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Effects of Plant Stress on Facultative Apomixis in Boechera (Brassicaceae)

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EFFECTS OF PLANT STRESS ON FACULTATIVE APOMIXIS IN *BOECHERA* (BRASSICACEAE)

by

Mayelyn Mateo de Arias

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ABSTRACT

Effects of Plant Stress on Facultative Apomixis in *Boechera* (Brassicaceae)

By

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Utah State University, 2015

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In angiosperms, apomixis is asexual reproduction by seed. In gametophytic apomixis, every nucleus in the female gametophyte is unreduced, and the embryo forms parthenogenetically. Different types of gametophytic apomixis occur depending on the origin of the embryo. In this research we documented that both aposporous and diplosporous apomixis occur in the genus *Boechera* (Brassicaceae). Our results from cytological analysis show that in *B. gunnisoniana*, *B. lignifera*, *B. retrofracta x exilis*, and *B. retrofracta x stricta* the type of apomixis is diplospory; and in *B. microphylla* it is apospory. Sexual reproduction was observed in *B. stricta*. By using quantitative real time PCR (qRT-PCR), we obtained the same pattern of expression of four stress-related genes and one ribosomal gene that had been previously documented in a project carried out with microarray analysis in our lab. In this part of the project, we could appreciate differences in gene expression in pistils and ovules of sexual and apomictic *Boechera* species. A major objective of this study was to evaluate the effects of stress on megasporogenesis, seed set, and gene expression in sexual and apomictic species of the

genus *Boechera*. Cytology of ovule development, flow cytometry of single seeds and qRT-PCR of pistil samples were conducted on the five apomictic *Boechera* species and the one sexual species mentioned above. Sets of plants from each species were grown under ideal control conditions and other sets were exposed to drought stress or drought plus heat stress. The cytological evaluation of ovules showed an increase in the frequency of sexual ovule development (meiosis instead of apomeiosis) under stress in some species. However, no alteration was detected in frequencies of sexual and apomictic seed set regardless as to whether the plants were grown with or without stress. Drought stress triggered changes in gene expression analyzed by qRT-PCR. While these changes may be related to the switch from apomeiotic to meiotic megasporogenesis, these changes did not cause an increase in sexual seed formation. Low frequency sexual seed formation (relative sexual sterility) may be the result of the interspecific or triploid nature of the apomictic plants being tested.

(128 pages)

PUBLIC ABSTRACT

Effects of Plant Stress on Facultative Apomixis in *Boechera* (Brassicaceae)

Mayelyn Mateo de Arias

In flowering plants, apomixis is asexual reproduction by seeds. Apomixis allows the production of offspring with the same genetic characteristics as the mother plant. Fertilization is not required. Apomixis could become a tool for naturally cloning high-yielding crop hybrids through their own seed. However, apomixis does not occur in major crop plants, except for citrus. In the present study, genes that might cause apomixis in naturally occurring apomictic plants were investigated. Sexual and apomictic species of the genus *Boechera* were exposed to stressed and non-stressed conditions. Effects of these treatments on the expression of apomixis was then measured. Stress triggered an increase in the frequency of sexual development in apomictic plants, but continuation of sexual development to form sexual seeds did not occur. Stress also triggered alterations in the expression of stress-related genes.

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Finally, my most special thanks to my heavenly Father for all my achievements, since He is the reason of my existence and the one who guides me in this life.

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CHAPTER 1

INTRODUCTION

Apomixis in flowering plants (angiosperms) is asexual reproduction by seed. Two major types of apomixis occur in plants, gametophytic and sporophytic. In gametophytic apomixis, every nucleus in the female gametophyte (embryo sac, ES), including the egg cell and the polar nuclei, is unreduced, and the embryo forms parthenogenetically. The unreduced ES originates in one of two ways, from a nucellar cell of the ovule wall (apospory) or directly from the megaspore mother cell (MMC) either before or during the early stages of meiosis (diplospory). Hence, all nuclei of the entire ES are unreduced, and the unreduced egg develops into an embryo without fertilization, i.e. parthenogenetically (Nogler, 1984). The endosperm in gametophytic apomicts forms pseudogamously or autonomously, i.e. it may or may not require fertilization of the ES central cell depending on the species (Ozias-Akins and van Dijk, 2007; Tucker and Koltunow, 2009; Yamada-Akiyama et al., 2009). In sporophytic apomixis, an abnormal unreduced parthenogenetically active cell (capable of embryogenesis without fertilization) of the ovule wall forms and undergoes embryo formation directly in a process referred to as adventitious or nucellar embryony. This clonal embryo uses the endosperm of the sexually derived ES for nutrition. Hence, sporophytic apomixis involves both gametophytic and sporophytic processes.

The term apomixis was first used by Winkler (1908) to describe “absence of mixis” (absence of meiotic recombination) followed by parthenogenesis. Some authors have expanded the definition to include all types of vegetative reproduction, e.g. the production of rhizomes, tillers and vegetative buds, etc., but this modification of

Winkler's original intension has fallen out of favor (Asker and Jerling, 1992; Carman, 1997; Nogler, 1984; Tucker and Koltunow, 2009). Today, apomixis refers to a two steps process: i) formation of an unreduced cell, gamete or gametophyte from cells or related tissues normally involved in reduced gamete or gametophyte formation (apomeiosis), and ii) parthenogenesis. Based on this definition, the term apomixis has been appropriately used to describe apomeiosis followed by parthenogenesis in all kingdoms of eukaryotes, i.e. it occurs in Protozoa (Bilinski et al., 1989; Asker and Jerling, 1992); in Chromista (Bilinski et al., 1989); in Animalia (Maniatsi et al., 2010; Dufresne et al., 2011; Robinson et al., 2011), including many insects (Rozek et al., 2009); as well as in Plantae and Fungi (Suomalainen et al., 1987; Asker and Jerling, 1992; Donahoo and Lamour, 2008; Carman et al., 2011; Hörandl and Hojsgaard, 2012). Therefore, apomixis occurs in every kingdom.

Because reproduction by apomixis produces clones of the mother plant, it may someday be a useful trait for crop improvement where it will cause progeny plants of a crop to express desired characteristics of the mother plant. This could be a great advantage with regard to hybrid seed formation particularly for cereals and legumes, and it includes inbred crops where hybridity could be very important but is not currently easy to achieve such as in rice, wheat, soybean and cotton (De Meeûs et al., 2007; Ozias-Akins and van Dijk, 2007; Tucker and Koltunow, 2009). It is first necessary to understand the genetic basis of apomixis in order to convert sexual crops to apomixis.

Because my research involved gametophytic apomixis, I review herein types of diplospory and apospory, which are differentiated by i) the type of apomeiosis involved and ii) the number and organization of nuclei in the subsequent ES. Before discussing

these forms, I first review sexual meiosis and ES formation. In sexual angiosperms, meiosis usually results in a linear or T-shaped tetrad of four spores with a single reduced nucleus (1n) in each. During pre-meiotic interphase, a single DNA replication (S phase) occurs such that each chromosome consists of two replicate sister chromatids. Meiotic chromosome pairing and crossing over (genetic recombination of chromatids from homologous chromosomes) occurs during prophase I. This is followed by metaphase I and anaphase I. The division that occurs (meiosis I) is unique to meiosis in that it is reductional, i.e., homologous chromosomes separate from each other. This produces two cells with only half the number of chromosomes (1n) in each. However, each chromosome still contains two chromatids (which possess recombined regions distil to crossover events) that are attached at their centromeres. The intervening interphase is unique to meiosis in that it does not include an S-phase (DNA replication). During anaphase II, the chromatids of each chromosome separate from each other. This mitotic-like division produces four spores with half the chromosome number (1n). Except in Oenothera, the three micropylar-most spores degenerate. The surviving megaspore at the chalazal-most end, or at the micropylar end in Oenothera (Johri et al., 1992), expands and undergoes three mitotic divisions to produce an 8-nucleate ES with an egg and two synergids at the micropylar end, two polar bodies (not to be confused with meiotic polar bodies, e.g. those formed during animal oogenesis) that fuse to form the 2n 1-nucleate central cell, and three antipodals at the chalazal end. This is the most common type of meiosis and ES formation observed in angiosperms and is referred to as the Polygonum type.

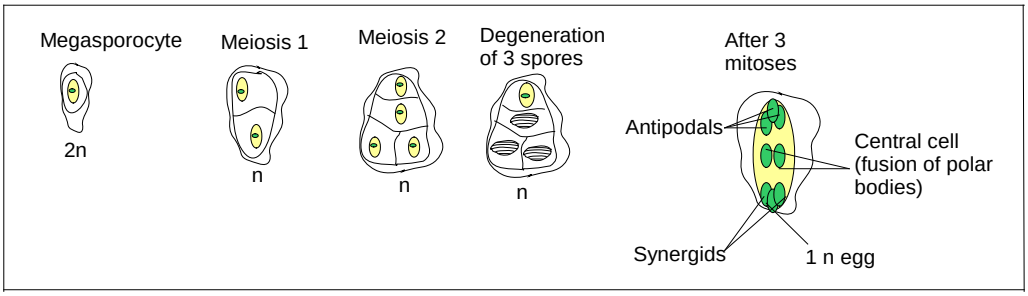

Figure 1.1 Polygonum type embryo sac development (sexual)

In some sexual species, the mature ES contains only four reduced nuclei or 16 to as many as 32 nuclei (Johri et al., 1992; Carman, 1997). In other sexual species, both karyokineses of meiosis occur, but the second cytokinesis does not. Hence, the product of meiosis is a dyad with each dyad member containing two genetically reduced nuclei. Because each spore contains two nuclei, this sexual mechanism is referred to as bispory. The micropylar member of the dyad degenerates, and the two reduced nuclei in the surviving dyad member undergo two (instead of three) mitotic divisions to produce an eight-nucleate sexual ES (Fig. 1.2), which otherwise resembles a Polygonum type ES. This bisporic type of meiosis and ES formation is referred to as the Allium type (Johri et al., 1992; Carman, 1997; Crane, 2001).

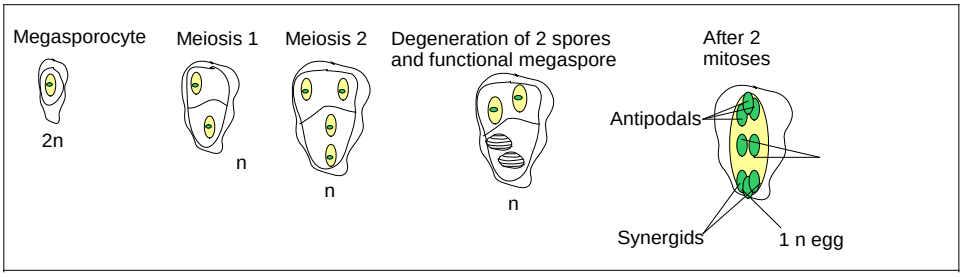

Figure 1.2 Allium type embryo sac development (sexual, bispory)

Additionally, in some sexual species of angiosperms, neither cytokinesis occurs such that the immediate product of meiosis is a single spore with four reduced nuclei.

This is referred to as tetraspory, and the four nuclei may *i*) immediately form a mature 4-nucleate sexual ES, *ii*) divide once to form a mature 8-nucleate sexual ES, *iii*) divide twice to form a mature 16-nucleate sexual ES, or *iv*) divide three times to form a mature 32-nucleate sexual ES (Johri et al., 1992; Carman, 1997).

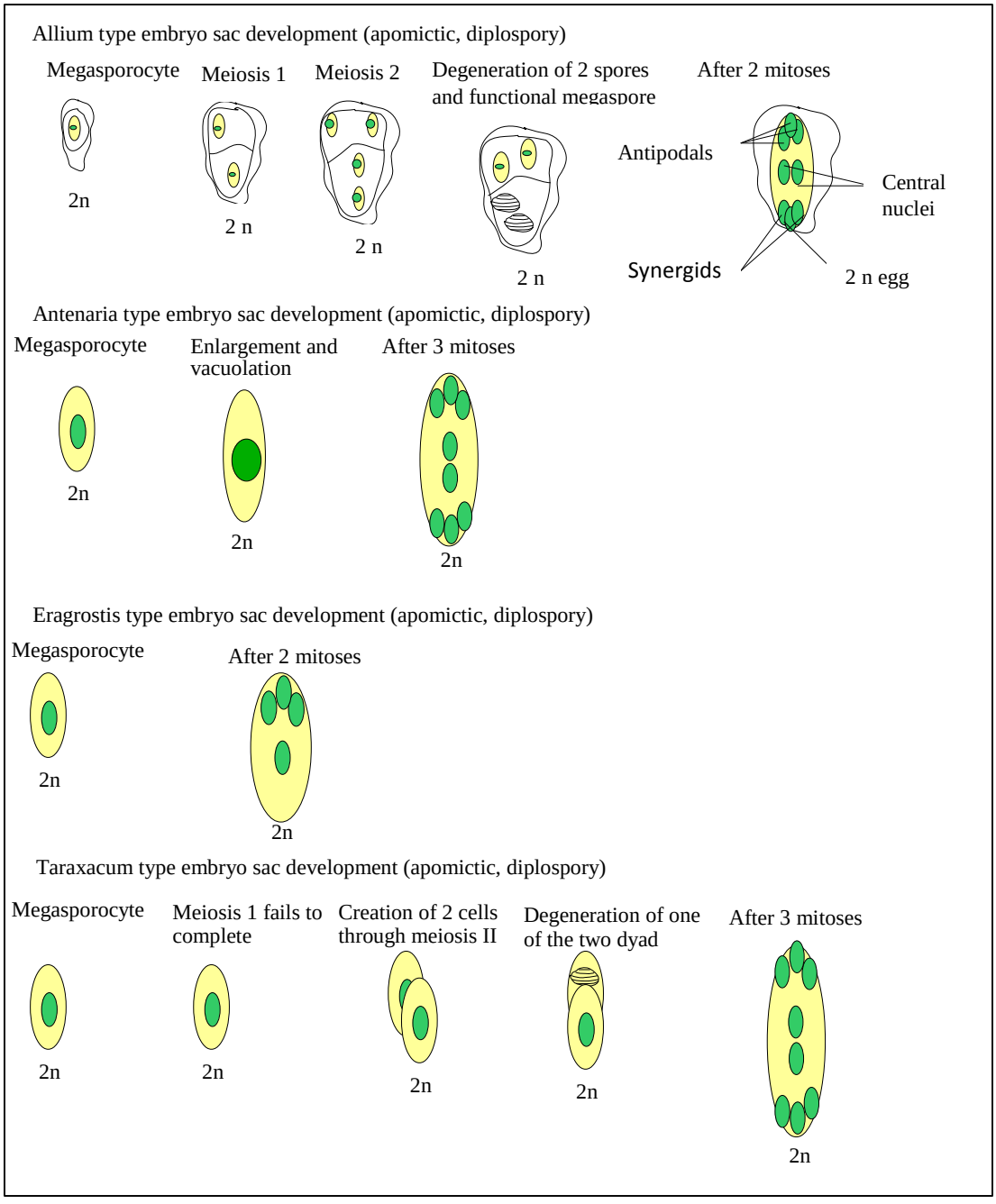

Figure 1.3. Forms of apomeiosis that directly involve the megaspore mother cell (MMC)

In most cases, the various types of gametophytic apomixis are conveniently described by comparing them to the more common Polygonum and Allium types of sexual ES formation. As mentioned above, forms of apomeiosis that directly involve the MMC are referred to as diplospory. These include the Allium odorum type, the Antennaria type, the Eragrostis type and the Taraxacum type (Fig. 1.3). The Elymus types are modifications of the Antennaria and Taraxacum types (Crane, 2001).

In Allium odorum type diplospory, two pre-meiotic S phases occur. This produces four chromatids per chromosome (instead of the normal two chromatids per chromosome), all four of which are joined at their centromeres (Fig. 1.4). A modified meiosis then occurs. Chromosome pairing is replaced by pseudo-chromosome pairing wherein all recombination is restricted to the four, genetically-identical sister chromatids of each chromosome. This intra-chromosome recombination (among sister chromatids) does not have genetic recombination implications because it involves sister chromatids that are identical replicates of each other. No recombination occurs between chromatids of homologous chromosomes, as would occur during normal meiosis. Metaphase I also differs from a normal meiosis in that all non-paired chromosomes align on the metaphase plate individually (Fig. 1.4). Thus, there are twice as many chromosomes on the metaphase plate during metaphase I (2n) as would occur during a normal meiosis I (n). During anaphase I, each chromosome (four interconnected chromatids) separates such that two interconnected chromatids move to each pole. The resulting cells are 2n. Like in a normal meiosis, no S phase occurs. During anaphase II, each chromosome divides again such that the two remaining chromatids of each chromosome migrate to their respective

poles. However, the second cytokinesis fails. This produces two nuclei that are genetically identical to the nuclei of undoubled somatic tissues of the sporophyte. Hence the products of Allium odorum type diplospory are a 2n 2-nucleate surviving megaspore at the chalazal end of the dyad and a 2n 2-nucleate spore that degenerates at the micropylar end (Fig. 1.4). The nuclei of the surviving spore divide twice to form an 8-nucleate genetically unreduced ES similar in design to a Polygonum type ES.

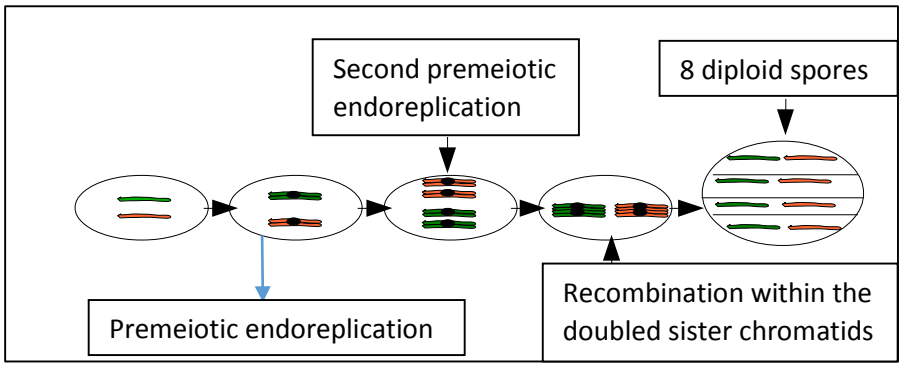

Figure 1.4. Meiosis in the genetically doubled MMC

Pre-meiotic chromosome doubling is rare among plants, occurring in *Allium* and possibly a few other genera (Carman, 1997), but it is a relatively common form of apomeiosis in animals where *i*) an unreduced egg forms and *ii*) the three unreduced polar bodies of oogenesis (analogous to the three degenerating megaspores of a sexual plant tetrad) degenerate (Suomalainen et al., 1987).

In *Antennaria* type diplospory, meiosis is completely eliminated, and the MMC forms an unreduced ES directly. Various meiosis-like processes occur in the other types of diplospory (Fig. 3). In all cases, the resulting ES is unreduced. Note that the mature ES is only 4-nucleate in the *Eragrostis* type (Fig. 1.3). Here, the central cell is not the product of polar body fusion but consists of a single polar body.

Types of apomeiosis that involve the formation of an unreduced ES from nucellar cells (rarely integument cells) are referred to as apospory (Fig. 1.5). These include *i*) the Hieracium type, which results in an unreduced 8-nucleate ES that is cytologically similar to a Polygonum type sexual ES, and *ii*) the Panicum type (fig. 1.6), which results in an unreduced 4-nucleate ES that is similar to a 4-nucleate Eragrostis type apomictic ES or a 4-nucleate Oenothera type sexual ES. Aposporous ES replace the female meiocyte or immature sexual ES in what appears cytologically to be a competitive process (Nogler, 1984). This is like the apomeiosis phase of sporophytic apomixis wherein nucellar cells produce embryos, but in apospory, a nucellar cell first produces an unreduced ES, and then parthenogenesis occurs from the unreduced egg in the aposporous ES.

