T.C.

ÇANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

UNRAVELING THE APOMIXIS-RELATED GENES IN BOECHERA GENUS: *BBM*, *APOLLO*, and *LEC2*

MASTER OF SCIENCE THESIS

BUKET ÜNER

Thesis Supervisor

PROF. DR. KEMAL MELİH TAŞKIN

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ETİK BEYAN

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Buket ÜNER

19.07.2023

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Buket ÜNER

Çanakkale, July 2023

ÖZET

BOECHERA CİNSİNDE APOMİKSİ İLE İLİŞKİLİ GENLERİN ORTAYA ÇIKARILMASI: BBM, APOLLO, VE LEC2

Buket ÜNER

Çanakkale Onsekiz Mart Üniversitesi

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Moleküler Biyoloji ve Genetik Anabilim Dalı Yüksek Lisans Tezi

Danışman: Prof. Dr. Kemal Melih Taşkın

19/07/2023, 60

Apomiksi, bitkilerde partenonegenez (cinsel üremenin döllenme aşamalarından kaçınma) ve apomayoz (mayozda anormallerin görülmesi ya da mayozun engellenmesi) sonucunda dişi ebeveyne özdeş embriyolar üreten bir eşeysiz üreme yöntemidir. Genetiği değiştirilmiş veya hibrit bitkiler için sabit bir genotipe sahip mahsuller üretmek önemli bir özelliktir. Apomiksinin arkasındaki moleküler mekanizma karmaşık olması ve genetik altyapısının tamamen anlaşılamamış olması ile birlikte, apomikt ve eşeyli türler arasında farklı şekilde düzenlenen veya mayoz gibi apomiksideki önemli süreçlerde rollerinin bulunduğu bilinen aday genler literatürde mevcuttur. *Apomiksis-Linked Locus (APOLLO)*, *Baby BOOM (BBM)* ve *Leafy Cotyledon 2* (*LEC2*) genleri, apomiktik gelişim için aday genler olarak tanımlanmışlardır. Bu çalışmanın amacı, apomik ve eşeyli üreme sırasında *BBM*, *LEC2* ve *APOLLO* genlerinin ekspresyon profillerindeki farklılıkların araştırılmasıdır. Ayrıca *BBM* ve *LEC2* genlerinin gen klonlama ve dizi analizleri gerçekleştirilmiştir. Bu çalımanın sonuçları, *APOLLO* geninin apo-alelinin *Boechera*'nın apomikt türlerinde, anter ve pistil dokularında apomayoz sırasında yüksek oranda gen anlatım göstermektedir ve eşeysel alelin mayoz sırasında eşeyli *Boechera*'da aynı sonuçları göstermektedir. Melezlemeden 5 gün sonra pistillerdeki gen anlatımında görülen keskin artışın, *APOLLO* geninin apo-aleli ile apomeiosis arasındaki ilişkiyi desteklediği gözlenmiştir. Melezlemeden

5 gün sonra sex-alelde bulunan aynı artışın, *APOLLO* geninin sex-aleli ile mayoz arasındaki ilişkiyi desteklediği gözlenmiştir. Ayrıca, *LEC2* gen anlatımının apomikt *B. divaricarpa* ve eşeyli *B.stricta* bitkileri arasında önemli bir fark göstermediği belirlenmiştir. *BBM* geni, hem apomikt hem de eşeyli *Boechera* bitkilerinde melezleme sonrası pistillerde anlamlı bir anlatım göstermemesine rağmen, *B. divaricarpa*'nın anter dokularında mayozdan sonra keskin bir şekilde azalma göstermiştir ve pistil dokularında yüksek bir şekilde ifadeye uğramıştır. Bu *BBM* geninin apomict *B. divaricarpa* bitkisinde otonom embryo oluşumunu destekleyebileceğini göstermiştir. Ayrıca gen klonlama çalışmalarımız sonucunda *B. divaricarpa* bitkilerinde *BBM* ve *LEC2* gen dizileri ortaya çıkarılmıştır. Bu çalışmanın bulguları, apomiktik yolların düzenleyici genlerinin keşfine önemli katkı sağlayacaktır.

Anahtar Kelimeler: Apomiksi, *Boechera*, Parthenogenez, Apomayoz, *BBM* , *LEC2*, *APOLLO*

ABSTRACT

UNRAVELING THE APOMIXIS-RELATED GENES IN BOECHERA GENUS: *BBM*, *APOLLO*, and *LEC2*

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School of Graduate Studies

Master of Science Thesis in Molecular Biology And Genetics

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Apomixis a way of asexual reproduction that generates embryos identical to the female parent as a result of the apomeiosis (partially or complete deficient from meiosis) and parthenonegenesis (avoiding fertilization stages of sexual reproduction) in plants. Apomixis is an important trait to produce hybrid plants with a fixed genotype . Although the molecular mechanisms behind apomixis are complex and many of its parts are still unknown, the candidate genes differently regulated between apomict and sexual species were reported in model species. Among these candidate genes, *Apomixis-Linked Locus (APOLLO), Baby BOOM (BBM)* and *Leafy Cotyledon 2* genes are the most promising genes. The aim of this study is investigation of differences in expression profile of *BBM*, *LEC2* and *APOLLO* genes during apomict and sexual reproduction. Gene spesific primer sequence were designed to perform gene expression analysis by real-time PCR. Besides, gene cloning analysis of *BBM* and *LEC2* genes were carried out. Our results suggest that apo-allele of *APOLLO* gene is highly expressed in anter and pistil tissues of apomict type of *Boechera* during apomeiosis ans sex-allele showed the same results in sexual *Boechera* during meiosis. Its sharp increase in expression in pistils of 5 days after pollunation (DAP) supported the relation between apo-allele of *APOLLO* gene and apomeiosis, and the relation between sex-allele of *APOLLO* gene and meiosis. Although the *LEC2* gene showed high expression in anter tissues, It didn't show any significant differences in gene expression between in both sexual *B. stricta* and apomict *B. divaricarpa* plants. It is also revealed that, *BBM* gene showed high expression in

B. stricta in anter tissues after meiosis and showed no expression in pistil tissues at the same stage. Interestingly, in apomict *B. divaricarpa*, *BBM* gene showed no expression in *B. divaricarpa* anter tissues and it showed high expression in pistil tissues. These results revealed the *BBM* gene would contribute the autonomous embryogenesis in apomict *B. divaricarpa*. Lastly, it is reported that the gene sequences of *BBM* and *LEC2* in *B. divaricarpa* and *B. stricta* plants as a result of our gene clonning studies. The findings of this study will contribute significantly to the discovery of the regulatory genes of apomictic pathways.

Keywords: Apomixis, *Boechera*, Parthenogenesis, Apomeiosis, *BBM* , *LEC2*, *APOLLO*

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MATERIALS AND METHODS

ABBREVIATIONS

TUBİTAK	The Scientific and Technological Research Council of Türkiye
TAIR	The Arabidopsis Information Resource
PCR	Polymerase Chain Reaction
cDNA	Complementary DNA
E. coli	*Escherichia coli*
CaCl2	Calcium chloride
M	Molarity (Molar concentration)
UV	Ultraviolet
LB	Luria-Bertani
BLAST	Basic Local Alignment Search Tool
DAP	Days After Pollination
MMC	Megaspore Mother Cell
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic acid
μl	Microliter
UPGMA	Unweighted Pair Group Method With Arithmetic Mean

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CHAPTER 1
INTRODUCTION

1.1. Apomixis

Apomixis is the mode of asexual reproduction that generates offsprings identical to the maternal plant by skipping meiosis and double fertilization (Barcaccia & Albertini, 2013; Brukhin, 2017; Koltunow, 1993; Nogler G.A, 1984). It was primarily revealed in *Alchornea ilicifolia* and it is defined in 400 species and 40 family from algae to flowering plants (Carman, 1997; Hojsgaard et al., 2014). 148 genres that produced adventitious embryony, 110 genres that produced apospory, and 68 genres that produced diplospory were documented (Carman, 1997; Hojsgaard et al., 2014)

Sexual and Apomictic Reproduction

In sexual plants, the megaspore mother cell (MMC) undergoes two meiosis in each ovule of the pistil (the female organ of flowering plants) and forms 4 haploid megaspores. One of these megaspores produces a haploid embryo sac (a female gametophyte, and the other three undergo apoptosis (Cresti, M., Blackmore, S., & Van Went, 1993; Drews et al., 1998). An embryo sac includes one haploid (n) egg and one diploid (2n) central cell, as well as two synergids and three antipodal cells. During pollination, the pollen tube grows towards the ovule and penetrates into the embryo sac. One of the sperm cells directly fertilizes the haploid egg cell to produce a diploid zygote; the other one fuses into the central cell to produce a triploid endosperm. This process is called "double fertilization". Meiosis of MMC and fusion of the female and male during sexual reproduction maintains diversity (Brukhin & Baskar, 2019; Cresti, M., Blackmore, S., & Van Went, 1993; Russel, 1992)

Apomixis is closely intertwined with sexual reproduction. Three key features characterized apomixis; apomeiosis, parthenogenesis, and endosperm formation. Apomeiosis involves the atypical behavior of the megaspore mother cell (MMC), either undergoing incomplete meiosis disregarding segregation and recombination or directly proceeding to mitosis (Albertini & Barcaccia, 2007; Koltunow et al., 1995). Parthenogenesis denotes the development of an embryo without fertilization. Endosperm development in

apomixis can occur through either fertilization of the central cell (pseudogamous) or autonomous embryo sac formation takes place respectively. These features distinguish apomixis from sexual reproduction.

Mechanisms of Apomixis

Apomixis can be classified into two main types: gametophytic and sporophytic apomixis. In the case of sporophytic apomixis, specifically adventitious embryony, the embryo is directly formed from the sporophytic cells of the ovule, which can be either nucellar or integumental cells (Naumova, 1993). It grows into the sexually generated endosperm to utilize the nutrients provided by endosperm for the viable seed formation. Sporophytic apomixis is the most common type of apomixis and has been identified in nearly 250 species of flowering plants, including agriculturally significant plants like *Citrus*, *Ribes*, and *Alnus* (Chaudhury et al., 1998; Koltunow et al., 1995; Naumova, 1993).

Gametophytic apomixis is a unique way plants reproduce without fertilization. It has two main mechanisms: apospory and diplospory (Koltunow et al., 1995). In aposporous apomicts, an embryo sac contains functional megaspore is originated from nucellar cell. In diplosporous apomicts, embryo sac is emerged from a MMC which bypass meiosis and form dyads rather than four megaspores. The embryo then develops independently (Asker, 1980; Barcaccia & Albertini, 2013; Nogler G.A, 1984; Savidan, 2001). Diplospory is reported in various species in various families such as Rosaceae, Solanaceae, Brassicaceae involving *Boechera* spp (Brukhin, 2017; Carman et al., 2019).

In both adventitious and gametophytic embryony, the development of a fertile seed is dependent on endosperm formation. The requirement of seed formation for the fertilization of the central cell is called pseudogamous apomixis. In pseudogamous apomixis, the endosperm development is triggered by a process similar to fertilization, involving the fusion of male and female nuclei or the involvement of pollen, even though egg cell fertilization does not occur. In autonomous apomixis, the central cell undergoes a transformation into endosperm in the absence of fertilization (Koltunow & Grossniklaus, 2003).
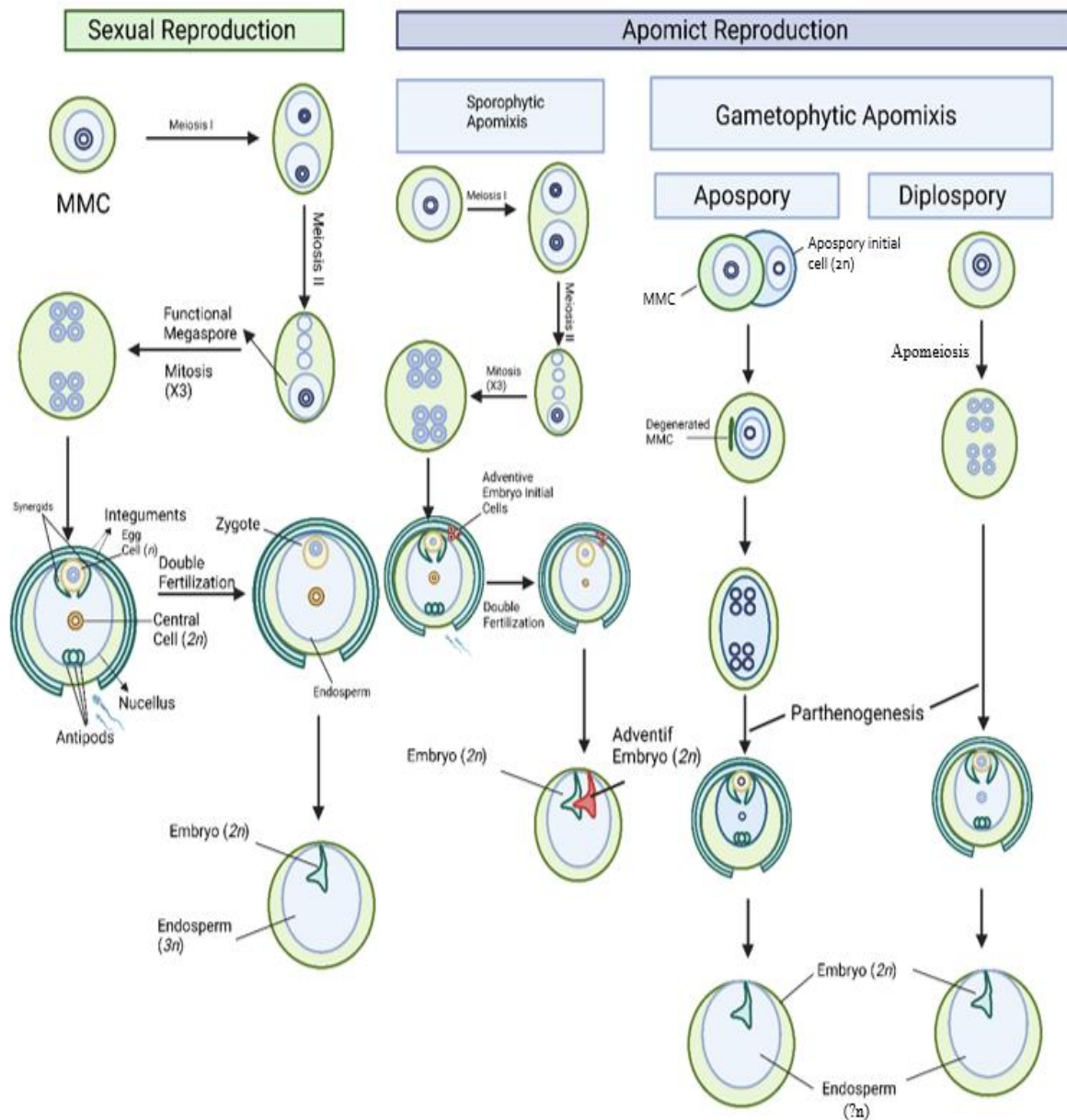
Figure 1: Mechanisms of Sexual and Apomict Reproduction

Agricultural Importance of Apomixis

Sexual reproduction in flowering plants generates population diversity through the segregation and recombination of genetic material via meiosis and pollination (Otto, 2008). Apomixis is an important trait for agricultural. (Hanna & Bashaw, 1987; Otto, 2008; Y. H. Savidan, 1986). It helps to fix the progeny of genetically modified or bred plants for

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conditions and hybrid vigor (Hanna & Bashaw, 1987; Y. H. Savidan, 1986; Spillane et al., 2004). Apomixis also helps to make agricultural sustainable by increasing crop yield by reducing agricultural field (Koltunow et al., 1995; Ortiz & Pessino, 2019).

Boechera spp.

The genus *Boechera* (Löve & Löve, 1976) is a biennial or perennial flowering plant of North America, including Canada and the northeastern United States. The chromosome number is n =7. Out of the 110 species in the Boechera genus, 71 have been identified as diploid, both sexual and apomictic species. (Al-Shehbaz, 2003; Brukhin et al., 2019; Schranz, Dobes, et al., 2005). Due to the presence of low ploidy levels in both sexual and apomict species and their close relationship with *Arabidopsis*, *Boechera* plants are appropriate for apomixis studies (Aliyu, Schranz, Sharbel, et al., 2010).

B. stricta is a diploid and sexual species, and it is the most widely distributed species within the Boechera genus. Due to its high potential for hybridization, it has been involved in the formation of numerous hybrids. One example of such a hybrid is *B. divaricarpa* which is a hybrid between *B. stricta x B. sparsiflora* (Rojek et al., 2018; Windham & Al-shehbaz, 2006). *B. divaricarpa* is an biennial apomict species mostly distributed in the Western United States (Aliyu, Schranz, Sharbel, et al., 2010; Schranz, Dobeš, et al., 2005; Windham & Al-Shehbaz, 2007). Apomict Boechera species, including *B. divaricarpa*, produce identical embryos to the maternal plant by *Taraxacum* type diplospory and the endosperm formation occurs psuedogamously (Carman et al., 2019; Rojek et al., 2018). B. divaricarpa is a facultative apomict plants that shows low frequency for the sexual seed formation (Aliyu, Schranz, Sharbel, et al., 2010).

1.6. Apomixis-Related Genes

Apomixis possesses a molecular mechanism that remains elusive in its complexity. It shares genetic mechanisms resembling sexual reproduction, except for the genes involved in three components of apomixis. When genes responsible for sexual reproduction undergo alterations or apomixis-specific genes are activated, apomixis surpasses infertility

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and generates seeds that are genetically identical to the female parent (Xu et al., 2022). Several genes have been identified as potentially associated with apomixis (Brukhin & Baskar, 2019; Koltunow & Grossniklaus, 2003; Xu et al., 2022).

In *Boechera*, the *APOLLO* (*apomixis-linked locus*) and *UPGRADE2* (*unreduced pollen grain development*) genes were reported to be associated with apomeiosis (Bakin et al., 2022; Corral et al., 2013). *APOLLO* encodes aspartate glutamate aspartate aspartate histidine exonuclease, which is downregulated in sexual ovules and is upregulated in apomeiotic ovules at the beginning of meiosis, implying that it has various sex-alleles and apo-alleles. Apomict alleles of *APOLLO* gene is highly expressed in the apomict ovules and down-regulated in the sexula ovules. Against this, sexual alleles of APOLLO genes were highly regulated in sexula ovules during meiosis and down-regulated in apomict ovules (Bakin et al., 2022; Corral et al., 2013).

