IDENTIFYING DNA-PROTEIN INTERACTIONS AND PROMOTER FUNCTION IN THE CANDIDATE APOMIXIS APOLLO GENE

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ABSTRACT

Among genes that control apomixis components, APOLLO is known for its strong linkage to apomeiosis. Previous studies on expression profiles of apomictic vs. sexual *Boechera* species revealed an ovule-specific expression for an apomictic allele which is characterized by a 20 bp Apo-insertion/Sex-deletion polymorphism conserved in the 5'UTR of APOLLO. In addition, there is allelic variation in *Boechera* species, as sexual individuals are homozygous for the Sex-alleles while apomicts are heterozygous for the Apo/Sex-alleles. I hypothesized that the apomixis-specific polymorphism (i.e., 20 bp Apo-insertion/Sex-deletion) in the 5′ untranslated region of Apo-allele (5'UTR; TGGCCCGTGAAGTTTATTCC) corresponds to specific transcription-factor binding sites (TF) which are absent in all Sex-alleles. To test this hypothesis a yeast one-hybrid assay was conducted by extracting RNAs of different tissues and creating corresponding cDNA libraries for both apomictic and sexual *Boechera* species. The libraries along with the bait sequences were sent to Hybrigenics (France) for the yeast one-hybrid assay experiment.

The results of the yeast one-hybrid assay demonstrated novel transcription factors belonging to the *APETALLA2* (*AP2*) family including *ERF15*, *ERF107*, *ERF5* for apomictic *Boechera* spp., and *ERF1B, ERF107* and *GLABROUS1* enhancer-binding protein for sexual *Boechera*. *AP2/ERF* proteins are known to have important functions in the transcriptional regulation of a variety of biological processes such as response to environmental clues and flower development. These data are in line with the results of a previous study in which the expression of *AP2* in sexual tetraploid pearl millet promoted both parthenogenesis and the production of haploid offspring.

To learn about the regulation of APOLLO in *planta*, the function of the APOLLO promoter was studied in transgenic lines. Firstly, native APOLLO promoters, including 1 kb upstream of the transcription start site (TSS) of both Apo- and Sex-alleles were cloned into Arabidopsis using a cassete carrying a GUS reporter gene. Then, 2 kb upstream of the TSS of both Apo- and Sex-alleles were cloned into both *Arabidopsis* and *Boechera* followed by analyzing their GUS activity. No GUS activity occurred in plants transgenic for the 1 kb APOLLO promoter, neither for Apo nor Sex-alleles. *Arabidopsis* transgenic for 2kb Apo promoter showed tissue-specific GUS activity in anther and *Boechera* transgenic for the same construct in the stigma of different

developmental stages, while the 2 kb Sex promoter did not show any GUS activity neither for *Arabidopsis* or *Boechera* transgenic lines. The data show that there are differences in GUS activity between Apo- vs. Sex-allele, though no GUS activity was observed in pre-meiotic ovules of Apo-allele transformants. This may mean that the 1 kb and 2 kb regions do not carry all needed regulatory elements and hence a longer promoter region can be tested for future study.

Finally, five different synthetic ~2 kb promoters were made to test Apo- vs. Sex-specific promoter components. A comparison of various flower developmental stages in transgenic lines containing the different constructs with the 2 kb native transgenic lines revealed that changes to the APOLLO promoter causes shifts in tissue- and developmental-stage specificity. In addition, it was shown that the 20 bp apomixis-specific polymorphism along with other sequences of the 5'UTR gives rise to ovule-specific GUS activity only when adjacent to 2 kb of the Sex-allele. Together, these findings will serve as a foundation for understanding the complex regulation of the APOLLO gene in *Boechera*.

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I would like to acknowledge my supervisor, Dr. Tim Sharbel. His kind and humble attitude as well as immense knowledge have always made me comfortable to express my ideas. I could not have imagined having a better advisor and mentor for my Ph.D. study. My gratitude extends to the members of my advisory committee Dr. Graham Scoles for his treasured support which was really influential in shaping my research methodology and critiquing my results. I also thank Drs. Curtis Pozniak, Hong Wang, Tom Warkentin, and Yuliang Wu for their invaluable advice. Their timely recommendations have had a huge effect on propelling my project to success Indeed.

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I am incredibly thankful to my loving husband for being by my side during the last four years. I would like to dedicate this thesis to my parents, Hayedeh Parviz and Ali Honari. They inspired me by training many generations of students and helping others consciously. Finally, I would like to dedicate it to my grandmother Morasse Parviz, who always encouraged me to be persistent in acquiring knowledge.

STATEMENT OF CONTRIBUTIONS

Great thanks to Joanne Ernest for her contributions to this research. Dr. Ernest performed the initial tissue collections and RNA extractions of apomictic and sexual *Boechera* which led to the preparation of yeast cDNA libraries sent to Hybrigenics Services, France (www.hybrigenics-services.com). The details of this experiment, including selected tissues and the extraction conditions, are shown in table 3.1. The yeast one-hybrid experiments were then conducted by Hybrigenics. The results of the Hybrigenics yeast one-hybrid assays are shown in Section 3.2 (p 51-55). The complete data sets from the yeast one-hybrid assays for *B. divaricarpa* bait vs. prey construct (*Boechera divaricarpa*_RP1) and for *B. stricta* bait vs. prey construct (*Boechera stricta*_RP1) are shown in figures 5.1 and 5.2 (appendices). For functional analysis of the pAPOLLO promoters, Dr. Ernest designed the 2 kb native Apo and Sex promoters, as well as the five synthetic promoter constructs shown in sections 4.2.21 and 4.2.22 (p 83-93; Figures 4.5-4.11, Tables 4.3, 4.4, 4.5). Dr. Ernest performed the overlap PCRs and cloning to produce these constructs, confirmed their sequences, and produced the respective *Arabidopsis* transgenic lines (shown in figures 5.9, 5.10, 5.11, 5.12, 5.13, 5.14, and 5.15 of the appendices).

Thanks also to Angie Li for her contribution. Ms. Li prepared, screened and maintained the pAPOLLO *Boechera* transformants described here. Ms. Li strived to optimize *Boechera* tissue culture condition which was important for making successful transgenic lines as can be seen in section 4.3.2 (p 99-100).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AG	Agamous
AIC	Apomictic Initial Cell
ANOVA	Analysis Of Variance
AP1	Apetala1
AP2	Apetala2
APOLLO	Apomeiosis-Linked Locuse
Asp	Aspartate
ASY1	Asynaptic1
BA	6-Benzylaminopurine
BBM	BABY BOOM
cDNA	Complementary DNA
CMEs	Chromatin-Modifying Enzymes
CYC	Cycloidea
DMSA	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EBE	ERF BUD ENHANCER
EBN	Endosperm Balance Number
EMSA	Electrophoretic Mobility Shift Assay
ERF	Ethylene Response Factors
GeBP	GLABROUS1 enhancer-binding protein
Glu	Glutamine
GUS	Beta Glucuronidase
HDG	homeodomain GLABROUS
IME	Intron-Mediated Enhancement
LOA	Loss of Apomeiosis
LOP	Loss of Parthenogenesis

MMC	Megaspore Mother Cell
MPS1	Multipolar Spindle 1
mRNA	Messenger RNA
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
ncRNA	non-coding RNAs
PBS	Predicted Biological Score
PCF	Proliferating Cell Factors
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RNAP	RNA polymerases
ROI	Region of Interest
ROS	Reactive Oxygen Species
SHP1	Shatterproof1
SID	Selected Interacting Domain
SOE	Splicing by Overlap Extension
STK	Seedstick
TDNA	Transfer DNA
TFBS	Transcription Factor Binding Sites
TFs	Transcription Factors
TSS	Transcription Start Site
UTRs	Untranslated Regions

CHAPTER 1. GENERAL INTRODUCTION

There are two main types of reproduction in plants, sexual and asexual. In sexual reproduction, male and female gametes must fuse to produce the first cell of the new embryonic plant, while in asexual reproduction no mixing (syngamy) of male and female gametes take place. In asexual reproduction through seed, also known as apomixis, progenies are identical to the maternal genotype and can be distinguished from vegetative reproduction in which the production of seeds or spores is hindered. Apomixis in plants is characterized by the formation of unreduced female gametes (apomeiosis) that give rise to an embryo independent of fertilization (parthenogenesis). The formation of a viable endosperm occurs either with fertilization by a sperm cell (*pseudogamy*) or without it (*autonomous endosperm*). In addition, based on the developmental origin of apomictically derived embryos, two main types of gametophytic and sporophytic apomixis are defined. Due to the potential of apomixis to fix hybrid vigor in crosses, it could be used in breeding strategies for fixation and propagation of any desired genotype. The molecular and breeding studies that have been focused on understanding the mechanisms controlling apomixis have been partially successful. Genetic analyses of sexual reproduction in plant model systems have identified genes that, when mutated, display elements of apomixis.

One of the model plants that has been widely used in studies of apomixis is *Boechera* which is a perennial member of the *Brassicaceae* family. *Boechera* species are adapted to a broad geographic distribution in North America by growing in environments ranging from deserts to rocky scree to moist alpine meadows (Figure 1.1). In addition, the genus is the most thoroughly characterized instance of diploid apomixis in angiosperms, which turns it into an alluring model plant for agricultural research. In addition to the mentioned characteristics, it has a small genome size of ~170–230 Mbp, which makes genomic studies easier (Rushworth *et al.*, 2011).

Figure 1.1 Species in the genus *Boechera* adapted to diverse habitats across the western United States. These habitats include the following: (top row) serpentine (Sierra County, California), sagebrush grassland (Custer County, Idaho) and Chihuahuan desert scrub (Eddy County, New Mexico); (bottom row) subalpine meadow (Ravalli County, Montana), rocky scree (Lemhi County, Idaho) and lava flow (Carrizozo Malpais, Lincoln County, New Mexico). Photographs of habitat in California and New Mexico courtesy of P. Alexander; all others, C. Rushworth. Molecular Ecology, Volume: 20, Issue: 23, Pages: 4843-4857, first published: 08 November 2011, https://doi.org/10.1111/j.1365-294X.2011.05340.x).

Comparing microarray data of microdissected ovules of sexual and apomictic *Boechera* has revealed a single differentially expressed gene (APOLLO) that is characterized by an apomixis-specific allele (Corral *et al.*, 2013). In fact, sexual *Boechera* are homozygous for the Sex-allele, while apomicts have both Apo- and Sex-alleles. There are structural differences between Apo and Sex-alleles. For example, the 5'UTR of the Apo-allele is shorter than Sex-allele, with no further sequence upstream of the 20 bp apomixis specific polymorphism, as identified by Corral *et al.* (2013) (Figure 1.2).The ovule-specific expression of the Apo-allele in apomictic *Boechera* is associated with a 20 bp Apo-insertion/8 bp Sex-deletion polymorphism in the 5'UTR of the Apo-allele. The APOLLO gene was found to code for a DEDD 3′→5′

exonuclease superfamily, which are characterized by four acidic residues, three Asp (D) and one Glu (E), distributed over three separate sequence segments (Corral *et al.*, 2013). The expression of this exonuclease in *A. thaliana* (AT1G74390), has been reported in flowering stages, mature plant embryo, petal differentiation and expansion stage, plant embryo bilateral stage, plant embryo cotyledonary stage and plant embryo globular stage (https://www.arabidopsis.org).

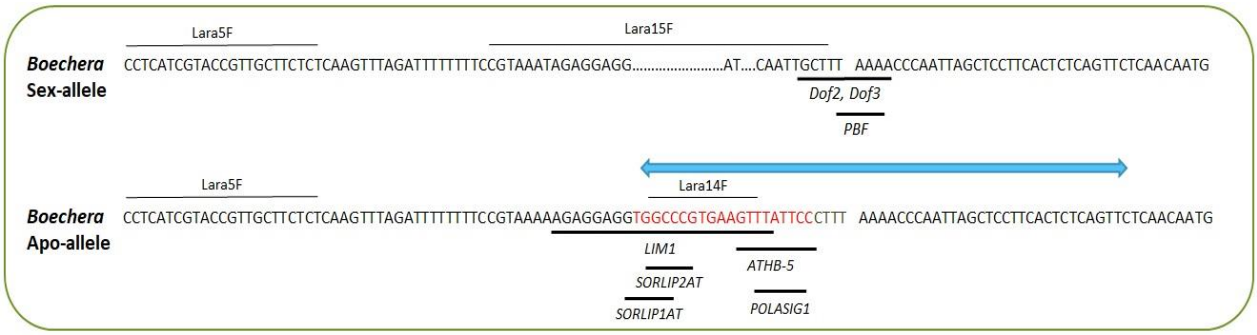


Figure 1.2 Comparing the 5'UTR of APOLLO of sexual versus apomictic *Boechera* spp. Fragment of the 5'UTR that shows the 20 bp Apo-insertion/Sex-deletion (red letters) in the context of *Boechera*'s Apo- and Sex-alleles. Blue line encompass the range of 5' limits of Apo-allele transcripts isolated and sequenced by Corral *et al.* (2013), while Sex-allele transcript start 350 bp upstream this region. Black lines under Lara primers show the position of PCR primers used. The binding site of putative TF predicted by Corral *et al.* (2013) located under each allele is shown with italic letters (Image taken from Corral *et al.*, 2013).

1.1. Research goals

The primary goal of this thesis is to identify DNA-protein interactions in the APOLLO 5'UTR using a yeast one-hybrid assay. Specific objectives addressed in this thesis were:

Objective 1: Identify the transcription factors and other proteins which interact with the 20 bp Apo-insertion/Sex-deletion in the 5'UTR of APOLLO apomixis alleles using a yeast one-hybrid assay.

The 20 bp apomixis-specific polymorphism (TGGCCCGTGAAGTTTATTCC) was characterized by a plus-strand transcription factor-binding site which is absent in all Sex-alleles. It was also mentioned that APOLLO is expressed in apomictic, but not sexual ovules (Corral *et al.*, 2013). In order to understand the mechanism underlying differential gene expression, identifying physical interactions between regulatory TF and their target genes is needed.

Objective 2: Identify promoter activity patterns for APOLLO.

In general, transcriptional expression is frequently regulated by promoter elements which are located immediately upstream of the 5'UTR and start codon (*cis*-elements; Klug and Ward, 2009). These elements and their associated expression patterns can be investigated by using a promoter-reporter construct. In these systems a putative promoter region which is cloned upstream of a reporter gene is normally transformed into the host plant. Expression of the reporter is subsequently assessed by fluorescent or colorimetric assay, and the most common of these reporter systems in plants is the β-glucuronidase (GUS) assay (Springer, 2000). The broad aim of this objective was a functional understanding of APOLLO by examining the expression patterns and promoter elements for Sex- and Apo-alleles.

CHAPTER 2. LITERATURE REVIEW

2.1. Evolution of plants from the reproductive point of view

The first evidence of plants on the earth is observed in fossils of unicellular photosynthetic organisms that divided by mitosis (Charlesworth, 1991). In more recent sediments of rock in southern Ontario, Canada, early prokaryotes in the form of blue-green algae were also found. Based on geological evidence, the reorganization of continental plates on earth has led to dramatic changes in sea level, and this was accompanied by the evolution of blue-green algae into more sophisticated multicellular plants at the end of the Cambrian era, for example by exploiting biochemical pathways developed in cyanobacteria that enabled respiration and photosynthesis (Bateman *et al.*, 1998).

Parallel to changes in the environment, plants began to develop mechanisms to spread their spores through water, and with the colonization of land plants developed mechanisms to produce and spread spores in dry environments (Raghavan, 2006). The first spores identified in the Ordovician era had a tetrahedral arrangement while others from younger sediments are found as isolated spores with a distinct trilete form. These structures provide strong evidence for meiotic division whereby a diploid cell produces four haploid cells (Pennington, 2002).

Furthermore, one of the earliest pieces of evidence of sexual reproduction was found in the fossil specimen *Isochadites* (*Codiaceae*), which show gametocytes with reproductive structures. Angiosperms later diverged with respect to the generation of seed producing embryos in the Jurassic era (Crane *et al.*, 1995). This was characterized by the emergence of the carpels, followed by the occurrence of double fertilization and hypotheses suggest that only after these two evolutionary steps occurred were mutations responsible for the appearance of the floral component derived (Raghavan, 2006).

2.2. Universality of double fertilization in flowering plants

Seed development in flowering plants occurs through several steps. First, the transition from the vegetative to reproductive phase occurs by formation of floral organs. After ovule and stamen formation, meiosis and gametogenesis occur in the ovules of the carpels and anthers of the stamens. Female gametes are produced from megaspore mother cells during mega-gametogenesis, whereby the functional megaspore undergoes three mitotic divisions to give rise

to a syncytium containing eight nuclei including antipodals, synergids, egg, and polar nucleus. In a phenomenon known as double fertilization (Figure 2.1), two sperm cells (via the pollen tube) fertilize both the egg cell to form the diploid embryo, and the binucleate central cell to produce the triploid endosperm, which becomes the primary energy source of the growing embryo. After fertilization, the ovule develops into the embryo (Tucker and Koltunow, 2009).

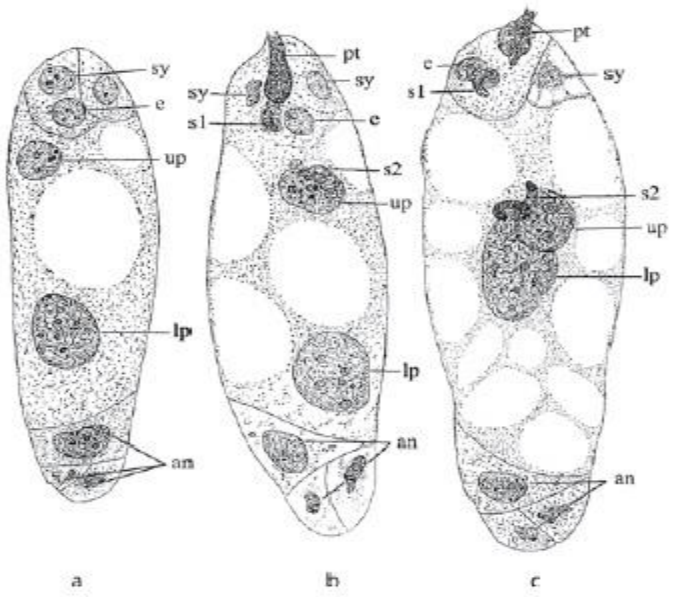


Figure 2.1 Double fertilization. a Mature embryo sac of *Lilium martagon* showing the egg apparatus, consisting of the egg and synergids, antipodals, upper polar nucleus, and lower polar nucleus. **b** Mature embryo sac after discharge of male gametes from the pollen tube. The nucleus one of sperm has entered the egg and that of the second sperm is in contact with the upper polar nucleus. **c** Double fertilization occurred. **an** Antipodals, **e** egg cell, **lp** lower polar nucleus, **pt** pollen tube, **s1** sperm that fuses with the egg, **s2** sperm that fuses with the polar nucleus, **sy** synergid, **up** upper polar nucleus (Image taken from Raghavan, 2006).

2.2.1. Double fertilization and the control of embryo initiation

The embryo and endosperm in a seed are derived from the fertilized egg and central cells of the embryo sac. Accessory cells in the embryo sac play an important role in the double fertilization process. The synergids guide the pollen tube through the micropyle into the embryo sac by producing a pollen tube attractant. For instance, in *Torenia fournieri*, the pollen tube is attracted to the embryo sac *in vitro*, suggesting that synergids produce diffusible signals, and at least one synergid cell is necessary for pollen tube attraction. The pollen tube penetrates the degenerating

synergid and after that releases two sperm cells (Higashiyama *et al.*, 2000; Higashiyama *et al.*, 2001).

Double fertilization was first identified as a ubiquitous feature in the reproductive biology of flowering plants in the nineteenth century by Navaschin (Kordyum, 2008). The discovery of double fertilization in liliaceous species, followed by confirmation of its existence in many other angiosperms, including both monocotyledons and dicotyledons, changed the way botanists understood plant reproductive biology. However, it was not until the observation of living material in *Monotropa hypopitys* that deep insight into isolated aspects of double fertilization was gained (Raghavan, 2003).

The evolutionary importance of the endosperm is due to its role in development of the seed (Crane, *et al.*, 1995). Following fertilization of the central cell, the endosperm not only differentiates into the nutritive tissue for the embryo, but it also plays an important role in the successful development of the embryo in many species (Brink and Cooper, 1947). Considering the importance of endosperm for embryo development, the failure of endosperm development in inter-ploidy crosses is enigmatic and has been attributed to differences in ploidy level *per se*, or to genome incompatibilities between species (Johnston *et al.*, 1980).

Nishimaya and Inomata (1966) postulated that the success of the endosperm depended primarily on a 2:1 ratio of the maternal to paternal genomes in the endosperm. In the other words, if embryo and endosperm ploidy increase without disrupting the 2 maternal: 1 paternal (2m: 1p) ratio, normal development of the embryo and endosperm ensues. Vinkenoog and Scott (2001) described this phenomenon in relation to what is known as imprinting. Genomic imprinting consists of mechanisms by which genes are expressed in a parent-of-origin manner. These mechanisms, either DNA methylation and/or histone modification during gametogenesis, and thereafter during endosperm development, would explain the specific expression of maternal versus paternal alleles (Curtis and Grossniklaus, 2008). In *Arabidopsis*, where imprinting can be manipulated through interploidy crosses (e.g., 2x × 4x; 2maternal: 4paternal), seed size variation with respect to endosperm development could be predicted, with gene dosage affecting the timing of cellularization of the endosperm and its proliferation potential (Vinkenoog and Scott, 2001).

To understand the phenomenon of endosperm development failure in interploidy crosses, Johonston *et al*. (1980) proposed an Endosperm Balance Number (EBN) hypothesis that later reported by other scientists in various species (Johnston *et al*. 1980; Parrott and Smith, 1986; Hawkes and Jackson, 1992). Based on this hypothesis, the genome of each species is assigned a specific value, which is not necessarily equal for the species of the same ploidy. It is EBN which determines the effective ploidy level in the endosperm and behaviour in intra- or inter-specific crosses. In most cases for a cross to be successful, the endosperm must have a ratio of two EBNs from the female parent to one EBN from the male parent. Any deviation from this ratio would be detrimental to endosperm development and give rise to either a deficient or dead embryo (Carputo *et al.*, 1999).

2.3. Transition from sexual reproduction to asexuality in plants

Asexual reproduction includes all the mechanisms that lead to production of genetic clones[1] that are identical to the mother plant. Based on geological evidence, the evolution of asexual organisms for the most part occurred during Pleistocene glaciations, as most extant asexual animals and plants are evolutionarily young and appear on the tips of phylogenetic trees (Schön *et al.*, 2009).

It is not clear, despite being evolutionary older, why sex is still a prominent mode of reproduction in nature. The first attempts to answer this question were not successful because for many years it was impossible to accurately identify the consequences of sexuality, or even to distinguish the concept of sexuality from both gender and reproduction (Stearns, 2017). To answer why sexual reproduction exists in nature, Darwin regarded the variation resulting from it as a necessary condition for evolution. He proposed that sexual reproduction exists due to the physiological advantages incurred during fertilization, which results in vigorous offspring (Schön *et al.*, 2009).

One of the leading characteristics of sexuality is the generation of genotypic diversity in progeny by chromosome assortment, meiotic recombination and syngamy. Theoretical studies confirm

[1] Clones are conspecific populations of asexual organisms grouped based on arbitrary degree of morphological resemblance.

[2] BBM is a member of AINTEGUMENTA-LIKE (AIL) subfamily of the APETALA2/ETHYLENE RESPONSE ELEMENT-BINDING FACTOR (AP2/ERF) family of transcription (Hortsman *et al*., 2015).

[3] The PlantPAN analysis navigator provide effective resources for detecting transcription factor binding sites (TFBS), corresponding TF, and other important regulatory elements (CpG islands and tandem repeats) in a promoter or a set of promoters in plants (Chow, Lee, *et al.*, 2018).The ”cross-species”

that genetic diversity, in terms of polymorphism or heterozygosity, may sometimes increase in asexual plants if clones are highly heterozygous (Balloux *et al.*, 2003). In this view, apomicts and sexual plants package genetic variation in different ways. Apomicts produce uniform but highly heterozygous progeny, all of which would be adapted to one environment. In contrast, sexuals produce various heterozygous progeny, each of which may be extremely fit in multiple environments (Stearns, 2017). The second prediction of sex for the individual is that, due to elimination of deleterious mutations (Muller's Rachet theory, Muller 1964) expressed in homozygotes of the next generation, the average heterozygosity per genome should decline (Stearns, 2017).

The prevalence of sexual reproduction is partially related to the expected lower fitness of asexuality, due to the accumulation of deleterious mutations (i.e., Muller's ratchet) (Keightley and Eyre-Walker, 2000). A large body of theory explains the rarity of asexuality among multicellular taxa by describing benefits created by sexual reproduction (Howard & Lively, 1994; Doncaster *et al.*, 2000). For example, when genetic associations produced in another time or location are no longer beneficial, sex and recombination can break these associations and increase the fitness by bringing together fit alleles that tend to be found in different individuals (Otto, 2009).

2.3.1. Costs of sex

The prevalence of sexual reproduction is still considered paradoxical because of several known deficiencies and costs of sexual compared to asexual reproduction (Gerber and Kokko, 2016). First, in contrast to the accepted role of sex in speeding up adaptive evolution, genetic recombination may break up favourable gene combinations to slow down adaptive evolution (Smith, 1978). Second, in organisms with male-female differentiation, there is a so called "two-fold cost" of sex (Hoekstra, 2005), whereby August Weismann (Weismann, 1889) proposed that a disadvantage to sexual reproduction was that two sexual individuals are needed to produce an offspring whereas one asexual individual is required to produce an offspring. This "two-fold cost" of sex hypotheses was then elaborated by Maynard Smith based on his experimental studies with parthenogenetic *Drosophila* in which parthenogenetic females can lay the same overall number of eggs in their lifetime as sexual females (Smith, 1958). In other words, males produced by sexual reproduction do not contribute to reproductive output, and thus the

reproductive output per individual for asexual species is twice that for sexual species, hence the twofold cost of sex (Otto and Lenormand, 2002).

Following recognition of the costs associated with sex, subsequent theoretical research has focused on finding a large benefit to sex to balance its significant costs. Of these, two are considered important; either sex increases the rate of adaptive evolution, and/or it prevents the accumulation of deleterious mutations (Muller, 1932). The most classic explanation in favour of sex is that it increases the rate of adaptive evolution by generating adaptive gene combinations (Butlin, 2002). This hypothesis that was first introduced by Weismann (Weismann, 1889) and then elaborated by (Muller, 1932), states that sex is beneficial by increasing genetic variation and allowing faster rates of adaptation by combining different beneficial mutations into the same genome (Roze, 2012). The fact that sexual populations adapt faster than asexual populations is consistent with these ideas and has been the subject of many studies (Malmberg, 1977; Rice & Chippindale, 2001; Goddard *et al*., 2005). On the other hand, because these studies indicate a population advantage to sex, and none of them directly compared sexual and asexual populations against one another during adaptation, they do not provide ample evidence for the maintenance of sex within a population (Becks and Agrawal, 2012).

According to Muller's ratchet theory (1964), unless a population has some way of eliminating deleterious mutations, they will begin to accumulate over time to continuously decrease viability. This continues until the mean absolute fitness of the population becomes less than one and cannot replace itself, and at this point, the asexual population is expected to quickly decline to extinction. However, recombination through sexual reproduction can decrease the deleterious effects of Muller's ratchet (Muller, 1964).

Further considerations of Muller's theory have led to the conclusions that sexual species are able to accumulate genetic mutations which act advantageously in combination with the genetic background of the population in which they arose (Muller, 1964). In short, the generation of individuals combining two or more independently derived mutations that are separately advantageous will be more probable in a sexual compared to an asexual population. In support of this, the rate of accumulation of an advantageous mutant gene in the population that undergoes recombination can be formulated and shows that the evolutionary advantage of sex is lost in sexual species which are obligate selfers. The same is true of organisms such as *Oenothera sp.*,

that have sexual reproduction, but no or very limited recombination between genomes (Muller, 1964).

2.3.2. Short vs. long term advantages of sexual reproduction

One short term advantage of sex is that sexual off-spring may have higher mean fitness than asexual ones. Note that sex and recombination can have no direct effect on mean fitness, rather they can only affect the variance of fitness, and accordingly the response to selection and mean fitness after selection (Burt, 2000). In contrast, long-term advantage is that sexually-derived progeny are more variable in fitness, leading to faster adaptation compared to their asexual counterparts (Becks and Agrawal, 2011). Nonetheless, this hypothesis has some deficiencies; for example, it is not clear that the heritable variance in fitness is significantly increased by sex (Barton and Charlesworth, 1998).

It has also been shown that sex evolves more easily when there is spatial heterogeneity in selection, because it helps break down maladaptive gene combinations introduced by migration. In other words, considering the benefits of sexual reproduction, spatially heterogeneous habitats are more favoured than homogenous ones (Becks and Agrawal, 2010), as sexual reproduction is advantageous in stressful environments ("abandon-ship hypothesis"; Agrawal *et al.*, 2005). Based on this hypothesis, which is mostly true for single-celled organisms such as bacteria, yeast, and short-lived multicellular invertebrates, increased levels of stress will lead to increased energy allocation to sexual reproduction (Griffiths and Bonser, 2013).

2.4. Apomixis mechanisms

While Mendel's work on genetic inheritance in *Pisum* is well-known, he actually identified alternative modes of inheritance in plants, including the *Hieracium* types. In the *Hieracium* type, F1 hybrids produce offspring like themselves, not like their parents, and later investigations into Mendel's research illustrated a distinct kind of sexual inheritance in which segregation was absent (Bateson and Mendel, 1909). Ostenfeld (1904) was the first to interpret the outcome of Mendel's crossing experiments on *Hieracium* as a result of parthenogenesis (i.e., the development of offspring without fertilization) (Bicknell *et al.*, 2016). The term apomixis was first used by Winkler (1908) to describe "absence of mixis" (absence of meiotic recombination) followed by parthenogenesis (Winkler, 1908). Furthermore, Juel (1989) found that embryos of

Antennaria alpina develop from unreduced (2n) egg cells with the complete chromosome sets of somatic cells, and today this type of reproduction by seed is known as apomixis (Nogler, 2006).

It seems that there is no significant difference between simple agamospermy or asexual reproduction through seed and vegetative reproduction through somatic cells such as stolons and apomixis. However, many botanists do not consider vegetative reproduction as similar to apomixis (Ramu *et al.*, 2017). Meristems are multicellular, and correspondingly mutations give rise to chimeric tissues. In contrast, apomicts go through a single-cell stage, which increases the possibility of mutation, and restricts the chance of virus transmission to the progeny (Briggs and Walters, 2017).

Apomixis in plants is described as the replacement or circumvention of the sexual pathway, and is characterized by three developmental steps: (i) the production of mitotically-unreduced gametes through a bypass of meiosis during embryo sac formation (*apomeiosis*), (ii) development of the unreduced gametes into an embryo independent of fertilization (*parthenogenesis*), and (iii) formation of viable endosperm either with (*pseudogamy*) or without fertilization (*autonomous endosperm*) by a sperm cell (Koltunow and Grossniklaus, 2003).

2.4.1. The importance of apomixis in agriculture

Apomixis is a natural form of asexual reproduction in plants and animals (typically referred to as parthenogenesis in the latter) and has received much attention because of its importance in agriculture for potentially fixing complex hybrid genotypes. Hence, understanding the molecular mechanisms involved in apomictic processes in natural species has attracted considerable interest (Tucker and Koltunow, 2009). Compared to other clonal propagation techniques such as somatic embryogenesis, apomixis avoids the need for tissue culture and production of artificial seeds which are mostly expensive (Ramulu *et al.*, 1999). Apomixis does not exist in crop plants, and strategies for introducing it from wild relatives into crops have up to now resulted in agronomically unsuitable, partially fertile apomictic plants (Vielle Calzada *et al*,1996). For example, the production of *Tripsacum* F1 hybrids by crossing maize (2n = 20) with tetraploid apomictic *T. dactyloides* (2n =72) as a pollen parent has been successful, but the F1 hybrid (2n = 46) backcrossed repeatedly to maize showed low male and female fertility, poor seed quality and seed set (Kindiger *et al.*, 1996). For these studies, the genus *Boechera* has become an interesting

model to understand apomixis because it has both diploid sexual and diploid apomictic species which are geographically and morphologically variable (Mau *et al.*, 2015).

2.4.2. Sexual reproduction is the default mode in many apomicts

It has been demonstrated that sexual reproduction is not completely eliminated in the apomictic subgenus *Pilosella* species. Instead, some of the apomictic plants produce rare sexually-derived progeny called 'off type', that comprise sexual hybrids and progeny exhibiting higher and lower ploidy states relative to parental apomicts (Bicknell *et al.*, 2003; Fehrer *et al.*, 2007). Flow cytometric seed screen analyses in the genus *Boechera* has similarly shown that apomixis is not always 100% penetrant (Aliyu *et al.*, 2010), thus, demonstrating that many apomicts are facultative and that there is interplay between sexual and apomictic pathways in these apomicts (Koltunow *et al.*, 2011). Facultative apomixis occurs when both sexual and apomictic reproduction occur in the same organism. In some cyclically apomictic plants, sex is favored in response to biotic and abiotic stress, whereas in the same individuals apomixis drives clonal fecundity during reproductively favorable conditions (Carman *et al.*, 2011).

Deletion mapping of genetic regions associated with apomixis in *Hieracium* demonstrated that apomixis in *H. caespitosum* is controlled by two principal loci. One locus regulates events associated with the avoidance of meiosis (LOA-Loss of Apomeiosis) while the other, unlinked locus controls the events associated with the avoidance of fertilization (LOP-Loss of Parthenogenesis; Catanach *et al.*, 2006). In order to elucidate their developmental roles during seed formation, apomictic mutants that had lost function in one or both loci were examined. Loss of both loci (LOA and LOP) led to loss of apomixis and complete reversion to sexual reproduction, suggesting that sexual reproduction seems to be the default reproductive mode in apomictic *H. praealtum* (Koltunow *et al.*, 2011).

Comparing morphological and molecular relationships of sexual and apomictic pathways in different species demonstrated that the events of sexual reproduction appear in ovules before development deviated towards apomixis. Thus, it can be inferred that the initiation of sexual reproduction is a prerequisite for apomixis, or that apomixis superimposed itself over the sexual system (Figure 2.2) (Tucker and Koltunow, 2009).

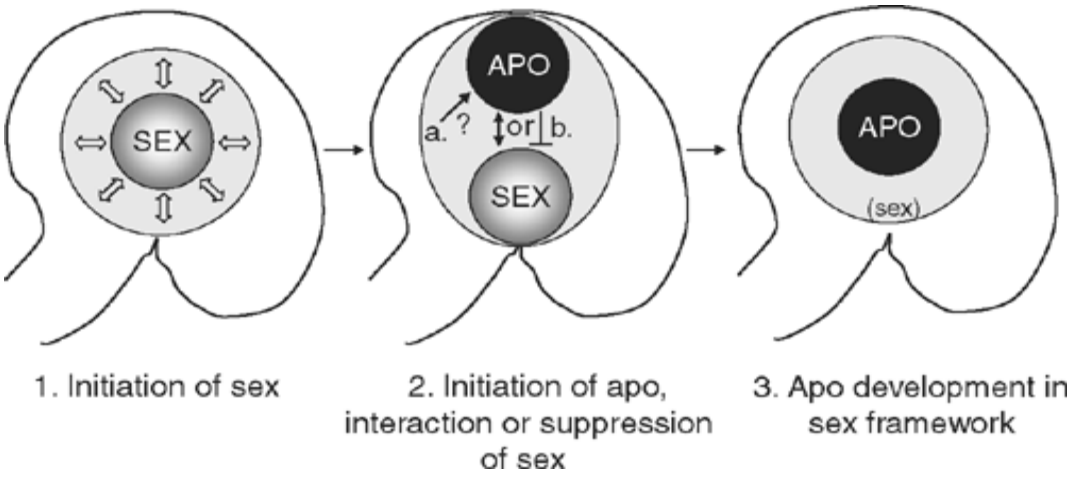


Figure 2.2 Initiation of apomixis. (1) The initiation of sexual reproduction is almost a necessary cue for apomixis. (2) Depending on spatial or temporal changes to the basic sexual process, it can lead to either the subsequent initiation of apomictic or sexual seeds. (3) Subsequent steps of apomictic process until embryo sac maturity take advantage of a basic sexual framework (Image taken from Tucker & Koltunow, 2009).

2.4.3. Developmental deviations from sexuality into apomixis

Apomixis occurs in many species and is assumed to have evolved independently and multiple times from sexual ancestors. Although the mechanisms that produce apomixis are diverse, they share common characteristics as apomixis arises through the spatial and temporal deregulation of developmental pathways leading to sexual seed formation (Grossniklaus, 2001). This hypothesis is supported by heterochrony during early megaspore formation in apomictic *Tripsacum* spp. (Grimanelli *et al.*, 2003). Evidence from the ecological distribution of asexual reproduction in clonal plants show that apomixis might be considered as a result of sexual failure rather than as a recipe for clonal success (Silvertown, 2008).

The fundamental aspects of sexual reproduction that are hindered in apomixis are meiosis and fertilization. A failure of meiosis accompanied with the production of diploid embryo sacs has been reported in *Datura* as the result of a recessive mutation produced through radium treated pollen which inhibited the second meiotic division (Satina and Blakeslee, 1935). Finally, expression profiling of 4 developmental stages of ovule development in sexual and apomictic *Boechera* demonstrated a heterochronic shift in global gene expression between reproductive forms (Sharbel *et al.*, 2010). Together, these observations point to heterochronic shifts of a developmental program in which embryo sac development can occur prior to completion of

meiosis, and has been suggested as a characteristic of apomictic development (Koltunow and Grossniklaus, 2003). Considering all aspects of apomixis, the inhibition of meiosis *per se* does not necessarily stimulate apomeiosis.

2.4.4. Sexual cues are required for apomixis initiation

Koltunow and others demonstrated that sexual cues arising during meiotic tetrad formation in ovules of *Hieracium* and *Pilosella* are necessary for somatic aposporous initial (AI) cell formation (Figure 2.3) (Koltunow *et al.*, 2011). These sexual cues range from photoperiod and nutrient levels to hormones, and might influence events in the ovule or contribute to the degree of apomixis (Koltunow, 1993). Even the molecular events surrounding sporophytic ovule tissues in *Arabidopsis,* for instance alterations in expression of specific proteins, may affect embryo sac, embryo, and endosperm development (Gasser *et al.*, 1998). Nemhauser *et al.* (2000) demonstrated that *rolB* oncogenes from *Agrobacterium rhizogenes* control the fundamental steps in morphogenesis by altering responses to auxin, which is an essential hormone for morphogenesis of gynoecium (Nemhauser *et al.*, 2000) and patterning effects of embryogenesis (Hardtke and Berleth, 1998). In other words, alterations in ovule structure related to auxin perception influence the frequency and timing of apomixis initiation in *Hieracium* (Koltunow *et al.*, 2001), developmental changes which may have a genetic and epigenetic basis.

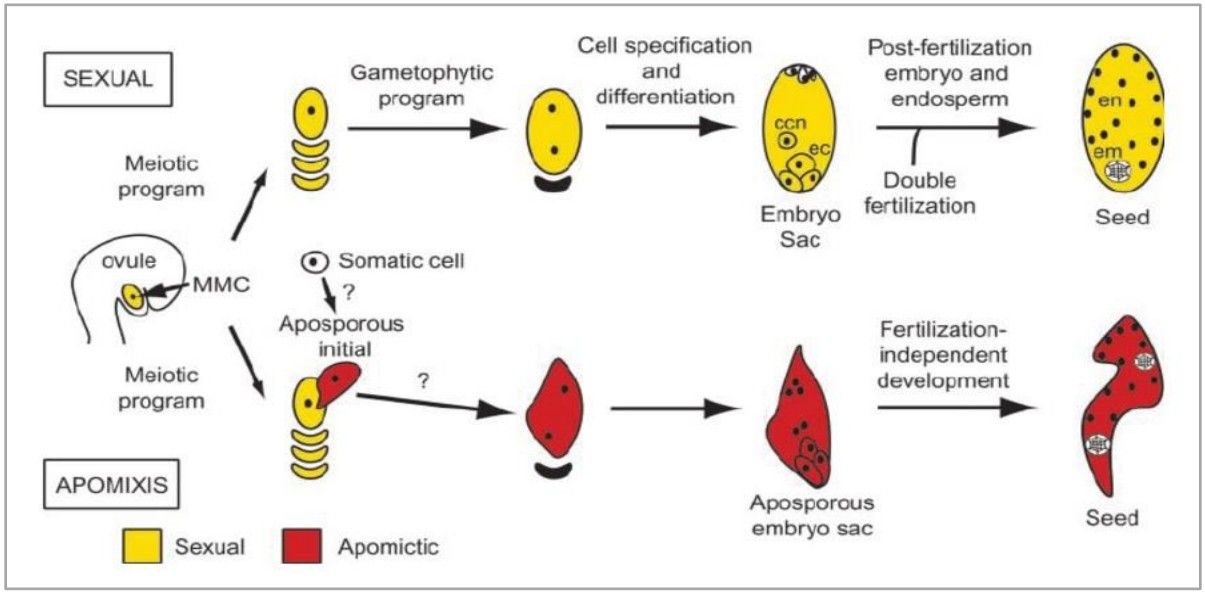


Figure 2.3 Mechanisms of apomixis and sexual pathways in *Hieracium*. Several lines of evidence suggest that epigenetic regulatory mechanisms are involved in reproduction and early

seed development (Baroux *et al.*, 2007; Curtis & Grossniklaus, 2008; Olmedo-Monfil *et al.*, 2010). In maize, inactivation of DNA methylation pathways give rise to multiple aposporous-like embryo sacs that do not undergo functional apomixis (Garcia-Aguilar *et al.*, 2010). Abbreviations: ccn, central cell nucleus Figure 2.3 1; ec, egg cell; em, embryo; en, endosperm (Image taken from Tucker *et al.*, 2003).

2.4.5. Sexual and apomictic development in plants

Apomixis can be subdivided into sporophytic and gametophytic forms, depending on the origin of the embryo (Koebner, 1994). In sporophytic apomixis, an embryo forms directly from nucellar or integument cells adjacent to a reduced embryo sac, and the fate of resulting adventitious embryos is dependent on the endosperm derived from double fertilization of the reduced embryo sac. In gametophytic apomixis, diplosporous embryos form from the megaspore mother cell (MMC), while aposporous embryos from other ovule cells (Bicknell and Koltunow, 2004).

To identify genetic factors that control apomixis and its differences from sexual reproduction, amplified fragment length polymorphism (AFLP) technology was used for either selecting molecular markers that co-segregate with apomixis, or for showing disequilibrium with apomixis in natural populations of *Hypericum perforatum* (Pupilli and Barcaccia, 2012). Using these markers, apomictically producing plants can be recognized from the sexual plants successfully (Schallau *et al.*, 2010).

Nogler (1984) stated that apomixis may occur when reproduction-specific gene expression is activated at the wrong time and/or place. In support of this, developmental heterochrony and apomixis-like meiotic non-reduction (apomeiosis), parthenogenesis and autonomous endosperm formation has been reported in several mutants of sexual species, suggesting that the expression and ultimate function of the genes critical for sexual development are mis-regulated in apomicts (Schallau *et al.*, 2010). For example, ovule formation is the same in apomictic and sexual *Hieracium*, but apomicts differ from sexuals in the timing of initiation of their embryo sac and in the way embryo sacs form within the ovule (Koltunow *et al.*, 1998).

2.4.6. *Boechera* is a model plant for studying apomixis

Many recent studies especially over the last two decades have focused on apomixis because of its potential to fix agriculturally valuable characteristics over many generations. The introduction of apomixis into crop plants would allow the permanent fixation of F1 hybrids often used in agriculture. This not only would benefit crop breeding and hybrid seed production, but also could

provide farmers the access to high-yielding hybrid crops (Grossniklaus *et al.*, 1998; Brukhin, 2017). However, the efforts to transfer apomixis into crops have not been successful because all of the factors related to the components of apomixis have not yet been fully characterized (Kumar *et al.*, 2017).

The genera *Boechera* and *Arabidopsis* belong to the same clade within the *Brassicaceae*. The North American *Boechera* genus is characterized by diploid sexuals, and diploid and triploid apomicts (Aliyu *et al.*, 2010). The genus comprises 50–80 species with remarkably high levels of polymorphism that are linked with polyploidy, aneuploidy, and interspecific hybridization. This variation in ploidy level, along with its adaptation to various habitats, diverse modes of reproduction, hybridization, and close relationship to *A. thaliana*, make it into a promising model for studies on speciation, apomixis, evolution, and phylogeography.

Understanding the origin of apomictic lineages in *Boechera sp*. has been the subject of many studies. Although the genome-wide effects of hybridization and polyploidy are known as the main evolutionary causes of apomixis they are not *de facto* requirements for the phenotypic expression of apomixis in *Boechera*. In fact, they are an indirect correlate of apomixis in *Boechera* sp. and hybridity and polyploidy may be beneficial as states in which deleterious mutations are hidden by multiple allelic variants of most genes (Lovell *et al.*, 2013). Many studies on other species show that apomixis is caused by the inheritance of discrete genetic factors (e.g., heterochromatic B-like chromosomes, Kantama *et al.*, 2007), or altered genetic architecture such as mutations and duplications (Carman, 1997).

Using a custom-made *Boechera*-specific microarray to compare gene expression differences in live micro-dissected ovules at the megaspore mother cell (MMC) stage, apomictic alleles (Apo-allele) of the APOLLO locus were shown to be exclusively expressed in apomictic ovules while Sex-alleles were not expressed in either sexual or apomictic ovules. The Apo-allele is also up-regulated in the leaves of apomictic individuals, but not anthers in comparisons of sexual versus apomictic *Boechera* (Figure 2.4 and Table 2.1) (Corral *et al.*, 2013). The conserved expression patterns of APOLLO in ovules of apomictic *Boechera,* based on qRT-PCR, is indicative of its importance in apomictic seed formation (Corral *et al.*, 2013). Thus, it was hypothesized that regulatory factors work specifically in apomictic ovules to induce allele-specific tissue expression, as a 20 bp conserved polymorphism (TGGCCCGTGAAGTTTATTCC) in the

5’UTR of apomixis-specific alleles imparts multiple putative transcription factor binding sites (Corral *et al.*, 2013).

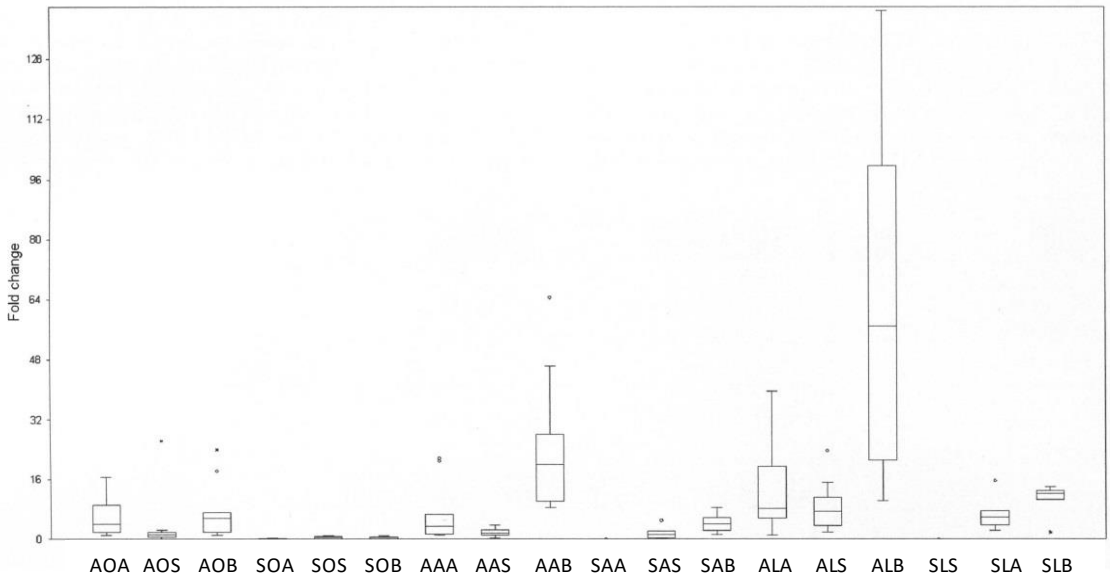


Figure 2.4 Quantitative reverse transcription-PCR validation of the APOLLO gene and Apo-allele and Sex-allele expression. AOA, apomictic ovule Apo-allele; AOS, apomictic ovule Sex-allele; AOB, apomictic ovule both alleles; SOA, sexual ovule Apo-allele; SOS, sexual ovule Sex-allele; SOB, sexual ovule both alleles; AAA, apomictic anther Apo-allele; AAS, apomictic anther Sex-allele; AAB, apomictic anther both alleles; SAA, sexual anther Apo-allele; SAS, sexual anther Sex-allele; SAB, sexual anther both alleles; ALA, apomictic leaf Apo-allele; ALS, apomictic leaf Sex-allele; ALB, apomictic leaf both alleles; SLA, sexual leaf Apo-allele; SLS, sexual leaf Sex-allele; SLB, sexual leaf both alleles (Image taken from Corral *et al.*, 2013).

Table 2.1 Summary of expression rate of APOLLO gene in apomictic versus sexual *Boechera* in different tissues that is shown with asterisks.

	Apomictic			Sexual		
	Ovule	Leaf	Anther	Ovule	Leaf	Anther
Apo-allele	***	*****	***	-	-	-
Sex-allele	-	***	*	-	**	*

2.5. Regulatory factors and apomixis

Studying gene regulation mechanisms in a multicellular organism will lead to a better understanding of cellular diversity. Although each cell contains the same genome, gene regulation is tissue specific and can be generally categorized as transcriptional or post-transcriptional regulation. In the transcriptional type of gene regulation, transcription factors bind to specific elements in DNA, while in post-transcriptional gene regulation they bind to RNA

(Jacob & Monod 1961; Jens & Rajewsky 2014). These two regulatory mechanisms can be further subdivided into many other layers of gene regulation, including: cell signaling; mRNA splicing, polyadenylation and localization; chromatin modifications; and mechanisms of protein localization, modification and degradation (Chen and Rajewsky, 2007).

2.5.1. Transcription factors involved in embryo development

In recent years, female gametophyte development has received increasing attention, and current data suggest that for the formation of functional female gametophytes orchestrated activation of several genes that regulate the establishment of cell fate is needed. In *Arabidopsis* for example, genes including *LACHESIS, CLOTHO, ATROPOS, BEL1-LIKE HOMEODOMAIN, AGAMOUS-LIKE80* and *AGL61* have been reported to control egg cell fate, and mutations in these genes give rise to abnormal embryo sacs along with female sterility (Groß-Hardt *et al*., 2007; Pagnussat *et al*., 2007; Moll *et al*., 2008; Steffen *et al*., 2008). Furthermore, transcriptome comparisons between wild-type egg-cells and the egg cell of parthenogenetic wheat provide valuable information about the transcription factors (RKD factors) involved in gametogenesis. This plant-specific family of TF normally exists in both *wheat* and *Arabidopsis*, but the predominant expression of them in *wheat* and *Arabidopsis* leads to the induction of an egg cell-like transcriptome. It was suggested that they are involved in the regulation of an egg cell transcriptional network as basis for egg cell specification and differentiation (Kőszegi *et al.*, 2011).

In eukaryotes, various RNA polymerases are responsible for the transcription of nuclear genes (Roeder & Rutter 1969). The transcriptional machinery in eukaryotes is mainly composed of RNA polymerase II, which is responsible for transcribing all protein-coding genes, as well as several genes that encode noncoding RNAs (Cramer *et al.*, 2008). RNA polymerase II promoters are composed of several discrete DNA sequences, including promoter elements, upstream promoter elements, and enhancers, and are binding sites of transcription factors (TFs). Transcription factors are proteins that influence the transcription of genes by binding to defined regions of the genome (Latchman, 1997). They have diverse and complicated effects on transcription and can increase the level of transcription initiation (activators) or reduce transcription levels (repressors)(Klug and Ward, 2009).

A common feature that all transcription factors share is that they contain DNA-binding domains that recognize specific sequences within the promoter regions of the genes they regulate (Kummerfeld and Teichmann, 2006). DNA-binding domains are named according to their structural characteristics. Based on the DNA binding domain they contain, transcription factors of plant and animal origin are classified into four major groups including: (1) basic domain (27.4%), (2) zinc–coordinating DNA-binding domain (15.8%), (3) helix-turn-helix (39.1 %), and (4) β-scaffold factors (Figure 2.5) (Latchman, 1997; Charoensawan *et al.*, 2010; Qian *et al.*, 2006).

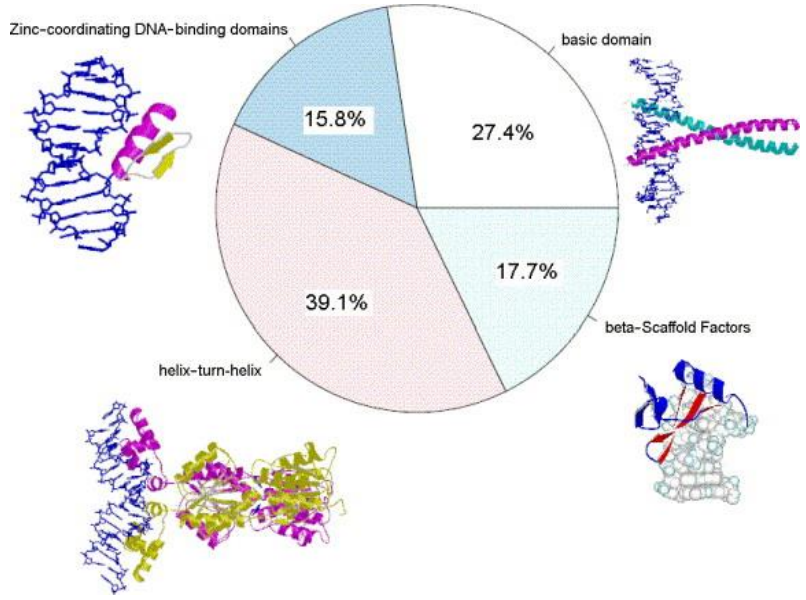


Figure 2.5 Transcription factor classification. Transcription factors are generally grouped into four distinct classes. Upper left, zinc-coordinating DNA-binding domains. Upper right, basic domains. Bottom left, helix-turn-helix. Bottom right β-scaffold factors (Image taken from Qian *et al.*, 2006).

2.5.1.1. TCP genes encode for transcription factors involved in ovule development

TCP is a family of transcription factors named after: teosinte branched 1 (*Zea mays* (Maize))(Doebley *et al.*, 1997), cycloidea (cyc)(Luo *et al.*, 1996) and *PROLIFERATING CELL FACTORS (PCF)* in rice (*Oryza sativa*) (Kosugi & Ohashi 1997; Ma *et al.*, 2016). *TCP* genes encode plant-specific transcription factors with a bLHL motifs that allow DNA binding and protein-protein interactions (Martín-Trillo and Cubas, 2010). They play an important role in regulating gametophytic development in orchid, rice and *Arabidopsis* species. This gene family codes plant-specific transcription factors that control diverse developmental traits (Sarvepalli and

Nath, 2011). The *TCP* family contains a conserved non-canonical helix-loop-helix (bHLH) domain, which is responsible for DNA binding and dimerization. Gene duplication and diversification has led to the establishment of two subfamilies: class I *(TCP-P)* and II *(TCP-C)*, based on the primary structure of the basic DNA domain (Cubas *et al.*, 1999). Class I and II proteins form dimers in solution and evidence supports that the *TCP* domain has a central function in this interaction. These two subfamilies antagonistically modulate plant growth and development through competition in binding to similar *cis*-regulatory modules called site II elements (Li, 2015).