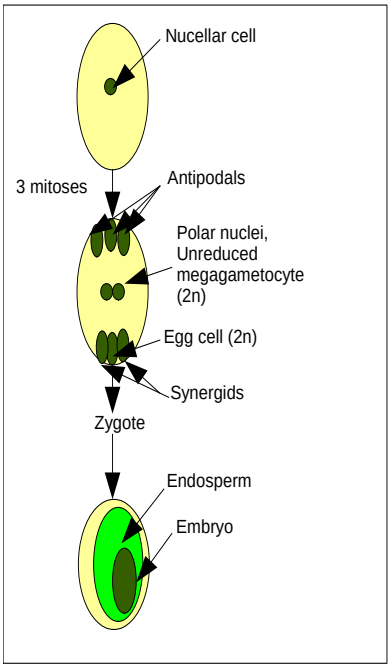

Figure 1.5. Apomeiosis that involves the formation of an unreduced embryo sac from nucellar cells or rarely from integument cells (Apospory)

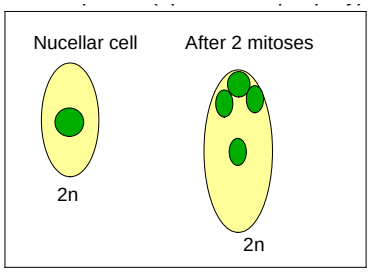

Figure 1.6. Panicum type embryo sac development (apomictic, apospory)

In the common sexual Polygonum type ES, the two central-most genetically reduced nuclei fuse to form the central cell, and this cell is fertilized by a single reduced nucleus (sperm) to produce an endosperm with a 2:1 maternal:paternal genome ratio (Fig. 1.7). By contrast, the maternal:paternal genome ratio is 1:1 for embryos. Modifications made to the 2:1 endosperm ratio, e.g., by crossing plants of different ploidy, disrupt endosperm formation usually leading to embryo and seed abortion (Tucker and Koltunow, 2009). In the Eragrostis and Panicum types of apomixis, the central cell of the mature ES contains only one unreduced maternal genome, which is usually fertilized by a genetically reduced sperm. This maintains the acceptable 2:1 endosperm balance ratio, and this may provide an advantage in the evolution of these types of apomixis. However, in most angiospermous apomicts, the ES central cell is a fusion product of two unreduced maternal nuclei, and it is usually fertilized by a reduced sperm to yield a 4:1 maternal:paternal genome ratio for the endosperm. Hence, the endosperm of apomicts tends to have an ability to tolerate unusual maternal:paternal genome ratios and to proceed in making functional seeds.

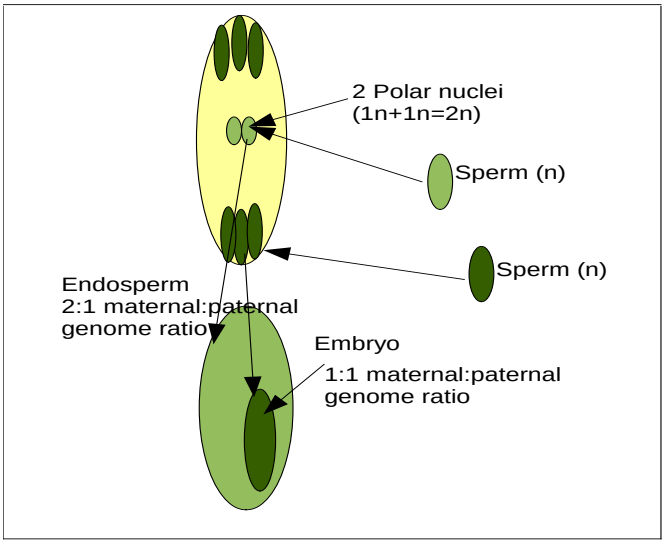

Figure 1.7. Sexual polygonum type embryo sac

In angiosperms, apomixis is a strictly female phenomenon. Paternal apomixis, where an apomeiotic (unreduced) and parthenogenetically competent pollen grain enters the female megagametophyte and undergoes embryogenesis (without syngamy), has been reported in the gymnosperm *Cupressus dupreziana* (Pichot et al., 2000; Nava et al., 2010). According to Pichot et al. (2000), this species is the only known member of the plant kingdom whose regular mode of reproduction is male apomixis. *Cupressus dupreziana* may provide opportunities for researching various aspects of apomixis.

Apomixis occurs in 78 angiospermous families, with adventitious embryony, apospory and diplospory occurring in 148, 110 and 68 genera, respectively (Hojsgaard et al., 2014). Apomixis is wide-spread in angiosperms, but it does not occur in major crop plants except for citrus (Ozias-Akins, 2006; van Dijk and Bakx-Schotman, 2004) and possibly mango (Koltunow et al., 2001). Apomixis is observed in many genera of the *Asteraceae, Poaceae* and *Rosaceae*; and it is almost exclusively restricted to perennial plants (Richards, 2003). Examples of well-known genera that contain apomictic species include *Crataegus* (hawthorns), *Amelanchier* (shadbush), *Sorbus* (whitebeams), *Rubus* (blackberries), *Hieracium* (hawkweeds), *Poa* (including Kentucky bluegrass), and *Taraxacum* (dandelions).

Apomixis may be obligate, cyclical or facultative. Obligate apomixis is when apomixis is the only mode of reproduction in a specific species. Cyclical apomixis occurs when the mode of reproduction in an individual organism alternates from asexual to sexual due to seasonal signals or other environmental changes. Facultative apomixis occurs when both sexual and apomictic reproduction occur in the same organism. Changes in facultativeness, i.e., the percentage of seeds formed apomictically vs.

sexually, have been observed to occur in response to specific environmental stressors. In general, stress increases the frequency of sexual reproduction in apomictic plants (Carman et al., 2011). Therefore, apomixis occurs in different ways, and sometimes it depends on the environment.

The origin of apomixis is unknown. According to Tucker and Koltunow (2009), apomixis has evolved many times during evolution, although the evolutionary origin of apomixis is not well understood. Lovell et al. (2013) assumed that gametophytic apomixis in *Boechera* (Brassicaceae) has some connection with hybridization and polyploidy, but these may not be the apomixis inducing factors (Voigt-Zielinski et al., 2012). Contrary to that assumption, Hörandl and Hojsgaard (2012) suggest that polyploidization and/or hybridization could activate the switch from sex to apomixis. Hybridization is a very important component that contributed to the evolution of species diversity in *Boechera* (Dobes et al., 2004; Koch et al., 2003; Sharbel et al., 2009). Carman (1997) suggested that the recurring processes of polyploidization and paleopolyploidization may be a key factor for the evolution of apomixis. According to Gustafsson (1947), apomixis might be the product of genetic interaction within polyploid taxa such that it would not be related to evolutionary change (by mutations, etc.) but to polyploidy itself. However, Sharbel et al. (2010) suggest that because most hybrids and polyploids are sexual, other genetic processes must be the key factors for the switch from sexual to asexual reproduction. In the same sense as Sharbel et al. (2010), and contrary to Gustafsson (1947), Nogler (1984) supposed that apomixis evolved by the evolution of certain genes. Once apomixis is established in a lineage, it may occasionally serve as a reproductively-stable springboard in the evolution of new groups of sexual plants,

possibly including new genera (Carman, 1997; Hörandl and Hojsgaard, 2012; Hojsgaard et al., 2014). Beck et al. (2011) show that some apomictic diploids of *Boechera* came from hybridization. But this analysis was done just in a small number of diploid species. For an angiospermous plant to be apomictic means that the embryo develops parthenogenetically from an unreduced somatic cell (sporophytic apomixis) or from an unreduced egg (gametophytic apomixis). Sharbel et al. (2009) suggested that each of these factors has its own genetic regulation. Hence, the odds for the evolution of these genetic controls occurring by random mutation from a sexual ancestor at the same time should be very rare. In this respect, Carman proposed that the basic apomictic mechanism (apomeiosis followed by parthenogenesis) is an ancient epigenetically-regulated program (conserved pathways hypothesis) that was inherited from angiospermous progenitors with innovations in apomictic types evolving thereafter (Carman et al., 2011; Hojsgaard et al., 2014). This is an important field of study with lots of work remaining to be done.

It has been proposed that various factors intervene in plants to induce apomixis. Gene expression profiling studies recently conducted in John Carman's lab have identified several biological processes that are strongly correlated with the onset of apomixis. These processes are regulated by specific genes that include environmental signal receptor and transduction genes and genes associated with retrograde signaling and circadian clock genes. These environmental signal reception and transduction genes could influence plants to be apomictic due to their function. Plants capture signals from the environment and use the information to control developmental responses epigenetically, including dormancy-breaking and germination (Penfield and Hall, 2009). Also, the shutdown of genes responsible for meiosis may be a factor that intervenes

during apomixis thus allowing apomeiosis to occur. According to Changbin et al. (2010), 68 genes function during meiosis in *Arabidopsis*, and some genes that were found greatly up-regulated in meiocytes of the same species are controlled by transposable elements. These results suggest that these genes might be involved in apomixis.

Genes responsible for retrograde and anterograde signaling might also affect apomixis. Retrograde genes are responsible for controlling the emission of signals that regulate the expression of nuclear genes (Eckardt, 2007); and anterograde genes are responsible for controlling organelle gene expression (Sota and Kinya, 2008). Both retrograde and anterograde regulations are associated with cytoplasmic male sterility. They affect fertilization by pollen and make pollen non-functional due to genome barriers between the nucleus and foreign mitochondria (Sota and Kinya, 2008). Retrograde signaling genes are also understood to regulate MADS box genes during cytoplasmic male sterility (Yang et al., 2008). In our expression profiling studies, some MADS box genes were differentially regulated between apomicts and sexual during early ovule development, including *PI*, *STK*, *AP3* and *SHP1*.

On another hand, circadian clock genes have been understood to control different processes in *Arabidopsis thaliana*. They coordinate metabolism and physiology with daily changes in the environment (McWatters et al., 2007). The circadian clock synchronizes physiological and developmental processes such as flowering time, with seasonal and diurnal times in most organisms (Más and Yanovsky, 2009). The circadian clock is also in charge of regulating several factors of development and growth, promoting plant fitness, and affecting signaling pathways necessary for plants to respond appropriately to the environment (Harmer, 2009). Circadian Clock Associated 1 (CCA1)

and Late Elongated Hypocotyl (LHY) together with Timing Of Cab Expression 1 (TOC1) constitute the central oscillator in plants (Green and Tobin, 2002; Zhaojun et al., 2007), and therefore, they control seasonal flowering and photomorphogenesis (Zhaojun et al., 2007). The circadian clock via histone methylation controls floral repressors to avoid premature flowering (Fekih et al., 2009). Another important gene in the circadian clock of *Arabidopsis* is the Early Flowering 4 (ELF4) locus. Due to its function, it has been found to be the central gene in the circadian clock (Kolmos and Seth, 2007). The function of this gene is entrainment to an environmental cycle and rhythm under constant conditions (McWatters et al., 2007). This gene is essential for oscillatory properties of the circadian clock. Due to the functions of all these genes, we think that they or some of them might participate in regulating apomixis.

Some other assumptions exist about what causes apomixis. Some researchers state that apomeiosis might occur because of the mutation of a single gene in a sexual plant. According to Olmedo-Monfil et al. (2010), female gamete formation in *Arabidopsis* is controlled by the mutation of the *Arabidopsis* protein ARGONAUTE 9 (AGO9). In the same sense, alteration of the *Arabidopsis* gene DYAD/SWITCH1 (SWI1) induces unreduced gamete formation (Ravi et al., 2008). On another hand, several Mendelian genetics factors make it difficult to understand the genetic basis of apomixis. These include epistatic gene interactions, or agents that modify gene expression, aneuploidy, polyploidy, segregation alteration, and repressed recombination, among others (Ozias-Akins and van Dijk, 2007). So, the situation is complicated, and much additional research is needed to understand what causes this phenomenon.

In the profiling studies carried out in Carman's lab, ovules and whole pistils from apomictic and sexual species of *Boechera* were analyzed and differences were found in the expression of genes whose functions are related to stress. Some of these genes were up-regulated either in apomictic species or in sexual species. In general, stress response genes and genes affiliated with the transduction of environmental stresses were up-regulated in ovules during sexual meiosis, in sexual *Boechera*, when compared to ovules in which apomeiosis was occurring in apomictic *Boechera*. A hypothesis tested by my research is that the ability to perceive environmental stresses during early ovule development is impaired in apomictic plants, and this absence of stress signals, which normally induces meiosis, is responsible for the occurrence of apomeiosis (Carman et al., 2011). To test this, I applied severe drought and temperature stresses to apomictic *Boechera* in an attempt to convert apomeiosis to sexual meiosis and apomictic seed set to sexual seed set. I also tested if shifts from apomeiosis and apomictic seed set to meiosis and sexual seed set are correlated with the expression of stress response genes as determined by quantitative real time PCR (qPCR).

Some of the genes discussed above were differentially expressed in the expression profiling studies conducted in our lab (manuscripts in preparation). My studies, which monitored effects of stress on reproduction-related gene networks, were conducted to find biological characteristics that differentiate these two forms of reproduction and to discover genes that may be involved. Specifically, my study determined effects of severe drought and high temperature stress on facultativeness of apomictic expression and on the expression of genes previously shown in our lab to be correlated with mode of reproduction in *Boechera*.

Several studies have been done to analyze the response of abiotic stress in plants due to different environmental agents. Some of these studies are based on alkaline stress, resulting in the change of gene expression due to changes in the expression of transcription factors. Metabolism genes, transcription regulators, genes involved in cell structure, energy, and protein synthesis as well as abscisic acid-mediated signaling factors and stress response genes were altered in plants of *Glycine soja* after being exposed to alkaline stress (Ge et al., 2011). Other stress factors that change gene expression in plants are temperature and drought. Drought triggers the up regulation of certain stress-response genes in *Arabidopsis* and *Boechera holboellii* species. According to Knight et al. (2006), some responsive proteins for desiccation, ABA and dehydration, as well as some transcription factors, the pentatricopeptide (PPR) genes and genes whose functions are unknown were up regulated when these species were submitted to water stress. On the same sense, the development of chickpea (*Cicer arietinum* L.) plants cultivated under chilling stress improved when the plants were treated with proline (Kaura et al., 2011). In *Arabidopsis*, the size and morphology of plants exposed to high temperature were altered (Liu et al., 2011). In addition, heavy metals can also cause changes in organisms. In *Arabidopsis*, it was demonstrated that uranium can alter morphology, physiology, type of nutrition and cellular redox equilibrium (Vanhoudt et al., 2008, 2011). Therefore, it is clearly understood that environmental factors trigger changes that can affect morphology, physiology and metabolic functions. As a consequence, such changes could alter the normal reproduction pathway in some plants, making sexual plants to reproduce asexually or vice versa.

Stress triggers changes in the function of genes and transcription factors in animals as well. In the apparent absence of stress, the expression of heat shock transcription factor 1 is nonetheless required for normal meiosis to occur in mice (Metchat et al., 2009). Stress is the direct regulator of mode of reproduction, i.e. sexual versus apomictic, in cyclically apomictic animals (Suomalainen et al., 1987). Hence, stress related genes should be investigated when investigating possible causes of apomixis.

How stress affects an organism might also depend on the stage and time of development of an organism as well as on the genotype. In *Vicia villosa* drought stress triggered changes in morphological and phonological characteristics as well as in productivity, but these changes were affected by time and stage of development in which the stress was applied (Andersen et al., 2002; Sun et al., 2004; Petraitytė et al., 2007; Aragón et al., 2008). In wheat, the effect of drought was shown to be dependent on genotype (Rampino et al., 2006) and on the stage of development the plant was in when drought was imposed (Zhu et al., 2005). C3 plants are particularly responsive to the level of drought stress (Araus et al., 2002). In rice it was shown that the effect also depends on the organ stressed (Zhou et al., 2007). Environmental stresses trigger changes in plant development because they affect mechanisms at the cellular, physiological and molecular levels (Barnabás et al., 2008).

In some eukaryotes, apomixis is cyclical or facultative, i.e., both sex and apomixis occur. In facultative apomicts, sex and apomixis occur simultaneously as is found among the numerous seeds of an inflorescence. In cyclical apomixis, the organism is apomictic in one season, generally under favorable conditions, and sexual generally during stressful

conditions (Suomalainen et al., 1987). Several plants tend to behave like cyclical apomixis in that the frequency of sexual seed set increases under stressful conditions. Stress also influences cell apoptosis and the cell cycle, although the mechanisms are not well understood (Nedelcu and Michod, 2010). Since apomixis is facultative in some species and dependent on stress, I imposed different types of stress in my study to advance our understanding of the responsible mechanisms.

Drought, high temperatures, salt and unusual photoperiods influence reproductive organs and related functions. Drought increases the senescence of reproductive organs (Sun et al., 2004). High temperature stress of *Brassica napus* during flowering reduced micro and megagametophyte fertility, caused fruit abortion and inhibited the further development of seeds (Young et al., 2004). In the same sense, salt stress inhibited microsporogenesis and stamen filament elongation and caused ovule abortion in *Arabidopsis* (Sun et al., 2004). Also, according to Nan et al. (2002), photoperiod influences the levels of hormones present in tissues of wheat. Submitting plants to more than one type of abiotic stress simultaneously can enhance the stress response. Hence, I used both heat and drought stress treatments in my study.

Stress response genes may cause changes in cascades of different genes. In *Arabidopsis,* mitogen-activated protein kinases (MAPKs) trigger changes in the cascades of different stress-response genes whose function is to defend the plant from different environmental stressors. Most environmental stress-response pathways converge into the H_2O_2 signaling pathway (Kovtun et al., 2000).

Closely linked genes on chromosomes are often co-regulated in that they share transcription regulators and are co-expressed. Proteins associated with the same function,

e.g., development of a particular tissue, are the products of co-expressed genes (Wuchty et al., 2006) and the expression of these interacting proteins is altered in a coordinated manner across species (Fraser et al., 2004). It is possible that with the change of one or more genes due to environmental stress, other genes from the same genome neighborhood may change. If such genes are linked to reproduction, stress might change the entire mechanism of reproduction. On another hand, according to Hörandl and Hojsgaard (2012), epigenetic modifications might cause a sexual plant to become apomictic after events such as polyploidization or hybridization. This suggestion is supported by Grimanelli (2012) who suggests that epigenetic modifications could produce parthenogenesis in apomictic plants. According to Kota and Feil (2010) epigenetic reprogramming of histone modifications and DNA methylation provide important functions during the production of germ cells in sexual plants. Hence, epigenetic reprogramming might trigger or influence the switch in mode of reproduction, especially when facultative apomictic plants are subjected to stress.

A reversion from apomeiosis to meiosis in anthers, as a result of stress, with some evidence of the same phenomenon occurring in ovules, was observed in plants of *Boechera* by Böcher (1951). However, because the apomictic *Boechera* were interspecific hybrids or triploids, meiosis was highly disturbed and did not produce viable pollen grains. Shifts from apomixis to mixis in response to stress have also been reported in other apomictic plants and animals (Suomalainen et al., 1987; Carman et al., 2011). Therefore, I exposed apomictic plants to different drought and high temperature stresses and evaluated the effects of these treatments on frequencies of sexual meiosis and seed set vs. apomeiosis and apomictic seed set.

Since genes causing apomixis are not known, my aim was to conduct plant stress experiments in facultatively apomictic *Boechera,* to evaluate the effect of stress on frequencies of sexual meiosis and seed set vs. apomictic meiosis (apomeiosis) and seed set and to correlate any changes in mode of reproduction with changes in the expression of certain genes that we have identified as candidates for the regulation of mode of reproduction. Sexually versus apomictically-produced seeds were differentiated by analyzing the ploidy of individual seeds, i.e., the embryo and endosperm components, using flow cytometry. To carry out the flow cytometry analyses, we analyzed the ploidy level of dried seed collected from each treatment. Seeds with a 2C embryo and 3C endosperm were considered sexual. Seeds with a 2C embryo and a 5-6C endosperm were considered apomictic. The methods used were those of Matzk et al. (2000). Additionally, effects of stress on megasporogenesis (meiotic versus apomeiotic) were quantified by embryology as in Carman et al. (2011).

Gene expression profiling of plants (excised immature pistils) subjected to stress versus a control was conducted using qPCR. This method uses the polymerase chain reaction to amplify and quantify a specific cDNA region of interest with a fluorescent dye spiked into the PCR cocktail. The fluorescence is measured after every PCR cycle, i.e., in real time.

Results of this research will be used in the future to define functional analyses of specific candidate genes by up and down regulating these genes transgenically and evaluating the effects on apomixis. If we can identify these genes, they could be used to convert sexual crops to apomictic crops thus fixing hybrid vigor and other desired characteristics.