Also, various genes would have been implicated in embryonic development (Brukhin & Baskar, 2019; Xu et al., 2022). *BBM* is an important gene that must be strictly regulated throughout sexual reproduction to avoid parthenogenesis. BBM gene is repressed in the early stages of embryos and it is expressed patternally in sexual ovules and its overexpression cause the parthenogenesis in sexual ovules (Chahal et al., 2022; J. A. Conner et al., 2017; Schmidt, 2020). BBM is identified in *Brassica napus*, and encodes AP2-domain transcription factor of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family (Boutilier et al., 2002; Hand & Koltunow, 2014). The *BBM* gene is upregulated in zygote and sperm cells in rice and maize and at the early stage of seed development in *A. thaliana* (Anderson et al., 2017; B. Chen et al., 2022; J. Chen et al., 2017). The ectopic expression of *BBM* induces parthenogenesis in *A. thailana* (Boutilier, 2002; B. Chen et al., 2022) and *B. napus* (Boutilier et al., 2002). In addition, *BBM* directly triggers additional transcription factors linked to the development and preservation of embryo characteristics, such as *LEAFY COTYLEDON1* (*LEC1*), *ABI3*, *FUSCA3*, and *LEC2* (Boutilier et al., 2002; Tian et al., 2020).

The *LEC2* gene is a B3-domain transcription factor that is the key regulator of embryogenesis in *A. thaliana* (Stone et al., 2001). Mutation of *LEC* genes terminates embryogenesis, and their ectopic expression induces embryogenesis in seedlings (Gaj et al., 2005). The *BBM* and *LEC2* genes might be correlated in the induction of parthenogenesis and adventive embryo formation (Koltunow & Grossniklaus, 2003).

The aim of this study is to characterize and compare the expression levels of *APO, BBM* and *LEC2* genes in various tissues (before and after meiosis and days after fertilization) obtained from sexual *B. stricta* and apomict *B. divaricarpa* species and perform the complementation analysis for *BBM* and *LEC2* genes in *A. thaliana* mutants.

In this thesis, several procedures were conducted. Initially, the length of anthers and pistils (both before and after meiosis) was measured, and RNA was isolated from these samples. Additionally, flower buds were emasculated, and the pistils were manually pollinated using pollen from the same plant. Siliques were collected at 1, 3, and 5 days after pollination, and RNA was isolated from these samples as well. Bioinformatic tools were employed to identify *APO*, *BBM*, and *LEC2* genes in *B. stricta* and *B. divaricarpa*, and suitable primers were designed for gene expression and cloning analysis. The expression levels of *APO*, *BBM*, and *LEC2* genes were evaluated using qPCR with three technical and three biological replicates, and gene expression values were analyzed using the ΔΔCt method. *BBM* and *LEC2* genes were amplified from cDNA obtained from *B. divaricarpa* buds and inserted into the pUC19 vector with a CaMV35S promoter and nopaline synthase (NOS) terminator sequences.

CHAPTER 2
PREVIOUS STUDIES

Asker (1980) defined gametophytic apomixis as the formation of embryos from unreduced embryo sacs and parthenogenesis of their egg cells. This work revealed the comparison of sexual and apomict reproduction, a model for the gametophytic apomixis, and had a further prediction for transferring apomixis to the sexual plants.

Koltunow (1993) reviewed the apomixis and its developmental stages and give a comparison to the sexual reproduction with summarizing mechanistic and genetic knowledge about apomixis. Apomixis is the asexual formation of embryogenesis in the ovule resulting with clonal offsprings of maternal plant. Apomixis has two mechanism called gametophytic and sporophytic apomixis. Gametophytic apomixis is divided into two mechanism; apospory and diplospory. Apomixis has three common feature in all mechanisms, apomeiosis, pathenogenesis, and endosperm formation.

Koltunow et al. (1995) reviewed the agricultural importance of apomixis. According to this review, clonal seed formation through apomixis can provide cheap and time-saving vegetative propagation in large-scale crop production. It is also suggested that hybrid plants cannot be sources of seeds for the next generation because of genetic segregation. Control of apomixis can fix the production of true breeding hybrids. It also supports sustainable agriculture by reducing the use of the agricultural landscape and producing more productive crops. Additionally, Hieracium and Arabidopsis are suggested as model plants for genetic research on apomixis. Arabidopsis is a suitable plant with its small genome size to visualize mutant screening for apomixis, and Hieracium species are suitable for comparative analysis because they consist of both sexual and apomic plants and show a wide range of ploidy.

(Al-Shehbaz, 2003) were listed all species known as *Arabis* species but then changed as the member of *Boechera* genus according to morphological, cytological and molecular analysis. All species were listed in *Boechera* genus and evaluated. In this list, It is reported

that *Arabis drummondii* were transferred into *Boechera* genus and called as a *Boechera Stricta*. In the same manner, *Arabis divaricarpa* were defined as a *Boechera divaricarpa* which is the hybrid of , *B. stricta* (as *A. drummondii*) and *B. holboellii*.

Schranz et al. (2006) were the first attempt of transfer of apomixis by crossing *B. stricta* and *B. divaricarpa*, and revealed that genetic and evolutionary control of apomixis might be a complex. F1, F2, F3 and test crosses of F3 plant with an natural apomict were grown and ploidy analysis, genome in situ hybridization and microsatelite analysis were carried out. F1 plants (egg cell of diploid sexual plant and pollen of the apomictic plant) showed apomixis that is coming from apomict parent.They also shown that parental plant had an abnormal chromosome which is parallel to the heterochromatic (Het) chromosome in other *Boechera* apomicts. F2 plants showed various progeny which indicates that there is no simple factor effecting inheritance. According to these results, apomixis has complex genetic control behind apomixis.

Ozias-Akins, (2006) reviewed the genetic and developmental processes of apomixis and indicated the importance of identification of genomes and similar genes associated with developmental stages of both apomict and sexual plants. It is also indicated that genetic background of both types of gametophytic apomixis (apospory and diplospory) is important to modify crop plants and many genes that could be related with the mechanisms of apomixis were listed (i.e. *BBM, LEC, WUS* and *SERK* genes were listed as embryo initial genes).

Aliyu et al. (2010) developed a high-throughput flow cytometric seed visualization technique to embryo measurement of 20.000 single seeds of 71 diploid and triploid accessions of *Boechera* genus including *B. holboellii, B. stricta* and *B. divaricarpa.* Sexual seed formation were observed in *B. stricta* and apomict seed formation were observed in *B. retrofracta* and *B. divaricarpa*. The examinations showed three interconnected features in *Boechera* genus which are low, high and obligate expression levels of apomeiosis, association between apomeiosis and parthenogenesis and genotype-spesific differences in seed formation of apomict plants. It is also postulated that low-level apomeiosis is diirectly

correlated ancestral features and high-level apomeiosis could be related with the regulatory changes through hybridization.

Lovell et al. (2013) carried out an initial analysis that revealed the formation of gametophytic apomixis through genetic traits and the association with polyploidy and hybridization indirectly to understand population genetic patterns of apomixis. It is revealed that while most of the diploid apomicts are correlated with intraspecific crossings, triploid apomicts are formed as a result of hybridization in *Boechera*.

Brukhin (2017) reviewed developmental stages and the related genes of apomixis. They defined the comparison of developmental stages and genetic mechanisms of sexual and apomict reproduction, as well as the effects of regulation and mutations of genes and genetic loci related to the apomixis. The *BBM* and *LEC2* genes are examples of possible candidate genes for apomixis and are expected to be regulators of embryogenesis in apomixis. It is also reviewed that the biallelic heritage of *APOLLO* can control the formation of female gametes asexually in apomictic plants.

Corral et al. (2013) established the *APOLLO* (*apomixis-linked locus*) gene that encodes an Aspartate Glutamate Aspartate Aspartate histidine exonuclease. Gene expression analysis of 10 apomictic (diploid and triploid) and 7 sexual (diploid) *Boechera* was carried out with microarray analysis, and the *APOLLO* gene was cloned from 18 genotypes and characterized by various levels of polymorphism between sexual and apomictic. Characterized apomixis-related polimorphisms were not found in sexual genotype, those polymorphisms defines as apo-alleles and the rest of them defined as sex-alleles. Gene expression profile of *APOLLO* gene demonstrate that is downregulated in sexual ovules and and upregulated in apomict ovules during meiosis.

The *BABY BOOM (BBM)* gene that encodes an AP2 domain transcription factor that belongs to the AP2/ERF transcription factor family were firstly identified by Boutilier et al. (2002). Subtractive hybridization was used to isolate genes between 8 and 32 stages of

microspore-derived embryogenesis. Two *BnBBM* genes and a single *AtBBM* gene were reported. The predicted protein sequences of *BnBBM* were 98 to 99% identical with *AtBBM* and 60 to 89% identical to other AP2 subfamily proteins. *BBM* transcripts were found between the globular and cotyledon stages of microspore-derived embryogenesis by RNA gel blot, RT-PCR, and mRNA in situ hybridization analysis. Ectopic expression of *BBM* genes in *Brassica napus* and *Arabidopsis thailiana* under the control of proper promoters causes dwarf plants that grow and flower slowly with rounded leaves and redundant rosette leaves. All these results reveal the importance of the role of the *BBM* gene in signal transduction during embryogenesis from somatic cells.

Conner et al. (2011) reported that the expression of *PsASGR-BABY BOOM-like* (*PsASGR-BBML*) in egg cells before fertilization induces parthenogenesis *Pennisetum squamulatum*. *PsASGR-BBML* gene were transferred into the sexual pearl millet under the control of its own promoter and terminator by using a biolistic particle delivery system. *PsASGR-BBML* gene promote embryo-like structure end of the sexual embryo sac that indicates parthenogenesis and absence of the fertilization of the central cell causes the deficiency of the endosperm formation. Endosperm formation was observed after 2 days when pollination occurred. Percentage of the occurance parthenogenesis were reported in the range between 35% and 36%. The PsASGR-BBML gene expression profile was confirmed by qPCR analysis in the parthenogenesis-formed mutants.

Worthington et al. (2016) supported the role of *ASGR-BBML* as a candidate gene for embryonic development without fertilization with their outcomes. The genomic organization of polyploid apomicts was revealed with the molecular maps of tetroploid *Brachiaria* species. The research revealed that the *ASGR-BBML* gene sequence is highly conserved in grass genera showing apospory called *Paniceae*.

Khanday et al. (2019) experimented the possible parthenogenic effect of BBM transgene in *Seratia italica*. Phylogenetic analysis were performed with sexual monocot plants and apomict species and three *BBML* protein were characterized in S. italica. All three identified *BBM* genes were transformed under the control of egg-cell sprecific promoter and

their expression levels were controlled by non-quantitative RT- PCR. All *SiBBM* transgenes induced the parthenogenetic embro development and see in haploid T_1 line.

Chen et al. (2022) investigated *BBM* gene expression in both *A. thailiana* and newly developed CRISPR mutants. The expression patterns were analyzed using GFP and GUS genes, and the results demonstrated that the *BBM* gene is expressed during all stages of embryogenesis, from zygote formation to endosperm formation. Crispr cassettes were constructed by the Golden Gate method, and subsequent transformations were performed on *A. tumefaciens*, *Arabidopsis*, *B. napus*, and *Solanum lycopesicon* (tomato). Findings demonstrated that the ectopic expression of the *BBM* gene in *Arabidopsis*, *B. napus*, and tomato is necessary to avoid fertilization in embryogenesis, which is proof of the importance of the *BBM* gene to engineering parthenogenesis in crops.

Meinke et al. (1994) demonstrated the morphological features of *lec2* mutant embryos. Observations suggest that embryos simultaneously germinate after the torpedo stage. The mutants are also sensitive to the absisic acid in culture. Mutant seeds were pigmented at maturity, desiccation-tolerant, and hypocotyls were white or pale green.

Stone et al. (2001) were identified the role of *LEC2* gene in embryo development as a central regulator of embryogenesis and seed formation. Clones containing *LEC2* gene were transferred into homozygous *lec2-1* mutants. In situ hybridization were performed to detect *LEC2* RNA with a spesific *LEC2* antisense RNA which resulted with *LEC2* expressed during embryo formation. *LEC2* gene is ectopically expressed under the control of CaMV35S in the *lec2* mutant plants and resulted with the somatic embryogenesis. Formed somatic embryos were morfologically similar to the zygotic embryo. Findings also suggest that *LEC2* encodes a B3 domain protein.

Harada (2001) reviewed the role of *LEC* genes in different phase of embryogenesis and suggested that *LEC* genes have central role in both morphogenesis phase and the late maturation phase. The distictive feature of *LEC* genes is functioning both early and late

stages of embryogenesis. It regulates the suspensors that are important for the pushing embryo into the embryo sac. It is also suggested that phenotype of *lec* genes mutants are proven the *LEC* genes importance in embryogenesis. It is postulated that it is important to determine how *LEC2* gene is regulated in embryo development.

Malik et al. (2007) observed 0h, 3d, 5d and 7d points of the microspore embryogenesis and constracted standard, normalized and substracted cDNA libraries and sequenced for al those 4 stages in *B. napus*. Datas demonstrated that 5d stage is the switch to the embryogenic state. They also analyzed expression of genes with Stekel and Falciani R test and results suggest that pollen-spesific pathways were predominant in 3d. After all, they reported 16 molecular marker genes including *BnBMM* and *BnLEC2* in microspore embryogenesis.

Horstman et al. (2017) demonstrated that *BBM* regulates *LAFL* genes (*FUSCA3* (*FUS3*), *ABSCISIC ACID INSENSITIVE3* (*ABI3*), *LEC1* and *LEC2*) as a transcription vector. *LEC2* is regulated by *BBM* directly, and *LEC1* and *FUS3* are essential in somatic embryogenesis. *LAFL* gene expression is also regulated by *Arabidopsis BBM-like* proteins i.e. *PLETHORA2*. They identified the genes network of *BBM* by using chromatin immunoprecipitation and next generation sequencing, detected that *BBM* binds to *LAFL* genes' (except *FUS3*) promoter region during somatic embryogenesis and also found that ectopic *BBM* expression stumulates expression of the *LAFL* genes in seed germination. Complementation analysis of lec2 mutant line with *BBM* transgene under the CaMV35S promoter fix the morphogeny of embryogenesis.

CHAPTER 3
MATERIALS AND METHODS

3.1. Characterizations of Genes And Expression Analysis

3.1.1. Primer Design

The Genome, transcriptome, and proteome sequences of *B. stricta* were obtained from the Phytozome database (https://phytozome-next.jgi.doe.gov/info/Bstricta_v1_2). These sequences were downloaded and imported into the Geneious R8 software (Kearse et al., 2012) to create a BLAST database. The reference protein sequences *AtBBM* and *AtLEC2* were used for comparison, and a protein BLAST analysis was performed against the *B. stricta* proteome. The top result with the best E value, identity, and coverage values was selected for further analysis. This selected result was then used to perform a BLAST against the B. stricta transcriptome. Subsequently, the best result obtained from the transcriptome analysis was blasted against the *B. stricta* genome. The resulting transcript and gene sequences were aligned using the MUSCLE alignment tool to obtain exon sequences.

For primer design, the primers were selected to have an optimum melting temperature (TM) of 60 ℃. Moreover, all primers were designed to bind specifically to exon junctions to prevent them from binding to any genomic region. In cases where no suitable primer pairs could be designed to target the exon junctions, primers were selected from the vicinity of the 5' end of the sequence or from upstream regions of introns that were longer than 1000 bp.

3.1.2. Primer Efficiency

RNA from *B. divaricarpa* buds was utilized to synthesize complementary DNA (cDNA), and pUC19::BBM and pUC19::LEC2 plasmids were employed as samples to assess the effectiveness of primers through standard curve analysis. The samples were serially diluted 10-fold for a total of six dilutions (as presented in Table 1). Each of these serially diluted samples served as a template for quantitative PCR (qPCR) reactions. The components required for the qPCR reactions are provided in Table 2. Each reaction consisted of a total volume of 10 μl, with 6 μl of Syber Green master mix combined with 4 μl of cDNA. All reactions were conducted using the Step One Plus Real-Time PCR System (Applied Biosystems, USA), with the temperatures specified in Table 3.

To obtain cDNA, RNA extracted from *B. divaricarpa* bud was utilized, and cloned plasmids were employed as a reference sample for evaluating the efficiency of the primers using a standard curve analysis. The samples were subjected to a 10-fold serial dilution process, repeated six times, as outlined in Table 1. Each of these diluted samples served as a template for conducting qPCR reactions. The reaction components are provided in Table 2. In each 10 μl reaction, 6 μl of Syber Green master mix was mixed with 4 μl of the cDNA. All reactions were performed using the Step One Plus Real-Time PCR System from Applied Biosystems, USA, with the temperatures specified in Table 3.

Table 1.

Serial Dilution of cDNA samples

Dilution Number	Dilution Amount of Sample	Final Concentrations	Volume of Nuclease Free Water
1	Stock of Sample	15 μl	45 μl
2	1/10	6 μl	54 μl
3	1/100	6 μl	54 μl
4	1/1000	6 μl	54 μl
5	1/10000	6 μl	54 μl
6	1/100000	6 μl	54 μl

Table 2.

Component of Syber Green Mix

Reaction components	Amount
Syber Green Master mix	5 μl
cDNA	1 μl
10 μM Forward Primer	0,3 μl
10 μM Reverse Primer	0,3 μl
Nuclease-free water	0,4 μl

Table 3.

Temperature and Cycles of Real Time PCR reaction

Step	Temprature	Time	Cycle
Initial Denaturation	95 °C	10 min	1
Denaturation	95 °C	15 sec	
Annealing	55 °C	30 sec	40
Extension	72 °C	1 min	

A trendline scatter plot was plotted by comparing the log value of each sample with the Ct values. Slope of graph and R^2 values are calculated. The percentage of efficiency was evaluated by using the formula below.

$$\text{Efficiency} = (10^{(-1/\text{slope})} - 1) \times 100$$

3.1.3. Tissue Collection

Professor Dr. Eric Schranz from Wageningen University, Holland, provided the seeds of both apomictic *B. divaricarpa* (ES9, 500209; BS) and diploid sexual *B.stricta* (ES6, ID 500206; DG; DQ013050). To initiate the growth process, the *Boechera* seeds underwent a vernalization treatment by placing them on moistened filter paper in Petri dishes and keeping them in darkness at a temperature of 4 °C for a duration of 3 weeks. Following vernalization, the seeds were transferred to a growth chamber with a light-dark cycle of 16 hours of light and 8 hours of darkness, at a temperature of 21 °C. Upon germination, the seedlings were transplanted into a soil:perlite mixture with a ratio of 1:4 and grown for 4 weeks. Subsequently, the plants were exposed to a temperature of 10 °C for a period of 8 weeks to induce flowering. Approximately 6 weeks after the cold treatment, flowering occurred, and the flowering plants were categorized into three distinct biological groups. To conduct further analyses, the flowers and unopened buds from each biological groups were carefully opened using fine forceps. Anthers and pistils were collected for length measurement. The collected anthers and pistils were immediately frozen with liquid nitrogen and stored at -80 °C until RNA isolation was performed for gene expression analysis.

