STUDIES ON MOLECULAR AND GENETIC CHARACTERIZATION OF THE GENES RESPONSIBLE FOR THE MULTICARPELLARY GYNOECIUM IN *Thermopsis turcica*

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STUDIES ON MOLECULAR AND GENETIC CHARACTERIZATION OF THE GENES RESPONSIBLE FOR THE MULTICARPELLARY GYNOECIUM IN *Thermopsis turcica*

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ABSTRACT

Thermopsis turcica is a critically endangered endemic plant species in Turkey. The main agricultural trait of *T. turcica* is having a gynoecium of 2-4 free carpellate-ovaries. *Vicia faba* is different from *T. turcica* due to having unicarpellate ovary. In this study, reciprocal crosses between *V. faba* and *T. turcica* were made. In the crosses in which *T. turcica* was used as the paternal parent, globular hybrid embryos were obtained but they were not rescued since embryos were too small for culturing separately from the endosperms. In histological analysis, it was found that pollen tube reached the ovary at the first day of pollination and ovule fertilization occurred on the fourth day of pollination. The data from RT-PCR and sequencing give the first molecular data on the identification of the putative partial homologues of *CLV*, *WUS* and *FAS* were isolated from *T. turcica*. Due to the sequence similarity, demonstrated that these new isolated partial genes were most probably *CLV*, *WUS*, and *FAS* homologues in *T. turcica*. In addition, dihydropyrimidine dehydrogenase (NADP+)-like partial sequence which has a phosphate binding domain in *T. turcica* was isolated. For detailed expression of each ortholog gene, quantitave real-time polymerase chain reaction was implemented and expression patterns were shown. These results might be used for other related crops of economic importance in order to obtain cultivars having polycarpellary feature and are the first scientific report about the molecular basis of the multicarpellary in *T. turcica*.

Thermopsis turcica'da ÇOKLU KARPEL OLUŞUMUNDAN SORUMLU OLAN GENLERİN MOLEKÜLER VE GENETİKSEL KARAKTERİZASYONU ÜZERİNE ÇALIŞMALAR

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ÖZET

Thermopsis. turcica yok olmak üzere olan Türkiye endemiği bitki türüdür. *T. turcica*'nın başlıca özelliği 2-4 serbest karpelli ovaryuma sahip olmasıdır. *Vicia. faba* tek karpelli ovaryuma sahip olması ile *T. turcica*'dan ayrılmaktadır. Bu çalışmada, melezlemeler *T. turcica* ve *V. faba* arasında yapılmıştır ve melezleme kombinasyonunda *T. turcica* hem ana hem de baba olarak kullanılmıştır. T. *turcica*'nın baba olarak kullanıldığı melezlemeler sonucunda, globular aşamada hibrit embriyolar elde edilmiş fakat izole embriyoların küçük olmaları ve endosperm ihtiyaçları nedeni ile kültürlerinin yapılmasında başarılı olunamamıştır. Histolojik analizler sonucunda, polen tüpünün polinizasyonun ilk gününde yumartalığa ulaştığı ve 4. günde ovül fertilizasyonun gerçekleştiği tespit edilmiştir. Polimeraz zincir reaksiyonu ve dizilime çalışmalarından elde edilen veriler çoklu karpel oluşumda sorumlu olduğu düşünülen *CLV*, *WUS*, and *FAS* genlerinin *T. turcica*'daki homologlarının belirlenmesine yönelik ilk moleküler veri olma özelliğindedir. Dizileme analizi sonucunda, *T. turcica*'dan dihydropyrimidine dehydrogenase gen bölgesine ait kısmi diziler tespit edilmiş olup, izole edilen kısmi gen bölgesinin fosfat bağlama domaini içerdiği görülmüştür. *T. turcica*'da tespit edilen gen ortologlarının ekspresyon düzeyleri kantitatif real-time polimeraz zincir reaksiyonu analizi ile belirlenmiştir. Elde edilen bu bulguların yeni çeşit geliştirme çalışmaları için diğer ekonomik öneme sahip türlere uygulanabileceği düşünülmekte olup tez çıktısının *T. turcica*'da çoklu karpel oluşumu üzerine uygulanan moleküler araştırmaları içeren ilk bilimsel rapor olma özelliği bulunmaktadır.

To my esteemed family,

To my best friends forever...

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
bp	Base pair
C. arietinum	*Cicer arietinum*
cDNA	Complementary Deoxyribonucleic Acid
CLV	CLAVATA
CTAB	N-Cetyl-N, N, N-Trimethyl-Ammonium Bromide
DNA	Deoxyribonucleic Acid
ss-cDNA	Single Strand Complementary DNA
°C	Degrees Celcius
DAP	Day After Pollination
DEPC	Diethylpyrocarbonate
EDTA	Ethylene Diamine Tetraacetic Acid
EtBr	Ethidium Bromide
EtOH	Ethanol
FAS	FASCIATA
g	Gram
GA_3	Gibberellic Acid
gDNA	Genomic DNA
h	Hour
IAA	Indole-3-Butyric Acid
l	Litre
L. japonicus	*Lotus japonicus*
mg	Miligram
M	Molar
mM	Milimolar
M. truncatula	*Medicago truncatula*
MEF	Mouse Embryonic Fibroblast
min	Minute
ml	Milliliter
mRNA	Messenger RNA
MS	Murashige and Skoog

µg	Microgram
µl	Microlitre
NCBI	National Center for Biotechnology
ng	Nanogram
NGBG	Nezahat Gokyigit Botanical Garden
ORF	Acronym for Open Reading Frame
PCR	Polymerase Chain Reaction
P. vulgaris	*Phaseolus vulgaris*
RNA	Ribonucleic Acid
RNA-Seq	RNA Sequencing
RT-PCR	Reverse Transcription Polymerase Chain Reaction
STP	Stamina Pistilloida
TBE	Tris-Boric Acid-EDTA
T. turcica	*Thermopsis turcica*
U	Unite
UV	Ultraviolet Light
V. faba	*Vicia faba*
V	Volt
v/v	Volume/volume
WUS	WUSCHEL
w/v	Weight/volume
ZEA	Zeatin