The *TCP* family was until recently limited to *CYCLOIDEA (CYC)* and *TEOSINTE BRANCHED1* , but two additional proteins with similarity in the bHLH region, including *PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR1 (PCF1)* and *PCF2*, have been found through database research. These proteins were isolated based on their ability to specifically bind to promoter elements of a rice gene for the *PROLIFERATING CELL NUCLEAR ANTIGEN* (*PCNA*), a protein that contributes to cell cycle and DNA replication (Jónsson and Hübscher, 1997). *PCNA* is known as an auxiliary protein of DNA polymerase δ, and is one of the factors that are essential for the synthesis of the leading strand during replication *in vitro* of simian virus 40 DNA (Kosugi *et al.*, 1995).

Studies on promoters of the *Arabidopsis PCNA-2* gene show that it is the target of at least five DNA-binding cellular protein extracts that belong to the *TCP* family of proteins (Cubas, 2002). *TCP* family transcription factors are regulatory proteins that bind to *cis*-acting elements named site II motifs in the promoter of the *PCNA* gene. These motifs have been shown as essential components for the proliferating cell-specific transcriptional activity of the gene, and were recognized by nuclear proteins that had near-identical binding sites (Kosugi *et al.*, 1995).

2.5.1.2. The TCP domain basic region binds DNA

It is the bHLH domain of *TEOSINTE BRANCHED 1, CYCLOIDEA, PCF1*(*TCP*) genes that facilitate its binding to DNA and is necessary for homo- and hetero-dimerization of these transcription factors (Kosugi and Ohashi, 1997). Prediction analyses, based upon the chemical and structural properties of DNA binding residues, identified the role of the residues in the basic region of the *TCP* domain in DNA interactions (Ahmad & Sarai, 2005; Andrabi *et al.*, 2009). However, in rice *PCF1* and *PCF2*, this basic region was not sufficient for DNA binding

(Aggarwal *et al.*, 2010). It has been shown that the basic region of the *TCP* domain influences the preferences and selectivity of *TCP* proteins. In addition, the nature of HLH motifs influences the capacity of the basic region to select among different target sequences (Viola *et al.*, 2012).

2.5.1.3. Interaction behaviors of class I and class II of *TCP* transcription factors

Several studies on *TCP* transcription factors have provided evidence that they can bind DNA as homodimers or heterodimers. Dimerization seems to be necessary for DNA binding, as protein deletions that prevent dimer formation also abolish DNA binding (Aggarwal *et al.*, 2010). To understand the role of different parts of *TCP* domains in *Arabidopsis*, the domain TCP4Δ6 (residues 87 to 103) that takes part in dimerization were deleted, and the truncated protein failed to bind to DNA (Aggarwal *et al.*, 2010). So far, the formation of heterodimers has been reported to occur between specific members of the same class of *TCPs* (Yang *et al.*, 2012; Danisman *et al.*, 2012; Aguilar-Martínez & Sinha, 2013). In one study where the likelihood of dimerization between *PCF1* and *PCF2* in rice was investigated, the yeast two-hybrid system was used to show that these two proteins preferentially bind DNA as a heterodimer rather than homodimer (Kosugi and Ohashi, 1997).

2.5.2. Regulatory networks involved in the determination of ovule identity

The ovule of *A. thaliana* represents an ideal system for ovule developmental studies in plants. To date, most molecular studies of ovule development have focused on dicot species such as *Arabidopsis* and *Petunia* (Tucker and Koltunow, 2009), and several genes are involved in primordium initiation, pattern formation and morphogenesis of ovules in *Arabidopsis*. The MADS box genes *APETALA1(AP1), SEEDSTICK(STK), SHATTERPROOF1(SHP1), SHP2*, and *AGAMOUS(AG)* redundantly control ovule identity (Pinyopich *et al.*, 2003). In one study that investigated the role of these genes in ovule identity, a quadruple mutant of *ap2 stk shp1 shp2* showed that all (95%) ectopic ovules were converted into carpelloid structures (Figure 2.6) while the remaining ovules did not develop into mature ovules (Pinyopich *et al.*, 2003).

Figure 2.6 Carpelloid structure of ovules. Ectopic ovule is largely absent from first-whorl organs of ap2 stk shp1 shp2 mutants (Image taken from Pinyopich *et al.,* 2003).

2.5.2.1. BELL1 transcription factor controls ovule development

The genetic network controlling ovule development has been identified and characterized (Colombo *et al*., 2008). Among different transcription factors involved in ovule development, *BELL1* is a major factor controlling ovule patterning, and determines the identity and development of integuments. A mutation in *BELL1* leads to a lack of the inner integument and formation of an organ with unknown identity in place of the outer integument. In fact, ovules of *Arabidopsis* show polarity in at least two axes of symmetry (proximal-distal and the adaxial-abaxial), and the P-D axis eventually exhibits considerable bending due to asymmetrical growth which occurs along the anterior-posterior (A-P) axis (Figure 2.7; Grossniklaus and Schneitz, 1998). In another experiment with unrestricted *AGAMOUS* expression in *BELL1* mutant ovules, or constitutive expression of an *AG* transgene in a transformant, the ovules were converted into a carpel (Ray *et al*., 1994). It was speculated that *BELL1* might have some effect on preventing ectopic gene expression of the MADS box gene *AGAMOUS* in ovules (Bowman *et al*., 1991), whereby it negatively controlled *AG*. Thus, it is the combinatory effects of regulatory mechanisms that define how an ovule develops from undifferentiated cells (Balasubramanian and Schneitz, 2002).

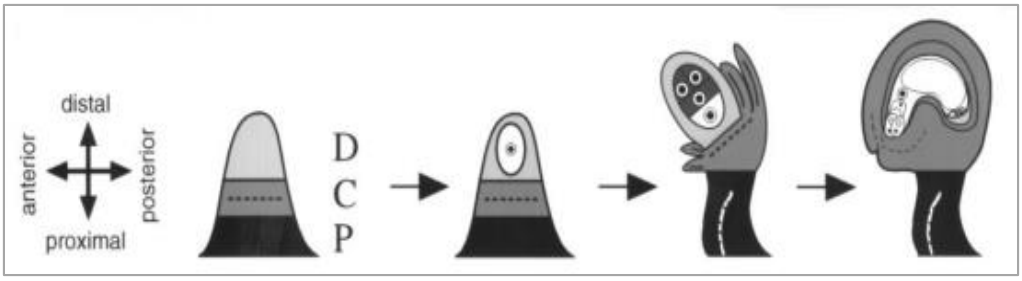


Figure 2.7 Asymmetrical growth of integument in *Arabidopsis*. Scheme to highlight the two axes of polarity and the postulated Proximal-distal (P-D) pattern elements, and asymmetrical growth of integument along the P-D axis (Image taken from Grossniklaus & Schneitz, 1998).

2.5.3. The stamen and carpel identity gene *AGAMOUS (AG)*

One characteristic of transcription factors, their mobility between cells and even organs, has been the subject of many studies in order to decipher the cellular components facilitating intercellular transport of proteins and RNAs (Jackson, 2002; Ruiz-Medrano *et al.*, 2004). Among the different MADS-box family of transcription factors, *AGAMOUS (AG)*, which contributes to carpel identity, has been known for its ability to move in the L1 layer of developing flowers as well as the inner cells of the L1 layer of the floral meristem. These kinds of cell-to-cell movements of *AG* might suppress the expression of the *WUSCHEL* gene in underlying layers (Urbanus *et al.*, 2010). It has been hypothesized that the ability of *AG* to move inwards into the floral meristem might lead to *AG-induced* termination of *WUSCHEL* expression, which results in the consumption of the last meristematic cells for the proper development of the pistil and ovule (Lohmann *et al.*, 2001; Sablowski 2007).

2.6. Epigenetic regulatory mechanisms during ovule development

Plant gametophyte development gives rise to several cell types with distinct fates following two or three divisions. It has been postulated that epigenetic differentiation of the mitotic daughter nuclei may take place in nuclei before cellularization, which defines cell fate. Several studies show that transitions during reproduction and early seed development are epigenetically regulated through dynamic changes in chromatin state (Xiao *et al.*, 2006; Baroux *et al.*, 2007; Curtis & Grossniklaus, 2008).

It has been hypothesized that chromatin-based regulation that controls the vegetative developmental transitions could lead to the differentiation between apomictic and sexual reproduction (Garcia-Aguilar *et al.*, 2010). Recent analyses of *Arabidopsis* plants defective in the

ARGONAUTE9 (AGO9) gene suggest this might be the case (Olmedo-Monfil *et al.*, 2010). *AGO9* is part of a non-cell-autonomous small RNA pathway expressed in the somatic tissues of the gametes. Mutations in *AGO9* affect the fate of the precursor gamete cells of the gametes in the *Arabidopsis* ovule and result in a sterile spore with multiple cells with an aposporous-like phenotype. This result shows that epigenome-level regulators are important in directing female germ cell development toward sexual reproduction. In one study in maize, in order to identify possible chromatin-level regulators of apomixis, the expression pattern of diverse Chromatin-Modifying Enzymes during reproduction in sexual and apomictic plants was compared. The results were indicative of different expression patterns of Chromatin-Modifying Enzymes in the ovule of apomictic vs. sexual maize during ovule development. The apomictic Maize was the F1 hybrid between maize and its apomictic relative *Tripsacum dactyloides* which express apomixis with high penetrance (>99%)(Garcia-Aguilar *et al.*,2010).

A significant amount of research has aimed to identify the genes that are expressed in the embryo sac of *Arabidopsis* through genome-wide transcriptional profiling experiments (Pina *et al.*, 2005) and whole flower and silique transcriptome analyses (Hennig *et al.*, 2004). The results show that the percentage of genes classified as transcriptional regulators from the complete embryo sac and pollen expressed transcriptomes was about 6% to 10% (Johnston *et al.*, 2007). The experiment with mutants shows that genes expressed in the embryo sac may play a crucial role during embryo sac development, including *HOG1*, which is of special interest due to its role in epigenetic control of embryo and endosperm development through DNA hypo-methylation (Rocha *et al.*, 2005).

2.7. Gene regulation through the 5'UTR

In addition to *cis*-regulatory elements that control gene expression, introns are also known for their function in gene expression, a mechanism known as intron-mediated enhancement (IME) of gene expression (Mascarenhas *et al.*, 1990). IME was first reported in the regulation of the *adh1* gene in maize. It was reported that expression of the *adhl* coding region and the CAT coding region was dramatically increased by the addition of an intron (Callis *et al.*, 1987). The importance of introns in enhancement of gene expression of plants, mammals, *Saccharomyces cerevisiae*, nematodes and insects has been clarified (Laxa *et al.*, 2016). Moreover, 79% of all *Arabidopsis* genes contain introns (Poczai *et al.*, 2000). IME is a complex phenomenon, and its

mechanisms are largely unknown. All introns that are involved in IME must be located in the correct orientation (Mun *et al.*, 2002; Maas *et al.*, 1991) within the transcribed sequence (Clancy *et al.*, 1994) and close to the transcription initiation start (Parra *et al.*, 2011).

Further studies have shown that the presence of an intron in the 5' region of coding sequence or UTR leads to enhanced accumulation of mRNA (Karthikeyan *et al.*, 2009; Rose 2004). The results of another study demonstrated that an intron may not just increase or enhance gene expression, but may also be involved in regulating spatial gene expression patterns (Samach *et al.*, 2000). Furthermore, one study on *A. thaliana* 5'UTRs shows that the presence of 5'UTR introns not only enhances gene expression, but also their length is correlated with the level of gene expression (Chung *et al.*, 2006). IME is affected by its nucleotide composition. However, IME cannot be assigned to one specific sequence element and is more likely a result of a combination of multiple factors. CT stretches have been identified as important elements for transcription in general (Jeong *et al.*, 2007; Clancy and Hannah, 2002).

CHAPTER 3. USING THE YEAST ONE-HYBRID ASSAY TO IDENTIFY THE TRANSCRIPTION FACTORS AND OTHER PROTEINS WHICH INTERACT WITH THE 20 BP INSERTION IN THE 5' UTR OF APOLLO APOMIXIS ALLELES.

3.1. Introduction

The APOLLO gene, which has been found by comparing sexual and apomictic flower transcriptomes of *Boechera sp.*, displays differential expression between apomictic and sexual pre-meiotic ovules. Sexual plants are homozygous for the APOLLO Sex-alleles while all apomictic plants are heterozygous for the Sex- and Apo-alleles (Corral *et al.*, 2013). Compared to apomicts, APOLLO was significantly down-regulated in sexual ovules. However, in apomictic plants, the Apo-allele of APOLLO was significantly ($P < 0.001$) up-regulated in apomictic ovules (Corral *et al.*, 2013).

Corral *et al.* (2013) identified several linked Apo-allele-specific polymorphisms. Among three types of polymorphisms observed in APOLLO, a single 20 bp Apo-insertion/Sex-deletion polymorphism in the 5′ untranslated region (5'UTR; TGGCCCGTGAAGTTTATTCC), which is absent in all Sex-alleles, can act as a binding site for specific transcription-factors (i.e., transcription factor binding sites-TFBS). This hypothesis is based on the detection of *cis*-regulatory elements by PlantPAN analysis of all sequenced APOLLO 5′ UTR regions. In the same study, sequence analysis for TFBS on the 5'UTR also revealed that the 20 bp polymorphism provides TBS for *ATHB*-5, *LIM1*, *SORLIP1AT*, *SORLIP2AT*, and *POLASIG1* in Apo-alleles, while the 5'UTR of the Sex-alleles's promoter contains specific TFBS for *Dof2*, *Dof3*, and *PBF* (Corral *et al.*, 2013).

In another study Sharbel (2010) reported dowonregulation of genes in early stages of ovule development in sexual versus apomictic *Boechera* accessions is mediated by transcriptional regulation, and that many genes display divergent patterns of gene expression through time in apomictic versus sexual ovules. Finally, it was hypothesized that accumulated DNA sequence variation in regulatory regions between different *Boechera* taxa may be the underlying reason for the switch from sexual to apomictic seed development during subsequent hybridization events (Sharbel *et al.*, 2010).

Unlike sexual plants that have the chance to fix beneficial mutations and purge deleterious mutations, asexual plants are expected to accumulate deleterious mutations over time (i.e., Muller's ratchet; Muller 1964). In support of this, it has been shown that apomictic *Boechera* accumulate mutations in conserved nucleotide positions such as synonymous sites that are phylogenetically constrained (e.g., regulatory factors) (Lovell *et al.*, 2017; Mau *et al.*, 2021). Despite this, the APOLLO Apo-allele demonstrates conservation in the DNA sequence in virtually all genetic backgrounds and geographic localities of apomictic *Boechera* (Corral *et al.*, 2013).

Other studies support the hypothesis that the APOLLO Apo-allele has had evolutionary success in *Boechera* through its hybridization-driven spread in an infectious manner into different sexual genetic backgrounds, likely through rare haploid pollen from different apomictic lineages (Mau, *et al.*, 2021). After that, recurrent polyploidy mediated by the production of meiotically unreduced gametes has enabled polyploid apomicts to diverge into novel niches (Mau *et al.*, 2015). Considering this conserved (insertion-deletion) motif in APOLLO alleles, as well as its possible role in containing binding sites for transcription factors, the goal of this study is to identify proteins that interact with the apomixis-specific polymorphism (Apo-insertion/Sex-deletion).

DNA-protein interactions are a very important phenomenon in living organisms. In fact, cellular functions such as gene regulation and chromosome repair, not to mention being critical developmental processes, depend mainly on DNA-protein interactions (Dey *et al.*, 2012). The role of transcription factors (TF) is to activate or repress transcription of their target genes by binding to *cis*-regulatory (i.e., linked) elements that are frequently located in a gene's promoter. They regulate transcription spatially or temporally and ensure plant survival with short- and long- term impacts on plant physiology and development.

With respect to the APOLLO specific insertion-deletion and other polymorphisms, it was hypothesized that there will be different TFs which interact with the Apo- vs. Sex-alleles (Corral *et al.*, 2013). To unravel the relationships between TF and *cis*-acting elements, several computational and experimental approaches have become available in recent years (Chow *et al.*, 2018). In this experiment, in order to understand the mechanisms underlying differential gene expression, a yeast one-hybrid assay was applied. In this system, protein-DNA interactions are

detected in eukaryotic cells, which provides an advanced approach for studies of TF functions in plants (Sun *et al.*, 2017).

Objective: Identify the transcription factors and other proteins which interact with the 20 bp insertion in the 5'UTR of the APOLLO apomixis alleles using a yeast one-hybrid assay

The 20-nucleotide apomixis-specific insertion (TGGCCCGTGAAGTTTATTCC) was characterized by a plus-strand transcription factor-binding site which is absent in all Sex-alleles. Furthermore, APOLLO is expressed in apomictic, but not sexual ovules (Corral *et al.*, 2013). In order to understand the mechanisms underlying differential gene expression, identifying physical interactions between regulatory TFs and their target genes is needed.

3.1.1. Principles of the yeast one-hybrid assay

In this study the yeast one-hybrid assay was used to unravel the interaction between the target DNA (APOLLO Apo-allele) and TFs. To date several different methods have been developed to study protein-DNA interactions, including gel-shift, DNase footprinting, and chromatin immunoprecipitation assays (Dey *et al.*, 2012). However, a cell-based yeast one-hybrid assay provides an in-depth understanding of the implications of these interactions on a holistic level.

The yeast one-hybrid system is similar to the yeast-two hybrid system in its main concepts, but protein-DNA interactions are tested instead of protein-protein interactions. These methods are because TFs have two physically separable domains, including an activation domain and a DNA-binding domain. If these two domains bind together, the result will be a functionally active transcription factor that can recruit RNA polymerase II at its corresponding promoter transcription start position (Dey *et al.*, 2012). It was first developed as a strategy to identify the gene that encodes the DNA-interacting subunit of the yeast origin recognition complex (Li and Herskowitz, 1993) and later adapted for higher throughput studies (Deplancke *et al.*, 2004). The yeast one-hybrid assay is a highly sensitive method and detects weak DNA-protein interactions since proteins are in their native configuration (Dey *et al.*, 2012).

The yeast one-hybrid system has two main components, including a reporter construct with a DNA of interest (i.e., bait construct, the APOLLO promoter in this experiment), cloned upstream of a reporter gene(s). The second is a hybrid expression library, constructed by fusing a transcriptional activation domain to random protein segments (prey construct). Both components

are introduced into a budding yeast strain, and the bait is used to "fish" for interacting prey (Figure 3.1). The DNA of interest used in bait construction can be short *cis*-regulatory elements (as single copy or tandem repeats) or longer and more complex DNA fragments such as promoters or enhancers (Reece-Hoyes *et al.*, 2009).

The reporter gene most used is HIS3, which enables yeast with positive interactions to grow in a medium lacking histidine. In order to make sure the growth of yeast is the result of a positive interaction rather than a basal level or "leaky" expression of HIS3, a competitive inhibitor 3-amino-1, 2, 4-triazole (3AT) is used in the medium. Hence, the higher level of expression can be attributed to cells showing positive interaction while false positives are eliminated (Alexander *et al.*, 2001).

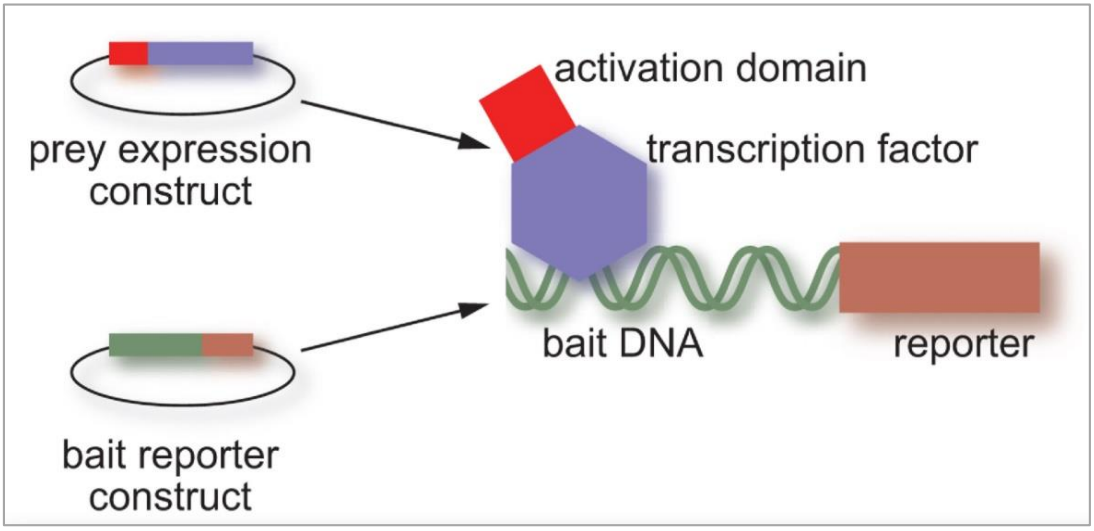


Figure 3.1 Yeast one-hybrid assay schematic. The interaction between the bait sequence and TF leads to the expression of the reporter gene (Image taken from Reece-Hoyes and Marian Walhout, 2012).

3.2. Materials and methods

(a) Preparation of material for the yeast one-hybrid assay performed by Hybrigenics

In order to conduct the yeast one-hybrid assay, the tissues (florets, young siliques and the whole seedlings) of 3-week-old seedlings were collected from *B. stricta* (sexual) and *B. divaricarpa* (apomictic). *Boechera stricta* is a sexual and predominantly self-fertilizing short-lived perennial (biennial) with the chromosome number of 2n=2X=14, and is native to Rocky Mountains in North America (Lee and Mitchell-Olds, 2011). *Boechera divaricarpa* is found especially in

California and Washington, and is an apomictic allodiploid biennial contatining genomes of *B. stricta* and *B. sparsiflora* with a chromosome number of 2n=2X=14 (Windham and Al-shehbaz, 2007). The total mass was ~0.2 g (fresh weight) of tissue for florets and young siliques (each), and 0.5 g for seedlings. The RNA was isolated separately and then pooled in the percentages shown in the library request forms as follow (Table 3.1).

Table 3.1 Library request form for *B. divaricarpa* and *B. stricta*. Table contains the tissue names and proportion of total RNA samples per tissue (in volume).

Species and Strain		*Boechera divaricarpa* (ES517)	*Boechera stricta* (ES718)
Tissue/cell Type (Proportion of total RNA sample %)	Whole seedling (3-week-old)	68%	61%
	Florets	17%	21%
	Young siliques	15%	18%
Kits used for extraction		Qiagen RNeasy Plant Kit	Qiagen RNeasy Plant Kit
Final buffer		ddH_2O	ddH_2O
RNA concentration (µg/µL)		0.742 µg/µL	0.769 µg/µL
Volume (µL)		1,550 µL	1,550 µL
RNA Quality (O.D, gel picture)		Bioanalyser – RIN = 9.3	Bioanalyser – RIN = 9.1
Comments		Tissue for both RNA extraction was collected over a 24-h time course to account for RNA variation with circadian cycles.	

Tissues were collected every three hours over a 24-h period, and RNAs were extracted from six different samples by using a Qiagen RNeasy mini kit. Then, the total RNAs for whole seedling, florets, and young siliques were pooled separately for *B. divaricarpa* and *B. stricta* before sending for cDNA library's preparation by Hybrigenics (www.hybrigenics-services.com). The promoter sequences (bait) of the Apo- and Sex-allele were screened against TF and proteins (prey) expressed in both *Boechera* cDNA libraries.

Two *Boechera* 5'UTR sequences were used as bait, including:

a. The 5'UTR region of the APOLLO Apo-allele (pApo-APOLLO) carried two copies of the 20 bp Apo-insertions and sequences surrounding it.

b. Two copies of the motifs spanning the same region of the Sex-allele (pSex-APOLLO; Figure 3.2). To conduct the yeast one-hybrid assay these DNA sequences were cloned upstream of a reporter gene to create a 'DNA bait-reporter' construct. The DNA bait attached to the reporter

was then cloned into a centromeric yeast one-hybrid vector (pB303). Finally, a cell-to-cell mating was carried out between the 'DNA bait - reporter' yeast strain and a yeast strain pre-transformed with the *Boechera* cDNA libraries (www.hybrigenics-services.com). Then the results of the cell-to-cell mating were screened for expression.

a.

AAAGAGGAGGTGGCCCGTGAAGTTTATTCCCTTTAAAACCAAAGAGGAGGTGGCCCGTGAAGTTTATTCCCTTTAAAACC

b.

TTTTCCGTAAAAAGAGGAGGATCAATTGCTTTAAAACCCATTTTCCGTAAAAAGAGGAGGATCGATTGCTTTAAAACCCA

Figure 3.2 The sequences used as bait in yeast one-hybrid assay experiment (5'-3'). a. The 5'UTR of Apo-allele (*B. divaricarpa*) contains the apomixis-specific polymorphism i.e., 20 bp Apo-insertion/Sex-deletion (red letters) twice. b. The 5'UTR of Sex-allele (*B. stricta*) contains a shorter Sex-specific motif (red letters) twice and there is a single nucleotide polymorphism (SNP) on it as shown with blue color.

The key benefits of the proprietary Hybrigenics ULTImate yeast one-hybrid assay used in this study was its rapid and exhaustive approach with the ability to screen millions of cell-to cell interactions, enabling the detection of even weak interactions as well as those in which their transcripts are rare. In addition, the Hybrigenics assay includes a bioinformatics analysis of the results which include confidence scores to evaluate the data.

3.2.1. Yeast one-hybrid assay protocol for 5'UTR of Sex-allele vs. *B. stricta* library

The sequence for the Sex-allele 5'UTR insertion was PCR-amplified and cloned into the vectors pB303 and pB304. pB303 was constructed by inserting the HIS3 gene into the pFL39 backbone (Bonneaud *et al.*, 1991). pB304 corresponds to pB304 but with a different sequence in front of

the HIS3 reporter gene. The constructs were checked by sequencing the entire inserts. Screening was performed against a random-primed *B. divaricarpa* cDNA library constructed into pP6 that derives from the original pGADGH (Bartel *et al.*, 1993) plasmid.

For the screen with the pB303 construct, 153 million clones (15-fold the complexity of the library) were screened using a mating approach with the YHGX13 (Y187 ade2-101::loxP-kanMX-loxP, matα) and Y1H300 (mata) strains as previously described (Fromont-Racine *et al.*, 1997). 366 HIS+ colonies were selected on a medium lacking leucine, tryptophan and histidine and supplemented with 50 mM 3-Aminotriazol to handle bait autoactivation. For the screen with the pB304 construct, 231 million clones (23-fold the complexity of the library) were screened using the same mating approach. 28 HIS+ colonies were selected on a medium lacking leucine, tryptophan, and histidine. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure. A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction as previously described (Formstecher *et al.*, 2005; Rain *et al.*, 2001; Wojcik *et al.*, 2002).

3.2.2. Yeast one-hybrid assay protocol for 5'UTR of Apo-allele vs. apomictic *B. divaricarpa* library

The sequence for the Apo-allele 5'UTR insertion was PCR-amplified and cloned into the vector pB303. pB303 was constructed by inserting the HIS3 gene into the pFL39 backbone (Bonneaud *et al.*, 1991). The construct was checked by sequencing the entire insert. Screening was performed against a random-primed apomictic *B. divaricarpa* cDNA library constructed into pP6 that derives from the original pGADGH (Bartel *et al.*, 1993) plasmid.

204 million clones (20-fold the complexity of the library) were screened using a mating approach with the YHGX13 (Y187 ade2-101::loxP-kanMX-loxP, matα) and Y1H300 (mata) strains as previously described (Fromont-Racine *et al.*, 1997). 29 His+ colonies were selected on a medium lacking leucine, tryptophan, and histidine, and supplemented with 100 mM 3-Aminotriazol to handle bait autoactivation. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated

procedure. A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction as previously described (Formstecher *et al.*, 2005; Rain *et al.*, 2001; Wojcik *et al.*, 2002).

(b) Characterization of the data obtained from Hybrigenics

The yeast one-hybrid assay carried out for bait constructs of Apo-APOLLO 5'UTR insertion and Sex-APOLLO 5'UTR insertion vs. prey constructs of apomictic *B. divaricarpa* and sexual *B. stricta* respectively.

In order to analyze yeast one-hybrid assay data the following parameters were used.

1. Type: 5p 3p
2. Contig's name
3. Gene name (Best Match)
4. Gene Bank ID (NCBI)
5. Gene ID (NCBI)
6. Global Predicted Biological Score (PBS): A, B, C, D, E, F, N/A
7. Additional gene notes
8. Start and stop positions
9. Frame
10. Sense (Sense or Anti-sense)
11. Percent Id 5p/3p
12. Raw experimental 5p SEQ
13. Post linker extraction 5p SEQ
14. Raw experimental 3p SEQ
15. Post linker extraction 3p SEQ
16. Fragment theoretical Sequence

The Global Predicted Biological Score (PBS) is computed to assess the interaction reliability. It is an E-value, primarily based on comparison between the number of independent prey fragments found for an interaction and the chance of finding them at random (background noise). The value varies between 0 and 1. Several thresholds have been arbitrarily defined in order to rank the results in 4 categories from A (the highest confidence rank) to D.

1. Very high confidence of interaction (A)
2. High confidence of interaction (B)
3. Good confidence in the interaction (C)
4. Moderate confidence in the interaction; it is important to consider this level of interaction can be either false positive or interactions hard to detect by the Y2H assay technique (D)
5. Interactions involving highly connected prey domains; it can be because of biological function of prey proteins that lead to their interactions or proteins with a prey interacting domain that contains a known protein interaction motif (E)
6. Experimentally proven technical artifact (F)
7. The confidence of interaction is not applicable for the following reasons: All the fragments of the same reference Coding sequences are antisense, the 5p sequence is missing, all the fragments of the same reference Coding sequences lie in the 5' or 3'UTR.

3.3. Results

3.3.1. Analyzing the yeast one-hybrid data

In order to interpret and analyze the yeast one-hybrid assay data, the first step was to examine the data and select TF with a significant amount of interaction as shown in (Appendix Figures 5.1 and 5.2). These "Domsight" data compare the bait fragment and the Selected Interacting Domain (SID) of the prey proteins with the functional and structural domains (PFAM, SMART, TMHMM, SignalP, Coil algorithms) on these proteins. Secondly, the full sequences of those TF were identified using BLASTn (https://blast.ncbi.nlm.nih.gov). This tool was applied to compare the yeast one-hybrid assay result with the available database and find the best matches among different species. Finally, they were organized into a graph and compared based on Query cover and E-value parameters to explore the significant homologies (Figure 3.4).

3.3.2. Summary of the yeast one-hybrid assay results

In the experiment with the *B. divaricarpa* (Apo) cDNA library, 39 independent clones were identified from a total of 204 million interactions. For the *B. stricta* (Sex) cDNA library, 366 independent clones with a total of 153 million interactions were analyzed. In a yeast one-hybrid assay experiment, HIS3 gene expression is normally measured by growing transformed yeast cells on selective media lacking leucine, uracil, and histidine. Activity of the HIS3 reporter is

then quantified as the survival rates of yeast transformants on plates containing some amounts of 3-aminotriazole (3-AT), a competitive inhibitor of the HIS3 protein as shown in (Table 3.2).

Table 3.2 Summary of yeast one-hybrid assay result. This table shows the number of processed clones per interaction, number of analyzed interactions, and 3AT concentration.

	B. divaricarpa	*B. stricta*
Nature	cDNA	cDNA
Reference Bait Fragment	Apo-APOLLO 5'UTR insertion	Sex-APOLLO 5'UTR insertion
Prey Library	*B. divaricarpa*	*B. stricta*
Vector	pB303 (centromeric vector)	pB303 (centromeric vector)
Processed clones	39	366
Analyzed Interactions	204 million	153 million
3AT concentration	100.0 mM	50.0 mM

The results show the different proteins that interact with bait sequences including structural and functional ones. The yeast one-hybrid assay data include proteins that interact with the *B. divaricarpa* Apo-allele 5'UTR insertion (Table 3.3), while the proteins that were detected for the *B. stricta* Sex-allele 5'UTR insertion as bait and *B. stricta* as prey library are shown in (Table 3.4).

Table 3.3 Proteins were detected for the *B. divaricarpa* Apo-allele 5'UTR insertion as bait and *B. divaricarpa* as prey library. pB303 used the centromeric yeast one-hybrid vector.

Protein ID	Protein Name	Confidence Of Interaction
PF00924	Mechanosensitive ion channel MscS	Moderate
PF00847/SM00380	AP2/ERF domain (ERF107, ERF5, ERF15)	Moderate
PF00696	Aspartate/glutamate/uridylate kinase	Moderate
PF00171	Aldehyde dehydrogenase domain	Moderate
PF00206	Fumarate lyase, N-terminal	Moderate
PF10415	Fumarase C, C-terminal	Moderate
PF03767	Acid phosphatase, class B-like	Weak

Table 3.4 Proteins were detected for the *B. stricta* Sex-allele 5'UTR insertion as bait and *B. stricta* as a prey library. pB303 was used as the centromeric yeast one-hybrid vector.

Protein ID	Protein Name	Confidence of Interaction
PF00262	Calreticulin/calnexin	Moderate
PF00847 / SM00380	AP2/ERF domain (ERF 107, ERF 1B)	High/Moderate
SM00382	AAA+ ATPase domain	Moderate
PF12848	ABC-transporter extension domain	Moderate
PF00005	ABC transporter-like	Moderate
SM00717	SANT/Myb domain	Moderate
PF01649	Ribosomal protein S20	Moderate
PF01798	snoRNA binding domain	Moderate
PF08156	NOP5, N-terminal	Moderate
SM00931	NOSIC	Moderate
PF07172	Glycine rich protein family	Moderate
PF01061	ABC-2 type transporter	Moderate
PF04889	Pre-mRNA splicing factor Cwf15/Cwc15.	Moderate
PF00109	Beta-ketoacyl synthase, N-terminal	Moderate
SM00825	Polyketide synthase, beta-ketoacyl synthase	Moderate
PF02801	Beta-ketoacyl synthase, C-terminal	Moderate
PF04504	GLABROUS1 enhancer-binding protein family	Moderate
PF00240/SM00213	Ubiquitin domain	Moderate

3.3.3. Identification of transcription factors that interact with the promoter motifs of the APOLLO promotor

BLASTn was used to annotate the yeast one-hybrid assay data, as it is suitable for short queries and cross-species comparisons (Hu and Kurgan, 2019). To interpret and analyze the data for each TF, the corresponding theoretical sequence was chosen from the results provided by Hybrigenics (www.Hybrigenics.com). The following sequence is one example chosen from the whole dataset and it is representative of a TF with the gene ID: 14419s0017.

(ACCGGAAGAAGAGAATAAACATTACAGAGGAGTGAGACAAAGACCGTGGGGAAAATTCGCGGCGGAGATAAGAGACCCGC
ATAAACGCGGATCTCGCGTTTGGCTCGGGACGTTTGATACAGCGATTGACGCGGCTAGAGCTTATGATAAAGCAGCGTTTAGA
CTTCGAGGATCCAAGGCGATTTTGAATTTCCCTCTTGAAGTTGGCAAGTGGAAACCACGCGCCGACGAAGGTGACAAGAAAC
GGAAGAGAGACGACGACGAAGATGAGAAAGTGACTGTTGTCCAGAAACAGAAAGTTAACGGTAACGGTGGAGAGACGTTTC
CGTTATGCGACTGGGACTTAACGGGGTTTCTTAACTTTCCGCTTCTCTCGCCGTTATCTCCTCATCCATCGTTTGGTTTTTCACAA
TTGACCGTTGTTTGANNNNNNNNNNNNNNNNNNNNNNNN)

BLASTn was applied to the mentioned sequence on (https://blast.ncbi.nlm.nih.gov) and the results were several hits. Then the BLASTn tool was used for comparing those similar hits based

on E-value and Query coverage. E-values quantify the number of hits one can expect to observe by chance, and query coverage is the fraction of the query sequence length that is covered by the aligned segments (Hu and Kurgan, 2019). Based on these characteristics, the most desirable hits are the ones with the highest query coverage and the lowest E-value. The corresponding TFs that were found by this method were *ERF5* as is shown in (Figure 3.4). The same method was used for the rest of the sequences and the other TFs including *ERF1*, *ERF107*, *ERF15*, and GLABROUS1 enhancer-binding protein (*GeBP*) (Tables 3.3 and 3.4).

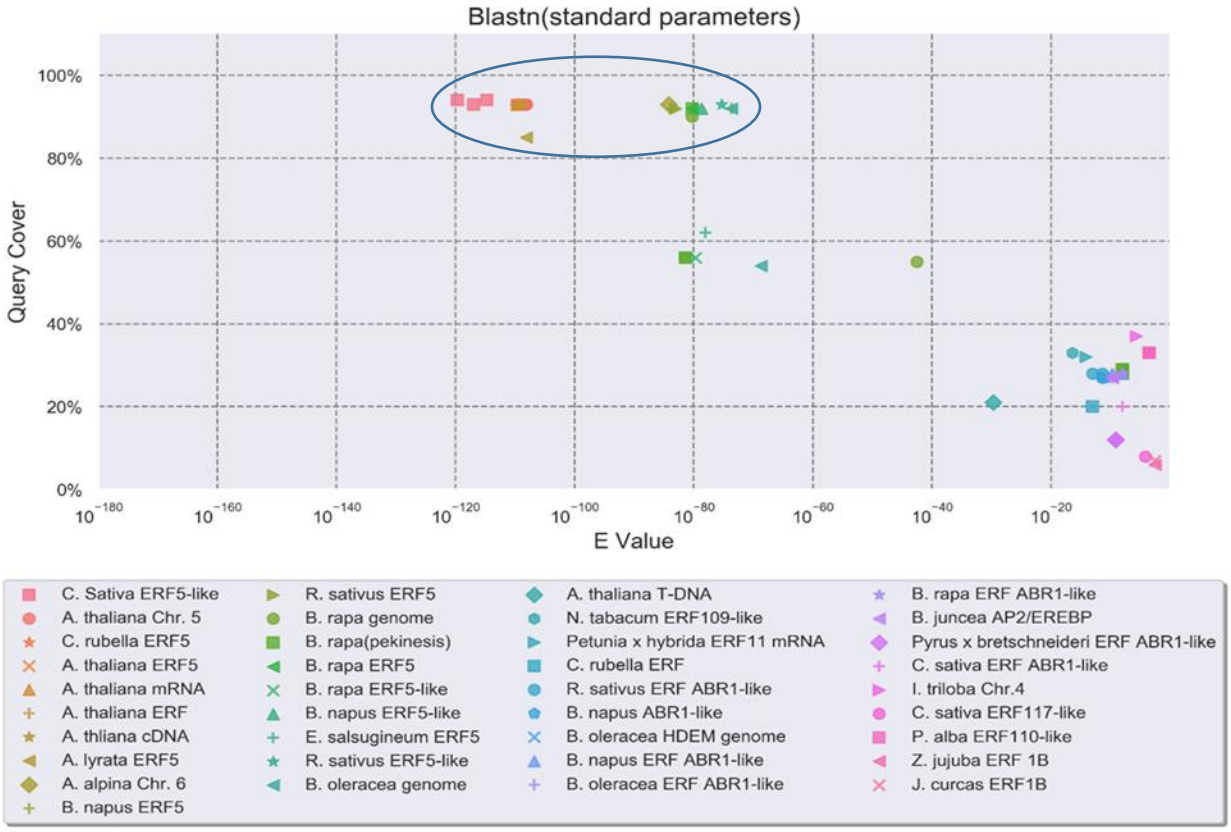


Figure 3.3 Blasting the *B. divaricarpa* (ID14419s0017.1) sequence to the NCBI viridiplantae database. Circled data shows that *ERF5* is the best candidate as it has the highest Query cover and the lowest E-value in a group of data belonging to different species.

3.4. Discussion and conclusions

3.4.1. Characterization of the proteins identified in the yeast one-hybrid assay.

(a) Interacting factors of the Sex-allele and the normal sexual pathway

Among the proteins detected from the *B. stricta* cDNA library about 5% belong to *AP2/ERFBP* family (*ERF1*, *ERF107*) and *GeBP* proteins with a known function of regulating reactive oxygen species (ROS) driven mechanisms (Shaikhali *et al.*, 2015). An earlier study introduced *GeBP* as the first member of a newly discovered protein with a nuclear localization that was involved in leaf primordia development (Curaba *et al.*, 2003). The rest of the proteins include: **(a)** Structural proteins such as an ABC-transporter extension domain, ABC-2 type transporter; **(b)** Enzymes such as an ATPase domain, Beta-ketoacyl synthase, polyketide synthase; **(c)** Functional protein such as calreticulin/calnexin that act as molecular chaperon; **(d)** Regulatory proteins such as ribosomal protein S20, snoRNA binding domain, NOP5, Pre-mRNA splicing factor Cwf15/Cwc15.

The *GeBP* had the highest query coverage and lowest E-value among the hits gained from blast of the data from the Sex promoter vs. Sex cDNA experiment (Table 3.4). Plants produce reactive oxygen species (*ROS*) as a result of photosynthetic electron transport and metabolism and control redox regulation by using oxidants and antioxidants as flexible integrators of metabolic and environmental signals (Dietz, 2003). To date, several redox regulated signaling systems have been shown to regulate gene expression by conveying information to the nucleus. Among them, the *GeBP* has a mediator activity in *A. thaliana* (Bäckström *et al.*, 2007) that binds promoters containing the *CryR2* element, 5'-ACATAWCT-3' (Shaikhali *et al.*, 2015). Its DNA-binding activity is decreased upon direct physical interaction with the mediator subunits, and is modulated by redox (oxidation-reduction) conditions, which modify both the *GeBP* structure and its DNA-binding activity through the interaction of the mediators (*MED10a, MED28* and *MED32*) (Shaikhali *et al.*, 2015).

Plants have innate systems for scavenging/detoxifying ROS such as the production of polyamines (putrescine, spermidine, and spermine) (Ha *et al.*, 1998). These metabolites generate a highly efficient antioxidant system that maintains a homeostasis between ROS elimination and overproduction (Hadacek *et al.*, 2010). Secondly, a higher activity of meiosis-specific proteins can initiate double-strand break formation and thus increase recombination frequency leading to

the repair of DNA damage caused due to oxidative stress (Bleuyard *et al*., 2006). It can be concluded that environmental stresses and the increased levels of ROS have been a major driving force for the shifting from sex to apomixis in facultatively apomictic eukaryotes (Hörandl and Hadacek, 2013). The reason why the *GeBP* transcription factor interacts with the APOLLO Sex-allele promoter's motifs might be due to its direct or indirect effect on meiosis initiation as a way of buffering ROS consequences for sexual *Boechera*. This conclusion is in accordance with the data represented by Corral *et al*. (2013) in which the APOLLO Sex-allele was up-regulated in somatic cells of both sexual and apomictic *Boechera*, while the APOLLO Apo-allele was only expressed in ovule (meiotic stage) of apomictic *Boechera*.

It can be postulated that the regulatory impact of *ERF1* is to keep the sexual *Boechera* population in a steady state during aggressive biotic/abiotic stresses. However, its exact mechanism has not been determined. In this context, the function of *ERF1* detected in the sexual prey library against sexual *B. stricta* (bait sequence) can be described by the Red Queen Hypothesis. According to this theory, parasite pressure maintains sexual reproduction in the host population by selecting for the ability to produce rare genotypes that are resistant to infection (Park and Bolker, 2019). In fact, the advantage of sexual reproduction in the host population comes from its ability to respond to cyclical changes in selection pressure caused by pathogens over time, and not just the generation of genetic diversity (Clay and Kover, 1996). Thus, despite the two-fold costs of sex, being sexual works in favor of plant survival when discussing the pathogen-host relationship. For example, clonally propagated crop species (e.g., sugarcane and bananas) are more susceptible to pathogen infection compared to self-fertilized crops, which in turn incur more losses than outcrossing crops (Stevens, 1948).

In one study conducted by Horstman *et al*. (2015) the *HOMEODOMAIN GLABROUS (HDG)* transcription factor family has been shown to interact *in vitro* and *in vivo* with *BABY BOOM (BBM)*[2] a gene associated with parthenogenesis in other plants. The *HDG* transcription factor is known for its role in root, shoot, and meristem differentiation as well as ectopic expression of margin cells leading to an increased ploidy, while BBM has the opposite role of decreasing

[2] BBM is a member of AINTEGUMENTA-LIKE (AIL) subfamily of the APETALA2/ETHYLENE RESPONSE ELEMENT-BINDING FACTOR (AP2/ERF) family of transcription (Hortsman *et al*., 2015).

cellular differentiation of cotyledon cells and reduced ploidy (Horstman *et al.*, 2015). It was suggested that *HDG/BBM* act antagonistically, with the balance between the two leading to either cell proliferation or cell differentiation (Nakamura *et al.*, 2006). It is plausible that the *AP2* transcription factors (*ERF1B* and *ERF107*) reported in this study (Table 3.4) for sexual *Boechera* have some type of antagonistic interaction with the *GeBP* as that reported by Horstman *et al.* (2015). The antagonistic interaction of these two proteins maintains the cell proliferation process which might be important in a normal meiosis event.

(b) Interacting factors of the Apo-allele and deviations from the sexual pathway

Among the proteins identified from the yeast one-hybrid assay of the apomictic *B. divaricarpa* cDNA library, about 8% belong to the *AP2/ERFEBP* family of transcription factors (Figure 3.4). They are known to have important functions in the transcriptional regulation of a variety of biological processes, such as response to environmental signals and flower development (Suzuki *et al.*, 2006). The rest of the proteins are structural proteins such as a mechanosensitive ion channel (MscS) and enzymes such as aspartate/glutamate/uridylate kinase, aldehyde dehydrogenase domain, fumarate lyase, N-terminal and fumarase C, C-terminal.

The TFs found in this study, *A. thaliana ERF107, AtERF15 (AtERF93), AtERF5 (AtERF10)* and *AtERF1* more specifically belong to the *IXc (B3)* sub-family of the *ERF* gene family (Figure 3.4). In general, the genes in this group are characterized by a highly conserved c-terminus *EDLL* motif (conserved glutamic acid (E), aspartic acid (D) and leucine (L) residues) motif which is classified into *CMIX-3*, *CMIX-2*, and *CMIX-1*, respectively. Several members of the *IXc* sub-family, including *ERF1 (AtERF92),* and *AtERF15 (AtERF93),* which contain a *CMIX-4* motif, have already been shown to function as activators of GCC box-dependent transcription in *Arabidopsis* leaves (Fujimoto *et al.*, 2000). In addition to the action of the *EDLL* peptide for targeted gene activation, it also can alter the regulation of flowering time in *Arabidopsis* (Tiwari *et al.*, 2012). *AtERF5* bears a putative MAP kinase phosphorylation site in the C-terminal region (Fujimoto *et al.*, 2000), while *AtERF#107* has no MAP kinase phosphorylation site.

The genes in group IX, such as *ERF1*, show high expression in response to biotic stress, unlike ROS which is abiotic (Berrocal-Lobo *et al.*, 2002; You and Chan, 2015). It has furthermore been shown that defense-related phytohormones such as ethylene, jasmonate, and salicylic acid

differentially induce the expression of genes in group IX (Oñate-Sánchez and Singh, 2002). Using their conserved DNA binding domain these types of TFs bind with the multiple *cis*-elements and trigger multiple responses (Shinozaki and Yamaguchi-Shinozaki, 2000). One probable reason for the variable response is differential binding to the promoter (Wang *et al.*, 2014; Phukan *et al.*, 2017).

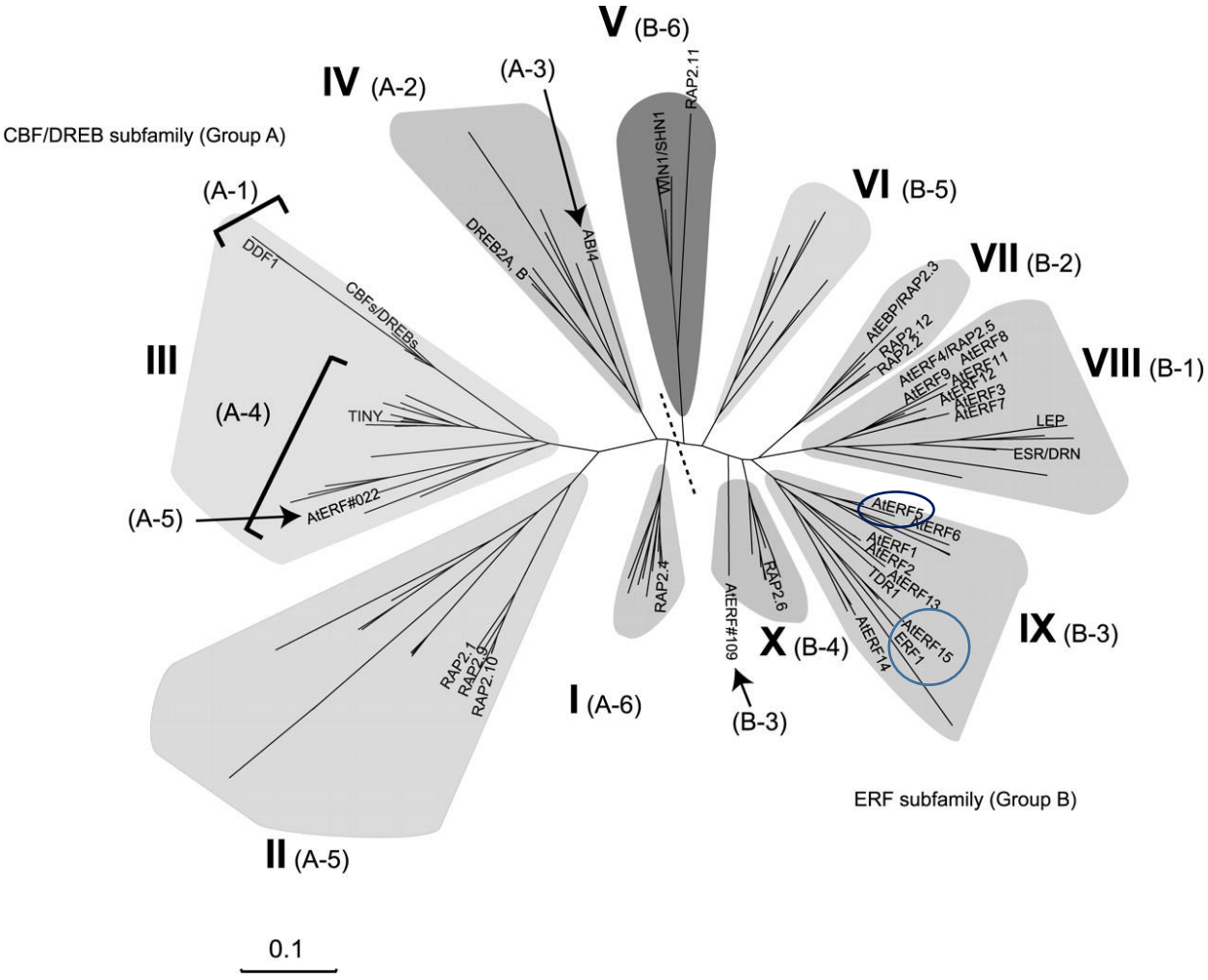


Figure 3.4 Diagrammatic representation of the location of *AP2* family of TFs identified in the yeast one-hybrid screen compared to the other known members of this family from different plants. *AtERF5*, *AtERF15*, and *ERF1* which are circled belong to group *IX (B3)* of the *ERF* subfamily. The so-called *CBF/DREB* and ERF subfamilies are divided with a broken line. Classification by (Sakuma *et al.*, 2002) is indicated in parentheses including subgroups of (A-1, A-2, A-3, A-4, A-5, A-6) and subgroups (B-1, B-2, B-3, B-4, B-5, B-6), some of the proteins has been described to belong to two different groups based on their characteristics as shown with arrows (Image taken from Suzuki *et al.*, 2006).

This is not the first report of a metabolic transcription factor associated with apomixis. In one study the expression of a *BABY BOOM (BBM)* gene *(APETALA2/ETHYLENE RESPONSE FACTOR, AP2/ERF* family of transcription factors), which was cloned from an apomictic grass (*Pennisetum squamulatum*), induced both parthenogenesis (embryo formation without fertilization) and haploid embryo production in sexual cereals such as rice and maize (Conner *et al.*, 2015). However, it was unsuccessful for the model dicot system *Arabidopsis* (Conner *et al.*, 2017). Furthermore, in a previous study on the genetic control of apomixis, a wild-type *BBM* gene from a sexually reproducing plant induced parthenogenesis when misexpressed in egg cells of transgenic *Arabidopsis*, suggesting that asexual reproduction could potentially evolve from the altered expression of existing genes within the sexual pathway (Conner *et al.*, 2017). It also suggests that metabolic factors, in addition to apomixis-specific mutations, regulate these phenomena (Khanday *et al.*, 2019).

The result of another study compared the seedling RNA-seq transcriptomes of an apomict and a sexual *Boechera*, both isolated from a drought-prone habitat (Shah *et al.*, 2016). The transcripts of apomictic versus sexual *Boechera* show that members of the (*APETALA2*/ethylene-responsive element binding proteins) transcription factor family predominated equally in both up-regulated and down-regulated genes in apomictic seedlings. In addition, down-regulation of two important meiotic regulators *ASYNAPTIC 1 (ASY1)* and *MULTIPOLAR SPINDLE 1 (MPS1)* was accompanied by activation of stress-responsive genes particularly belonging to the *NAC-DOMAIN CONTAINING (NAC)* transcription factor. The author then postulated that the adaptive traits (response to drought) associated with the evolutionary history of apomicts co-adapted with meiotic gene deregulation at an early developmental stage. In other words, there is a correlation between upregulation of stress-responsive genes, and down regulation of meiotic genes and both are affected by epi-genetic changes (Shah *et al.*, 2016).

Referring to the Shah *et al*. (2016) study and based on yeast one-hybrid data, a scenario can be developed in which environmental signals lead to epigenetic changes in both *AP2* genes and putative meiotic regulators. The detection of *AP2* in cDNA libraries interacting with the APOLLO promoter may imply a switch in the metabolic cycle which causes a stress response in apomictic *Boechera*. However, direct connections between stress response and regulation of meiotic genes in apomicts have not been the subject of many studies. The stress-co-adapted and

highly heterozygous apomictic genomes, similarly to the genomes of polyploids (Chandra and Dubey, 2010; Syamaladevi *et al.*, 2016), generally, are considered to have the capacity of buffering mutation load in the absence of meiotic recombination (Lynch, 1984; Shah *et al.*, 2016). It also has been proposed that because the positive association between heterozygosity and fitness tends to be stronger under stressful conditions than under favorable ones, stress itself may promote the persistence of genetic variation (Hoffmann and Hercus, 2000). It can be inferred that apomictic *Boechera* due to its heterozygous genome and the lack of recombination has the capacity to adapt to environmental changes more rapidly (*B. divaricarpa's* genome heterozygosity rate is around 2.5% as estimated by GenomeScope; Brukhin *et al.*, 2019).

Rather than a response to stress, apomixis could act as a repressor of meiotic regulators. This could occur through DNA methylation or other mechanisms responsible for the disruption or inhibition of meiosis in megaspore mother cells (MMC) in early stages of seed development (Rojek *et al.*, 2018). It also has been proposed that, compared to sexuals, broadly adapted apomicts may more efficiently buffer mild abiotic stress. Therefore, cellular oxidative stress and the formation of ROS that result in DNA damage are limited, and there is thus no need for meiosis and recombination in the apomicts (Figure 3.5). This conclusion is based on a hypothesis in which repair of oxidative damage on nuclear DNA has been introduced as a major driving force in the evolution of meiosis (Hadany and Beker, 2003; Hörandl and Hadacek, 2013).