Additionally, anthers from unopened buds were extracted using fine forceps. The anthers were emasculated, and the stigma was pollinated with pollen from the same plant. Siliques were collected 1, 3, and 5 days after pollination and promptly frozen with liquid nitrogen. The frozen siliques were stored at -80 °C until RNA isolation was conducted for gene expression analysis.

For efficiency and cloning studies, unopened buds and flowers were collected and immediately frozen with liquid nitrogen. These samples were stored at -80 °C until RNA isolation could be performed.

3.1.4. RNA Isolation

To prevent contamination, all laboratory materials such as mortar, pestle, and forceps, were submerged in a 1% Diethyl Pyrocarbonate (DEPC) solution. They were kept in the DEPC solution overnight. The next day, the DEPC solution was discarded, and all materials were autoclaved at 121°C for 15 minutes to sterilize them. During RNA isolation, all equipment used in the process was cooled down with liquid nitrogen to maintain the integrity of the RNA.

The collected tissues from both *B. divaricarpa* and *B. stricta* plants were homogenized. The homogenized tissues were treated with TRIzol (15596026-Thermo Fisher), and after centrifugation, the resulting supernatant was transferred into a clean tube that was free from any RNase contamination.

The supernatant obtained from the previous step was then treated with a 1:1 solution of chloroform and phenol. After centrifugation, the upper phase of the solution, which contains the RNA, was carefully transferred into another clean tube. An equal volume of 70% ethanol was added to the RNA-containing solution.

The RNA isolation protocol was continued using the Purelink RNA Mini Kit (12183-018A – Invitrogen), following the manufacturer's instructions. This kit provides a standardized procedure for efficient RNA isolation.

To assess the quality of the isolated RNA, 1% agarose gel electrophoresis was performed. This gel electrophoresis helps to visualize the RNA bands and ensure its integrity.

Furthermore, the concentration of the isolated RNA samples was measured using a Qubit 2.0 Fluorometer (Q32866 - Life Technologies, Carlsbad, CA, USA). This instrument utilizes fluorescent dyes to accurately quantify the concentration of RNA in the samples.

3.1.5. cDNA Synthesis

To remove any potential DNA contamination, 1 µg of RNA was treated with DNAse I (M0303S-New England Biolabs) at 37°C for 10 minutes. The reaction components used for this step are listed in Table 4. Following the DNAse treatment, 1 µl of EDTA (50 mM), 2 µl of random primer, and 0.8 µl of dNTP mix were added to the reaction mix. The reaction was then incubated at 75 °C for 10 minutes.

Table 4.

DNAse Reaction Components

Component	Amount
10X Dnase Buffer	1 µl
RNA	1000 ng
DNAse I	0.1 µl
Nuclease-free Water	up to 10 µl

After the initial steps, the reaction mix was completed by adding the components of the High Capacity cDNA Reverse Transcription Kit (4368814-Applied Biosystems, Fisher Scientific – USA), as specified in Table 5. The cDNA synthesis was performed with the temperature cycles outlined in Table 6. These cycles involve specific temperatures and time intervals for the synthesis of cDNA from the RNA template.

Table 5.

cDNA Synthesis Reaction Components

Component	Amount
Reaction Buffer	2 μl
Reverse Transcriptase	1 μl
Nuclease-Free Water	3.2 μl
Final volume	6.2 μl

Table 6.

PCR Temprature Cycles of cDNA Synthesis

Temprature (°C)	Time (min)
25	10
37	120
85	5
4	∞

3.1.6. Real Time Polymerase Chain Reaction

The gene expression analysis of *APOLLO*, *BBM*, and *LEC2* genes in *B. stricta* and *B. divaricarpa* was conducted using the Step One Plus Real-Time PCR System from Applied Biosystems, USA. For each well, a 10 μl qPCR reaction was prepared, consisting of 6 μl of

Syber-Green Master Mix and 4 µl of diluted cDNA (1:4). The composition of the reaction is provided in Table 2.

The qPCR analysis aimed to compare the gene expression profiles of *APOLLO*, *BBM* and *LEC2* genes between the sexual *B. stricta* and apomictic *B. divaricarpa* species. Anther and pistil tissues, both before and after meiosis, as well as siliques collected at 1, 3, and 5 days after pollination (DAP), were used as samples. Three biological replicates were utilized, with three technical replicates for each group of samples. The reference gene used for normalization as an endogenous control was *Polyubiquitin* (*UBQ*), following the study by Pellino et al., 2011. The reaction was performed with the temperature conditions provided in Table 3.

After the completion of the reaction, a Melt Curve Analysis was conducted between 60-95°C to identify the presence of non-specific products or primer dimers. The gene expression analysis was performed using the ΔΔCt method, which enables the comparison of gene expression levels between different samples and conditions.

3.2. Clonning Studies

3.2.1. Primer Design For *APOLLO*, *BBM* and *LEC2* genes

Primer Tm values were deliberately selected to fall within the range of 57°C and 63°C, and the primer length was carefully chosen to be between 25 to 28 base pairs. Moreover, the GC ratio of the primers was maintained at a level between 30% to 80%. To enhance the efficiency of the restriction reaction, four random bases (GATC) were incorporated at the 5' end of the primer extension.

Following the four random bases, restriction sites were strategically positioned. This approach facilitated the fusion of the coding sequence of the gene of interest with the pUC19 vector.

As for the reverse primer, after the four random bases, the appropriate restriction sites were selected. For each gene, the selected primers and the corresponding chosen restriction enzymes were detailed in Table 7.

Table 7.

Cloning Primer Sequences

Primer Name	Primer Sequence	Restriction Enzyme
BBM_F	ATCGTCTAGAATGAACTCAATGAATAAC TGGTTAGG	XbaI
BBM_R	ATCGGAGCTCCTAACTGTCGTTCCAAACT GTAAAC	SacI
LEC2_F	ATCGTCTAGAATGGATAACTTCTTGCCCT TTTCC	XbaI
LEC2_R	ATCGGAGCTTCACCACACAAAGTCGTTA AAGC	SacI
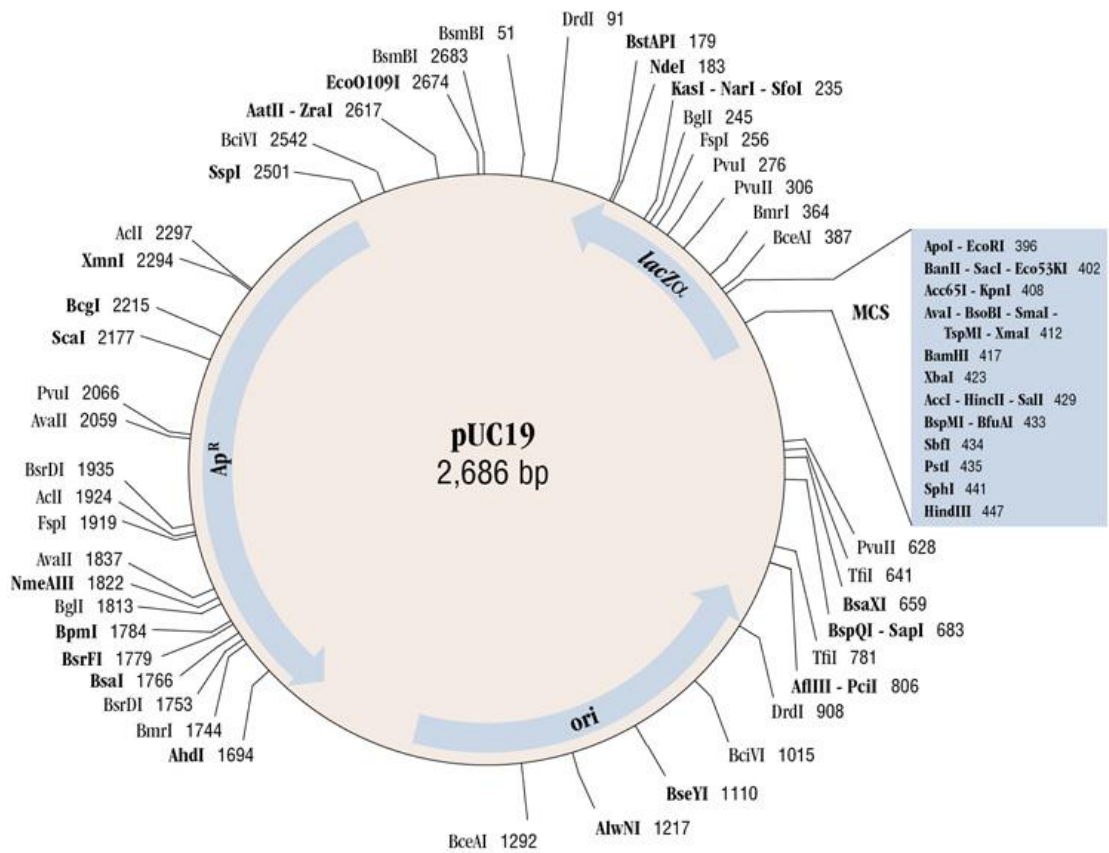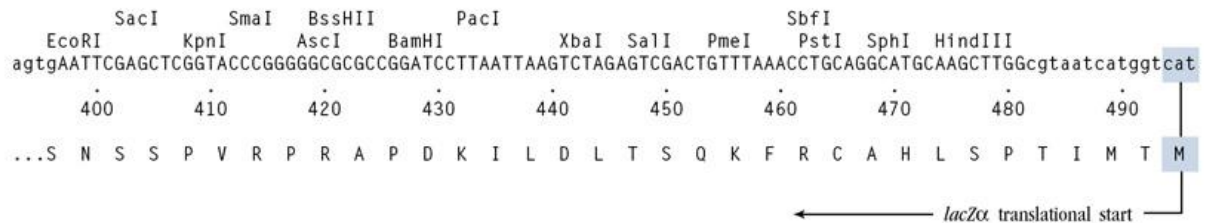
Figure 2: Plasmid Map of pUC19 (https://international.neb.com/products/n3041-puc19-vector)

3.2.2. Polymerase Chain Reactions (PCR)

The complete coding sequences of the *BBM*, and *LEC2* genes were amplified from their respective cDNAs. This amplification was carried out using gradient-PCR, and the Phusion High-Fidelity DNA Polymerase Enzyme Kit (F530L, Thermoscientific, USA) was utilized for this purpose. The gradient-PCR approach allowed for the determination of the optimal annealing temperature for the designed cloning primers. The primers were tested over a temperature range of 54°C to 62°C to identify the most suitable annealing temperature.

The reaction contents for the gradient-PCR experiments are provided in Table 8, while the corresponding temperature settings used during the PCR reactions are listed in Table 9. Following the PCR amplification, the resulting DNA fragments were analyzed by running them on a 1% agarose gel electrophoresis to visualize and confirm the successful amplification of the target sequences.

Coding sequences of genes were amplified from cDNAs amplified from unopened buds RNA of apomict *B. divaricarpa*. Primer binding temprature were determined at 58.3°C. Amplified DNAs were run in 1% agarose gel electrophoresis.

Table 8.

Content of Polymerase Chain Reaction

Component	Amount
5X Phusion High-Fidelity Buffer	5 µl
dNTP	0.5 µl
Reverse Primer	1 µl
Forward Primer	1 µl
Template DNA	1 µl
Phusion DNA Polymerase Enzyme	0.25 µl
Nuclease-Free Water	16.25 µl

Table 9.

Temprature Cycles of Polymerase Chain Reaction

PCR Stages	Temperature	Time	Cycle
Initial Denaturation	98 °C	30 s	1
Denaturation	98 °C	10 s	
Annealing	54-62 °C	30 s	35
Extension	72 °C	1 min	
Final Extension	72 °C	10 min	1

3.2.3. DNA Gel Electrophoresis

A 1% agarose gel containing 1X TAE buffer and 10 mg/mL ethidium bromide was used to run the PCR products in electrophoresis. The samples were run at 120 volts electrical voltage and 100 A electrical current for approximately 1 hour. The size of the PCR products was determined using the 1 kb marker. Electrophoresis results were photographed using a UV gel imaging system. PCR products that could not be visualized at the desired purity were purified using the Monarch® PCR&DNA Cleanup Kit (T1030S- New England Biolabs). The concentrations of purified PCR products were measured with Quibit (Qubit 2.0, Invitrogen Q32866).

3.2.4. Compotent Cell Preperation

To construct the gene cassette, the genes of interest were inserted into the pUC19 vector. Competent cells were prepared from *E. coli* DH10B. Single colony *E. coli* DH10B stock was inoculated into 50 mL of LB broth (0.5 g Bacto-tryptone, 0.25 g yeast extract, 0.5 g NaCl) and sterilized in an autoclave at 121°C for 15 minutes. 500 µl of overnight culture was added to sterilized LB broth. The new culture was incubated at 37 °C in a shaker incubator until its OD600 value was measured between 0.3-0.4. After that, the cells were incubated in ice for 10 min. Then, the cells were centrifugated at 4 °C at 14.000 rpm for 4 min. Pelleted cells were dissolved with 10 mL 0.1 M calcium chloride solution and incubated

for 30 min. The cells were centrifugated at 4 °C with 14.000 rpm for 4 min. The Pellet of the cells was dissolved with 2 mL calcium chloride solution and stored at 4 °C until its usage.

3.2.5. Restriction Enzyme Digestion and Ligation

The sequence of gene of interest, which were amplified using the PCR method, were subjected to restriction enzyme digestion to construct the gene of interest in the pUC19 vector.

To ensure accurate and efficient insertion of the gene cassette into the vector, restriction enzymes that produce complementary sticky ends were chosen. This helps prevent incorrect insertion and promotes the correct alignment of the gene cassette with the vector. Two different restriction enzymes were selected, one for each end of the gene of interest.

The digestion reactions were set up as double restriction reactions. This means that both ends of the gene cassette fragments were treated with the appropriate restriction enzymes simultaneously. The reaction contents for these double restriction reactions can be found in Table 10.

The restriction enzyme digestion reactions were incubated at 37°C for 15 minutes to activate the enzymes, allowing them to cleave the DNA at their specific recognition sites. After the digestion, the reactions were then incubated at 80°C for 20 minutes to deactivate the restriction enzymes, preventing any further cleavage and ensuring the stability of the DNA fragments.

Once the gene cassette fragments were appropriately digested, they were ready to be ligated into the pUC19 vector. Restiricted fragments were ligated by using T4 DNA ligase enzyme (EL0011, Thermo ScientificTM). Reaction was set up with components were given on Table 11 and were incubated 15 min at room temperature.

Table 10.

Restriction Reaction Component

Component	Amount
10X T4 DNA Ligase Buffer	2 μl
T4 DNA Ligase	0.2 μl
Vector	20-100 ng
Insert DNA	1:1 – 5:1 molar ratio to vector
Nuclease Free Water	up to 20 μl

Table 11.

 Ligation Reaction Components

Component	Amount
DNA	1 μg
10X rCutSmart Buffer	5 μl
Enzyme 1	1 μl
Enzyme 2	1 μl
Nuclease Free Water	up to 50 μl

3.2.6. Transformation

In this experiment, the ligation reaction containing the gene of interest and the pUC19 vector was added to 200 μl of competent cells. The mixture was then incubated on ice for 30 minutes to allow the DNA to be taken up by the competent cells.

After that, the cells were subjected to a heat shock by transferring them at 42°C for 90 seconds, followed by immediate cooling on ice for 2 minutes. This thermal treatment helps the DNA to enter the cells more effectively.

Following the heat shock, the treated cells were mixed with 800 µl of SOC medium, which is a nutrient-rich recovery medium containing yeast extract, KCl, MgCl2, triptone, NaCl, MgSO4, and glucose. The cells were then incubated in a shaker incubator at 37°C for 1 hour and 30 minutes. This incubation period allows the transformed cells to recover and express the antibiotic-resistance genes carried by the vector.

After the incubation, the cells were spread onto selective LB (Luria-Bertani) agar plates containing specific antibiotics. For pUC19-containing cells, the antibiotic used was 50 mg/mL ampicillin. The cells were spread in four different amounts (10 µl, 20 µl, 35 µl, and 50 µl) to determine the optimal cell concentration for efficient transformation.

The plates were then incubated at 37°C, allowing the transformed cells to grow while non-transformed cells are inhibited by the antibiotics. The resulting colonies on the selective plates represent the successfully transformed cells containing the gene of interest in the respective vector (pUC19). These transformed cells can be used for further experiments or applications.

3.2.7. Colony PCR

Colony PCR was employed to confirm successful gene transfer following the insertion of each gene cassette into the vector, and subsequent transformation of the vectors into cells. To conduct the PCR, cells were collected from chosen colonies on petri dishes that had been incubated overnight with LB solid medium containing the appropriate antibiotic. These cells were then suspended in 50 µl of nuclease-free water.

For the Colony PCR reactions, Taq DNA polymerase enzyme (2236785 - Thermo-Fisher Scientific) was used. The reaction components can be found in Table 12. To initiate

each PCR reaction, 1 µl of the suspended colonies was utilized as a template. Universal M13 primers were employed for all reactions.

The PCR reactions were carried out with specific temperature cycles, as detailed in Table 13. These temperature cycles allowed for the amplification of the DNA fragments and enabled the verification of gene transfer in the transformed cells.

Component	Amount
10X PCR Buffer -MgCl2	2.5 µl
50 mM MgCl2	0.75 µl
10 mM dNTP	0.5 µl
10 mM Forward Primer	1.25 µl
10 mM Reverse Primer	1.25 µl
Template DNA	1-500 ng
Taq DNA Polymerase (5U/ µl)	0.1 µl
Nuclease free water	Up to 25 µl

Table *12*.

Colony PCR reaction content

Table 13.

Colony PCR Cycles

PCR Cycles	Temprature	Time	Cycle
First Denaturation	94 °C	3 min	1
Denaturation	94 °C	45 sec	
Annealing	55 °C	30 sec	35
Extension	72 °C	90 sec	
Final Extension	72 °C	10 min	1

After the Colony PCR reactions, the resulting products were separated and analyzed using 1% agarose gel electrophoresis. The gel was then subjected to UV light, which allowed the visualization of the DNA fragments. Colonies that showed the correct size of the gene cassette were identified based on their band patterns on the gel.

Subsequently, the colonies with the correct gene cassette size were selected and inoculated into 10 mL of LB broth containing the appropriate antibiotics. These inoculated cultures were then incubated overnight at 37 °C in a shaker incubator. The incubation process provided the optimal conditions for the bacterial cells to grow and multiply in the liquid medium.

3.2.8. Plasmid Isolation

Plasmids were isolated using the GeneJET Plasmid Miniprep Kit (Thermo ScientificTM, K0503) following the manufacturer's instructions. The isolation process was carried out from bacterial cultures that had been inoculated in 50 mL of sterile LB broth

containing the appropriate antibiotic. These cultures were incubated overnight at 37 °C with continuous shaking to allow the bacteria to grow and propagate.