The following nomenclature is used in this thesis:

Protein names are written in upper case letters, e.g. FAS
Gene names are written in upper case italic letters, e.g. *FAS*

CHAPTER 1

INTRODUCTION

1.1. Context and Motivation

Thermopsis turcica is a perennial herbaceous, endemic and rare flower crop in Turkey. Except for these characteristics, the most important feature of this species is to have a 2-4 free carpellary ovary (Figure 1.1). Carpels are the structural units of the female organ in a plant. In carpels, an internal morphological space that permits the development of additional organs, as well as ovules, can be presented [1–4].

Figure 1.1: Three free carpellary ovary of *T. turcica*

Plant family Fabaceae with nearly 700 genera and 18,000 species has agronomically important plants [5]. In this dissertation, *Vicia faba* (faba bean), which is one of the oldest crops used for human and animal consumption, and *T. turcica*, which is endemic endangered plant species, are selected to understand the mechanism of multicarpellary feature in Fabaceae by molecular analysis and to search the possibility of increasing yield in edible crops by crossing.

Sexual hybridization was conducted between *V. faba* and *T. turcica*. We aimed to analyze the crossability between two species; *V. faba* and *T. turcica* in this study. Interspecific and intergeneric-reciprocal crosses and self-pollinations were implemented using both populations of *T. turcica*, Eber, and Aksehir, and *V. faba* and observations were made on F1 plants.

As a model for understanding of polycarpellary ovarium in Fabaceae, it is believed that the different mechanisms responsible for carpel multiplication like in *Pisum sativum*, *Medicago truncatula* [2] in the subfamily Mimosoideae of Fabaceae [6,7] and in tribe Swartzieae of subfamily Papilionoideae [8,9]. We focused primarily on the genetic mechanisms underlying flower development in *T. turcica.* We tried to determine which mechanism (floral meristem increase- *CLAVATA-WUSCHEL* distortions or homeosis-ABC model) causes carpel multiplication in *T. turcica.* Orthologs of target genes (*CLV*, *WUS*, and *FAS*) related to multicarpellary ovary in developing flowers of *T. turcica* are isolated, and their expression patterns are examined in the framework of this dissertation.

1.2. Structure of the Thesis

This thesis is divided into four chapters that contain a brief summary of the most relevant results of our research.

In Chapter 1, motivations and organizations of the dissertation are introduced.

Chapter 2 presents a brief introduction about *T. turcica*, theoretical concepts and provides detailed information about intergeneric hybridization and embryo culture. The chapter presents the few studies conducted on *T. turcica* in the literature. Literature related to morphology, ecology, micropropagation, identification of mineral, dry matter, alkaloid content and antioxidants of *T. turcica* followed by the question of the possibility of obtaining hybrids from *T. turcica* and its distant relatives. Then, the results of histological analysis on pollinated samples are presented. In the second part of the chapter, *in vitro* propagation from the embryos of *T. turcica* is introduced. This part of Chapter 2 is based on the following paper, which will be referred in Chapter 2.

- Tekdal D., Cetiner S. 2014. In-Ovule Embryo Culture of *Thermopsis turcica*. *Journal of Animal and Plant Sciences,* 26(6): 1673-1679.

In Chapter 3, isolation and characterization of the genes related to multicarpellary gynoecium in *T. turcica* are provided. First of all, total RNA isolation and cDNA synthesis from isolated RNAs are discussed. Then, used orthologs of desired genes found in GenBank for primer design are presented. After that, degenerate primer design is introduced. The second part of this chapter covers RT-PCR analysis, gel extraction, sequencing, and sequence analysis. In the last part, the quantitative real-time polymerase chain reaction (qRT-PCR) analysis is presented.

Chapter 4 concludes the main body of the thesis and provides future directions. The perspectives for future studies on the multicarpellary feature in *T. turcica* are also presented in this chapter.

Numerous tables, figures, and drawings are included in this dissertation to facilitate comprehension of the presented research. The appendix part further increases the accessibility of the information.

CHAPTER 2

CLASSICAL HYBRIDIZATION

2.1. General Introduction

2.1.1. Fundamental Aspects of *Thermopsis turcica*

The family of Fabaceae consists of three subfamilies: Caesalpinioideae, Mimosoideae, and Papilionoideae [5]. The genus of *Thermopsis* which belongs to subfamily Papilionoideae of the Fabaceae (Leguminosae) family has 28 genera and 400 endemic plant species in Turkey [10,11]. *Thermopsis* are mainly naturally found in mountainous areas of Asia and North America, and only endemic species of this genus in Turkey is *Thermopsis turcica* and named by Turkish botanists in 1983 [12].

The uniform occurrence of at least two free carpellary ovaries of *T. turcica* is the first record in the subfamily Papilionoideae (=Faboideae) of Fabaceae [13–15]. *T. turcica* (Fabaceae) is found at very low population numbers and is naturally distributed in a very narrow area located around Konya, Afyonkarahisar, Eber and Aksehir lakes and their surroundings (Figure 2.1). It has been classified as a critically endangered (CR) plant in "Red Data Books of Turkish plant" and is taken under conservation [12,14,16]. However, due to unidentified seed predators that utilize *T. turcica* seeds for larval development and excessive agricultural practices in plant's habitat, virtually all populations of this important rare plant species, *T. turcica*, are under serious threat [17].