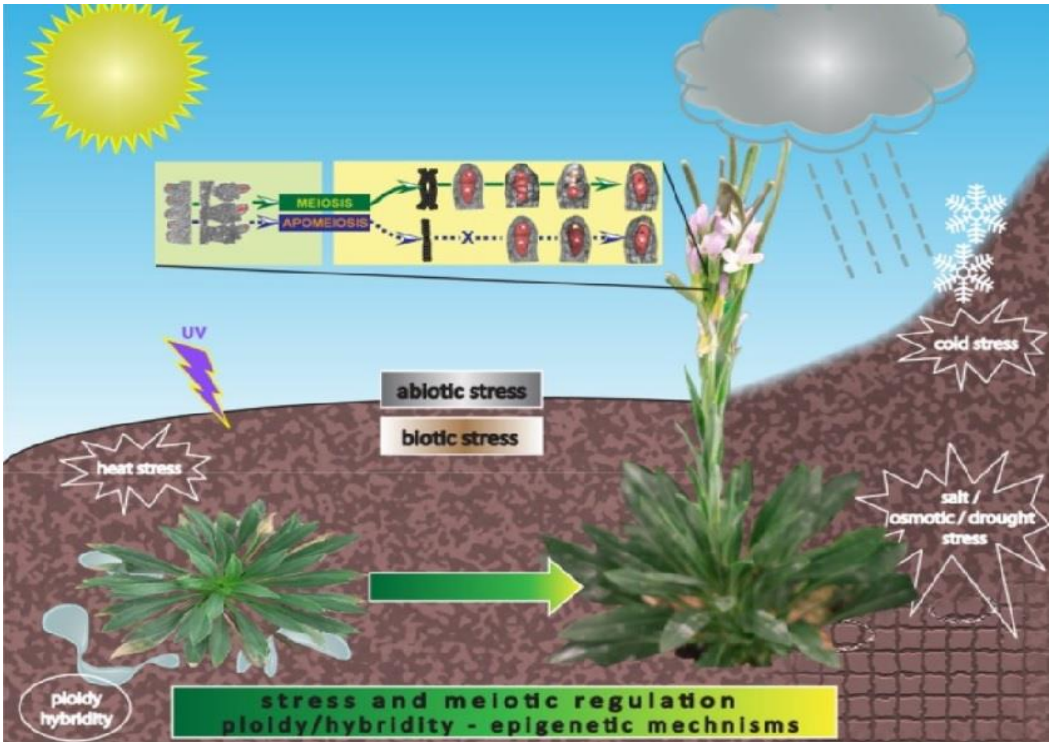


Figure 3.5 Apomictic *Boechera* could have evolved from sexual ancestors under harsh environmental conditions. Both genetic and epigenetic control of stress and apomeiotic regulation throughout plant development could be viewed as co-adapted gene responses during stress recovery acting as an efficient buffer to tolerate the mutation load in the absence of meiotic recombination (Image taken from Shah *et al.*, 2016).

Corral *et al*. (2013) predicted that the APOLLO gene is an exonuclease (DEDDh) that regulates apomeiosis by its catalytic activity (cleaver of transcripts). A scenario in which *AP2* transcription factors binding to the APOLLO Apo-allele lead to the increased level of its transcripts is plausible, as that could initiate the degradation of meiotic regulator transcripts through its catalytic activities. This model developed based on the Shah *et al*. (2016) study in which deregulation of meiotic regulators are accompanied by the activation of stress-responsive genes such as *NAC* and *AP2*. However, he assumed that meiotic regulator repression in apomictic *Boechera* is a consequence of the global DNA methylation which acts as epigenetic modifiers (Shah *et al*., 2016).

3.4.2. Analyzing technical aspects of the yeast one-hybrid assay

Despite structural differences between the Sex- versus Apo-allele motifs used for the two yeast one-hybrid experiments, they have one transcription factor (*ERF107*) in common (Tables 3.3 and 3.4), although with a higher interaction confidence for the Sex-allele promoter. The higher

interaction of confidence found for the TF in the sexual cDNA library may mean that competition exists between *ERF107* and *GeBP* for binding to the Sex-allele motif, leading to an antagonistic relationship on the level of their transcriptional activity and function. Alternatively, maintaining *AP2* as an interacting partner with the APOLLO gene may be important, however, it may not have triggered the same cascade of events in sexual versus apomictic *Boechera*. In line with this is the fact that the *AP2* transcription factors have highly degenerate Transcription Factor Binding Sites (TFBS) that trigger multiple responses in plants (Phukan *et al.*, 2017).

Comparing the TFs predicted in the previous study (Corral *et al.*, 2013) and those found here, discrepancies could be due to the unsophisticated motif mapping of PlantPAN[3] and similar tools which generate high numbers of false positive predictions (Kulkarni and Vandepoele, 2020). Because of the novelty of the protein-DNA interactions identified in the yeast one-hybrid assay, an electrophoretic mobility shift assay (EMSA) is suggested for their validation (Taylor-Teeples *et al.*, 2015). It is anticipated that this finding would pave the way to our understanding of regulation of the APOLLO gene.

The data gained from the yeast one-hybrid assay can also be assessed from a different perspective such as the Apo- vs. Sex-allele motifs used for "fishing" TFs. Firstly, the complexity of the bait sequences can be studied, and they can be "simple" or "complex" based on how many potential binding sites they have. A simple bait has few potential binding sites as it is generally less than 30 bp for a gene of interest and may be cloned as a single copy or a tandem repeat, while a complex bait has more sites as it is generally longer (generally an entire promoter) and normally one copy is screened (Reece-Hoyes and Marian Walhout, 2012). Thus, the Apo- and Sex-allele bait used in this study are simple motifs because they are less than 30 bp and are cloned as a single copy.

The reporter gene used for yeast one-hybrid experiment is HIS3, which allows the growth of yeast cells with positive interactions in a medium lacking HIS3. Adding a proper concentration

[3] The PlantPAN analysis navigator provide effective resources for detecting transcription factor binding sites (TFBS), corresponding TF, and other important regulatory elements (CpG islands and tandem repeats) in a promoter or a set of promoters in plants (Chow, Lee, *et al.*, 2018).The "cross-species" option of it make it suitable to study conservation of TFBS in promoters of homologous genes (Kulkarni and Vandepoele, 2020).

of 3 amino-1, 2, 4-triazole (3AT) prevents the leaky expression of HIS3 reporter gene. However, adding higher concentration may suppress true but weak interactions (false positive) becaue a higher expression level of HIS3 is required for growth (Dey et al., 2012). Knowing that 3AT stringency used in *B. divaricarpa* experiment was 50 mM higher than that of *B. stricta* (Table 3.2), it has a higher chance of creating false positive data.

When generating reporter constructs for the yeast one-hybrid assays the concern is that the junctions between the bait sequence and the vector sequence create new "artificial" potential binding sites. In screening simple baits, (especially in tandem where even more artificial sites occur between the repeats) this can be even more critical since the junctions create a much larger proportion of potential sites compared to larger baits. Thus, the tandem repeats used for the Sex-allele is more prone to create extra sites for fishing TFs and generating false-positive data due to their smaller sizes. To validate this data a mutated version can be interrogated in which only the junctions are maintained to identify protein-DNA interactions (PDIs) that occur at these artificial sites (Reece-Hoyes *et al.*, 2009). However, in order to validate if a protein or mixture of proteins can bind to the bait used here, or to determine if more than one protein is involved in the binding complex, a proper validation method such as Electrophoretic Mobility Shift Assay (EMSA) is proposed.

3.4.3. Analyzing APOLLO promoter polymorphism from an evolutionary view

The polymorphism of the APOLLO gene used as a motif in the yeast one-hybrid assay (i.e., 20 bp Apo-insertion/Sex-deletion) can be studied from an evolutionary view. Conservation of this Apo-specific polymorphism (Mau *et al.*, 2015) implies that it is under selective pressure to maintain function. It has been proposed that in species with larger population sizes, a positive correlation between rates of recombination and levels of polymorphism is expected as a result of natural selection (Corbett-Detig *et al.*, 2015). In addition, under neutrality, there is no relationship between levels of polymorphism and recombination because the frequency of neutral mutations is not influenced by recombination (Hudson, 1983), while in the presence of selection, levels of polymorphism are reduced by an amount proportional to the strength of selection and the recombination rate (Maynard and Haigh, 2007). Together, this means that in regions with a lower recombination rate, fewer polymorphisms would be observed (Corbett-Detig *et al.*, 2015). In contrast, it seems that in asexual populations with minimum recombination

rates, higher levels of polymorphisms can be explained by Muller's ratchet theory. Based on this theory, lack of recombination and variation in populations reduces the efficiency of selection against deleterious mutations, leading to an accumulation of mutations and a gradual increase in mutational load (Muller's ratchet) (Hojsgaard and Hörandl, 2015).

Corral *et al*. (2013) also suggested that the APOLLO Apo-allele is monophyletic, having arisen in a single ancestral population followed by its transition to different sexual backgrounds. Monophyly of the Apo-allele is furthermore strongly supported by the presence of the 20-bp 5′ UTR Apo-allele polymorphism in *A. thaliana* and *B. rapa*. (Corral *et al*., 2013). However, its presence does not mean that it has the same function as that in apomicitc *Boechera* since these plants are more sexual. It also points to the fact that the "Apo-allele" existed even before the appearance of apomictic *Boechera*, followed by its association with apomixis and spread into the different populations seen today. One explanation might be that the 20 bp polymorphism is composed of a series of point mutations that occurred one after another, or the whole 20 bp insertion is the result of one mutational event.

It has been proposed that in apomictic lineages, evolution occurs due to both genetic drift and natural selection (Brukhin *et al.*, 2019). Lovell *et al*. (2014) proposed that the strength of natural selection was different in sexual versus apomictic *B. spatifolia* populations (Lovell *et al.*, 2014). Another study on *B. stricta* using recombinant inbred lines subjected to lab and field experiments showed that flowering time is under directional selection (Anderson *et al*., 2011). These studies show that sexual populations are more prone to directional selection compared to asexual populations (Shah *et al.*, 2016). Corral *et al*. (2013) also stated that co-evolutionary balance between APOLLO and regulatory factors (through binding site homology) has been attained early in the evolutionary history of sexual *Boechera* spp., as "normal" sexual seed formation would be under strong selection pressure.

Whether the APOLLO promoter is under directional selection or genetic drift, it seems that it plays a role in the regulation of APOLLO by providing TF binding sites that are determined here in this study. Duveau *et al*. (2017) found that mutations in the promoter of a yeast metabolic gene often display environment-dependent effects on gene expression and that these effects have been influenced by selection in natural populations (Duveau *et al.*, 2017). Analyzing the binding profiles of TFs to DNA using a mathematical model, Hoffman (2010) demonstrated that over

evolutionary time, a clear excess of mutations with lower rate was fixed in promoters. This data was consistent with most changes being neutral, however, this is not consistent across all promoters, and some promoters have shown more rapid divergence (Hoffman and Birney, 2010). Interestingly, different classes of genes, as determined by Gene Ontology annotations show that the promoter of genes involved in developmental processes as well as those involved in phosphorylation and the cell cycle are more prone to mutations (Gene ontology Consortium, 2006).

Discussing the APOLLO polymorphism from an evolutionary point of view, the coevolution of its corresponding TFs cannot be excluded. Knowing that the 20 bp polymorphism introduced into *Boechera* spp. through hybridization between two closely related taxa lead to the spread of apomixis into different sexual populations (Corral *et al.*, 2013; Mau *et al.*, 2021), the interaction of the Apo-allele and trans-acting TFs led to the deregulation of APOLLO during MMC formation. Though the TFs detected in this experiment are mostly *AP2* with the known role of response to environmental signals, it seems that meiotic regulatory factors are also needed to affect MMC misregulation.

In conclusion, two main types of transcription factors have been identified in this study which may play a role in the induction of apomixis through their interactions with the 5'UTR of the APOLLO gene. The *APETALA2* family of TFs is up-regulated mostly in response to hormonal changes by binding to GC-rich motifs. Among this protein family, *ERF 5* and *ERF15* demonstrated a moderate interaction with the Apo-allele, while *ERF1B* shown a moderate interaction with the Sex-allele (Tables 3.3 and 3.4). The second prominent protein reported in this study is *GLABROUS1 ENHANCER-BINDING PROTEIN* (*GeBP*) with a demonstrated moderate interaction with the Sex-allele, thus suggesting its role in the regulation of sexual *Boechera* through *ROS* driven mechanisms. Regulation of oxidative stress machinery is proposed to be a part of a switch between meiotic reproduction, as a mechanism of DNA repair, versus apomeiosis in organisms such as *Boechera* spp. with a mixed (facultative) reproductive mode. These data are consistent with the previous studies in which high levels of *AP2* transcripts were reported in apomictic *Boechera*. This may mean that the role of *AP2* in buffering harsh environmental conditions in *B. divaricarpa* is correlated with its affinity to the APOLLO

promoter. However, more studies are needed to unravel the functional role of APOLLO in arresting meiosis and initiation of apomeiosis.

CHAPTER 4. FUNCTIONAL ANALYSIS OF APOLLO, USING THE 1 KB NATIVE PROMOTER REGION OF THE APOLLO APO- AND SEX-ALLELES AND FIVE DIFFERENT SYNTHETIC PROMOTER CONSTRUCTS.

4.1. Introduction

Promoters are the most crucial element in the process of transcription initiation and regulation in prokaryotes and eukaryotes. Promoters in general, and eukaryotic promoters specifically, have conserved sequences (core promoter) which provide binding sites for RNA polymerases (RNAP) and associated sigma factors (Wang *et al.*, 2018). Upstream of the core promoter region are the proximal and distal regions of promoters which contain different regulatory sequences such as enhancers, silencers, insulators and repressors (Juven-Gershon *et al.*, 2008). For a given gene, the untranslated regions, including the 5′ and 3′ untranslated regions (UTRs) and introns are the major regions involved in the regulation of expression. The core promoter expanded ~80 bp upstream of the transcription start site (TSS) contains elements such as the TATA box and CpG motifs, which play roles in transcription initiation (Barrett *et al.*, 2012). The physical lengths of the upstream genomic regions that contribute to a "full promoter" are phylogenetically less conserved, although the core promoter is typically in a fixed position relative to the TSS. Thus, the size of the active, fully functional promoter depends directly on the positions of the *cis*-acting elements and, their interactions present in both the proximal and distal regions (Hernandez-Garcia and Finer, 2014).

There are two main approaches for functional analysis of promoters, (1) adding an uninterrupted promoter sequence upstream of a reporter gene, or more importantly (2) using manipulated promoters (Hernandez-Garcia and Finer, 2014). In these systems a putative promoter region which is cloned upstream of a reporter gene is normally transformed into the host plant. Expression of the reporter is subsequently assessed by a fluorescent or colorimetric assay, and the most common of these reporter systems in plants is the β-glucuronidase (GUS) assay (Warnatz *et al.*, 2010). The GUS reporter system utilizes the uidA gene of *Escherichia coli*, which codes for the enzyme, β-glucuronidase (Blanco *et al.*, 1982). When incubated with specific colorless or non-fluorescent substrates, this enzyme can turn them into stable colored or fluorescent product. The presence of the GUS-induced color is indicative of the subcellular locality of an active gene expression (Jefferson *et al.*, 1986).

In order to understand the role of various regions of the promoter, 5'UTR deletion lines can be created, and expression of a downstream gene be assessed (Cho *et al.*, 2017). Other studies focus on synthetic promoters in which promoter fragments are fused with putative *cis*-elements to determine element functionality, and these can differ tremendously from native promoters because they can provide expression profiles that do not exist in nature (Rushton *et al.*, 2002; Hernandez-Garcia and Finer, 2014). In one study, the generation of variant promoters in a single cloning step was used to facilitate the simultaneous investigation of the entire promoter without any deletions (Remans *et al.*, 2005).

There is differential gene expression between the APOLLO Apo-allele vs. Sex-allele, and the Apo-allele shows expression in pre-meiotic ovules of apomictic *Boechera's* flowers (Corral *et al.*, 2013). In addition, there are structural differences between Apo- and Sex-alleles. For example, the 5'UTR of the Apo-allele is shorter than Sex-allele, with no further sequence upstream of the 20 bp apomixis specific polymorphism, as identified by Corral *et al.* (2013) using RACE data of six different *Boechera* genotypes.

It was hypothesized by Corral *et al.* (2013) that differential gene expression between apomictic and sexual individuals is the result of structural variations between the alleles, including the Apo-allele specific polymorphism and the differences in the length of their 5'UTR. The aim of this study was to understand gene function by analyzing the expression patterns and promoter elements for Apo- and Sex-alleles of the APOLLO gene. Thus, transgenic lines with five different synthetic promoters were made, each carrying different Apo- vs. Sex-allele fragment combinations to investigate the importance of the apomixis-specific polymorphisms and their upstream promoter elements.

In this study, first 1 kb and then 2 kb upstream sequences, which hypothetically contain the native APOLLO promoters from sexual and apomictic *Boechera,* were first cloned and their functions were characterized by detecting the expression level of the β-glucuronidase (GUS) gene in transgenic *Arabidopsis* and an apomictic *Boechera*. Then the role of different promoter components on APOLLO expression, including the 20 bp Apo-insertion, 5'UTR, and the upstream 2 kb region were studied. The reason for choosing these segments was due to structural variation between the Apo- vs. Sex-allele promoters (Figure 4.1). Thus, synthetic promoters were made to study the impact of the APOLLO promoter components on three levels including: **a.**

constructs with the 20 bp Apo-allele insertion vs. constructs with the Sex-allele deletion, **b.** constructs carrying 5'UTR of Apo-allele vs. 5'UTR of Sex-allele, **d.** constructs with 2 kb upstream region (i.e., promoter) of the Apo vs. Sex-alleles.

Synthetic promoter constructs were made using overlapping PCR, each carrying different Apo vs. Sex-allele components, to understand their function and unravel their combinatory effects. Compared to other techniques that add one or multiple *cis*-elements into a reporter construct, this method has the benefit of adding larger fragments of DNA from two different genetic resources in a desired order.

4.2. Material and methods

4.2.1. Plant Material for isolation of DNA sequences

The plant materials used for this experiment were apomictic *B. divaricarpa* (ES517) and sexual *B. stricta* (ES718). *Boechera stricta* is a sexual and predominantly self-fertilizing short-lived perennial (biennial) with the chromosome number of 2n=2X=14, which is native to the Rocky Mountains in North America (Lee and Mitchell-Olds, 2011). *Boechera divaricarpa* is found especially in California and Washington, and is an allodiploid apomictic biennial allodiploid containing genomes of *Boechera stricta* and *Boechera sparsiflora*, with a chromosome number of 2n=2X=14 (Windham and Al-Shehbaz, 2007).

The seeds of apomictic and sexual individuals were stratified in wet soil separately at 4 °C for 4 weeks as a vernalization treatment and then grown together in racks filled with Sunshine mix #8 under 16-h days and 20 °C ambient temperature (Lee and Mitchell-Olds, 2013) (Figure 4.1). For the GUS activity analysis of the allele-specific APOLLO transformants and promoter assays, leaf tissue of two-month-old *Boechera* and three-week-old *A. thaliana* plants were harvested, frozen with liquid nitrogen and kept at −80 °C for total DNA extraction.

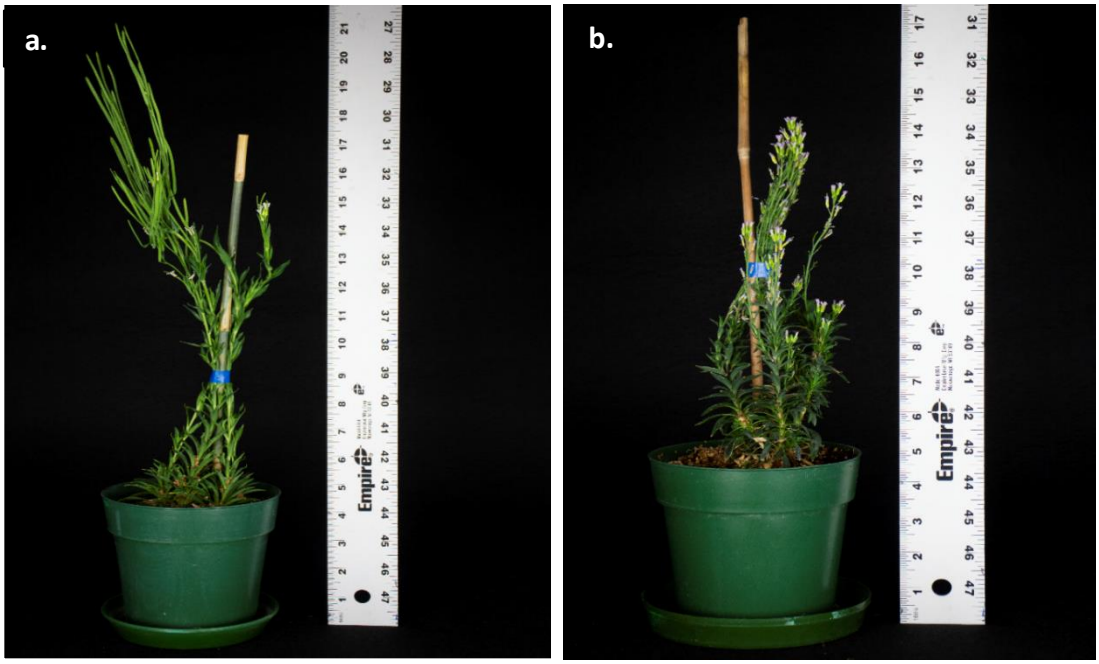


Figure 4.1 Photos of the sexual and apomictic *Boechera* accessions used in this study. a. *Boechera stricta*, **b.** *Boechera divaricarpa*.

4.2.2. APOLLO promoter constructs

In this study different APOLLO promoter constructs were made, including: 1 kb upstream of the TSS, 2 kb upstream of the TSS, and five different synthetic promoter constructs. The first part of this chapter describes the construction of the 1 kb APOLLO promoter construct and the second part includes the 2 kb APOLLO promoter construct and five different synthetic promoter constructs.

4.2.3. Plasmids used for cloning

Two different plasmids were used in this experiment including an entry vector (reporter gene construct) and an expression vector as follows.

PENTR/D-TOPO (http://tools.thermofisher.com/) was used as an entry vector. It is a bacterial plasmid in the form of dsDNA with the kanamycin resistance gene. This Gateway type vector (lambda att-type) is a recombinational cloning system entry (master) vector for TOPO cloning (Figure 4.2).

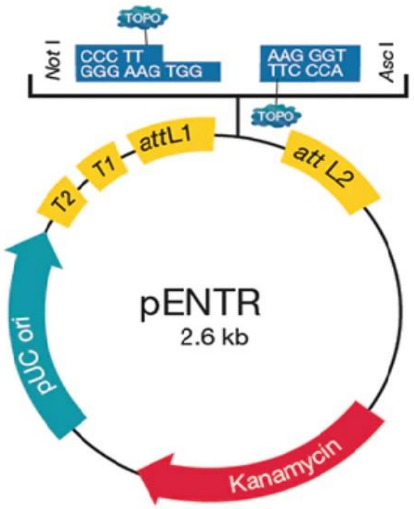


Figure 4.2 pENTR vector for directional TOPO cloning. It contains a Kanamycin resistance gene, PUC ori, T1 and T2 (Terminators), and an insertion site between flanking recombination sequences of attL1 and attL2.

PBGWFS7 was used as the expression vector for the promoter analysis study. In these vectors, a frame fusion between the regions coding for *EgfpER* and β-glucuronidase (GUS) were cloned downstream of the GATEWAY™ cassette. The vector's backbone has a spectinomycin resistance gene for bacterial selection and a BASTA gene as plant selectable marker (Karimi *et al.*, 2002)(Figure 4.3).

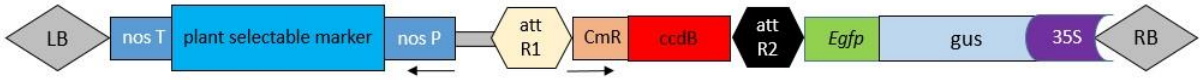


Figure 4.3 Promoter analysis vector (gfp-gus fusion) pBGWFS7. Abbreviations: LB-Left border of TDNA, RB- Right border of TDNA, nosT-nopaline synthase terminator, nos P-nopaline synthase promoter, GATEWAY cassette (attR1 with or without CmR,ccdB,R2 orientation) Egfp-green fluorescence promoter, 35S Cauliflower mosaic virus terminator, GFP cassette (Egfp,35S) (http://www.psb.ugent.be/gateway).

4.2.4. Primer design for TOPO cloning of the 1 kb and 2 kb upstream regions

Specific primers were designed based on BAC Apo- and Sex-allele libraries (Corral *et al.*, 2013) using the online Primer3Plus software (http://www.bioinformatics.nl/ cgi-bin/primer3plus/primer3plus.cgi). Then CACC ends were added to those primer sequences and ordered from Thermo fisher (https://www.thermofisher.com). These ends are complementary with the GGTG of the TOPO vector, enabling PCR product to be cloned directionally in the

TOPO vector. The primers used for amplification of the 1 kb promoter region of the APOLLO gene are shown in (Table 4.1).

Table 4.1 Primers used for the amplification of 1 Kb promoters of Apo and Sex-alleles. The TPS primer is used as reverse primer for both Apo- and Sex-alleles.

Genes	Targets	Primer name	Type	Size	Sequences (5'-3')
APOLLO	Sex promoter	Sex800-**CACC**	Forward	1 kb	5'-**CACC**TGACCCCGCAAGAATAAATC-3'
APOLLO	Apo promoter	Apo3-**CACC**	Forward	1 kb	5'-**CACC**AGGGGCACGTACCCTATACT-3'
APOLLO	Apo promoter	TPS	Reverse	1 kb	5'-GATAGCCCCAAACTCCAAAATCGC -3'

4.2.5. Preparation of DNA insert and plasmid vector for cloning

After harvesting, about 1.0 cm^2 of leaf sample was stored for one night at –80 °C, followed by a Geno-grinder (SPEX Sample Prep, 2010 Geno Grinder) at 3220 RPM for 30 seconds. Total DNA was extracted and purified from the leaf material using a DNeasy Plant Mini Kit (www.qiagen.com) as follows.

1. 1-400 µL Buffer Ap1 and 4 µL RNase were added to the leaf samples followed by vortexing and incubating for 10 min at 65 °C (Invert the tube 2-3 times during incubation).
2. 130 µL Buffer P3 was added, mixed and centrifuged for 5 min at (14,000 rpm).
3. The lysate was pipetted into a QIAshredder spin column placed in a 2 mL collection tube and centrifuged for 2 min at 14,000 rpm.
4. The flow-through was transferred into a new tube without disrupting the pellet followed by adding 1.5 volumes of Buffer AW1 and mixing by pipetting. 650 µL of the mixture was transferred into a DNeasy Mini spin column placed in a 2 mL collection tube and centrifuged for 1 min at ≥8000 rpm.
5. The spin column was placed into a new 2 mL collection tube and 500 µL Buffer AW2 was added, centrifuged for 1 min at ≥ 6000 x g, and finally the flow through was discarded.
6. Another 500 µL Buffer AW2 was added and centrifuged for 2 min at 20,000 g.
7. The spin column was transferred to a new 1.5 mL or 2 mL micro-centrifuge tube.
8. 100 µL Buffer AE was added for elution and incubated for 5 min at room temperature (15-25 °C) followed by centrifugation for 1 min at ≥ 6000 x g.

After DNA isolation the polymerase chain reaction (PCR) was carried out in a total volume (25 µL) containing <100 ng of DNA template, 1 µL each of the primers of (Table 4.1) at 5-7.5 pmol, and 12.5 µL Clone Amp HiFi PCR Premix. The standard PCR was carried out by denaturing the DNA at 98 °C for 1 min, followed by amplification for 30 cycles with secondary denaturation at 98 °C for 30 s, annealing at 55 °C for 5-15 s, and extension at 72 °C for 30-60 s, with a final extension at 72 °C for 5 min. The PCR product was analyzed by electrophoresis on a 1.0% agarose gel stained with SYBR safe (Invitrogen), and the gel image was documented. A 1000-bp size hyper ladder (https://www.fishersci.ca) was used as a marker to indicate the sizes of the amplicons. The fragments containing the 1 kb of promoter amplification product were then purified using a GeneJET Genomic DNA Purification Kit (Thermofisher scientific).

4.2.6. Ligation and transformation of bacterial strains with a recombinant plasmid

The gel-purified insert DNA was diluted to the appropriate concentration for ligation and a ligation reaction mix was set up using 100 ng of vector and an insert with a vector molar ratio of 0.5:1 to 2:1 as follows:

1. 0.5 to 4 µL of fresh PCR product
2. 1 µL of salt solution
3. Water to a final volume of 5 µL
4. 1 µL of TOPO vector

The ligation reaction was carried out at room temperature for 30 min and the reaction mix was used to transform *One Shot* chemically competent *E. coli* cells (Thermofisher scientific, Catalog number C404003). The ligation mix then was used to transform *E. coli* $DH5_{\alpha}$ cells, and once a positive clone was confirmed, the recombinant plasmid was used to transform *Agrobacterium tumefaciens* cells for plant transformation by electroporation procedure as follows.

4.2.7. Transformation of *E. coli* $DH5_{\alpha}$

Frozen cells from -80 °C were thawed on ice for 10 min. The full volume (2 µL) of each ligation mixture was added to individual tubes and each tube was mixed briefly and placed on ice for 30 min. The mixture was heat shocked in a 42 °C thermal block for 30 seconds and transferred back to ice immediately. 250 µL of SOC media was added to each tube and the mixture was incubated at 37 °C for 1 h. The mixture was spun down at 14,000 rpm in a microcentrifuge for 10 min and

the cell pellet was resuspended in 100 µL LB medium. Cells were plated onto solid LB plates with 50 mg/l kanamycin and placed at 37 °C overnight.

4.2.8. Screening for positively transformed bacteria.

Positive colonies were selected by picking individual colonies with adequate growth. Single colonies were picked and used to inoculate 10 mL of LB containing 50 mg/l kanamycin and the cultures were incubated overnight with shaking at 37 °C.

LB (Luria-Bertani) liquid medium recipe

H_2O	950 mL
Tryptone	10 g
NaCl	10 g
Yeast extract	5 g

Reagents were combined and shaken until the solutes dissolved and pH was adjusted to 7.0 with 5N NaOH (~0.2mL). The final volume of the solution was adjusted to 1.0 L with H_2O followed by autoclave sterilization for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.

4.2.9. Small scale plasmid DNA miniprep for restriction fragment analysis and DNA sequencing

The Wizard *Plus* SV Minipreps DNA Purification System (www.Promega.com) provided a simple and rapid isolation of plasmid DNA. The miniprep method followed the method recommended by the manufacturer with some modifications for handling large plasmids and used the proprietary solutions provided by the manufacturer. Ten mL of 16-h (LB) culture was centrifuged at 3,000 rpm for 10 min and the pelleted bacterial cells were resuspended in 250 µL of the cell resuspension solution. The cell suspension was lysed with 250 µL of cell lysis buffer and allowed to stand at room temperature for 5 min followed by adding 10 µL of alkaline protease. The solution was neutralized with 350 µL of neutralization solution and centrifuged for 10 min at 14,000 rpm in a microcentrifuge at 4 °C. The supernatant was applied to a QIAprep® spin column, centrifuged for 1 min and the flow through was discarded. The column was washed with 750 µL of PB buffer and centrifuged for 1 min. The column was washed again with 250 µL of buffer PE and centrifuged for 2 min. The flow through was discarded and the column was dried by further centrifugation for 1 min. The plasmid DNA then was eluted with 50 µL of

nuclease free water applied to the column, followed by a brief 1 min centrifugation. The purified plasmid DNA was quantified with a spectrophotometer and used for plasmid restriction digest screening and DNA sequencing.

4.2.10. Screening of plasmid DNA for recombinant DNA using restriction enzymes

Positive plasmids were screened by restriction enzyme digest of the plasmid in order to verify the presence of the recombinant DNA insert for both APOLLO Apo- and Sex-alleles. 1 µL of plasmid DNA was digested in a total volume of 20 µL with 1 µL of each restriction enzyme flanking the insert and 2 µL restriction enzyme buffer. The reaction was carried out for 15 min at 37 °C, then stopped by heating for 30 min at 65 °C. The digested DNA was separated on a 0.8% agarose gel to confirm the presence of the inserted DNA with the correct molecular weight. The plasmid DNA was also sequenced to confirm the insert DNA as well as the regions flanking the insert. Another method for screening the plasmids used in this study was applying appropriate primers targeting the specific areas of inserted DNA (M13 forward & TSP reverse). Positive *E. coli* clones confirmed by restriction digest and DNA sequencing were selected for transformation into *Agrobacterium tumefaciens*. Using the Geneious software (https://www.geneious.com, Geneious version 10.2.6) it was predicted that the PvuII and HindIII enzymes would generate three DNA fragments (560 bp, 882 bp, and 1,942 bp) from the 1 kb APOLLO Apo promoter.

4.2.11. Screening of plasmid DNA for recombinant DNA by using PCR

PCR was performed using the M13-F and TSP-R primers on the pENTR/D-TOPO vector as template with an Eppendorf Mastercycler pro gradient thermal cycler, using 1 µL each of the primers at 20 pmol, 9.5 µL H_2O, and 12.5 µL Promega Go Taq DNA polymerase (https://www.fishersci.ca). Standard PCR was carried out by denaturing the DNA at 95 °C for 10 min, followed by amplification for 30 cycles with denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1.5 min, with a final extension at 72 °C for 10 min.

4.2.12. Sequence determination and assembly of PCR fragments for sequence alignment

The gel-purified PCR product was diluted to 100 ng/µl in sterile distilled water and sent for sequencing along with sequencing primers diluted to 5 pmole/µl, The DNA was sequenced by

the Sanger sequencing lab (NRC building, Saskatoon) and the results were further analyzed using the Geneious software (Geneious 10.2.6).

4.2.13. Gateway recombination cloning

The Gateway system (www.ThermoFisher.com) reactions were applied to take advantage of its capacity of the site-specific recombination reactions in which the bacteriophage λ can both integrate into and excise itself out of a bacterial chromosome (Hartley *et al.*, 2000). This recombination reaction between flanking recombination sequences (*attL* and *attR* sites) is called the LR reaction and generates an expression clone. To conduct the LR reaction, an LR Clonase II enzyme mix was used with the following components.

1. 1-7 µL entry clone (50-150 ng)
2. 1 µL destination vector or pBGWFS7 (150 ng/µl)
3. TE buffer (pH-8.0), to 8 µL

About 2 µL of LR Clonase II enzyme mix was added to the above reaction, mixed and incubated at 25 °C for 1 h. Then 1 µL of Proteinase K solution was added to each sample to terminate the reaction, and samples were incubated for 10 min at 37 °C. Finally, aliquots (2 µL) of each reaction were transformed into *Agrobacterium* GV3010 competent cells by electroporation, then placed on LB medium containing 50 µg /ml of each (Rifampicin, Spectinomycin) and plates incubated at 29 °C overnight.

4.2.14. Screening of plasmid DNA for recombinant DNA using primers.

PCR was performed using the Lara5-F (5'-CCTCATCGTATCGTTGCGTCTCTC-3') and GFP-R primers (5'-CGACCACTACCAGCAGAACAC-3') and pBGWFS7 plasmid as template on an Eppendorf Mastercycler pro gradient thermal cycler using 1 µL each of the primers at 20 pmol, 9.5 µL H_2O, 12.5 µL Go Taq DNA polymerase (www.Promega.ca). Standard PCR was carried out by denaturing the DNA at 95 °C for 10 min, followed by amplification for 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1.5 min, with a final extension at 72 °C for 10 min.

4.2.15. Screening for positive transformed bacteria using digestion enzymes

Putative pApo: pBGWFS7 and pSex: pBGWFS7 lines were first digested with PvuIII enzyme to confirm the presence of both Apo- and Sex promoter constructs. 1 µL of plasmid DNA was

digested in a total volume of 20 µL, with 1 µL of each restriction enzymes flanking the insert and 2 µL restriction enzyme buffer. The reaction was carried out for 15 min at 37 °C, then stopped by heating for 30 min at 65 °C. The digested DNA was separated on a 0.8% agarose gel to confirm the presence of the inserted DNA with the correct molecular weight.

4.2.16. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*

4.2.16.1. Growth of plants for transformation

For functional analysis of the 1 kb and 2 kb promoters and making transgenic lines of five different synthetic promoter constructs, *A. thaliana* (Col-0) was used. Their seeds were stratified at 4 °C for 48 h and then transferred to pots containing Sunshine mix 8 and grown in the phytotron (National Research Council. Saskatoon, SK, Canada). The plants were grown under 16 h days and 20 °C ambient temperature, and the 8-week-old seedlings were then used to make transgenic lines.

4.2.16.2. Inoculation medium with *Agrobacterium*

The *Agrobacterium* strain GV3010, which harbored the different expression vectors, was grown overnight in 30 mL of LB medium with 50 µg/ml rifampicin and spectinomycin, and then added to 300 mL of fresh medium with the same antibiotics and grown to the stationary phase (OD600 - 2.0). Cells were harvested by centrifuging at 5500g for 20 min, and the pellet was re-suspended in infiltration medium (0.5x MS, 5% sucrose, 0.05% Silwett L-77) and measured for the desired density (OD600 of 0.8 or >2.0). Cells were inoculated by drop inoculation onto every flower, then covered with black plastic bags and placed in darkness for 24 h (Clough and Bent, 1998). Seeds were collected when all siliques were mature and dry.

4.2.17. A rapid protocol for selection of transformed plants

Seeds produced by inoculated plants were surface sterilized by immersion in 70% (v/v) ethanol for 5 min, followed by immersion in a 10% (v/v) sodium hypochlorite solution (Fisher Scientific) for 10 min. Seeds were then washed four times with sterile distilled water and sown on a medium containing 0.5× MS Basal Salt Mixture, 1% (w/v) sucrose, 0.8% and 1.2% (w/v) agar supplemented with 50 µg/ml BASTA (glufosinate ammonium). Plated seeds were incubated in the dark for 48 h at 4 °C for stratification and transferred to a growth chamber (National Research Council, Saskatoon, SK, Canada) for 4-6 h at 22 °C in continuous white light (120

μmol m^{-2} s^{1}) in order to stimulate germination. The plates were then wrapped in aluminum foil and incubated for 2 d at 22 °C. After removing the foil, seedlings were incubated for 48 h at 22 °C in white light (120 μmol m^{-2} s^{-1}). Two-week-old seedlings then were transferred to pots filled with Sunshine mix 8, and once they started flowering were used for the GUS activity assay.

4.2.18. Histochemical GUS assay

Transgenic *Arabidopsis* plants containing the clones were analyzed for expression of the GUS gene. Plant tissues such as leaves, flowers and stems were collected in order to determine the level and tissue specificity of GUS. In addition, changes in temporal expression of the GUS gene were determined by observing GUS expression during different developmental stages in transgenic plants.

4.2.19. Histochemical GUS assay of plant tissues and clearing of tissues.

Histochemical GUS staining was performed following the protocol of Jefferson (1987) that was optimized by Dr. Hong Wang at the University of Saskatchewan. Mature *Arabidopsis* flowers were divided into 12 developmental stages defined by landmark events based on examining the shape, size, and surface features of developing floral organs, and in addition the duration of each stage was also estimated (Smyth *et al*., 1990). A similar method for dividing *Boechera* ovules into different developmental stages was developed by Rojek *et al.* (2018).

Plant tissues such as leaves, flowers and stems were harvested from transformants growing for 32 days in soil mix, and fixed in cold 90% acetone for 20 min. Tissues were washed with 50 mM sodium phosphate (pH 7.0) and then incubated in a solution containing GUS staining buffer [0.2 M Disodium phosphate, 0.2 M Monosodium phosphate, 0.1 M potassium ferrocyanide, 0.1 M potassium ferricyanide,0.5 M EDTA disodium salt (pH 8.0), H2o], X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) solution [20 mg of X-Gluc dissolved in 1 mL of dimethyl sulfoxide (DMSO)] and 0.1% Triton X-100. After a vacuum infiltration treatment of 15 min to facilitate the penetration of the dying solution, the immersed tissues were incubated in 37 °C for 48 h.

Tissues were cleared following the protocol of Malamy and Benfey (1997). For observation of whole mounts, samples were transferred to small petri dishes containing solution 1 (0.24 N HCl in 20% methanol) and incubated on a 57 °C heat block for 15 min. This was poured off and

solution 2 (7% NaOH, 7% hydroxylamine-HCl in 60% ethanol) was added for 15 min at room temperature, followed by rehydration for 5 min each in 40%, 20% and 10% ethanol, and infiltration for 15 min in 5% ethanol, 25% glycerol. Samples were mounted in 50% glycerol on glass microscope slides. Three plants were used in each treatment and untreated *A. thaliana* var. Columbia was used as a control. Promoter activity in the leaves of *A. thaliana* was measured at noon by a GUS fluorometric assay at two- and five-days post-treatment.

4.2.20. Construction of five different synthetic promoter constructs using the 2 kb native promoter

The GUS activity of the 2 kb native (Apo- and Sex-) promoter region and five different synthetic promoter constructs were then analyzed to test different Apo- versus Sex-specific promoter components. These five different spliced promoters were prepared using a modified protocol based upon the "gene Splicing by Overlap Extension" method (SOE) (Horton *et al.*, 1989). This method allows segments from two different genes to be recombined or "spliced" together by overlap extension in which the copy of a DNA strand developed by the first PCR reaction can then serve as a template for an extension from a second primer in the opposite orientation (Figure 4.4). This method enabled the production of different constructs which contained different components of the Apo- or the Sex-allele (Table 4.2) and (Figures 4.6 and 4.7).

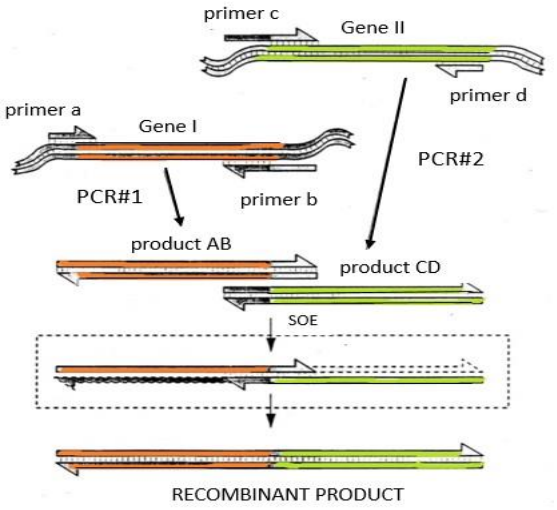


Figure 4.4 An illustration of the concept of SOE (gene Splicing by Overlap Extension). PCR#1 amplifies Gene I fragment AB, and PCR #2 amplifies Gene II into the fragment CD. Primers **b** and

c are the SOE primers, and they are used to modify the end of the two PCR products so that they have the same sequence. When these PCR products are mixed, denatured and reannealed under PCR conditions, the top strand of AB and the bottom strand of CD overlap and act as primers on one another, leading to the formation of a recombinant product (as shown inside the broken rectangle). Inclusion of the outside primers **a**, **d** in the SOE reaction causes the recombinant product to be PCR amplified right after it is formed (Image taken from Horton *et al.*, 2013).

Table 4.2 Native promoter (2 kb) and synthetic promoter constructs. Two native (pC1 and pC2) and five synthetic promoter constructs were created to test the importance of different apomictic- versus sex-specific polymorphism identified in *Boechera* (all have the same length of ~2 kb).

Construct name	Promoter type core	20 bp Apo-insertion	5' UTR	Transgenic lines
pC1	Apo promoter	**+**	Apo	*Arabidopsis/Boechera*
pC2	Sex promoter	**-**	Sex	*Arabidopsis/Boechera*
pC3	Apo promoter	**-**	Apo	*Arabidopsis*
pC4	Sex promoter	**+**	Sex	*Arabidopsis*
pC5	Apo promoter	Random insertion	Apo	*Arabidopsis*
pC6	Apo promoter	**-**	Sex	*Arabidopsis*
pC7	Sex promoter	**+**	Apo	*Arabidopsis*

4.2.21. Construction of 2 kb Apo and Sex native promoters and five different synthetic promoter constructs

Apollo promoter variants were cloned by overlap PCR (CloneAmp proofreading enzyme) by using two rounds of PCR. In PCR round one (PCR1), promoter fragments were amplified from apomictic (ES517) or sexual (ES718) *Boechera* genomic DNA. For making constructs pC1 and pC2 (apomictic and sexual, respectively), the entire 2 kb promoter regions, including the native 5'UTRs, were amplified without alteration (Figures 4.5 and 4.6) (Table 4.3). Primers for these PCRs did not include overhangs, aside from the CACC tag used for TOPO directional cloning as shown in (Table 4.5).

Figure 4.5 Construct pC1. Primer pairs of pAPOLLO AF -2 kb and pAPOLLO 1R were used to amplify 1,945 bp of DNA insert containing 71 bp 5'UTR of Apo promoter with 20 bp Apo-insertion (yellow).

Figure 4.6 Construct pC2. Primer pairs of pAPOLLO SF -2 kb and pAPOLLO 1R were used to amplify 1,970 bp DNA insert containing 104 bp 5'UTR of Sex promoter (gray).

For pC3, pC4, and pC5 the 2 kb promoter region up to, but not including, the 20 bp Apo-insertion site contained within the 5'UTR, was spliced with an alternate version of the 5'UTR and/or Apo-insertion. To do this, the two spliced pieces of DNA were amplified separately (CloneAmp proofreading enzyme) with complementary overhangs incorporated into the internal primers (Table 4.3). For pC3, primer overhangs were designed to "skip" amplification of the Apo-specific insertion, removing it from the final sequence. For pC4, primer overhangs incorporated the Apo-specific insertion as the overlapping region so that it would be incorporated into the final sequence. For pC5, primer overhangs were designed to incorporate a randomized insertion into the final promoter sequence (Figures 4.7 to 4.9).

The randomized insertion was created to check if any changes in the 20 bp Apo-insertion sequences order create a new GUS activity pattern. In order to make a randomized sequence the first step was to design the random 20 bp insertion using the following website (https://www.bioinformatics.org/sms2/shuffle_dna.html). Firstly, the 20 bp apomixis-specific polymorphism was entered as the raw sequence. Secondly, the randomized sequence was used to design the overlap primer the same was used for other constructs.

For pC6 and pC7, the 2 kb promoter region up to, but not including, the 5'UTR, was spliced with the alternate 5'UTR (Table 4.4). As above, the two fragments were amplified independently with complementary overhangs incorporated into the primers. Beyond this splicing, no additional mutations or deletions were made (Figures 4.10 and 11)

Table 4.3 Primer pairs and templates used for the first round of overlap PCR. Native constructs pC1 and pC2 were amplified using one set of primers, while two sets of internal and overhang primers were used for constructs pC3 to pC7. Constructs pC1 and pC2 only have internal primers.

PCR1						
Construct	**F primer**	**R primer**	**Template**	**F primer**	**R primer**	**Template**
pC1	pAPOLLO AF -2 kb	pAPOLLO 1R	ES517 (Apo)			
pC2	pAPOLLO SF -2 kb	pAPOLLO 1R	ES718 (Sex)			
	Internal	**Overhang**		**Internal**	**Overhang**	
pC3	pAPOLLO AF -2 kb	pAPOLLOa -64R +oh3	ES517 (Apo)	pAPOLLOa -44F +oh3	pAPOLLO 1R	ES517 (Apo)
pC4	pAPOLLO SF -2 kb	pAPOLLOs -58R +oh4	ES718 (Sex)	pAPOLLOs -58F +oh4	pAPOLLO 1R	ES718 (Sex)
pC5	pAPOLLO AF -2 kb	pAPOLLOa -64R +oh5	ES517 (Apo)	pAPOLLOa -44F +oh5	pAPOLLO 1R	ES517 (Apo)
pC6	pAPOLLO AF -2 kb	pAPOLLOa -71R +oh6(s)	ES517 (Apo)	pAPOLLOs -104F +oh6(a)	pAPOLLO 1R	ES718 (Sex)
pC7	pAPOLLO SF -2 kb	pAPOLLOs -104R +oh7(a)	ES718 (Sex)	pAPOLLOa -71F +oh7(s)	pAPOLLO 1R	ES517 (Apo)

In order to conduct the second round of PCR (PCR2), an approximately equimolar ratio of a pair of purified PCR products from the first round of PCR (PCR1) was created with the same forward and reverse primers used to create pC1 and pC2 to amplify the fully-spliced sequences.

Table 4.4 Primer pairs and templates used for (PCR2) of overlap PCR.

PCR2			
	F primer	**R primer**	**Template**
pC3	pAPOLLO AF -2 kb	pAPOLLO 1R	PCR1 3a+3b
pC4	pAPOLLO SF -2 kb	pAPOLLO 1R	PCR1 4a+4b
pC5	pAPOLLO AF -2 kb	pAPOLLO 1R	PCR1 5a+5b
pC6	pAPOLLO AF -2 kb	pAPOLLO 1R	PCR1 6a+6b
pC7	pAPOLLO SF -2 kb	pAPOLLO 1R	PCR1 7a+7b

Table 4.5 Primer sequences used for overlap PCR. The CACC overhang of the first two primers and the overhangs of the other primers are highlighted in bold.

Primer Name	Primer Sequence
pAPOLLO Apo F -2 kb	**CACC**ACGAGGGATCCGGACAAAGATAC
pAPOLLO Sex F -2 kb	**CACC**CTCTGTTTCTTTCGTCCCGGTATTT
pAPOLLO 1R	TGTTAAGAACTGAGAGTGAAGGAG
pAPOLLOa -44F +oh3	**TTTTTTCCGTAAAAAGAGGAGG**CTTTAAAACCCACCAATTAGC
pAPOLLOa -58F +oh4	**TGGCCCGTGAAGTTTATTCC**ATCGATTGCTTTAAAACCCACC
pAPOLLOa -44F +oh5	**AAGTCTATAGCACGTGCATC**CTTTAAAACCCACCAATTAGC
pAPOLLOa -64R +oh3	**GCTAATTGGTGGGTTTTAAAG**CCTCCTCTTTTTACGGAAAAAA
pAPOLLO -58R +oh4	**GGAATAAACTTCACGGGCCA**CCTCCTCTTTTTACGGAAA
pAPOLLOa -64R +oh5	**GATGCACGTGCTATAGACTT**CCTCCTCTTTTTACGGAAAAAA
pAPOLLOa -104F +oh6	**TTTAGATTTTTTTCCGTAAAAA**TCGTACCGTTGCTTCTCTCAAG
pAPOLLOa -71R +oh6	**GAGAGAAGCAACGGTACGATTT**TTACGGAAAAAAATCTAAACTTG
pAPOLLOa -71F +oh7	**ATGACGCAAGATAAACCTCA**GAGGAGGTGGCCCGTGAAGTT
pAPOLLOa -104R +oh7	**AACTTCACGGGCCACCTCCTC**CTTGAGAGAAGCAACGGTACGA

In order to make construct pC3, pAPOLLO AF -2 kb (internal) and pAPOLLOa -64R +oh3 (overhang) were used as primer pairs and ES517 was used as DNA sample to amplify the 2 kb upstream of the Apo promoter up to the 20 bp Apo-insertion/Sex-deletion site. Secondly, pAPOLLOa -44F +oh3 (overhang) and pAPOLLO 1R (internal) were used as primer pairs and ES 517 as DNA sample to amplify 44 bp of the 5'UTR of Apo-allele promoter by removing the 20 bp Apo-insertion. Finally, to amplify the full spliced sequences an approximately equimolar ratio of a pair of purified PCR products from the first round one of PCR (PCR1) used as DNA samples for the second round of PCR (PCR2) with primer pairs of pAPOLLO AF -2 kb and pAPOLLO 1R (Figure 4.7).

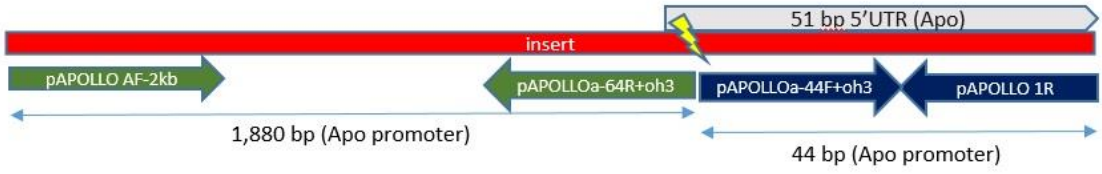


Figure 4.7 Construct pC3. 2 kb DNA insert created using SOE; Primer pairs used to amplify 1,880 bp of Apo promoter are shown in green, and primer pairs used to amplify 44 bp of 5'UTR (gray) by deleting the 20 bp Apo-insertion (yellow) are shown in dark-blue.

In order to make construct pC4 primer pairs of pAPOLLO SF-2 kb (internal) and pAPOLLOs -58R +oh4 (overhang) and DNA of ES718 (Sex) were used to amplify the 2 kb upstream of Sex promoter by incorporating the 20 bp Apo-insertion. Secondly, the overhang primer pairs of pAPOLLOa -58F +oh4 (overhang) and pAPOLLO 1R (internal) and DNA of ES718 were used to amplify 52 bp of the 5'UTR of Sex promoter by incorporating the 20 bp Apo-insertion. Finally, to amplify the full spliced sequences an approximately equimolar ratio of a pair of purified PCR products from the first round one of PCR (PCR1) were used as DNA samples for the second round of PCR (PCR2) with primer pairs of pAPOLLO SF -2 kb and pAPOLLO 1R. In fact, the 20 bp Apo- insertion incorporated into the products of PCR1 (i.e., 4a & 4b) in Table (4.4) were used as recombination sites for the PCR2 and eventually became part of the final product (Figure 4.8).

Figure 4.8 Construct pC4. 2 kb insert created using SOE; Primer pairs used to amplify 1,917 bp of Sex promoter, are shown in green, and primer pairs used to amplify 52 bp of Sex promoter by incorporating the 20 bp Apo-insertion (yellow) in dark-blue; The 5'UTR of Sex promoter is in gray.

In order to make construct pC5, primer pairs of pAPOLLO AF -2 kb (internal) and pAPOLLOa -64R +oh5 (overhang) and DNA of ES517 (Apo) were used to amplify the 1,917 upstream of the Apo promoter by incorporating a random 20 bp insertion. Secondly, the primer pairs of pAPOLLOa -44F +oh5 (overhang) and pAPOLLO 1R (internal) and DNA of ES517 (Apo) were used to amplify 52 bp of the 5'UTR of Apo promoter by incorporating a random 20 bp insertion. Finally, to amplify the full spliced sequences an approximately equimolar ratio of a pair of purified PCR products from the first round one of PCR (PCR1) were used as DNA samples for the second round of PCR (PCR2) with primer pairs of pAPOLLO AF -2 kb and pAPOLLO 1R. In fact, the 20 bp random insertion incorporated into the products of PCR1 (i.e., 5a & 5b) in Table (4.4) were used as recombination sites for the PCR2 and eventually became part of the final product (Figure 4.9).

Figure 4.9 Construct pC5. 2 kb DNA insert created using SOE; Primer pairs used to amplify 1,880 bp of the Apo promoter are shown in green, and primer pairs used to amplify 44 bp of Apo promoter by incorporating a random 20 bp insertion (yellow) in dark-blue; the 5'UTR of the Apo promoter is in gray.

In order to make construct pC6, pAPOLLO AF -2 kb (internal) and pAPOLLOa -71R +oh6 (overhang) were used as primer pairs and ES517 (Apo) as DNA sample to amplify the 1,870 bp upstream of the Apo promoter up to, but not including the 20 bp Apo-insertion/Sex-deletion. Secondly, primer pairs of pAPOLLOa -104F +oh6 (overhang) and pAPOLLO 1R (internal), and DNA sample of ES718 (Sex) were used to amplify 104 bp of the 5'UTR of Sex promoter. Finally, to amplify the full spliced sequences an approximately equimolar ratio of a pair of purified PCR products from the first round one of PCR (PCR1) were used as DNA samples for the second round of PCR (PCR2) with primer pairs of pAPOLLO AF -2 kb and pAPOLLO 1R (Figure 4.10).

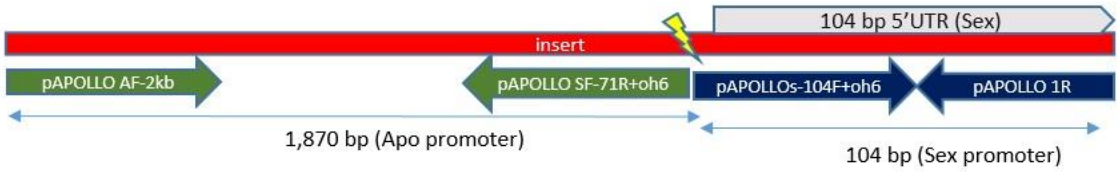


Figure 4.10 Construct pC6. 2 kb DNA insert created using SOE; Primer pairs used to amplify 1,870 bp of the Apo-promoter are shown in green, and primer pairs were used to amplify 104 bp of the Sex promoter in dark-blue; the 5' UTR of the Sex promoter is in gray.