Table 1.1 Terminology important to my study.

Term	Definition
Sexual reproduction	The production of gametes through meiosis followed by the formation of embryos through syngamy (fertilization).
Apomixis	In animals, reproduction without mixis of gametes (Suomalainen et al. 1987). Apomixis in angiosperms involves three processes: (1) formation of an unreduced, embryogenically-competent cell, either in an unreduced ES by apospory or diplospory wherein the egg assumes the embryogenic fate (gametophytic apomixis) or from a nucellar cell, which assumes the embryogenic fate (sporophytic apomixis); (2) embryo formation without fertilization (parthenogenesis) from the embryogenically-competent egg or nucellar cell; (3) endosperm formation in an ES with or without fertilization of the central cell, i.e., pseudogamously or autonomously.
Apomeiosis	Without meiosis: it is usually defined as formation of an unreduced egg. In Dr. Carman`s lab, it is defined as the process of producing a parthenogenetically-competent unreduced egg in the ES, as a part of gametophytic apomixis, or producing a parthenogenetically-competent nucellar cell, as a part of sporophytic apomixis. The resulting cells are genetically identical to the parent plant.
Parthenogenesis	Embryogenesis from a reduced or unreduced egg cell or from a nucellar cell without fertilization.
Facultative Apomixis	The occurrence of both sexual and apomictic reproduction within the same organism, e.g., where frequencies of sexual and apomictic seeds produced in an apomict may vary depending on environmental conditions.
Obligate Apomixis	Obligate apomixis is when apomixis is the only mode of reproduction in a specific species under any environmental condition or season.
Cyclical Apomixis	Cyclical apomixis occurs when the mode of reproduction in an individual organism is alternated from asexual to sexual or vice versa due to seasonal or other environmental changes.

Terms important to my study are defined in Table 1.1. I conducted plant stress experiments in facultatively apomictic and completely sexual *Boechera* and evaluated the effects of these treatments on (1) frequencies of apomeiotic versus meiotic megasporogenesis, (2) frequencies of sexual seed set vs. apomictic seed set, and (3) the

expression of a particular set of genes previously shown to be differentially expressed at different stages of ovule development between sexual and apomictic *Boechera*. Specifically, I determined effects of environmental stresses (drought and temperature) on:

- frequency of sexual vs. apomictic seed set as determined by flow cytometry
- frequency of sexual vs. apomictic megasporogenesis as determined cytologically
- expression of stress-related apomixis candidate genes as determined by qRT-PCR

My experiments were conducted using five species of facultatively apomictic *Boechera* and one sexual species. My null hypothesis was that alterations in environmental stress will have no effect on frequencies of meiotic vs. apomeiotic megasporogenesis, frequencies of sexual vs. apomictic seed set, or on expression levels of stress related candidate genes in pistils.

Based on the literature reviewed above, I expected to observe an increase in the frequency of sexual seed set of apomictic *Boechera* as a response to stress and to correlate this with changes in candidate gene expression. I observed that the frequency of sexual (meiotic) megasporogenesis in facultatively apomictic plants was greatly increased by stress. However, ovules in which sexual meiosis occurred did not produce seed. In contrast, ovules that did produce seed were those in which megasporogenesis had occurred apomeiotically. This result opens the door for future research to identify genes responsible for the stress-induced switch from apomeiosis to meiosis. Once we know the genes and gene networks responsible for apomixis, future research will include introducing appropriate modifications in genes and gene networks in crops that will permit apomixis to be harnessed for agricultural benefits. This could greatly advance

agriculture by reducing investments in hybrid seed production and by the conversion of currently inbred crops to superior-yielding hybrid crops.

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CHAPTER 2

DIPLOSPOROUS AND APOSPOROUS APOMIXIS IN BOECHERA (BRASSICACEAE)

ABSTRACT

Boechera encompasses approximately 70 sexual diploid species and hundreds of apomictic diploid and polyploid hybrids. Methods other than cytological investigations of the female developmental pathway have generally been used to identify apomixis in *Boechera*. While these methods are definitive, they do not identify the type of apomixis expressed. The embryology of only three apomictic species of *Boechera* has been reported, *B. holboellii*, *B. gunnisoniana* and *B. retrofracta*. These undergo Taraxacum-type diplospory, in which the first division of the megaspore mother cell fails and is followed by the second meiotic division and the formation of two unreduced megaspores. Herein, we document, using differential interference contrast microscopy, Taraxacum-type diplospory in diploid accessions of *B. exilis* x *retrofracta*, *B. lignifera*, and *B. retrofracta* x *stricta*. We also document Hieracium-type apospory as the major form of reproduction in several accessions of *B. microphylla*. This is the first formal report of apospory being a major mechanism of reproduction in *Boechera* and in the entire Brassicaceae. Apospory involves the formation of an unreduced embryo sac from a nucellar cell adjacent to the degenerating megaspore mother cell or its meiotic products. Sexual reproduction, involving 8-nucleate Polygonum-type embryo sac formation, was also documented for *B. stricta*.

INTRODUCTION

Boechera has become an important model genus for the study of apomixis (Aliyu et al., 2010, 2013; Sharbel et al., 2010; Carman et al., 2015). It belongs to the family Brassicaceae, which contains 340 genera (Schranz et al., 2005). *Boechera* contains 110 species, which are primarily distributed in Greenland, in the Russian Far East, and in North America (Al-Shehbaz and Windham, 2010). Its greatest diversity occurs in the Western United States (Mitchell-Olds, 2001) where many species are widely distributed in deserts, rocky scree and moist alpine meadows (Rushworth et al., 2015). Among the species found in North America, about 70 are sexual (Alexander et al., 2013), and 38 are apomictic (Al-Shehbaz and Windham, 2006, 2007a, 2007b). *Boechera* is the only genus in the Brassicaceae that contains verified apomictic species (Schranz et al., 2005, 2006). Taxonomically, it was separated from *Arabis* in 1975 by Love and Love, mainly due to differences in chromosome numbers (Dorn, 2003). Much work remains to be done in this genus to obtain a final classification, not only taxonomically, but also in terms of understanding the origins of apomixis in the genus and the species involved.

Boechera ($2n = 2x = 14$) is closely related to *Arabidopsis* ($2n = 2x = 10$). According to Koch and Haubold (2001) and Schranz et al. (2007), the ancestors of these two genera diverged about 20 million years ago, and high levels of conservation in gene synteny and gene sequence homology exist. This allows researchers to investigate *Boechera* using the highly advanced genomic tools and information developed for *Arabidopsis* (Mitchell-Olds, 2001; Schmidt et al., 2014; and Carman et al., 2015). Triploid ($2n = 21$) and tetraploid ($2n = 28$) apomictic *Boechera* are also common, as are diploid interspecific hybrid apomicts and apomictic aneuploids. A common aneuploid

number for near-diploid apomicts is 15 ($2n = 2x + 1 = 15$) (Schranz et al., 2006; Kantama et al., 2007).

Four papers have reported the type of apomixis expressed in *Boechera*. Taraxacum-type diplospory was reported in each. Here, the megaspore mother cell (MMC) undergoes an apomeiotic M_I restitution and a normal M_{II} that produces a dyad of unreduced megaspores. The micropylar megaspore degenerates, and the chalazal megaspore develops into a genetically-unreduced 8-nucleate Polygonum-type embryo sac. Unreduced eggs in diplosporous ovules of *Boechera* develop into embryos parthenogenically, but fertilization is required for endosperm formation, which is required for embryo development and healthy seed formation (pseudogamy). Taraxacum-type diplospory has previously been documented in diploid and triploid accessions of *B. holboellii* (Böcher, 1951; Naumova et al., 2001), diploid accessions of *B. retrofracta* (Sharbel et al., 2010) and triploid hybrids involving *B. gunnisoniana* (Taskin et al., 2004). The occurrence of apospory in *Boechera microphylla* was reported in a book chapter (Carman, 2007), but details were not provided. Apospory involves degeneration of the sexual MMC or its meiotic products coupled with unreduced embryo sac formation from a somatic cell of the ovule. Herein I provide details of apospory in multiple accessions of *B. microphylla* and of diplospory in accessions of *B. gunnisoniana*, *B. lignifera*, *B. retrofracta x exilis*, and *B. retrofracta x stricta*. I also document sexual reproduction in *B. stricta*.

MATERIALS AND METHODS

Plant materials --Genotypes studied in this investigation were originally collected by John Carman and his students as live specimens (Table 2.1). Seeds harvested from these plants were placed on moist filter paper in petri dishes, stratified at 4 °C for 3 weeks, and planted in cone-shaped pots (68 mm diameter x 255 mm tall, 600 mL volume) or square pots (85 mm wide x 95 mm tall, 350 mL volume), which were filled with Sunshine Mix #1 potting soil (Sun Gro Horticulture Canada Ltd, Vancouver, BC). Thirty to 40 plants of each genotype were established. After 6 weeks, seedlings (8-12 leaves) were vernalized for 10-12 weeks in a cold room (4-6 °C) with minimal lighting (8/16 day/night photoperiod) from soft-white fluorescent bulbs. The vernalized plants were then transferred to a controlled-environment greenhouse where a 16/8 h day/night photoperiod was maintained using supplemental light provided by 1000 W high-pressure sodium-vapor lamps. These provided a minimum photosynthetic photon flux of 600 µmol m^{-2} sec^{-1} at the tops of the canopies. Day/night temperatures were maintained at 22/16 °C, and plants were watered regularly with a 15:20:20 (250 mg L^{-1}) nutrient solution.

Table 2.1. Accession numbers and collection information for *Boechera* species and species hybrids evaluated embryologically and by single seed flow cytometry

Taxon	Accession	Location	GPS
B. gunnisoniana hybrid	CO11005	CO, Gunnison Co., hillside East of Highway 50	38º 31' 38.6" N 106º 48' 51.7" W
B. lignifera	WY05001	WY, Sweetwater Co., bluffs South of Green River	41º 33' 06" N 109º 31' 31" W
B. microphylla	UT10003	UT, Millard Co., shaded N-facing slopes, Oak Creek Canyon	39º 21' 00" N 112º 15' 50" W
B. retrofracta x exilis	UT11004	UT, Utah Co., Right Fork, Hobble Creek Canyon	40º 00' 46" N 109º 13' 19" W
B. retrofracta x stricta	CO11010	CO, Rio Blanco Co., drainage near Cow Creek Access Road	39º 42' 10.4" N 107º 59' 54.8" W
B. stricta	UT10007	UT, Duchesne Co., North Fork of Duchesne River (west side)	40º 33' 21.4" N 110º 53' 30.3" W

Embryological analyses -- For studies of megasporogenesis, clusters of floral buds at the late pre-anthesis stage and younger were fixed in formalin acetic acid alcohol (FAA) for 48 h and then transferred to 70 % ethanol for storage at room temperature. In preparation for analysis, buds were cleared in a series of solutions ending with a 2:1 concentration of benzyl benzoate and dibutyl phthalate (Crane and Carman, 1987). Thirty to forty pistils were then excised for each genotype, and those ranging from 0.7-3.0 mm in length were mounted such that pistils of similar length occurred in separate columns on the slides. The pistils were mounted in a small volume of clearing solution, up to 16 per slide, and covered with a coverslip. Ovaries inside the cleared pistils were studied using a BX53 microscope (Olympus, Center Valley, PA, USA) equipped with differential interference contrast (DIC) optics. Details of ovule development were photographed using a MicroFire 599809 camera (Olympus).

Single Seed Flow Cytometry -- Nuclei were isolated from individual mature seeds of each genotype using a mortar, pestle and a few drops of Partec (Partec North America, Inc., Swedesboro, NJ) buffer, which contained DAPI (4,6-diamidino-2-phenylindole). Pestles were used only to crack the seeds open. The seed fragments were not ground. The seed fragments were exposed to the Partec buffer solution for several minutes. The nuclei-containing solutions were then filtered through 30 µm nylon filters into 1.2 mL tubes. Nuclear fluorescence values for each sample were determined using a Partec I flow cytometer per the manufacturer's instructions. Relative fluorescence intensities of nuclei were analyzed using Partec software. Embryo and endosperm ploidy levels were determined from 50 individually-analyzed seeds per genotype. Sexual seeds were identified by a 2:3 C embryo to endosperm ratio. Apomictic seeds were identified by a 2:5, 2:6, 2:7 or 3:9 C embryo to endosperm ratio (Matzk et al., 2000).

RESULTS AND DISCUSSION

Apomixis has been documented in *Boechera* based on the presence of unreduced pollen (Mulligan, 1995; Sharbel and Mitchell-Olds, 2001; Koch et al., 2003; Schranz et al., 2005; Taskin et al., 2009), progeny tests (Roy, 1995), and embryo to endosperm genome ratios (Aliyu et al., 2010). However, these methods, do not reveal the type of apomixis expressed. Herein, types of apomixis expressed for several species and species hybrids are reported. Also reported is extent of facultativeness for female apomeiosis (determined cytologically, Table 2.2), and extent of facultativeness for apomictic seed set (determined by single-seed flow cytometry, Table 2.3). Sexual reproduction was

observed in *B. stricta*, Hieracium-type apospory was observed in *B. microphylla*, and Taraxacum-type diplospory was observed in the remaining taxa (Fig. 2.1-2.3).

Table 2.2. Frequencies of sexual tetrad and sexual or diplosporous dyad formation and frequencies of aposporous (AS) embryo sac formation by taxon as determined by DIC microscopy of ovules in the meiocyte (M) to early embryo sac (ES) formation stages.

Taxon	M to early ES	Tetrad	Dyad	AS
	No.	-------- % --------		
B. gunnisoniana hybrid	424	4	96	0
B. lignifera	190	16	84	0
B. microphylla	176	35	0	65
B. retrofracta x exilis	24	42	58	0
B. retrofracta x stricta	11	45	55	0
B. stricta	197	98	2	0

Table 2.3. Reproductive pathways by taxon as determined by single-seed embryo:endosperm C values (2C:3C sexual, others apomictic)

	Embryo		Endosperm					
Taxon	2C	3C	3C	5C	6C	7C	9C	Not detected
B. gunnisoniana hybrid		50					7	43
B. lignifera	50		2		45	1		2
B. microphylla	50		2	44		1		3
B. retrofracta x exilis	50				24			26
B. retrofracta x stricta	50				2			48
B. stricta	50		12					38

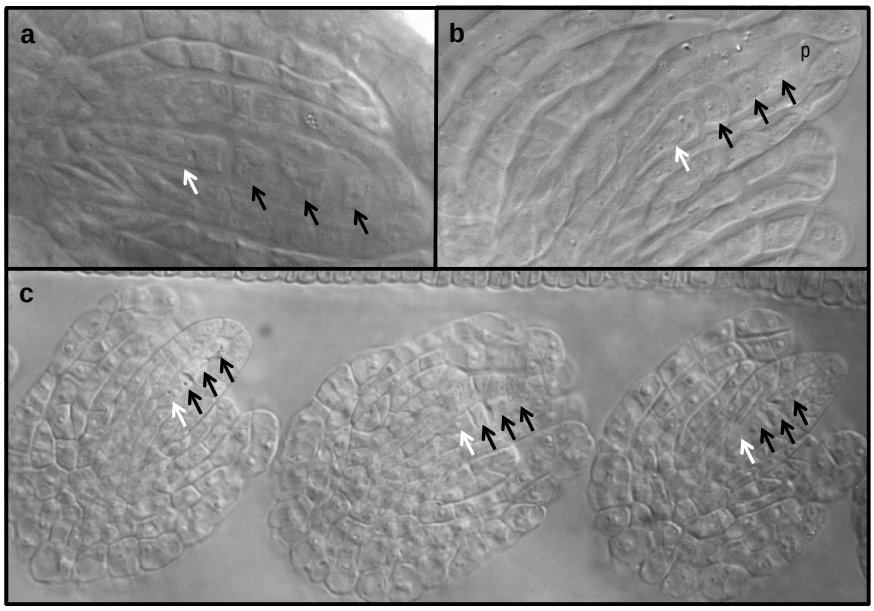

Figure 2.1. Sexual megasporogenesis of the Polygonum type in *B. stricta*. **a-c:** tetrads of genetically-reduced megaspores; white arrows, surviving megaspores; black arrows, degenerating megaspores; p, parietal cells.

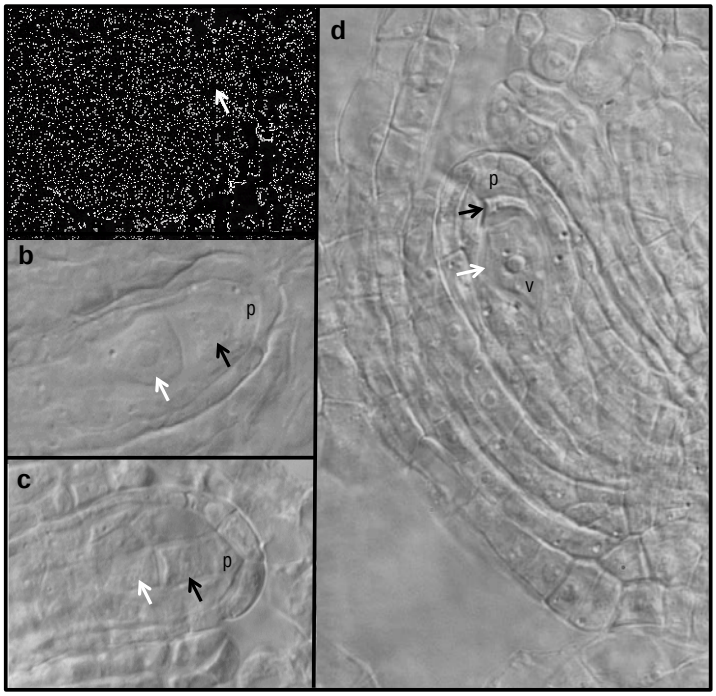

Figure 2.2 Diplosporous megasporogenesis of the Taraxacum type in apomictic *B. lignifera*. **a-c:** dyads of genetically-unreduced megaspores; **d:** rapidly-growing 1-nucleate unreduced embryo sac and degenerating megaspore; white arrows, surviving megaspores or embryo sacs; black arrows, degenerating megaspores; p, parietal cells; v, vacuoles.