The conservation of this threatened unusual plant species is being co-ordinated by Nezahat Gokyigit Botanical Garden, a few universities in Turkey and Turkish Ministry Forestry and Water Affairs. The taxonomic hierarchy of this species has been revised and is as follows:

Kingdom: Plantae
Subkingdom: Tracheobionta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Rosidae
Order: Fabales
Family: Fabaceae
Genus: *Thermopsis*
Species: *Thermopsis turcica*, Kit Tan et al. [18,19]

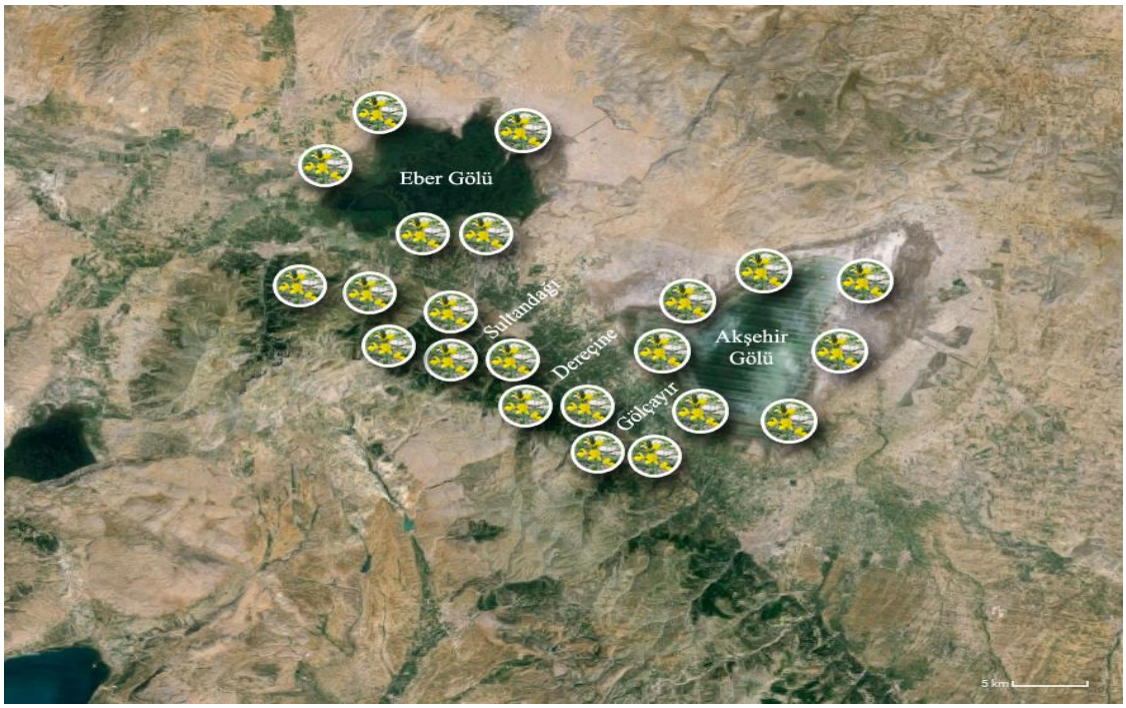

Figure 2.1. Distribution of *T. turcica* over Turkey (around Eber and Aksehir lake)

T. turcica possesses hermaphrodite golden yellow flowers and a 2-3 seeded legume fruit [13]. It is perennial with a long rhizome and ten free stamens [12,13,16]. The pollination period of *T. turcica* is from May to June in Turkey [11].

The plants of *T. turcica* initiate flowers after four years from the time of planting when grown from seed and two years when grown from rhizomes [11,13,20]. Furthermore, the main agricultural trait of *T. turcica* is having a gynoecium of 2-4 free carpellate-ovaries, each containing ten ovules (Figure 2.2). *T. turcica* is commonly known as 'aci piyan, Eber sarisi, and sari meyan' among locals [11].

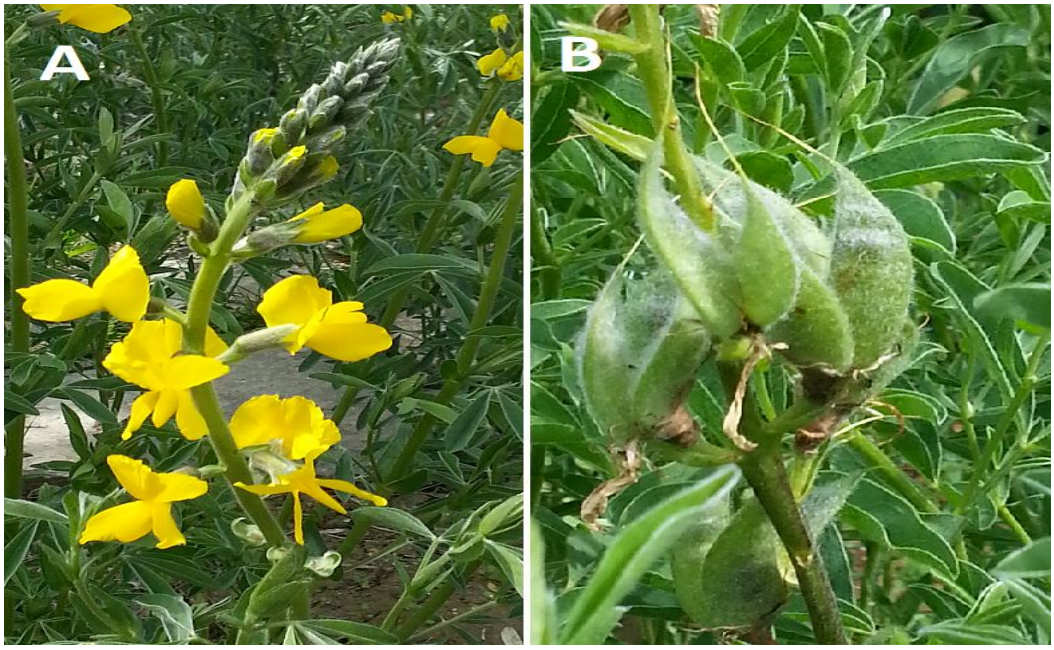

Figure 2.2. (A) General view of *T. turcica*'s flowers, (B) General view of *T. turcica*'s fruits

