE$ (columns)

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<tr>
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<tr>
<td>cc</td>
<td>5</td>
<td>119</td>
<td>124</td>
</tr>
<tr>
<td>dt</td>
<td>7</td>
<td>40</td>
<td>47</td>
</tr>
<tr>
<td>total</td>
<td>64</td>
<td>252</td>
<td>316</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th></th>
<th>value</th>
<th>df</th>
<th>prob</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>likelihood ratio chi-square</td>
<td>52.531</td>
<td>4.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>
CURRICULUM VITAE

Lei Gao
(435)9942933
lei.gao@aggiemail.usu.edu

QUALIFICATIONS

• Highly motivated and innovative botanist with Ph.D. experience in plant science and horticulture.
• Extensive background in plant reproduction especially in studying the mechanism of apomixis by sequencing techniques.
• Hands-on experience in designing, identifying and inducing meiosis/apomeiosis in apomictic/sexual plant.
• Written and oral fluency in Mandarin & English.

EDUCATION

2018     Ph.D. in Plant Science, Plant, Soils and climate Department, Utah State University.

AWARDS

2014-2018  Doctorate student tuition award from Utah State University.
2009-2013  High-level university scholarship from China Scholarship Council.

TEACHING & RESEARCH

2017     Lab assistant for Plant Propagation class
2016     Supervised/guided high school student in Dr. Carman’s lab.
2013     Lab assistant for Plant Propagation class
2012     Lab assistant for Plant Propagation class
2011     Supervised/guided high school student in Dr. Carman’s lab.
2010     Supervised/guided high school student in Dr. Carman’s lab.

2009-2018  Research assistant in Dr. Carman’s Lab.