In order to make construct pC7, primer pairs of pAPOLLO SF -2 kb (internal) and pAPOLLOa -104R +oh7 (overhang) and sample of ES718 (Sex) were used to amplify the 1,865 bp upstream of the Sex promoter up to, but not including the 5'UTR. Secondly, primer pairs of pAPOLLOa -71F +oh7 (overhang) and pAPOLLO 1R (internal), and DNA sample of ES518 (Apo) were used to amplify 71 bp of the 5'UTR of the Apo promoter. Finally, to amplify the full spliced sequences an approximately equimolar ratio of a pair of purified PCR products from the first round one of PCR (PCR1) used DNA samples for the second round of PCR (PCR2) with primer pairs of pAPOLLO SF -2 kb and pAPOLLO 1R (Figure 4.11).

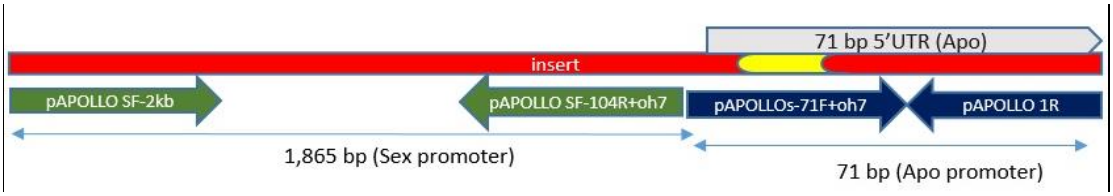


Figure 4.11 Construct pC7. 2 kb DNA insert created using SOE; Primer pairs used to amplify 1,865 bp of the Sex promoter are shown in green, and primer pairs used to amplify 71 bp 5'UTR of the Apo promoter carrying he Apo-insertion (yellow) is in dark-blue; The 5'UTR of the Apo promoter is in gray color.

4.2.22. Transgenic lines were made using 2 kb native Apo and Sex promoters and five synthetic promoter constructs

The pAPOLLO Apo -2 kb and pAPOLLO Sex -2 kb forward primers included a CACC tag for subsequent cloning of all promoter variants into pENTR/D-TOPO (www.thermofisher.com). All promoter constructs were verified by sequencing and sub-cloned into the pBGWFS7 reporter vector by the LR reaction as described before. *Agrobacterium* strains GV3103 and MP90 were transformed by electroporation, followed by transforming into *Arabidopsis* and apomictic *Boechera* by a floral dip method. To make stable transgenic lines for *Boechera*, tissue culture methods were developed in the Sharbel lab using an apomictic accession (JL107). It takes at least two months for successful tissue culture and transformation from the beginning to end as described in (Table 4.6).

Table 4.6 The timeline for successful *Boechera* tissue culture.

Week	Action
0.5	Stratify seeds on plants
1	Germination in light
2	Excision, pre-culture
3	Transformation, co-cultivation
3	End of co-cultivation, selection
5	Transfer to new plates
6	Secondary transfers (every two weeks)
8	Rooting (when shoot is ready)

4.2.23. Histochemical staining of β-Glucuronidase activity and its spatial quantification

The same method of GUS staining was used successfully for both *Arabidopsis* and *Boechera* samples as follows. Thirty-two-day-old *Arabidopsis* flowers were divided into 12 developmental

stages defined by landmark events based on examining the shape, size, and surface features of developing floral organs, and in addition the duration of each stage was also estimated (Smyth *et al.*, 1990). A similar method was developed for dividing *Boechera* ovules into different developmental stages by Rojek *et al.* (2018).

Harvested transformants grown for 32 days were fixed in cold 90% acetone for 20 min (to standardize sampling conditions tissue collection was conducted at noon for all samples). Tissues were washed with 50 mM sodium phosphate (pH 7.0) and then incubated in a solution containing GUS staining buffer [0.2 M Disodium phosphate, 0.2 M Monosodium phosphate, 0.1 M potassium ferrocyanide, 0.1 M potassium ferricyanide,0.5 M EDTA disodium salt (pH 8.0), H_2O], X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) solution [20 mg of X-Gluc dissolved in 1 mL of dimethyl sulfoxide (DMSO)] and 0.1% Triton X-100. After a vacuum infiltration treatment of 15 min to facilitate the penetration of the dying solution, the immersed tissues were incubated at 37 °C for 48 h.

For clearing tissue, the protocol of Malamy and Benfey (1997) was used. For observation of whole mounts, samples were transferred to small petri dishes containing solution 1 (0.24 N HCl in 20% methanol) and incubated on a 57 °C heat block for 15 min. This was poured off and solution 2 (7% NaOH, 7% hydroxylamine-HCl in 60% ethanol) was added for 15 min at room temperature, followed by rehydration for 5 min each in 40%, 20% and 10% ethanol, and infiltration for 15 min in 5% ethanol 25% glycerol. Samples were mounted in 50% glycerol on glass microscope slides. Three plants were used in each treatment. Untreated *A. thaliana* var. Columbia was used as a control. Promoter activity in the leaves of *A. thaliana* was measured by a GUS fluorometric assay at two- and five-days post-treatment in a minimum of three flowers per developmental stage. The same procedure was used for *Boechera* samples with some modification in the final stage. After the rehydration stage (i.e., ethanol and infiltration) samples were maintained in 25% glycerol for one night and 50% glycerol for two nights at incubator (37 °C) for completion of bleaching process.

Finally, in order to quantify GUS activity, the open access image and analysis software Image J (https://rsb.info.nih.gov/ij/) was applied. First images showing blue X-Gluc staining in cleared tissues were converted from color (RGB) to gray (HSB). To measure the GUS positive areas

the linear region of interest (ROI) method was used, whereby a straight line is drawn through cell images to depict the mean intensity of the color (Béziat *et al.*, 2017).

4.3. Results

The results section is divided into two parts, the first part is related to transgenic lines for the 1 kb native promoters (Sex- and Apo-allele), and the second part contain the data for 2 kb native promoters as well as five synthetic different promoter constructs.

4.3.1. Transformation and expression with the 1 kb native promoter of the Apo- and Sex-allele

4.3.1.1. Native promoter amplification

To amplify the native promoter construct, a CACC was added to the 5' end of the primers designed to amplify APOLLO gene promoters and to allow for gateway cloning, and the expected promoter size for the Apo- and Sex-allele promoters (804 bp and 814 bp) were identified. Promoter regions for the Apo- and Sex-alleles were successfully PCR-amplified from wild-type *B. divaricarpa* and *B. stricta* for the Apo- and Sex-allele respectively. Finally, these promoter fragments were cloned into binary pBGWFS7 vectors for *in planta* analysis (Figure 4.12).

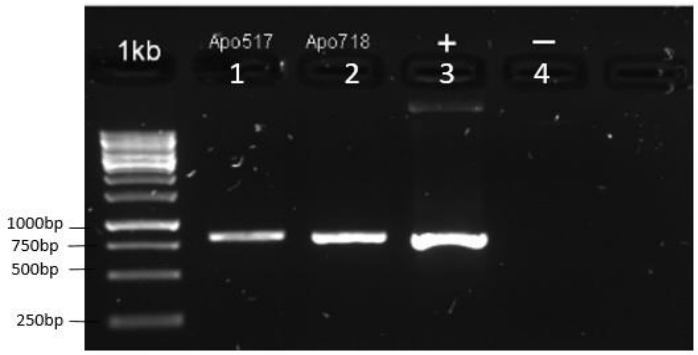


Figure 4.12 Native (1 kb) promoter amplification. Promoters amplified from apomictic and sexual *Boechera* showing expected sizes for all constructs. Expected band sizes are: 1. Apo promoter 804 bp, 2. Sex promoter 814 bp, 3. Positive control 800 bp, 4. Negative control. Primer pairs Apo3caccF and TSPR were used for the Apo-allele and Apo800caccF and TSPR for Sex-alleles. 1% agarose, stained with Cybersafe and Gene Ruler^{TM} 1 kb DNA ladder was used (www.thermofisher.com).

4.3.1.2. Cloning of the 1 kb Apo and Sex native promoter into the pENTR/D-TOPO vector

To clone the inserts into pBGWFS7, the amplified inserts were first cloned into pENTR/D-TOPO® (www.ThermoFisher.com). Validation of correct insertions was then made using PCR, restriction analysis and sequencing. Standard PCR using the M13F and TSPR primer pairs was applied to confirm the presence of insertions in the TOPO vector, with the predicted size of 949 bp and 962 bp for pApo (Figure 4.13) and pSex (Figure 4.14) respectively. Enzyme digestion of putative positive colonies was used to confirm the presence of the Apo promoter construct (pApo) using PvuII and HindIII, and the expected band sizes were observed in at least five out of eleven pENTR/D clones (Figure 4.15). Similarly, PvuI and EcoRI were used to validate the Sex-promoter (pSex) and the expected band sizes were observed in two clones out of nine (Figure 4.16).

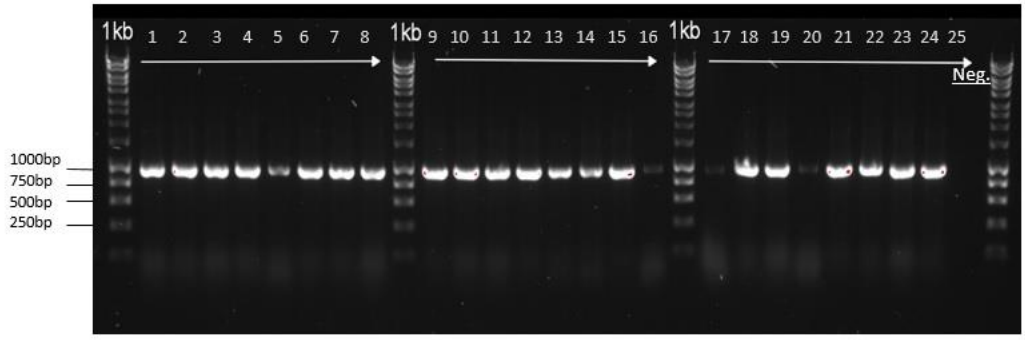


Figure 4.13 PCR Confirmation for the 1 kb Apo promoter for use in transgenic lines. Using M13F and TSPR, 21 lines were confirmed for pApo: pENTR construct (949 bp) using primers M13F and TSPR. 1% gel stained with Cybersafe and Gene Ruler^{TM} 1 kb DNA ladder was used (www.thermofisher.com).

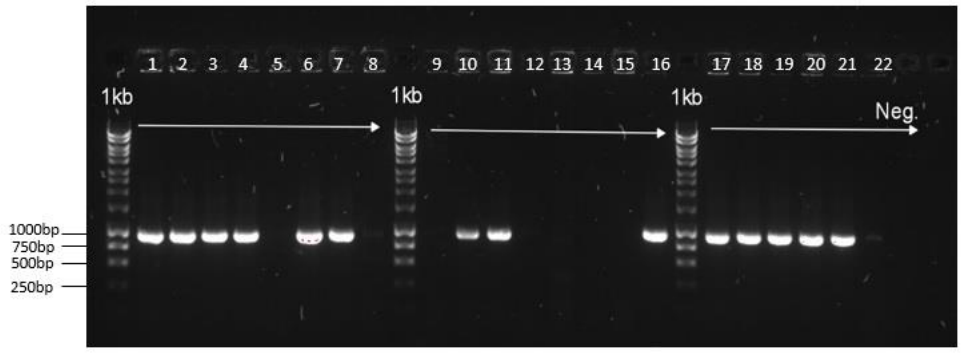


Figure 4.14 PCR Confirmation for the 1 kb Sex promoter for use in transgenic lines. Using primers M13F and TSPR, 14 lines were confirmed positive for pSex: pENTR (962 bp) in pSex lines. 1% agarose gel stained with Cybersafe. Line 22 is a negative control and Gene Ruler^{TM} 1 kb DNA ladder was used (www.thermofisher.com).

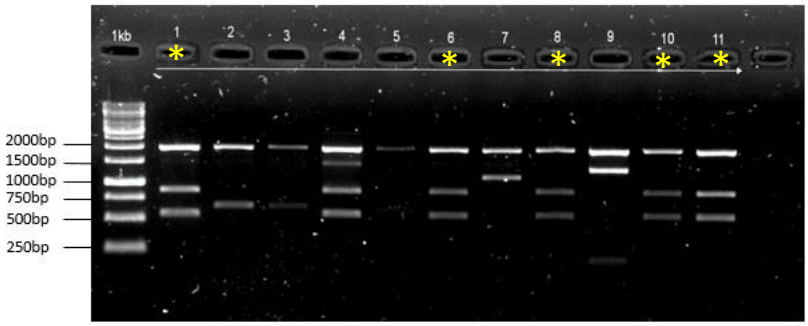


Figure 4.15 pApo: pENTR restriction analysis. Eleven lines positive for the pApo: pENTR construct were analyzed using PvuII and HindIII restriction analysis. The predicted banding pattern (560, 882 and 1,942 bp) was observed for at least 5 clones (stars). 1% agarose gel stained by Cybersafe and Gene Ruler^{TM} 1 kb DNA ladder was used (www.thermofisher.com).

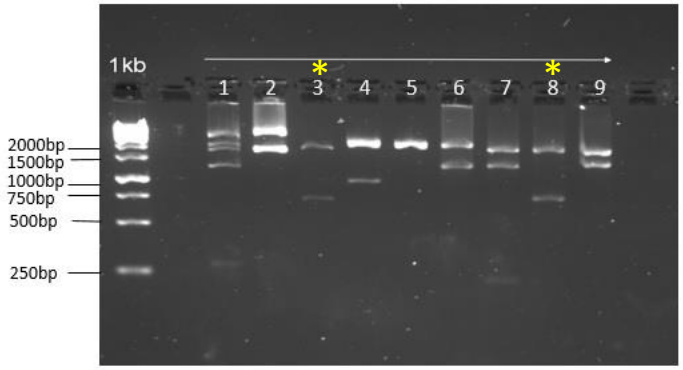


Figure 4.16 pSex:pENTR restriction analysis. Nine lines positive for the pSex:pENTR construct were analyzed using PvuI and EcoRI restriction analysis. The predicted banding pattern (970 and

2,385 bp) was observed for 2 clones (stars). 1% agarose gel stained by Cybersafe and Gene Ruler^{TM} 1 kb DNA ladder was used (www.thermofisher.com).

The DNA of clones was run on a 1% agarose gel to identify plasmids carrying insert DNA. Five out of 11 pApo: pENTR and 3 out of 7 pSex: pENTR had the correct sizes of 3,380 bp and 3,390 bp respectively (Figure 4.17). These clones were sequenced (Sanger sequencing facility, National Research Council, Saskatoon), edited for quality and aligned then against *Boechera* BAC genome sequences (Corral *et al.*, 2013). The alignments demonstrated that pApo had 99.1% similarity with the Bsp 9 isolate BAC2b_4 APOLLO gene (Figure 4.18), while the clone of pSex had 98.9% similarity with *Boechera* spp. IPK Bsp 9 isolate BAC5_7 APOLLO gene (Figure 4.19).

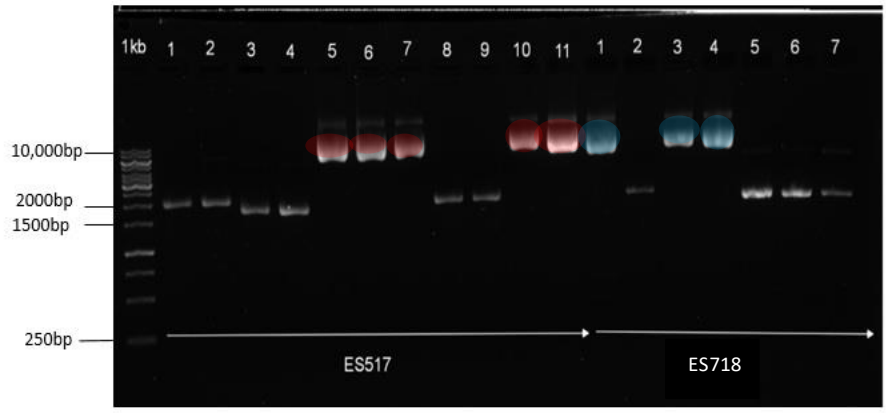


Figure 4.17 pNat:pENTR Sequencing. Positive (colored bands) clones of Apo- and Sex-promoter constructs were prepared for sequencing. Uncolored bands contained lines with empty vectors that were not sent for sequencing. Red color used to show bands with pApo:pENTR construct (3,380 bp), blue color used to show bands with pSex:pENTR construct (3,390 bp), both of which were selected for further cloning and promoter analysis.

Cybersafe stained, 1% agarose gel and Gene Ruler^{TM} 1 kb DNA ladder was used (www.thermofisher.com).

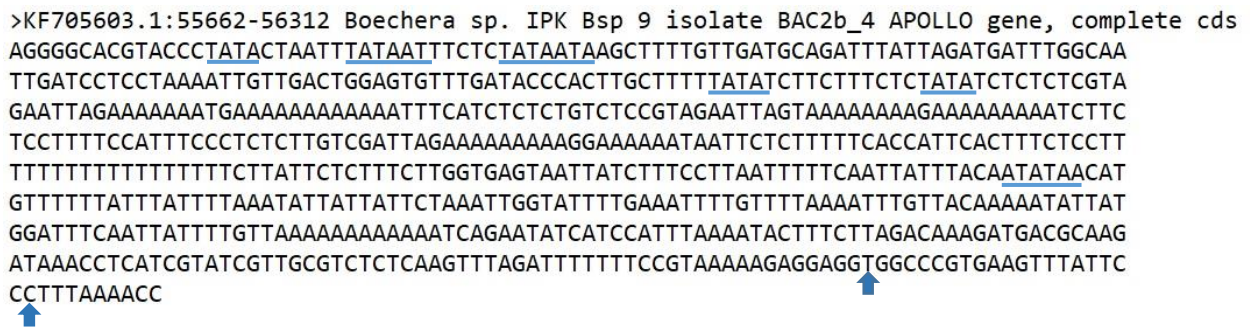


Figure 4.18 Nucleotide sequences of 1 kb Apo promoter construct. This includes the fragment of the APOLLO 5'UTR containing the 20 bp apomixis-specific insertion defined between both two arrows. Blue arrows show the position of TATA-box binding motifs distributed in the Apo promoter.

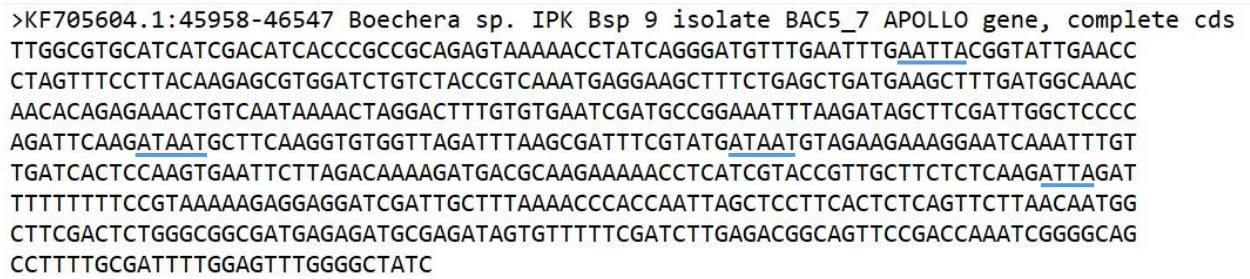


Figure 4.19 Nucleotide sequences of 1 kb Sex promoter construct. Blue lines show the position of TATA-box binding motifs distributed in the Sex promoter.

4.3.1.3. Cloning of 1 kb Apo and Sex native promoters into pBGWFS7 (*Agrobacterium tumefaciens*)

In order to transform *A. thaliana* Col-0 with the native promoter constructs (pNat: pBGWFS7) for *in planta* analysis, the confirmed constructs were first transferred into *A. tumefaciens* GV3101. Putative constructs were subsequently isolated and validated by PCR and restriction enzyme analysis.

4.3.1.4. Screening for promoter containing bacteria using PCR

In order to confirm the positive bacterial colonies, PCR was used followed by enzyme digestion for both pApo: pBGWFS7 and pSex: pBGWFS7. Firstly, PCR was used to determine the presence of the promoter construct after transformation into *A. tumefaciens*. Primer pairs of Lara5 and eGFP were used to amplify the expected product sizes of 847 bp and 876 bp for Apo- and Sex promoters respectively (Figures 4.20 and 4.21). Among the *Agrobacterium* clones that

were positive based on PCR, one confirmed line was then randomly selected for transformation into *A. thaliana* for *in planta* analysis.

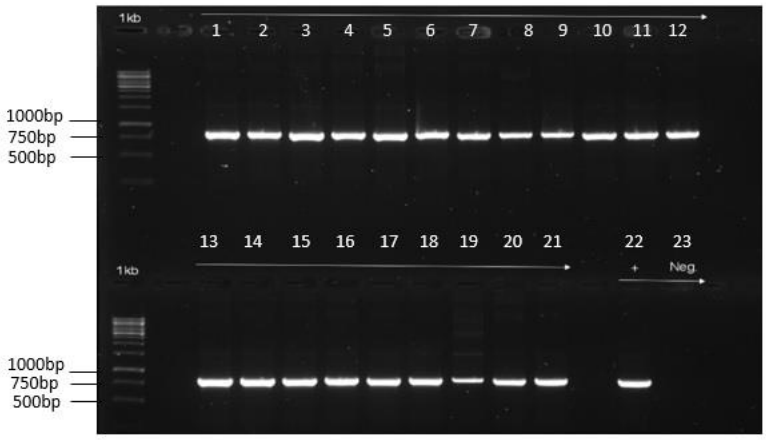


Figure 4.20 pApo: pBGWFS7 PCR Confirmation (*A. tumefaciens*). The PCR confirmations of pApo *A. tumefaciens* lines are shown. All promoter constructs showed at least one clone with the expected band size (847 bp) using primer pairs Lara5F and eGFPR. Line 22, Positive control. Line 23, Negative control. Stained with Cybersafe and run on 1% agarose and Gene RulerTM 1 kb DNA ladder was used (www.thermofisher.com).

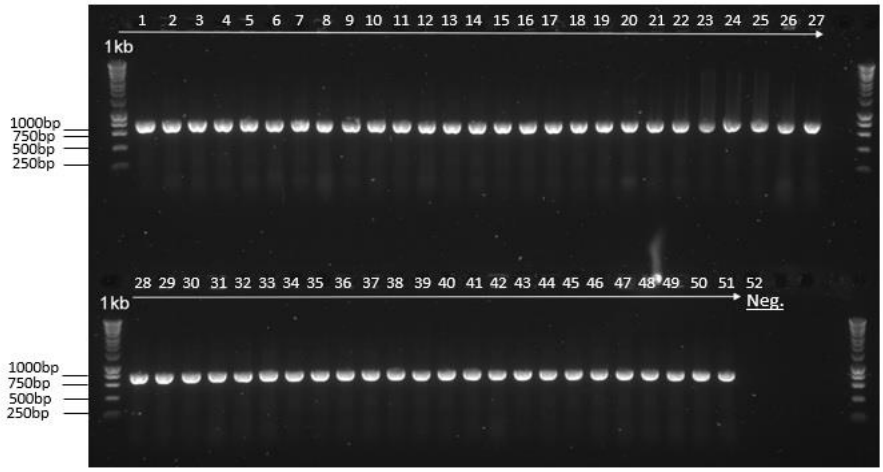


Figure 4.21 pSex: pBGWFS7 PCR Confirmation (*A. tumefaciens*). The PCR confirmation of pSex *A. tumefaciens* clones are shown with the expected band size (876 bp) using primer pairs Lara5F and eGFPR. Line 52, Negative control. Stained with Cybersafe and run on 1% agarose and Gene Ruler^{TM} 1 kb DNA ladder was used (www.thermofisher.com).

4.3.1.5. BASTA selection and transgenic line verification

To analyze the promoter constructs using the *in-planta* assays, the transformed *A. thaliana* were first screened to select for putative transgenic lines. All putative pApo and pSex lines were selected using the BASTA herbicide, whereby after the 2 h dark period all cotyledons appeared yellow and closed for both transgenic and wild types, while a 24 h light period was sufficient for BASTA-resistance plants to accumulate chlorophyll and continue growth, leading to positive transformants showing a longer hypocotyl and more expanded and intense green cotyledons. Non-transformants remained pale, with either closed or unexpanded cotyledons (Figure 4.22). After two weeks the transformants were transferred to the soil for further analysis on their GUS activity.

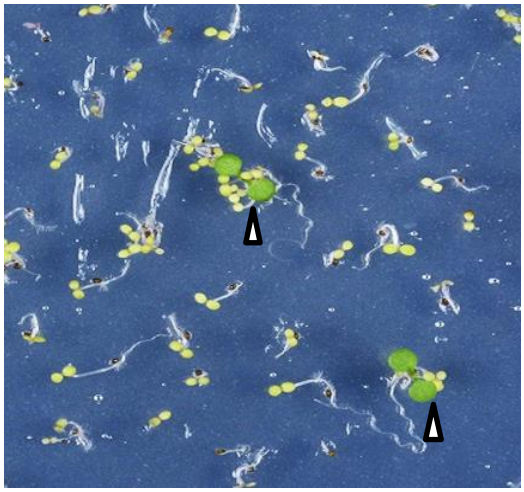


Figure 4.22 Selection of BASTA-resistance *A. thaliana* Col-0 seedlings. T1 Seedlings from *A. thaliana* Col-0 after floral dip transformation using *A. tumefaciens* strain GV3101 harboring the binary plasmid pBGWFS7. Arrows show BASTA-resistance *Arabidopsis* seedlings which are larger and darker compared to untransformed ones.

4.3.1.6. PCR confirmation of transgenic lines

To confirm that the selected transgenic lines contained the promoter construct of interest, PCR confirmation was performed. For the pNat transgenic lines, a total of 25 lines were confirmed to contain the pApo 1 kb construct (Figure 4.23). The Lara5 forward and eGFP506 reverse primer pairs produced the expected product size of 847 bp. The same primers were used for the

confirmation of pSex transgenic lines and generated the expected size of 876 bp from 32 confirmed lines (Figure 4.24).

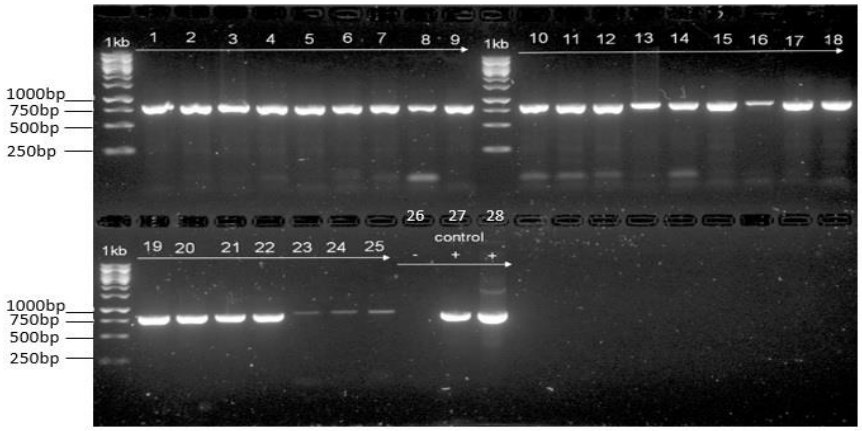


Figure 4.23 PCR confirmation of transgenic lines for the 1 kb pApo promoter. A total of 25 lines (1-25) were confirmed to contain the 1 kb pApo construct. Expected band size (847 bp) was generated using primer pairs Lara5F and eGFP506R. Line 26, Negative control. Lines 27 and 28, Positive control. Stained with Cybersafe and run on 1% agarose and Gene Ruler^{TM} 1 kb DNA ladder was used (www.thermofisher.com).

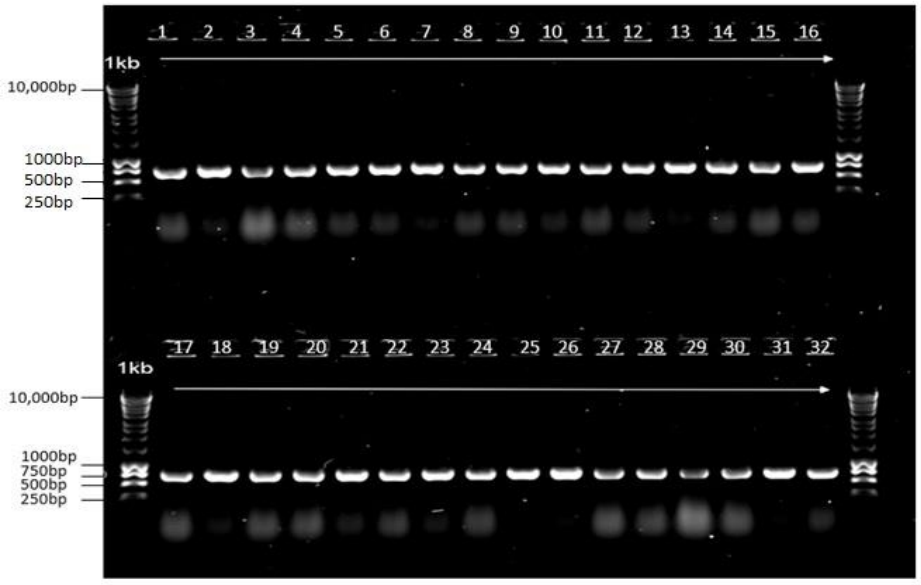


Figure 4.24 PCR confirmation of transgenic lines for 1 kb Sex promoter. A total of 32 lines (1-32) were confirmed to contain the 1 kb pSex construct, expected band size is 876 bp primer pairs using Lara5F and eGFP506R. Stained with Cybersafe and run on 1% agarose and Gene Ruler^{TM} 1 kb DNA ladder was used (www.Thermofisher.com).

4.3.1.7. Histochemical GUS assay of 1 kb Apo and Sex native promoters in *Arabidopsis*

No GUS expression was detected in the leaves and flowers of 25 lines containing the 1 kb pApo construct, and 32 lines containing the 1 kb pSex promoter (Figure 4.25).

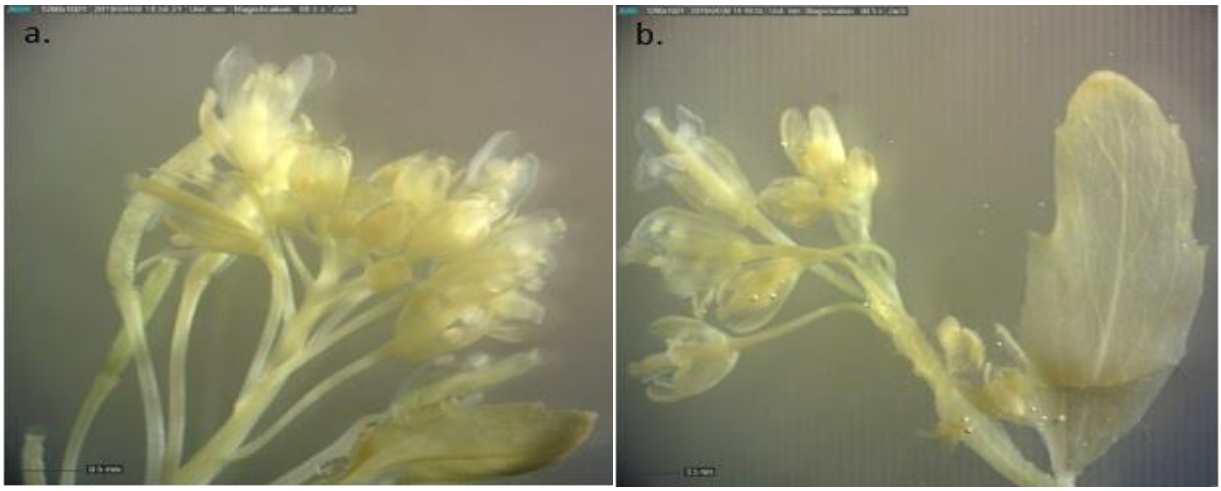


Figure 4.25 GUS expression of 1 kb native promoters in inflorescence of transgenic *Arabidopsis*. a. No Gus activity was observed for 1kb (pApo::GUS), **b.** No GUS activity observed for 1 kb (pSex::GUS), Images are taken by Digital Stereo Microscope (Nikon), Scale bar=0.5 mm.

4.3.2. Transformation and expression with the 2 kb native promoters of the Apo- and Sex-alleles

4.3.2.1. Transformation of 2 kb native promoter to *Arabidopsis* and *Boechera*

For transforming the 2 kb native promoters to *Arabidopsis*, the same protocol for transforming 1kb native promoters was used. In addition, transformation in *Boechera* was conducted followed by its tissue culture, using a method under development in the Sharbel lab (University of Saskatchewan, Saskatoon, SK, Canada). In this experiment the following conditions were used.

Medium: MS (Murashige and Skoog**)** medium

Genotype: JL107 (apomictic diploid)

Explant: Hypocotyl

Selection method: Glufosinate ammonium (BASTA)

Hormonal regime: 0.1 mg/l (0.5 µM) NAA (1-Naphthaleneacetic acid) as auxin and 2.0 mg/l (8.8 µM) BA (6-Benzylaminopurine) as cytokine

The tissue culture success for the above hormonal treatment was 25 out of 120 explants (21%) for pC1, and 23 out of 120 explants (19%) for pC2 constructs. After subjecting the explants to the hormonal treatments and selection medium 25 explants (63 plants) for pC1 and 23 explants (48 plants) for pC2 were grown successfully.

4.3.2.2. Histochemical GUS activity of 2 kb Apo and Sex native promoters in *Arabidopsis*

4.3.2.2.1. GUS activity of 2 kb native promoters for 2-week-old seedlings

Positive GUS activity for root, shoots, and leaves of 2-week-old seedlings for lines transgenic for the construct pC1 carrying 2 kb Apo native promoter was observed. The GUS activity was measured for 33 lines of 2-week-old seedlings each with 4 repeats. All 33 lines showed positive GUS activity (Figure 4.26a) except for 3 lines with no GUS activity in roots, shoots, and leaves. Five out of 33 lines were negative only for roots (Table 4.7). No tissue-specific GUS activity was observed for 31 lines of 2-week-old seedlings transgenic for the construct pC2, carrying 2 kb Sex native promoter (Figure 4.26b).

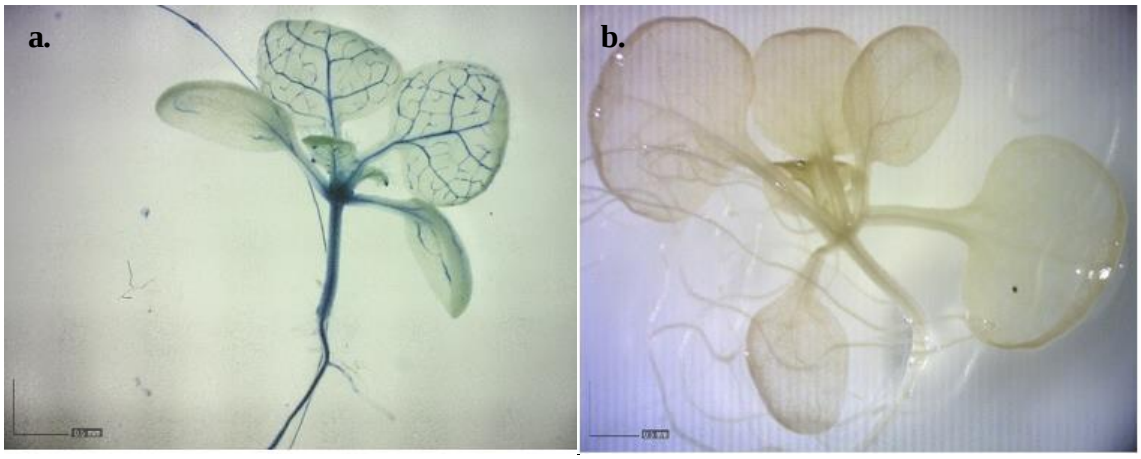


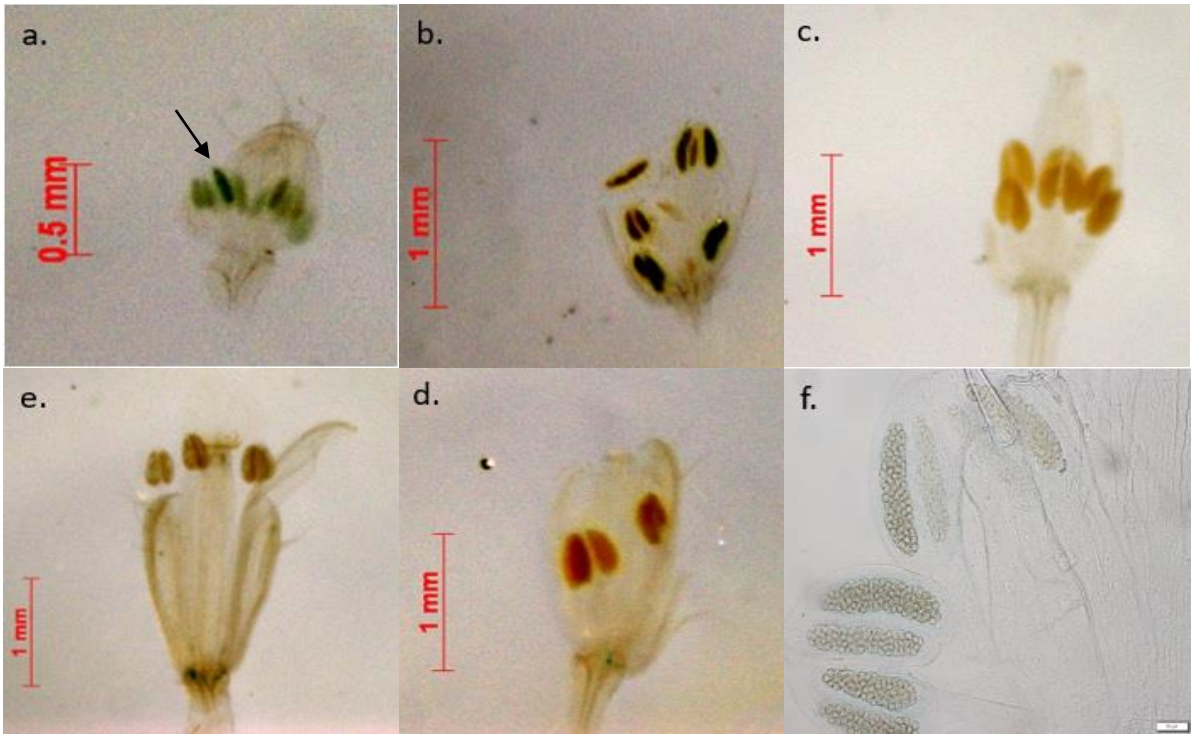
Figure 4.26 Expression patterns of *Boechera* native promoter genomic GUS constructs in *Arabidopsis*. a. GUS distribution of 2 kb native pApo promoter in seedling, b. No GUS activity observed in 2 kb native pSex promoter, Images are taken by a Digital Stereo Microscope (Nikon), Scale bar=0.5 mm.

Table 4.7 GUS activity in 2-week-old seedlings of 33 lines carrying the 2 kb native pApo promoter in *Arabidopsis*. Numbers show relative gene expression levels (average rate among leaf, hypocotyl, and root) based on personal observation (1 low to 4 high).

Sample T_1/T_2	Leaf	Hypocotyl	Root	Average Rate
pApo 1.1	Pos.	Pos.	Pos.	3
pApo 1.3	Pos.	Pos.	Pos.	4
pApo 1.6	Pos.	Pos.	Pos.	2
pApo 1.9	Neg.	Neg.	Neg.	_
pApo 1.10	Pos.	Pos.	Pos.	2
pApo 1.18	Pos.	Pos.	Pos.	2
pApo 1.20	Pos.	Pos.	Pos.	3
pApo 1.21	Pos.	Pos.	Pos.	3
pApo 1.22	Pos.	Pos.	Pos.	2
pApo 1.38	Pos.	Pos.	Neg.	1
pApo 1.39	Pos.	Pos.	Pos.	1
pApo 1.40	Pos.	Pos.	Pos.	1
pApo 1.42	Pos.	Pos.	Neg.	2
pApo 1.43	Pos.	Pos.	Neg.	1
pApo 1.44	Pos.	Pos.	Pos.	3
pApo 1.45	Pos.	Pos.	Pos.	2
pApo 1.46	Pos.	Pos.	Neg.	1
pApo 1.47	Pos.	Pos.	Pos.	2
pApo 1.48	Pos.	Pos.	Pos.	2
pApo 1.49	Pos.	Pos.	Pos.	3
pApo 1.53	Neg.	Neg.	Neg.	_
pApo 1.54	Pos.	Pos.	Pos.	3
pApo 1.55	Pos.	Pos.	Pos.	2
pApo 1.56	Pos.	Pos.	Pos.	3
pApo 1.57	Pos.	Pos.	Pos.	1
pApo 1.58	Pos.	Pos.	Pos.	2
pApo 1.59	Pos.	Pos.	Pos.	1
pApo 1.60	Neg.	Neg.	Neg.	_
pApo 1.61	Pos.	Pos.	Pos.	3
pApo 1.62	Pos.	Pos.	Neg.	1
pApo 1.63	Pos.	Pos.	Neg.	1
pApo 1.64	Pos.	Pos.	Pos.	3
pApo 1.65	Pos.	Pos.	Pos.	2

4.3.2.2.2. GUS activity of 2 kb native Apo- and Sex promoters for 6-week-old plants

GUS activity in 6-week-old *Arabidopsis* seedlings from 3 lines transgenic for construct pC1 was also measured. Some GUS activity was observed for anthers in the pre-meiotic stage (Figure 4.27a). No tissue-specific GUS activity was observed at all for 10 lines of 6-week-old *Arabidopsis* seedlings transgenic for pC2.



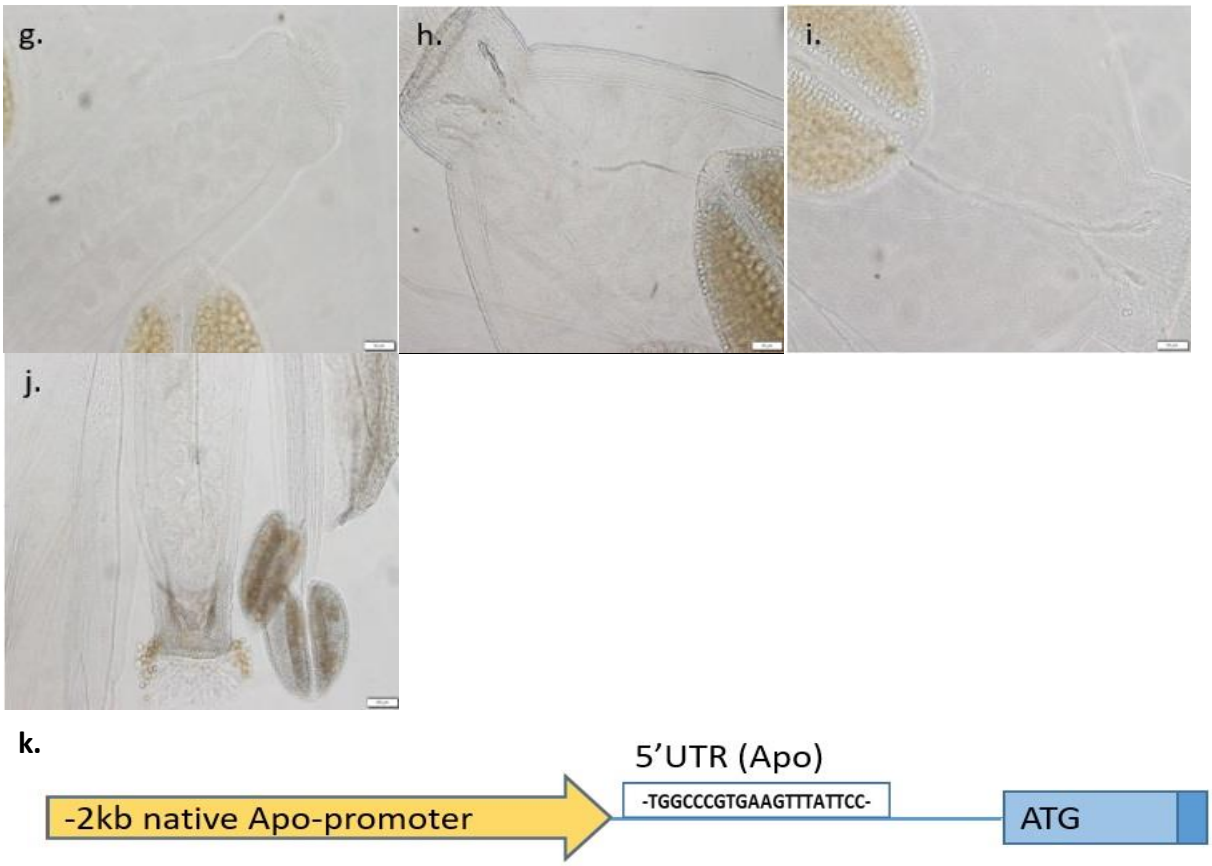


Figure 4.27 Expression patterns of 2kb native Apo promoter GUS constructs in *Arabidopsis* flowers. GUS distribution of pC1 in different developmental stages of mature flowers, a. 0.5 mm flower with GUS activity (arrow) in anthers Scale bar=0.5 mm, b. 1.0 mm flower with GUS activity in anthers, c. 1.5 mm flower, d. 2.0 mm flower, e. 3.0 mm flower, (b-d) Scale bar=1.0 mm, f. 0.7 mm flower with minimal GUS activity in anther wall layer, g. 1.0 mm flower, h. 1.5 mm flower, i. 2.0 mm flower, j. 3.0 mm flower. For (f-j) Scale bar=50 µm (Olympus BX61 microscope), Scale bar=0.5 mm, k. schematic of construct pC1, the box contains 20 bp Apo-insertion motif.

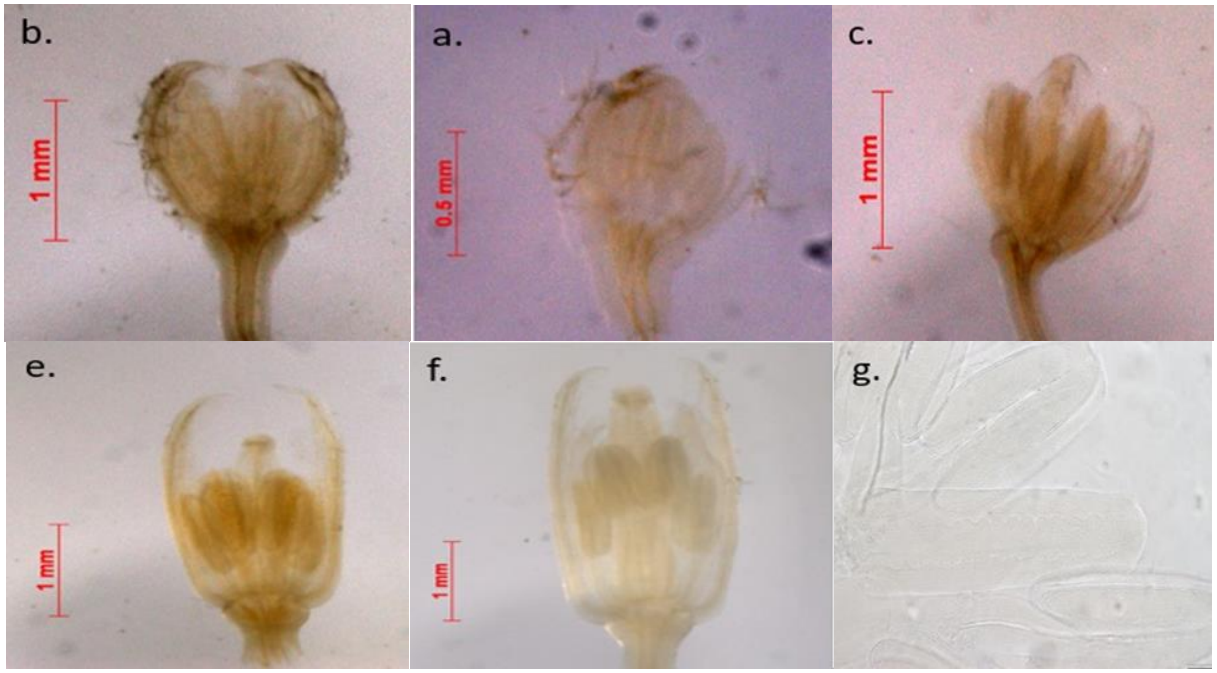
4.3.2.3. Histochemical GUS activity of 2 kb Sex and Apo native promoters in *Boechera*

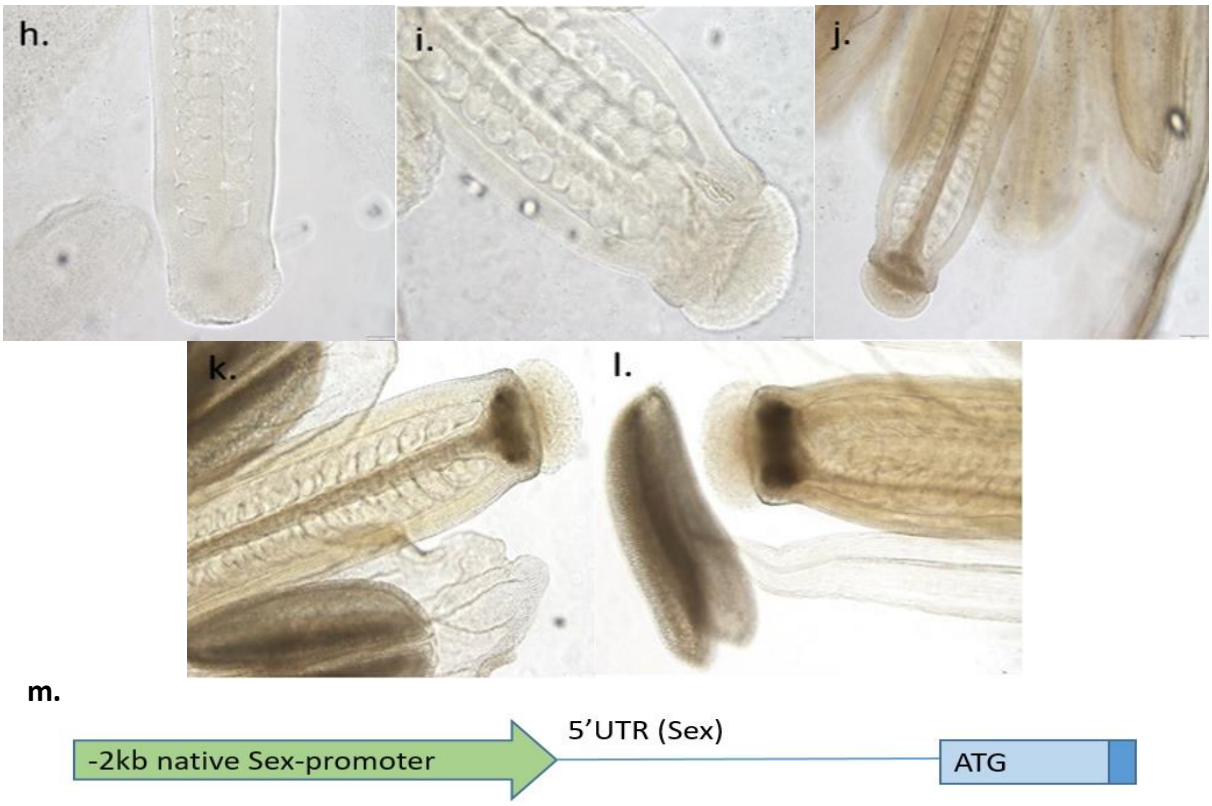
To investigate the promoter activities of APOLLO alleles, two promoter fragments containing 2 kb upstream of the 5'UTR of the APOLLO Apo- and Sex-alleles from *Boechera* were cloned using PCR. These promoter regions were fused to an *E. coli* reporter gene encoding a β-glucuronidase (GUS) reporter enzyme and introduced into *Boechera* through *Agrobacterium*-mediated gene transfer (see Materials and Methods). The DNA of both 2 kb Apo- and Sex-alleles

were sent for sequencing followed by comparing them with the BAC library sequences using NCBI (https://ncbi.nlm.nih.gov).

The 2 kb promoter is considered a long fragment for sequencing by only one pair of primers. Thus, three primer pairs were used, and three fragments were produced accordingly for each 2 kb Apo- and Sex-promoter and assembled using the Geneious software (version 10.2.6) as shown in appendix Figures 5.9 and 5.10. Finally, the T_2 transgenic *Boechera* plants were validated by PCR and subjected to histochemical staining for GUS activity.

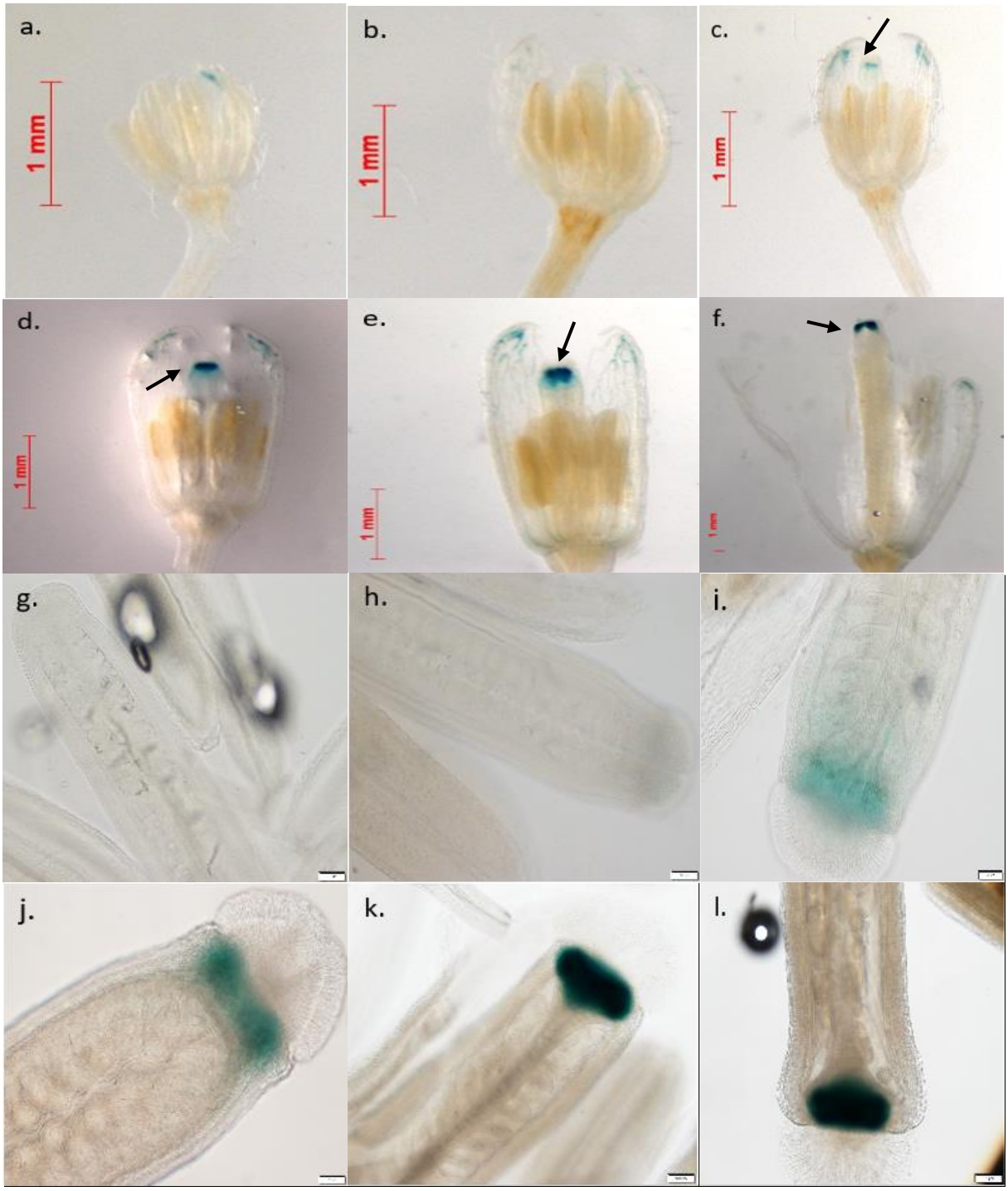
Histochemical and fluorometric analyses of transgenic plants revealed that there was no tissue-specific GUS activity for transgenic *Boechera* carrying the 2 kb native Sex promoter (Figure 4.28). Interestingly, the "Apo" promoter drove GUS activity in *Boechera's* flowers in a tissue-specific manner. Figure 4.29 shows the expression profiles of pApo: GUS's transgenes in different developmental stages of an apomictic *Boechera* (JL107). These data are representative of GUS activity in the stigma of twenty-five transgenic lines. In order to quantify the GUS activity data, Image J software was used that enabled us to turn the GUS' blue hue into grey pixels followed by conversion to digits (Figure 4.30).





m.