The distinguishing features of *T. turcica* are described in Table 2.1

Table 2.1. Particular characteristics of *T. turcica* [11,13,21]

	Thermopsis turcica
Habit	Perennial herbaceous
Flower colour	Yellow
Flowering period	May-June
Fruit time	July-August
Fruit type	Legume
Perfume	Not-scented

In 2009, the samples from Aksehir and Eber populations were collected and were preserved at Istanbul Ali Nihat Gokyigit Foundation Nezahat Gokyigit Botanical Garden (ANG Foundation NGBG). Since *T. turcica* is a critically endangered plant species and for the efficient conduct of the project, the study was carried out on Aksehir and Eber populations that are still in NGBG. The workers of NGBG have been growing two populations of *T. turcica*, Eber, and Aksehir. In this thesis, these two populations collected from Eber lake and its surroundings and from Aksehir lake and its surroundings are referred to as Eber and Aksehir populations, respectively.

2.2. Literature Review

The current literature covering subject of this doctorate thesis is as follows:

2.2.1. To date Conducted Studies Related to *Thermopsis turcica*

According to literature review regarding with *T. turcica*, no research was conducted at molecular level studying the multicarpellate feature of *T. turcica*, although this is a valuable agronomic character. Otherwise few but highly important studies were carried out on *T. turcica* regarding with its micropropagation [14,15,21–25], ecology [26,27], morphology [13,26–29], mineral and dry matter content [26,30], alkaloid content [31–33], antimicrobial activity [34–37], phylogenetic relationship of *T. turcica* [38,39] and self-compatible status [40].

2.2.2. Aims of the Study

Because of the appearance of a 2-3 free carpellate-ovary (Figure 2.3), *T. turcica* has a valuable character in the breeding of legumes.

Figure 2.3. The appearance of a 2-3 free carpellate-ovary showed in the black circles

The research was planned with following major aims:

- The primary objective of Chapter 1 was to investigate the possibility gene transfer from *T. turcica* to another species within the same family, Fabaceae, by classical hybridization. In addition, *T. turcica* was used as a recipient in hybridization combinations to search if *T. turcica* may be open-pollinated. Furthermore, cross compatibility, that is to say possibility of cross pollination of species beloning to a same or different group, between two populations of *T. turcica* and *V. faba* was searched in this dissertation. To date, no study has been conducted on fertilization biology of *T. turcica*. The present dissertation constitutes the first study on this issue.

- The second aim of this chapter was to addresses another area that has received little attention in the literature: setting the *in vitro* propagation of *T. turcica.* Effective regeneration protocol is necessary to preserve this Turkish endemic, rare plant species *in vitro*. In addition, we aim to provide the maintenance of this endemic, rare plant species, *T. turcica*, by *in vitro* tissue culture techniques as embryo culture. Embryo culture technique was also searched for this species for the first time in this research.

2.3. Intergeneric Hybridization of *Thermopsis turcica* and *Vicia faba*

2.3.1. Introduction

Fabaceae family constitute the majority of the world's main food crops. Increased yield in legume crops can be achieved by understanding the multicarpellary mechanisms [5,41]. To produce the plants having more than two free carpellary ovary is a major purpose in crosses of *T. turcica. T. turcica* is a valuable source for the attainment of desirable traits in crop improvement since it has a gynoecium with 2-4 functional pistils per flower (Figure 2.4).

Figure 2.4. General view of the fruit pod of *T. turcica*

The trait that provides multicarpellary gynoecium in *T. turcica* would be interesting if transferred to other species in Fabaceae by intergeneric hybridization. Intergeneric hybridization is used to transfer of desirable traits from one species to another in a different genus. Although intergeneric hybridization could be a source for crop improvement, intergeneric reproduction requires more efforts to overcome pre- and post-fertilization barriers.

Crosses between species lead to an incompatibility that may occur before and/or after fertilization. Pre-fertilization barriers are the failure of pollen germination and of pollen tube growth, and of not passing to the micropyle and/or embryo sac of pollen, whereas postfertilization barriers consists of abnormal development of endosperm or embryo that is referred to as ‘somatoplastic sterility’ [42].

The majority of crop plants are readily capable of sexual reproduction. The members of Fabaceae are good examples such of sexual production of progeny. To gain the knowledge on the fertilization biology of *T. turcica*, the hybridization should be conducted. Selection of potential male and female parents is important to obtain hybrids in a crossing program. Crosses between *V. faba* and *T. turcica* were made and in the crossing combinations of *T. turcica* were used as a maternal and also a paternal parent to search its crossability.

V. faba is the world’s largest feed legume crop. This species is also known as faba bean, broad bean, field bean and is produced by 58 countries in North Africa, Southwest and South Asia, and Mediterranean. It is an annual forage herb characterized by white flowers with dark purple spots and also a biofactory of nitrogen by fixing 130 to 160 kg N/ha. Flowers are white with dark purple markings. Nearly 30% of the population of *V. faba* are cross-pollinated [43].

The wild progenitor of *V. faba* has 2n=14 chromosomes whereas cultivated ones possess a chromosome number of 2n=12. *T. turcica* has diploid 2n=18 chromosomes [13,43,44]. There is a distinct difference between *V. faba* and *T. turcica* in terms of their gynoecium structure. *T. turcica* has 2-4 free carpellary ovary whereas *V. faba* has a solitary gynoecium (Figure 2.5). For this reason, crossing between *T. turcica* and *V. faba* is of particular interest in increasing yield in leguminous crops. *T. turcica* has greater potential as a source of obtaining more yield per flower due to its multicarpellary feature.