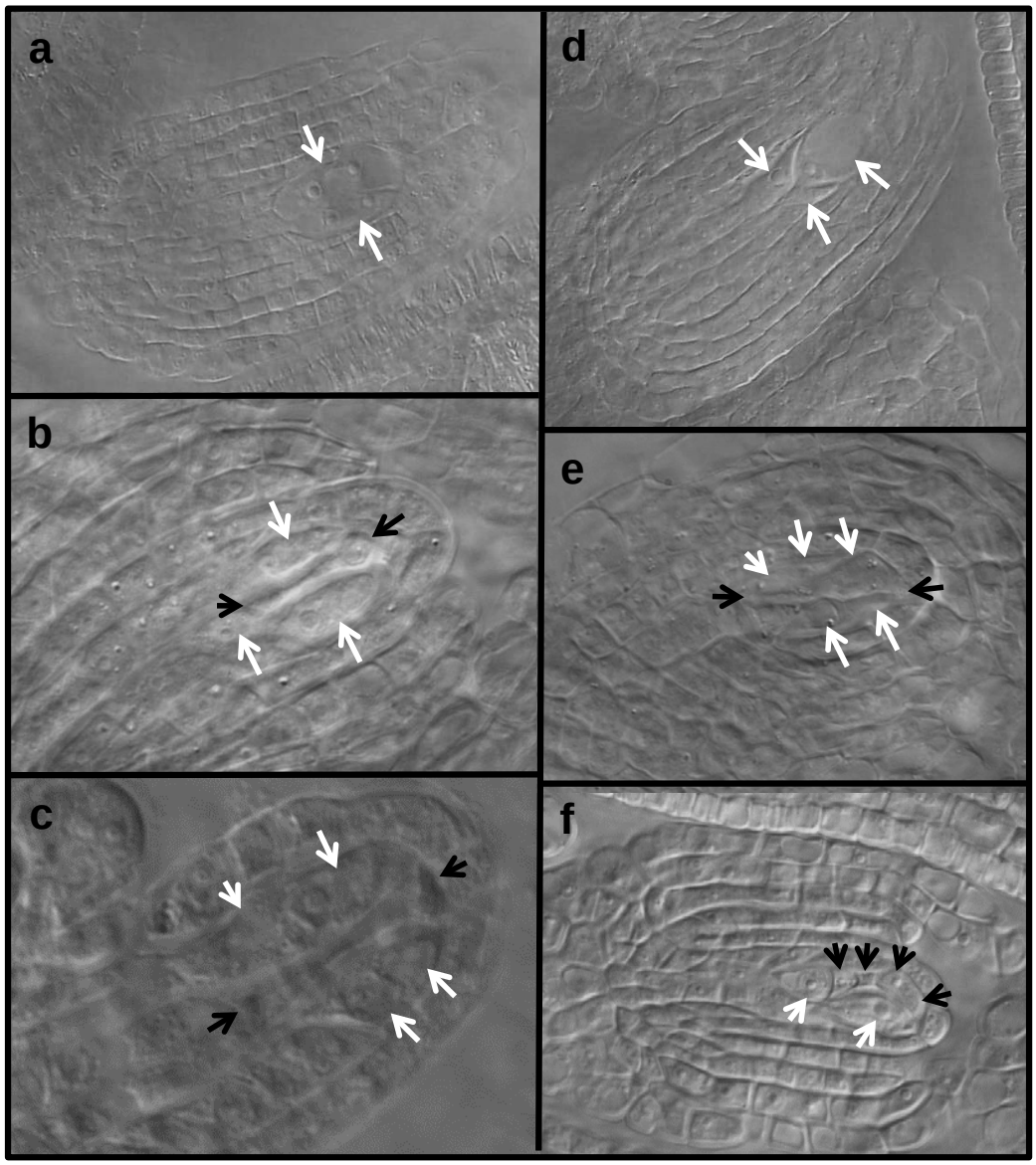

Figure 2.3. Aposporous embryo sac formation of the Hieracium type in apomictic *B. microphylla*. **a-f:** white arrows point to aposporous initials or 1-2 nucleate genetically-unreduced aposporous embryo sacs; **b-c, e:** black arrows point to degenerating spores from meiotically-produced megaspores; **f:** black arrows point to chalazal and micropylar-most ends of degenerating meiotically-produced tetrads; **a, d:** meiotic products completely degenerated

Determining the mode of reproduction by flow cytometry of single seeds depends on an ample supply of nuclei from both embryo and endosperm cells within single seeds. In the taxa studied, plenty of embryo nuclei were available in individual seeds to

determine the ploidy level of the embryo. However, *Boechera* seeds at maturity, like *Arabidopsis* seeds, contain only a single layer of endosperm cells, which are compressed against the ovule wall by the nutrient-storage-containing cotyledons of the mature embryo. The results of the present study suggest that nuclei in these endosperm cells degenerate rapidly upon seed maturation. In this respect, ploidy levels of endosperm were difficult to obtain by single seed flow cytometry using old seeds. Even in freshly-harvested mature seeds, some species yielded favorable endosperm ploidy level results while others did not (Table 2.3).

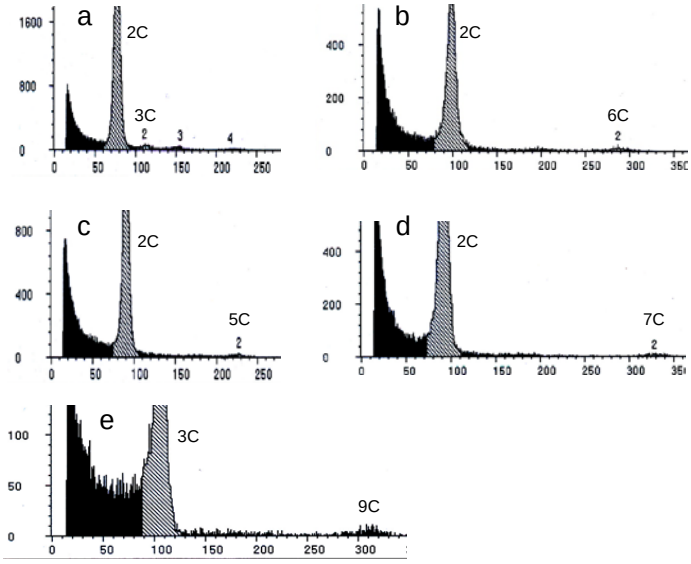

Figure 2.4. Representative flow-cytometry histograms derived from single seeds of various *Boechera* species. **a:** *B. stricta*, 2C:3C; **b:** *B. lignifera*, 2C:6C; **c-d:** *B. microphylla*, 2C:5C and 2C:7C, respectively; **e:** *B. gunnisoniana*, 3C:9C.

Seeds of *B. lignifera* and *B. microphylla* yielded favorable endosperm ploidy results, from which frequencies of sexually versus apomictically-produced seeds could be estimated (Fig. 2.4). Interestingly, the tabulated results (Table 2.3) were not consistent with those based on embryology (Table 2.2). In the case of *B. lignifera*, 16 % of ovules

were undergoing sexual megasporogenesis, but only 4 % of seeds were produced sexually. High-frequency abortion of seeds forming sexually presumably accounted for this discrepancy. Likewise, 35 % of *B. microphylla* ovules, in the meiocyte to early embryo-sac-formation stages, were observed to contain what appeared to be viable tetrads without obvious aposporous embryo sacs or aposporous initials (Table 2.2). Again, only about 4 % of flow-cytometry-tested *B. microphylla* seeds were being produced sexually (Table 2.3). In both cases, the data suggest these taxa are highly sterile sexually but that apomixis has provided an escape from sexual sterility. Sexual sterility is understandable because both taxa are considered to be interspecific hybrids, *B. lignifera* being derived from B. kelseyana and B. thompsonii, and B. microphylla being derived from *B. yellowstonensis*, *B. imnahaensis* and *B. thompsonii* (Fig. 2.5).

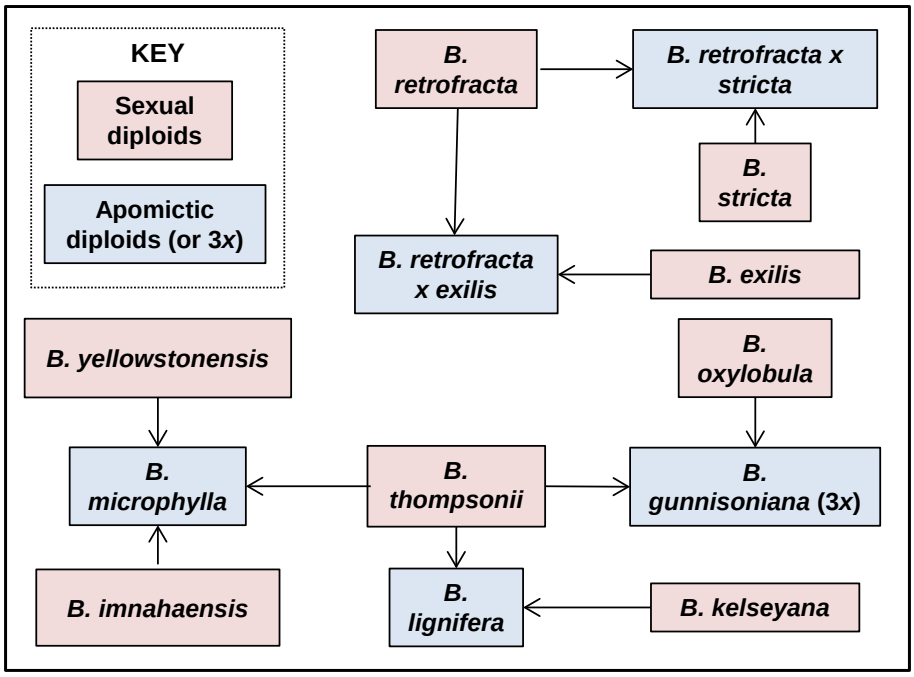

Figure 2.5. Sexual diploid progenitors of the apomictic interspecific *Boechera* hybrids evaluated herein. These are tentative phylogenies based on unpublished molecular marker data (personal communication to John G. Carman from Michael D. Windham, 2015). The relationship between *B. lignifera* and *B. kelseyana* is according to Windham et al. (submitted).

The high frequency of presumably-functional sexual tetrads recorded for *B. microphylla* (Table 2.2) may be misleading. Such tetrads might have been completely replaced by late-forming aposporous embryo sacs had the pistils continued to develop, i.e., had not been killed and fixed for cytology. Additionally, as discussed above for *B. lignifera*, many sexual tetrads that formed in *B. microphylla* ovules (if not replaced by late-forming aposporous embryo sacs) were likely sterile due to genetic imbalances coincident with meiosis occurring in this interspecific hybrid.

In many plants, including *Arabidopsis* (Kradolfer et al., 2013), a close relative of *Boechera*, endosperm formation fails or is greatly weakened when the maternal to paternal genome ratio differs from the normal 2:1, i.e., two maternal genomes (central cell) to one paternal genome (sperm nucleus). This endosperm balance number requirement is maintained in some apomicts by developmental modifications during embryo sac formation such that the mature embryo sac contains only four instead of eight nuclei, e.g., the 4-nucleate Panicum-type embryo sac wherein the central cell is derived from a single polar nucleus, or by modifications of the fertilization system. Examples of the latter include *i*) reduced sperm nuclei fertilizing unfused and unreduced polar nuclei and *ii*) unreduced central cells (fusion products of unreduced polar nuclei) being fertilized by unreduced pollen. In other apomicts, the 2:1 genome balance number requirement is completely relaxed such that maternal to paternal genome ratios, in endosperm cells, are highly divergent from the normal requirement of 2:1. Such ratios include 4:1 and 8:0 (Spielman et al., 2003).

A relaxation of the endosperm balance number requirement in apomicts is also observed in *Boechera* (Aliyu et al., 2010), which is confirmed in the present study. The

normal 2:1 ratio was observed in diploid sexual *B. stricta*, where it produced a 3C endosperm (Fig. 2.4a), and in diploid apomictic *B. lignifera*, where it produced a 6C endosperm (Fig. 2.4b). In the latter case, apomeiosis occurs on the male side to produce unreduced pollen (Windham et al. submitted). Thus, central cells in ovules of this species are 4C, which are fertilized by 2C sperm to produce 6C endosperm (maintaining the 2:1 maternal to paternal genome ratio). The 2:1 ratio was also observed for the triploid *B. gunnisoniana* hybrid, in which the genome contributions were 6C from the central cell and 3C from unreduced pollen (Fig. 2.4e). Deviations from the 2:1 endosperm balance number rule were observed in *B. microphylla*. A 4:1 ratio was observed in most seeds, 4C from the central cell and 1C from the sperm, but a presumably 4:3 ratio (7C) was observed in one seed, and this 4:3 ratio was also observed in one seed of *B. lignifera* (Table 2.3). Both species were grown adjacent to each other and to the triploid *B. gunnisoniana* hybrid, which produces unreduced (3C) pollen. Hence, the likely origin of the endosperm in the 2C:7C seeds (Table 2.3) is a 4C central cell being fertilized by a 3C *B. gunnisoniana* sperm.

When correctly identified, *B. gunnisoniana* is a diploid interspecific hybrid between sexual *B. thompsonii* and sexual *B. oxylobula* (Fig. 2.5). However, our *B. gunnisoniana*, which was collected at the type location for this species (Table 2.1), is triploid (Fig. 2.4e). It may be a tri-specific hybrid involving diploid *B. gunnisoniana* and another unknown *Boechera* species. As a triploid, the formation of viable male and female gametes by sexual meiosis is not expected. Here again, apomixis, on both the male and female side, appears to have provided an escape from the sterility that accompanies unbalanced male and female gametes in interspecific hybrids. Embryo and

endosperm ploidy levels were determined for only seven seeds of *B. gunnisoniana*. All of these had been produced by apomixis in which unreduced central cells (6C) were fertilized by unreduced sperm (3C) to produce a 3C to 9C embryo to endosperm ratio (Fig. 2.4e). This 3C:9C ratio was also consistently observed by Schmidt et al. (2014) for a different accession of triploid *B. gunnisoniana*.

The *B. retrofracta* x *exilis* and *B. retrofracta* x *stricta* apomicts were only weakly apomictic embryologically (Table 2.2), but no sexually-produced seeds were detected (Table 2.3). This again indicates sexual sterility due to interspecific hybridity. The high degree of facultativeness at the cytological level (Table 2.2) might reflect a recent hybridization-induced induction of apomixis in these taxa. In this respect, older apomicts are expected to be more nearly obligate apomicts than youthful apomicts due to acquisition in the older apomicts of a genetic background, over time, by fortuitous mutations and recombination that support the high seed fertility (fecundity) offered by near-obligate apomixis (Hair, 1956; Carman, 2007).

This report of apospory in *B. microphylla* is the first formal documentation of apospory as the primary mode of reproduction in the genus as well as in the entire Brassicaceae. It brings the number of angiospermous genera that contain aposporous species to 111 and the number of genera that contain both aposporous and diplosporous species to 18, 110 and 17, respectively, were previously documented (Hojsgaard et al., 2014). Aposporous embryo sac formation also occurs in high frequencies in certain *Antennaria*, which is generally diplosporous (Carman, 2007), and occasionally in *Tripsacum* (Carman, personal observations). This suggests that differences in the regulation of apospory and diplospory may be more subtle than previously thought. That

apospory and diplospory occur at the diploid level reinforces the conclusion that *Boechera* is highly unique among angiosperms. *Boechera* is an important genus for studying apomixis, and a vast amount of research is still needed to understand it at the evolutionary, phylogenetic and molecular levels. Such studies are aided by the fact that *Boechera* is closely related to *Arabidopsis*. *Boechera* are easy to grow, and of course its double mode of reproduction, apomixis and sex, make it appealing for molecular studies. In the present investigation we found that *Boechera* has the capability to reproduce aposporously and diplosporously, depending on the species. These findings will be important for future investigations, especially as they relate to types of apomixis.

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CHAPTER 3

DIFFERENTIALLY-EXPRESSED GENES IN PISTILS AND OVULES OF SEXUAL AND APOMICTIC *BOECHERA* (BRASSICACEAE)

ABSTRACT

Previous microarray studies in our laboratory using mRNA from sexual and apomictic *Boechera* have provided evidence that cells in pistils and ovules of apomictic *Boechera* are insensitive to stress. Herein, we chose four stress response genes that were differentially expressed in the previous studies and one ribosome gene. Our objective was to verify the microarray findings by quantitative real time PCR (qPCR) using an additional apomictic *Boechera* species, *B. retrofracta* x *exilis,* and two formerly-used species, sexual *B. stricta* and apomictic *B. lignifera*. Three sets of pistil samples were collected from each species, at the megaspore mother cell to early embryo sac formation stages, and these were analyzed by qPCR. For three of the five genes, expression differences followed those observed by the previous microarray studies. Expression valued for the two remaining genes were in the same direction as observed in the microarray studies, but the differences were not statistically significant. Further studies are required to determine whether other sexual and apomictic *Boechera*, which may be more phylogenetically divergent than those studied here, will also demonstrate the same differences in gene expression. Additional studies are also required to determine if these differences are at all involved in regulating mode of reproduction.

INTRODUCTION

Being rooted in soil, plants are unable to escape environmental stresses. Thus, plants tolerate stress by homeostasis adjustments. During these adjustments, developmental processes may be altered including the alteration of normal physiological schedules, and these alterations often depend on stress intensity. Cells subjected to high levels of stress (intensity or duration) experience greater changes in developmental and physiological programs (Khandelwal et al., 2008). How stress affects an organism might also depend on the stage and time of development of the organism as well as on the genotype.

In angiosperms, the time interval from the vegetative to the reproductive stage is an especially vulnerable period, with the environment contributing negatively or positively to flowering (Jiang et al., 2009). In the presence of certain stresses, chromatin may suffer changes, allowing differences in the transcription of genes (Wang et al., 2008; Jiang et al., 2009). In *Vicia villosa*, drought stress triggered changes in morphological characteristics as well as in productivity, but these changes were affected by time and stage of development in which the stress was applied (Andersen et al., 2002; Sun et al., 2004; Petraitytė et al., 2007; Aragón et al., 2008). In wheat, the effects of drought are highly dependent on genotype (Rampino et al., 2006) and on the stage of development in which the plant is exposed to drought (Zhu et al., 2005). C3 plants are particularly responsive to drought stress (Araus et al., 2002). In rice, it was shown that the effect of drought also depends on the organ stressed (Zhou et al., 2007). Environmental stresses trigger changes in plant development because they affect mechanisms at the cellular, physiological and molecular levels (Barnabás et al., 2008).

Abiotic and biotic stresses often affect the expression of the same stress response genes. The expression of stress response and defense genes were modified in *Arabidopsis* by exposure to RNA viruses (Whitham et al., 2003) and to the fungus *Botrytis cinerea* (AbuQamar et al., 2006). According to Wullschleger et al. (2009), drought stress triggered a deep modification in the transcriptome in *Populus*. Drought increases the senescence of reproductive organs (Sun et al., 2004). High temperature stress of *Brassica napus* during flowering caused fruit abortion and inhibited the further development of seeds (Young et al., 2004). In the same way, salt stress caused ovule abortion in *Arabidopsis* (Sun et al., 2004). In sexual *Arabidopsis* and in apomictic *Boechera holboellii*, drought triggers the up regulation of certain stress-response genes. Liu et al. (2011) reported that in *Arabidopsis*, the size and morphology of plants were changed when exposed to high temperature. Thus, submitting plants to one or more abiotic or biotic stressors often causes morphological and biological alterations.

Microarray studies conducted in our laboratory using mRNA from sexual and apomictic *Boechera* have provided strong evidence that cells in pistils and ovules of apomictic *Boechera* are insensitive to stress. Stress response genes were up-regulated in pistils and ovules of sexual *Boechera* compared to apomictic *Boechera*. The objective of the present study was to verify the microarray findings by qPCR using additional sexual and apomictic *Boechera*. To do this, four stress-related genes and one ribosomal gene were selected and their expression in pistils of two facultatively-apomictic *Boechera* species and one sexual species were analyzed.

MATERIALS AND METHODS

Plant materials -- Two diploid apomictic taxa were studied, *B. retrofracta* x *exilis* and *B. lignifera*, and one sexual species, *B. stricta* (Chapter 2). Seeds of these plants were placed on moist filter paper in petri dishes, stratified at 4 °C for 3 weeks, and planted in cone-shaped pots (68 mm diameter x 255 mm tall, 600 mL volume) or square pots (85 mm wide x 95 mm tall, 350 mL volume), which were filled with Sunshine Mix #1 potting soil (Sun Gro Horticulture Canada Ltd, Vancouver, BC). Thirty to 40 plants of each genotype were established. After 6 weeks, seedlings (8-12 leaves) were vernalized for 10-12 weeks in a cold room (4-6 °C) with minimal lighting (8/16 day/night photoperiod) from soft-white fluorescent bulbs. The vernalized plants were then transferred to a controlled-environment greenhouse where a 16/8 h day/night photoperiod was maintained using supplemental light provided by 1000 W high-pressure sodium-vapor lamps. These provided a minimum photosynthetic photon flux of 600 µmol m^{-2} sec^{-1} at the tops of the canopies. Day/night temperatures were maintained at 22/16 °C, and plants were watered regularly with a 15:20:20 (250 mg L^{-1}) nutrient solution.

Embryological staging of pistils for sample collection -- Pistils and ovules analyzed by the previous microarray studies were collected from the megaspore mother cell stage through the mid embryo sac formation stage. The intention of the present study was to obtain pistil samples at the same stage of development. To accomplish this, clusters of floral buds at the late pre-anthesis stage and younger were fixed in formalin acetic acid alcohol (FAA) for 48 h and then transferred to 70 % ethanol for storage at room temperature. The buds were then cleared in a series of solutions ending with a 2:1 concentration of benzyl benzoate and dibutyl phthalate (Crane and Carman, 1987). Thirty

to 40 pistils were then excised for each genotype, and those ranging from 0.7-3.0 mm in length were mounted such that pistils of similar length occurred in separate columns on the slides. The pistils were mounted in a small volume of clearing solution, up to 16 per slide, and covered with a coverslip. Ovaries inside the cleared pistils were studied using a BX53 microscope (Olympus, Center Valley, PA, USA) equipped with differential interference contrast (DIC) optics.