Figure 4.28 Expression patterns of native 2 kb (pSex::GUS) constructs in *Boechera* flowers. No tissue specific GUS activity was observed for 2 kb native Sex promoter a. 0.9 mm flower, b. 1.5 mm flower, c. 1.8 mm flower, d. 2.5 mm flower, e. 3.0 mm flower, f. 4.0 mm flower (a-f) Scale bar=1.0 mm (Zeiss Lumar.V12 Stereoscope), g. 0.9 mm, h. 1.5 mm flower, i. 1.8 mm flower, j. 2.5 mm flower, k. 3.0 mm flower, l. 4.0 mm flower, (g-l) Scale bar=100 µm (Olympus BX61 microscope), m. Schematic of 2 kb native Sex promoter fused to a GUS reporter gene without 20 bp Apo-insertion.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m.

Figure 4.29 Expression patterns of native 2 kb promoter pApo GUS constructs in *Boechera* flowers. Tissue specific GUS activity of the 2 kb native Apo promoter observed in stigmas of flowers in different developmental stages, a. 0.9 mm flower, b. 1.5 mm flower, c. 2.5 mm flower with GUS activity on stigma, d. 2.7 mm flower with GUS activity on stigma, e. 3.5 mm flower with GUS activity on stigma, (a-f) Scale bar=1.0 mm (Zeiss Lumar.V12 Stereoscope) f. 4.0 mm flower, g. 0.9 mm, h. 1.5 mm flower with minimal GUS activity on stigma, i. 1.5 mm flower with GUS activity on stigma, j. 2.5 mm with GUS activity on stigma, k. 3.5 mm with GUS activity on stigma, l. 4.0 mm flower with GUS activity on stigma, (g-l) Scale bar=100 µm (Olympus BX61 microscope), m. Schematic of 2 kb native pApo with the 20 bp Apo-insertion. There was also some dispersed GUS activity in petals and sepals in different developmental stages that did not follow a specific pattern. Thus, they were excluded for the quantification of GUS activity.

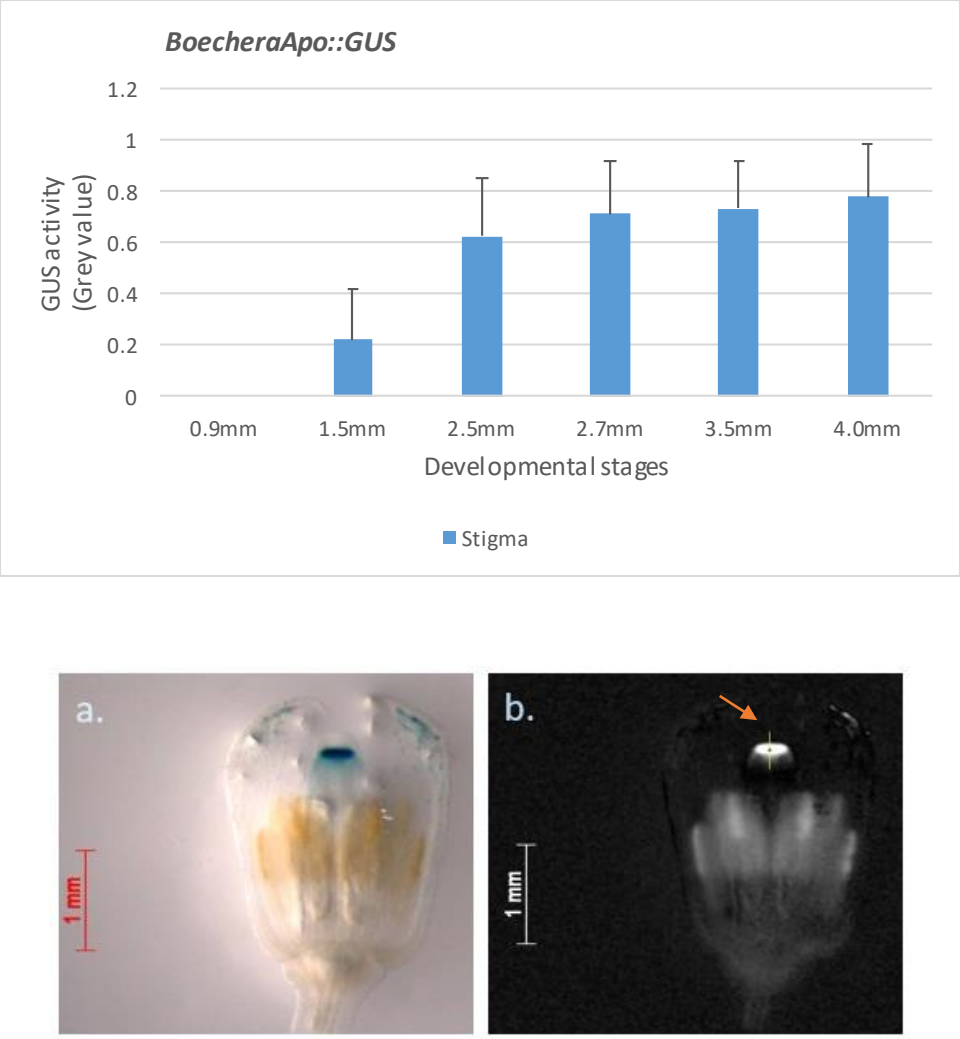


Figure 4.30 Quantification of GUS histochemical staining in flowers of pApo::GUS transgenic *Boechera*. a. RGB mode of image b. HSB mode of image showing histochemical staining of pApo::GUS's expression in stigma. Drawings in HSB images represent linear Regions of Interest (ROIs). Graph above shows the mean GUS intensity (SD) measured within the (ROIs) depicted in HSB images. The first stage of flower development (pre-meiotic) is significantly different P ≤0.001 from others (Tucky test; n=18 flower buds). The data were normalized using the Min-Max method. X-axis line represents different developmental stages of flower (based on the carpel height in mm).

4.3.3. The results of five different synthetic promoter constructs transformed into *Arabidopsis*.

4.3.3.1. PCR amplification to confirm the transgenic lines of five different APOLLO constructs in *Arabidopsis*.

To confirm that the positively selected transgenic lines contained the promoter construct of interest, PCR confirmation was performed on the genomic DNA using promoter-specific primers. Using Lara 5 and eGFP560R primers, the promoter construct (pC3) was confirmed for 12 lines with the expected band size of 713 bp (Figure 4.31), while promoter construct (pC4) was confirmed in 43 lines with the expected product size of 742 bp (Figure 4.32). For promoter construct (pC5), among 27 BASTA selected lines 2 were lost due to phytotron conditions and 23 lines were confirmed to contain the expected 733 bp fragment (Figure 4.33). Nine lines were confirmed for promoter construct (pC6), with the expected product size of 722 bp (Figure 4.34). While 42 lines were confirmed for promoter construct (pC7), with the expected product size of 1,275 bp (Figure 4.35).

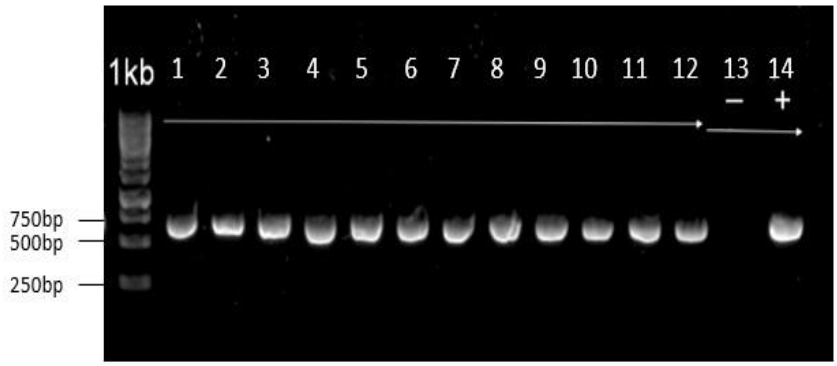


Figure 4.31 Confirmation of insertion for construct pC3. The PCR confirmation of 12 positive pC3 *A. tumefaciens'* lines are shown. Expected band size is 713 bp using primer pairs Lara5F and eGFP560R. Stained with Cybersafe and run on 1% agarose. Line 13, Negative control. Line 14, Positive control. Gene Ruler™ 1 kb DNA ladder was used (www.Thermofisher.com).

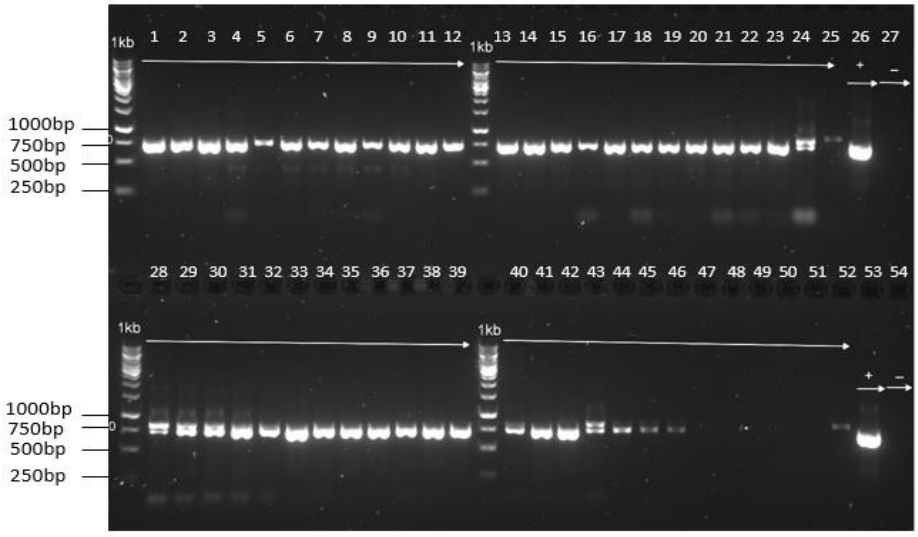


Figure 4.32 Confirmation of insertion for Construct pC4. The PCR confirmation of pC4 *A. tumefaciencs'* lines are shown. Expected band size is 742 bp using primer pairs Lara5F and eGFP 560R. Lines 26 and 53, Positive control. Lines 27 and 54, Negative control. Stained with Cybersafe and run on 1% agarose and Gene Ruler^{TM} 1 kb DNA ladder was used (www.Thermofisher.com).

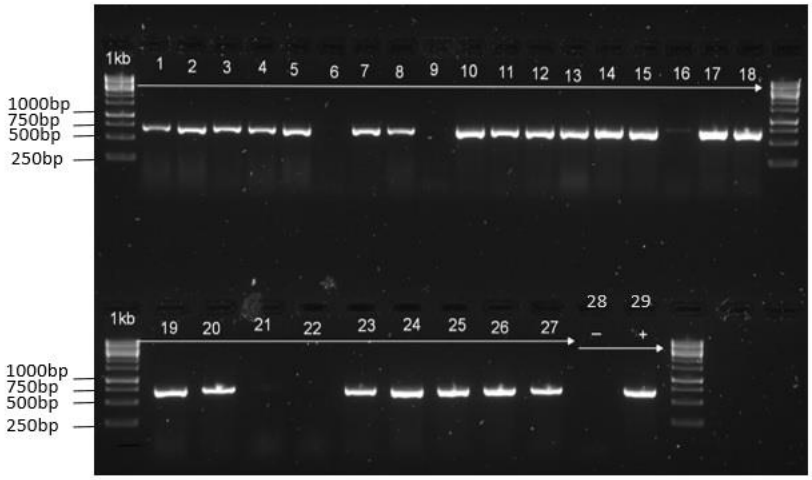


Figure 4.33 Confirmation of insertion for construct pC5. The PCR confirmation of pC5 *A. tumefaciens'* lines are shown. Expected band size is 733 bp using primer pairs Lara5F and eGFP560R. Lines 28, Negative control. Line 29, Positive control. Stained with Cybersafe and run on 1% agarose and Gene Ruler^{TM} 1 kb DNA ladder was used (www.Thermofisher.com).

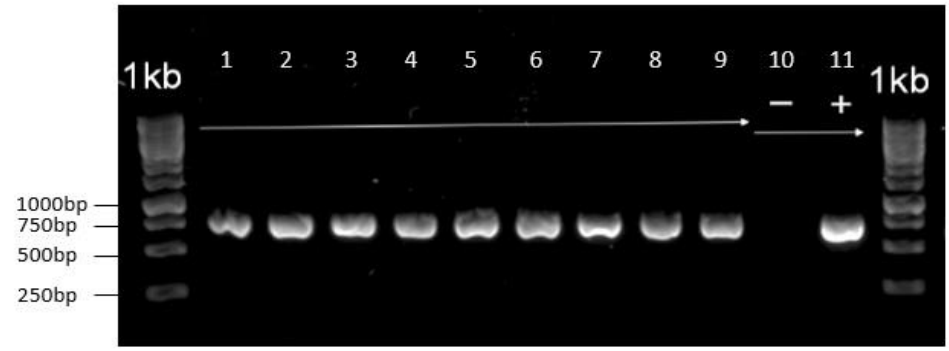


Figure 4.34 Confirmation of insertion for construct pC6. The PCR confirmation of pC6 *A. tumefaciens'* lines are shown. All promoter constructs showed the expected banding pattern. Expected band size is 722 bp using primer pairs Lara5F and eGFP560R. Line 10, Negative control. Line 11, Positive control. Stained with Cybersafe and 1% agarose and Gene Ruler^{TM} 1 kb DNA ladder was used (www.Thermofisher.com).

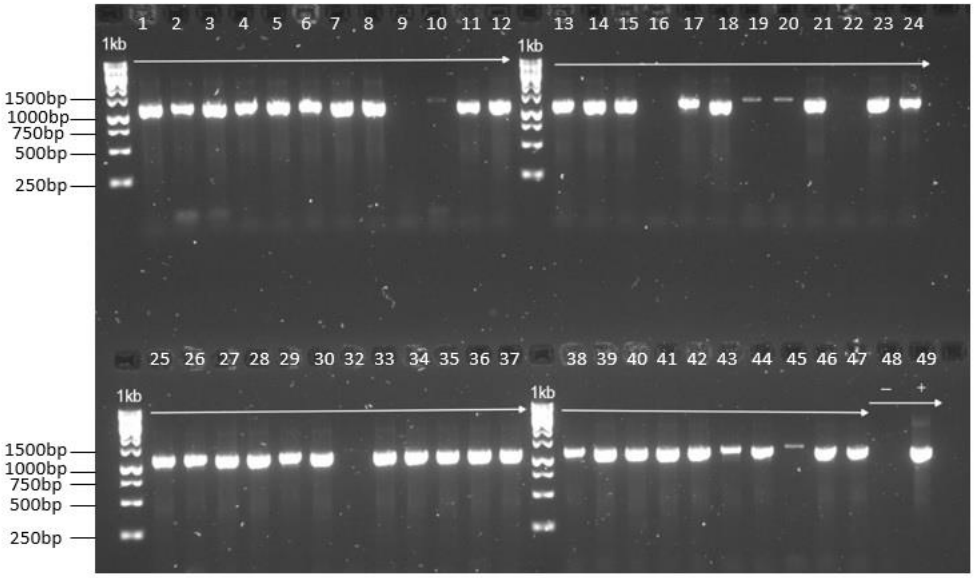
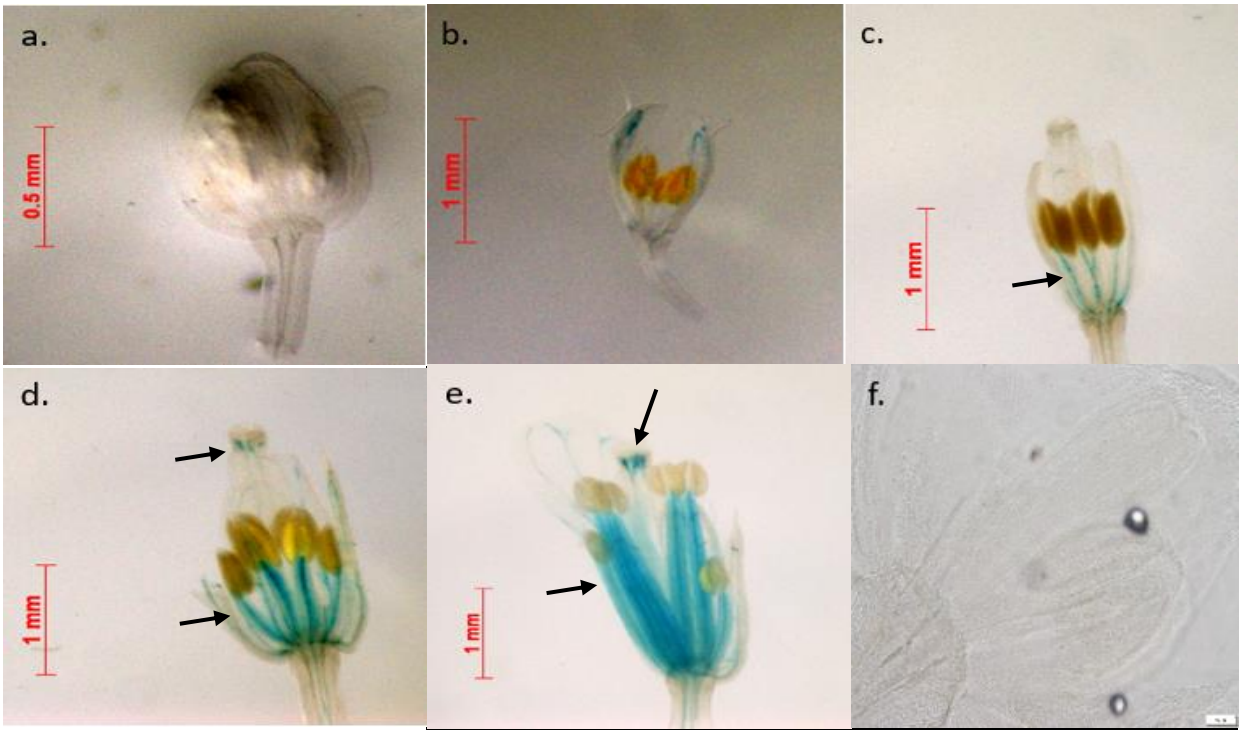


Figure 4.35 Confirmation of insertion for construct pC7. The PCR confirmations of pC7 *A. tumefaciens'* lines are shown. Promoter constructs in 43 lines showed the expected banding pattern. Expected band size is 1,275 bp using primer pairs Apo800F and eGFP560R. Line 48, Negative control. Line 49, Positive control. Stained with Cybersafe and run on 1% agarose and Gene Ruler Thermo scientific 1 kb DNA ladder was used (www.Thermofisher.com).

4.3.3.2. Histochemical GUS activity of five APOLLO synthetic promoter constructs in *Arabidopsis*

4.3.3.3. Histochemical GUS activity for construct pC3 changes reproductive tissue-specificity of expression

This construct carries the 2 kb native pApo promoter, the 5'UTR of Apo-allele, without the 20 bp Apo- insertion. The Image J quantification of GUS activity for construct (pC3) was carried out for 8 transgenic lines each of which has five different developmental stages (total of 40 flower buds). No GUS activity was observed for both stigma and ovule in the pre-meiotic (0.7 mm) and post meiotic (1.0 mm) stages, while positive GUS activity was observed for stages 3, 4, and 5 in stigma and filament with an increasing trend toward the final stages (Figure 4.36c, d, e). Dispersed GUS activity in petals was excluded because they did not show a constant pattern for all samples. Finally, the data was quantified using a two-way analysis of variance (two-way ANOVA) and the final two stages of flower development were significantly different from other stages ($P \leq 0.05$, Tucky test) (Figure 4.37).



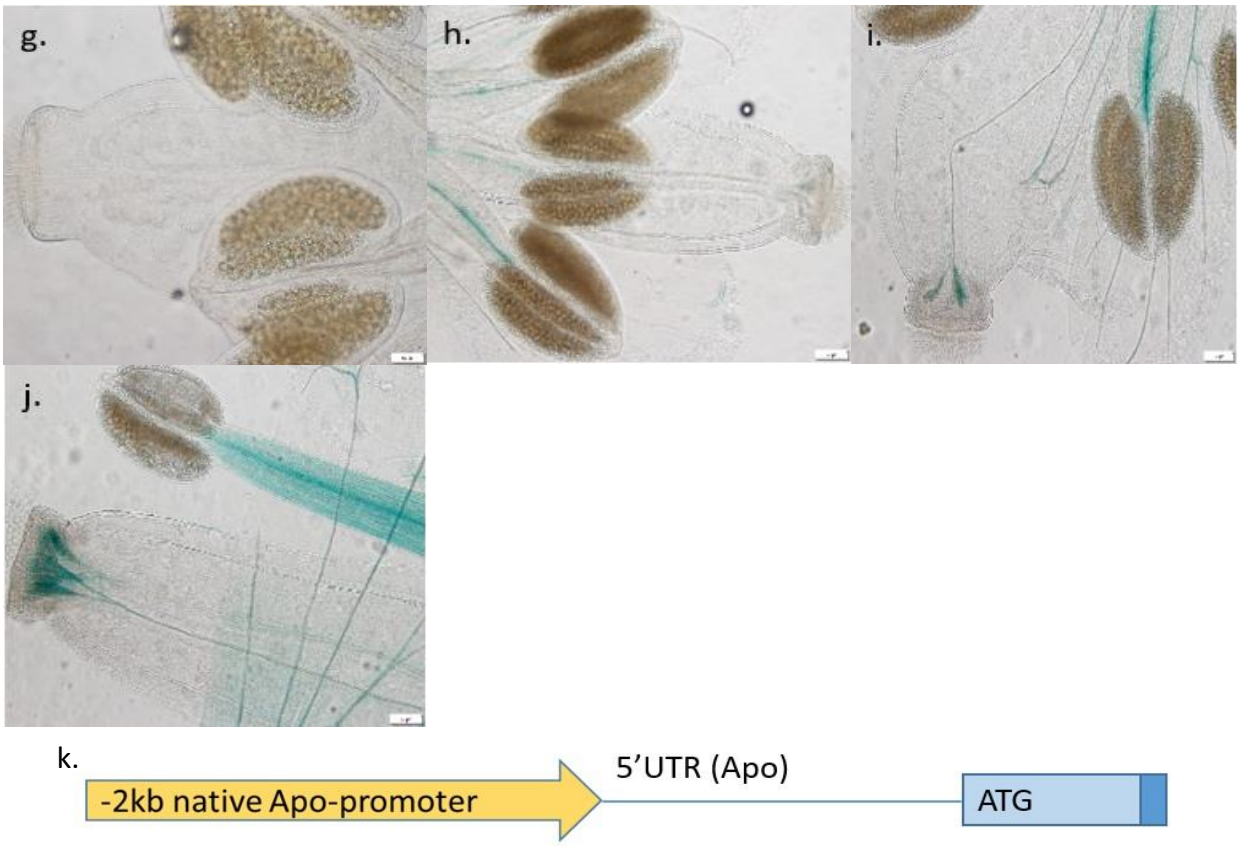


Figure 4.36 Expression patterns of synthetic promoter construct (pC3: GUS) in *Arabidopsis* flowers. Construct pC3 promoter in different developmental stages of flowers, a. 0.7 mm flower Scale bar=0.5 mm, b. 1.0 mm flower with GUS activity on petals, c. 1.5 mm flower with GUS activity on filament, d. 2.0 mm flower with GUS activity on filament and stigma. e. 3.0 mm flower with GUS activity on filament and stigma, (b-e) Scale bar=1.0 mm (Zeiss Lumar.V12 Stereoscope), f. 0.7 mm, g. 1.0 mm flower, h. 1.5 mm flower, i. 2.0 mm flower, j. 3.0 mm flower, (f-j) Scale bar=100 µm (Olympus BX61 microscope), k. Schematic of construct 3 contains the 1,866 bp Apo promoter, 51 bp 5'UTR of Apo-allele without 20 bp Apo-insertion fused to a GUS reporter gene.

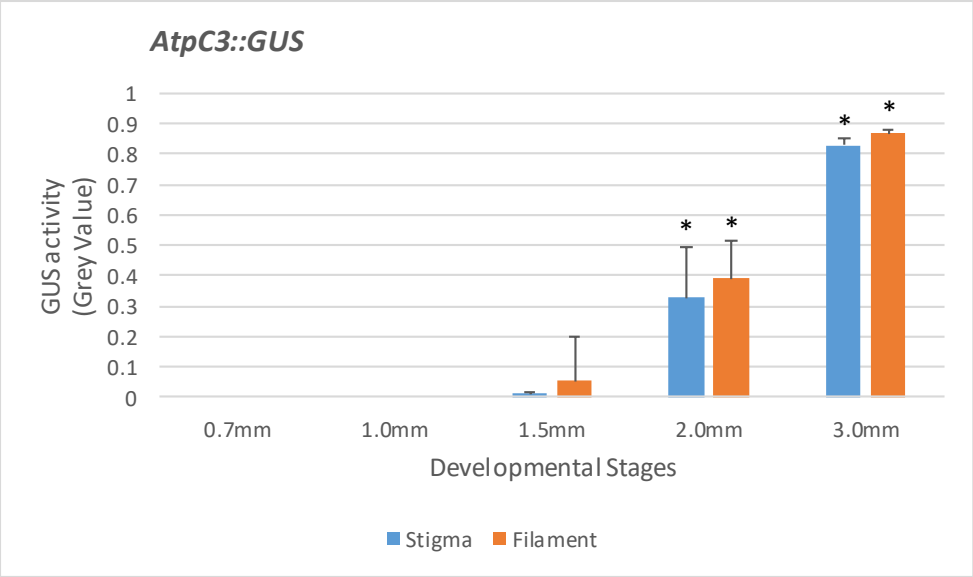


Figure 4.37 Quantification of GUS histochemical staining in flowers of pC3:GUS transgenic *Arabidopsis*. Graph showing the mean GUS intensity (SD) measured in stigma and filament for five developmental stages within flowers. Asterisks indicate two final developmental stages are significantly different P≤0.05 from others (Tucky test; n=40 flower buds). The data were normalized using the Min-Max method. X-axis line represents different developmental stages of flower (based on the carpel height in mm). AtpC3: GUS means *Arabidopsis* transgenic for pC3: GUS.

4.3.3.4. Histochemical GUS activity for construct pC4 changes reproductive tissue-specificity of expression

This construct carried the 2 kb native pSex promoter, the 5'UTR of the Sex-allele, with the 20 bp Apo-insertion. The GUS activity for 7 transgenic pC4 lines, each of which had five different developmental stages (total of 35 flower buds) was measured. No GUS activity in leaf or flower tissues was detected for those lines as shown in (Figure 4.38).

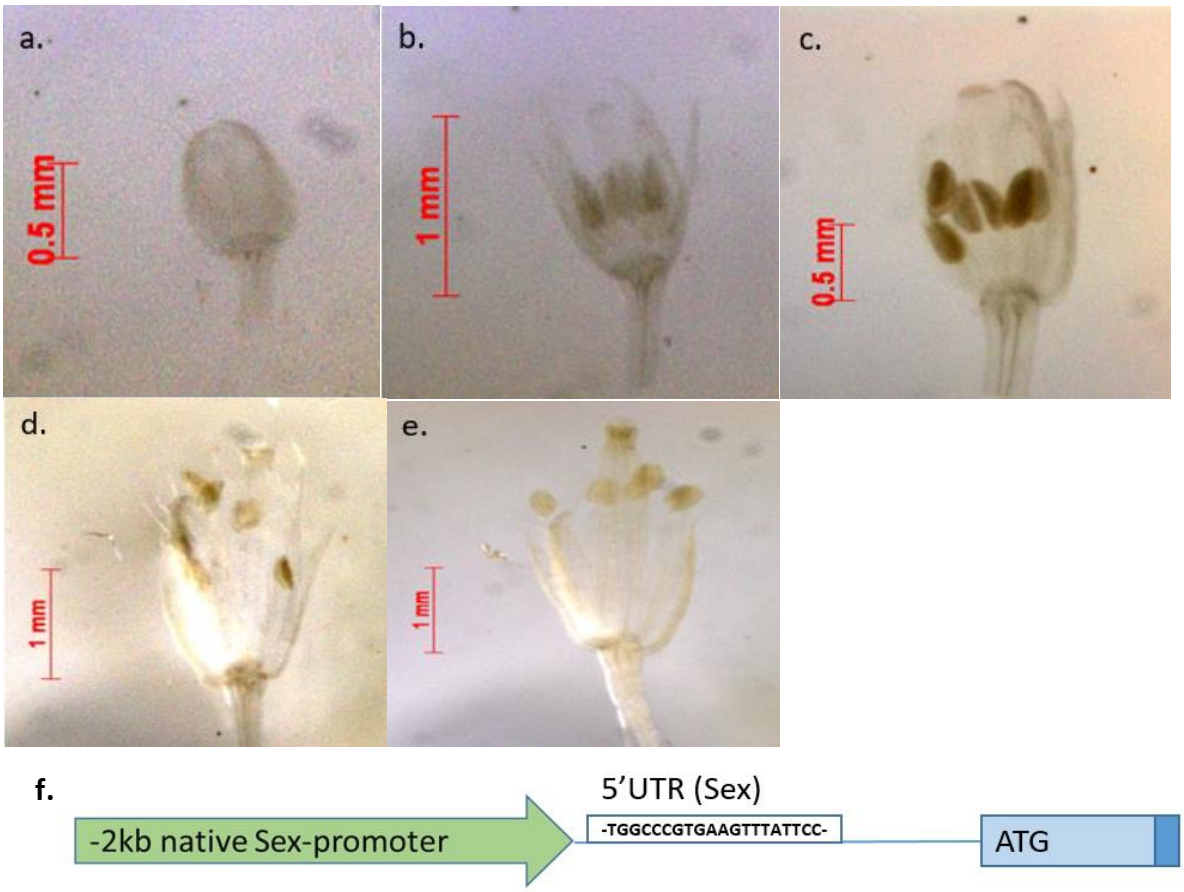
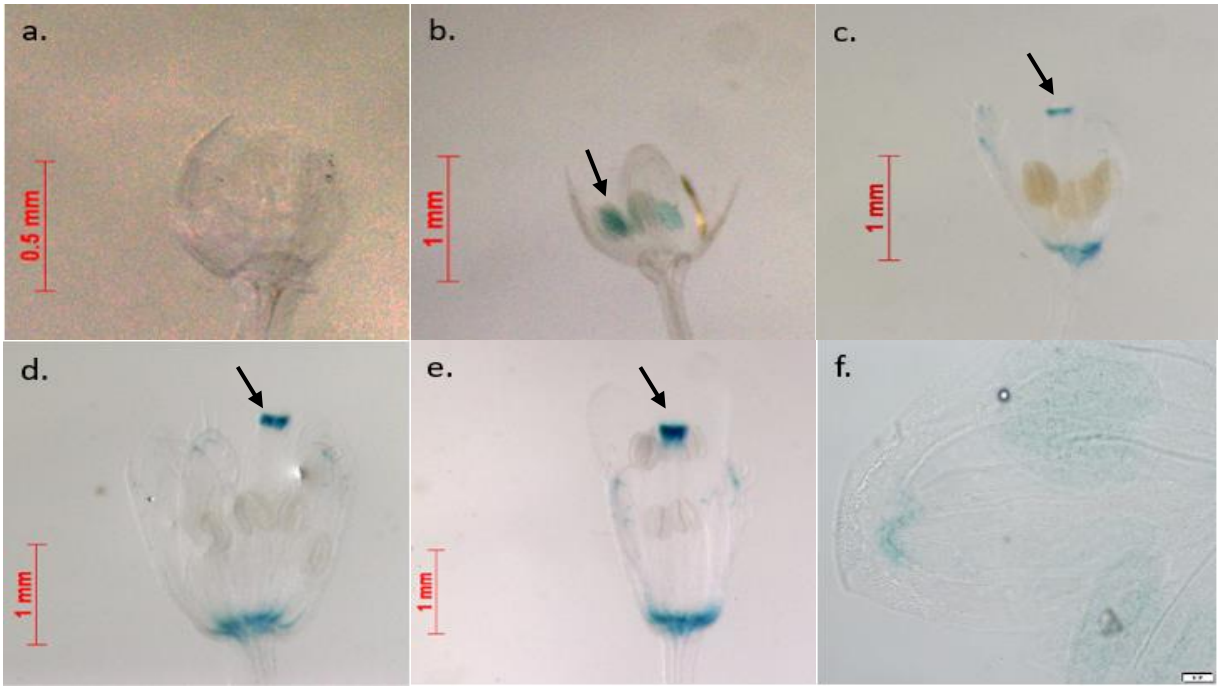


Figure 4.38 Expression patterns of spliced promoter construct (pC4:GUS) in *Arabidopsis* flowers. Construct pC4 promoter in different developmental stages of flowers, a. 0.7 mm flower Scale bar=0.5 mm, b. 1.0 mm flower, c. 1.5 mm flower, d. 2.0 mm flower, e. 3.0 mm flower, (b-f) Scale bar=1.0 mm. No GUS activity observed in any stages. Schematic of construct 4 contains the 1,866 bp Sex promoter, 124 bp 5'UTR of Sex-allele carrying 20 bp Apo-insertion (box) fused to a GUS reporter gene.

4.3.3.5. Histochemical GUS activity for construct pC5 changes reproductive tissue-specificity of expression

This construct was composed of the 2 kb native pApo promoter plus the 5'UTR of the Apo-allele, but a 20 bp random insertion was substituted for the apomixis-specific 20 bp insertion. The image J quantification of GUS activity for construct (pC5) was carried out for 6 transgenic lines each of which had five different developmental stages (total of 30 flower buds). A minimum GUS activity was observed in anther and receptacle for the pre-meiotic stage (Figure 4.39a), as well as some anther-specific GUS activity for stages 2 and stigma-specific GUS activity for stages 3, 4, and 5 (Figure 4.39b, c, d, and e). There is similarity between this

construct and pC1 in terms of stigma pattern. In addition, some GUS activity was observed in the receptacle of flowers for all stages, and petals that were excluded for quantification because they did not show a constant pattern for all samples. Finally, the data was quantified using analysis of variance (ANOVA) and pre-meiotic stage showed a significant difference from other stages ($P \leq 0.05$, Tucky test) (Figure 4.40).



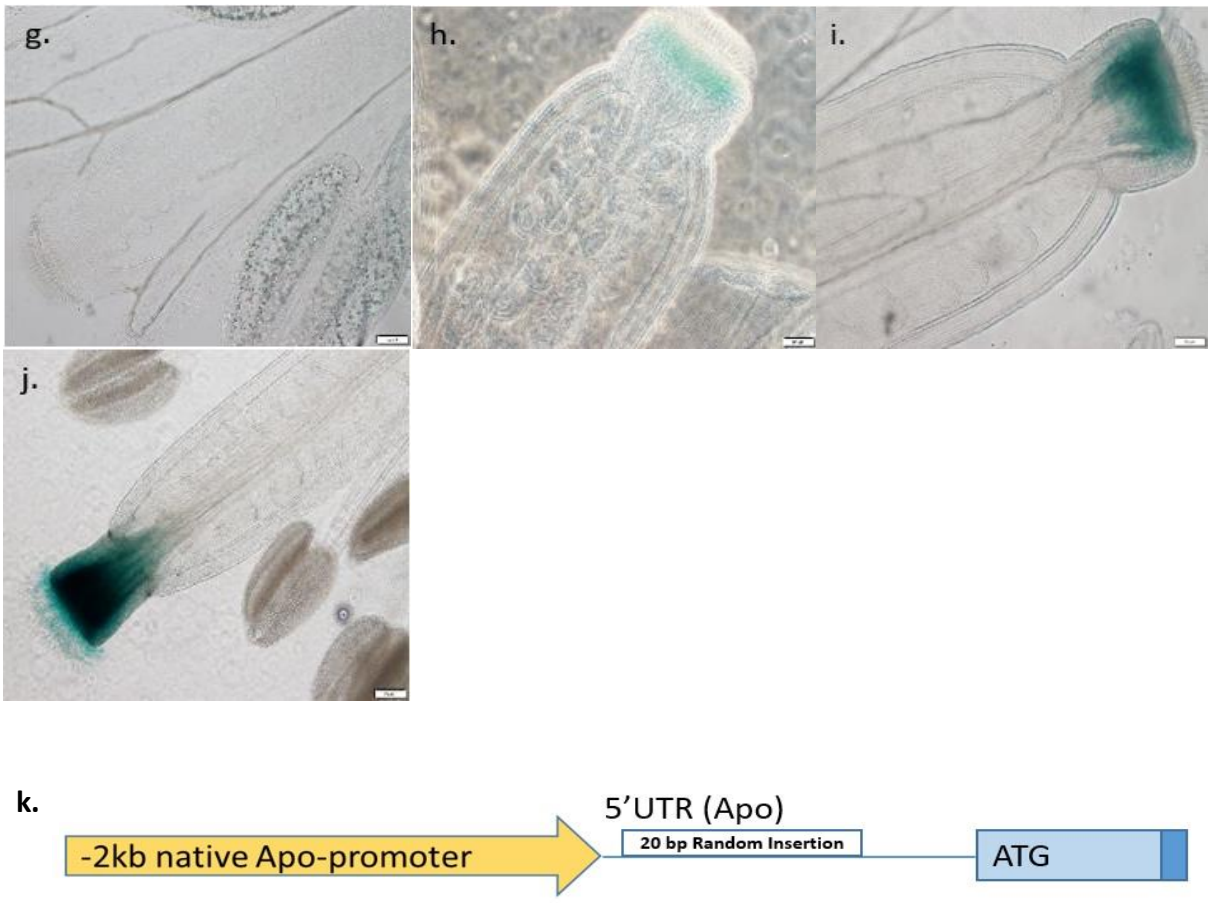


Figure 4.39 Expression patterns of spliced promoter construct (pC5: GUS) in *Arabidopsis* flowers. Construct pC5 promoters in different developmental stages of flowers, a. 0.7 mm flower Scale bar=0.5 mm, b. 1.0 mm flower with GUS activity in anther and receptacle, c. 1.5 mm flower with GUS activity in stigma and receptacle, d. 2.0 mm flower with GUS activity in stigma and receptacle, e. 3.0 mm flower with GUS activity in stigma and receptacle, (a-e) Scale bar=1.0 mm (Zeiss Lumar.V12 Stereoscope), f. 0.7 mm flower with GUS activity in anther and receptacle, g. 1.0 mm flower with GUS activity in anthers, h. 1.5 mm flower with GUS activity in stigma and receptacle, i. 2.0 mm flower with GUS activity in stigma and receptacle, j. 3.0 mm flower with GUS activity in stigma and receptacle, (g-k) Scale bar=50 µm (Olympus BX61 microscope), k. Construct 5 contains the 1,874 bp Apo promoter, 71 bp 5'UTR of Apo-allele with a random insertion (box) fused to a GUS reporter gene.

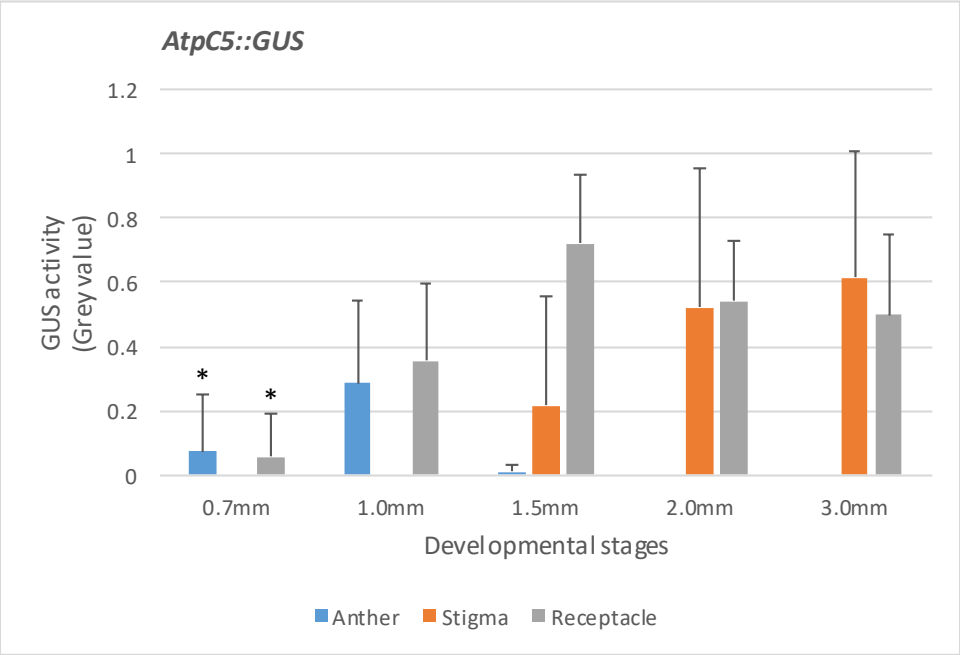
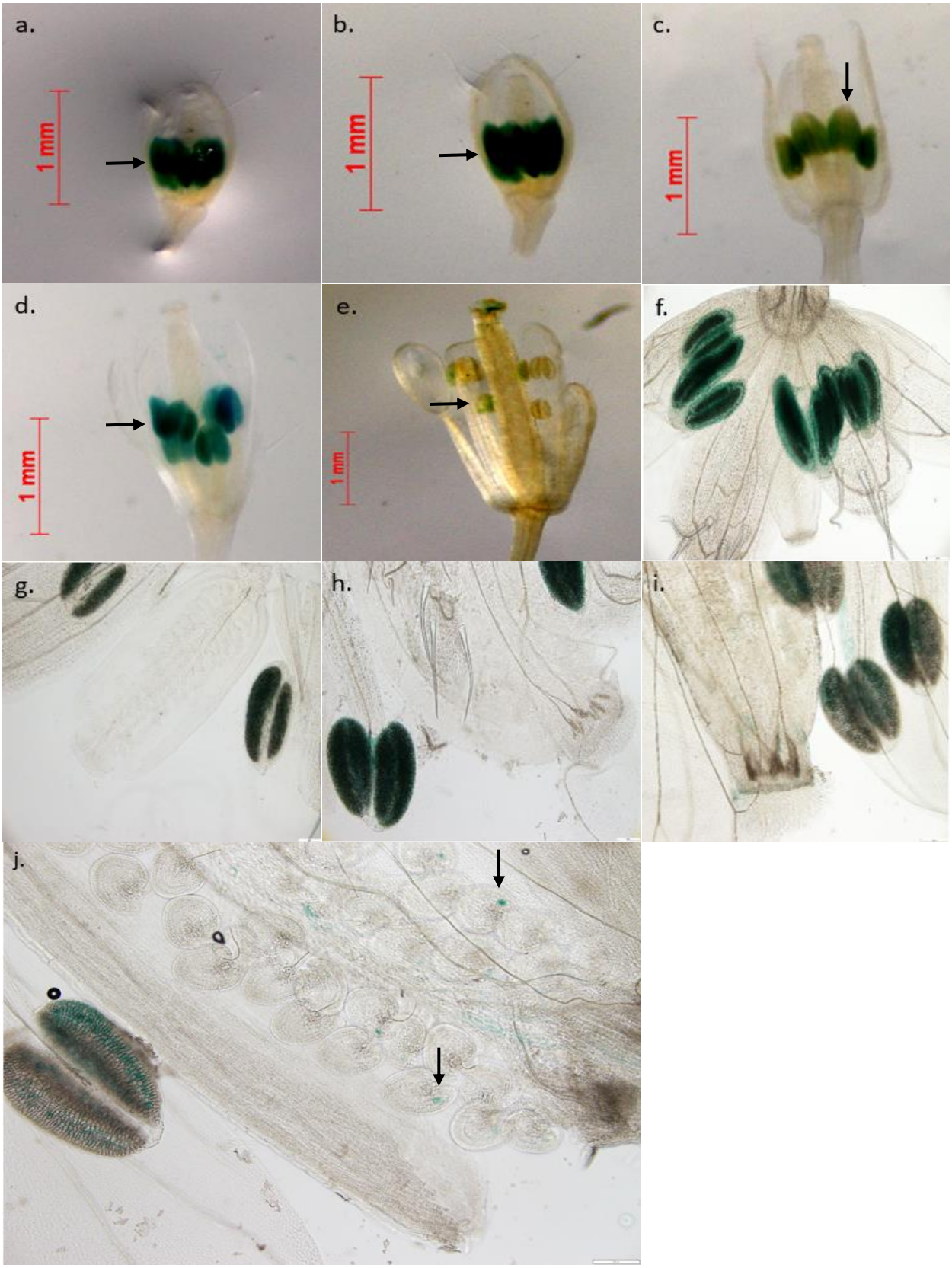


Figure 4.40 Quantification of GUS histochemical staining in flowers of pC5:GUS transgenic *Arabidopsis*. Graph showing the mean GUS intensity (SD) measured in different tissues (i.e., anther, stigma, and receptacle) and developmental stages within transgenic flowers. Asterisk indicates the first developmental stage is significantly different P≤0.05 from others (Tucky test, n=90 flower buds). The data were normalized using the Min-Max method. X-axis line represents different developmental stages of flower (based on the carpel height in mm). AtpC5:GUS means *Arabidopsis* transgenic for pC5:GUS.

4.3.3.6. Histochemical GUS activity for construct pC6 changes reproductive tissue-specificity of expression

This construct carried the 2 kb of pApo promoter, and the native 5'UTR of Sex-allele without the 20 bp Apo-insertion. The image J quantification of GUS activity for construct (pC6) was carried out for 4 transgenic lines each of which had five different developmental stages (total of 20 flower buds). Some anther-specific GUS activity with a varied intensity was observed for all developmental stages (Figure 4.41a, b, c, d, e), as well as what appears to be zygotic embryo cell-specific GUS activity in the final stage of flower development (after fertilization) (Figure 4.41j). Finally, the data was quantified using analysis of variance (ANOVA), and the second stage showed a significant difference from the other stages (P≤0.05; Tucky test) (Figure 4.42).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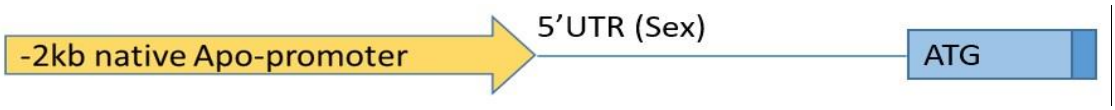


Figure 4.41 Expression patterns of spliced promoter construct (pC6: GUS) in *Arabidopsis* flowers. Construct pC6 promoters in different developmental stages of flowers, a. 0.7 mm flower with GUS activity in anther, b. 1.0 mm flower with GUS activity in anther, c. 1.7 mm flower with GUS activity in anther, d. 2.0 mm flower with GUS activity in anther, e. 3.0 mm flower with GUS activity in anthers, (a-e) Scale bar=1.0 mm (Zeiss Lumar.V12 Stereoscope), f. 0.7 mm flower with GUS activity in anther, g. 1.0 mm flower with GUS activity in anthers, h. 1.5 mm flower with GUS activity in anthers, i. 2.0 mm flower with GUS activity in anthers, j. 3.0 mm flower with GUS activity in anther in addition to zygotic embryo activity (black arrowheads)(f-j) Scale bar=50 µm (Olympus BX61 microscope), k. Schematic of construct pC6 contains 1,874 bp of Apo promoter, 104 bp 5'UTR of Sex-allele without the 20 bp Apo-insertion fused to a GUS reporter gene.

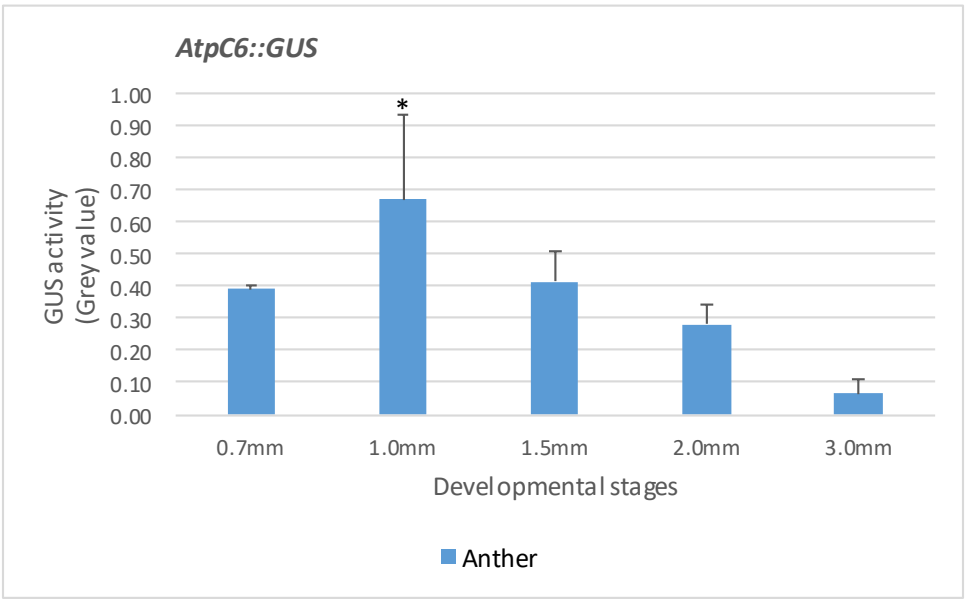
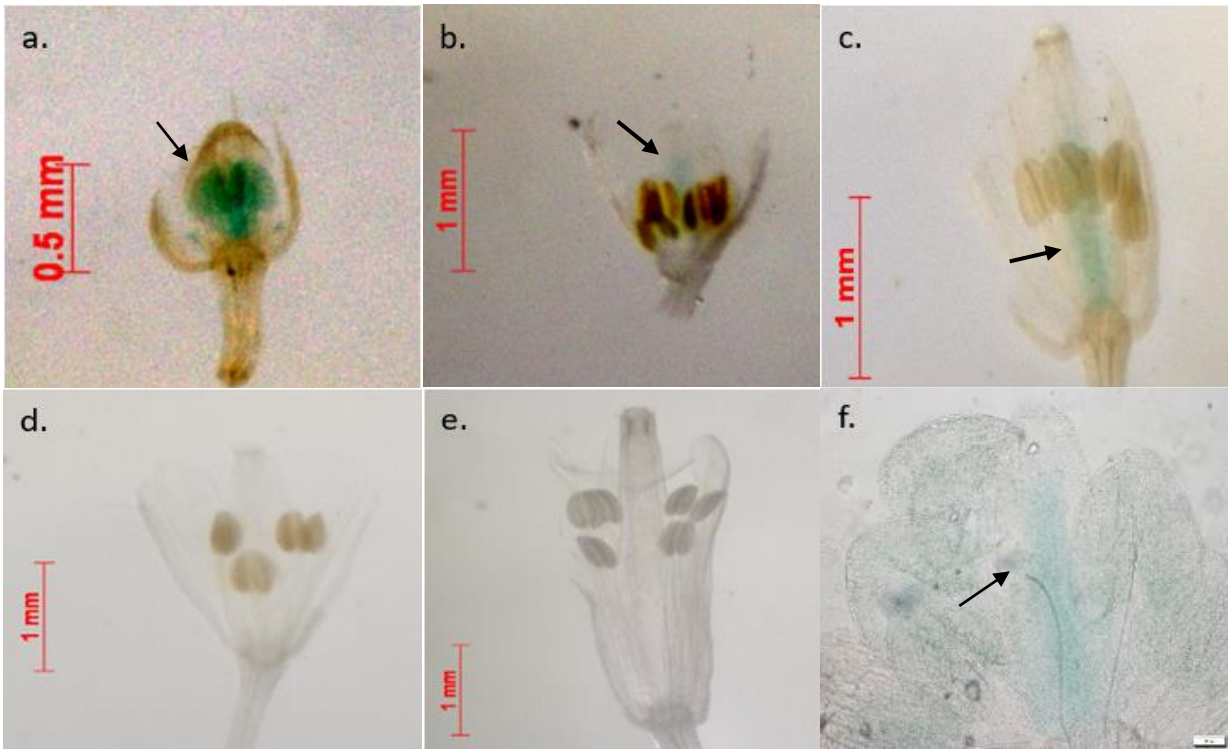
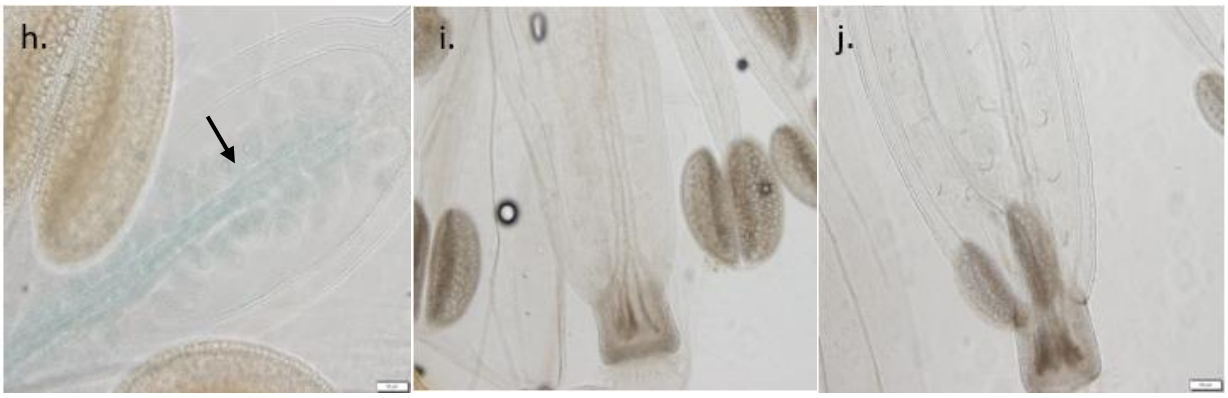


Figure 4.42 Quantification of GUS histochemical staining in flowers of PC6:GUS transgenic *Arabidopsis*. Graph showing the mean GUS intensity (SD) measured in anther of different developmental stages. Asterisk indicates the second developmental stage is significantly different P≤0.05 from others (Tucky test; n=20 flower buds). The data were normalized using the Min-Max method. X-axis line represents different developmental stages of flower (based on the carpel height in mm). AtpC6:GUS means *Arabidopsis* transgenic for pC6:GUS.

4.3.3.7. Histochemical GUS activity for construct pC7 changes reproductive tissue-specificity of expression

This construct carried the 2 kb of pSex promoter, the 5'UTR of Apo-allele, and the 20 bp Apo-insertion. The image J quantification of GUS activity for construct (pC7) was carried out for 6 transgenic lines each of which had five different developmental stages (total of 30 flower buds). Some GUS activity with a varied intensity was observed in pre-meiotic ovules and anthers for the first three developmental stages (Figure 3.43a, b, c, d, e). Finally, the data was quantified using analysis of variance (ANOVA), and stage 1 and 2 showed a significant difference from the other stages ($P \leq 0.05$; Tucky test) (Figure 4.44). To gain a better understanding of the GUS activity of different constructs and compare them based on different developmental stages, the summary results are shown in (Tables 4.8 and 4.9).





k.

Figure 4.43 Expression patterns of spliced promoter construct (pC7: GUS) in *Arabidopsis* flowers. Construct pC7 promoters in different developmental stages of flowers, a. 0.7 mm flower with GUS activity in anther and ovules, b. 1.0 mm flower with GUS activity in anther and ovules, c. 1.5 mm flower with GUS activity in anther and ovules, d. 2.0 mm flower, e. 3.0 mm flower, (a-f) Scale bar=0.5 and 1.0 mm (Zeiss Lumar.V12 Stereoscope), f. 0.6 mm flower with GUS activity (arrow) in anthers and ovules, g. 1.0 mm flower with GUS activity (arrow) in anther and ovules, h. 1.5 mm flower with GUS activity in anther and ovules, i. 2.0 mm flower, j. 3.0 mm flower, (g-l) Scale bar=100 µm (Olympus BX61 microscope), k. Schematic of construct 7 contains 1,866 bp Sex promoter, 71 bp 5'UTR of Apo including 20 bp Apo-insertion (box).