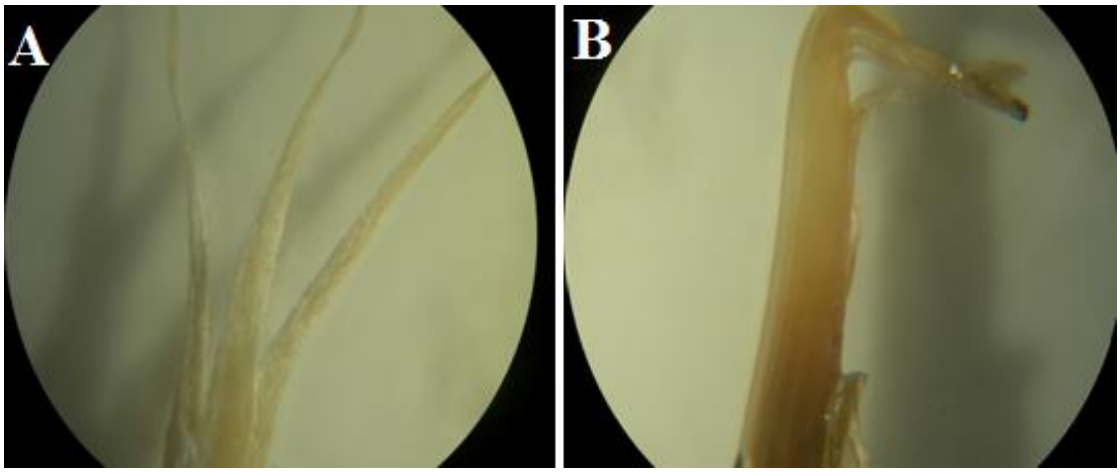

Figure 2.5. The appearance of the ovary of *T. turcica* (A) and *V. faba* (B)

In this chapter, whether embryo formation occurred in hybrid candidates crossed *T. turcica* and *V. faba* using histological techniques was searched. The development of hybrids is affected by the interactions among environmental, physiological, genetic and molecular events in the life cycle of an embryo.

2.3.2. Materials and Methods

2.3.2.1 Materials

2.3.2.1.1 Plant Material

In the present study, Eber and Aksehir populations of *T. turcica* and one *Vicia* genotype (*Vicia faba*) were used. Three species belong to the family of Fabaceae. For clearly understanding whether gene transfer is possible between *T. turcica* and *V. faba*, crosses were implemented with classic techniques. For this purpose, selected species and crossing combinations were determined in Table 2.2 and 2.3, respectively. A total of 500 pollinations were made between *T. turcica* and *V. faba*, including their reciprocals and free-pollinations of both populations of *T. turcica*.

Table 2.2. List of Selected Species

Number of Selected Species	Name of Selected Species
1	*T. turcica* (Eber Population)- rhizomes collected from Eber Lake by the workers of NGBG were used
2	*T. turcica* (Aksehir Population)- rhizomes collected from Aksehir Lake by the workers of NGBG were used
3	*V. faba*- non-hybrid seeds provided from breeders around Adana were used

Table 2.3. Combinations of Classical Hybridization

	♀		♂
1	*T. turcica* (Eber Population)	X	*T. turcica* (Eber Population)
2	*T. turcica (*Eber Population)	X	*T. turcica (*Aksehir Population)
3	*Vicia faba*	X	*T. turcica* (Eber Population)
4	*T. turcica* (Aksehir Population)	X	*T. turcica* (Aksehir Population)
5	*T. turcica* (Aksehir Population)	X	*T. turcica* (Eber Population)
6	*Vicia faba*	X	*T. turcica* (Aksehir Population)
7	*Vicia faba*	X	*Vicia faba*
8	*T. turcica* (Eber Population)	X	*Vicia faba*
9	*T. turcica* (Aksehir Population)	X	*Vicia faba*
10	*T. turcica (*Eber Population) (Free)		
11	*T. turcica (*Aksehir Population) (Free)		

2.3.2.1.2. Research Area

Plants were grown from seeds (*V. faba*) and rhizomes (two populations of *T. turcica*). The rhizomes were harvested by NGBG workers from around of Eber and Aksehir lakes at the end of August 2012 and kept at room temperature for planting. Just before the replanting, the rhizomes were dipped into CaO for a second to eliminate any pests.

Collected rhizomes of *T. turcica* were planted with the workers of Nezahat Gokyigit Botanical Garden in September 2012 and provided the seeds of *V. faba* sown in April 2013 to coordinate pollination period of both two plants, *T. turcica,* and *V. faba* (Figure 2.6). To protect the pollinated flowers from rain and other contaminants, planted area was covered with a plastic material (Figure 2.7).