Quantitative Real Time PCR (qPCR) -- Appropriately-staged pistils (80-150 per sample, three samples per species) were excised, placed immediately in RNALater (Qiagen), and stored at -80 °C. For qPCR process we used three biological replicates and two technical replicates from all the species and treatments. We extracted total RNA from pistils at different stage development using Qiagen RNeasy Plant Mini Kit, and then the RNA was treated with DNaseI before the conversion to cDNA. 1.8 µg of RNA from sample was converted to cDNA using the Qiagen Easy First Strand kit in a 14-µL reaction. Primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi); and the size of the amplicons used was between 163 and 230 bases, and an average melting temperature of 60 degrees. Additionally, we included a housekeeping gene for normalization, BoechACT2/Actin 2, which had previously been used in qPCR studies of Boechera mRNA (Pellino et al., 2011). Primers were tested for amplification of the expected cDNA region using Boechera gDNA. All samples concentrations were analyzed using Nanodrop 2000 UV visible spectrophotometer (Thermo Scientific, USA).The qPCR reaction was carried out in in 20-µL reaction volumes having QuantiTect SYBR Green PCR kit (Qiagen), 250 nM of both forward and reverse primers, and 3 µL of a 1:20 dilution of cDNA. The reaction cycles used were as:

50 ºC, 2 min; 95 ºC, 15.00 min; 40 cycles of 94 ºC, 15sec; 58 ºC, 30 sec; 72 ºC, 30 sec. qPCR were performed using a DNA Engine, Opticon 2, Continuous Fluorescence Detection System (Bio-Rad), in MicroAmp Optical 96-well 12 reaction plates with optical covers ANOVA was used to analyze the expression of each gene in the different species.

Table 3.1. Primer pairs used in the present study

Gene name	Primer	Tm	Product size
AT2G40140	AT2G40140_Forward:CAGATGTGTCGTGGGTGAAC	60	163
	AT2G40140_Reverse:TATGCCACAATCTGCTGCTC	59.98	
AT4G02730	AT4G02730_Forward:TCGCATTACACTTGCTCTGC	60.17	167
	AT4G02730_Reverse:ATCGTCTCATCGAACGATCC	60.04	
AT3G52940	AT3G52940_Forward:TTCAGGCTATTGGGGAATTG	59.89	163
	AT3G52940_Reverse:TCGAACCTCGTCTCTTCGTT	59.99	
AT3G13580	AT3G13580_Forward: TCATCTGCGTTGAGGATCTG	59.94	170
	AT3G13580_Reverse: GATGAAGTTCTCGCGGTTTC	59.82	
AT2G48020	AT2G48020_Forward:AGTTGACAGAGCCGGAAGAA	59.99	179
	AT2G48020_Reverse:CCATTCCTGCTGAAAACGAT	60.07	

RESULTS AND DISCUSSION

Gene selection -- The present study was designed to test whether relationships previously observed between specific gene networks and modes of reproduction, i.e., sexual or apomictic, based on microarray analyses of mRNA in *Boechera* pistils and ovules (in preparation), are also observed in additional *Boechera* taxa. The previous microarray studies involved two apomictic species, diplosporous *B. lignifera* and aposporous *B. microphylla*, and two sexual species, *B. formosa* and *B. stricta*. Over 5000 genes were identified in these microarray studies as being differentially expressed in ovules or pistils between sexual and apomictic species, and these genes were responsible

for hundreds of gene ontology (GO) categories being enriched (significantly more differentially expressed genes within a GO category than expected by chance). Many of the same enriched GO categories were observed for both the pistil-only and ovule-only analyses. These included many stress-response-related and ribosome-related GO categories. Consequently, for further analysis with additional *Boechera* taxa, we chose stress-response genes and ribosome-related genes that were differentially expressed in both pistils and ovules based on microarray analyses.

Criteria used in narrowing down differentially-expressed genes selected for the present qPCR study included their responsiveness to stress. Based on stress responsiveness, as observed in other species, seven genes were selected: AT3G03190 (glutathione S-transferase F11), AT2G40140 (zinc finger (CCCH-type) family protein), AT4G02730 (transducing/WD40 repeat-like superfamily protein), AT3G52940 (Ergosterol biosynthesis ERG4/ERG24 family), AT3G51300 (RHO-related protein from plants), AT3G21175 (ZIM-like 1), and AT2G48020 ((major facilitator superfamily protein). We also considered genes that encode ribosome components that differentially methylate mRNAs thus post-transcriptionally regulating the expression of other genes. These genes were AT3G13580 (ribosomal protein L30/L7 family protein) and AT2G44120 (ribosomal protein L30/L7 family protein).

Because the PCR primers used were designed from the *Arabidopsis* sequence, some failed to amplify genomic DNA of the *Boechera* species tested. Five of the nine originally-selected genes provided favorable amplifications: AT2G40140, AT4G02730, AT3G52940, AT3G13580 and AT2G48020, and these were used for qPCR in the present experiment. In previous experiments, our success rate in designing primers for *Boechera*

based on *Arabidopsis* sequence information has been about 80%, which is similar to what we observed here. The degree of differential expression for the chosen genes as observed in the previous microarray experiments is shown in Table 3.2.

Table 3.2. Genes differentially-expressed in ovule and pistil microarray experiments and chosen for qPCR analyses.

Gene	Tissue	Fold change	
		Up in sexuals	Up in apomicts
AT2G48020	Ovules	9.5	
	Pistils	2.6	
AT2G40140	Ovules	9.9	
	Pistils	2.0	
AT4G02730	Ovules		3.7
	Pistils		2.1
AT3G52940	Ovules		3.2
	Pistils		2.0
AT3G13580	Ovules		94.6
	Pistils		60.4

qPCR analyses -- The directions of differential expression for genes analyzed by qPCR appeared identical to those observed by microarray analyses (Table 3.2, Fig. 3.1). However, differential expression levels determined by qPCR were not significant for two of the genes, AT2G40140 and AT4G0273 (Fig. 3.1, Table 3.3). Note also that the sample variable was significant for all but one of the analyses (Table 3.3). Samples of pistils were taken from the same sets of plants, and some taxa produced enough pistils to complete all three sets of samples within a few days. Collection of samples from other taxa, because of growth differences, occurred over a period of several weeks. This likely introduced variability in the data, and it may be the major reason why stronger significant

differences were not observed. Also, the levels of significance for differentially expressed genes were much high in ovules than in pistils (Table 3.2). This suggests that the gene expression differences are more highly restricted to the germline and its associated tissues in ovules, with pistil tissue having a dilutive effect in the present analysis.

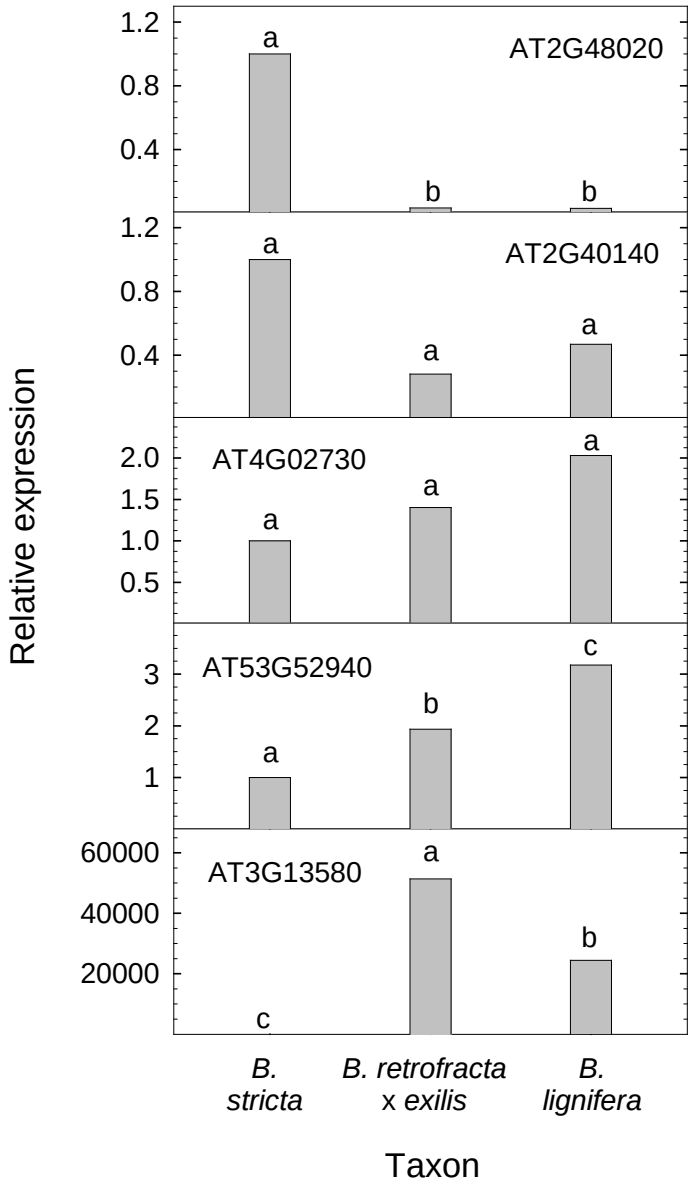

Figure 3.1. Differential gene expression as determined by qPCR. Data were adjusted so that expression levels are relative to those observed in sexual *B. stricta*.

Table 3.3. Analysis of variance statistics by gene

Source of variation	DF	Sum of squares	Mean squares	F-ratio	Significance level
AT2G48020					
Taxa (T)	2	96.953	48.476	211.243	0.000
Samples(T) (S(T))	6	8.074	1.343	5.864	0.010
AT2G40140					
T	2	10.189	5.094	1.061	0.386
S(T)	6	97.203	16.200	3.375	0.050
AT4G02730					
T	2	3.132	1.566	1.658	0.239
S(T)	6	10.866	1.811	1.948	0.177
AT3G52940					
T	2	8.387	4.193	46.345	0.000
S(T)	6	12.030	2.005	22.159	0.000
AT3G13580					
T	2	916.965	458.482	5921.305	0.000
S(T)	6	6.194	1.032	13.332	0.001

The most significantly different gene in terms of expression was AT3G13580. This gene encodes a ribosomal protein of 80 S and 60 S ribosomes in *Arabidopsis* (Giavalisco et al., 2005; Carroll et al., 2008; Abbasi et al., 2010). It is a L30/L7 family protein that functions in regulating transcription and influencing translation. It is expressed during several growth stages, including flowering and maturation of embryos; and it is expressed in flowers, seeds, embryos, among other organs and tissues (Tair.org.). Ribosomal proteins are influenced by stress, which changes their expression. In maize, the expression of ribosomal proteins at the transcriptional and post-transcriptional levels was affected by the length of stress, with anaerobic proteins being produced under hypoxic stress (Bailey-Serres and Freeling, 1990). Hence, this gene likely influences protein synthesis.

The next most significantly different gene in terms of expression was AT2G48020. This gene encodes a sugar transporter (Weber et al., 2005, Coupe et al., 2006, and Carter et al., 2004) known as ZIF2, Zinc-Induced Facilitator 2 and ERD6 (Early Response to Dehydration)-like 7 (tair.org; Remy et al., 2014). It is expressed during flowering, particularly during the globular and mature embryo stages. It is also expressed in roots and most other tissues of *Arabidopsis* (tair.org, Remy et al., 2014). Its participation in embryo and seed development make it important for the present study.

The next most significant gene in terms of expression was AT3G52940. It is known as ELL1, EXTRA-LONG-LIFESPAN 1, FACKEL, FK and HYD2 and is linked to brassinosteroids biosynthesis, embryo and seed development, among others. It is expressed in flowers, embryos, seeds, pollen and other organs (Tair.org). In animals, this gene encodes proteins responsible for sterol production and transcriptional and post-transcriptional regulation processes that affect meiosis, apoptosis and other cellular activities (Edwards and Ericsson, 1999; He et al., 2003). It is also involved in regulating sterol production in plants, including brassinosteroids, sitosterol and stigmasterol. In *Arabidopsis,* sterols influence embryonic and post-embryonic development, and FACKEL mutants produce irregular embryos in *Arabidopsis*, possibly because of poor brassinosteroid synthesis (Jang et al., 2000; Clouse, 2000; Schrick et al., 2000, Souter et al., 2002; Lindsey et al., 2003; Schaller, 2003; Pullen et al., 2010; Clouse, 2011; Qian et al., 2013). For brassinosteroids to work correctly and contribute to normal organ formation, it is important to maintain a normal cellular homeostasis (Tanaka et al., 2005). Therefore, an apparent insensitivity to stress, as putatively observed in *Boechera* apomicts, may cause considerable changes in the expression of this gene (Clouse, 2011).

Because of its importance on embryo development, its relation to stress, and its differential expression observed herein between pistils of sexual and apomictic plants, it is a good gene for further study.

AT2G40140 is a stress response gene, a CCCH-type zinc finger transcription factor and an ankyrin-repeat protein (Jae-Heung et al., 2004; Kaplan et al., 2006; tair.org). It is also known as SZF1 and SZF2 (Dong Hwan et al., 2008), AtSZF2 (Sun et al., 2007) or CZF1 (Vergnolle et al., 2005). It is regulated by other zinc-finger transcription factors that regulate multiple genes associated with stress. It also regulates various morphogenesis and signal transduction processes (Sun et al., 2007; Lin et al., 2011; Guohua et al., 2012), and its action involves hormone responses linked to development (Bogamuwa and Jang, 2013). It has motifs characterized by cysteines and histidines that are involved in multiple functions (Guohua et al., 2012), including growth, regulation of genes associated with abscisic acid (ABA) and gibberellic acid (GA) production, seed development, maturation and germination, embryo development (Li and Thomas, 1998; Lin et al., 2011; Bogamuwa and Jang, 2013), and light and oxidative stresses (Davletova et al., 2005). In *Arabidopsis,* AT2G40140 was down-regulated under high light stress (Khandelwal et al., 2008), and there was an alteration of its expression due to stress by virus infection (Whitham et al., 2003), as well as an alteration due to fungi (AbuQamar et al., 2006). In the same way, the expression of this gene showed an alteration when exposed to low temperature (Vogel et al., 2005; tair.org). Different expressions of this gene were found for orthologues of the *Arabidopsis* gene (Dong et al., 2008), including *Populus trichocarpa* (Guohua et al., 2012). In *Populus*, the response of this gene to drought was different between two different genotypes, and it yielded

different responses depending on the time of day (Wilkins et al., 2009). In the same way, Hamanishi et al. (2010) found in a study of six *Populus* species that the closer two genotypes were phylogenetically the more similar their responses to stress were, confirming that there are differences in how this gene responds to drought stress among genotypes. Sun et al. (2007) also reported that this gene is a salt stress response regulator in *Arabidopsis*. Therefore, this gene shows to be very important for plant development, and biotic and abiotic stresses are very important since they may influence plant metabolism and plant reproduction.

AT4G02730 encodes a protein expressed during the mature embryo stage, globular embryo stage, the flowering stage and others (tair.org). It is named ATWDR5B in humans. In *Arabidopsis*, WDR5 (WD40 REPEAT) Homolog B (WDR5B) up-regulates Flowering Locus C (FLC) (Jiang et al., 2009). Hence, it is an interesting choice with regard to the expression of apomixis.

The present study provides evidence that the differential expression of genes observed previously by microarray analyses of pistils and ovules among specific sexual and apomictic species of *Boechera* may be extended to the apomictic hybrid *B. retrofracta* x *exilis*. The data provide evidence for the hypothesis that sexual and apomictic pistils perceive their environment differently. Whether this perception is at all responsible for apomixis will require further study.

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CHAPTER 4

EFFECTS OF STRESS ON MEGASPOROGENESIS, SEED SET, AND GENE EXPRESSION IN SEXUAL AND APOMICTIC *BOECHERA* (BRASSICACEAE)

ABSTRACT

Boechera is an important genus for the study of apomixis. This importance is due to its proximity to *Arabidopsis*, easiness to reproduce and its advantage of having facultative apomictic species within the genus. Drought stress and drought plus heat stress were used to evaluate the effects of the stresses on five apomictic Boechera species and one sexual. Cytology, flow cytometry and qRT-PCR were used to analyze the samples. The cytological evaluation of ovules showed changes in the frequency of apomictic and sexual ovules, increasing the frequency of sexual ovules under stress in some species. Not changed was found in the frequencies of sexual and apomictic seeds grown with or without stress. Finally, gene expression profiling with qRT-PCR showed changes in some species and genes due to stress. However, these changes are not shown to be related to mode of reproduction.

INTRODUCTION

Facultative apomixis has been observed to occur in response to specific environmental stressors. Stress increases the frequency of sexual reproduction in apomictic plants (Böcher, 1951; Carman et al., 2011). Facultative apomixis occurs in response to specific environmental stressors. The frequency of sexual reproduction of seeds in facultative apomictic species increases when the plants are grown under stress conditions (Böcher, 1951; Carman et al., 2011); and this phenomenon has also been reported in animals (Suomalainen et al., 1987). Therefore, to study gene expression profiling on facultative apomictic species developed under different environment is a great tool to understand causes of apomixis on plants.

Biological characteristics and expression of stress response genes may be affected by several biotic and abiotic stressors some of them being drought and heat. Drought and heat stresses trigger alteration in the metabolism of different species. Drought stress was reported to affect wheat, depending on the genotype and stage of development (Zhu et al., 2005; Rampino et al., 2006). Due to drought, morphological characteristics and productivity were modified in *Vicia villosa*, rice, C3 plants and Populus (Andersen et al., 2002; Araus et al., 2002; Sun et al., 2004; Petraitytė, 2007; Zhou et al., 2007; Aragón et al., 2008; Wullschleger et. al., 2009). It has been reported that the combination of drought and heat to stress plants trigger different alteration on plants that when plants are subjected under either heat or drought. This phenomenon occurs due to the intervention of two multigene defense pathways (Rizhsky et al., 2004). In Tobacco, a combination of drought + heat stress triggered the suppression of photosynthesis and closure of stomata; and it triggered the induction of some transcripts and the suppression of others, that

would not be suppressed or induced when subjected to these stresses individually, and not combined (Rizhsky et al., 2002). Therefore, combination of these stresses is also good to analyze in Boechera, and see if it has a significant effect on stress response genes, and influences the mode of reproduction in facultative apomictic Boechera species.

Several methodologies have been used to identify apomixis in Boechera. Some of the methodologies used are cytology, flow cytometry, genomic in situ hybridization, microarray and RNA-Seq Analyses (Schmidt et al., 2014). These methods, besides karyotype analysis, were used to analyze apomixis in the diploid apomictic *B. divaricarpa* and the diploid sexual *B. stricta* (Schranz et al., 2006). Therefore, different methodologies work for identification of apomixis.

In this experiment we used flow cytometry, cytology and gene expression profiling with qPCR to analyze the effect of drought and drought plus heat stress in apomictic species of Boechera. For this research, we selected four stress-related responses genes and one ribosomal gene: AT2G40140, AT4G02730, AT3G52940, AT3G13580 and AT2G48020. AT2G40140 is a protein coding and stress response gene, reported as a CCCH-type zinc finger protein and an ankyrin-repeat protein (Jae-Heung et al., 2004; Kaplan et al., 2006; tair.org). AT4G02730 is a protein-coding and stress response gene. AT3G52940 is also protein-coding and stress response gene, also known as ELL1, EXTRA-LONG-LIFESPAN 1, FACKEL, FK, and HYD2, which is linked to brassinosteroids and sterol biosynthetic process, embryo and seed development, among other processes. AT3G13580 is a coding-protein gene and a Ribosomal protein L30/L7 family protein. Finally, AT2G48020 is another protein-coding and stress response gene. It is also known as ZIF2, ZINC-INDUCED FACILITATOR 2 and ERD6 (Early

Response to Dehydration)-like 7 (tair.org; Remy et al., 2014). The reason for the selection of these genes is that they were differentially expressed in ovules and pistils of one apomictic and one sexual species in a previous microarray analysis carried out at Carman`s lab. In addition, with exception of AT3G13580, these genes are stress response related, and they are expressed during stages such as embryo, seed and flower development. On another hand, AT3G13580 is a ribosomal protein L30/L7 family protein that encodes ribosome components and influences expression of other genes.