For the plasmid isolation, 5 mL of the bacterial culture was used as the starting material. The GeneJET Plasmid Miniprep Kit facilitated the extraction of plasmids from the bacterial cells.

Following isolation, the obtained plasmids were subjected to restriction enzyme digestion using one of the restriction enzymes previously used for ligation. The restriction enzyme digestion aimed to confirm the presence and integrity of the desired gene cassette within the plasmids. After digestion, the plasmids were separated using electrophoresis on a 1% agarose gel and visualized under a UV system (Figure 5).

The correctly sized plasmids, indicating successful incorporation of the gene cassette, were identified based on their appearance on the gel and were subsequently stored at -20 °C for further use and analysis.

3.2.9. Sequencing and Phylogenetic Analysis

The PCR amplicons of *BBM* and *LEC2* genes were transformed into the *E. coli* cells with pUC19 vector. After colony PCR and restriction digestion, the sequences of *B. divaricarpa BBM* and *LEC2* genes were sequenced by MEDSANTEK, Türkiye with the Sanger method. The sequencing files (ab1) were transferred to the Geneious v.8.1 software for further analysis. All chromatogram sequences were assembled and mapped to reference sequences for each of *BBM* and *LEC2* gene and consensus sequences of each gene were extracted.

The consensus sequence of both genes were blasted through BLASTn program against the plant species in the NCBI and all sequences were used to plot phylogenetic tree through neighbor-joining method.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Bioinformatic Analysis and Primer Design

Bioinformatic analyses were conducted to obtain the *BBM* and *LEC2* gene sequences of B. stricta. Genome, transcriptome, and proteome sequences were retrieved from the Phytozome database. Protein sequences of *AtBBM* (AT5G17430.1) and *AtLEC2* (AT1G28300.1) genes from *Arabidopsis thaliana* were obtained from the TAIR database and then compared to the *B. stricta* proteome using Geneious v8.1.9 software.

For the *LEC2* gene, the best match in the blastp results was the sequence Bostr.15697s0149.1.p, which showed 100% coverage and 81.2% similarity. All other sequences had lower similarity rates, below 51.3%. The analysis continued with the highest blastp result (Bostr.15697s0149.1.p). Sequences coding for the protein of interest were acquired through tblastn and blastn analyses.

According to tblastn results, the Bostr.15697s0149.1 transcript exhibited 100% similarity and coverage with the Bostr.15697s0149.1.p protein sequence. After blastn analysis, more than one hit sequence with 100% identity was observed. The sequence with the closest E value to zero and the highest coverage (45.33%) was selected. This sequence was then aligned with the transcript sequence to obtain exon and intron sequences using MUSCLE alignment. The *LEC2* gene was found to have six exon regions, and the reverse primer sequence was designed at the first exon junction to prevent binding to genomic sequences. The count of exon junctions is six for the *LEC2* gene in *Arabidopsis thailiana* and *Manihot esculenta* (Brand et al., 2019). The primer sequences are provided in Table 17.

Similarly, for the *BBM* gene, the sequence Bostr.26527s0471.1.p showed the highest similarity in the blastp results with 100% coverage and 89.1% similarity. All other sequences had similarity rates lower than 51.6%. Sequences coding for the protein of interest were obtained through tblastn and blastn analyses. The Bostr.15697s0149.1.p

sequence was then blasted against the *B. stricta* transcriptome using tblastn. The highest hit sequence (Bostr.26527s0471.1) exhibited 100% similarity and coverage with the Bostr.26527s0471.1.p protein sequence. After blastn analysis, multiple hit sequences with 100% identity and zero E-value were observed. The sequence with the highest coverage (41.55%) was selected and aligned with the transcript sequence to obtain exon and intron sequences using MUSCLE alignment. The *BBM* gene was found to have an 8-exon region, and the forward primer sequence was designed at the first exon junction to prevent binding to genomic sequences. The count of exon junctions and alleles varies among different species. For instance, in *Setaria italica*, the *BBM* gene has three alleles: *SiBBM1* with 8 exons, *SiBBM2* with 9 exons, and *SiBBM3* also with 9 exons (Chahal et al., 2022). Meanwhile, *Brassica napus* exhibits a 9 exon region, and *Arabidopsis thaliana* displays an 8 exon region for the same gene (Chen et al., 2022). In *Pennisetum squamulatum*, the *PsASGR-BBML* gene comprises eight exons (J. A. Conner et al., 2015). The primer sequences are also listed in Table 17.
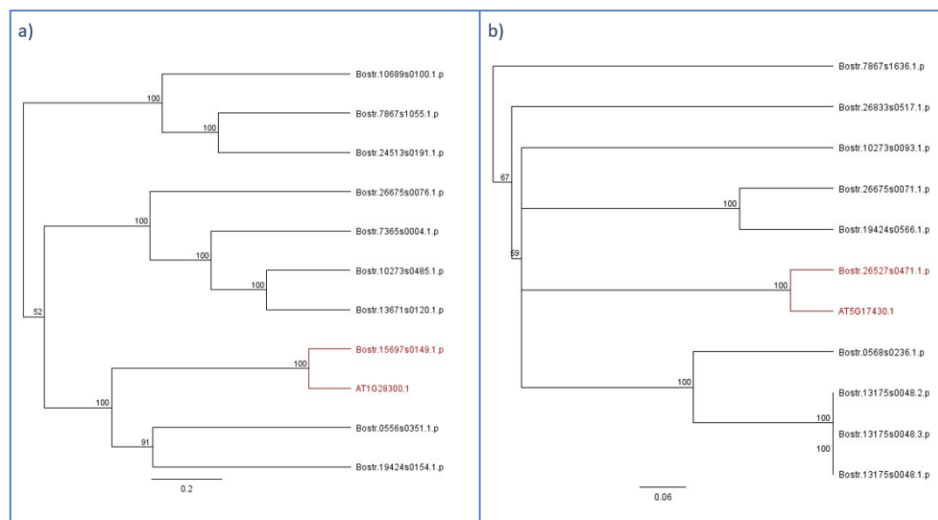
Figure 3: Phlogenetic trees (UPGMA) plotted with *BBM* and *LEC2* protein sequences a) Phlogenetic tree (UPGMA) of *BBM* b) Phlogenetic tree (UPGMA) of *LEC2*

Figure 4: Blast analysis results of *B. stricta BBM* ve *LEC2* genes a) blastp results of *LEC2* protein sequence of *A. thaliana* against *B. stricta* proteome b) tblastn results of *LEC2* protein sequence of *B. stricta* against *B. stricta* transcriptome c) BLASTn results of *LEC2* transcript sequence of *B. stricta* against *B. stricta* genome d) blastp results of *BBM* protein sequence of *A. thaliana* against *B. stricta* proteome e) tblastn results of *BBM* protein sequence of *B. stricta* against *B. stricta* transcriptome f) BLASTn results of *BBM* transcript sequence of *B. stricta* against *B. stricta* genome.

Table 14.

qPCR Primer sequences

Gene Name	Primer Name	Primer Sequence
APOLLO (Apo-allele)	A_APOLLO_F	5′-GTGGCCCGTGAAGTTTATTCCCT-3′
	APOLLO_R	5′-GATAGCCCCAAACTCCAAAATCGC-3′
APOLLO (Sex-allele)	S_APOLLO_F	5′-CCGTAAATAGAGGAGGATCAATTGCTT-3′
	APOLLO_R	5′-GATAGCCCCAAACTCCAAAATCGC-3′
BBM	BBM_F	5'-ACAATAGTCACTCCCGAGATTGG-3'
	BBM_R	5'-TCAAGCTTTGGTCCATTTTGTTCA-3'
LEC2	LEC2.3_F	5'- GATGGCAGCGGTGATGAATC-3'
	LEC2.3_R	5'-ATTCACTTCCGGTTTGCGTAAC-3'
UBQ	BOECHUBQ_F	5'-GGCTAAGATCCAGGACAAGGAAGGTAT- 3'
	BOECHUBQ_R	5'- CTGGATGTTATAGTCAGCCAAAGTGCG - 3'

Gene sequences of 5 apomict allele and 5 sex allele of *APOLLO* in various *Boechera* plants were obtained from the (Corral et al., 2013a) and aligned by using MUSCLE alignment. The nucleotides differ in sex-allele and apo-allele of *APOLLO* gene were demonstrated on Figure 6. The primer pairs for both apomict and sexual allele of *APOLLO* also obtained from (Corral et al., 2013a). A phylogenetic tree constructed for the *APOLLO* gene using the UPGMA method. In this tree,It is observed that the apomict alleles of the *APOLLO* gene from various *Boechera* species formed a single cluster, indicating their close genetic relationship. Similarly, the sex alleles of the *APOLLO* gene from different *Boechera* species also clustered together, suggesting their shared genetic ancestry. On the other hand, all the other sequences from various sexual and apomict plants branched out separately from these two clusters. This indicates that these other sequences have distinct genetic differences from the apomict and sex alleles of the *APOLLO* gene. In summary, the phylogenetic tree revealed that the apomict alleles and sex alleles of the *APOLLO* gene in *Boechera* species share a common genetic origin and are distinct from other sequences, as they form separate clusters in the tree.
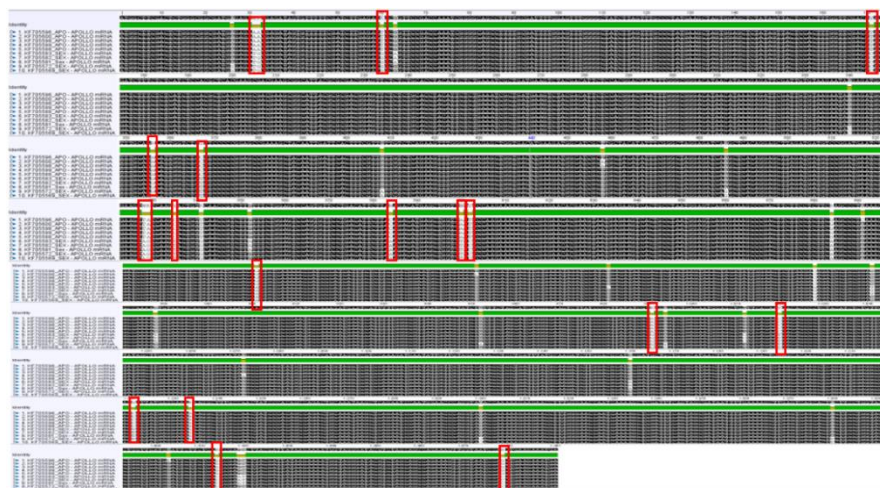
Figure 5: Sequence alignment of apomict and sexual allele of *APOLLO* gene in various *Boechera* species
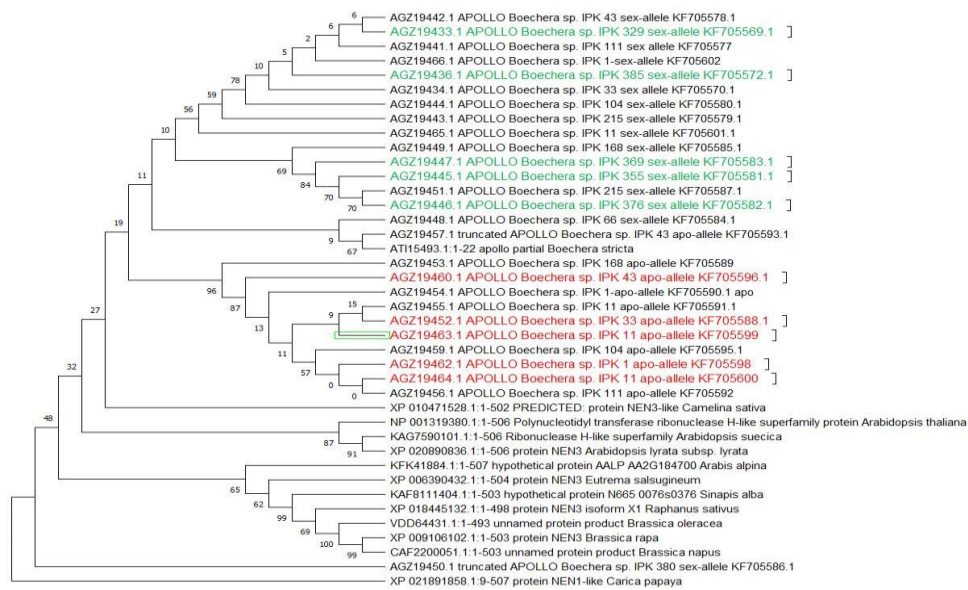
Figure 6: Phylogenetic Tree (UPGMA) of *APOLLO* gene

4.2. Plant Materials and RNA Isolation

In this study, both apomictic *B. divaricarpa* and sexual *B. stricta* plants were used. Anther and pistil tissues were collected from buds and flowers at different developmental stages, specifically before and after meiosis.

The lengths of the buds, anthers, and pistils from all the collected tissues were measured. The mean (average) values of the bud lengths were calculated and are provided in Table 15. For *B. divaricarpa* plants, anthers with a length between 0.6 mm and 0.8 mm were considered to be in the meiosis stage, while anthers with a length of 0.8 mm and longer were categorized as being in the after meiosis stage (Taskin et al., 2009).

In the case of *B. stricta*, anthers from unopened buds were considered to be in the meiosis stage, while anthers from fully developed flowers of *B. stricta* were classified as being in the after meiosis stage.

Table 15.

Mean values of bud, anther and pistil lengths

	B. divaricarpa	*B. stricta*	**Developmental Stages**
Unopened Bud	3,4 (±1,6) mm	4,3 (±0,9) mm	Meiosis/Apomeiosis
Anther	0,7 (±0,2) mm	1.1 (±1,2) mm	Meiosis/Apomeiosis
Gynocium	2,4 (±0,1) mm	2.7 (±0,7) mm	Meiosis/Apomeiosis
Flower	5,2 (±1.5) mm	8.8 (±1,8) mm	After Meiosis
Anther	0.6 (±0,2) mm	0.7 (±0,2) mm	After Meiosis
Silique	3.4 (±1) mm	4.1 (±1,3) mm	After Meiosis

In this experiment, RNA isolation was conducted using pistil and anther tissues from both before and after meiosis. Additionally, pistil tissues were collected at different time points after pollination (1, 3, and 5 days after pollination).

The quality of the extracted RNA was assessed using 1% agarose gel electrophoresis, and the presence of distinct 28S and 18S rRNA bands indicated good quality RNA (as shown in Figure 1).

To quantify the RNA, Quibit analysis was performed, and the results are provided in Table 16. The Quibit values, which measure the RNA concentration, fell within the range of 150-1500 ng/µl for anther and pistil tissues (before and after meiosis). For the pollinated tissues, the Quibit values ranged from 100-874 ng/µl (Appendix 19).

To prevent DNA contamination, the RNA samples were treated with DNAse I. Subsequently, cDNA synthesis was carried out using 1000 ng of RNA from each sample. This step involves converting the RNA into complementary DNA (cDNA), which can be used for various downstream applications, such as gene expression analysis.
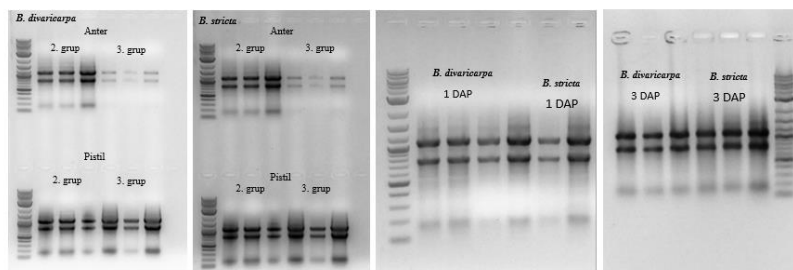
Figure 7: Agarose Gel Electrophoresis results of RNA samples

4.3.   Polymerase Chain Reactions

In this experiment, cDNA was synthesized from the RNA extracted from young buds of *B. divaricarpa*. *BBM* and *LEC2* genes are showed efficient amplification at all three different temperatures: 56 °C, 58 °C, and 60 °C on gradient PCR. To determine the optimal annealing temperature for PCR amplification, the, using gradient PCR.

After amplification, the products were separated and analyzed using 1% agarose gel electrophoresis and visualized under UV light. Remarkably, the bands observed in Figure 6 corresponded to the expected lengths of the target genes. Specifically, the coding sequence of the *BBM* gene was observed at 1725 base pairs, while the *LEC2* gene appeared at 1092 base pairs.

These results demonstrate the successful amplification of the *BBM* and *LEC2* genes at different annealing temperatures. The bands at the expected lengths indicate that the PCR was effective in specifically amplifying the target genes, confirming the presence and integrity of the gene sequences in the cDNA obtained from the *B. divaricarpa* young bud RNA.
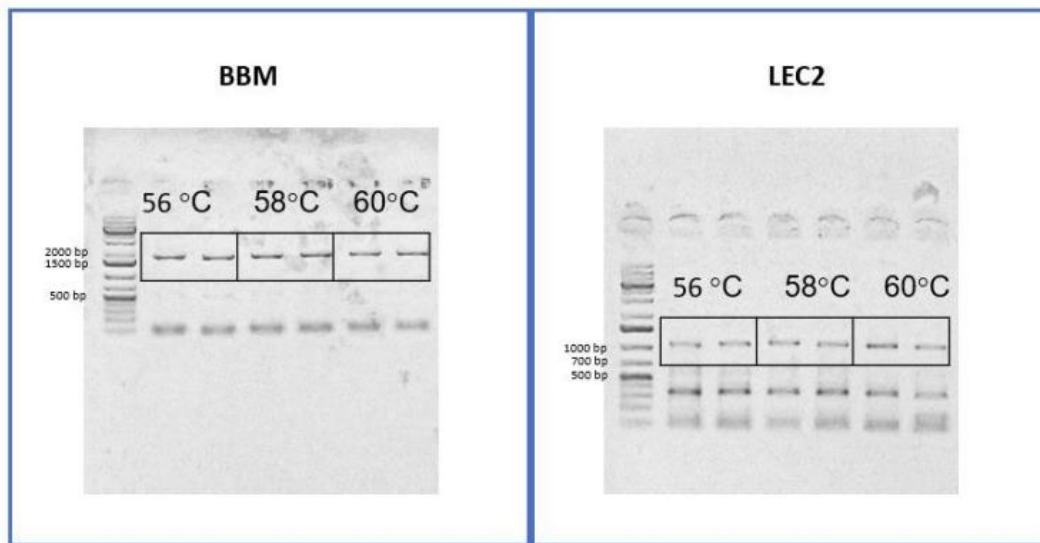
Figure 8: Gradient PCR results of *BBM* and *LEC2* genes

BBM and *LEC2* amplicons that had an accurate length were purified by using the GeneJet Gel Extraction Kit (K0692, ThermoScientific) from sliced agarose gel fragments. Extracted *BBM* and *LEC2* amplicons were visualized on agarose gel again and visualized with a UV light transilluminator for validation.