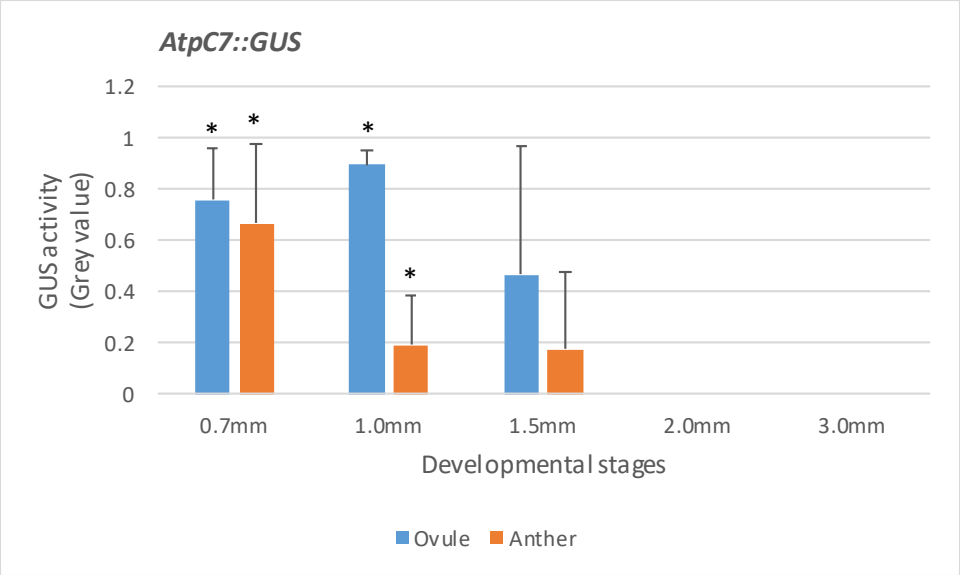


Figure 4.44 Quantification of GUS histochemical staining in flowers of pC7: GUS transgenic *Arabidopsis*. Graph showing the mean GUS intensity (SD) measured in different tissues and developmental stages within transgenic flowers. Asterisks indicate the first two developmental stages are significantly different P≤0.05 from others (Tucky test; n=30 flower buds). X-axis line represents different developmental stages of flower (based on the carpel height in mm). The data were normalized using the Min-Max method. AtpC7:GUS means *Arabidopsis* transgenic for pC7:GUS.

Table 4.8 The results of *Arabidopsis* and *Boechera* (JL107) transgenic for 2 kb native promoter and *Arabidopsis* transgenic for five different synthetic constructs.

Construct	Promoter	5'UTR	20 bp Apo-insertion	GUS leaf	GUS flower	Transformant	length
pC1	Apomictic	Apomictic	Positive	+	+	*At./Boechera*	2 kb
pC2	Sexual	Sexual	Negative	-	-	*At./Boechera*	2 kb
pC3	Apomictic	Apomictic	Negative	+	+	*At.*	2 kb
pC4	Sexual	Sexual	Positive	-	-	*At.*	2 kb
pC5	Apomictic	Apomictic	Random insertion of 20 bp	+	+	*At.*	2 kb
pC6	Apomictic	Sexual	Negative	+	+	*At.*	2 kb
pC7	Sexual	Apomictic	Positive	-	+	*At.*	2 kb

Table 4.9 Comparing the GUS activity in different tissues of all constructs based on different developmental stages. Only stage 1 represents the pre-meiotic stage of flower development.

	Stage 1	Stage 2	Stage 3	Sage 4	Stage 5
pC1	-	Stigma	Stigma	Stigma	Stigma
pC2	-	-	-	-	-
pC3	-	-	Filament/Stigma	Filament/Stigma	Filament/Stigma
pC4	-	-	-	-	-
pC5	Anther/Receptacle	Anther/ Receptacle	Anther/Stigma/Receptacle	Stigma/Receptacle	Stigma/Receptacle
pC6	Anther	Anther	Anther	Anther	Anther
pC7	Ovule/Anther	Ovule/Anther	Ovule/Anther	-	-

4.4 Discussion and conclusions

A comparison of gene expression in the micro-dissected ovules of apomictic versus sexual *Boechera* led to the discovery of the APOLLO gene. The heterozygous nature of APOLLO, as well as the presence of apomixis-specific polymorphism were proposed to be responsible for the differential gene expression between pre-meiotic ovules of apomictic versus sexual *Boechera* (Corral *et al.*, 2013). Although genomic and transcriptome data support this hypothesis, the APOLLO gene is still uncharacterized, and its function is only inferred in general terms. In this study a systematic survey of expression of native and synthetic APOLLO promoters fused to a GUS reporter gene was performed. The results with constructs regulated by native promoters demonstrated that there is a difference in gene expression between the pApo and pSex promoters, and the GUS activity in reproductive tissues shown here provides more resolution to the conclusions drawn by Corral *et al.* (2013).

(a) 1 kb native *Boechera* promoters do not drive gene expression in *Arabidopsis*

In the experiment with the 1 kb promoter, no GUS activity was observed for APOLLO Apo- or Sex-allele in transgenic *Arabidopsis* lines. Knowing that 1 kb of the eukaryotic promoter carries a TATA box (Figure 4.18) and/or other sequences needed for attaching RNA polymerase, which is necessary for transcription initiation, there may be some other elements needed to induce gene expression that are located upstream of the 1 kb region of the APOLLO promoter. Other possibilities are that APOLLO is regulated post-transcriptionally or the role of the 5'UTR and the 20 bp Apo-insertion in gene regulation is not as predicted by Corral *et al.* (2013). Thus, transgenic lines that contain a longer APOLLO promoter region, up to 2 kb, were made to check how they affect the gene expression.

(b) Expression of 2 kb native pApo in stigma and not ovules as predicted

Boechera transgenic lines for the 2 kb native pApo promoter showed stigma-specific GUS activity which started in the second stage of flower development and increased toward the final stages (Figure 4.29). Thus, there is a discrepancy between the data observed here and the ovule-specific Apo-allele gene expression that was predicted by Corral *et al.* (2013). An intriguing explanation might be that mRNA produced in stigma is then transported to the ovules in apomictic *Boechera* (Figure 4.29). Studies have shown that both messenger RNAs and small RNAs are able to move from cell to cell via plasmodesmata, membrane-lined, nanoscale

channels through the cell wall (Tilsner *et al.*, 2016). These moving RNAs have defined regulatory functions in development, nutrient homeostasis, stress adaptation, and plant-microbe and plant-plant interactions. The so-called mobile mRNAs traffic through the phloem to regulate many developmental processes, but despite the burgeoning discovery of them, little is known about the mechanism underlying the intracellular sorting of these mRNAs (Luo *et al.*, 2018). In the reproduction context, small RNAs appear to move from the central cell to the egg cell and synergids of the female gametophyte prior to fertilization (Erdmann *et al.*, 2017).

In addition, the absence of GUS activity in pre-meiotic ovules for the 2 kb native Apo-allele transformants as predicted by Corral *et al.* (2013) may mean that the promoter is not long enough for its induction. Another explanation might be that APOLLO is not regulated transcriptionally (e.g., post transcriptional down regulation). Thus, making transgenic lines with a longer Apo promoter such as 4 kb or longer is suggested.

(c) No GUS activity observed in apomictic *Boechera* transgenic for 2 kb native Sex-promoter

While there was GUS activity in the stigma for the 2 kb native Apo promoter in the apomictic *Boechera* transformant, none was observed for the 2 kb Sex promoter, and this is consistent with the observations of Corral *et al.* (2013). The introgression of a sexual allele containing the 20 bp Apo-insertion from a sexual ancestor into other *Boechera* species through hybridization, followed by its fixation due to the lack of recombination (i.e., apomixis) has led to the establishment of the heterozygosity for APOLLO in apomictic *Boechera* (Corral *et al.*, 2013). It is hypothesized that the trans-acting effects of TFs on Sex- versus Apo-alleles led to their altered GUS activity in apomicts relative to sexuals (Corral *et al.*, 2013).

It may be that architectural differences between the Apo- and Sex-allele result in either the recruiting of different TFs, or the same TFs with a divergent function. This is supported by the yeast one-hybrid assay data produced by Hybrigenics (see chapter 3), whereby the TFs detected for apomictic *Boechera* were different from those of sexual *Boechera* with only one transcription factor in common (*ERF107*; Tables 3.3 and 3.4). One implication of these data is that the *ERF107* has a diverged function from apomictic versus sexual *Boechera*.

(d) Differences observed in GUS activities for the native 2 kb Apo promoter between *Boechera* and *Arabidopsis* transgenic lines

Conservation of the 20 bp apomixis-specific insertion in the 5'UTR of Apo-alleles of genetically and geographically diverse *Boechera* spp., an insertion which shares more than 80% sequence similarity with *A. thaliana* and *B. rapa*, implies its single evolutionary origin from a common ancestor (Corral *et al.*, 2013). In addition, it was hypothesized that interaction between the orthologues of APOLLO and their regulatory factors in *A. thaliana* and *Brassica* spp. leads to normal sexual seed formation (Corral *et al.*, 2013). In support of this, the GUS activity observed for *Arabidopsis* lines transgenic for the 2 kb native Apo-allele promoter was different from that of apomictic *Boechera* transgenic lines. This variation is likely related to species-specific differences between their regulatory factors, as apomictic *Boechera* has been hypothesized as having a diverged set of regulatory factors related to the hybridization event (Corral *et al.*, 2013).

The similar GUS activity patterns in *Boechera* and *Arabidopsis* which are transgenic for the Apo-allele is expected considering the monophyletic origin of the Apo insertion in *Boechera* spp. and other *Brassicaceae*, compared to the Sex-allele which is phylogenetically-younger (Corral *et al.*, 2013). Due to the difficulty of tissue culture and transforming *Boechera*, only apomictic transgenic lines were possible at the time of this thesis. The GUS activity observed in *Boechera* transformed with the Apo-allele are reflective of the "wild type" genetic background, since the Apo-promoter is equipped with the regulatory machinery needed for Apo-allele gene expression. Ideally a sexual accession should be used for making transgenic lines for the Sex-allele, which was not possible in this study due to the difficulties of tissue culture.

(e) GUS activity for five different synthetic promoters in *Arabidopsis*

The second part of the experiment was using synthetic APOLLO promoter constructs to study different components of the Apo vs. Sex-alleles located around 2 kb upstream of the transcription start site. A comparison of GUS activity in *Arabidopsis* for the same developmental stage in all 5 constructs demonstrated that changes to the APOLLO promoter produced shifts in tissue and developmental stage specificity when compared to the 2 kb native promoter constructs. Though transgenic lines carrying the native 2 kb Apo promoter which were used in this study did not result in ovule-specific GUS activity as expected by Corral *et al.* (2013), it was a base for comparison with other constructs.

(e.1) GUS activity for *Arabidopsis* lines transgenic for pC3

Transgenic pC3 lines carry the 2 kb native pApo promoter and the 5'UTR of the Apo-allele without 20 bp Apo-insertion (i.e., the Sex-allele deletion), or in other words pC3 is a pC1 construct with the 20 bp Apo-insertion deleted (Figure 4.7). Compared to the transgenic lines for pC1 (2 kb native pApo promoter) where their GUS activity was limited to the stigma, pC3 showed GUS activity in filaments and stigma in earlier and later stages of flower development respectively. Thus, the absence of the 20 bp Apo-insertion changes expression from female to male tissue, in addition to the timing of expression.

Deletion of the 20 bp Apo-insertion thus likely leads to the loss of the recognition site for regulating interaction elements that control APOLLO expression in specific floral tissues, for example *ERF5* and *ERF15* (Chapter 3; Table 3.4). In addition, the GUS activity observed in transgenic lines for pC3 showed a shift from filament to stigma. This observation raises the question of how the shifting in GUS activity occurs. The GUS activity observed in the stigma in later stages of flower development (after fertilization) may mean that the localization of GUS activity for pC3 is affected by specific signals from pollen.

(e.2) GUS activity for *Arabidopsis* lines transgenic for pC4

Transgenic pC4 lines carry the 2 kb native pSex promoter, and the 5'UTR of Sex with the 20 bp Apo-insertion. In other words, pC4 is a pC2 (2 kb native Sex promoter) with the addition of the 20 bp Apo-insertion, and no GUS activity was observed as was the case with the pC2 transgenic lines (Figures 4.28 and 4.38). Knowing that except for the 20 bp Apo-insertion every other part of this construct belongs to Sex-allele, it can be inferred that the APOLLO specific polymorphism itself is not sufficient to produce any GUS activity, or the 2 kb upstream promoter region of the Sex-allele is not enough to drive expression.

(e.3) GUS activity for *Arabidopsis* lines transgenic for pC5

Transgenic pC5 lines carry the 2 kb native pApo promoter, and the 5'UTR of Apo-allele with a random 20 bp insertion (i.e., Apo insertion but different sequence). In other words, pC5 is a pC1 (2 kb native Apo-promoter) with its 20 bp Apo-insertion substituted by a random insertion. It showed GUS activity in anthers, stigma, and receptacle (Figure 4.39), and the expression patterns observed in stigma are to some extent similar to that of detected in pC1 transgenic lines

(Figure 4.29). Thus, the insertion of the random 20 bp down-stream from the Apo promoter and 5'UTR of Apo changes the GUS tissue-specificity pattern. This observation is similar to that of pC3, in that the absence of 20 Apo-insertion has led to the loss of recognition sites for Apo interacting factors. Importantly, the observation of pC5 transgenic lines in which manipulation of the 20 bp insertion led to stigma, receptacle, and anther-specific GUS activity (Figure 4.39), points to the fact that regulatory elements which are specific to the 20 bp Apo-insertion are tied to the deviation from exclusive stigma-specific GUS activity observed for pC1 transgenic lines (Figure 4.29).

During the initial stages of *Arabidopsis* flower development, the primordium is delineated morphologically into a distal floral meristem and a proximal pedicel. As flower development continues, a receptacle is defined as the point of attachment of the flower with the underlying stem (pedicle) tissues, and the cell differentiation at this junction has the same pattern as that of the distal floral meristem (Douglas and Riggs, 2005). Thus, the GUS activity observed at the receptacle may point to the role of *AP2*, one of the TFs that interact with APOLLO Apo-allele, on floral development (Table 3.4). As mentioned in chapter three *AP2* participates in the A, B and C model of flower development in which genes specify floral organ identity through involvement in the cell cycle and flower development. The results of other studies show that mutations in floral meristem identity genes such as *LFY* and *AP1* hinder distal pedicel elaboration, meaning that its formation is tied to floral fate (Douglas and Riggs, 2005).

(e.4) GUS activity for *Arabidopsis* lines transgenic for pC6

Transgenic pC6 lines carry 2 kb of the Apo promoter and the 5'UTR of the Sex-allele (i.e., with a 20 bp deletion). Histochemical GUS staining yielded a prominent signal in anthers which was observed in both pre-meiotic and post-meiotic floral buds (Figure 4.41). As the native Apo-allele promoter (pC1) drives expression in *Arabidopsis* while the native Sex-allele promoter (pC1) does not, the data here demonstrate that the Apo-promoter upstream of the 5'UTR drives flower-specific expression, while the 5'UTR is responsible for male- or female-specific tissue expression. The observation of this pattern in pre-meiotic and meiotic stages can be interpreted as the association of pC6 with an unknown meiotic function in male tissues. In addition, what appears to be a zygotic embryo-specific GUS activity observed in later stages of flower development (Figure 4.41) demonstrates that the localization of pC6 GUS activity may be

affected by fertilization. It also may mean that there is a correlation between the function of UPGRADE, another gene known for induction of apomixis through pollen, and APOLLO. Alternative microscopic methods are required to confirm GUS activity in egg-cells

(e.5) GUS activity for *Arabidopsis* lines transgenic for pC7

Finally, transgenic pC7 lines carry the 2 kb native Sex promoter, 5'UTR of the Apo-allele with the 20 bp Apo-insertion. This construct shows both ovule and anther-specific GUS activity in earlier stages of flower development (Figure 4.43). As the native Sex-allele promoter (pC2) showed no activity in *Arabidopsis* flowers (Figure 4.28), these data demonstrate that the Apo 5'UTR can drive both male- and female-specific expression of APOLLO. This is consistent with the data of Corral *et al*. (2013), which showed that the Apo-allele 5'UTR of *Boechera* is relatively similar in sequence to *Arabidopsis* and *Brassica* compared to the Sex-allele 5'UTR, and supports the hypothesis that the Apo-allele 5'UTR is phylogenetically-older, having originally been derived from sexually-reproducing species.

It is unclear whether the pC7 promoter achieved its novel GUS activity pattern through positive and/or negative gene regulation associated with elements residing in the 2 kb Sex promoter and Apo-allele 5'UTR (with Apo-insertion). Furthermore, this construct is the only one that shows GUS activity in pre-meiotic ovules (Figure 4.43), which implies the 5'UTR of Apo-allele underlies its regulation. An explanation for why 20 bp Apo-insertion with its 5'UTR did not produce such pattern in transgenic lines for the native 2kb Apo-promoter may reside in the 3D structure of the promoter. Enhancers can communicate their defined activities across large genomic distances by physically contacting distal promoters via chromatin folding, and this regulatory landscape can drive a complex and flexible patterns of transcriptional activity (Robson *et al.*, 2019). One possibility is that the 2kb Sex promoter has some motifs similar to sequences located in the distal Apo promoter, with this leading to ovule-specific GUS activity in pC7, although additional data from (eg.) ovule microdisections is need to determine the exact locality of GUS activity

Another explanation for the pC7 construct data is that the substitution of the Apo- with the Sex-promoter has limited access of the regulatory machinery to only the 5'UTR of the Apo-allele (carrying 20 bp Apo-insertion), whereby the 2 kb Sex promoter did not contribute to expression. Thus, it can be implied that the 5'UTR of the Apo-allele is the main player driving

ovule-specific GUS activity. Another possible explanation is that the 2 kb Sex promoter contains motifs that actively contributed to the observed GUS activity pattern, although this is not consistent with the negative native Sex promoter activities in *Arabidopsis* and *Boechera* (pC2; Figures 4.26 and 4.28).

It seems that the 20 bp Apo-insertion is providing transcription factor binding sites which contribute to tissue-specificity of GUS activity, and the data from chapter 3 support this conclusion. In addition, it has been suggested that the 20 bp Apo-insertion could lead to repression or activation of APOLLO in apomictic MMC (Corral *et al.*, 2013), and the data from chapter 3 points to loss of a recognition site for a negatively regulating interaction elements such as *ERF1B* and *GeBP* (Table 3.4) or through interaction with positive regulatory elements such as *ERF5*, *ERF107*, and *ERF15* (Table 3.3).

In addition, the data presented in this study show that the enhancing activity of the 20 bp Apo-insertion is influenced by its nearby sequences, such as the 5'UTR. Despite the importance of the nearby sequences, the existence of distal enhancers cannot be ruled out. Studies on chromosome conformation show that even distal enhancers are commonly in physical proximity with their target genes. In fact, in higher eukaryotic cells, regulatory elements can reside up to hundreds of kilobases away from the genes they control, but can be brought into spatial proximity through chromatin loop formation (Deng and Blobel, 2017). Therefore, another comprehensive study by using techniques such as Hi-C (Lieberman-Aiden *et al.*, 2009) could shed light on the importance of distal enhancers on the APOLLO gene expression.

In summary it can be inferred that deletion and randomization of 20 bp Apo-insertion led to changes in tissue-specificity patterns of GUS activity compared to that of native promoters. It can be concluded that the 20 bp Apo-insertion affects the specificity of GUS activity in female versus male organs. For instance, the absence of the 20 bp Apo-insertion from the 2 kb native Apo promoter (pC1) which formed construct pC3, produced additional filament-specific GUS activity. This may mean that the Apo-insertion provides regulatory binding sites for TFs such as *ERF5*, *ERF15*, and *ERF107* (Table 3.4) that counter negatively controlled APOLLO expression in male tissue. The addition of the 20 bp Apo-insertion into the native Sex 5'UTR to create construct pC4 did not change the GUS activity from that of pC2 (i.e., no GUS activity observed for both). Finally, the randomization of the 20 bp in native Apo promoter to create pC5 lead to

changes in timing and locality of GUS activity indicating that the Apo-insertion is an important factor in APOLLO Apo-allele regulation.

Constructs pC6 and pC7 had different structures from previous synthetic promoters. pC6, with the Sex-allele 5'UTR combined with the 2 kb of Apo promoter showed a prominent anther-specific GUS activity which started in meiotic stages and shifted to zygotic embryos in later stages of flower development. pC7, with the Apo-allele 5'UTR combined with the 2 kb Sex promoter showed prominent ovule and anther-specific GUS activity in early stages of flower development. It can be inferred that the original 5'UTR of Sex- or Apo-allele can cause male and female-specific GUS activities respectively, and importantly this happens early on during flower development. It can be concluded that the 20 bp Apo-insertion including its surrounding sequences are correlated with female-specific GUS activity, or that the 20 bp Apo-insertion *per se* is not sufficient to confer reporter expression in female tissues.

The analysis of these synthetic constructs showed that specific parts of Apo-promoter, specifically the 5'UTR and 20 bp Apo-insertion, are associated with expression of APOLLO in female tissues. In this light, the behavior of the APOLLO allele promoters is similar to that of genes in sex-determining regions (SDR) of sex chromosome (Renner and Müller, 2021). In sex chromosomes, genes with male function accumulate on the Y chromosome over time, and likewise, genes with the female-biased function will accumulate on the X chromosome (Harkess *et al.*, 2020). The data gained from the study on seven promoter constructs demonstrated that Apo-promoter sequences, specifically the 5'UTR and the 20 bp Apo-insertion, drive GUS activities in female tissues (e.g., stigma for construct pC2 & ovule for construct pC7), while the Sex promoter 5'UTR contributes to driving expression in male tissues (anther). Considering that apomixis is *per se* a female trait, female-specific expression of genes controlling apomixis is expected and supported by the data presented here.

Another characteristic of sex chromosomes reflected in the APOLLO gene is that the crossing-over is suppressed in the segments of sex chromosomes which contain the SDR genes (Darlington, 1931; Westergaard, 1958). Crossing over is by definition suppressed around the 20 bp Apo-insertion located in the promoter of the APOLLO Apo-allele as a result of apomixis. Thus, it can be implied that APOLLO Apo- and Sex-promoters possibly act similarly to that of sex-linked genes. Based on this model, the Sex promoter contributes to normal meiosis and

sexual development, while the Apo-promoter participates in apomeiotic ovule development in *Boechera*, a female-linked characteristic. Another example of sex-linked genes in plants is *NO TRANSMITTING TRACT* (*WIP2/NTT*) in *Arabidopsis*, which encodes a CH2H/C2HC zinc finger transcription factor that is specifically expressed in the transmitting tract and funiculus of ovules, playing a key role in fruit development (Harkess *et al.*, 2020).

A biological model can be developed from this study based on the data gained from the yeast one-hybrid assay and other studies (Shah *et al*, 2016). It has been proposed that the interaction of *AP2* and *GeBP* with motifs of the Sex promoter and/or their own antagonistic interactions initiate events leading to a normal meiosis in sexual *Boechera*. In contrast, the interaction of *AP2* (*ERF5*, *ERF107*, and *ERF15*) with the 20 bp Apo-insertion drives APOLLO Apo-allele (exonuclease) expression, leading to down regulation of meiosis regulators and initiation of apomeiosis in apomictic *Boechera*. Recent whole-genome sequencing of sexual and apomictic *Boechera* further support (and validate) these findings (T.F. Sharbel, unpublished data), although a discussion of these data is beyond the scope of this study.

(f) Hybridization is associated with the differential gene expression between apomictic versus sexual *Boechera*

The elevated heterozygosity in diploid apomictic *Boechera* can be explained by widespread hybridization associated with the transition from sexuality to asexuality, or it could be a byproduct of apomixis itself. In apomictic individuals the overall heterozygosity is expected to increase over generations due to the lack of recombination and mutation accumulation (Meselson effect; Hojsgaard and Hörandl, 2015). Based on the result of one study on an apomictic *Boechera* population, there was clear evidence for the hybrid origin of the highly heterozygous apomicts (Beck *et al.*, 2012). In addition, mutation accumulation on a genomic level in apomictic *Boechera* has clearly been shown in conserved non-coding sequences (Lovell *et al*., 2017).

Unlike apomictic *Boechera* the sexual diploid species such as *B. stricta*, are typically less heterozygous and are divergent only at neutral loci (Beck *et al.*, 2012). The interspecific "collision" of different sexual genomes, may produce apomictic *Boechera* with some structural changes in the genome such as homeologous chromosome substitutions and aneuploidy (Kantama *et al.*, 2007). If divergence extends to the regulatory genes, apomixis as a novel characteristic could result from the novel pattern of gene expressions created by the interaction

of divergent transcriptional regulators (Carman, 2001). The data reported in this study show that the variation observed in GUS activity between transgenic lines for pApo native vs. pSex promoters is consistent with the divergent theory of transcriptional regulators and supports the phylogenetic hypothesis proposed in Corral *et al.* (2013).

The similarity of the APOLLO gene in *Boechera* with that in *A. thaliana* was one of the reasons that transgenic lines of *Arabidopsis* were used for studying synthetic promoters (i.e., monophyletic origin of APOLLO; Corral *et al.*, 2013). The difference reported in GUS activity between *Arabidopsis* and *Boechera* spp. for the 2 kb native pApo construct is likely associated with species-specific regulatory machinery, although the differences in their TF networks is minor, such that *Arabidopsis* can reliably be used for studying APOLLO functionality.

(g) The APOLLO function in *Boechera* probably is associated or is parallel with cyclins or other meiotic regulators

The variation of the GUS activity patterns observed for native and synthetic constructs may mean that their expression is associated with the cell cycle. For example, in construct pC7 ovule-specific GUS activity was observed in the pre-meiotic stage which decreased over time. The phase-dependency and tissue-specificity of GUS activity reported here may imply that there is a correlation between the function of APOLLO with cyclins or other meiotic regulators. Cyclins are a family of proteins that control cell cycle progression by activating cyclin-dependent kinase (CDK) enzymes or groups of enzymes required for synthesis (Galderisi *et al.*, 2003). It has been demonstrated that the duplication and evolution of cyclin genes in plants was also accompanied by functional diversification (Bulankova *et al.*, 2013).

The role of *AP2* in somatic embryogenesis and cell proliferation has been also the subject of other studies (Boutilier *et al.*, 2002; Koltunow *et al.*, 2003). However, no expression was detected for *AP2* in the zygote analyzed at such early stages suggesting that they may not be directly involved in embryo initiation (Gagliardini & U. Grossniklaus, unpublished data; Koltunow *et al.*, 2003). Their overexpression possibly causes some type of stress or activation response that leads to the de-differentiation of specific cells (Koltunow *et al.*, 2003). Klucher *et al.* (1996) speculated that *AP2/ERF* domain proteins, being unique to plants, might have coevolved with plant-specific pathways such as hormone signal transduction (Boutilier *et al.*, 2002). The result of one study on the role of *AP2* transcription factors on cell proliferation,

differentiation and growth of *Arabidopsis* leaves revealed that these alterations are through expression changes in genes such as *RBR*, *TCP*, cyclines (CYCD), as well as genes involved in cell expansion (ADF5) (Marsch-Martinez *et al*., 2006). Thus, it can be inferred that *AP2* reported in this study not only interacts with APOLLO, but also can lead to the up-regulation of cyclines or other genes.

The data gained from another study which compared gene expression profiles of the Apomictic Initial Cell (AIC) and the Megaspore Mother Cell (MMC) between apomictic *Boechera* and sexual *Arabidopsis* suggests that protein ubiquitinylation and degradation, as well as cell cycle control, may be differentially regulated between the MMC and AIC (Schmidt, 2020). The manipulation of cell cycle progression or meiotic genes has also been shown to lead to the formation of unreduced gametophytes (d'Erfurth *et al.*, 2009). Finally, an enrichment in expression of cell cycle related genes was also documented in *Hieracium prealtum* AIC and embryo sacs (Okada *et al.*, 2013).

5. General discussion concluding remarks and future perspectives

The previous study conducted by (Corral *et al.*, 2013) compared the microarray data of sexual and apomictic *Boechera* and identified APOLLO as an apomixis candidate gene followed by suggesting that *cis*- regulatory elements in its promoter are responsible for APOLLO's differential expression. Although microarray data are informative to provide gene expression signatures for tissues under various experimental conditions, these data fail to reveal the transcriptional regulatory mechanisms that control differential gene expression. One reason might be that the TFs binding to *cis*-regulatory elements may not have been experimentally characterized. In addition, many TFs belong to larger families with members that can share similar DNA-binding domains and overlapping DNA recognition elements. Finally, mRNA levels measured by microarrays may not be a good indication of the transcription levels, but the result of a balance between direct and indirect transcriptional effects that lead to the differences in mRNA levels. Thus to gain a better understanding of transcriptional mechanisms that lead to differential gene expression, identifying TFs that interact with those genes of interest is necessary (Deplancke *et al.*, 2004).

In order to identify protein interactions with APOLLO promoters' motifs including the Apo and Sex-alleles, a yeast one-hybrid assay was used in this study. In this experiment a number of TFs were identified which putatively interacted with the 5'UTR motifs of Apo- and Sex-alleles used as the bait constructs. These TFs belong to the *APETALATA2/ERF* family, and were also reported to regulate apomixis through metabolic factors rather than apomixis-specific mutations (Khanday *et al.*, 2019). These findings may be helpful for understanding the regulatory mechanisms that control APOLLO expression, but do not provide any evidence on the role of enhancers or other factors as they can be located either upstream or downstream of the promoter, on the same or even on different chromosomes. The proteins detected for the yeast one-hybrid assay for both sexual and apomictic libraries belong to enzymatic, structural and regulatory families. Among the TFs detected in the *B. stricta* library *GeBP* activity can diminish the ROS consequences for a plant through the production of metabolites such as polyamines (putrescine, spermidine, and spermine), or through DNA repair during meiosis (Bleuyard *et al.*, 2006). The result of another study comparing the transcriptomes between the MMC of *Arabidopsis* and AIC of apomictic *Boechera gunnisoniana* showed that polyamine and spermine/spermidine metabolism is only enriched upon initiation of the apomictic germline (Schmidt *et al.*, 2014). It

can be implied that *GeBP* regulatory function in sexual *Boechera* in the absence of polyamines might be through the mere meiosis function on DNA repair. In addition, *ERF1* detected in the sexual prey library against *B. stricta* (bait sequence) can be associated with sexual reproduction in the host population through selection of rare genotypes that are resistant to infection (Red Queen theory) (Park and Bolker, 2019).

The detection of *AP2* in cDNA libraries interacting with the APOLLO promoter suggest a switch in metabolic cycles leading to a response to stress in apomictic *Boechera*. In this light, apomicts with highly heterozygous genomes which are co-adapted with environmental stresses could potentially tolerate mutation load in the absence of recombination. The enrichment of polyamines in the early phase of apomictic germlines as reported by Schmidt *et al*. (2014) may mean that apomictic *Boechera* harnesses oxidative damage, decreasing the need for meiosis and recombination in the apomicts. It is possible that the APOLLO gene in apomictic *Boechera* can decrease the effects of meiotic regulators by its exonuclease activity, or its DHHD activity could be limited to the degradation of small interfering RNAs predicted by Corral *et al*. (2013). A model that describes the mechanisms of response to stress in apomictic and sexual *Boechera* is shown in Figure (4.45).

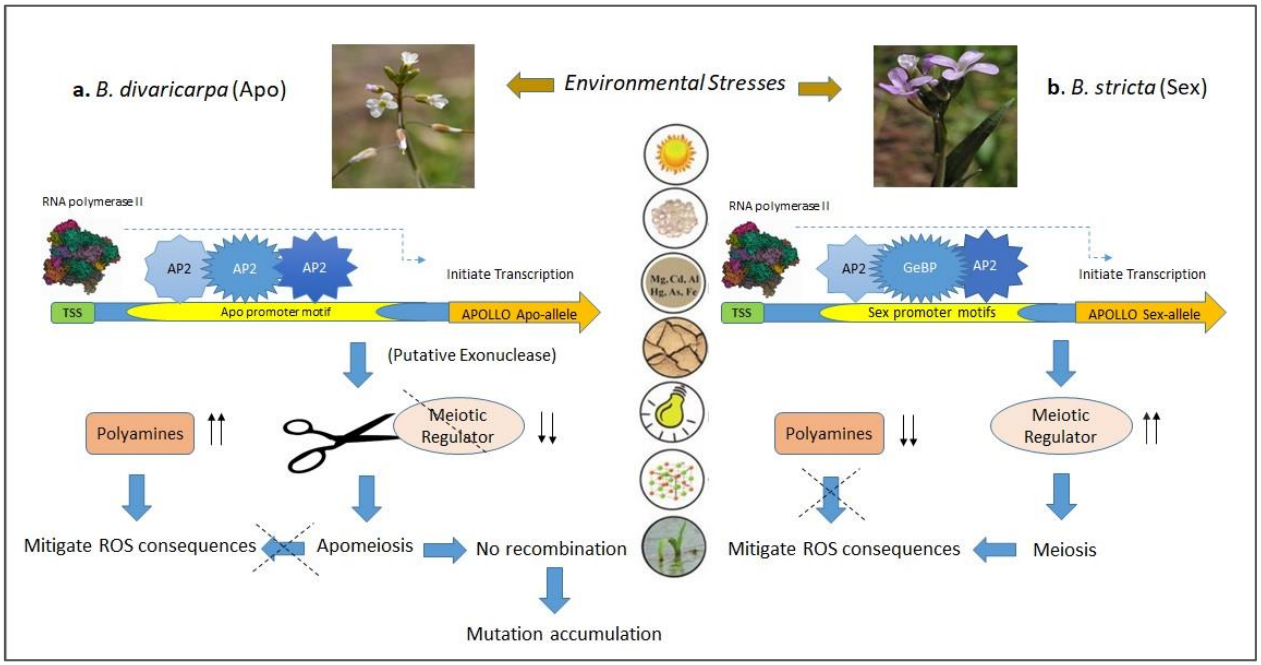


Figure 5.1 A model that describes the events that occur in apomictic versus sexual *Boechera* while they are imposed on environmental stresses. a. The TFs binding to the 20 bp Apo-

insertion/Sex-deletion lead to the initiation of transcription for Apo-allele which results in down-regulation of meiotic regulators and apomeiosis. The high amounts of polyamines in turn harness the consequences of ROS for apomictic *B. divaricarpa*. b. The TFs binding to motifs of the Sex promoter leads to initiation of transcription which results in meiosis. Meiosis in turn harnesses the consequences of ROS for sexual *B. stricta* in the absence of polyamines. Abbreviations, TSS means Transcription Start Site.

The above model can explain a series of events promote shifting from sexual to apomictic reproduction in facultative *Boechera* under variable environmental conditions. However, it does not describe wether it would happen in one single step or through a longer (evolutionary) time span. In addition, APOLLO is associated with the production of unreduced gametes (apomeiosis), and not parthenogeneis. Nontheles, it is plausible that a common key regulator control both components in apomictic *Boechera*. In fact, the genetic control of parthenogenesis and apomeiosis can be hypothesized to rely on a single gene (Mogie, 1992). However, parthenogenesis can clearly segregate from apomeiosis (Vijverberg *et al.,* 2019), including in *Boechera* (Mau *et al.,* 2021).

A study on the transcriptional changes occurring in the ovule nucellus before the failure of meiosis and differentiation of aposporous initials in *Hypericum perforatum* showed strong differences in the expression of several genes encoding transposable elements (Galla *et al.*, 2019). In addition, transcriptional differences in the ovule nucleus and pistil terminal developmental stages were also found for a subset of genes encoding for potentially interacting proteins involved in pre-mRNA splicing (Galla *et al.*, 2019). Furthermore, differential expression in genes operating in RNA silencing, RNA-mediated DNA methylation (RdDM) and histone and chromatin modifications was observed in sexual versus apoporous ovule transcriptome (Galla *et al.*, 2019). These *Hypericum* data point to similar mechanisms, such as RNA splicing and RNA-directed DNA methylation, might occur in the ovule of apomictic *Boechera* which are correlated with the failure of meiosis.

The *in vitro* results presented in chapter 3 demonstrated that the *ERF* family of transcription factors interacts with the 20 bp Apo-insertion of the Apo-allele and motif present in the regulatory regions of Sex-allele. To further confirm the importance of the TF interactions with its target genes *in vivo*, transgenic lines were used that express the β-glucuronidase (GUS) reporter gene driven by 2 kb of the 5′ upstream promoter and untranslated regions of APOLLO (*pApo*::*GUS*). The data revealed tissue-specific GUS activity in flowers and leaves

and this pattern can be explained by the fact that the 20 bp Apo-insertion with its 5'UTR changes GUS activity from that of the Sex-allele. The data gained here is not totally in accordance with the ovule expression pattern as predicted by Corral *et al*. (2013). Although, the differential gene expression patterns obsrved points to the fact that structural changes reside in APOLLO promoter has an evolutionary function. Further studies such as using Hi-C (Lieberman-Aiden *et al.*, 2009) can reveal if other factors upstream of Apo promoter can affect the APOLLO gene expression.

Based on the genomic collision model, gene regulation in an allopolyploid species such as *B. divaricarpa* is governed by *cis-trans* regulatory relationships. This includes the intra-genome interactions derived from each of the parental diploids, in addition to newly formed inter-genome interactions (Hu and Wendel, 2019). The hybridization event leading to the introduction of the apomixis-specific polymorphism into *B. divaricarpa* also coincided with the divergence of the regulatory machinery. It can be inferred that TFs of the ancestral sexual *B. stricta* acting in trans on the newly derived apomictic genome, that is to say the APOLLO Apo-allele, underly differential gene expression between the Apo- and Sex-alleles.

The GUS activity patterns observed in this study also can be described through the ABC model of gene expression in the context of the TFs. Based on this model of gene expression three classes of homeotic functions (A, B and C) genes specify floral organ identity in a combinatorial way. *APETALA2* is thought to provide the A-function which specifies the identity of perianth organs (sepals and petals in eudicots) (Wu *et al.*, 2017). In addition to its main role, *AP2* is often involved in repression of other class-C genes (*AG*) that control sepal-to-carpel and petal-to-stamen homeotic conversion (Monniaux and Vandenbussche, 2018). It can be inferred that *AP2* in combination with other TFs can produce variable gene expression in floral tissues, as shown here in transgenic lines for the 2 kb native Apo-promoter and synthetic constructs.

In addition, *ERF BUD ENHANCER* (*EBE*; At5g61890) which encodes a member of the (*AP2/ERF*) transcription factor superfamily, is strongly overexpressed in proliferating cells (Mehrnia *et al.*, 2013). In fact, EBE activates several genes involved in cell cycle regulation and dormancy breaking and its effect on shoot branching likely results from an activation of the mentioned genes (Mehrnia *et al.*, 2013). Thus, it is not far from imagination that the *AP2* reported here plays such a role in up-regulation and/or down-regulation of cyclins (Marsch-

Martinez *et al.*, 2006). Overexpression of EBE in cells which are under stress causes cellular dedifferentiation, which leads to a stem-cell-like fate before cells adopt a new fate. In fact, exposure to stress leads to a reduction in protein synthesis (Dhindsa and Cleland, 1975; Grafi *et al.*, 2011) that facilitates the switch to the quiescent state of stem cells. Second, chromatin remodeling, a fundamental theme of cellular dedifferentiation, is induced following exposure of plants to various stress conditions (Grafi *et al.*, 2011).

It is hypothesized that in apomictic *Boechera* which experience harsh environmental conditions, those archesporial cells that are supposed to turn into MMCs undergo reprograming which leads to apomeiosis. Another scenario might be that *AP2* has two targets including APOLLO and cyclins, controlling an orchestrated regulatory network that leads to apomixis in *Boechera*. The result of a comprehensive transcriptional analysis between apomictic *Boechera gunnisoniana* versus sexual *Arabidopsis* also suggested that key regulatory mechanisms are differentially regulated, involving hormone pathways, cell cycle control, signal transduction, and epigenetic regulatory processes (Schmidt *et al.*, 2014). Thus, the induction of apomixis seems to be the result of a variety of mechanisms which are not mutually exclusive.

The difference in GUS activities observed between the 2 kb Apo vs. Sex native promoter constructs may also be influenced by structural variations that selectively bind particular regulatory elements, leading to varying levels of expression. Furthermore, protein diversity between APOLLO alleles could arise through alternative splicing, in which a variety of exon combinations are used to generate multiple mRNAs from a single gene (Pajares *et al.*, 2007; Davuluri *et al.*, 2008). An important example is the hemoglobin γ A gene (*HBG1*) with TATA-box-lacking and TATA-box-containing alternative promoters that are used at different developmental stages (Duan *et al.*, 2002). In this way, variation between APOLLO Apo- and Sex-alleles could have accumulated after a loss in recombination capacity as explained by the Meselson effect[4] (Hojsgaard and Hörandl, 2015). This diversity has been fixed in some motifs such as the 20 bp insertion of Apo-allele, and could produce differential gene expression in a mechanism like that of alternative promoters.

[4] When two alleles or copies of a gene, within an asexual diploid individual evolve independently of each other, they become increasingly different over time by accumulation of neutral mutations. This phenomenon of allelic divergence is commonly known as the Meselson effect.

In addition, diploid heterozygous apomictic individuals could take advantage of complex gene regulation mechanisms arising from bidirectional promoter activity. In practice, bidirectional promoters can produce variable products and relative numbers of transcripts produced via several regulatory steps during the transcription cycle (initiation, elongation, and termination). It was shown that in cases of bidirectional promoters, many of the promoter segments between two bidirectional genes initiate transcription in both directions and contain shared elements that regulate both genes (Trinklein *et al.*, 2004). Bidirectional transcription itself is classified into those affecting the bidirectional promoter, neighboring protein-coding genes, or more distal genes (Wei *et al.*, 2011). According to this model and based on the fact that APOLLO gene is heterozygote (for both Apo- and Sex-allele), there is a possibility that transcription starts from a point in the Apo-allele promoter and continues toward the Sex-allele or vice versa and consequently gives rise to variable transcriptions for apomictic *Boechera*. Further studies will reveal if APOLLO has a bidirectional promoter and how it can affect its expression.

Transgenic sexual *Boechera* lines containing the 2kb native Sex promoter would shed more light on its expression profile changes in sexual versus apomictic genome backgrounds. In addition, the 2kb native Apo promoter can be cloned upstream of other genes as a potent biotechnology tool for promoting the switch of expression of other (e.g., reproductive) tissues. Knocking out specific parts of the Apo-allele identified here is the next logical step to investigate its role in apomeiosis induction in apomictic *Boechera*. Synthetic promoters can drive constitutive, spatiotemporal, inducible, and even unique combinations of transgene expression patterns, depending on the included elements. All synthetic promoters used in this experiment generated unique spatiotemporal[5] expression patterns induced by the combination of elements used in their structures.

In conclusion, future studies are required to shed light on the mechanism of tissue-specific transcription activation and/or repression; for instance, if there is any interaction with tissue-specific transcriptional cofactors that either activate or repress expression or may function in a combinatorial manner with other TFs. In order to learn about the interaction between recognized

[5] Spatiotemporal promoters by providing a more specific control over native genes and transgenes, restrict gene expression to certain cells, tissues, organs, or developmental stages (Hernandez-Garcia and Finer, 2014).

TFs and their targets, different motifs can be designed and used in an Electrophoretic Mobility Shift Assay (EMSA). In addition, the ERF family of TFs found in this study often bind GC-rich motifs, and since we have a GCC motif in the 20 bp Apo-insertion, this could be a site of mutagenesis to deter binding specificity in future studies. Finally, the complex regulatory architecture of apomixis could also include non-coding RNAs (ncRNA) and bidirectional promoters, and thus understanding whether the APOLLO has bidirectional promoter activities in sexual and apomictic *Boechera* would provide further insights into its regulation.

APPENDICES

These data are the hits gained from *Boechera divaricarpa* and *Boechera stricta* libraries. The sequences mentioned here are limited to the TF with highest confidence that were significant based on our research question and all sequences are not included.

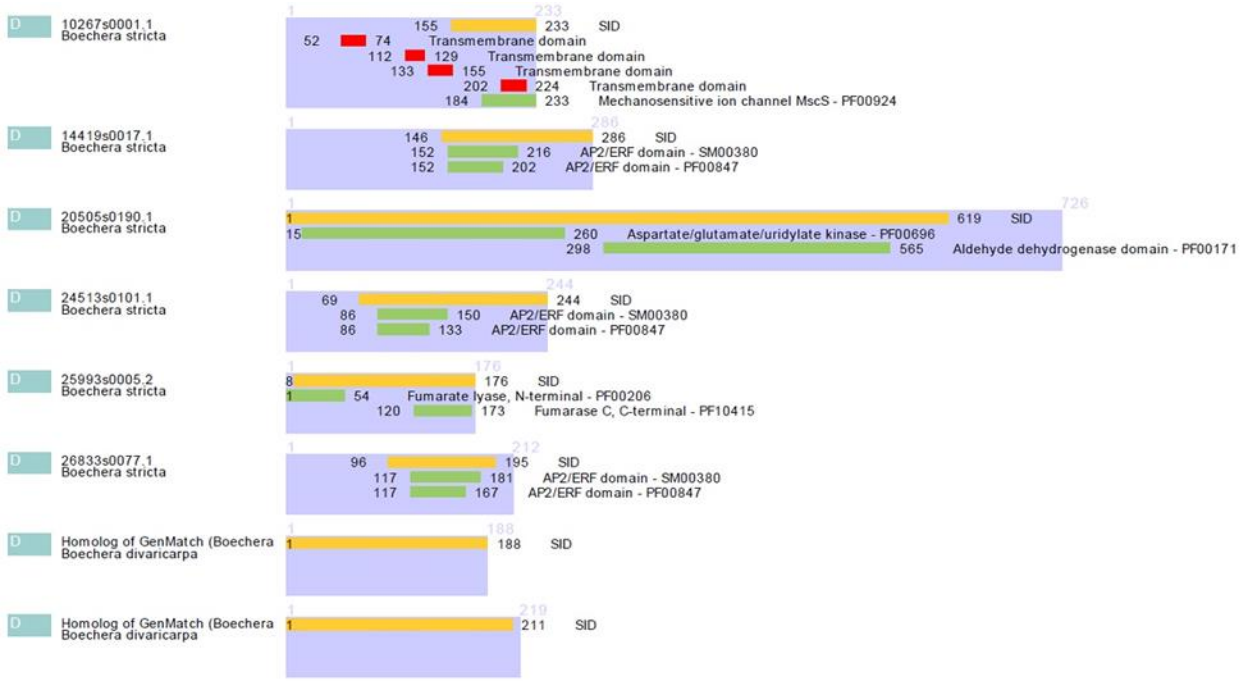
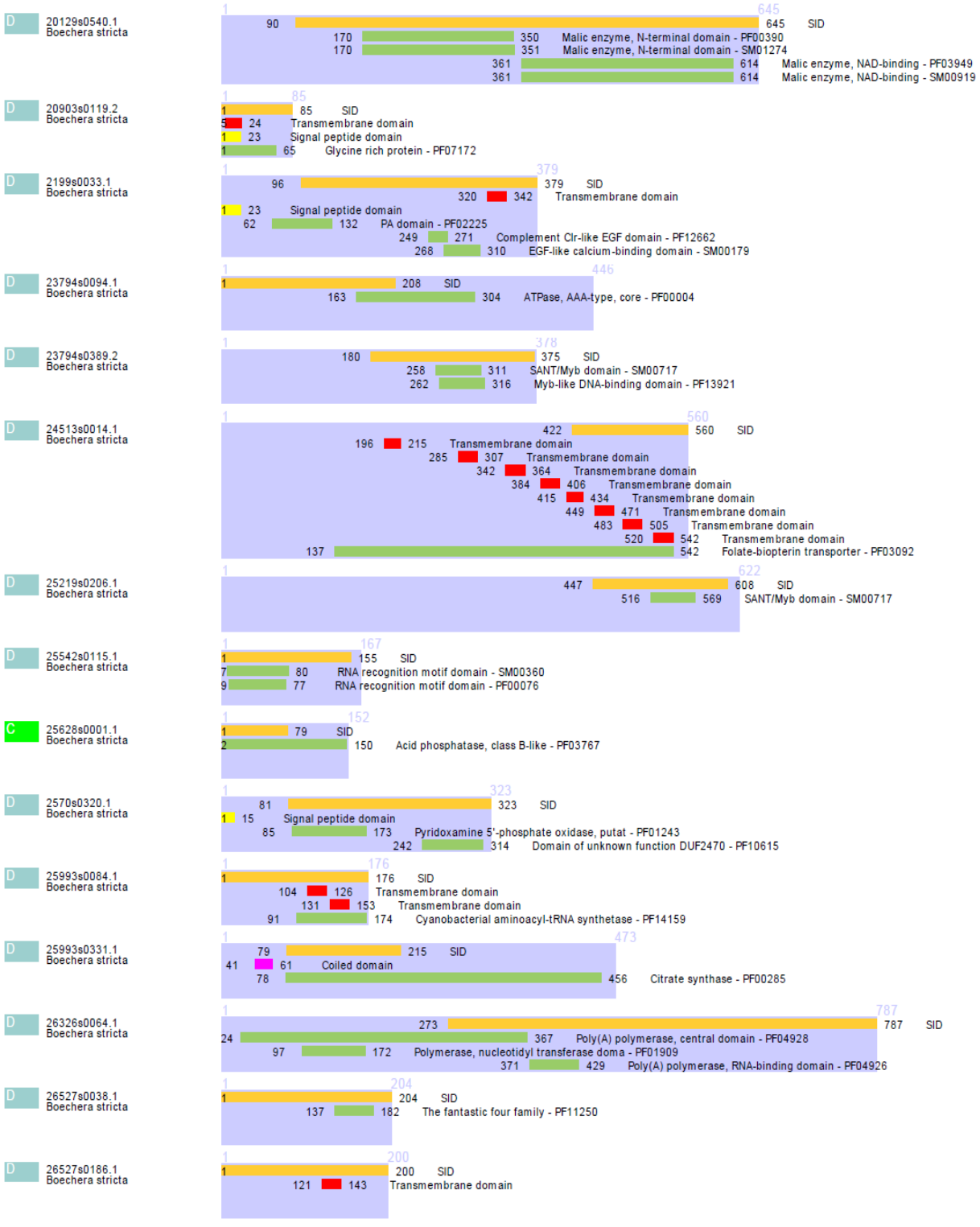


Figure 1 The result of yeast one-hybrid assay for *B. divaricarpa* bait (BODIA_RP1_hgx4970v1) vs. prey construct (*Boechera divaricarpa*_RP1). Proteins are leveled based on their confidence of interaction. Orange color demonstrates Simple Intra-sequence Difference (SID) fragment, green color demonstrates Pfam[6] or SMART domain, red color means transmembrane domain (TMHMM)[7].