The hypothesis for this research is that apomictic plants do not have the ability or capability to perceive environmental stresses during ovule development, and this absence of stress signals, which normally triggers meiosis, is responsible for the occurrence of apomeiosis (Carman et al., 2011). We applied drought and temperature stresses to facultative apomictic Boechera species seeking to convert apomictic to sexual seeds set. My study determined the effects of drought and high temperature on facultativeness of apomictic expression and on the expression of the genes chosen.

MATERIALS AND METHODS

Plant materials and treatments -- Five apomictic *Boechera* were investigated in the present study, three are diplosporous diploids, *B. lignifera*, *B. retrofracta* x *exilis* and *B. retrofracta* x *stricta*, one is a diplosporous triploid, *B. gunnisoniana*, and one is an aposporous diploid, *B. microphylla*. A diploid sexual species was also studied, *B. stricta*. Details concerning collection locations and embryology are reported in Chapter 2.

Seeds of the six taxa were placed on moist filter paper in petri dishes, stratified at 4 °C for 3 weeks, and planted in cone-shaped pots (68 mm diameter x 255 mm tall, 600

mL volume) or square pots (85 mm wide x 95 mm tall, 350 mL volume), which were filled with Sunshine Mix #1 potting soil (Sun Gro Horticulture Canada Ltd, Vancouver, BC). Thirty to 40 plants of each genotype were established. After 6 weeks, seedlings (8-12 leaves) were vernalized for 10-12 weeks in a cold room (4-6 °C) with minimal lighting (8/16 day/night photoperiod) from soft-white fluorescent bulbs. Sets of vernalized control plants, drought-treated plants, and heat and drought-treated plants, 8-12 replicates of each were then selected at random for each species.

We had previously observed that vernalized 8-12-leafed seedlings would begin to flower within several days of removal from vernalization. Hence, immediately upon removal from the vernalization chamber, control plants and drought-stressed plants were placed in a controlled-environment greenhouse, and drought and heat-stressed plants were placed in a controlled-environment growth chamber. The control and drought-stressed plants in the greenhouse were exposed to a 16/8 h day/night photoperiod that was maintained using supplemental light provided by 1000 W high-pressure sodium-vapor lamps. These provided a minimum photosynthetic photon flux of 600 µmol m^{-2} sec^{-1} at the tops of the canopies. Day/night temperatures were maintained at 22/16 °C. The functioning of the greenhouse was continuously monitored using a Campbell Scientific (Logan, UT) CR1000 data logger that was connected to thermistors in the greenhouse.

Drought treatments in the greenhouse were imposed by growing the plants at a transpiration rate that was maintained at 50% of the control plants. Transpiration rate was not controlled, but it was replaced by weighing the pots. This was accomplished by *i*) adding a layer of perlite to the surface of all pots, to inhibit evaporation directly from the soilless potting mix, *ii*) obtaining a field capacity weight for each pot (pot weight 2 h

after drenching), *iii*) adding enough water to the controls, measured by pot weight, each day to bring them back to field capacity, *iv*) and by adding a certain amount of water to the drought-stressed plants each day to equal 50% of the transpiration rate of the control plants. From our preliminary tests, *Boechera* plants grown with less than 50% of control water failed to flower and eventually died. The heat and drought-stressed plants in the growth chamber were exposed to a continuous 32 °C environment and a photosynthetic photon flux of 500-700 µmol m^{-2} sec^{-1} (16/8 day/night photoperiod). Drought treatments in the growth chamber were imposed as for those in the greenhouse.

Embryological analyses -- Megasporogenesis in control and treated plants was investigated to evaluate the effects of stress on the mode of reproduction. Clusters of floral buds at the late pre-anthesis stage and younger, of control and treated plants, were fixed in formalin acetic acid alcohol (FAA) for 48 h and then transferred to 70 % ethanol for storage at room temperature. The buds were then cleared in a series of solutions ending with a 2:1 concentration of benzyl benzoate and dibutyl phthalate (Crane and Carman, 1987). Thirty to 40 pistils were excised for each genotype, and those ranging from 0.7-3.0 mm in length were mounted such that pistils of similar length occurred in separate columns on the slides. The pistils were mounted in a small volume of clearing solution, up to 16 per slide, and covered with a coverslip. Ovaries inside the cleared pistils were studied using a BX53 microscope (Olympus, Center Valley, PA, USA) equipped with differential interference contrast (DIC) optics. Details of ovule development were photographed using a MicroFire 599809 camera (Olympus).

Frequencies of sexual and apomictic ovule development were evaluated by restricting analyses to ovules in a diagnostic stage. For plants exhibiting sexual or

diplosporous megasporogenesis, numbers of dyad and tetrads were recorded. The dyad to tetrad ratio in sexual plants is low, because the sexual dyad stage in sexual plants is brief. In contrast, the dyad to tetrad ratio in diplosporous plants is high, because the diplosporous dyad stage is long. For plants exhibiting aposporous apomixis, the presence of aposporous initials (AI) or aposporous embryo sacs (AES) were recorded for ovules in the meiocyte to early embryo sac formation stages. By the 2-nucleate embryo sac stage, it is difficult to tell with certainty whether the embryo sac is sexual or aposporous. Hence, for quantification purposes, earlier-staged ovules were used.

Single seed flow cytometry -- Nuclei were isolated from individual mature seeds produced from control and treated plants using a mortar, pestle and a few drops of Partec (Partec North America, Inc., Swedesboro, NJ) buffer, which contained DAPI (4,6-diamidino-2-phenylindole). Pestles were used only to crack the seeds open. The seed fragments were not ground. The seed fragments were exposed to the Partec buffer solution for several minutes. The nuclei-containing solutions were then filtered through 30 µm nylon filters into 1.2 mL tubes. Nuclear fluorescence values for each sample were determined using a Partec I flow cytometer per the manufacturer's instructions. Relative fluorescence intensities of nuclei were analyzed using Partec software. Embryo and endosperm ploidy levels were determined from 50 individually-analyzed seeds per genotype. Sexual seeds were identified by a 2:3 C embryo to endosperm ratio. Apomictic seeds were identified by a 2:5, 2:6, 2:7 or 3:9 C embryo to endosperm ratio (Matzk et al., 2000).

qRT-PCR -- For qPCR, appropriately staged pistils (ovules in the MMC to early embryo sac formation stages) were excised from control and drought-stressed sexual *B.*

stricta and diplosporously-apomictic *B. lignifera* and *B. retrofracta* x *exilis*. Three samples of 80-150 pistils each were collected for each set of control plants and for each set of drought-stressed plants. The pistils were immediately placed in RNALater (Qiagen), and stored at -80 °C. RNA extraction, PCR primers (for five genes) and qPCR were as previously reported (Chapter 3). Briefly, RNA was extracted and treated with DNaseI, cDNA was synthesized, and qPCR reactions were performed using a DNA Engine, Opticon 2, Continuous Fluorescence Detection System (Bio-Rad). For each gene, two technical replicates of each sample were analyzed (three samples per treatment by taxa combination). This provided six qPCR expression values per treatment by taxa combination for each gene. Each gene by taxa by treatment combination also included six technical replicates of the BoechACT2/Actin 2 housekeeping gene (Pellino et al., 2011), which were used to normalize the results. qPCR expression values were calculated for each gene as the difference between PCR cycle values and the average of the technical replicates of the housekeeping gene (delta Ct method). Delta Ct values were then normalized against the mean value for the sexual *B. stricta* control (delta delta Ct method). The data were subjected to analyses of variance with samples nested within treatments and taxa (SYSTAT II, 2004). The $P \leq 0.05$ level was chosen to represent significance. Relative expression values were determined by raising 2 to the power of the negative delta-delta Ct mean values for each treatment-by-taxon combination.

RESULTS AND DISCUSSION

Stress decreased the frequency of occurrence of diplosporous megasporogenesis (dyad formation leading to unreduced embryo sac formation) in diplosporous *B.*

gunnisoniana and *B. lignifera*, and it reduced the occurrence of unreduced aposporous embryo sac formation in *B. microphylla*. For *B. gunnisoniana* and *B. lignifera*, drought plus heat caused a greater shift from apomeiotic to meiotic megasporogenesis than drought alone (Fig. 4.1-4.3; Appendices II, III). These observations are consistent with those of Böcher (1951), who reported a stress-induced shift during microsporogenesis, in apomictic *B. holboellii*, from apomeiotic to meiotic. Böcher (1951) provided only minimal evidence that this stress-induced shift from apomeiotic to meiotic development occurred on the female side as well. Herein, we document extreme shifts from apomeiotic to meiotic development on the female side as well.

The side-by-side existence of sex and apomixis in facultative apomicts with sex being induced by stress indicates the existence of a sex apomixis switch that responds to environmental conditions. If sensitivity to the environment has been lost, the switch remains on or off (apomictic or sexual). If some sensitivity remains, then the plant may respond accordingly, as appears to be the case with the *Boechera* species observed herein. Percentage apomictic seed set in such plants is correlated with optimal growth/flowering conditions. As growth conditions deteriorate, percentage sexual development, often including sexual seed set, increases. In the case of *Boechera*, reversions from apomeiotic to meiotic development in young ovules do not guarantee that seeds will develop. Böcher (1951) noted that most meiotic microspores were sterile because the *Boechera* taxa were interspecific hybrids or triploids. The same appears to be the case with our apomictic *Boechera* species, which are of interspecific hybrid origin or are triploids (Chapter 2). As detailed below, sexual seed set in our apomictic plants remained low regardless of the increase in meiotic development induced by stress. Other

plants in which development reverts from apomeiotic to meiotic in response to stress include *Calamagrostis purpurea* (Nygren, 1951), *Ageratina riparia* (Sparvioli, 1960), *Limonium transwallianum* (Hjelmqvist and Grazi, 1964), *Dichanthium aristatum* (Knox and Heslop-Harrison 1963; Knox, 1967), *Themeda australis* (Evans and Knox, 1969), *Dichanthium intermedium* (Saran and de Wet, 1976), *Paspalum cromyorrhizon* (Quarin, 1986), and *Brachiaria brizantha* (Lutts et al., 1994).

An important point to make is that *B. gunnisoniana* is a triploid obligate apomictic species. Therefore, although it cannot produce sexual seeds, yet the process to produce sexual seeds may start in the ovules developed under stress conditions. It is good to clarify that sample sizes were small for the *B. retrofracta* hybrids, so these were combined for Figure 4.1. Even with combining them, stress did not shift the mode of reproduction. Interestingly, these plants, and presumably the apomixis that they express, appear to be of very recent origin. Thus, it is possible that their high facultativeness (nearly 50:50 apomeiotic to meiotic in the controls) is the reason they do not shift in response to stress.

Using single seed flow cytometry, we determined that percentage sexual seed set did not increase with stress (Table 4.1) even though the frequency of ovules undergoing meiosis had increased (Fig. 4.1). The data we obtained confirmed that the five apomictic taxa used in this study are strongly apomictic in terms of seed set. In the same sense, we confirm sexuality for *B. stricta*. Some species did not survive to the drought + heat stress, and therefore, we were not able to collect seeds for analysis. In addition, the concentration of the endosperm was very low in most of the samples, and this was a disadvantage to measuring ploidy level. However, following the pattern of the ones that

could be detected, we may conclude that mode of seed formation generally remained apomictic even though many ovules had reverted to meiosis.

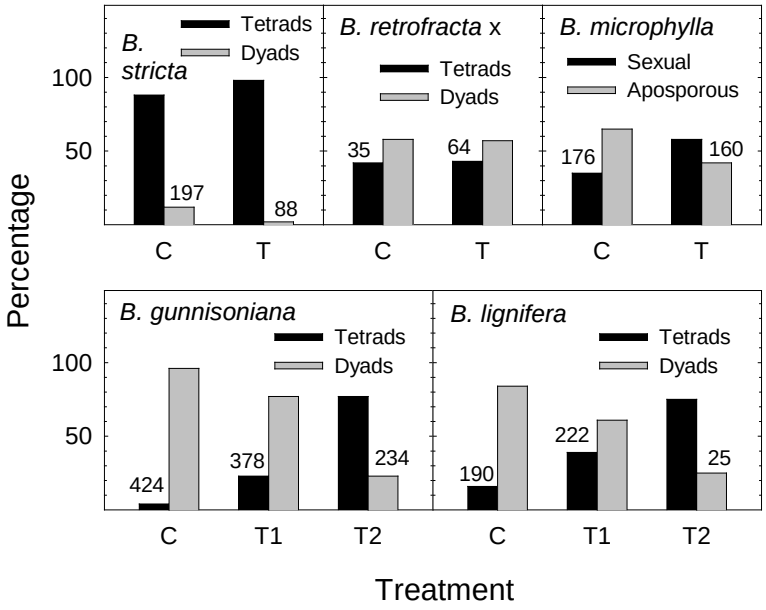

Figure 4.1. Stress treatment effects on tetrad and dyad formation frequencies in sexual *B. stricta*, diplosporous *B. gunnisoniana* and diplosporous *B. retrofracta* x *exilis* and *B. retrofracta* x *stricta* (*B. retrofracta* x) and on frequencies of sexual and aposporous embryo sac formation in aposporous *B. microphylla*. Numbers by pairs of bars represent ovules observed that were in a diagnostic stage. C, control; T, drought and drought and heat treatments combined; T1, drought treatment; T2, drought and heat treatment.

For *B. microphylla*, the switch from aposporous apomeiosis to only sexual apomeiosis was not as pronounced as in the well-established diplosporous species. Again, this might reflect the high level of facultativeness observed in the control plants. For this species, as well as for *B. stricta*, an evaluation of ovules from treatment 2 was not carried out because the plants did not survive both drought and heat stress. As a conclusion, we demonstrated that growing facultative apomictic species of *Boechera* under stress increases the frequency of sexual meiosis in ovules. Meiotic ovules in the apomicts apparently aborted, thus reducing seed set per silique. Abortion of meiotic ovules in these

apomicts is understandable because of interspecific hybridity and triploidy. Thus, apomixis in these species is truly an escape from sexual sterility.

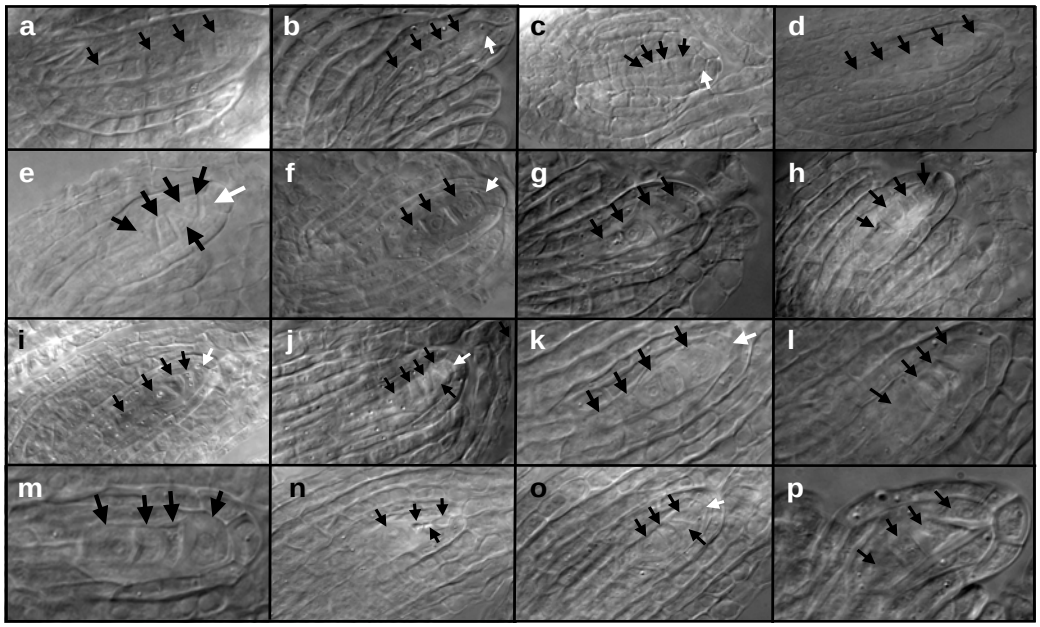

Figure 4.2. Megaspores derived by meiotic processes in sexual and apomictic Boechera. Meiotically-reduced tetrads of megaspores from non-stressed (a) and drought-stressed (b) sexual B. stricta; tetrads and pentads of genetically-reduced but potentially-unbalanced megaspores of drought and heat-stressed apomictic diploid B. retrofracta x exilis (c), non-stressed apomictic triploid B. gunnisoniana (d), drought stressed apomictic triploid B. gunnisoniana (e-h), drought and heat-stressed apomictic triploid B. gunnisoniana (i, j), drought-stressed apomictic diploid B. lignifera (k-o), and drought-stressed apomictic diploid B. microphylla (p). Black arrows indicate megaspores. White arrows indicate the occasional occurrence of subepidermal cells between the nucellar epidermis and the micropylar-most megaspores.

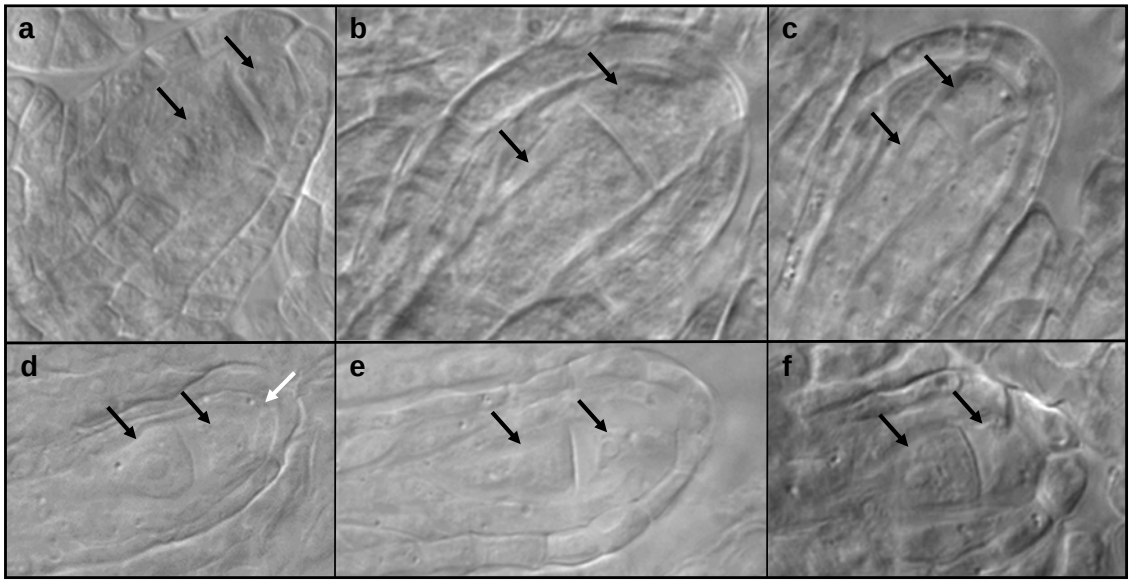

Figure 4.3. Unreduced apomeiotic dyads of megaspores from non-stressed apomictic B. retrofracta x exilis (a), drought and heat-stressed B. retrofracta x stricta (b), drought and heat-stressed B. lignifera (c), non-stressed apomictic triploid B. gunnisoniana (d), drought-stressed apomictic triploid B. gunnisoniana (e) and drought-stressed apomictic B. lignifera (f). Black arrows indicate megaspores. White arrow indicates a sub-epidermal layer between the nucellar epidermis and the dyad.

In triploid *B. gunnisoniana*, an obligate apomict, the embryos were 3C, as expected, and endosperm 9 C (Table 4.1), in both control and treatment 1, with exception of one seed found to have 5C endosperm. This shows that in this species endosperm formation is pseudogamous (requiring fertilization of the central cell) were the two 3C polar nuclei fuse to produce a 6C central cell that is fertilized by a 3C sperm (unreduced sperm) to form the 9C endosperm. The results obtained with *B. gunnisoniana* in this research agree with what was obtained by Taskin et al. (2004). In their study, seeds had 6C endosperm but one had a 5C endosperm. There plant was apparently diploid, because it had 2C embryos. Taskin et al., 2004 reported that this 2C embryo correspond to an asexual 2C embryo developed from an unreduced egg cell; and the 5C endosperm formed from 2 unreduced polar nuclei fertilized by a reduced sperm. As we obtained 3C for all embryos, we conclude that the 5C peak might be the result of contamination.