4.4. Restriction Reactions ve Ligation
4.4.1. Construction of pUC19:: LEC2

Coding sequence of the *LEC2* gene was amplified from *B. divaricarpa* young bud cDNA by PCR. SphI (GCATG'C) (R3182S, New England Biolabs) and SacI-HF (C'TCGAG) (R3156S, New England Biolabs) restriction enzymes were used to produce sticky overhangs on both the pUC19 vector and the coding sequence of *LEC2*. Restricted sequences were ligated by using T4 DNA ligase (EL0011, Thermo ScientificTM). Ligated pUC19::LEC2 vector was transformed into E. coli and grown on ampicillin-selective LB medium.

Four of the colonies were selected to perform colony PCR. The length of the colony-PCR amplicon of the vector containing the *LEC2* gene is 1906 bp. The colony PCR amplicon of pUC19 828 bp. It is observed that one of the four selected colonies contain *LEC2* gene in pUC19 after UV light visualization of agarose gel (Figure 8)

Selected colonies (Figure 9-a) were incubated in 10 ml of liquid LB at 37 °C overnight, and plasmid isolation was performed. Isolated plasmids are restricted by SacI and SphI restriction enzymes. Figure 9-b showed that 35S::LEC2::NOS were succesfully constructed with pUC19.
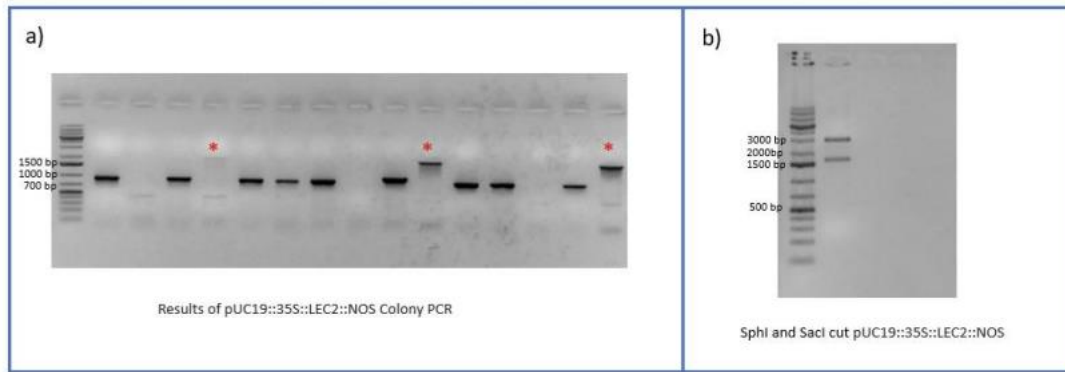
Figure 9: Agarose Gel Electrophoresis Results of pUC19::LEC2 construction a) Agarose Gel Electrophoresis Visualization of colony PCR results pUC19::LEC2 b) Agarose Gel Electrophoresis Visualization of SphI and SacI cut pUC19::LEC2

4.4.2. Construction of pUC19::BBM

Coding sequence of the *BBM* gene was amplified from *B. divaricarpa* young bud cDNA by PCR. *XbaI* (T'CTAGA) (R0145S, New England Biolabs) and *SacI-HF* (GAGCT'C) (R3156S, New England Biolabs) restriction enzymes were used to produce sticky overhangs on the pUC19 vector and the coding sequence of *BBM*. Restricted sequences were ligated by using T4 DNA ligase (EL0011, Thermo ScientificTM). Ligated pUC19::BBM construction was transformed into E. coli and grown on kanamycin-selective LB medium.

Seven of the colonies were selected to perform colony PCR. The length of the colony-PCR amplicon of the vector containing the *BBM* is bp. The colony PCR amplicon

of pUC19 is 828 bp. It is observed that all of the seven selected colonies contain conding sequence of *BBM* after UV light visualization of agarose gel (Figure 15-a).

Selected colonies (Figure 15-b) were incubated in 10 ml of liquid LB at 37 °C overnight, and plasmid isolation was performed. Isolated plasmids are restricted by SalI-HF restriction enzymes. Figure 15-b showed that coding sequence of *BBM* was succesfully constructed with pUC19.
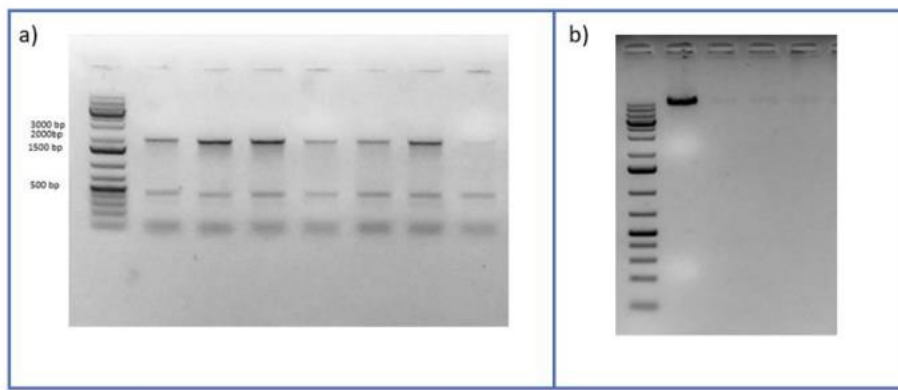
Figure 10: Agarose gel electrophoresis results of pUC19::BBM a) pUC19::BBM colony PCR results b) SalI restricted pUC19::BBM

4.5. Sequencing Results of *BBM* and *LEC2* genes

Sequencing results confirmed the entry of the *LEC2* and *BBM* gene into pUC19. We amplified the full coding sequence of *LEC2* and *BBM* from *B. divaricarpa* buds and cloned it into the pUC19. Sequencing and alignments showed that the inserted sequences are in the correct size. Consensus sequence of *BBM* gene was blasted with BLASTn against plant species through NCBI. The length of *BdBBM* gene was correct with 1725 bp.
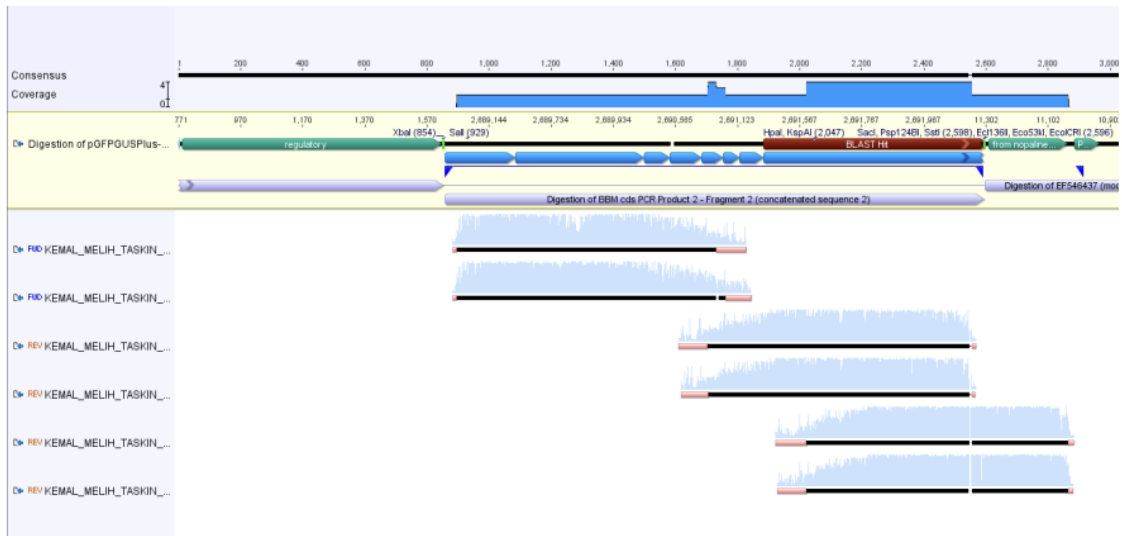
Figure 11: *BBM* consensus sequences and assembled chromatogram sequences

Consensus sequence of *BdLEC2* gene was blasted with BLASTn against plant species through NCBI. The length of *BdLEC2* gene was correct with 1092 bp.
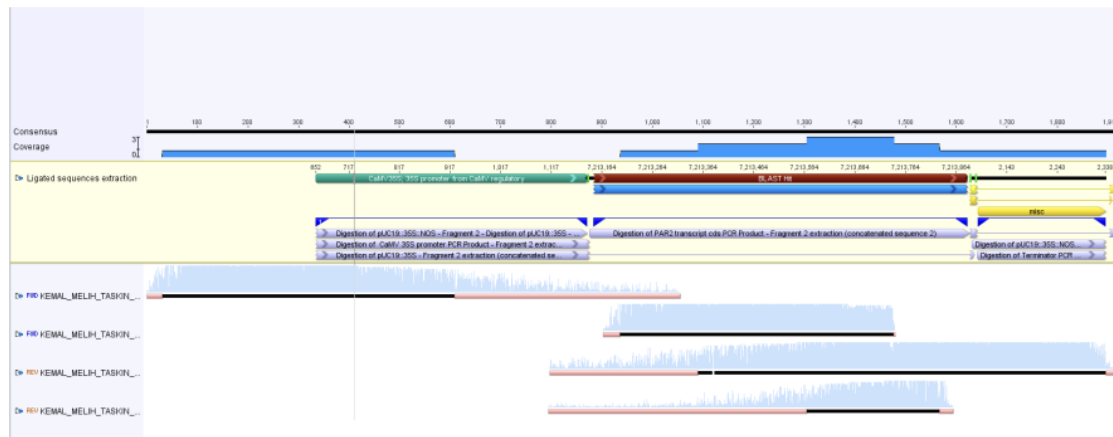
Figure 12: *LEC2* consensus sequences and assembled chromatogram sequences

A phylogenetic tree were plotted with the results of Blastn analysis of consensus sequences of both *BdBBM* and *BdLEC2* by using neighbor-joining method (Figure 17). Phylogenetic tree and Blastn results reveals the similarity between close species' *BBM* and *LEC2* genes and *BdBBM* and *BdLEC2* genes. *BdBBM* gene is showed the highest identity score against *Camelina sativa* with the 94.10%, 0 E-Value and 100% coverage, *Capsella*

rubella with the 92.86%, 0 E-Value and 99% coverage (Appendix 1). It shows homology with the close relative species such as *Brassica Napus*, and *A. thailiana*. *BdLEC2* gene is showed the highest identity score against *Camelina sativa* with the 90.74%, 0 E-Value and 100% coverage, *Arabidopsis lyrata subsp. Lyrata* with the 90.28%, 0 E-Value and 100% coverage (Appendix 2). It shows homology with the close relative species such as *Brassica Napus*, and *A. thailiana*.
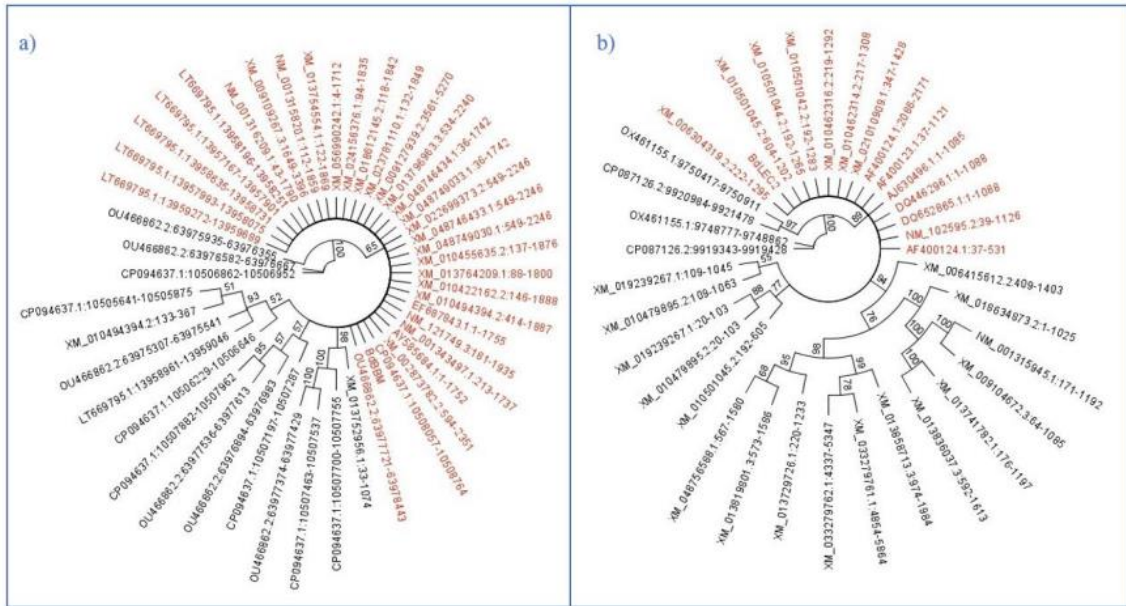
Figure 12: Phylogenetic tree (Neighbor-joining) of consensus sequences of *BBM* and *LEC2* genes a) Phylogenetic tree (Neighbor-joining) of consensus sequence of *BBM* gene b) Phylogenetic tree (Neighbor-joining) of consensus sequence of *LEC2*

4.6. Efficiency of Primers

The primer efficiency plot was generated using the CT values obtained from qPCR experiments conducted with serially diluted cDNA samples. The efficiency of the *LEC2* gene primer set, LEC2.3_F and LEC2.3_R, was determined using the standard curve, slope, and coefficient of determination (R^2). The efficiency of the *LEC2* primer was calculated to be 91.2%. This value indicates how well the primer set amplifies the target gene during qPCR. An efficiency of 100% means that the target gene is doubling in quantity with each PCR cycle. In this case, an efficiency of 91.2% suggests that the primer set is very effective in amplifying the *LEC2* gene.

Additionally, the coefficient of determination (R^2) was found to be 1. The R^2 value represents the goodness of fit of the standard curve to the data points. A value of 1 indicates a perfect fit, meaning that the data points closely follow the standard curve. This further supports the suitability of the primers for qPCR analysis, as the results show a high level of accuracy and precision in measuring the gene expression.

Overall, these results demonstrate that the LEC2.3_F and LEC2.3_R primers are suitable for qPCR analysis of the *LEC2* gene, with high efficiency and a perfect fit to the standard curve and they were firstly used in a qPCR analysis. This makes them reliable tools for quantifying gene expression levels in the studied samples.

Dilutions	Copy Numbers	Log	Ct Values	Dilution (X) =	10
1.	3000000	6,477121	14,2683417		
2.	300000	5,477121	17,82078743		
3.	30000	4,477121	21,37975375	Target =	LEC2
4.	3000	3,477121	24,91914749	Sample =	B. divaricarpa flower buds
5.	300	2,477121	28,46862793	Slope =	-3,553853989
6.	30	1,477121	32,04873657	Efficiency (%) =	91,2
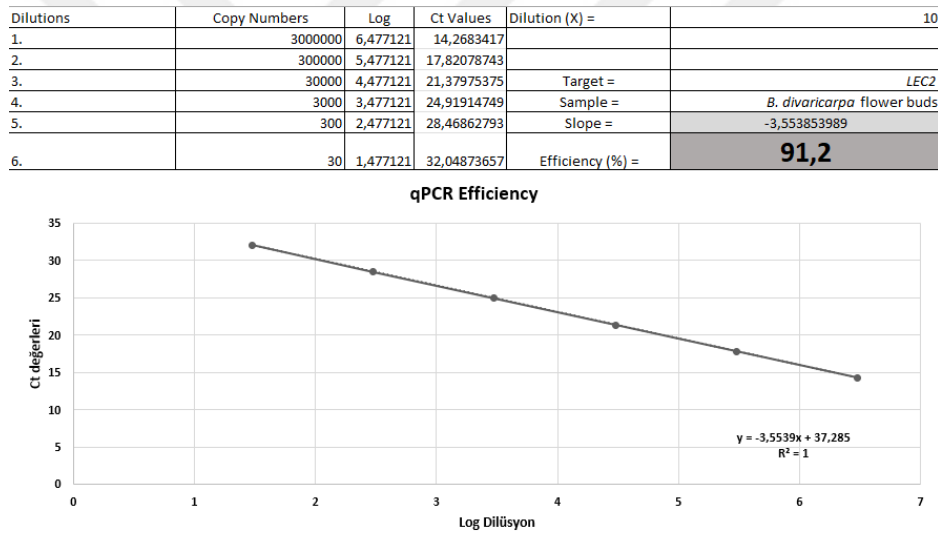
Figure 14: Efficiency results of *LEC2* primer pairs

The efficiency of the *BBM* gene primer set, BBM_F and BBM_R, was determined using the standard curve, slope, and coefficient of determination (R^2). The efficiency of the *BBM* primer was calculated to be 94.7%. In this case, an efficiency of 94.7% suggests that the primer set is very effective in amplifying the *BBM* gene.

Additionally, the coefficient of determination (R^2) was found to be 0.99. A value of 0.99% indicates a perfect fit, meaning that the data points closely follow the standard curve.

This further supports the suitability of the primers for qPCR analysis, as the results show a high level of accuracy and precision in measuring the gene expression.

Overall, these results demonstrate that the BBM_F and BBM_R primers are suitable for qPCR analysis of the *BBM* gene, with high efficiency and a perfect fit to the standard curve and they were firstly used in a qPCR analysis. This makes them reliable tools for quantifying gene expression levels in the studied samples.

Dilutions	Copy Numbers	Log	Ct Values	Dilution (X) =	10
1.	3000000	6,477121	13,68462626		
2.	300000	5,477121	17,07721583		
3.	30000	4,477121	20,63195801	Target =	BBM
4.	3000	3,477121	24,13370832	Sample =	B. divaricarpa flower buds
5.	300	2,477121	27,49126053	Slope =	-3,455465181
6.	30	1,477121	30,92410564	Efficiency (%) =	**94,7**

qPCR Efficiency

y = -3,4555x + 36,067
R² = 0,9999
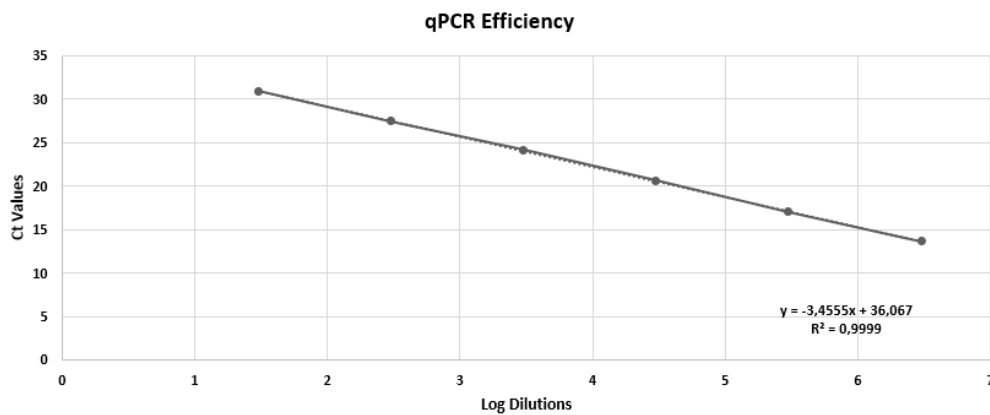
Figure 15: Efficiency results of *BBM* primer pairs

The efficiency of the *APOLLO* gene primer set for the apomict alleles, A_APOLLO_F and APOLLO_R, was determined using the standard curve, slope, and coefficient of determination (R^2). The efficiency of the primer was calculated to be 101.6%. In this case, an efficiency of 101.2% suggests that the primer set is very effective in amplifying the *APOLLO* gene.