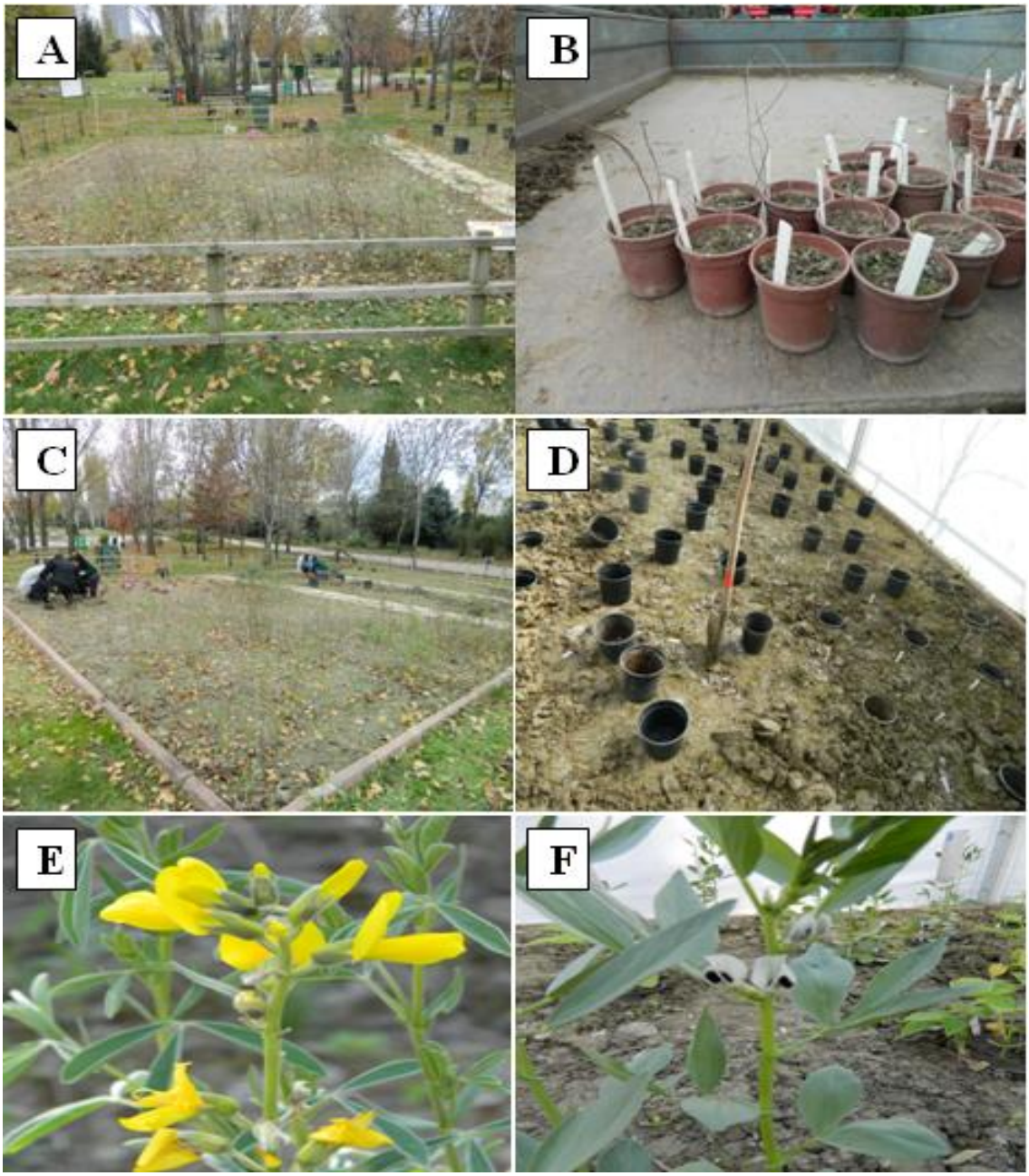

Figure 2.6. A: The area where classical hybridization study at NGBG was conducted; B: Rhizomes of *T. turcica* were collected for the present study by workers of NGBG from different villages near Konya in Turkey, C: Planting of collected *T. turcica* rhizomes in September 2012; D: Planted the seeds of *V. faba* in April 2013 to catch the same flowering time with *T. turcica* after covering the area; E and F: Flowers of *T. turcica* and *V. faba*, respectively

Figure 2.7. A: The area of planted rhizomes of *T. turcica* (Aksehir population) and seeds of *V. faba;* B: The area of planted rhizomes of *T. turcica* (Eber population) and seeds of *V. faba* at NGBG

2.3.2.1.3. Chemicals

All the chemicals used in this thesis are listed in Appendix A.

2.3.2.1.4. Equipment

All the equipments used in this thesis is listed in Appendix B.

2.3.2.1.5. Solutions

Acetocarmine solution (1%): 1 g carmine dye was dissolved in boiling 100 ml glacial acetic acid (45%), cooled rapidly, and then taken into a dark bottle. Stored at room temperature.

FPA-70: For 1 L, 900 ml Ethanol-70%, 50 ml Formaldehyde, 50 ml Propionic acid were mixed and stored at +4°C.

8N NaOH: For 100 ml, 32 g NaOH was dissolved and stored at room temperature.

Aniline Blue Dye: 0.1% aniline blue was dissolved in 0.1N K_3PO_4 and stored at +4°C. During the usage, the solution was diluted 1:3 with ddH_2O.

Johansen solutions:

Table 2.4 The conten of Johansen solutions

Solutions	Constituents		
	Distilled water (ml)	Ethyl alcohol (96%)	Tertiary butyl alcohol (TBA) (ml)
Johansen-1 (70%)	300	500	200
Johansen -2 (85%)	150	500	350
Johansen -3 (95%)	-	450	550
Johansen -4 (100%)	-	200	800

Haematoxylin stain:

Table 2.5. The content of Haematoxylin stain

Constituents	
Haematoxylin	1 g
Ethyl alcohol	6 ml
Potassium alum	12,5 g
KMnO4	0, 18 g
Glycerin	25 ml
Methanol	25 ml
Distilled water	100 ml

Haematoxylin is solved in ethyl alcohol. Potassium alum is dissolved in distilled water, and then $KMnO_4$ is added. Other reagents are added in the order given. The prepared solution is covered with a loose cotton wool and exposed to light for 4 to 6 weeks to enable its oxidation or ripening.