[6] Pfam or protein family data base is a database of protein families that includes their annotations and multiple sequence alignments generated using hidden Markov models (http://pfam.janelia.org) (Finn *et al.*, 2014)

[7] TMHMM means transmembrane protein topology described based on hidden Markov model (Krogh *et al.*, 2001)


D 20129s0540.1 Boechera stricta
1 645
90 645 SID
170 350 Malic enzyme, N-terminal domain - PF00390
170 351 Malic enzyme, N-terminal domain - SM01274
361 614 Malic enzyme, NAD-binding - PF03949
361 614 Malic enzyme, NAD-binding - SM00919
D 20903s0119.2 Boechera stricta
1 85
1 85 SID
5 24 Transmembrane domain
1 23 Signal peptide domain
1 65 Glycine rich protein - PF07172
D 2199s0033.1 Boechera stricta
1 379
96 379 SID
320 342 Transmembrane domain
1 23 Signal peptide domain
62 132 PA domain - PF02225
249 271 Complement Clr-like EGF domain - PF12662
268 310 EGF-like calcium-binding domain - SM00179
D 23794s0094.1 Boechera stricta
1 446
1 208 SID
163 304 ATPase, AAA-type, core - PF00004
D 23794s0389.2 Boechera stricta
1 378
180 375 SID
258 311 SANT/Myb domain - SM00717
262 316 Myb-like DNA-binding domain - PF13921
D 24513s0014.1 Boechera stricta
1 560
422 560 SID
196 215 Transmembrane domain
285 307 Transmembrane domain
342 364 Transmembrane domain
384 406 Transmembrane domain
415 434 Transmembrane domain
449 471 Transmembrane domain
483 505 Transmembrane domain
520 542 Transmembrane domain
137 542 Folate-biopterin transporter - PF03092
D 25219s0206.1 Boechera stricta
1 622
447 608 SID
516 569 SANT/Myb domain - SM00717
D 25542s0115.1 Boechera stricta
1 167
1 155 SID
7 80 RNA recognition motif domain - SM00360
9 77 RNA recognition motif domain - PF00076
C 25628s0001.1 Boechera stricta
1 152
1 79 SID
2 150 Acid phosphatase, class B-like - PF03767
D 2570s0320.1 Boechera stricta
1 323
81 323 SID
1 15 Signal peptide domain
85 173 Pyridoxamine 5'-phosphate oxidase, putat - PF01243
242 314 Domain of unknown function DUF2470 - PF10615
D 25993s0084.1 Boechera stricta
1 176
1 176 SID
104 126 Transmembrane domain
131 153 Transmembrane domain
91 174 Cyanobacterial aminoacyl-tRNA synthetase - PF14159
D 25993s0331.1 Boechera stricta
1 473
79 215 SID
41 61 Coiled domain
78 456 Citrate synthase - PF00285
D 26326s0064.1 Boechera stricta
1 787
273 787 SID
24 367 Poly(A) polymerase, central domain - PF04928
97 172 Polymerase, nucleotidyl transferase doma - PF01909
371 429 Poly(A) polymerase, RNA-binding domain - PF04926
D 26527s0038.1 Boechera stricta
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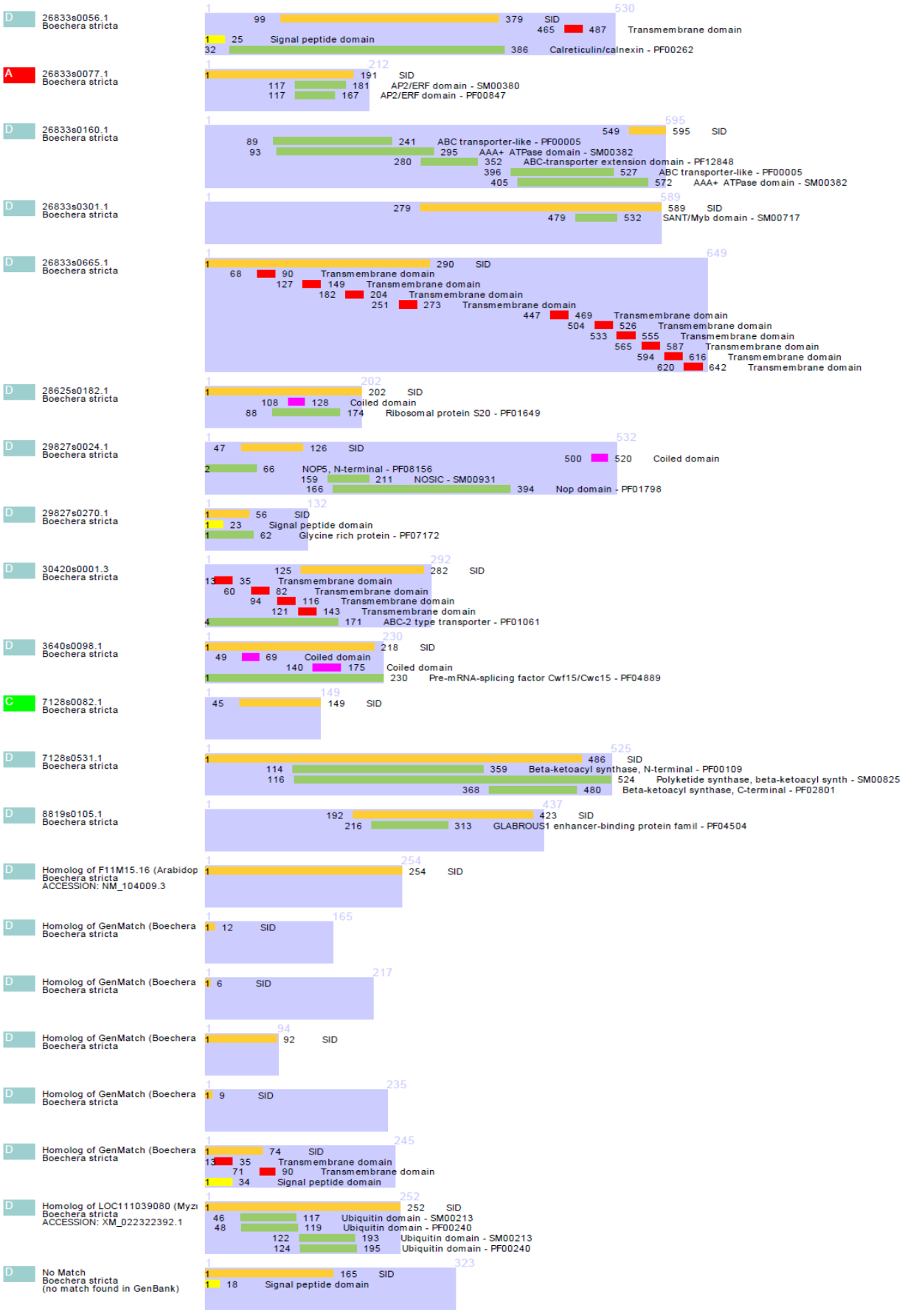
1 204 SID
137 182 The fantastic four family - PF11250
D 26527s0186.1 Boechera stricta
1 200
1 200 SID
121 143 Transmembrane domain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1 530
99 379 SID
465 487 Transmembrane domain
1 25 Signal peptide domain
32 386 Calreticulin/calnexin - PF00262
A 26833s0077.1 Boechera stricta
1 212
1 191 SID
117 181 AP2/ERF domain - SM00380
117 167 AP2/ERF domain - PF00847
D 26833s0160.1 Boechera stricta
1 595
549 595 SID
89 241 ABC transporter-like - PF00005
93 295 AAA+ ATPase domain - SM00382
280 352 ABC-transporter extension domain - PF12848
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479 532 SANT/Myb domain - SM00717
D 26833s0665.1 Boechera stricta
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1 290 SID
68 90 Transmembrane domain
127 149 Transmembrane domain
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504 526 Transmembrane domain
533 555 Transmembrane domain
565 587 Transmembrane domain
594 616 Transmembrane domain
620 642 Transmembrane domain
D 28625s0182.1 Boechera stricta
1 202
1 202 SID
108 128 Coiled domain
88 174 Ribosomal protein S20 - PF01649
D 29827s0024.1 Boechera stricta
1 532
47 126 SID
500 520 Coiled domain
2 66 NOP5, N-terminal - PF08156
159 211 NOSIC - SM00931
166 394 Nop domain - PF01798
D 29827s0270.1 Boechera stricta
1 132
1 56 SID
1 23 Signal peptide domain
1 62 Glycine rich protein - PF07172
D 30420s0001.3 Boechera stricta
1 292
125 282 SID
13 35 Transmembrane domain
60 82 Transmembrane domain
94 116 Transmembrane domain
121 143 Transmembrane domain
4 171 ABC-2 type transporter - PF01061
D 3640s0098.1 Boechera stricta
1 230
1 218 SID
49 69 Coiled domain
140 175 Coiled domain
1 230 Pre-mRNA-splicing factor Cwf15/Cwc15 - PF04889
C 7128s0082.1 Boechera stricta
1 149
45 149 SID
D 7128s0531.1 Boechera stricta
1 525
1 486 SID
114 359 Beta-ketoacyl synthase, N-terminal - PF00109
116 524 Polyketide synthase, beta-ketoacyl synth - SM00825
368 480 Beta-ketoacyl synthase, C-terminal - PF02801
D 8819s0105.1 Boechera stricta
1 437
192 423 SID
216 313 GLABROUS1 enhancer-binding protein famil - PF04504
D Homolog of F11M15.16 (Arabidop Boechera stricta ACCESSION: NM_104009.3
1 254
1 254 SID
D Homolog of GenMatch (Boechera Boechera stricta
1 165
1 12 SID
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1 92 SID
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1 9 SID
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1 245
1 74 SID
13 35 Transmembrane domain
71 90 Transmembrane domain
1 34 Signal peptide domain
D Homolog of LOC111039080 (Myzı Boechera stricta ACCESSION: XM_022322392.1
1 252
1 252 SID
46 117 Ubiquitin domain - SM00213
48 119 Ubiquitin domain - PF00240
122 193 Ubiquitin domain - SM00213
124 195 Ubiquitin domain - PF00240
D No Match Boechera stricta (no match found in GenBank)
1 323
1 165 SID
1 18 Signal peptide domain

Figure 2 The result of yeast one-hybrid assay for *B. stricta* bait (BODIA_RP1_hgx4970v1) vs. prey construct (*Boechera stricta*_RP1). Proteins are leveled based on their confidence of interaction. Orange color demonstrates Simple Intra-sequence Difference (SID) fragment, green color demonstrates Pfam or SMART domain, red color means transmembrane domain, pink color mean Coiled-coil domain and yellow color means Signal peptide (SignalP).

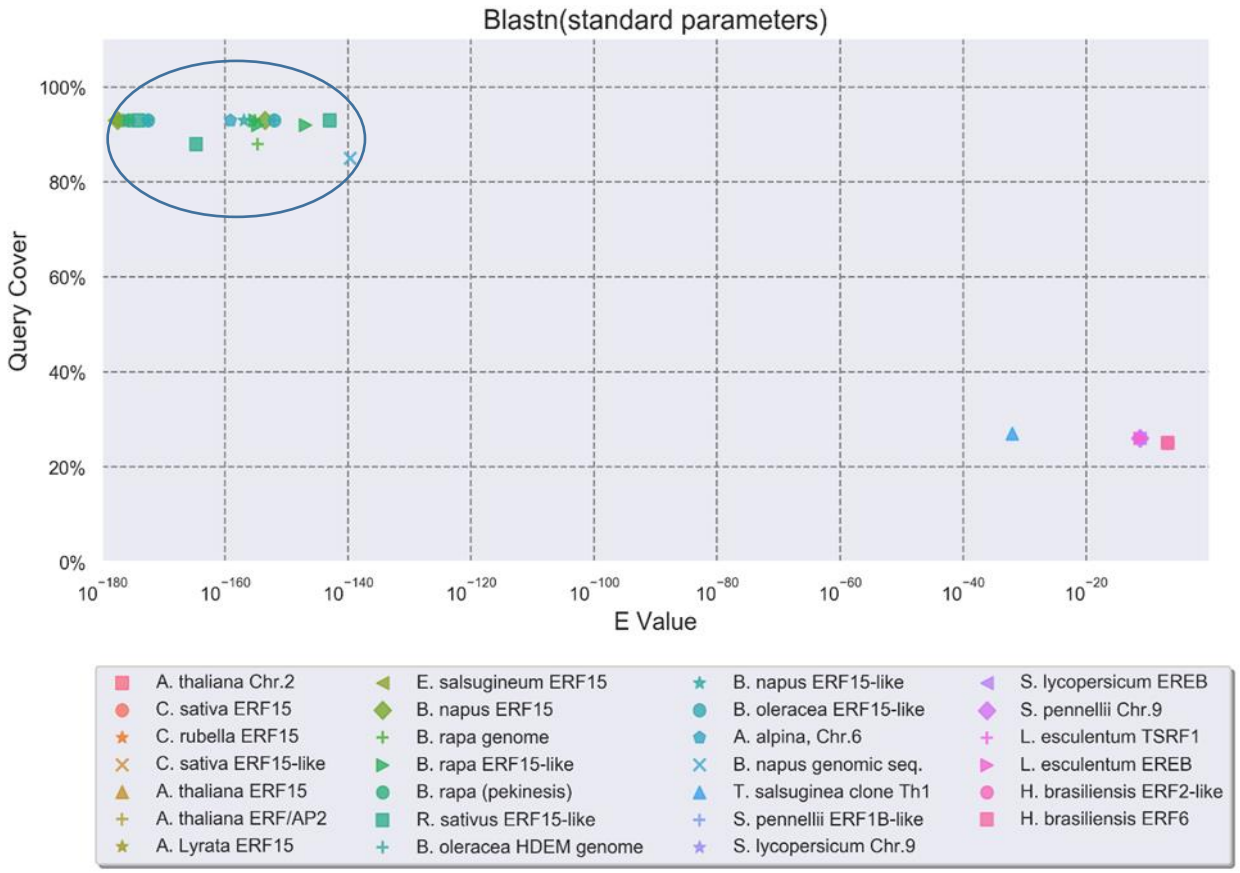

Figure 3 Blasting the *B. divaricarpa*, (ID24513s0101.1) sequence to the NCBI viridiplantae database. Circled data shows that *ERF15* is the best candidate with the highest Query cover and the lowest E-value.

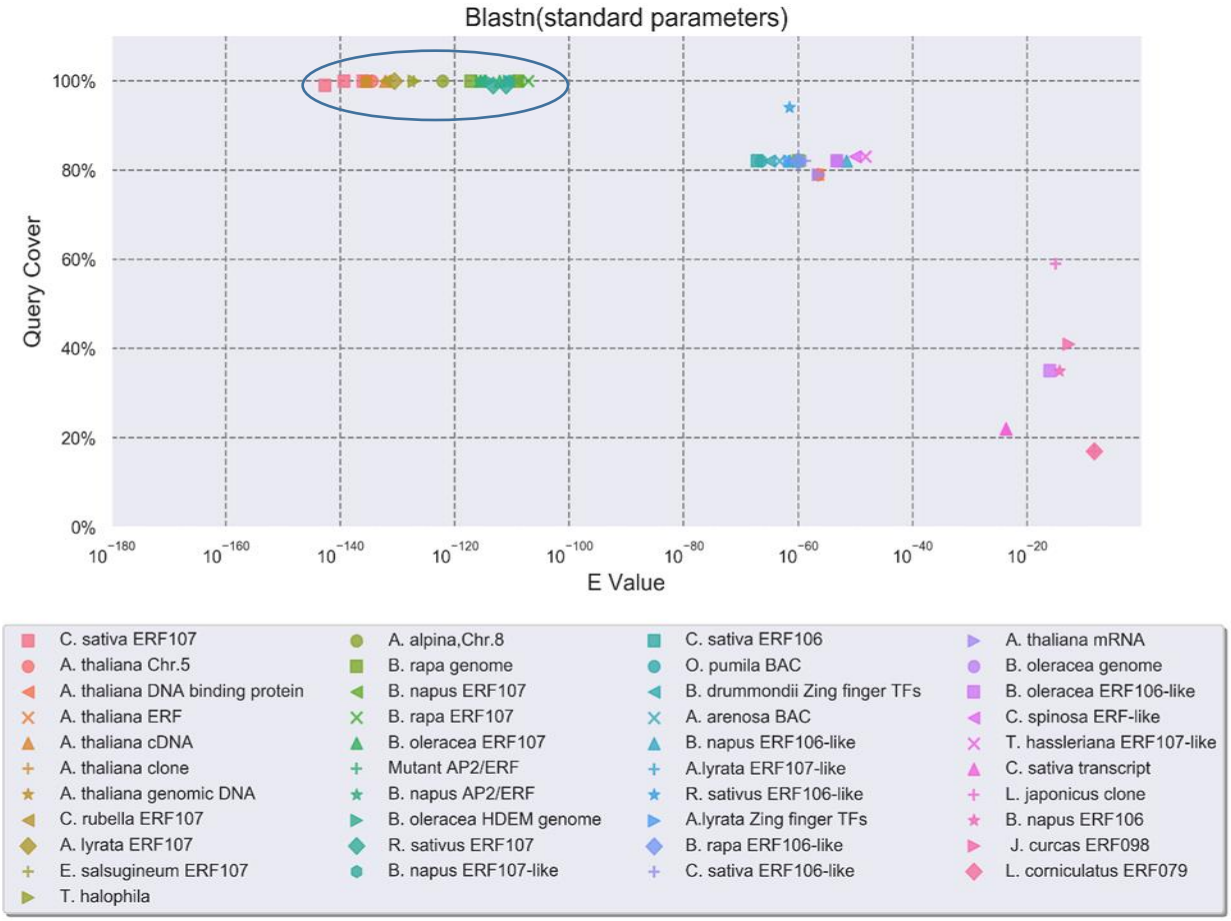

Figure 4 Blasting the *B. divaricarpa*, ID26833s0077.1 sequence to the NCBI vi-ridiplantae database. Circled data shows that *ERF107* is the best candidate with the highest Query cover and the lowest E-value.

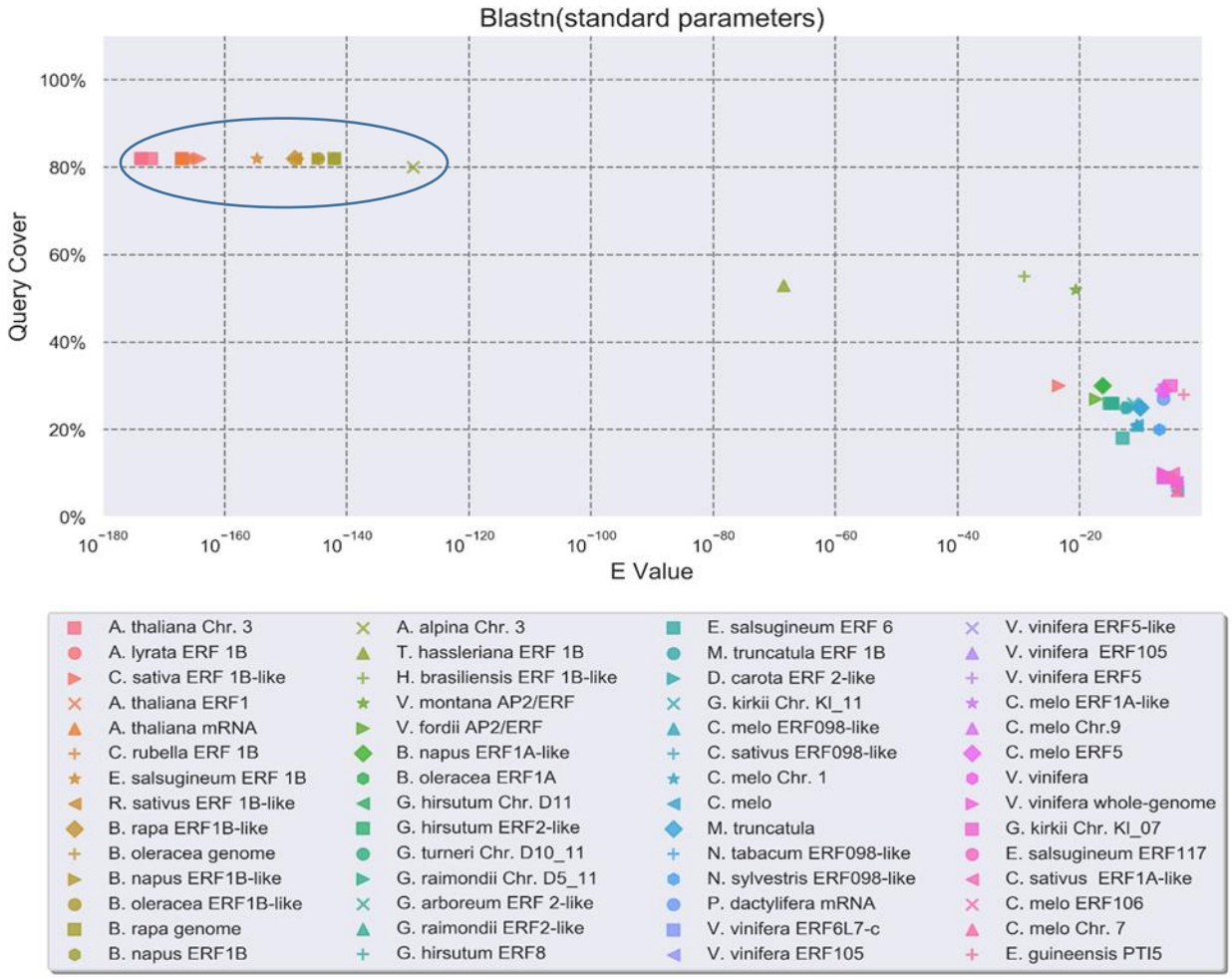

Figure 5 Blasting the *B. stricta*, ID19424s0319.1 sequence to the NCBI viridiplantae database. Circled data shows that *ERF1B* is the best candidate with the highest Query cover and the lowest E-value.

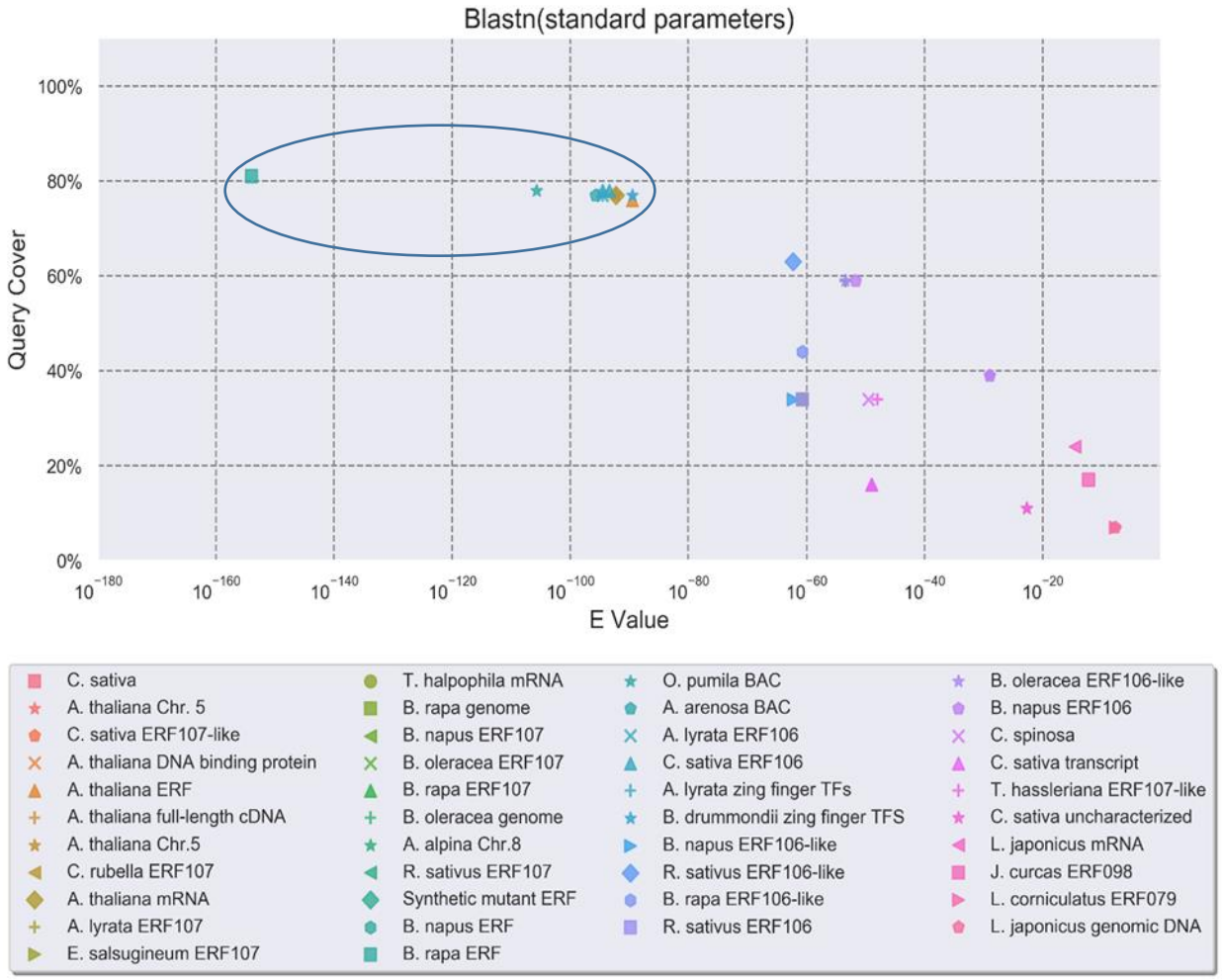

Figure 6 Blasting the *B. stricta*, ID26833s0077.1 sequence to the NCBI viridiplantae database. Circled data shows that *ERF107* is the best candidate with the highest Query cover and the lowest E-value.

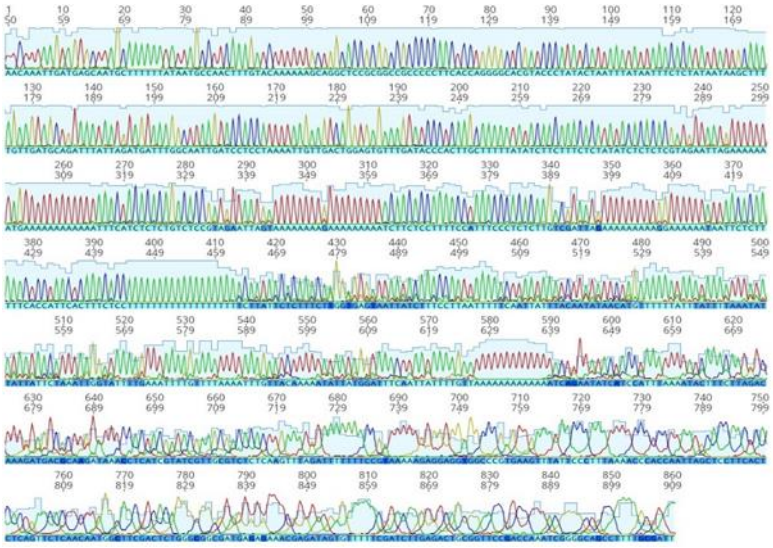

Figure 7 Densitogram of 1 kb Apo promoter in pENTR/D vector. This sequence has 99.1% similarity with *Boechera* sp. IPK Bsp 9 isolate BAC2b_4 APOLLO gene. This clone was used for recombination with Expression vector (pBGWFS7).

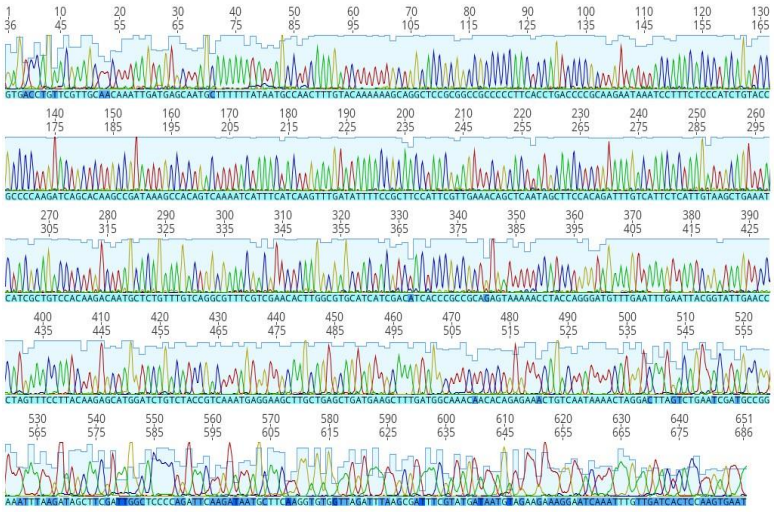

Figure 8 Densitogram of 1 kb Sex promoter in pENTR/D vector. This sequence has 98.9% similarity with *Boechera sp.* IPK Bsp 9 isolate BAC5_7 APOLLO gene. This clone was used for recombination with expression vector (pBGWFS7).

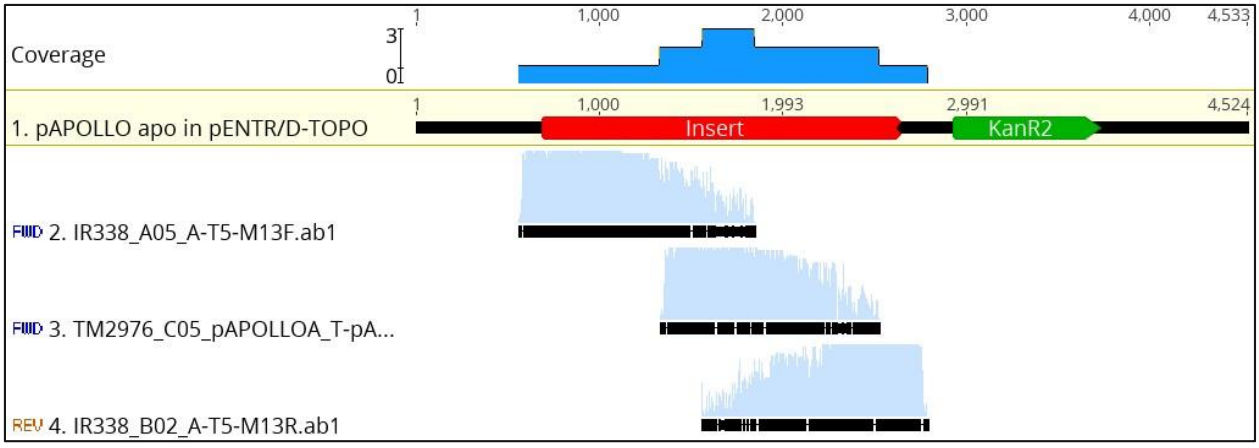

Figure 9 sequencing data for pC1. Three reads mapped to pAPOLLO Apo in pENTR/D- TOPO using Geneious software version 10.2.6 with pairwise identity 86.6%.

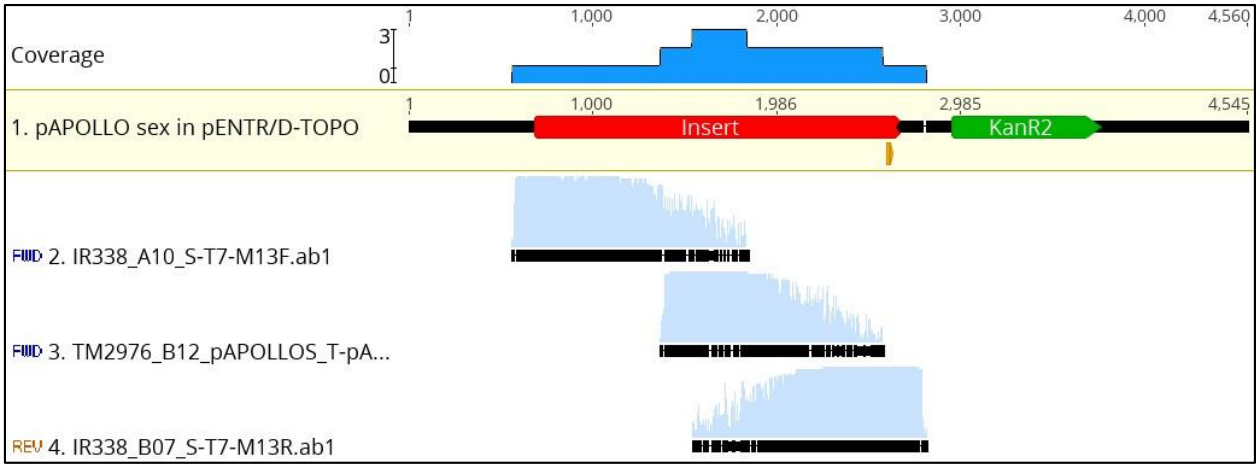

Figure 10 Sequencing data for pC2. Three reads mapped to pAPOLLO Sex in pENTR/D- TOPO using Geneious software version 10.2.6 with pairwise identity 85.5%.

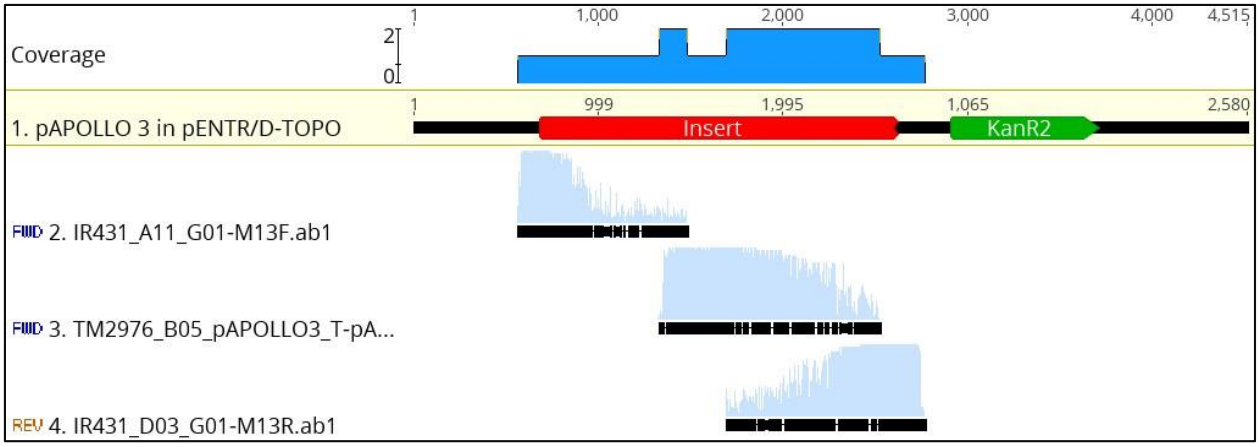

Figure 11 Sequencing data for pC3. Three reads mapped to pAPOLLO3 in pENTR/D- TOPO using Geneious software version 10.2.6 with pairwise identity 78.5%.

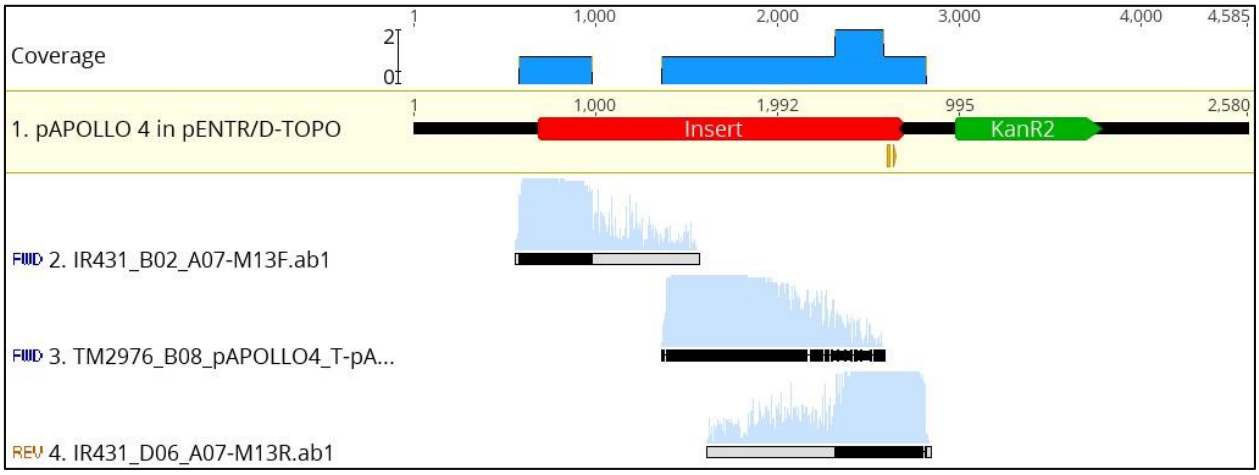

Figure 12 Sequencing data for construct pC4. Three reads mapped to pAPOLLO4 in pENTR/D-TOPO using Geneious software version 10.2.6 with pairwise identity 91.1%.

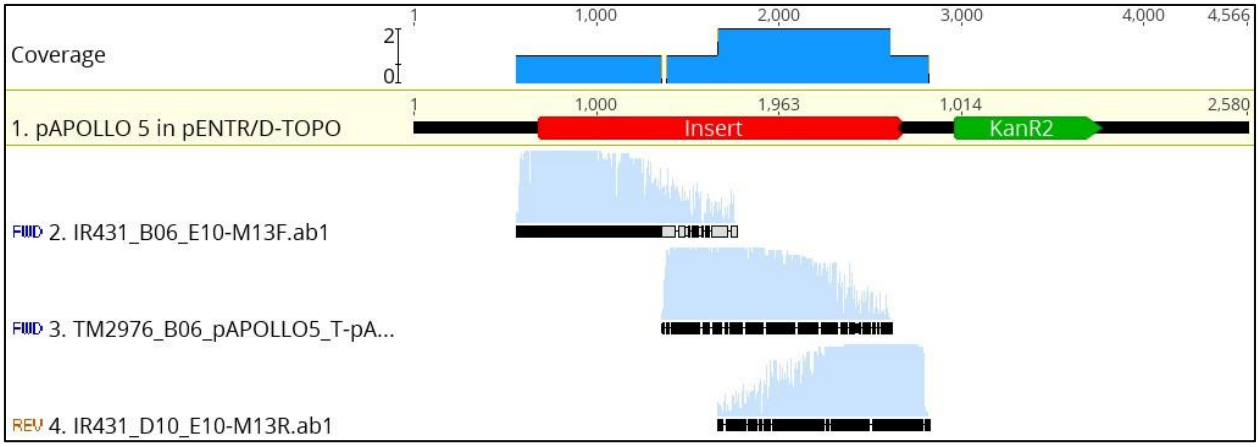

Figure 13 Sequencing data for construct pC5. Three reads mapped to pAPOLLO5 in pENTR/D-TOPO using Geneious software version 10.2.6 with pairwise identity 88%.

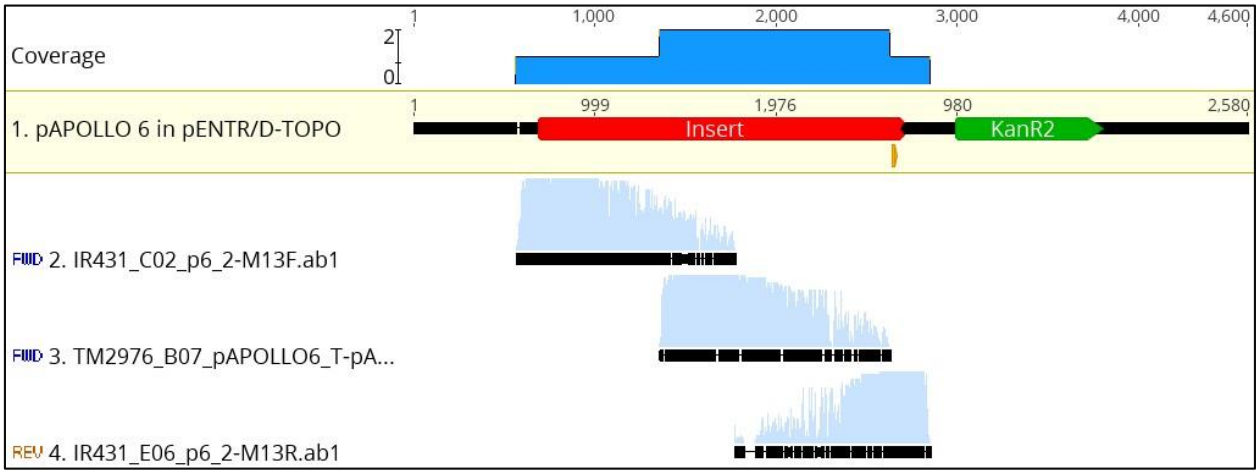

Figure 14 Sequencing data for construct pC6. Three reads mapped to pAPOLLO6 in pENTR/D-TOPO using Geneious software version 10.2.6 with pairwise identity 78.6%.

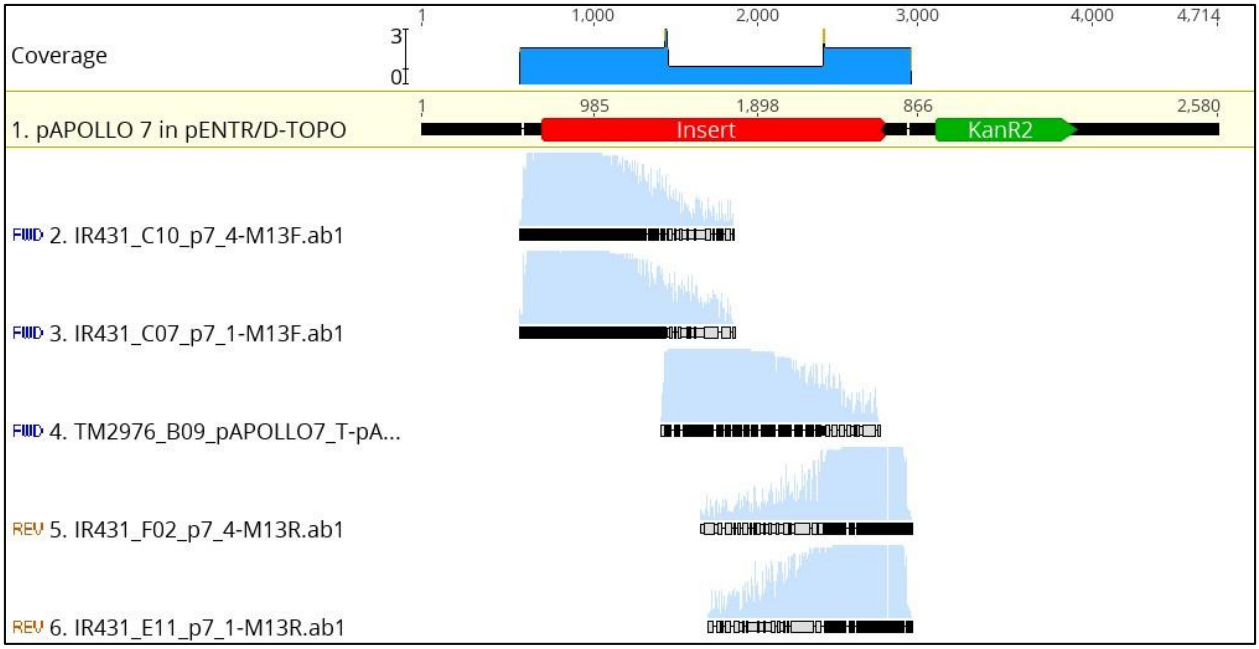

Figure 15 Sequencing data for construct pC7. Five reads mapped to pAPOLLO7 in pENTR/D-TOPO using Geneious software version 10.2.6 with pairwise identity 98.4%.

REFERENCES

Aggarwal, P., Gupta, M. D., Joseph, A. P., Chatterjee, N., Sinivasan, N., & Nath, U. (2010). Identification of specific DNA binding residues in the TCP family of transcription factors in Arabidopsis. Plant Cell, 22(4), 1174–1189. https://doi.org/10.1105/tpc.109.066647

Agrawal, A. F., Hadany, L. and Otto, S. P. (2005). The Evolution of Plastic Recombination, Genetics, 171(2), 803 – 812. https://doi.org/10.1534/genetics.105.041301

Aguilar-Martlnez, J. A., & Sinha, N. (2013). Analysis of the role of Arabidopsis class I TCP genes AtTCP7, AtTCP8, AtTCP22, and AtTCP23 in leaf development. Frontiers in Plant Science, 4, 406. https://doi.org/10.3389/fpls.2013.00406

Ahmad, S. and Sarai, A. (2005). PSSM-based prediction of DNA binding sites in proteins, BMC Bioinformatics, 6(1), 33. https://doi.org/10.1186/1471-2105-6-33

Alexander, M. K., Bourns, B. D. and Zakian, V. A. (2001). One-Hybrid Systems for Detecting Protein-DNA Interactions BT - Two-Hybrid Systems: Methods and Protocols, in MacDonald, P. N. (ed.). Totowa, NJ: Humana Press, 177, 241–259. https://doi.org/10.1385/1-59259-210-4:241

Aliyu, O. M., Schranz, M. E. and Sharbel, T. F. (2010). Quantitative variation for apomictic reproduction in the genus Boechera (Brassicaceae)., American journal of botany, 97(10), 1719–31. https://doi.org/10.3732/ajb.1000188

Anderson, J. T., Lee, C.-R. and Mitchell-Olds, T. (2011). Life-history QTLS and natural selection on flowering time in Boechera stricta, a perennial relative of Arabidopsis, Evolution; international journal of organic evolution, 65(3), 771–787. https://doi.org/10.1111/j.1558-5646.2010.01175.x

Andrabi, M., Mizuguchi, K., Sarai, A., & Ahmad, S. (2009). Prediction of mono- and di-nucleotide-specific DNA-binding sites in proteins using neural networks. BMC Structural Biology, 9(1), 30. https://doi.org/10.1186/1472-6807-9-30

Bäckström, S. et al. (2007). Purification of a Plant Mediator from Arabidopsis thaliana Identifies PFT1 as the Med25 Subunit. Molecular Cell, 26(5), 717–729. https://doi.org/10.1016/j.molcel.2007.05.007

Balasubramanian, S., & Schneitz, K. (2002). NOZZLE links proximal-distal and adaxial-abaxial pattern formation during ovule development in Arabidopsis thaliana. Development, 129(18), 4291-4300. https://doi.org/10.1242/dev.129.18.4291

Balloux, F., Lehmann, L., & De Meeûs, T. (2003). The population genetics of clonal and partially clonal diploids. Genetics, 164(4), 1635–1644. https://doi.org/10.1093/genetics/164.4.1635

Baroux, C., Pien, S. and Grossniklaus, U. (2007). Chromatin modification and remodeling during early seed development. Current Opinion in Genetics & Development, 17(6), 473–479. https://doi.org/10.1016/j.gde.2007.09.004

Barrett, L. W., Fletcher, S. and Wilton, S. D. (2012). Regulation of eukaryotic gene expression by the untranslated gene regions and other non-coding elements. Cellular and molecular life sciences, 69(21), 3613–3634. https://doi.org/10.1007/s00018-012-0990-9

Bartel, P. L., Chien, C.-T., Sternglanz, R., and Fields, S. (1993). Using the two- hybrid system to detect protein-protein interactions, in Cellular Interactions in Development: A Practical Approach (Hartley, D. A., ed.). Oxford University Press, 153–179

Barton, N. H., & Charlesworth, B. (1998, September 25). Why sex and recombination? Science. 281(5385), 1986-1990. https://doi.org/10.1126/science.281.5385.1986

Bateman, R. M., Crane, P. R., Dimichele, W. A., Kenrick, P. R., Rowe, N. P., Speck, T., & Stein, W. E. (1998). Early evolution of land plants: Phylogeny, physiology, and ecology of the primary terrestrial radiation. Annual Review of Ecology and Systematics, 29, 263–292. https://doi.org/10.1146/annurev.ecolsys.29.1.263

Bateson, W., & Mendel, Gregor, (1909). Mendel's principles of heredity / by W. Bateson. Cambridge : University Press, 1-450. https://doi.org/10.5962/bhl.title.44575

Beck, J. B., Alexander, P. J., Allphin, L., Al-Shehbaz, I. A., Rushworth, C., Bailey, C. D., & Windham, M. D. (2012). Does hybridization drive the transition to asexuality in diploid Boechera? Evolution, 66(4), 985–995. https://doi.org/10.1111/j.1558-5646.2011.01507.x

Becks, L., & Agrawal, A. F. (2010). Higher rates of sex evolve in spatially heterogeneous environments. Nature, 468(7320), 89–92. https://doi.org/10.1038/nature09449

Becks, L., & Agrawal, A. F. (2012). The evolution of sex is favoured during adaptation to new environments. PLoS Biology, 10(5), e1001317. https://doi.org/10.1371/journal.pbio.1001317

Becks, L. & Agrawal, A. F. (2011). The effect of sex on the mean and variance of fitness in facultatively sexual rotifers, Journal of Evolutionary Biology, 24(3), 656–664. https://doi.org/10.1111/j.1420-9101.2010.02199.x

Berrocal-Lobo, M., Molina, A. and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi, The Plant Journal, 29(1), 23–32. https://doi.org/10.1046/j.1365-313x.2002.01191.x

Béziat, C., Kleine-Vehn, J. and Feraru, E. (2017). Histochemical Staining of β-Glucuronidase and Its Spatial Quantification BT - Plant Hormones: Methods and Protocols. in Kleine-Vehn, J. and Sauer, M. (eds). New York, NY: Springer New York, 73–80. https://doi.org/10.1007/978-1-4939-6469-7_8

Bicknell, R., Catanach, A., Hand, M., & Koltunow, A. (2016). Seeds of doubt: Mendel's choice of Hieracium to study inheritance, a case of right plant, wrong trait. Theoretical and Applied Genetics. Springer Verlag. 129(12), 2253–2266. https://doi.org/10.1007/s00122-016-2788-x

Bicknell, R. A. and Koltunow, A. M. (2004). Understanding Apomixis: Recent Advances and Remaining Conundrums, The Plant Cell, 16(1), S228–S245. https://doi.org/10.1105/tpc.017921

Bicknell, R. A., Lambie, S. C., & Butler, R. C. (2003). Quantification of progeny classes in two facultatively apomictic accessions of Hieracium. Hereditas, 138(1), 11–20. https://doi.org/10.1034/j.1601-5223.2003.01624.x

Bleuyard, J.-Y., Gallego, M. E. and White, C. I. (2006). Recent advances in understanding of the DNA double-strand break repair machinery of plants. DNA Repair, 5(1), 1–12. https://doi.org/10.1016/j.dnarep.2005.08.017

Blanco, C., Ritzenthaler, P., & Mata-Gilsinger, M. (1982). Cloning and endonuclease restriction analysis of uidA and uidR genes in Escherichia coli K-12: determination of transcription direction for the uidA gene. Journal of bacteriology, 149(2), 587–594. https://doi.org/10.1128/jb.149.2.587-594.1982

Bonneaud, N., Ozier-Kalogeropoulos, O., Li, G., Labouesse, M., Minvielle-Sebastia, L., & Lacroute, F. (1991). A family of low and high copy replicative, integrative and single-stranded S. cerevisiae/E. coli shuttle vectors. Yeast, 7(6), 609–615. https://doi.org/10.1002/yea.320070609

Boutilier, K., Offringa, R., Sharma, V. K., Kieft, H., Ouellet, T., Zhang, L., … Van Lookeren Campagne, M. M. (2002). Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *Plant Cell*, 14(8), 1737–1749. https://doi.org/10.1105/tpc.001941

Bonser, S. P. (2013). Is Sex Advantageous in Adverse Environments? A Test of the Abandon-Ship Hypothesis. The American Naturalist, 182(6), 718–725. https://doi.org/10.1086/673476

Bowman, J. L., Drews, G. N. and Meyerowitz, E. M. (1991). Expression of the Arabidopsis floral homeotic gene AGAMOUS is restricted to specific cell types late in flower development. The Plant Cell, 3(8), 749 – 758. https://doi.org/10.1105/tpc.3.8.749

Briggs, D., & Walters, S. (2016). Plant Variation and Evolution (4th ed.). Cambridge: Cambridge University Press. https://doi.org/10.1017/CBO9781139060196

Brink, R. A. and Cooper, D. C. (1947). The endosperm in seed development, The Botanical Review, 13(9), 479–541. https://doi.org/10.1007/BF02861549

Brukhin, V. (2017). Molecular and genetic regulation of apomixis, Russian Journal of Genetics, 53(9), 943–964. https://doi.org/10.1134/S1022795417090046

Brukhin, V., Osadtchiy, J. V., Florez-Rueda, A. M., Smetanin, D., Bakin, E., Nobre, M. S., & Grossniklaus, U. (2019). The Boechera genus as a resource for apomixis research. Frontiers in Plant Science. 10, 392. https://doi.org/10.3389/fpls.2019.00392

Bulankova, P., Akimcheva, S., Fellner, N., & Riha, K. (2013). Identification of Arabidopsis Meiotic Cyclins Reveals Functional Diversification among Plant Cyclin Genes. PLoS Genetics, 9(5), e1003508. https://doi.org/10.1371/journal.pgen.1003508

Burt A. (2000). Perspective: sex, recombination, and the efficacy of selection--was Weismann right?. Evolution; international journal of organic evolution, 54(2), 337–351. https://doi.org/10.1111/j.0014-3820.2000.tb00038.x

Butlin R. (2002). Evolution of sex: The costs and benefits of sex: new insights from old asexual lineages. Nature reviews. Genetics, 3(4), 311–317. https://doi.org/10.1038/nrg749

Callis, J., Fromm, M. and Walbot, V. (1987). Introns increase gene expression in cultured maize cells., Genes & development, 1(10), 1183–1200. https://doi.org/10.1101/gad.1.10.1183

Carman, J. G., Jamison, M., Elliott, E., Dwivedi, K. K., & Naumova, T. N. (2011). Apospory appears to accelerate onset of meiosis and sexual embryo sac formation in sorghum ovules. BMC Plant Biology, 11. https://doi.org/10.1186/1471-2229-11-9

Carman, J. G. (1997). Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetraspory, and polyembryony. Biological Journal of the Linnean Society, 61(1), 51–94. https://doi.org/10.1111/j.1095-8312.1997.tb01778.x

Carman, J. G. (2001). The gene effect: genome collisions and apomixis. The Flowering of Apomixis: from Mechanisms to Genetic Engineering. 95-110.

Carputo, D., Monti, L., Werner, J. E., & Frusciante, L. (1999). Uses and usefulness of endosperm balance number. Theoretical and Applied Genetics, 98(3), 478–484. https://doi.org/10.1007/s001220051095

Catanach, A. S., Erasmuson, S. K., Podivinsky, E., Jordan, B. R., & Bicknell, R. (2006). Deletion mapping of genetic regions associated with apomixis in Hieracium. Proceedings of the National Academy of Sciences of the United States of America, 103(49), 18650–18655. https://doi.org/10.1073/pnas.0605588103

Chandra, A. & Dubey, A. (2010). Effect of ploidy levels on the activities of Δ1-pyrroline-5-carboxylate synthetase, superoxide dismutase and peroxidase in Cenchrus species grown under water stress. Plant Physiology and Biochemistry, 48(1), 27–34. https://doi.org/10.1016/j.plaphy.2009.10.003

Charlesworth B. (1991). The evolution of sex chromosomes. Science (New York, N.Y.), 251(4997), 1030–1033. https://doi.org/10.1126/science.1998119

Charoensawan, V., Wilson, D. & Teichmann, S. A. (2010). Genomic repertoires of DNA-binding transcription factors across the tree of life, Nucleic Acids Research, 38(21), 7364–7377. https://doi.org/10.1093/nar/gkq617

Chen, K., & Rajewsky, N. (2007). The evolution of gene regulation by transcription factors and microRNAs. Nature Reviews Genetics. 8, 93–103. https://doi.org/10.1038/nrg1990

Cho, S. H., Haning, K., Shen, W., Blome, C., Li, R., Yang, S., & Contreras, L. M. (2017). Identification and characterization of 5' untranslated regions (5'utrs) in zymomonas mobilis as regulatory biological parts. Frontiers in Microbiology, 8, 2432. https://doi.org/10.3389/fmicb.2017.02432

Chow, C. N., Chiang-Hsieh, Y. F., Chien, C. H., Zheng, H. Q., Lee, T. Y., Wu, N. Y., Tseng, K., Hou, P., & Chang, W. C. (2018). Delineation of condition specific Cis- and Trans-acting elements in plant promoters under various Endo- and exogenous stimuli. BMC Genomics, 19(2), 85. https://doi.org/10.1186/s12864-018-4469-4

Chow, C. N., Lee, T. Y., Hung, Y. C., Li, G. Z., Tseng, K. C., Liu, Y. H., Kuo, P., Zheng, H., & Chang, W. C. (2019). Plantpan3.0: A new and updated resource for reconstructing transcriptional regulatory networks from chip-seq experiments in plants. Nucleic Acids Research, 47(D1), D1155–D1163. https://doi.org/10.1093/nar/gky1081

Chung, B. Y. W., Simons, C., Firth, A. E., Brown, C. M., & Hellens, R. P. (2006). Effect of 5′UTR introns on gene expression in Arabidopsis thaliana. BMC Genomics, 7(1), 120. https://doi.org/10.1186/1471-2164-7-120

Clancy, M., Vasil, V., Curtis Hannah, L., & Vasil, I. K. (1994). Maize Shrunken-1 intron and exon regions increase gene expression in maize protoplasts. Plant Science, 98(2), 151–161. https://doi.org/10.1016/0168-9452(94)90005-1

Clancy, M. and Hannah, L. C. (2002). Splicing of the Maize Sh1 First Intron Is Essential for Enhancement of Gene Expression, and a T-Rich Motif Increases Expression without Affecting Splicing, Plant Physiology, 130(2), 918 – 929. https://doi.org/10.1104/pp.008235

Clay, K. and Kover, P. X. (1996). The Red Queen hypothesis and plant/pathogen interactions. Annual Review of Phytopathology, 34(1), 29–50. https://doi.org/10.1146/annurev.phyto.34.1.29

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium -mediated transformation of Arabidopsis thaliana. The Plant Journal, 16(6), 735–743. https://doi.org/10.1046/j.1365-313x.1998.00343.x

Colombo, L., Battaglia, R. and Kater, M. M. (2008). Arabidopsis ovule development and its evolutionary conservation. Trends in Plant Science, 13(8), 444–450. http://dx.doi.org/10.1016/j.tplants.2008.04.011

Conner, J. A., Mookkan, M., Huo, H., Chae, K., & Ozias-Akins, P. (2015). A parthenogenesis gene of apomict origin elicits embryo formation from unfertilized eggs in a sexual plant. Proceedings of the National Academy of Sciences of the United States of America, 112(36), 11205LP–11210. https://doi.org/10.1073/pnas.1505856112

Conner, J. A., Podio, M. and Ozias-Akins, P. (2017). R-BBML transgenesHaploid embryo production in rice and maize induced by PsASG. Plant Reproduction, 30(1), 41–52. https://doi.org/10.1007/s00497-017-0298-x

Gene Ontology Consortium (2006). The Gene Ontology (GO) project in 2006. Nucleic acids research, 34(Database issue), D322–D326. https://doi.org/10.1093/nar/gkj021

Corbett-Detig, R. B., Hartl, D. L. and Sackton, T. B. (2015). Natural selection constrains neutral diversity across a wide range of species, PLoS biology, 13(4), e1002112–e1002112. https://doi.org/10.1371/journal.pbio.1002112

Corral, J. M., Vogel, H., Aliyu, O. M., Hensel, G., Thiel, T., Kumlehn, J., & Sharbel, T. F. (2013). A conserved apomixis-specific polymorphism is correlated with exclusive exonuclease expression in premeiotic ovules of apomictic Boechera species. Plant Physiology, 163(4), 1660–1672. https://doi.org/10.1104/pp.113.222430

Cramer, P., Armache, K. J., Baumli, S., Benkert, S., Brueckner, F., Buchen, C., Damsma, G.,Dengl, S., Geiger, S., Jasiak, A., Jawhari, A., Jennebach, S., Kamenski, T., Kettenberger, H., Kuhn, C., Lehmann, E., Leike, K., Sydow, J., & Vannini, A. (2008). Structure of eukaryotic RNA polymerases. Annual Review of Biophysics. 37, 337-352. https://doi.org/10.1146/annurev.biophys.37.032807.130008

Crane, P., Friis, E.M., & Pedersen, K.R. (1995). The origin and early diversification of angiosperms. Nature, 374(6517), 27-33. https://doi.org/10.1038/374027A0

Cubas, P., Lauter, N., Doebley, J., & Coen, E. (1999). The TCP domain: A motif found in proteins regulating plant growth and development. Plant Journal, 18(2), 215–222. https://doi.org/10.1046/j.1365-313X.1999.00444.x

Cubas, P. (2002). Role of TCP genes in the evolution of morphological characters in angiosperms, in Developmental Genetics and Plant Evolution. CRC Press (Systematics Association Special Volumes), 247–266. https://doi.org/10.1201/9781420024982.ch13.