Additionally, the coefficient of determination (R^2) was found to be 0.94. A value of 0.94% indicates a good fit, meaning that the data points closely follow the standard curve.

This further supports the suitability of the primers for qPCR analysis, as the results show a high level of accuracy and precision in measuring the gene expression.

Overall, these results demonstrate that the A_APOLLO_F and A_APOLLO_R primers are suitable for qPCR analysis of the *APOLLO* gene, with high efficiency and a perfect fit to the standard curve. This makes them reliable tools for quantifying gene expression levels in the studied samples.

Dilutions	Copy Numbers	Log	Ct Values	Dilution (X) =	10
1.	1000000	6	28,06883303		
2.	100000	5	31,01533763		
3.	10000	4	33,95572853	Target =	*APOLLO*
4.	1000	3	40	Sample =	*B. Divaricarpa* flower buds
5.	100	2	40	Slope =	-3,284699631
6.	10	1		Efficiency (%) =	**101,6**

qPCR Efficiency

y = -3,2847x + 47,747
R² = 0,9444
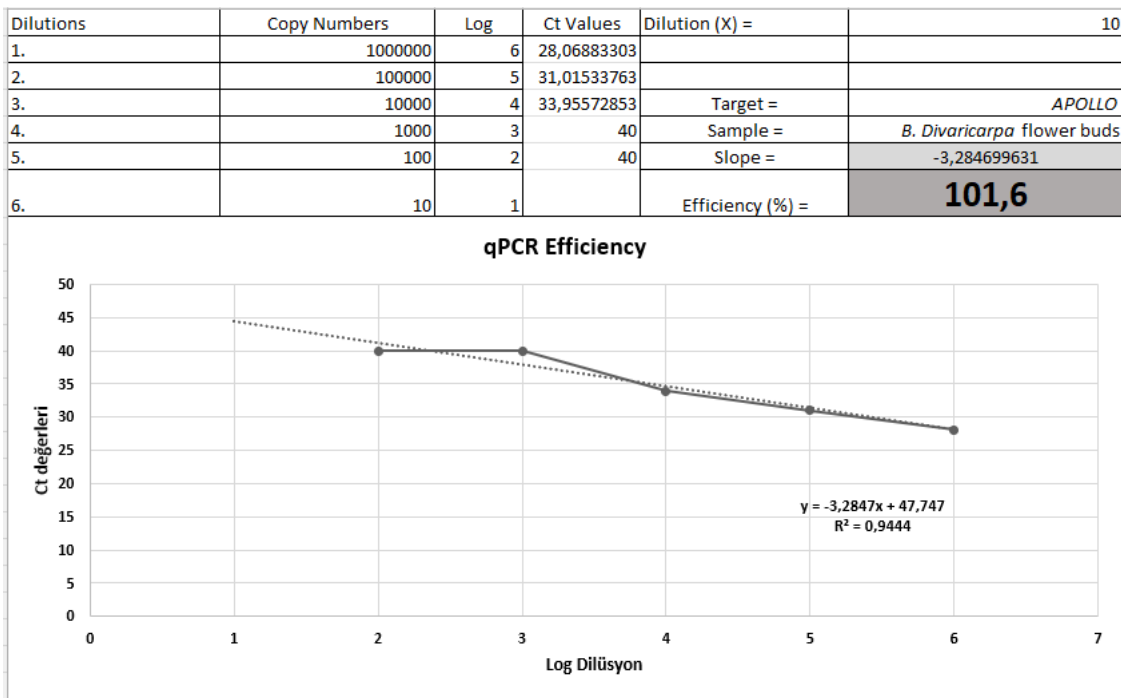
Figure 16: Efficiency results of apomict allele of *APOLLO* primer pairs

The efficiency of the *APOLLO* gene primer set for the sexual alleles, S_APOLLO_F and APOLLO_R, was determined using the standard curve, slope, and coefficient of determination (R^2). The efficiency of the primer was calculated to be 106.7%. In this case, an efficiency of 106.7% suggests that the primer set is effective in amplifying the *APOLLO* gene.

Additionally, the coefficient of determination (R^2) was found to be 0.98. A value of 0.98% indicates a perfect fit, meaning that the data points closely follow the standard curve.

This further supports the suitability of the primers for qPCR analysis, as the results show a high level of accuracy and precision in measuring the gene expression.

Overall, these results demonstrate that the S_APOLLO_F and A_APOLLO_R primers are suitable for qPCR analysis of the *APOLLO* gene, with high efficiency and a perfect fit to the standard curve. This makes them reliable tools for quantifying gene expression levels in the studied samples. These primers for both apomict and sexual alleles were obtained from (Corral et al., 2013) and also used before for the gene expression analysis (qPCR) of *APOLLO* gene in 18 apomictic and sexual individuals of *Boechera* spp. The primers named Lara14F and TSP1-R for the apomict allele and Lara15F and TSP1-R for sexual allele of *APOLLO* gene. This is also proven the quality of primer sequences in the qPCR results.

Dilüsyonlar	Temsili Kopya sayıları	Log	ct değerleri	Sulandırma Katsayısı =	10
1.	1000000	6	20,67573675		
2.	100000	5	23,36205292		
3.	10000	4	25,95086002	Hedef =	APOLLO sex-allele
4.	1000	3	30,3842659	Sample =	B. Stricta flower buds
5.	100	2		Eğim =	-3,171439457
6.	10	1		Verimlilik (%) =	**106,7**
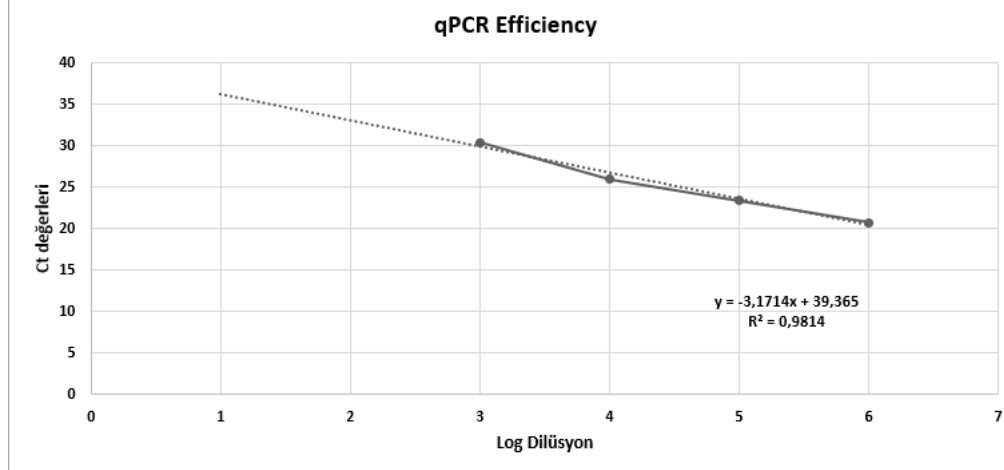
Figure 17: Efficiency results of sexual allele of *APOLLO* primer pairs

4.7.   Gene Expression Analysis of *BBM* gene

In this study, we focused on the *BBM* gene which is a most promising candidate gene or the   parthenogenesis in apomicts. It encodes AP2-domain transcription factor of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family that regulates the early

embryogenesis and endosperm development (Boutilier, 2002; Chen et al., 2022) . It is suggested that the *BBM* gene is highly regulated in both zygote and sperm cells during embryogenesis in various plant species (Anderson et al., 2017; B. Chen et al., 2022; J. Chen et al., 2017). Since it is reported that its ectopic expression of BBM *gene* causes parthenogenesis, ıt is a strong candidate gene for the parthenogenesis stage of apomixis and it's expression is firmly regulated to prevent the parthenogenesis during sexual embryogenesis (Brukhin & Baskar, 2019; Chen et al., 2022; Koltunow & Grossniklaus, 2003; Ozias-Akins, 2006; Schmidt, 2020).
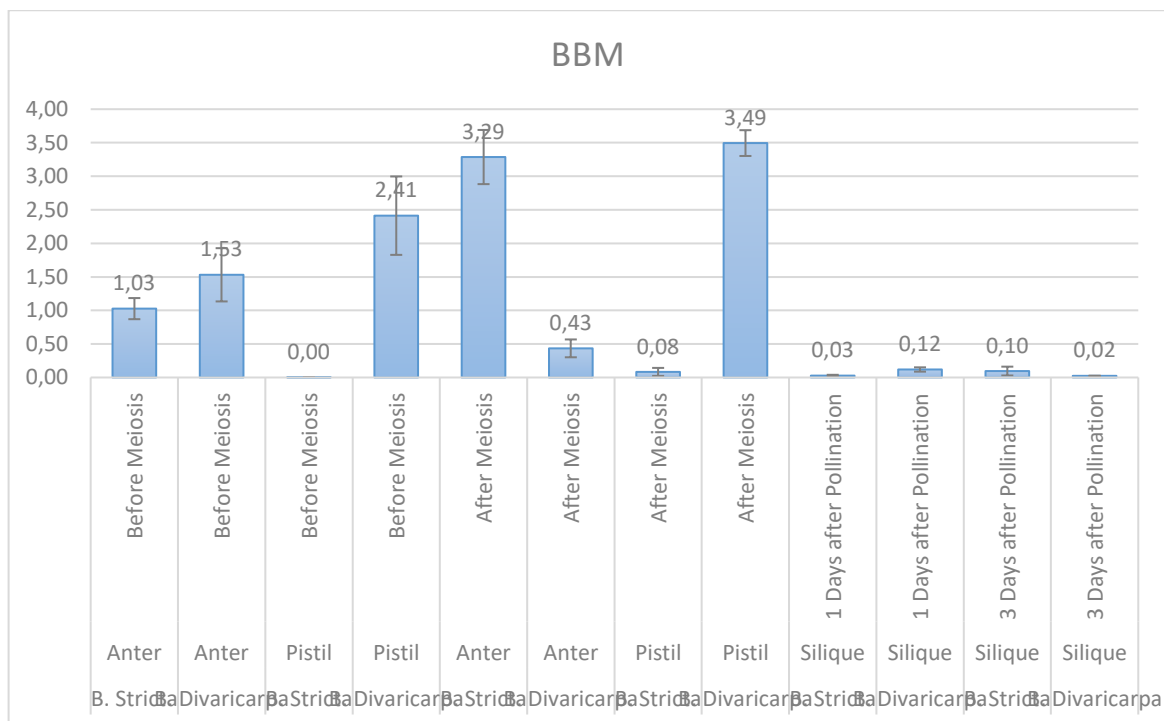
Figure 18: *BBM* gene expression in both sexual *B. stricta* and apomict *B. divaricarpa*

To further explore the role of the *BBM* gene in apomictic reproduction, we conducted an evaluation of its expression profile in both sexual and apomictic Boechera plants at various developmental phases. This investigation aims to gain a better understanding of the gene's involvement in apomixis and shed light on its potential significance in reproductive processes. By examining the expression of the *BBM* gene in sexual and apomict species and stages, we target to uncover valuable insights into the molecular mechanisms underlying apomixis.

The findings of our study align with the previous research conducted by Khanday et al. (2019) that demonstrated the *OsBBM1* is highly regulated in sperm cells and it shows any expression in egg cells in rice (*Oryza sativa*). In the current study, it was observed that the BBM gene in B. stricta was upregulated after meiosis stages in anthers, whereas there was no significant expression in the anther tissues of B. divaricarpa during the same stage.

It is also shown *BBM* gene is highly regulated after meiosis stage in pistil tissues of *B. divaricarpa*. These results indicates an important difference of expression profile of *BBM* gene in between sexual *B. stricta* and apomict *B. divaricarpa*. These results also indicate that *BBM* gene could be parentally expressed in sexual plants during the early embryogenesis as it is demonstrated in rice and maize before (Khanday et al., 2019) and they indicated that it is important to express *BBM* gene for autonomous embryo formation in the absence of fertilization. It is also reported that *BBM* is sufficient for the parthenogenesis and its over expression in sexual plants resulted with parhenogenesis or somatic embryo formation (Boutilier et al., 2002; Chahal et al., 2022; J. Conner et al., 2011; J. A. Conner et al., 2015, 2017). In relation to these, in *B. divaricarpa*, the expression profile of *BBM* gene indicates the role of in autonomous embryo development.

BBM gene did not showed any significant expression in pistil tissues of *B.stricta* in any stage of meiosis and embryogenesis (Dunnet's T3 test, $p > 0.05$). Altough, the *BBM* gene did not showed any significant expression during meiosis in B. stricta in the same stage, it is highly-regulated in pistil tissues of *B. divaricarpa* during meiosis.

Homogenity results of Tukey HSD (Honestly Significant Difference) (Appendix 7 and Appendix 9) and Dunnet's T3 test results (Appendix 8 and Appendix 10) were given in Appendix. Since our datas did not show homogenity, detection of relative gene expression analysis were run out by using Dunnet's T3 test. There is no significant gene expression detected in *B. stricta* and all the results except relative gene expression between before and after meiosis pistil in *B. divarcarpa* were significant.

4.8. Gene Expression Analysis of *LEC2* gene

In this study, we focused on the *LEC2* gene, which is considered a potential candidate gene for apomixis. This gene encodes a B3 domain transcription factor and plays a crucial role in regulating embryogenesis in *A. thaliana* (Stone et al., 2001). Several studies have suggested that LEC2 is a critical regulator of somatic embryogenesis (Boutilier, 2002; Gaj et al., 2005; Horstman et al., 2017). Additionally, there is a hypothesis that the *LEC2* gene may be associated with the parthenogenesis stage of apomixis (Brukhin & Baskar, 2019; Koltunow & Grossniklaus, 2003; Ozias-Akins, 2006; Schmidt, 2020).

To further explore the role of the *LEC2* gene in apomictic reproduction, we conducted an evaluation of its expression profile in both sexual and apomictic *Boechera* plants at various developmental phases. This investigation aims to gain a better understanding of the gene's involvement in apomixis and shed light on its potential significance in reproductive processes. By examining the expression of the *LEC2* gene in sexual and apomict species and stages, we target to uncover valuable insights into the molecular mechanisms underlying apomixis.

The findings in previous research by Stone et al. (2001) indicated that the *LEC2* gene is crucially involved in both early and late embryogenesis in *A. thaliana*. In our investigation of sexual *B. stricta* and apomict *B. divaricarpa*, we did not observed the similar results. Although the *LEC2* gene upregulated in anter tissues during meiosis and after meiosis, It did not show any significant differences between both *B. stricta* and *B. divaricarpa* plants.
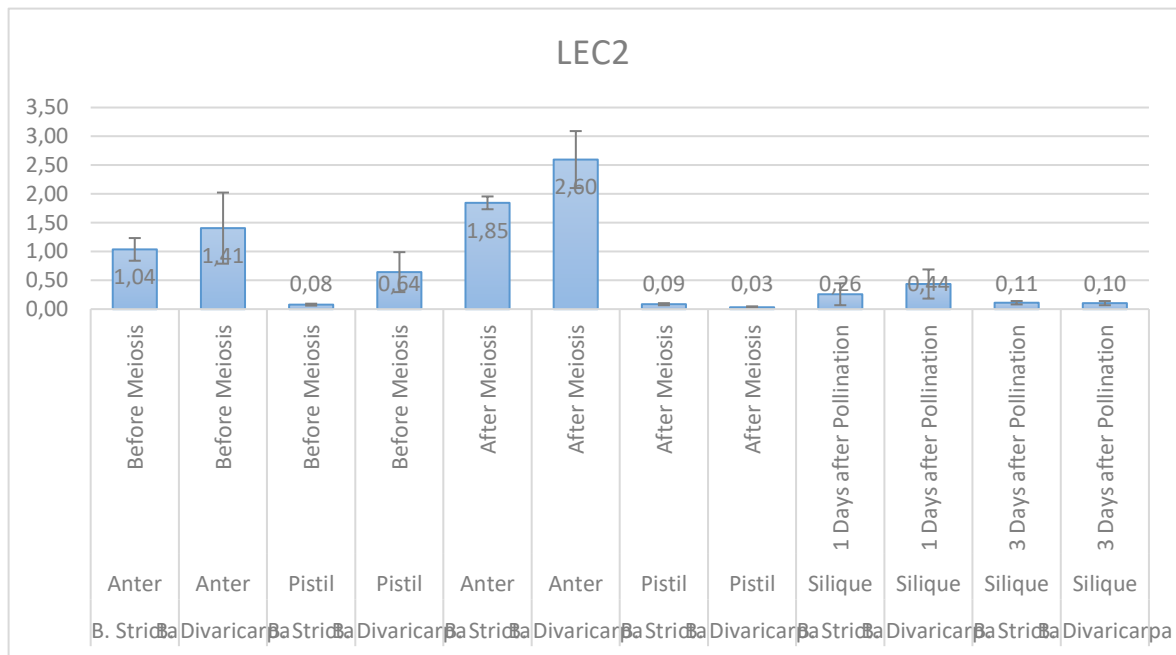
Figure 19: *LEC2* gene expression in both sexual *B. stricta* and apomict *B. divaricarpa*

Interestingly, in apomictic *B. divaricarpa*, the expression pattern of the *LEC2* gene was distinct. It exhibited with a low level expression before meiosis, but there was no observable expression during the post-meiosis stage. These results indicate that the LEC2 gene does not have a role in the reproductive processes of both sexual and apomictic *Boechera* plants. Further investigation into the regulatory mechanisms and functions of the *LEC2* gene in *Boechera* with more detail would provide insights into its significance in apomixis and sexual reproduction.

4.9. Gene Expression Analysis of *APOLLO* gene

In this study, we investigated the expression profile of the *APOLLO* gene, an apomixis-linked gene, in Boechera species. The *APOLLO* gene encodes an Aspartate Glutamate Aspartate Aspartate histidine exonuclease and is known to be associated with apomeiosis (Corral et al., 2013; Bakin et al., 2022).

The *APOLLO* gene has two different alleles that exhibit distinct expression profiles in sexual and apomictic plants. The apo-alleles of this gene are regulated in ovules of apomictic *Boechera* plants, and the expression analysis reveals up-regulation in apomictic ovules. Additionally, it is noted that apomictic genotypes are heterozygous for apo-alleles, while sexual genomes are homozygous for sex-alleles (Bakin et al., 2022; Corral et al., 2013; Kliver et al., 2018).