2.3.2.2 Methods

2.3.2.2.1 Pollinations and morphological analysis

For crossing program, selfing, reciprocal crosses, and crosses among plants from two populations of *T. turcica* (Eber and Aksehir) and *V. faba* were done at Nezahat Gokyigit Botanical Garden during pollination period of May and June, 2013. The area of planting covers 30 m^2. In this area, 31 individual plants of Eber population and 52 individual plants of Aksehir populations were planted. In addition, in the same area, 600 seeds from each of *V. faba* and *Phaseolus vulgaris* were sown. Due to less flowering of *T. turcica* and the necessity of much more labor force, cross-hybridization was implemented mostly between *V. faba* and *T. turcica*. No data is presented on *P. vulgaris* in this dissertation. Pistil samples were collected from 1 to 10 days after the pollination without damaging the population after hybridization.

Pollinations:

Pollination and histological analysis procedures were carried out as described in the previous study [45]. All pollinations were implemented using the fresh pollen. For obtaining fresh pollen, at least 60 flowers were collected just at preanthesis of each population (Eber and Aksehir populations) and *V. faba*, and their petals and pistils were removed (Figure 2.8 and 2.9).

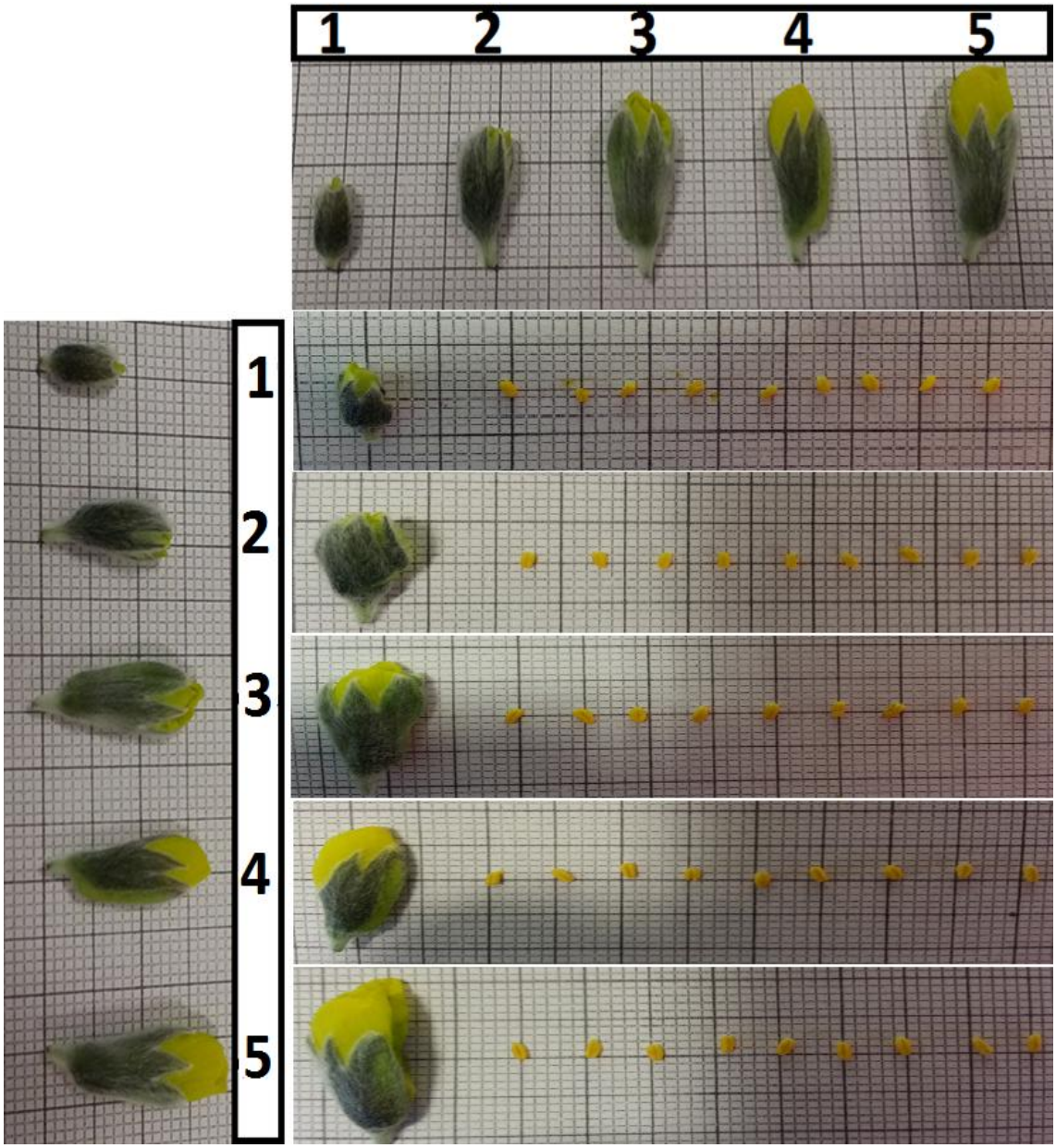

Figure 2.8. Insufficient pollen level for pollination studies

Figure 2.9. Flower buds described in number 3, 4 and 5 were used for pollinations

For hybridization study, the age of female and donor parents are critical criteria for successful hybridization. A female organ must be receptive (Figure 2.10).