Curaba, J., Herzog, M., & Vachon, G. (2003). GeBP, the first member of a new gene family in Arabidopsis, encodes a nuclear protein with DNA-binding activity and is regulated by KNAT1. Plant Journal, 33(2), 305–317. https://doi.org/10.1046/j.1365-313X.2003.01622.x

Curtis, M. D., and Grossniklaus, U. (2008). Molecular control of autonomous embryo and endosperm development. Sexual Plant Reproduction. 21(1), 79–88. https://doi.org/10.1007/s00497-007-0061-9

D'Erfurth, I., Jolivet, S., Froger, N., Catrice, O., Novatchkova, M., & Mercier, R. (2009). Turning meiosis into mitosis. PLoS Biology, 7(6), e1000124. https://doi.org/10.1371/journal.pbio.1000124

Danisman, S., van der Wal, F., Dhondt, S., Waites, R., de Folter, S., Bimbo, A., van Dijk, A. D., Muino, J. M., Cutri, L., Dornelas, M. C., Angenent, G. C., & Immink, R. G. (2012). Arabidopsis class I and class II TCP transcription factors regulate jasmonic acid metabolism and leaf development antagonistically. Plant physiology, 159(4), 1511–1523. https://doi.org/10.1104/pp.112.200303

Darlington, C. D. (1931). Meiosis in diploid and tetraploid Primula sinensis. Journal of Genetics, 24(1), 65–96. https://doi.org/10.1007/BF03020823

Davuluri, R. V., Suzuki, Y., Sugano, S., Plass, C., & Huang, T. H. M. (2008). The functional consequences of alternative promoter use in mammalian genomes. Trends in Genetics. 24(4), 167-177. https://doi.org/10.1016/j.tig.2008.01.008

Deng, W., & Blobel, G. A. (2017). Detecting Long-Range Enhancer-Promoter Interactions by Quantitative Chromosome Conformation Capture. Methods in molecular biology (Clifton, N.J.), 1468, 51–62. https://doi.org/10.1007/978-1-4939-4035-6_6

Deplancke, B., Dupuy, D., Vidal, M., & Walhout, A. J. (2004). A gateway-compatible yeast one-hybrid system. Genome research, 14(10B), 2093–2101. https://doi.org/10.1101/gr.2445504Dey

Dey, B., Thukral, S., Krishnan, S., Chakrobarty, M., Gupta, S., Manghani, C., & Rani, V. (2012). DNA-protein interactions: Methods for detection and analysis. Molecular and Cellular Biochemistry, 365(1), 279–299. https://doi.org/10.1007/s11010-012-1269-z

Dhindsa, R. S. and Cleland, R. E. (1975). Water Stress and Protein Synthesis. Plant Physiology, 55(4), 782 – 785. https://doi.org/10.1104/pp.55.4.782

Dietz, K.-J. B. T.-I. R. of C. (2003). Redox Control, Redox Signaling, and Redox Homeostasis in Plant Cells, in. Academic Press, 228, 141–193. https://doi.org/10.1016/S0074-7696(03)28004-9

Doebley, J., Stec, A., & Hubbard, L. (1997). The evolution of apical dominance in maize. Nature, 386(6624), 485–488. https://doi.org/10.1038/386485a0

Doncaster, C. P., Pound, G. E., & Cox, S. J. (2000). The ecological cost of sex. Nature, 404(6775), 281–285. https://doi.org/10.1038/35005078

Douglas, S. J. and Riggs, C. D. (2005). Pedicel development in Arabidopsis thaliana: Contribution of vascular positioning and the role of the BREVIPEDICELLUS and ERECTA genes. Developmental Biology, 284(2), 451–463. https://doi.org/10.1016/j.ydbio.2005.06.011

Duan, Z. J., Fang, X., Rohde, A., Han, H., Stamatoyannopoulos, G., & Li, Q. (2002). Developmental specificity of recruitment of TBP to the TATA box of the human gamma-globin gene. Proceedings of the National Academy of Sciences of the United States of America, 99(8), 5509–5514. https://doi.org/10.1073/pnas.072084499

Duveau, F., Yuan, D. C., Metzger, B., Hodgins-Davis, A., & Wittkopp, P. J. (2017). Effects of mutation and selection on plasticity of a promoter activity in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America, 114(52), E11218–E11227. https://doi.org/10.1073/pnas.1713960115

Erdmann, R. M., Hoffmann, A., Walter, H. K., Wagenknecht, H. A., Groß-Hardt, R., & Gehring, M. (2017). Molecular movement in the Arabidopsis thaliana female gametophyte. Plant reproduction, 30(3), 141–146. https://doi.org/10.1007/s00497-017-0304-3

Fehrer, J., Gemeinholzer, B., Chrtek, J., & Bräutigam, S. (2007). Incongruent plastid and nuclear DNA phylogenies reveal ancient intergeneric hybridization in Pilosella hawkweeds (Hieracium, Cichorieae, Asteraceae). Molecular Phylogenetics and Evolution, 42(2), 347–361. https://doi.org/10.1016/j.ympev.2006.07.004

Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., … Punta, M. (2014, January 1). Pfam: The protein families database. Nucleic Acids Research. 42(D1), D222-D230. https://doi.org/10.1093/nar/gkt1223

Formstecher, E., Aresta, S., Collura, V., Hamburger, A., Meil, A., Trehin, A., Reverdy, C., Betin, V., Maire, S., Brun, C., Jacq, B., Arpin, M., Bellaiche, Y., Bellusci, S., Benaroch, P., Bornens, M., Chanet, R., Chavrier, P., Delattre, O., Doye, V., … Daviet, L. (2005). Protein interaction mapping: a Drosophila case study. Genome research, 15(3), 376–384. https://doi.org/10.1101/gr.2659105

Fromont-Racine, M., Rain, J.-C. and Legrain, P. (1997). Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens. Nature Genetics, 16(3), 277–282. https://doi.org/10.1038/ng0797-277

Fujimoto, S. Y., Ohta, M., Usui, A., Shinshi, H., & Ohme-Takagi, M. (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. The Plant cell, 12(3), 393–404. https://doi.org/10.1105/tpc.12.3.393

Galderisi, U., Jori, F. P. and Giordano, A. (2003). Cell cycle regulation and neural differentiation. Oncogene, 22(33), 5208–5219. https://doi.org/10.1038/sj.onc.1206558

Galla, G., Basso, A., Grisan, S., Bellucci, M., Pupilli, F., & Barcaccia, G. (2019). Ovule Gene Expression Analysis in Sexual and Aposporous Apomictic Hypericum perforatum L. (Hypericaceae) Accessions. Frontiers in plant science, 10, 654. https://doi.org/10.3389/fpls.2019.00654

Garcia-Aguilar, M., Michaud, C., Leblanc, O., & Grimanelli, D. (2010). Inactivation of a DNA methylation pathway in maize reproductive organs results in apomixis-like phenotypes. The Plant cell, 22(10), 3249–3267. https://doi.org/10.1105/tpc.109.072181

Gasser, C. S., Broadhvest, J. and Hauser, B. A. (1998). Genetic analysis of ovule development, Annual Review of Plant Physiology and Plant Molecular Biology, 49(1), 1–24. https://doi.org/10.1146/annurev.arplant.49.1.1

Gerber, N. and Kokko, H. (2016). Sexual conflict and the evolution of asexuality at low population densities, Proceedings of the Royal Society B: Biological Sciences, 283(1841). https://doi.org/10.1098/rspb.2016.1280

Goddard, M. R., Godfray, H. C., & Burt, A. (2005). Sex increases the efficacy of natural selection in experimental yeast populations. Nature, 434(7033), 636–640. https://doi.org/10.1038/nature03405

Grafi, G., Chalifa-Caspi, V., Nagar, T., Plaschkes, I., Barak, S., & Ransbotyn, V. (2011). Plant response to stress meets dedifferentiation. Planta. Springer Verlag, 233(3), 433–438. https://doi.org/10.1007/s00425-011-1366-3

Griffiths, J., Bonser, S., & Associate Editor and Editor: Susan Kalisz. (2013). Is Sex Advantageous in Adverse Environments? A Test of the Abandon-Ship Hypothesis. The American Naturalist, 182(6), 718-725. https://doi.org/10.1086/673476

Grimanelli, D., García, M., Kaszas, E., Perotti, E., & Leblanc, O. (2003). Heterochronic expression of sexual reproductive programs during apomictic development in Tripsacum.Genetics, 165(3), 1521–1531. https://doi.org/10.1093/genetics/165.3.1521

Groß-Hardt, R., Kägi, C., Baumann, N., Moore, J. M., Baskar, R., Gagliano, W. B., Jürgens, G., & Grossniklaus, U. (2007). LACHESIS restricts gametic cell fate in the female gametophyte of Arabidopsis. PLoS Biology, 5(3), 0494–0500. https://doi.org/10.1371/journal.pbio.0050047

Grossniklaus, U. (2001). From sexuality to apomixis: molecular and genetic approaches. Edited by Y. Savidan, J. G. Carman, and T. Dresselhaus. Mexico: CIMMYT, IRD European Commission DG VI (FAIR). 168-211. ISBN 970-648-074-9. xii, 243 p

Grossniklaus, U., Koltunow, A. and van Lookeren Campagne, M. (1998). A bright future for apomixis. Trends in Plant Science, 3(11), 415–416. https://doi.org/10.1016/S1360-1385(98)01338-7

Grossniklaus, U., & Schneitz, K. (1998). The molecular and genetic basis of ovule and megagametophyte development. Seminars in cell & developmental biology, 9(2), 227–238. https://doi.org/10.1006/scdb.1997.0214

Ha, H. C., Sirisoma, N. S., Kuppusamy, P., Zweier, J. L., Woster, P. M., & Casero, R. A. (1998). The natural polyamine spermine functions directly as a free radical scavenger. Proceedings of the National Academy of Sciences of the United States of America, 95(19), 11140–11145. https://doi.org/10.1073/pnas.95.19.11140

Hadacek, F., Bachmann, G., Engelmeier, D., & Chobot, V. (2010). Hormesis and a Chemical Raison D'être for Secondary Plant Metabolites. Dose-response, 9(1), 79–116. https://doi.org/10.2203/dose-response.09-028.Hadacek

Hadany, L. and Beker, T. (2003). Fitness-associated recombination on rugged adaptive landscapes. Journal of Evolutionary Biology, 16(5), 862–870. https://doi.org/10.1046/j.1420-9101.2003.00586.x

Hardtke, C. S. and Berleth, T. (1998). The Arabidopsis gene MONOPTEROS encodes a transcription factor mediating embryo axis formation and vascular development. The EMBO journal, 17(5), 1405–1411. https://doi.org/10.1093/emboj/17.5.1405

Harkess, A., Huang, K., van der Hulst, R., Tissen, B., Caplan, J. L., Koppula, A., Batish, M., Meyers, B. C., & Leebens-Mack, J. (2020). Sex Determination by Two Y-Linked Genes in Garden Asparagus. The Plant cell, 32(6), 1790–1796. https://doi.org/10.1105/tpc.19.00859

Hartley, J. L., Temple, G. F. and Brasch, M. A. (2000). DNA cloning using in vitro site-specific recombination. Genome research, 10(11), 1788–1795. https://doi.org/10.1101/gr.143000

Hawkes, J. G. & Jackson, M. T. (1992). Taxonomic and evolutionary implications of the Endosperm Balance Number hypothesis in potatoes. Theoretical and Applied Genetics, 84(1), 180–185. https://doi.org/10.1007/BF00223998

Hennig, L., Gruissem, W., Grossniklaus, U., & Köhler, C. (2004). Transcriptional programs of early reproductive stages in Arabidopsis. Plant physiology, 135(3), 1765–1775. https://doi.org/10.1104/pp.104.043182

Hernandez-Garcia, C. M. and Finer, J. J. (2014). Identification and validation of promoters and cis-acting regulatory elements. Plant Science, 217–218, 109–119. https://doi.org/10.1016/j.plantsci.2013.12.007

Higashiyama, T., Kuroiwa, H., Kawano, S., & Kuroiwa, T. (2000). Explosive discharge of pollen tube contents in Torenia fournieri. Plant physiology, 122(1), 11–14. https://doi.org/10.1104/pp.122.1.11

Higashiyama, T., Yabe, S., Sasaki, N., Nishimura, Y., Miyagishima, S. Y., Kuroiwa, H., & Kuroiwa, T. (2001). Pollen tube attraction by the synergid cell. Science, 293(5534), 1480–1483. https://doi.org/10.1126/science.1062429

Hoekstra R. F. (2005). Evolutionary biology: why sex is good. Nature, 434(7033), 571–573. https://doi.org/10.1038/434571a

Hoffman, M. M. & Birney, E. (2010). An effective model for natural selection in promoters. Genome research, 20(5), 685–692. https://doi.org/10.1101/gr.096719.109

Hoffmann, A. A. and Hercus, M. J. (2000). Environmental Stress as an Evolutionary Force. BioScience, 50(3), 217–226. https://doi.org/10.1641/0006-3568(2000)050[0217:ESAAEF]2.3.CO;2

Hojsgaard, D., & Hörandl, E. (2015). A little bit of sex matters for genome evolution in asexual plants. Frontiers in Plant Science, 6, 82. https://doi.org/10.3389/fpls.2015.00082

Hörandl, E. and Hadacek, F. (2013). The oxidative damage initiation hypothesis for meiosis, Plant reproduction. 26(4), 351–367. https://doi.org/10.1007/s00497-013-0234-7

Horstman, A., Fukuoka, H., Muino, J. M., Nitsch, L., Guo, C., Passarinho, P., Sanchez-Perez, G., Immink, R., Angenent, & G., Boutilier, K. (2015). AIL and HDG proteins act antagonistically to control cell proliferation. Development (Cambridge), 142(3), 454–464. https://doi.org/10.1242/dev.117168

Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., & Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene*, 77(1), 61–68. https://doi.org/10.1016/0378-1119(89)90359-4

Horton, R. M., Cai, Z., Ho, S. N., & Pease, L. R. (2013). Gene splicing by overlap extension: Tailor-made genes using the polymerase chain reaction. *BioTechniques*, 54(3), 528–535. https://doi.org/10.2144/000114017

Howard, R. S., & Lively, C. M. (1994). Erratum: Parasitism, mutation accumulation and the maintenance of sex. Nature, 367(6463), 554-557. https://doi.org/10.1038/368358a0

Hu, G. and Kurgan, L. (2019). Sequence Similarity Searching. Current Protocols in Protein Science. 95(1), e71. https://doi.org/10.1002/cpps.71

Hu, G. and Wendel, J. F. (2019). Cis–trans controls and regulatory novelty accompanying allopolyploidization. New Phytologist, 221(4), 1691–1700. https://doi.org/10.1111/nph.15515

Hudson, R. R. (1983). Properties of a neutral allele model with intragenic recombination, Theoretical Population Biology. 23(2), 183–201. https://doi.org/10.1016/0040-5809(83)90013-8

Initiative, T. A. G. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature, 408, 796-815. https://doi.org/10.1038/35048692

Jackson, D. (2002). Double labeling of KNOTTED1 mRNA and protein reveals multiple potential sites of protein trafficking in the shoot apex. Plant physiology, 129(4), 1423–1429. https://doi.org/10.1104/pp.006049

Jacob, F. and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. Journal of Molecular Biology, 3(3), 318–356. https://doi.org/10.1016/S0022-2836(61)80072-7

Jefferson, R. A., Burgess, S. M., & Hirsh, D. (1986). Beta- Glucuronidase from *Escherichia coli* as a gene-fusion marker. PNAS, 83(22), 8447-8451. https://doi.org/10.1073/pnas.83.22.8447

Jefferson, R. A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Molecular Biology Reporter, 5(4), 387–405. https://doi.org/10.1007/BF02667740

Jens, M. and Rajewsky, N. (2014). Competition between target sites of regulators shapes post-transcriptional gene regulation. Nature Reviews Genetics, 16(2), 113-126. https://doi.org/10.1038/nrg3853.

Jeong, Y. M., Mun, J. H., Kim, H., Lee, S. Y., & Kim, S. G. (2007). An upstream region in the first intron of petunia actin-depolymerizing factor 1 affects tissue-specific expression in transgenic Arabidopsis (Arabidopsis thaliana). Plant Journal, 50(2), 230–239. https://doi.org/10.1111/j.1365-313X.2007.03053.x

Johnston, A. J., Meier, P., Gheyselinck, J., Wuest, S. E. J., Federer, M., Schlagenhauf, E., Becker, J., Grossniklaus, U. (2007). Genetic subtraction profiling identifies genes essential for Arabidopsis reproduction and reveals interaction between the female gametophyte and the maternal sporophyte. Genome Biology, 8(10). https://doi.org/10.1186/gb-2007-8-10-r204

Johnston, S. A., den Nijs, T. P. M., Peloquin, S. J., & Hanneman, R. E. (1980). The significance of genic balance to endosperm development in interspecific crosses. Theoretical and Applied Genetics, 57(1), 5–9. https://doi.org/10.1007/BF00276002

Jónsson, Z. O. and Hübscher, U. (1997). Proliferating cell nuclear antigen: More than a clamp for DNA polymerases. BioEssays, 19(11), 967–975. https://doi.org/10.1002/bies.950191106

Juel, H. O., (1898). Parthenogenesis bei Antennaria alpina (L.). R. Br. Bot. Centralblatt 74(13), 369–372.

Juven-Gershon, T., Hsu, J. Y., Theisen, J. W., & Kadonaga, J. T. (2008). The RNA polymerase II core promoter - the gateway to transcription. Current Opinion in Cell Biology, 20(3), 253-9, https://doi.org/10.1016/j.ceb.2008.03.003

Kantama, L., Sharbel, T. F., Schranz, M. E., Mitchell-Olds, T., de Vries, S., & de Jong, H. (2007). Diploid apomicts of the Boechera holboellii complex display large-scale chromosome substitutions and aberrant chromosomes. Proceedings of the National Academy of Sciences of the United States of America, 104(35), 14026–14031. https://doi.org/10.1073/pnas.0706647104

Karimi, M., Inzé, D. and Depicker, A. (2002). GATEWAYTM vectors for Agrobacterium-mediated plant transformation. Trends in Plant Science, 7(5), 193–195. https://doi.org/10.1016/S1360-1385(02)02251-3

Karthikeyan, A. S., Ballachanda, D. N. and Raghothama, K. G. (2009). Promoter deletion analysis elucidates the role of cis elements and 5'UTR intron in spatiotemporal regulation of AtPht1;4 expression in Arabidopsis. Physiologia Plantarum, 136(1), 10–18. https://doi.org/10.1111/j.1399-3054.2009.01207.x

Keightley, P. D., & Eyre-Walker, A. (2000). Deleterious mutations and the evolution of sex. Science (New York, N.Y.), 290(5490), 331–333. https://doi.org/10.1126/science.290.5490.331

Khanday, I., Skinner, D., Yang, B., Mercier, R., & Sundaresan, V. (2019). A male-expressed rice embryogenic trigger redirected for asexual propagation through seeds. Nature, 565(7737), 91–95. https://doi.org/10.1038/s41586-018-0785-8

Kindiger, B., Sokolov, V., & Dewald, C. (1996). A comparison of apomictic reproduction in eastern gamagrass (Tripsacum dactyloides (L.) L.) and maize-Tripsacum hybrids. Genetica, 97(1), 103–110. https://doi.org/10.1007/BF00132586

Klucher, K. M., Chow, H., Reiser, L., & Fischer, R. L. (1996). The AINTEGUMENTA gene of Arabidopsis required for ovule and female gametophyte development is related to the floral homeotic gene APETALA2. *The Plant cell*, 8(2), 137–153. https://doi.org/10.1105/tpc.8.2.137

Klug, W. S., Ward, S. M., Spencer, C.A., & Palladino, M. A. (2009). Concepts of genetics. 9th ed.. San Francisco: San Francisco : Pearson Benjamin Cummings. 48(4), M220–M220. https://doi.org/10.1136/mp.48.4.m220-b

Koebner, R. (1994). Apomixis in Plants. By S. E. Asker and L. Jerling. Boca Raton and London: CRC Press (1992), 298, ISBN 0-8493-4545-6. Experimental Agriculture, 30(1), 107. https://doi.org/10.1017/S0014479700023954

Koltunow, A. M. (1993). Apomixis: Embryo Sacs and Embryos Formed without Meiosis or Fertilization in Ovules. The Plant cell, 5(10), 1425–1437. https://doi.org/10.1105/tpc.5.10.1425

Koltunow, A. M., Johnson, S. D., Lynch, M., Yoshihara, T., & Costantino, P. (2001). Expression of rolB in apomictic Hieracium piloselloides Vill. causes ectopic meristems in planta and changes in ovule formation, where apomixis initiates at higher frequency. Planta, 214(2), 196–205. https://doi.org/10.1007/s004250100612

Koltunow, A. M. G., Johnson, S. D., Rodrigues, J. C. M., Okada, T., Hu, Y., Tsuchiya, T., Wilson, S., Fletcher, P., Ito, K., Suzuki, G., Mukai, Y., Fehrer, J., & Bicknell, R. A. (2011). Sexual reproduction is the default mode in apomictic Hieracium subgenus Pilosella, in which two dominant loci function to enable apomixis. Plant Journal, 66(5), 890–902. https://doi.org/10.1111/j.1365-313X.2011.04556.x

Koltunow, A. M. and Grossniklaus, U. (2003). Apomixis: A developmental perspective. Annu Rev Plant Biol, 54, 547-574. https://doi.org/10.1146/annurev.arplant.54.110901.160842

Koltunow, A. M., Johnson, S. D. and Bicknell, R. A. (1998). Sexual and apomictic development in Hieracium. Sexual Plant Reproduction, 11(4), 213–230. https://doi.org/10.1007/s004970050144

Kordyum, E. L. (2008). Double fertilization in flowering plants: 1898–2008. Cytology and Genetics, 42(3), 147–158. https://doi.org/10.3103/S009545270803002X

Kosugi, S. and Ohashi, Y. (1997). PCF1 and PCF2 specifically bind to cis elements in the rice proliferating cell nuclear antigen gene., The Plant Cell, 9(9), 1607 – 1619. https://doi.org/10.1105/tpc.9.9.1607

Kosugi, S., Suzuka, I. and Ohashi, Y. (1995). Two of three promoter elements identified in a rice gene for proliferating cell nuclear antigen are essential for meristematic tissue-specific expression, The Plant Journal, 7(6), 877–886. https://doi.org/10.1046/j.1365-313X.1995.07060877.x

Köszegi, D., Johnston, A. J., Rutten, T., Czihal, A., Altschmied, L., Kumlehn, J., Wust, S., Kirioukhova, O., Gheyselinck, J., Grossniklaus, U., & Bäumlein, H. (2011). Members of the RKD transcription factor family induce an egg cell-like gene expression program. Plant Journal, 67(2), 280–291. https://doi.org/10.1111/j.1365-313X.2011.04592.x

Krogh, A., Larsson, B., Von Heijne, G., & Sonnhammer, E. L. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. Journal of Molecular Biology, 305(3), 567–580. https://doi.org/10.1006/jmbi.2000.4315

Kulkarni, S. R. and Vandepoele, K. (2020). Inference of plant gene regulatory networks using data-driven methods: A practical overview. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 1863(6), 194447. https://doi.org/10.1016/j.bbagrm.2019.194447

Kumar, S., Saxena, S. and Gupta, M. G. (2017). Marker-assisted screening of breeding populations of an apomictic grass Cenchrus ciliaris L. segregating for the mode of reproduction. Crop Breeding and Applied Biotechnology. scielo, 17(1), 10–17. https://doi.org/10.1590/1984-70332017v17n1a2

Kummerfeld, S. K., & Teichmann, S. A. (2006). DBD: a transcription factor prediction database. Nucleic Acids Research, 34(1), D71-D84. https://doi.org/10.1093/nar/gkj131

Latchman, D. S. (1997). Transcription factors: An overview. The International Journal of Biochemistry & Cell Biology, 29(12), 1305–1312. http://dx.doi.org/10.1016/S1357-2725(97)00085-X

Laxa, M., Müller, K., Lange, N., Doering, L., Pruscha, J. T., & Peterhänsel, C. (2016). The 5'UTR intron of arabidopsis GGT1 aminotransferase enhances promoter activity by recruiting RNA polymerase II. Plant Physiology, 172(1), 313–327. https://doi.org/10.1104/pp.16.00881

Lee, C.-R. and Mitchell-Olds, T. (2013). Complex trait divergence contributes to environmental niche differentiation in ecological speciation of Boechera stricta. Molecular Ecology, 22(8), 2204–2217. https://doi.org/10.1111/mec.12250

Lee, C. R., & Mitchell-Olds, T. (2011). Quantifying effects of environmental and geographical factors on patterns of genetic differentiation. Molecular ecology, 20(22), 4631–4642. https://doi.org/10.1111/j.1365-294X.2011.05310.x

Li, J. J., & Herskowitz, I. (1993). Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. Science, 262(5141), 1870 –1874. https://doi.org/10.1126/science.8266075

Li, S. (2015). The Arabidopsis thaliana TCP transcription factors: A broadening horizon beyond development. Plant Signaling & Behavior, 10(7), 1044192. https://doi.org/10.1080/15592324.2015.1044192

Lieberman-Aiden, E., Van Berkum, N. L., Williams, L., Imakaev, M., Ragoczy, T., Telling, A., Amit, I., Lajoie, B., Sabo, P., Dorschner, M., Sandstrom, R., Bernstein, B.,Bender, M.,Groudine, M., Gnirke, A., Stamatoyannopoulos, J., Mirny, L., Lander, E., & Dekker, J. (2009). Comprehensive mapping of long-range interactions reveals folding principles of the human genome. Science, 326(5950), 289–293. https://doi.org/10.1126/science.1181369

Lohmann, J. U., Hong, R. L., Hobe, M., Busch, M. A., Parcy, F., Simon, R., & Weigel, D. (2001). A molecular link between stem cell regulation and floral patterning in Arabidopsis. Cell, 105(6), 793–803. https://doi.org/10.1016/S0092-8674(01)00384-1

Lovell, J. T., Aliyu, O. M., Mau, M., Schranz, M. E., Koch, M., Kiefer, C., Song, B. H., Mitchell-Olds, T., & Sharbel, T. F. (2013). On the origin and evolution of apomixis in Boechera. Plant reproduction, 26(4), 309–315. https://doi.org/10.1007/s00497-013-0218-7

Lovell, J. T., Grogan, K., Sharbel, T. F., & McKay, J. K. (2014). Mating system and environmental variation drive patterns of adaptation in Boechera spatifolia (Brassicaceae). Molecular Ecology, 23(18), 4486–4497. https://doi.org/10.1111/mec.12879

Lovell, J. T., Williamson, R. J., Wright, S. I., McKay, J. K., & Sharbel, T. F. (2017). Mutation Accumulation in an Asexual Relative of Arabidopsis. PLoS Genetics, 13(1). 1006550. https://doi.org/10.1371/journal.pgen.1006550

Luo, D., Carpenter, R., Vincent, C., Copsey, L., & Coen, E. (1996). Origin of floral asymmetry in Antirrhinum. Nature, 383(6603), 794 –799. https://doi.org/10.1038/383794a0

Luo, K.-R., Huang, N.-C. and Yu, T.-S. (2018). Selective Targeting of Mobile mRNAs to Plasmodesmata for Cell-to-Cell Movement. Plant Physiology, 177(2), 604 – 614. https://doi.org/10.1104/pp.18.00107

Lynch, M. (1984). Destabilizing hybridization, general-purpose genotypes and geographic parthenogenesis. The Quarterly Review of Biology, 59(3), 257–290. https://www.jstor.org/stable/2827008

Ma, X., Ma, J., Fan, D., Li, C., Jiang, Y., & Luo, K. (2016). Genome-wide Identification of TCP Family Transcription Factors from Populus euphratica and Their Involvement in Leaf Shape Regulation. Scientific Reports, 6. https://doi.org/10.1038/srep32795

Maas, C., Laufs, J., Grant, S., Korfhage, C., & Werr, W. (1991). The combination of a novel stimulatory element in the first exon of the maize Shrunken-1 gene with the following intron 1 enhances reporter gene expression up to 1000-fold. Plant Molecular Biology, 16(2), 199 –207. https://doi.org/10.1007/BF00020552

Malamy, J. E. and Benfey, P. N. (1997). Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development, 124(1), 33 – 44. http://dev.biologists.org/content/124/1/33.abstract

Malmberg, R. L. (1977). The evolution of epistasis and the advantage of recombination in populations of bacteriophage T4. Genetics, 86(3), 607–621. https://doi.org/10.1093/genetics/86.3.607

Martín-Trillo, M., & Cubas, P. (2010). TCP genes: a family snapshot ten years later. Trends in plant science, 15(1), 31–9. https://doi.org/10.1016/j.tplants.2009.11.003

Marsch-Martinez, N., Greco, R., Becker, J. D., Dixit, S., Bergervoet, J. H., Karaba, A., de Folter, S., & Pereira, A. (2006). BOLITA, an Arabidopsis AP2/ERF-like transcription factor that affects cell expansion and proliferation/differentiation pathways. *Plant molecular biology*, 62(6), 825–843. https://doi.org/10.1007/s11103-006-9059-1

Mascarenhas, D., Mettler, I. J., Pierce, D. A., & Lowe, H. W. (1990). Intron-mediated enhancement of heterologous gene expression in maize. Plant Molecular Biology, 15(6), 913–920. https://doi.org/10.1007/BF00039430

Mau, M., Lovell, J. T., Corral, J. M., Kiefer, C., Koch, M. A., Aliyu, O. M., & Sharbel, T. F. (2015). Hybrid apomicts trapped in the ecological niches of their sexual ancestors. Proceedings of the National

Academy of Sciences of the United States of America, 112(18), 2357–2365. https://doi.org/10.1073/pnas.1423447112

Mau, M., Liiving, T., Fomenko, L., Goertzen, R., Paczesniak, D., Böttner, L., & Sharbel, T. F. (2021). The spread of infectious asexuality through haploid pollen. New Phytologist, 230(2), 804–820. https://doi.org/10.1111/nph.17174

Maynard, J. and Haigh, J. (2007). The hitch-hiking effect of a favourable gene. Genetics Research, 89(5–6), 391–403. https://doi.org/10.1017/S0016672308009579

Mehrnia, M., Balazadeh, S., Zanor, M. I., & Mueller-Roeber, B. (2013). EBE, an AP2/ERF transcription factor highly expressed in proliferating cells, affects shoot architecture in Arabidopsis. Plant physiology, 162(2), 842–857. https://doi.org/10.1104/pp.113.214049

Mogie, M. (1992). The Evolution of Asexual Reproduction in Plants. London: Chapman & Hall.

Moll, C., Von Lyncker, L., Zimmermann, S., Kägi, C., Baumann, N., Twell, D., Grossniklaus, U., & Groß-Hardt, R. (2008). CLO/GFA1 and ATO are novel regulators of gametic cell fate in plants. Plant Journal, 56(6), 913–921. https://doi.org/10.1111/j.1365-313X.2008.03650.x

Monniaux, M., & Vandenbussche, M. (2018). How to evolve a perianth: A review of cadastral mechanisms for perianth identity. Frontiers in Plant Science. Frontiers Media S.A. 9, 1573. https://doi.org/10.3389/fpls.2018.01573

Muller, H. J. (1932). Some Genetic Aspects of Sex. The American Naturalist, 66(703), 118–138. https://doi.org/10.1086/280418

Muller, H. J. (1964). The relation of recombination to mutational advance, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1(1), 2–9. https://doi.org/10.1016/0027-5107(64)90047-8

Mun, J. H., Lee, S. Y., Yu, H. J., Jeong, Y. M., Shin, M. Y., Kim, H., Lee, I., & Kim, S. G. (2002). Petunia actin-depolymerizing factor is mainly accumulated in vascular tissue and its gene expression is enhanced by the first intron. Gene, 292(1–2), 233–243. https://doi.org/10.1016/S0378-1119(02)00646-7

Nakamura, M., Katsumata, H., Abe, M., Yabe, N., Komeda, Y., Yamamoto, K. T., & Takahashi, T. (2006). Characterization of the class IV homeodomain-Leucine Zipper gene family in Arabidopsis. Plant physiology, 141(4), 1363–1375. https://doi.org/10.1104/pp.106.077388

Nakano, T., Suzuki, K., Fujimura, T., & Shinshi, H. (2006). Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant physiology, 140(2), 411–432. https://doi.org/10.1104/pp.105.073783

Nemhauser, J. L., Feldman, L. J. and Zambryski, P. C. (2000). Auxin and ETTIN in Arabidopsis gynoecium morphogenesis, Development, 127(18), 3877 – 3888. http://dev.biologists.org/content/127/18/3877

Nishiyama, I., and Inomata, N. (1966). Embryological studies on cross-incompatibility 2x and 4x in Brassica. The Japanese Journal of Genetics, 41(1), 27-42. https://doi.org/10.1266/JJG.41.27

Nogler G.A. (1984) Gametophytic Apomixis. In: Johri B.M. (eds) Embryology of Angiosperms. Springer, Berlin, Heidelberg. 475-518. https://doi.org/10.1007/978-3-642-69302-1_10

Nogler G. A. (2006). The lesser-known Mendel: his experiments on Hieracium. Genetics, 172(1), 1– 6. https://doi.org/10.1093/genetics/172.1.1

Okada, T., Hu, Y., Tucker, M. R., Taylor, J. M., Johnson, S. D., Spriggs, A., Tsuchiya, T., Oelkers, K., Rodrigues, J. C., & Koltunow, A. M. (2013). Enlarging cells initiating apomixis in Hieracium praealtum transition to an embryo sac program prior to entering mitosis. Plant physiology, 163(1), 216 –231. https://doi.org/10.1104/pp.113.219485

Olmedo-Monfil, V., Durán-Figueroa, N., Arteaga-Vázquez, M., Demesa-Arévalo, E., Autran, D., Grimanelli, D., Slotkin, R. K., Martienssen, R. A., & Vielle-Calzada, J. P. (2010). Control of female gamete formation by a small RNA pathway in Arabidopsis. Nature, 464(7288), 628–632. https://doi.org/10.1038/nature08828

Oñate-Sánchez, L. and Singh, K. B. (2002). Identification of Arabidopsis Ethylene-Responsive Element Binding Factors with Distinct Induction Kinetics after Pathogen Infection, Plant Physiology, 128(4), 1313 – 1322. https://doi.org/10.1104/pp.010862

Ostenfeld, C. H. (1904). Weitere Beiträge zur Kenntnis der Fruchtentwicklung bei der Gattung Hieracium, 22, 537–541, https://doi.org/10.1111/j.1438-8677.1904.tb05282.x

Otto S. P. (2009). The evolutionary enigma of sex. The American naturalist, 174 (1), S1–S14. https://doi.org/10.1086/599084

Otto, S. P., & Lenormand, T. (2002). Resolving the paradox of sex and recombination. Nature Reviews Genetics. 3, 252–261. https://doi.org/10.1038/nrg761

Pagnussat, G. C., Yu, H.-J. and Sundaresan, V. (2007). Cell-Fate Switch of Synergid to Egg Cell in Arabidopsis eostre Mutant Embryo Sacs Arises from Misexpression of the BEL1-Like Homeodomain Gene BLH1. The Plant Cell, 19(11), 3578–3592. https://doi.org/10.1105/tpc.107.054890

Pajares, M. J., Ezponda, T., Catena, R., Calvo, A., Pio, R., & Montuenga, L. M. (2007). Alternative splicing: an emerging topic in molecular and clinical oncology. The Lancet. Oncology, 8(4), 349–357. https://doi.org/10.1016/S1470-2045(07)70104-3

Park, S. W., & Bolker, B. (2019). Bridging the gap between theory and data: the Red Queen Hypothesis for sex. BioRxiv, 637413. https://doi.org/10.1101/637413

Parra, G., Bradnam, K., Rose, A. B., & Korf, I. (2011). Comparative and functional analysis of intron-mediated enhancement signals reveals conserved features among plants. Nucleic acids research, 39(13), 5328–5337. https://doi.org/10.1093/nar/gkr043

Parrott, W. A., & Smith, R. R. (1986). Evidence for the existence of endosperm balance number in the true clovers (Trifolium spp.). Canadian Journal of Genetics and Cytology, 28(4), 581–586. https://doi.org/10.1139/g86-085

Pennington, R. T. (2002). Willis, K.J., McElwain, J.C. The evolution of plants. Annals of Botany, 90(5), 678–679. https://doi.org/10.1093/aob/mcf232.

Phukan, U. J., Jeena, G. S., Tripathi, V., & Shukla, R. K. (2017). Regulation of Apetala2/Ethylene Response Factors in Plants. Frontiers in plant science, 8, 150. https://doi.org/10.3389/fpls.2017.00150

Pina, C., Pinto, F., Feijó, J. A., & Becker, J. D. (2005). Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. Plant physiology, 138(2), 744–756. https://doi.org/10.1104/pp.104.057935

Pinyopich, A., Ditta, G. S., Savidge, B., Liljegren, S. J., Baumann, E., Wisman, E., & Yanofsky, M. F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature, 424(6944), 85–88. https://doi.org/10.1038/nature01741

Poczai, P., Cernák, I., Varga, I., & Hyvönen, J. (2014). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Genetic Resources and Crop Evolution, 61(1), 796–815. https://doi.org/10.1134/S1022795411020074

Pupilli, F. and Barcaccia, G. (2012). Cloning plants by seeds: Inheritance models and candidate genes to increase fundamental knowledge for engineering apomixis in sexual crops. Journal of Biotechnology, 159(4), 291–311. https://doi.org/10.1016/j.jbiotec.2011.08.028

Qian, Z., Cai, Y.-D. and Li, Y. (2006). Automatic transcription factor classifier based on functional domain composition. Biochemical and Biophysical Research Communications, 347(1), 141–144. http://dx.doi.org/10.1016/j.bbrc.2006.06.060

Raghavan, V. (2003). Some reflections on double fertilization, from its discovery to the present. New Phytologist. 159(3), 565-583. https://doi.org/10.1046/j.1469-8137.2003.00846.x

Raghavan, V. (2006). Double Fertilization: Embryo and Endosperm Development in Flowering Plants. Springer Berlin Heidelberg, 1-237. https://doi.org/10.1007/3-540-27792-7

Rain, J. C., Selig, L., De Reuse, H., Battaglia, V., Reverdy, C., Simon, S., Lenzen, G., Petel, F., Wojcik, J., Schächter, V., Chemama, Y., Labigne, A., & Legrain, P. (2001). The protein-protein interaction map of Helicobacter pylori. Nature, 409(6817), 211–215. https://doi.org/10.1038/35051615

Ramu, P., Esuma, W., Kawuki, R., Rabbi, I. Y., Egesi, C., Bredeson, J. V., Bart, R., Verma, J., Buckler, E., & Lu, F. (2017). Cassava haplotype map highlights fixation of deleterious mutations during clonal propagation. Nature Genetics, 49(6), 959–963. https://doi.org/10.1038/ng.3845

Ramulu, K. S., Sharma, V. K., Naumova, T. N., Dijkhuis, P., & Van Lookeren Campagne, M. M. (1999). Apomixis for crop improvement. Protoplasma, 208(1), 196–205. https://doi.org/10.1007/BF01279090

Ray, A., Robinson-Beers, K., Ray, S., Baker, S. C., Lang, J. D., Preuss, D., Milligan, S. B., & Gasser, C. S. (1994). Arabidopsis floral homeotic gene BELL (BEL1) controls ovule development through negative regulation of AGAMOUS gene (AG). Proceedings of the National Academy of Sciences of the United States of America, 91(13), 5761–5765. https://doi.org/10.1073/pnas.91.13.5761

Reece-Hoyes, J. S., Deplancke, B., Barrasa, M. I., Hatzold, J., Smit, R. B., Arda, H. E., Pope, P. A., Gaudet, J., Conradt, B., & Walhout, A. J. (2009). The C. elegans Snail homolog CES-1 can activate gene expression in vivo and share targets with bHLH transcription factors. Nucleic acids research, 37(11), 3689–3698. https://doi.org/10.1093/nar/gkp232

Reece-Hoyes, J. S. and Marian Walhout, A. J. (2012). Yeast one-hybrid assays: a historical and technical perspective, Methods (San Diego, Calif.). 2012/08/03, 57(4), 441–447. https://doi.org/10.1016/j.ymeth.2012.07.027

Remans, T., Grof, C. P. L., Ebert, P. R., & Schenk, P. M. (2005). Functional promoter analysis using an approach based on an in vitro evolution strategy. BioTechniques, 38(2), 209–216. https://doi.org/10.2144/05382ST01

Renner, S. S. and Müller, N. A. (2021). Plant sex chromosomes defy evolutionary models of expanding recombination suppression and genetic degeneration. Nature Plants, 7(4), 392–402. https://doi.org/10.1038/s41477-021-00884-3

Rice, W. R., & Chippindale, A. K. (2001). Sexual recombination and the power of natural selection. Science (New York, N.Y.), 294(5542), 555–559. https://doi.org/10.1126/science.1061380

Robson, M. I., Ringel, A. R., & Mundlos, S. (2019). Regulatory Landscaping: How Enhancer-Promoter Communication Is Sculpted in 3D. *Molecular cell*, 74(6), 1110–1122. https://doi.org/10.1016/j.molcel.2019.05.032

Rocha, P. S., Sheikh, M., Melchiorre, R., Fagard, M., Boutet, S., Loach, R., Moffatt, B., Wagner, C., Vaucheret, H., & Furner, I. (2005). The Arabidopsis HOMOLOGY-DEPENDENT GENE SILENCING1 gene codes for an S-adenosyl-L-homocysteine hydrolase required for DNA methylation-dependent gene silencing. The Plant cell, 17(2), 404–417. https://doi.org/10.1105/tpc.104.028332

Roeder, R. G., & Rutter, W. J. (1969). Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. Nature, 224(5216), 234–237. https://doi.org/10.1038/224234a0

Rojek, J., Kapusta, M., Kozieradzka-Kiszkurno, M., Majcher, D., Górniak, M., Sliwinska, E., Sharbel, T. F., & Bohdanowicz, J. (2018). Establishing the cell biology of apomictic reproduction in diploid Boechera stricta (Brassicaceae). Annals of botany, 122(4), 513–539. https://doi.org/10.1093/aob/mcy114

Rose, A. B. (2004). The effect of intron location on intron-mediated enhancement of gene expression in Arabidopsis. The Plant Journal, 40(5), 744–751. https://doi.org/10.1111/j.1365-313X.2004.02247.x

Roze, D. (2012). Disentangling the benefits of sex. PLoS Biology, 10(5). https://doi.org/10.1371/journal.pbio.1001321

Ruiz-Medrano, R., Xoconostle-Cazares, B. and Kragler, F. (2004). The plasmodesmatal transport pathway for homeotic proteins, silencing signals and viruses, Current opinion in plant biology, 7(6), 641-650. https://doi.org/10.1016/j.pbi.2004.09.012

Rushton, P. J., Reinstädler, A., Lipka, V., Lippok, B., & Somssich, I. E. (2002). Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. The Plant cell, 14(4), 749 –762. https://doi.org/10.1105/tpc.010412

Rushworth, C. A., Song, B. H., Lee, C. R., & Mitchell-Olds, T. (2011). Boechera, a model system for ecological genomics. Molecular Ecology. 20(23), 4843-4857. https://doi.org/10.1111/j.1365-294X.2011.05340.x

Sablowski, R. (2007). Flowering and determinacy in Arabidopsis, Journal of Experimental Botany, 58(5), 899–907. https://doi.org/10.1093/jxb/erm002

Sakuma, Y., Liu, Q., Dubouzet, J. G., Abe, H., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2002). DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochemical and Biophysical Research Communications, 290(3), 998–1009. https://doi.org/10.1006/bbrc.2001.6299

Samach, A., Onouchi, H., Gold, S. E., Ditta, G. S., Schwarz-Sommer, Z., Yanofsky, M. F., & Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science, 288(5471), 1613–1616. https://doi.org/10.1126/science.288.5471.1613

Sarvepalli, K. and Nath, U. (2011). Hyper-activation of the TCP4 transcription factor in Arabidopsis thaliana accelerates multiple aspects of plant maturation. The Plant Journal, 67(4), 595–607. https://doi.org/10.1111/j.1365-313X.2011.04616.x

Satina, S.& Blakeslee, A. F. (1935). Cytological Effects of a Gene in Datura which Causes Dyad Formation in Sporogenesis, Botanical Gazette, 96(3), 521–532. https://doi.org/10.1086/334498

Schallau, A., Arzenton, F., Johnston, A. J., Hähnel, U., Koszegi, D., Blattner, F. R., Altschmied, L., Haberer, G., Barcaccia, G., & Bäumlein, H. (2010). Identification and genetic analysis of the APOSPORY locus in Hypericum perforatum L., Plant Journal, 62(5), 773–784. https://doi.org/10.1111/j.1365-313X.2010.04188.x

Schmidt, A., Schmid, M. W., Klostermeier, U. C., Qi, W., Guthörl, D., Sailer, C., Waller, M., Rosenstiel, Ph., & Grossniklaus, U. (2014). Apomictic and Sexual Germline Development Differ with Respect to Cell Cycle, Transcriptional, Hormonal and Epigenetic Regulation. PLoS Genetics, 10(7). https://doi.org/10.1371/journal.pgen.1004476

Schmidt, A. (2020). Controlling Apomixis: Shared Features and Distinct Characteristics of Gene Regulation. Genes, 11(3), 329. https://doi.org/10.3390/genes11030329

Schön, I., Martens, K. and Dijk, P. (eds) (2009). Lost Sex. Dordrecht: Springer Netherlands. https://doi.org/10.1007/978-90-481-2770-2

Shah, J. N., Kirioukhova, O., Pawar, P., Tayyab, M., Mateo, J. L., & Johnston, A. J. (2016). Depletion of Key Meiotic Genes and Transcriptome-Wide Abiotic Stress Reprogramming Mark Early Preparatory Events Ahead of Apomeiotic Transition. Frontiers in plant science, 7, 1539. https://doi.org/10.3389/fpls.2016.01539

Shaikhali, J., Davoine, C. É., Brännström, K., Rouhier, N., Bygdell, J., Björklund, S., & Wingsle, G. (2015). Biochemical and redox characterization of the mediator complex and its associated transcription factor GeBPL, a GLABROUS1 enhancer binding protein. Biochemical Journal, 468(3), 385–400. https://doi.org/10.1042/BJ20150132

Sharbel, T. F., Voigt, M. L., Corral, J. M., Galla, G., Kumlehn, J., Klukas, C., Schreiber, F., Vogel, H., & Rotterd, B. (2010). Apomictic and sexual ovules of Boechera display heterochronic global gene expression patterns. Plant Cell, 22(3), 655–671. https://doi.org/10.1105/tpc.109.072223

Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Current Opinion in Plant Biology, 3(3), 217–223. https://doi.org/10.1016/S1369-5266(00)80068-0

Silvertown, J. (2008). The Evolutionary Maintenance of Sexual Reproduction: Evidence from the Ecological Distribution of Asexual Reproduction in Clonal Plants. International Journal of Plant Sciences, 169(1), 157–168. https://doi.org/10.1086/523357

Smith, J. M. (1958). The Effects of Temperature and of Egg-Laying on the Longevity of Drosophila subobscura. Journal of Experimental Biology, 35(4), 832 – 842. http://sageke.sciencemag.org/cgi/content/abstract/sageke;2002/37/cp15

Smith, J. M. (1978). The evolution of sex. Cambridge (England: Cambridge University Press).

Smyth, D. R., Bowman, J. L. and Meyerowitz, E. M. (1990). Early flower development in Arabidopsis. The Plant cell. 2(8), 755–767. https://doi.org/10.1105/tpc.2.8.755

Springer, P. S. (2000). Gene traps: Tools for plant development and genomics. Plant Cell, 12(7), 1007–1020. https://doi.org/10.1105/tpc.12.7.1007

Stearns, S. C. (2017). The masterpiece of nature: The evolution and genetics of sexuality. Ethology and Sociobiology, 5(1), 73–75. https://doi.org/10.1016/0162-3095(84)90043-8

Steffen, J. G., Kang, I. H., Portereiko, M. F., Lloyd, A., & Drews, G. N. (2008). AGL61 interacts with AGL80 and is required for central cell development in Arabidopsis. Plant Physiology, 148(1), 259–268. https://doi.org/10.1104/pp.108.119404

Stevens, N. E. (1948). Disease Damage in Clonal and Self-Pollinated Crops1. Agronomy Journal, 40(9), 841–844. https://doi.org/10.2134/agronj1948.00021962004000090010x

Sun, Y., Li, Y., Huang, G., Wu, Q., & Wang, L. (2017). Application of the yeast one-hybrid technique to plant functional genomics studies. Biotechnology and Biotechnological Equipment. Taylor and Francis Ltd. https://doi.org/10.1080/13102818.2017.1378595

Syamaladevi, D. P., Meena, S. S. and Nagar, R. P. (2016). Molecular understandings on "the never thirsty" and apomictic Cenchrus grass. Biotechnology Letters, 38(3), 369–376. https://doi.org/10.1007/s10529-015-2004-0

Taylor-Teeples, M., Lin, L., de Lucas, M., Turco, G., Toal, T. W., Gaudinier, A., Young, N. F., Trabucco, G. M., Veling, M. T., Lamothe, R., Handakumbura, P. P., Xiong, G., Wang, C., Corwin, J., Tsoukalas, A., Zhang, L., Ware, D., Pauly, M., Kliebenstein, D. J., Dehesh, K., Tagkopoulos, I., Breton, G., Pruneda-Paz, J. L., Ahnert, S. E., Kay, S. A., Hazen, S. P., & Brady, S. M. (2015). An Arabidopsis gene regulatory network for secondary cell wall synthesis. Nature, 517(7536), 571–575. https://doi.org/10.1038/nature14099

Tilsner, J., Nicolas, W., Rosado, A., & Bayer, E. M. (2016). Staying Tight: Plasmodesmal Membrane Contact Sites and the Control of Cell-to-Cell Connectivity in Plants. Annual Review of Plant Biology, Annual Reviews Inc. 67, 337-364. https://doi.org/10.1146/annurev-arplant-043015-111840

Tiwari, S.B., Belachew, A., Ma, S.F., Young, M., Ade, J., Shen, Y., Marion, C.M., Holtan, H.E., Bailey, A., Stone, J.K., Edwards, L., Wallace, A.D., Canales, R.D., Adam, L., Ratcliffe, O.J. and Repetti, P.P. (2012). The EDLL motif: a potent plant transcriptional activation domain from AP2/ERF transcription factors. The Plant Journal, 70, 855-865. https://doi.org/10.1111/j.1365-313X.2012.04935.x

Trinklein, N. D., Aldred, S. F., Hartman, S. J., Schroeder, D. I., Otillar, R. P., & Myers, R. M. (2004). An abundance of bidirectional promoters in the human genome. Genome research, 14(1), 62–66. https://doi.org/10.1101/gr.1982804

Tucker, M. R., Araujo, A. C. G., Paech, N. A., Hecht, V., Schmidt, E. D. L., Rossell, J. B., … Koltunow, A. M. G. (2003). Sexual and apomictic reproduction in Hieracium subgenus Pilosella are closely interrelated developmental pathways. Plant Cell, 15(7), 1524–1537. https://doi.org/10.1105/tpc.011742

Tucker, M. R., & Koltunow, A. (2009). Sexual and asexual (apomictic) seed development in flowering plants: molecular, morphological and evolutionary relationships. Functional plant biology, 36(6), 490–504. https://doi.org/10.1071/FP09078

Urbanus, S.L., Martinelli, A.P., (Peter) Dinh, Q.D., Aizza, L.C.B., Dornelas, M.C., Angenent, G.C. and Immink, R.G.H. (2010). Intercellular transport of epidermis-expressed MADS domain transcription factors and their effect on plant morphology and floral transition. The Plant Journal, 63, 60-72. https://doi.org/10.1111/j.1365-313X.2010.04221.x

Vielle Calzada, J. P., Crane, C. F., & Stelly, D. M. (1996). Apomixis: The asexual revolution. Science. https://doi.org/10.1126/science.274.5291.1322

Vijverberg, K., Ozias-Akins, P., & Schranz, M. E. (2019). Identifying and Engineering Genes for Parthenogenesis in Plants. Frontiers in plant science, 10, 128. https://doi.org/10.3389/fpls.2019.00128

Vinkenoog, R. and Scott, R. J. (2001). Autonomous endosperm development in flowering plants: how to overcome the imprinting problem?. Sexual Plant Reproduction, 14(4), 189–194. https://doi.org/10.1007/s00497-001-0106-4

Viola, I. L., Reinheimer, R., Ripoll, R., Manassero, N., & Gonzalez, D. H. (2012). Determinants of the DNA binding specificity of class I and class II TCP transcription factors. The Journal of biological chemistry, 287(1), 347–356. https://doi.org/10.1074/jbc.M111.256271

Wang, S., Cheng, X., Li, Y., Wu, M., & Zhao, Y. (2018). Image-based promoter prediction: a promoter prediction method based on evolutionarily generated patterns. Scientific Reports, 8(1). https://doi.org/10.1038/s41598-018-36308-0

Wang, X. H., Li, Q. T., Chen, H. W., Zhang, W. K., Ma, B., Chen, S. Y., & Zhang, J. S. (2014). Trihelix transcription factor GT-4 mediates salt tolerance via interaction with TEM2 in Arabidopsis. BMC Plant Biology, 14(1). https://doi.org/10.1186/s12870-014-0339-7

Warnatz, H. J., Querfurth, R., Guerasimova, A., Cheng, X., Haas, S. A., Hufton, A. L., Manke, Th., Vanhecke, D., Nietfeld, W., Vingron, M., Janitz, M., Lehrach, H., & Yaspo, M. L. (2010). Functional analysis and identification of cis-regulatory elements of human chromosome 21 gene promoters. Nucleic Acids Research, 38(18), 6112–6123. https://doi.org/10.1093/nar/gkq402

Wei, W., Pelechano, V., Järvelin, A. and Steinmetz, L. (2011). Functional consequences of bidirectional promoters. Trends in Genetics, 27(7), 267–276. https://doi.org/10.1016/j.tig.2011.04.002

Westergaard, M. (1958). The Mechanism of Sex Determination in Dioecious Flowering Plants. Advances in Genetics, 9, 217–281. https://doi.org/10.1016/S0065-2660(08)60163-7

Weismann, A. (1889). Essays upon heredity and kindred biological problems. https://doi.org/10.5962/bhl.title.28066

Windham, M. D., & Al-Shehbaz, I. A. (2007). New and noteworthy species of Boechera (Brassicaceae) III: additional sexual diploid and apomictic hybrids. Harvard Papers in Botany, 12(1), 235–257. https://doi.org/10.3100/1043-4534(2007)12[235:nansob]2.0.co;2

Winkler, H. (1908). "Über Parthenogenesis und Apogamie im Pflanzenreich". Progressus Rei Botanicae. 2 (3), 293–454.

Wojcik, J., Boneca, I. G. and Legrain, P. (2002). Prediction, Assessment and Validation of Protein Interaction Maps in Bacteria. Journal of Molecular Biology, 323(4), 763–770. https://doi.org/10.1016/S0022-2836(02)01009-4

Wu, F., Shi, X., Lin, X., Liu, Y., Chong, K., Theißen, G., & Meng, Z. (2017). The ABCs of flower development: mutational analysis of AP1/FUL-like genes in rice provides evidence for a homeotic (A)-function in grasses. The Plant journal : for cell and molecular biology, 89(2), 310–324. https://doi.org/10.1111/tpj.13386

Xiao, W., Custard, R. D., Brown, R. C., Lemmon, B. E., Harada, J. J., Goldberg, R. B., & Fischer, R. L. (2006). DNA methylation is critical for Arabidopsis embryogenesis and seed viability. Plant Cell, 18(4), 805–814. https://doi.org/10.1105/tpc.105.038836

Yang, X., Pang, H., Liu, B., Qiu, Z., Gao, Q., Wei, L., Dong, Y. and Wang, Y. (2012). Evolution of Double Positive Autoregulatory Feedback Loops in CYCLOIDEA2 Clade Genes Is Associated with the Origin of Floral Zygomorphy, The Plant Cell, 24(5), 1834–1847. https://doi.org/10.1105/tpc.112.099457

You, J., & Chan, Z. (2015). ROS Regulation During Abiotic Stress Responses in Crop Plants. Frontiers in plant science, 6, 1092. https://doi.org/10.3389/fpls.2015.01092