To explore the expression pattern of the *APOLLO* gene in *B. stricta* and *B. divaricarpa*, we conducted qPCR analysis using tissues from anthers and pistils, both before and after meiosis. We also analyzed pistil tissues at different time points after pollination (1 DAP, 3 DAP, and 5 DAP).

By examining the expression of the *APOLLO* gene in these different tissues and stages, we aim to gain insights into its role in the reproductive processes of both sexual and apomictic *Boechera* species.

Our results corralated with the previous studies, Corral et al., 2013 were found that there are sex and apo alleles of *APOLLO* gene in various natural apomict and sexual *Boechera* plants. Likewise, the results in this study suggest that, gene expression profile of alleles of *APOLLO* gene differs in sexual and apomic plants. The sex-allele of *APOLLO* gene is highly expressed in both anther and pistil tissues of sexual *B. stricta*. It is slightly upregulated in anter and pistil tissues in *B. divaricarpa* during meiosis. It is nearly six fold higher expressed in sexual *B. stricta* than apomict *B. divaricarpa* before meiosis and decreased after meiosis. It is not showed any significant expression after meiosis and after pollination. Exceptionally, It showed up-regulation in expression in 5 DAP of sexual *B. stricta*. The expression were higher than in sexual *B. stricta* in comparison with *B. divaricarpa* (Bakin et al., 2022).

Homogenity results of Tukey HSD (Honestly Significant Difference) (Appendix 15 and Appendix 16) and Dunnet's T3 test results (Appendix 17 and Appendix 18) were given

in Appendix. Since our datas did not show homogenity, detection of relative gene expression analysis were run out by using Dunnet's T3 test.
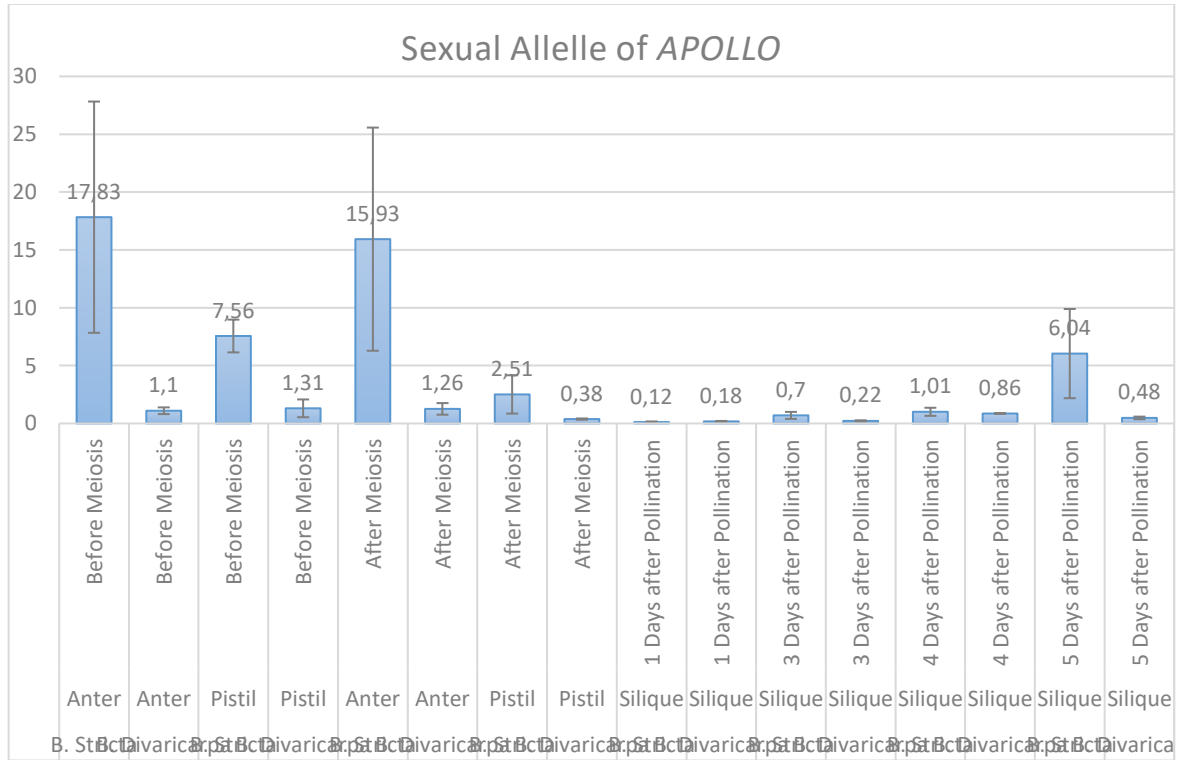
Figure 20: Sexual allele of *APOLLO* expression in both sexual *B. stricta* and apomict *B. divaricarpa*

The findings from our study indicate that the Apo-allele of the *APOLLO* gene is expressed at higher levels in the anter and pistil tissues of *B. divaricarpa* compared to sexual *B. stricta*. During meiosis, the apo-allele is significantly upregulated in apomict *B. divaricarpa*, showing a 1.5-fold increase when compared to *B. stricta*. In contrast, the apo-allele is only slightly expressed in sexual *B. stricta* during meiosis.

After meiosis, the expression of the apo-allele sharply decreases in both plant species until 5 DAP. However, at 5 DAP, a remarkable increase in expression is observed in *B. divaricarpa* pistils, reaching a level seven times higher than before. On the other hand, as expected, no expression is observed in sexual *B. stricta* at 5 DAP (Bakin et al., 2022).

These results strongly suggest that the apo-allele of the *APOLLO* gene is specifically upregulated during meiosis in apomict *B. divaricarpa*, while being downregulated in sexual *B. stricta.* This is consistent with the findings of previous studies, which indicated a correlation between the Apo-allele expression and apomixis in *Boechera* species (Bakin et al., 2022; Corral et al., 2013).

Overall, our study provides valuable insights into the differential expression patterns of the apo-allele of the *APOLLO* gene in sexual and apomictic *Boechera* species during meiosis and post-meiosis stages. This information contributes to our understanding of the molecular mechanisms underlying apomixis and its regulation in these plants.

Homogenity results of Tukey HSD (Honestly Significant Difference) (Appendix 15 and Appendix 16) and Dunnet's T3 test results (Appendix 17 and Appendix 18 ,) were given in Appendix. Since our datas did not show homogenity, detection of relative gene expression analysis were run out by using Dunnet's T3 test.
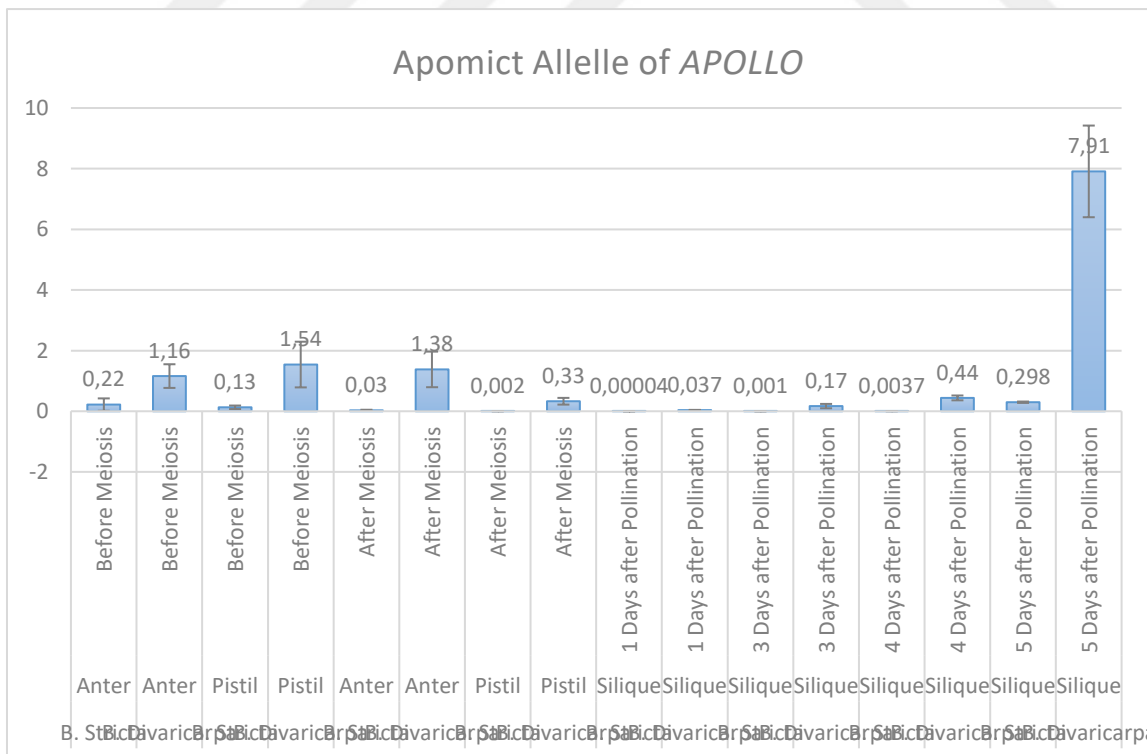
Figure 21: Apomictic allele of *APOLLO* expression in both sexual *B. stricta* and apomict *B. divaricarpa*

CHAPTER 5

CONCLUSION

In this study, the main objective was to investigate the expression differences of candidate genes that could play crucial roles in regulating apomixis at various developmental stages of embryogenesis in both apomictic and sexual *Boechera* plants. To achieve this, we collected anther and pistil tissues from unopened buds and flowers, as well as pollinated pistils at different time points (1, 3, and 5 days after pollination) in both *B. stricta* and *B. divaricarpa* plants. Through our research, we successfully revealed the expressional differences and identified the most expressed stage for both alleles of the *APOLLO* gene, *BBM* gene, and *LEC2* gene, providing novel insights into these genes' roles in apomixis.

While the molecular mechanisms of apomixis are complex and many stages remain unknown, several candidate genes that could be differentially regulated during stages of apomeiosis, parthenogenesis, and endosperm formation, contributing to clonal seed production from maternal plants, have been proposed in previous studies (Brukhin & Baskar, 2019; Schmidt, 2020). Among these candidates, the *APOLLO* gene has been identified as an apomixis-related gene locus, containing both sexual and apomictic alleles and known to regulate apomeiosis (Bakin et al., 2022; Corral et al., 2013). Additionally, the *BBM* and *LEC2* genes, which act as transcription factors, have been found to play critical roles during both early and late embryogenesis (Boutilier et al., 2002; Stone et al., 2001). Furthermore, *BBM* has been shown to regulate the *LEC2* gene during embryogenesis (Horstman et al., 2017; Tian et al., 2020). Both of these genes have also been associated with parthenogenesis during apomixis (Boutilier et al., 2002; Gaj et al., 2005).

In this study, we demonstrated the expression profiles of the *APOLLO*, *BBM*, and *LEC2* genes in both *B. stricta* and *B. divaricarpa*. Our results revealed the existence of two alleles of the *APOLLO* gene: one being the apo-allele, which is highly expressed in the anter and pistil tissues of apomictic *Boechera* during apomeiosis, particularly during meiosis. The subsequent decrease in expression after meiosis further emphasizes the association between the apo-allele and apomeiosis. The other allele is the sex-allele, which is expressed in sexual

Boechera species at the same stage of meiosis. The significant difference in expression levels of the sexual allele of *APOLLO* between *B. stricta* and apomic species indicates an interaction between meiosis and apomixis.

BBM gene is not expressed during meiosis in gynocium and highly regulated in siliques after meiosis stage in *B. divaricarpa* plants. Oppositely, it is highly regulated in anther tissues in the same stage of *B. stricta* and not expressed in siliques. These result could confirm the higher expression of *BBM* in early stages of embryogenesis can cause the autonomous embryogenesis.

LEC2 gene is highly regulated in anter tissues of both *B. divaricarpa* and *B. stricta* and shows no significant expression difference between two species.

Overall, our findings provide valuable information about the expression patterns of these key candidate genes in relation to apomixis in *Boechera* species. This study contributes to our understanding of the molecular mechanisms underlying apomixis and its regulation during different stages of embryogenesis.

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APPENDIX

	Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	PREDICTED: Camelina sativa AP2-like ethylene-responsive transcription factor BBM (...	Camelin...	false flax	90675	2628	2628	100%	0.0	94.10%	2048	XM_010455635.2
☑	PREDICTED: Camelina sativa AP2-like ethylene-responsive transcription factor BBM (...	Camelin...	false flax	90675	2599	2599	100%	0.0	93.77%	2089	XM_010422162.2
☑	PREDICTED: Capsella rubella AP2-like ethylene-responsive transcription factor BBM (...	Capsell...	NA	81985	2483	2483	99%	0.0	92.86%	2013	XM_023781110.1
☑	PREDICTED: Arabidopsis lyrata subsp. lyrata AP2-like ethylene-responsive transcriptio...	Arabido...	NA	81972	2396	2396	99%	0.0	91.64%	2679	XM_002873782.2
☑	Arabidopsis thaliana Integrase-type DNA-binding superfamily protein (BBM), mRNA	Arabido...	thale cr...	3702	2377	2377	100%	0.0	91.43%	2084	NM_121749.3
☑	Arabidopsis thaliana ecotype C24 BABY BOOM (BBM) mRNA, complete cds	Arabido...	thale cr...	3702	2377	2377	100%	0.0	91.43%	1755	EF687843.1
☑	Arabidopsis thaliana clone at5g17430 AP2/EREBP transcription factor mRNA, complet...	Arabido...	thale cr...	3702	2368	2368	100%	0.0	91.37%	1752	AY585684.1
☑	PREDICTED: Camelina sativa AP2-like ethylene-responsive transcription factor BBM (...	Camelin...	false flax	90675	2224	2576	98%	0.0	94.05%	2043	XM_010494394.2
☑	Arabidopsis thaliana Integrase-type DNA-binding superfamily protein (BBM), mRNA	Arabido...	thale cr...	3702	2076	2076	86%	0.0	91.57%	1878	NM_001343497.1
☑	PREDICTED: Eutrema salsugineum AP2-like ethylene-responsive transcription factor B...	Eutrem...	NA	72664	1953	1953	99%	0.0	87.29%	1938	XM_024156376.1
☑	PREDICTED: Brassica rapa AP2-like ethylene-responsive transcription factor BBM2 (L...	Brassic...	field mu...	3711	1821	1821	99%	0.0	85.88%	3601	XM_009109267.3
☑	Brassica napus AP2-like ethylene-responsive transcription factor BBM2 (LOC10638562...	Brassic...	rape	3708	1816	1816	99%	0.0	85.82%	1992	NM_001316209.1
☑	Brassica napus AP2-like ethylene-responsive transcription factor BBM1 (LOC10642617...	Brassic...	rape	3708	1810	1810	99%	0.0	85.81%	2014	NM_001315820.1
☑	PREDICTED: Brassica oleracea var. oleracea AP2-like ethylene-responsive transcriptio...	Brassic...	NA	109376	1799	1799	99%	0.0	85.70%	1926	XM_013754554.1
☑	PREDICTED: Raphanus sativus AP2-like ethylene-responsive transcription factor BBM...	Raphan...	radish	3726	1760	1760	99%	0.0	85.51%	2500	XM_056990242.1
☑	PREDICTED: Brassica napus AP2-like ethylene-responsive transcription factor BBM2 (...	Brassic...	rape	3708	1674	1674	99%	0.0	84.75%	2953	XM_048746433.1
☑	PREDICTED: Brassica napus AP2-like ethylene-responsive transcription factor BBM2 (...	Brassic...	rape	3708	1674	1674	99%	0.0	84.75%	2375	XM_022699373.2
☑	PREDICTED: Brassica napus AP2-like ethylene-responsive transcription factor BBM2 (...	Brassic...	rape	3708	1674	1674	99%	0.0	84.75%	2936	XM_048749030.1
☑	PREDICTED: Brassica rapa AP2-like ethylene-responsive transcription factor BBM2 (L...	Brassic...	field mu...	3711	1640	1640	99%	0.0	84.38%	5857	XM_009127939.2

Appendix 1: BLASTn results of consensus sequence of *BBM* gene

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	PREDICTED: Camelina sativa B3 domain-containing transcription factor L...	Camelina ...	1452	1452	100%	0.0	90.74%	1428	XM_010462314.2
☑	PREDICTED: Camelina sativa B3 domain-containing transcription factor L...	Camelina ...	1424	1424	100%	0.0	90.28%	1430	XM_010501042.2
☑	PREDICTED: Camelina sativa B3 domain-containing transcription factor L...	Camelina ...	1363	1363	100%	0.0	89.55%	1412	XM_010462316.2
☑	PREDICTED: Arabidopsis lyrata subsp. lyrata B3 domain-containing transc...	Arabidopsi...	1341	1341	99%	0.0	89.06%	1642	XM_021010909.1
☑	PREDICTED: Camelina sativa B3 domain-containing transcription factor L...	Camelina ...	1336	1336	100%	0.0	89.10%	1412	XM_010501044.2
☑	Arabidopsis thaliana mRNA for hypothetical protein, clone At1g28300	Arabidopsi...	1334	1334	99%	0.0	88.91%	1089	AJ630496.1
☑	Arabidopsis thaliana LEAFY COTYLEDON 2 mRNA, complete cds	Arabidopsi...	1334	1334	99%	0.0	88.91%	1332	AF400123.1
☑	Arabidopsis thaliana clone 0000013165_0000009489 unknown mRNA	Arabidopsi...	1330	1330	99%	0.0	88.85%	1092	DQ652865.1
☑	Arabidopsis thaliana clone pENTR221-At1g28300 transcriptional factor B3 ...	Arabidopsi...	1330	1330	99%	0.0	88.85%	1092	DQ446296.1
☑	Arabidopsis thaliana AP2/B3-like transcriptional factor family protein (LEC2...	Arabidopsi...	1330	1330	99%	0.0	88.85%	1317	NM_102595.2
☑	PREDICTED: Camelina sativa B3 domain-containing transcription factor L...	Camelina ...	1245	1368	94%	0.0	90.29%	1306	XM_010479895.2
☑	PREDICTED: Camelina sativa B3 domain-containing transcription factor L...	Camelina ...	1151	1274	94%	0.0	88.83%	1288	XM_019239267.1
☑	PREDICTED: Capsella rubella B3 domain-containing transcription factor L...	Capsella r...	1098	1098	97%	0.0	85.65%	1493	XM_006304319.2
☑	PREDICTED: Eutrema salsugineum B3 domain-containing transcription fac...	Eutrema s...	1064	1064	90%	0.0	86.28%	1623	XM_006415612.2
☑	PREDICTED: Brassica oleracea var. oleracea B3 domain-containing transc...	Brassica o...	850	850	94%	0.0	82.03%	1302	XM_013729726.1
☑	PREDICTED: Brassica napus B3 domain-containing transcription factor LE...	Brassica n...	845	845	94%	0.0	81.93%	1777	XM_048756588.1
☑	PREDICTED: Brassica napus B3 domain-containing transcription factor LE...	Brassica n...	845	845	94%	0.0	81.93%	1680	XM_013819801.3
☑	PREDICTED: Camelina sativa B3 domain-containing transcription factor L...	Camelina ...	830	1326	93%	0.0	91.65%	1349	XM_010501045.2
☑	PREDICTED: Brassica napus B3 domain-containing transcription factor LE...	Brassica n...	821	821	94%	0.0	81.39%	1811	XM_013836037.3