In *B. stricta*, embryos were 2C and endosperms 3C (Fig. 4.4, Table 4.1), as was expected, thus verifying that *B. stricta* is sexual. In *B. retrofracta* x *stricta* and *B. retrofracta* x *exilis*, all samples detected were 2C for the embryos and 6C for the endosperms (Table 4.1), thus showing pseudogamous (fertilized) endosperm formation (Fig. 4.5, 4.6). In *B. lignifera*, embryos were 2C and most endosperms 6C (Fig. 4.7), again showing pseudogamous endosperm formation. It is good to point out that in the control of *B. lignifera*, two seeds of 50 produced a 3C endosperm, which is strong evidence for infrequent sexual reproduction in this species. Finally, in *B. microphylla*, embryos were 2C and most of the endosperms were 5C (Fig. 4.8). This establishes that endosperm formation is pseudogamous, were the two 2C central cells (unreduced polar nuclei) fused with a 1C reduced sperm to form 5C endosperm. This species was originally considered sexual because it produces reduced pollen (Windham, personal communication). Our embryological studies confirm that it is apomictic, and that it is an aposporous apomictic. Two seeds of *B. microphylla* also had 3C endosperms, showing that sexuality may occasionally occur in this species as well. Therefore, sexuality happens in facultative apomictic *Boechera* grown under normal conditions. Other peaks in the histograms corresponded to endoreduplication of genetic material from embryos or endosperm.

Table 4.1. Reproductive pathways by taxon as determined by single-seed embryo:endosperm C values (2C:3C sexual, others apomictic) of both treatment and control.

	Embryo		Endosperm					
Taxon	2C	3C	3C	5C	6C	7C	9C	Not detected
B. gunnisoniana hybrid control		50					7	43
B. gunnisoniana hybrid treatment 1		50		1			3	46
B. lignifera control	50		2		45	1		2
B. lignifera treatment 1	50				49			1
B. microphylla control	50		2	44		1		3
B. microphylla treatment 1	150			101		6		43
B. retrofracta x exilis control	50				24			26
B. retrofracta x exilis treatment 1	50				11			39
B. retrofracta x exilis treatment 2	50				34			16
B. retrofracta x stricta control	50				2			48
B. retrofracta x stricta treatment 1	50				33			17
B. retrofracta x stricta treatment 2	50				18			32
B. stricta control	50		12					38
B. stricta treatment 1	50		26					24

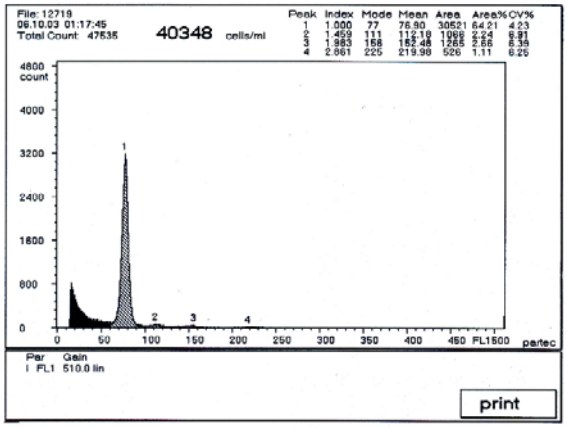

Figure 4.4. Histogram of diploid sexual B. stricta with peaks 2C, 3C, for embryo and endosperm; and 4C and 6C for endoreplication of embryo and endosperm, correspondingly.

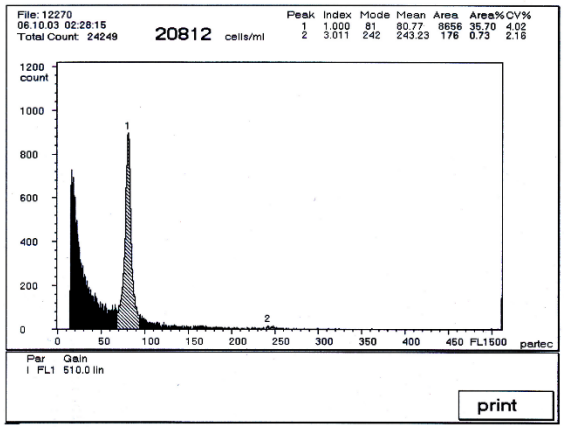

Figure 4.5. Histogram of diploid apomictic B. retro x stricta, showing peaks 2C and 6C, for embryo and fertilized endosperm, correspondingly.

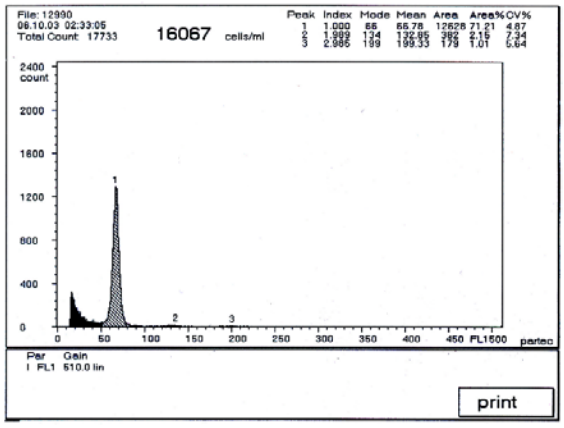

Figure 4.6. Histogram of diploid apomictic B. retrofracta, showing peaks 2C, 4 C and 6C, for embryo, endoreplication of embryo and fertilized endosperm, correspondingly.

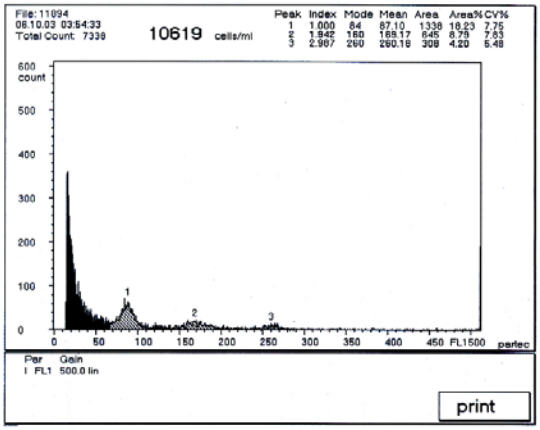

Figure 4.7. Histogram of diploid apomictic B. lignifera, showing peaks 2C, 4 C and 6C, for embryo, endoreplication of embryo and fertilized endosperm, correspondingly.

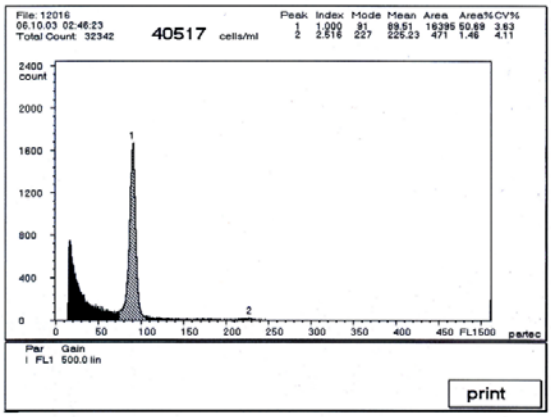

Figure 4.8. Histogram of diploid apomictic B. microphylla with peaks 2C and 5C, for embryo and fertilized endosperm, correspondingly.

The taxa we used for gene expression profiling were sexual *B. stricta* and diplosporous *B. retrofracta* x *exilis* and *B. lignifera*. The genes studied, to determine if stress affects their expression, were AT2G40140, AT4G02730, AT3G52940, AT3G13580 and AT2G48020. For AT2G48020, the effect of stress treatments was not significant at $P \leq 0.05$, but the taxa effect was highly significant (Table 4.2). Specifically,

gene expression was higher in the sexual *B. stricta* regardless of treatment (Fig. 4.9). These results match with those of the microarray performed in our lab (Chapter 3).

Treatments and taxa significantly influenced the results with AT2G40140 (Table 4.2). Again, gene expression was lower in the apomicts, and this expression in both the sexual and the apomicts was reduced by stress (Fig. 4.9). For sure, there was an effect on the gene because of the stress. While this correlation is consistent with a shift from apomeiosis to meiosis, it does not mean that this change in gene expression triggered an approach to sexual meiosis. It may just be a coincidence. We assume that if a gene triggers a switch in mode of reproduction as part of the stress signaling pathway, its expression would be closer in what we observe in the sexual species. In the present case, the shift in expression does not appear to be moving in that direction. We would expect a proximity among them if the mode of reproduction of *B. lignifera* or *B. retrofracta* x *B. stricta* (apomictic) would have become closer to B. *stricta* which is sexual, since the frequency of facultative apomixis is expected to increase sexual reproduction under stress conditions. Thus, while there was a change on the regulation of this gene due to stress, being down-regulated in the treatment of apomictic species, the change probably is not related to the observed shift to meiosis.

Gene expression differences due to stress were not detected for AT4G02730, but stress in *B. lignifera* enhanced the expression of AT3G52940 and AT3G13580 (Fig. 4.9); significant main effects of taxa and treatments (Table 4.2). Each of these genes were upregulated in apomicts in previous microarray studies (Chapter 3). AT3G13580 showed

a much higher expression in the apomictic species. This suggests that although an alteration is shown to occur due to stress in the apomictic species, the pattern is still the same of microarray previous analysis, where this gene was upregulated on apomictic species. The alteration seems not to be related to mode of reproduction. Only AT3G52940 showed an approach to what we might expect of a gene involved in the switch to apomeiosis, i.e., depression in gene expression was more severe in the apomicts than in the sexual, but this interaction was not significant (Table 4.2). Nevertheless, this gene, FACKEL, in animals encodes proteins responsible for sterol production and transcriptional and post-transcriptional regulation processes that affect meiosis (Edwards and Ericsson, 1999; He et al., 2003). Hence, it is of interest to the present study. In plants, it is involved in regulating sterol production, including brassinosteroids, and these influence embryonic and post-embryonic development.

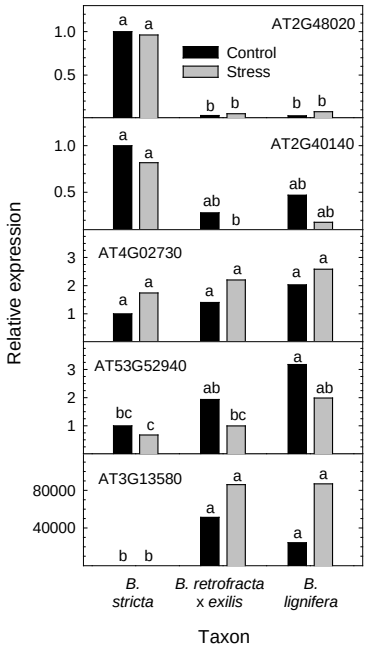

Figure 4.9. Differential gene expression as determined by qPCR. Data were adjusted so that expression levels are relative to those observed in the control plants of sexual *B. stricta*.

Table 4.2. Analysis of variance statistics by gene.

Source of variation	DF	Sum of squares	Mean squares	F-ratio	Significance level
AT2G48020					
Treatment (T)	1	3.709	3.709	3.629	0.071
Lines (L)	2	154.942	77.471	77.102	0.000
T*L	2	2.799	1.399	1.393	0.274
Samples(T*L) (S(T*L))	12	20.354	1.696	1.688	0.153
AT2G40140					
T	1	22.219	22.219	8.964	0.008
L	2	61.439	30.720	12.394	0.000
T*L	2	11.368	5.684	2.293	0.130
S(T*L)	12	123.368	10.281	4.148	0.003
AT4G02730					
T	1	3.231	3.231	3.736	0.069
L	2	3.790	1.895	2.192	0.141
T*L	2	0.321	0.160	0.185	0.832
S(T*L)	12	19.231	1.603	1.853	0.115
AT3G52940					
T	1	4.849	4.849	27.608	0.000
L	2	15.617	7.808	44.459	0.000
T*L	2	0.243	0.122	0.693	0.513
S(T*L)	12	14.971	1.248	7.103	0.000
AT3G13580					
T	1	19.058	19.058	5.262	0.034
L	2	1768.742	884.371	244.173	0.000
T*L	2	2.266	1.133	0.313	0.735
S(T*L)	12	63.756	5.313	1.467	0.224

Our hypothesis was that the ability to perceive environmental stresses during ovule development is impaired in apomictic plants. Thus, an absence of stress signals, which can normally induce meiosis, causes apomeiosis. The strong stress imposed by our experiments confirm that the stressing of *Boechera* apomicts shifts apomeiosis to meiosis. However, apomeiosis is only part of apomixis. For complete apomixis to occur, the surviving megaspore must be fertile. In the present experiments, interspecific hybridity and triploidy likely prevented fertility of sexually-derived ovules. Our results

are fully consistent with those of Böcher (1951), who observed stress-induced shifts from male apomeiosis to male meiosis, but as in our experiments, the male gametes were sterile because of the interspecific nature of the *Boechera* germplasm he used. Our results indicate that a switch to meiosis is not sufficient to produce viable seed. The meiotic products must also be viable, which was not the case for the *Boechera* taxa we used. More studies using other genotypes, and maybe other stresses, are required in order to find genes able to switch the mode of reproduction in facultative apomictic species.

In our experiment, important results were obtained confirming the effect of stress on gene expression. The above results showed that stresses produce changes on stress related genes. Those changes depend on the type of stress, the magnitude of stress and also on the species or genotype in consideration (Andersen et al., 2002; Sun et al., 2004; Rampino et al., 2006; Petraitytė, 2007; Aragón et al., 2008; Khandelwal et al., 2008). The samples of the species under study were submitted to the same stress, and the methodologies to do the analyses were the same. However, not all the species had the same response to the stress. The alteration of gene expression observed does not show to be related to mode of reproduction with the possible exception of FACKEL. In another hand, making a comparison with previous microarray analyses carried out at Carman`s lab, the expressions of these genes largely confirm previous results. Both types of stress, drought and heat, had significant effects on the mode of reproduction as manifested by frequencies of sexual and apomictic ovule development, which were determined cytologically. In the triploid *B. gunnisoniana* and the diploid *B. lignifera*, the frequency of apomictic ovule formation was reduced from an average of 90 % for control plants to an average of 70 % for the drought-stress-treated plants, and finally to an average of 23

% for the drought and heat-stressed plants. For both species, *B. gunnisoniana* and *B. lignifera*, drought plus heat caused a greater effect than drought alone. In *B. stricta*, the frequency of observed dyads decreased under drought stress. As *B. stricta* is a repeatedly confirmed sexual species, this observation may be a result of a more rapid meiosis during stress such that the dyad stage is shorter in duration and thus less likely to be observed. The dyads observed in *B. stricta* were likely sexual in control plants and drought-treated plants. Our results with facultatively-apomictic Boechera are consistent with previous findings with male apomeiosis in *Boechera* (Böcher, 1951) and with findings of others that facultatively apomictic species may switch their mode of reproduction to sexual when submitted to stress conditions (Carman et al., 2011). Not all the species showed the same behavior with the stresses. Therefore, this result is showing that the effect may be depended on the genotype, with highly facultative taxa possibly being less sensitive to stress. As a conclusion, we demonstrated that growing facultative apomictic species of *Boechera* under stress may increase the frequency of sexual ovule development.

As far as frequencies of sexual and apomictic seeds are concerned, when growing these *Boechera* species under stress conditions, several points need to be discussed. In this research, we confirmed the sexuality or asexuality of the species under study; and we also conclude that the frequencies of sexual and apomictic seed set did not change with the stress. These findings demonstrate that although the frequency of sexual ovules increased when the plants were submitted to stress, being greater the stronger the stress was, those ovules that changed their mode of reproduction did not develop into seeds. Therefore, we assume that seeds that started to form sexually, as verified by cytology,

subsequently aborted, and that is why they did not show up in the flow cytometry analysis.

The results of qPCR indicate that drought and heat cause changes in the expression of some of the stress related genes studied. Those changes depended on the type of stress, the magnitude of stress and also on the species or genotype in consideration (Andersen et al., 2002; Sun et al., 2004; Rampino et al., 2006; Petraitytė et al., 2007; Aragón et al., 2008; Khandelwal et al., 2008). Some genes showed alterations due to stress (AT2G40140 and AT3G13580), but the others did not. The alteration in gene expression may or may not be related to mode of reproduction.

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CHAPTER 5

SUMMARY

Boechera is an important genus for studying apomixis, a topic that needs more investigation in order to understand factors that intervene in the apomictic reproduction pathway. This genus has several advantages for the study of apomixis, which include its relationship with *Arabidopsis*, easiness to produce, and the capability to reproduce sexually and asexually. In this investigation we confirmed different types of apomixis (apospory and diplospory); and we also investigated the effects of drought stress on gene expression, emphasizing the effects of stress on mode of reproduction.

Concerning apospory and diplospory, our results through cytology demonstrated that diplospory is present in the triploid species *B. gunnisoniana*, and in the diploid species *B. lignifera, B. retrofracta x stricta,* and *B. retrofracta* x *exilis*; but in *B. microphylla* the type of apomixis present is apospory. This finding in *B. microphylla* confirms Carman's (2007) report. We also conclude that apomixis occurs at the diploid level in *Boechera*, and that apospory as well as diplospory are present in this genus.

To evaluate the effect of stress on mode of reproduction in *Boechera*, drought and drought plus heat were imposed. Both stresses significantly affected mode of reproduction as shown in the frequencies of sexual and apomictic ovules produced. Not all species showed the same behavior. In *B. lignifera* the frequency of apomictic ovules were reduced from approximately 84 % in the control to 61 % in drought stress, and 25 % in the drought plus heat stress; showing that in this species the effect of drought plus heat was greater than the effect of drought only. Similar responses occurred in *B. stricta* in treatment 1, but here the decrease in frequency of sexual dyads observed may have been

caused by meiosis, and hence the dyad stage, occurring more rapidly during stress. These results agree with papers reviewed by Carman et al. (2011) that reported the frequency of sexual seed formation increases when facultative apomictic plants are grown under certain stress conditions. In *B. gunnisoniana*, a triploid, and therefore an obligate apomict, although it cannot produce sexual seeds, yet the process to produce sexual seeds may start in the ovules developed under stress conditions. In the control of this species, 96 % of ovules produced dyads (apomictic reproduction); and this frequency was reduced to 77 % and 23 % in treatments 1 and 2, respectively. In *B. microphylla*, the switch in mode of reproduction was less pronounced; and in *B. retrofracta* x *stricta* combined with *B. retrofracta* x *exilis* the frequency of apomictic ovules was reduced in treatment 1 but increased slightly in treatment 2, although it is good to point out that for these two taxa, only small ovules were analyzed, and this may have biased the result toward dyad formation. Nevertheless, the data indicate that genotype is a very important factor affecting facultativeness in *Boechera* and the effect that stress has on facultativeness. Similar conclusions were reached by Rampino et al. (2006). As a conclusion for this part of the experiment, we demonstrated that growing facultative apomictic species of *Boechera* under drought or under drought plus heat stress increases the frequency of sexual ovule development.

Another methodology used to evaluate the effect of stress on reproductive processes was flow cytometry, with which we analyzed the stress response on the frequency of sexual and apomictic seed set. In this study we confirmed the sexuality or asexuality of the species under study; however, we found that the frequency of sexual and apomictic seed set did not change with the stresses. In triploid *B. gunnisoniana*, embryos were 3C

and most of the endosperms were 9C (endosperm peaks shown not by peaks detected by the machine, but by the agglomeration of many black dots at the 9C location in the histograms). No sexual seeds were detected, demonstrating that although the frequency of sexual ovules increased when the plants were submitted to stress, those ovules, that had changed their mode of reproduction, did not developed into seeds. If stress causes a reversion to sexual megasporogenesis, there will be an increase of the frequency of aborted seeds. The seeds that reach a complete formation are formed from apomictic ovules only; and the ones that started to form from sexual ovules are aborted. As a result, the frequency of aborted seeds (per silique) increases when apomicts are stressed, and this increase in aborted seeds may largely be due to the sterility of sexually-derived embryo sacs, because of genetically-unbalanced gamete formation in interspecific hybrids and triploids. Seeds that started to form from sexual ovules, in this investigation, aborted. That is the reason why although the frequency of sexual ovules of stressed samples increased (shown in the cytology analysis), sexual seeds did not appear in the flow cytometry analysis. It is good to point out that in the control of *B. lignifera* and *B. microphylla*, 2 seeds out of 50 were sexual. Therefore, sexuality happens within facultative apomictic *Boechera* species grown under normal conditions; however stress produced sexual ovules, but it did not produce sexual seeds.