Appendix 2: BLASTn results of consensus sequence of *LEC2* gene

RGE

	Stages	N	Subset for alpha = 0.05
			1
Tukey HSD[a,b]	3 Days After Pollination	7	31,8246
	1 Days After Pollination	8	31,8959
	Before Meiosis	8	31,9202
	After Meiosis	9	31,9297
	Sig.		,996

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7,937.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix 3: Tukey HSD (Honestly Significant Difference) test results of LEC2 gene expression in *B. divaricarpa* pistils

Multiple Comparisons

Dependent Variable: RGE

Dunnett T3

(I) Stages	(J) Stages	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Before Meiosis	After Meiosis	-,00949	,51955	1,000	-1,6283	1,6093
	1 Days After Pollination	,02427	,28314	1,000	-,8422	,8908
	3 Days After Pollination	,09556	,40383	1,000	-1,1755	1,3666
After Meiosis	Before Meiosis	,00949	,51955	1,000	-1,6093	1,6283
	1 Days After Pollination	,03376	,49606	1,000	-1,5530	1,6205
	3 Days After Pollination	,10505	,57357	1,000	-1,6367	1,8468
1 Days After Pollination	Before Meiosis	-,02427	,28314	1,000	-,8908	,8422
	After Meiosis	-,03376	,49606	1,000	-1,6205	1,5530
	3 Days After Pollination	,07130	,37313	1,000	-1,1514	1,2940
3 Days After Pollination	Before Meiosis	-,09556	,40383	1,000	-1,3666	1,1755
	After Meiosis	-,10505	,57357	1,000	-1,8468	1,6367
	1 Days After Pollination	-,07130	,37313	1,000	-1,2940	1,1514

Appendix 4: Dunnet's T3 test results of *LEC2* gene expression in *B. divaricarpa* pistils

Homogeneous Subsets

RGE

	Stages	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a,b]	1 Days After Pollination	9	30,5263	
	After Meiosis	8		33,3818
	Before Meiosis	9		33,5091
	3 Days After Pollination	7		33,5945
	Sig.		1,000	,976

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8,162.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix 5: Figure 3: Tukey HSD (Honestly Significant Difference) test results of *LEC2* gene expression in *B. stricta* pistils

Multiple Comparisons

Dependent Variable: RGE

Dunnett T3

(I) Stages	(J) Stages	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Before Meiosis	After Meiosis	,12737	,30878	,998	-,8524	1,1071
	1 Days After Pollination	2,98287*	,41807	,000	1,6444	4,3214
	3 Days After Pollination	-,08537	,60453	1,000	-2,2251	2,0543
After Meiosis	Before Meiosis	-,12737	,30878	,998	-1,1071	,8524
	1 Days After Pollination	2,85550*	,48102	,000	1,4007	4,3103
	3 Days After Pollination	-,21273	,64966	1,000	-2,3622	1,9368
1 Days After Pollination	Before Meiosis	-2,98287*	,41807	,000	-4,3214	-1,6444
	After Meiosis	-2,85550*	,48102	,000	-4,3103	-1,4007
	3 Days After Pollination	-3,06824*	,70816	,007	-5,2951	-,8414
3 Days After Pollination	Before Meiosis	,08537	,60453	1,000	-2,0543	2,2251
	After Meiosis	,21273	,64966	1,000	-1,9368	2,3622
	1 Days After Pollination	3,06824*	,70816	,007	,8414	5,2951

*. The mean difference is significant at the 0.05 level.

Appendix 6: Dunnet's T3 test results of LEC2 gene expression in *B. stricta* pistils

Homogeneous Subsets

RGE

	Stages	N	Subset for alpha = 0.05			
			1	2	3	4
Tukey HSD[a,b]	After Meiosis	9	24,2950			
	Before Meiosis	9		25,1560		
	1 Days After Pollination	8			32,6054	
	3 Days After Pollination	6				34,9733
	Sig.		1,000	1,000	1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7,784.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix 7: Tukey HSD (Honestly Significant Difference) test results of *BBM* gene expression in *B. divaricarpa* pistils

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RGE

Dunnett T3

(I) Stages	(J) Stages	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Before Meiosis	After Meiosis	,86101	,30331	,082	-,0873	1,8093
	1 Days After Pollination	-7,44942*	,26779	,000	-8,2921	-6,6068
	3 Days After Pollination	-9,81729*	,18582	,000	-10,3925	-9,2420
After Meiosis	Before Meiosis	-,86101	,30331	,082	-1,8093	,0873
	1 Days After Pollination	-8,31043*	,36203	,000	-9,3954	-7,2254
	3 Days After Pollination	-10,67831*	,30640	,000	-11,6367	-9,7199
1 Days After Pollination	Before Meiosis	7,44942*	,26779	,000	6,6068	8,2921
	After Meiosis	8,31043*	,36203	,000	7,2254	9,3954
	3 Days After Pollination	-2,36788*	,27129	,000	-3,2240	-1,5118
3 Days After Pollination	Before Meiosis	9,81729*	,18582	,000	9,2420	10,3925
	After Meiosis	10,67831*	,30640	,000	9,7199	11,6367
	1 Days After Pollination	2,36788*	,27129	,000	1,5118	3,2240

*. The mean difference is significant at the 0.05 level.

Appendix 8: Dunnet's T3 test results of *BBM* gene expression in *B. divaricarpa* pistils

Homogeneous Subsets

RGE

	Stages	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a,b]	After Meiosis	7	29,4675	
	1 Days After Pollination	6	32,9813	32,9813
	3 Days After Pollination	7		34,0766
	Before Meiosis	7		34,1105
	Sig.		,055	,818

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6,720.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix 9: Tukey HSD (Honestly Significant Difference) test results of *BBM* gene expression in *B. stricta* pistils

Post Hoc Tests

Multiple Comparisons

Dependent Variable: RGE

Dunnett T3

(I) Stages	(J) Stages	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Before Meiosis	After Meiosis	4,64308	1,37295	,051	-,0234	9,3095
	1 Days After Pollination	1,12922	,68287	,503	-1,0181	3,2766
	3 Days After Pollination	,03396	1,12317	1,000	-3,6750	3,7429
After Meiosis	Before Meiosis	-4,64308	1,37295	,051	-9,3095	,0234
	1 Days After Pollination	-3,51386	1,36981	,161	-8,1839	1,1562
	3 Days After Pollination	-4,60913	1,63450	,084	-9,7130	,4948
1 Days After Pollination	Before Meiosis	-1,12922	,68287	,503	-3,2766	1,0181
	After Meiosis	3,51386	1,36981	,161	-1,1562	8,1839
	3 Days After Pollination	-1,09526	1,11934	,892	-4,8086	2,6181
3 Days After Pollination	Before Meiosis	-,03396	1,12317	1,000	-3,7429	3,6750
	After Meiosis	4,60913	1,63450	,084	-,4948	9,7130
	1 Days After Pollination	1,09526	1,11934	,892	-2,6181	4,8086

Appendix 10: Dunnet's T3 test results of *BBM* gene expression in *B. stricta* pistils

RGE

	Stages	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a,b]	3 DAP	9	25,2428		
	After Meiosis	9	26,2725	26,2725	
	1 DAP	9	26,9783	26,9783	
	Before Meiosis	8		28,3087	28,3087
	4 DAP	8		28,4906	28,4906
	5 DAP	8			30,5287
	Sig.		,523	,256	,255

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8,471.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix 11: Tukey HSD (Honestly Significant Difference) test results of sex allele of *APOLLO* gene expression in *B. divaricarpa* pistils

RGE

	Stages	N	Subset for alpha = 0.05
			1
Tukey HSD[a,b]	4 DAP	8	27,1271
	1 DAP	8	28,3171
	3 DAP	8	28,4612
	After Meiosis	9	28,9024
	5 DAP	9	29,8748
	Before Meiosis	7	32,1947
	Sig.		,081

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8,107.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix 12: Tukey HSD (Honestly Significant Difference) test results of sex allele of *APOLLO* gene expression in *B. stricta* pistils

(I) Stages	(J) Stages	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound
Before Meiosis	After Meiosis	2,03619	,63115	,071	-,1173
	1 DAP	1,33041	,47217	,203	-,4764
	3 DAP	3,06596*	,46059	,002	1,2578
	4 DAP	-,18185	,55022	1,000	-2,1117
	5 DAP	-2,21994	1,74552	,931	-9,0148
After Meiosis	Before Meiosis	-2,03619	,63115	,071	-4,1897
	1 DAP	-,70578	,46765	,839	-2,4357
	3 DAP	1,02977	,45595	,414	-,6953
	4 DAP	-2,21804*	,54634	,016	-4,0988
	5 DAP	-4,25613	1,74431	,340	-11,0500
1 DAP	Before Meiosis	-1,33041	,47217	,203	-3,1372
	After Meiosis	,70578	,46765	,839	-1,0242
	3 DAP	1,73555*	,18025	,000	1,1172
	4 DAP	-1,51226*	,35084	,019	-2,8048
	5 DAP	-3,55034	1,69328	,512	-10,3767
3 DAP	Before Meiosis	-3,06596*	,46059	,002	-4,8741
	After Meiosis	-1,02977	,45595	,414	-2,7548
	1 DAP	-1,73555*	,18025	,000	-2,3539
	4 DAP	-3,24782*	,33509	,000	-4,5304
	5 DAP	-5,28590	1,69009	,153	-12,1158
4 DAP	Before Meiosis	,18185	,55022	1,000	-1,7480
	After Meiosis	2,21804*	,54634	,016	,3373
	1 DAP	1,51226*	,35084	,019	,2197
	3 DAP	3,24782*	,33509	,000	1,9652
	5 DAP	-2,03808	1,71668	,953	-8,8447
5 DAP	Before Meiosis	2,21994	1,74552	,931	-4,5749
	After Meiosis	4,25613	1,74431	,340	-2,5378
	1 DAP	3,55034	1,69328	,512	-3,2760
	3 DAP	5,28590	1,69009	,153	-1,5440
	4 DAP	2,03808	1,71668	,953	-4,7685

Appendix 13: Dunnet's T3 test results of sex allele of *APOLLO* gene expression in *B. divaricarpa* pistils

(I) Stages	(J) Stages	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound
Before Meiosis	After Meiosis	3,29233*	,65808	,011	,7519
	1 DAP	3,87758*	,92698	,014	,6400
	3 DAP	3,73350	2,59130	,866	-6,4226
	4 DAP	5,06765	1,61824	,124	-1,0052
	5 DAP	2,31994	1,03773	,399	-1,2751
After Meiosis	Before Meiosis	-3,29233*	,65808	,011	-5,8328
	1 DAP	,58524	,74977	,999	-2,2488
	3 DAP	,44117	2,53332	1,000	-9,7521
	4 DAP	1,77532	1,52367	,958	-4,2846
	5 DAP	-,97239	,88303	,975	-4,2568
1 DAP	Before Meiosis	-3,87758*	,92698	,014	-7,1151
	After Meiosis	-,58524	,74977	,999	-3,4193
	3 DAP	-,14408	2,61609	1,000	-10,2930
	4 DAP	1,19007	1,65765	,999	-4,9145
	5 DAP	-1,55763	1,09817	,892	-5,3086
3 DAP	Before Meiosis	-3,73350	2,59130	,866	-13,8895
	After Meiosis	-,44117	2,53332	1,000	-10,6344
	1 DAP	,14408	2,61609	1,000	-10,0048
	4 DAP	1,33415	2,93314	1,000	-9,1519
	5 DAP	-1,41355	2,65735	1,000	-11,5621
4 DAP	Before Meiosis	-5,06765	1,61824	,124	-11,1405
	After Meiosis	-1,77532	1,52367	,958	-7,8352
	1 DAP	-1,19007	1,65765	,999	-7,2946
	3 DAP	-1,33415	2,93314	1,000	-11,8202
	5 DAP	-2,74771	1,72203	,797	-8,9332
5 DAP	Before Meiosis	-2,31994	1,03773	,399	-5,9150
	After Meiosis	,97239	,88303	,975	-2,3120
	1 DAP	1,55763	1,09817	,892	-2,1933
	3 DAP	1,41355	2,65735	1,000	-8,7350
	4 DAP	2,74771	1,72203	,797	-3,4378

Appendix 14: Dunnet's T3 test results of sex allele of *APOLLO* gene expression in *B. stricta* pistils

RGE

	Stages	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a,b]	4 DAP	7	38,7660	
	Before Meiosis	8		40,0000
	After Meiosis	8		40,0000
	1 DAP	9		40,0000
	3 DAP	8		40,0000
	5 DAP	8		40,0000
	Sig.		1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7,958.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix 15: Tukey HSD (Honestly Significant Difference) test results of apomict allele of *APOLLO* gene expression in *B. divaricarpa* pistils

RGE

	Stages	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a,b]	4 DAP	7	38,7660	
	Before Meiosis	8		40,0000
	After Meiosis	8		40,0000
	1 DAP	9		40,0000
	3 DAP	8		40,0000
	5 DAP	8		40,0000
	Sig.		1,000	1,000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7,958.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix 16: Tukey HSD (Honestly Significant Difference) test results of apomict allele of *APOLLO* gene expression in *B. stricta* pistils

(I) Stages	(J) Stages	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound
Before Meiosis	After Meiosis	,94883	,45412	,481	-,5993
	1 DAP	-1,74172*	,32293	,006	-2,9778
	3 DAP	1,85935*	,32370	,004	,6232
	4 DAP	-2,25283*	,47391	,005	-3,8928
	5 DAP	-3,50091*	,36953	,000	-4,8045
After Meiosis	Before Meiosis	-,94883	,45412	,481	-2,4970
	1 DAP	-2,69055*	,34940	,000	-3,9930
	3 DAP	,91052	,35012	,261	-,3923
	4 DAP	-3,20166*	,49233	,000	-4,8852
	5 DAP	-4,44974*	,39288	,000	-5,8195
1 DAP	Before Meiosis	1,74172*	,32293	,006	,5057
	After Meiosis	2,69055*	,34940	,000	1,3881
	3 DAP	3,60107*	,14366	,000	3,1159
	4 DAP	-,51111	,37476	,899	-1,9651
	5 DAP	-1,75918*	,22893	,000	-2,5968
3 DAP	Before Meiosis	-1,85935*	,32370	,004	-3,0955
	After Meiosis	-,91052	,35012	,261	-2,2134
	1 DAP	-3,60107*	,14366	,000	-4,0862
	4 DAP	-4,11218*	,37543	,000	-5,5661
	5 DAP	-5,36026*	,23002	,000	-6,1992
4 DAP	Before Meiosis	2,25283*	,47391	,005	,6129
	After Meiosis	3,20166*	,49233	,000	1,5182
	1 DAP	,51111	,37476	,899	-,9429
	3 DAP	4,11218*	,37543	,000	2,6583
	5 DAP	-1,24808	,41559	,132	-2,7418
5 DAP	Before Meiosis	3,50091*	,36953	,000	2,1973
	After Meiosis	4,44974*	,39288	,000	3,0799
	1 DAP	1,75918*	,22893	,000	,9216
	3 DAP	5,36026*	,23002	,000	4,5213
	4 DAP	1,24808	,41559	,132	-,2457

Appendix 16: Dunnet's T3 test results of apomict allele of *APOLLO* gene expression in *B. divaricarpa* pistils

(I) Stages	(J) Stages	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound
Before Meiosis	After Meiosis	,00000	,00000	.	,0000
	1 DAP	,00000	,00000	.	,0000
	3 DAP	,00000	,00000	.	,0000
	4 DAP	1,23402	,79868	,809	-2,1804
	5 DAP	,00000	,00000	.	,0000
After Meiosis	Before Meiosis	,00000	,00000	.	,0000
	1 DAP	,00000	,00000	.	,0000
	3 DAP	,00000	,00000	.	,0000
	4 DAP	1,23402	,79868	,809	-2,1804
	5 DAP	,00000	,00000	.	,0000
1 DAP	Before Meiosis	,00000	,00000	.	,0000
	After Meiosis	,00000	,00000	.	,0000
	3 DAP	,00000	,00000	.	,0000
	4 DAP	1,23402	,79868	,809	-2,1804
	5 DAP	,00000	,00000	.	,0000
3 DAP	Before Meiosis	,00000	,00000	.	,0000
	After Meiosis	,00000	,00000	.	,0000
	1 DAP	,00000	,00000	.	,0000
	4 DAP	1,23402	,79868	,809	-2,1804
	5 DAP	,00000	,00000	.	,0000
4 DAP	Before Meiosis	-1,23402	,79868	,809	-4,6485
	After Meiosis	-1,23402	,79868	,809	-4,6485
	1 DAP	-1,23402	,79868	,809	-4,6485
	3 DAP	-1,23402	,79868	,809	-4,6485
	5 DAP	-1,23402	,79868	,809	-4,6485
5 DAP	Before Meiosis	,00000	,00000	.	,0000
	After Meiosis	,00000	,00000	.	,0000
	1 DAP	,00000	,00000	.	,0000
	3 DAP	,00000	,00000	.	,0000
	4 DAP	1,23402	,79868	,809	-2,1804

Appendix 18: Dunnet's T3 test results of apomict allele of *APOLLO* gene expression in *B. stricta* pistils

Appendix 19: Quibit Values of RNA Samples

Species	Sample Name	Quibit values (ng/ μl) for each biological replicate		
		1.	**2.**	**3.**
B. divaricarpa	Pistil - Before Meiosis	890	550	1000
B. divaricarpa	Pistil – After Meiosis	1000	740	726
B. divaricarpa	Anther - Before Meiosis	496	159	558
B. divaricarpa	Anther – After Meiosis	274	206	250
B. stricta	Pistil - Before Meiosis	306	172	356
B. stricta	Pistil – After Meiosis	472	490	690
B. stricta	Anther - Before Meiosis	264	132	216
B. stricta	Anther – After Meiosis	100	110	116
B. divaricarpa	Pistil - 1 DAP	181	243	112
B. stricta	Pistil – 1 DAP	112	112	181
B. divaricarpa	Pistil – 3 DAP	580	250	778
B. stricta	Pistil – 3 DAP	812	406	200
B. divaricarpa	Pistil – 5 DAP	159	620	454
B. stricta	Pistil – 5 DAP	692	874	580