The results of qPCR show that drought and heat stresses changed the expression of some genes. One of these genes was a ribosomal protein gene, and the other one was a stress-response gene. Alteration happened according to the type of stress, the magnitude of stress and the species or genotype in consideration (Andersen et al., 2002; Sun et al., 2004; Rampino et al., 2006; Petraitytė, 2007; Aragón et al., 2008; Khandelwal et al.,

2008). The expression of genes AT2G40140 and AT3G13580 was altered due to the stress. But, stress did not trigger any alteration on genes AT4G02730, AT3G52940 and AT2G48020. Nevertheless, the alteration of gene expression on the species that were affected by the stress may not be related to mode of reproduction. In another hand, the results are basically in agreement with those of previous microarray analyses carried out at Carman`s lab. We conclude that drought stress triggered alterations in the expression of some of the genes, but it has not been shown that these genes affect mode of reproduction.

Our hypothesis was that the ability to perceive environmental stresses during ovule development is impaired in apomictic plants, and this absence of stress signals, which may often induce reproduction, including meiosis, is responsible for the occurrence of apomeiosis. We have clearly seen that stress triggered an alteration in the frequency of sexual and apomictic ovule formation, increasing the frequency of sexual ovules coming from plants developed under stress conditions. As a result, we conclude that the stresses triggered changes in mode of reproduction. Stress also significantly affected the expression of the chosen genes. The main effect of stress was significant for all genes and the $P \leq 0.10$ level and was significant for three of the five genes at the $P \leq 0.05$ level. The results suggest that it is very important to continue evaluating additional stress-response genes related to mode of reproduction in future investigations. There is no contradiction between the results obtained by the different methodologies, cytology, flow cytometry and qPCR. The switch detected in mode of reproduction by cytology was not seen by flow cytometry, probably because sexual seeds aborted. As a suggestion, it is important to carry out more studies using probably other genotypes, stresses or genes, in order to

find specific genes able to switch the mode of reproduction of facultative apomictic species with the final aim of finding genes responsible for apomixis.

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APPENDIXES

APPENDIX I

Gene sequences with left and right primers identified for qRT-PCR

1. AT2G40140: >AT2G40140.1 | Symbols: CZF1, ZFAR1, SZF2, ATSZF2 | zinc finger (CCCH-type) family protein | chr2:16772073-16774657 FORWARD LENGTH=2310

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3. AT3G52940: >AT3G52940.1 | Symbols: FK, HYD2, ELL1 | Ergosterol biosynthesis ERG4/ERG24 family | chr3:19630198-19633296 REVERSE LENGTH=1465

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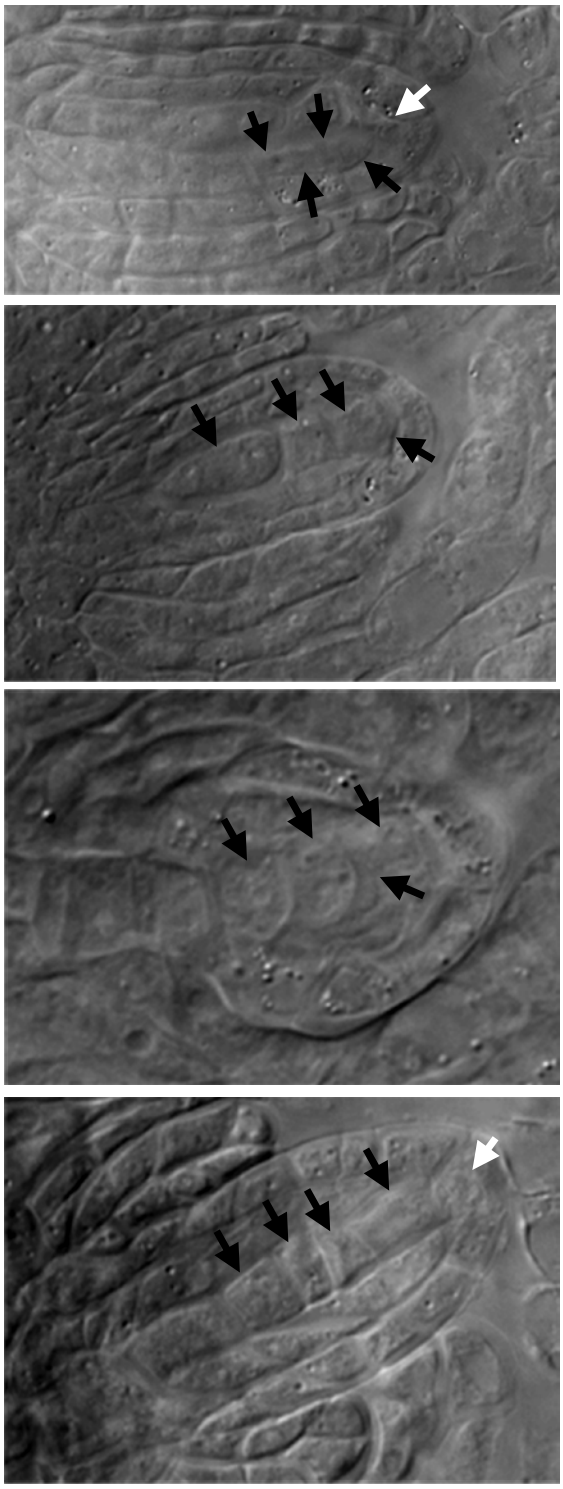
APPENDIX II

Additional Photomicrographs of Tetrads in Sexual and Apomictic *Boechera*

Drought-stressed sexual *B. stricta*



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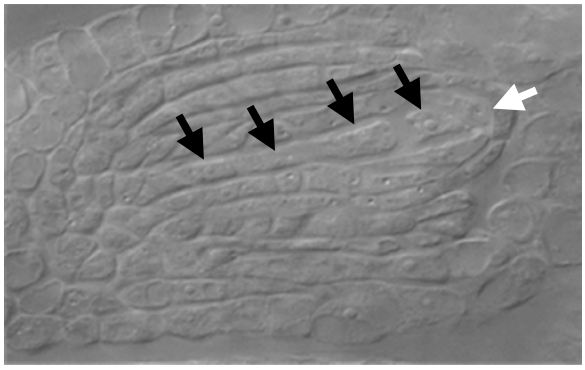

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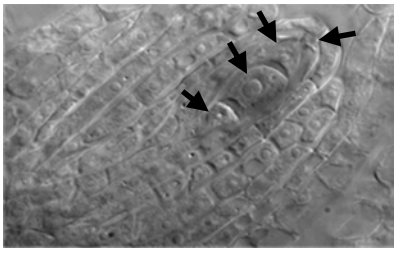
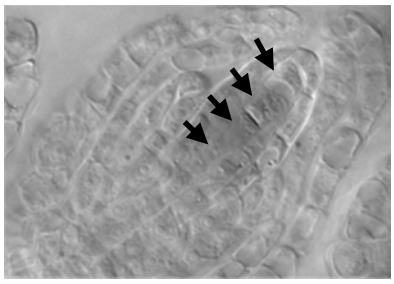
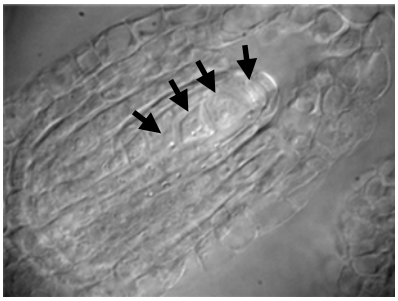
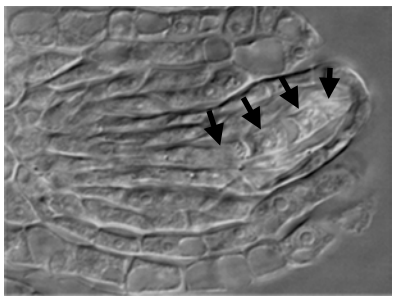
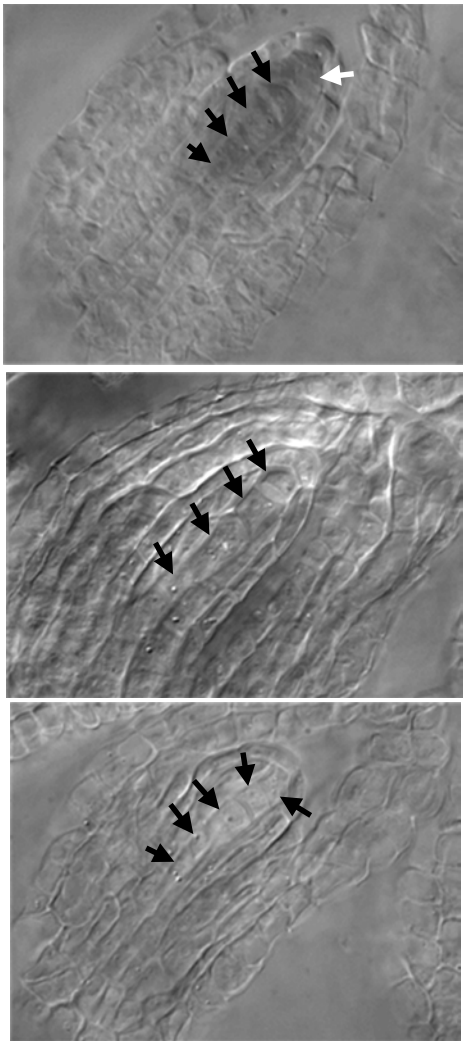




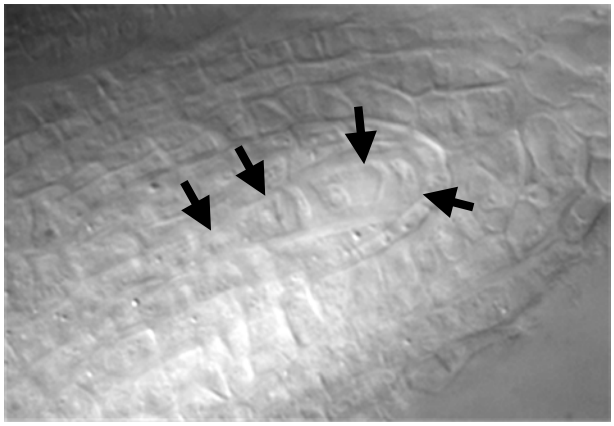




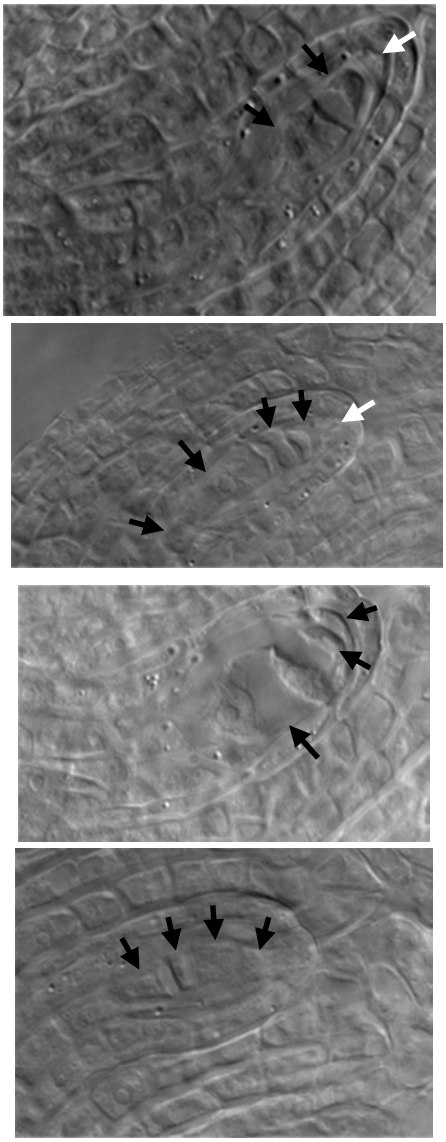
Drought and heat-stressed apomictic triploid *B. gunnisoniana*



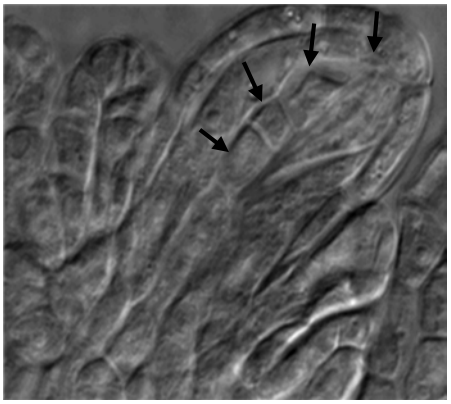
Non-stressed apomictic diploid *B. lignifera*



Drought-stressed apomictic diploid *B. lignifera*



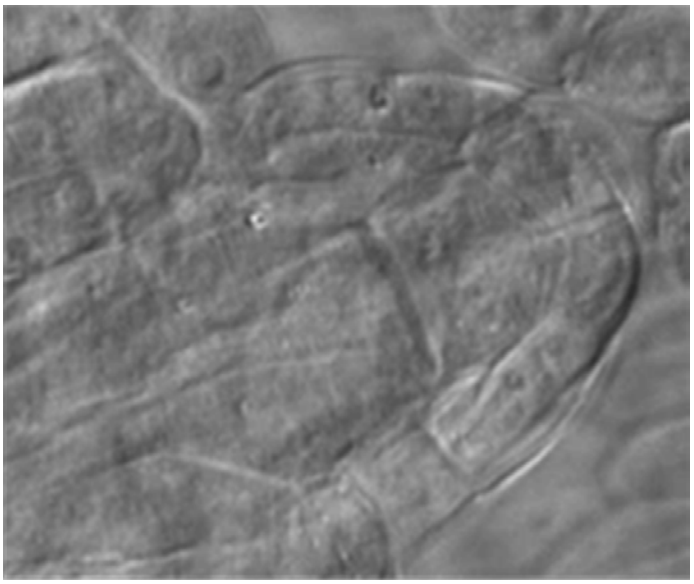
Drought-stressed apomictic diploid *B. microphylla*



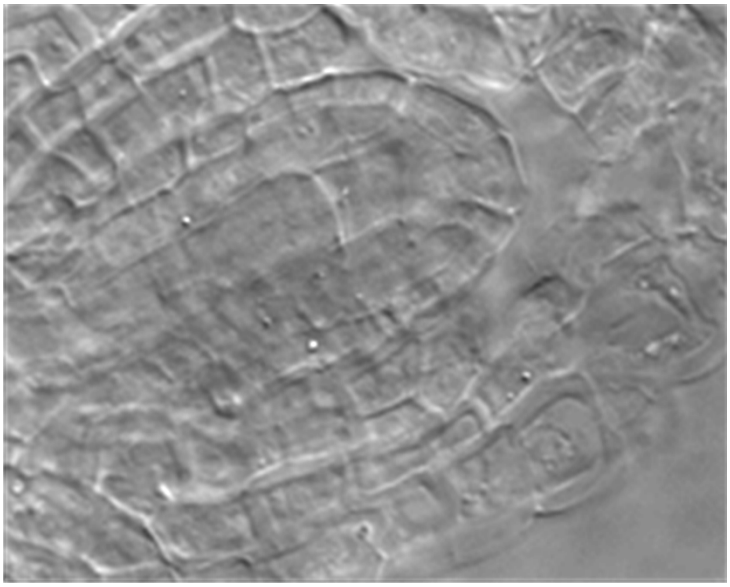
APPENDIX III

Additional Photomicrographs of Apomeiotic Dyads in Apomictic *Boechera*

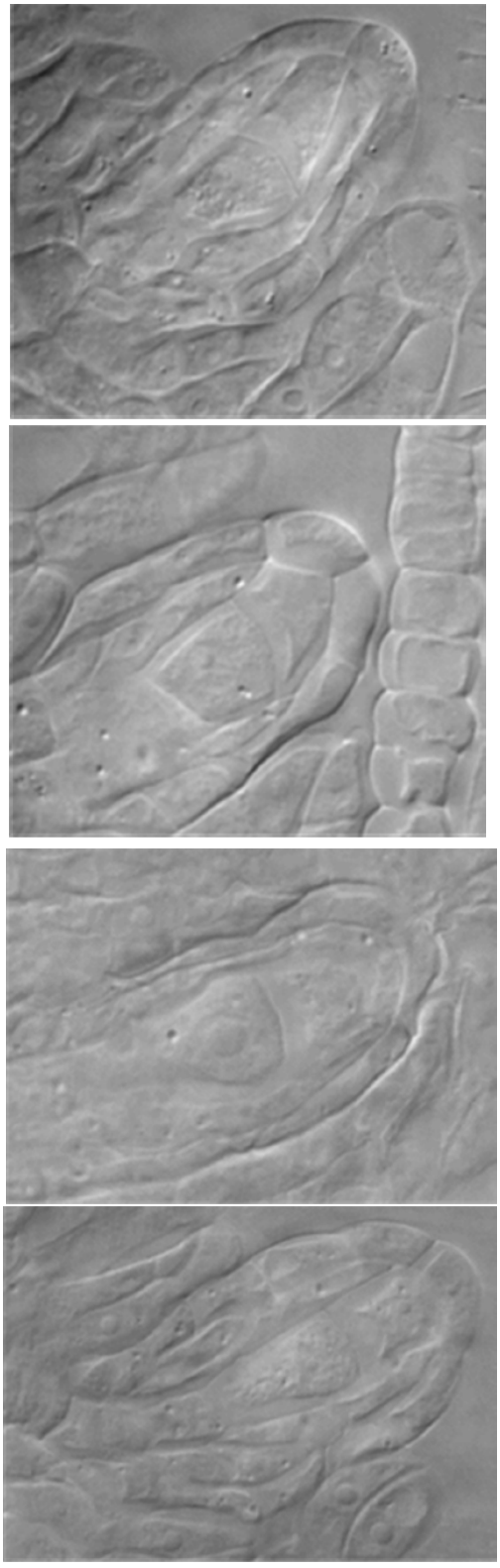
Drought-stressed diploid apomictic *B. retrofracta* x *exilis*



Drought and heat-stressed diploid apomictic *B. retrofracta* x *exilis*

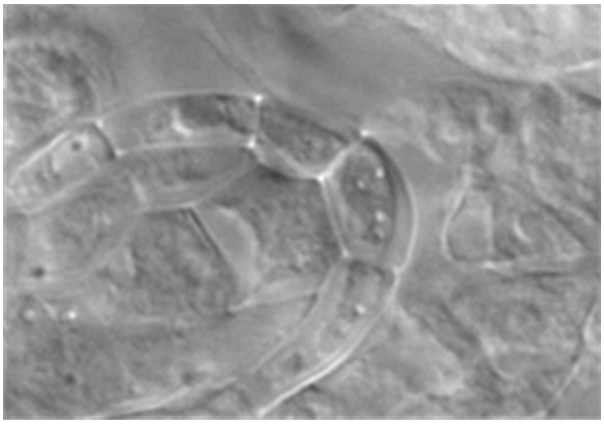


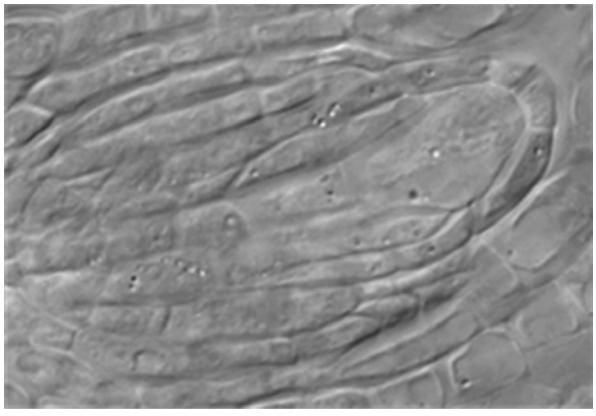
Non-stressed triploid apomictic *B. gunnisoniana*



Drought-stressed triploid apomictic *B. gunnisoniana*

Drought and heat-stressed triploid apomictic *B. gunnisoniana*





Drought-stressed diploid apomictic *B. lignifera*