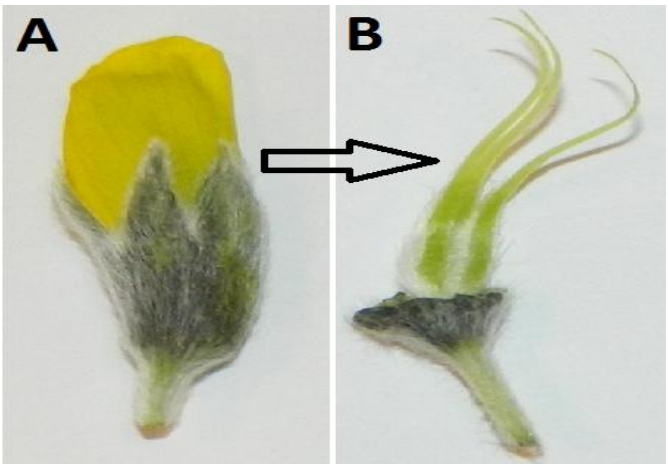

Figure 2.10. Selected development age of a bud used as a female parent for hybridization; A: bud, B: receptive pistil

The anthers were left to dehisce for overnight at room temperature of about 24°C. Fresh pollen was used for pollination. Pollen viability was tested just before pollination via colorimetric test, that is, acetocarmine. To test the pollen viability different tests such as pollen germination test and colorimetric test can be used, but colorimetric test is much better than the other since it is easier, faster and the effects of some external factors like environmental conditions including temperature, humidity, and light are minimized and media usage is sufficient [46–48].

Removed anthers were placed on a glass slide. With a proper forcep, pollen grains were pinched from the anthers. Each slide was stained with a solution of acetocarmine for 7 min. Slides were analyzed under a fluorescence microscope, and pollens with bold red color were accepted as viable and useful for pollination (Figure 2.11). The shape of the pollen differs in *T. turcica* and *V. faba*. The pollen of *T. turcica* is medium size, noticeably smaller than *V. faba* type pollen, circular, and pollens are linked up by corpus extensions, however, pollen shape of *V. faba* is prolate and the pollen is large (Figure 2.12).

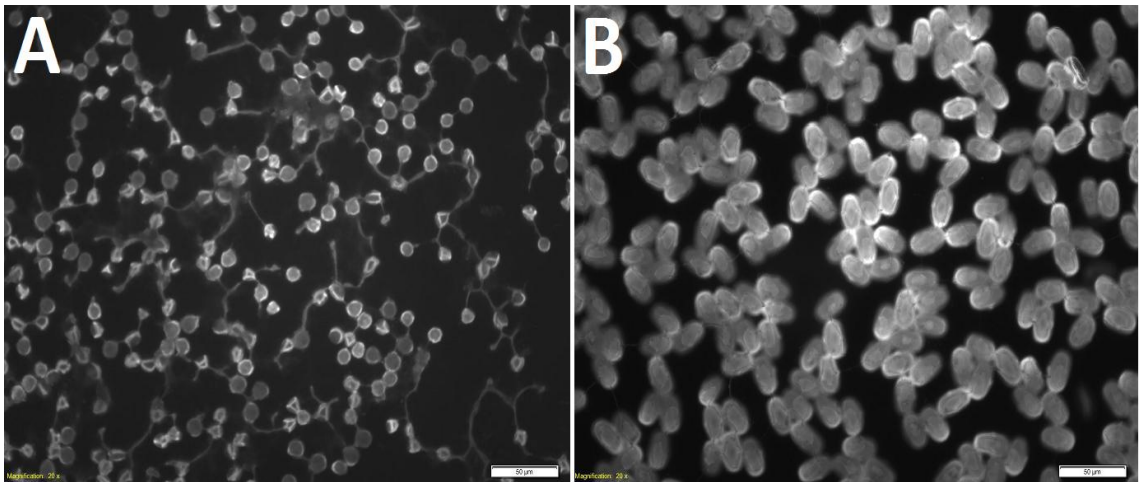

Figure 2.11. General observation of pollens (x20) belongs to A: *T. turcica* and B: *V. faba* under a fluorescence microscopy using acetocarmine (taken photo as black and white; ocular measurement is 50 micrometer (µm))

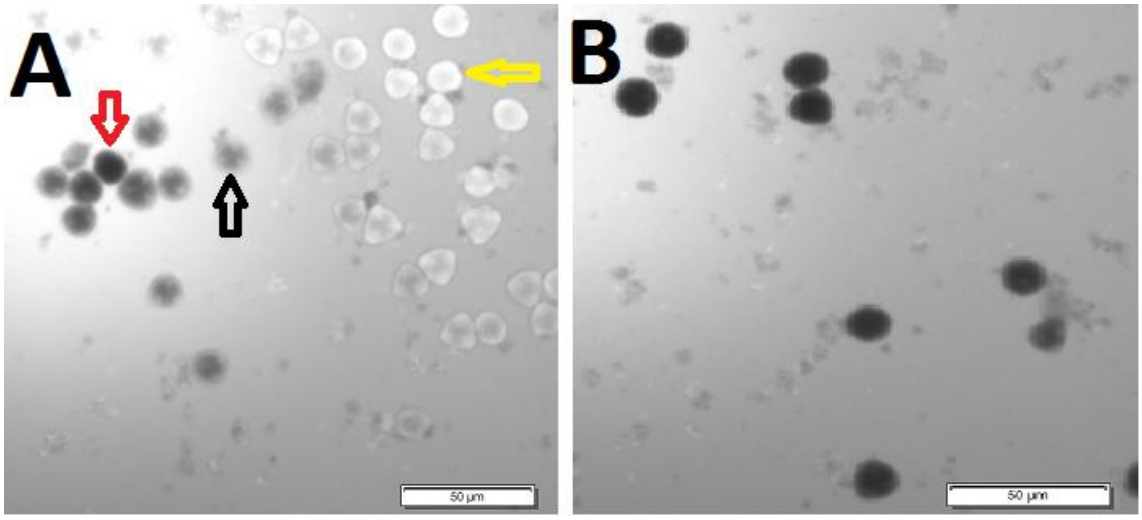

Figure 2.12. Testing of pollen viability according to staining level by colorimetric analysis using acetocarmine dye, A: Trinucleate pollens (x20) of *T. turcica* (arrows: red arrow shows viable pollen in bold red colour, yellow arrow shows nonviable pollen in colourless, black arrow shows moribund pollens; B: Selected pollens (x20) with bold red colour for pollination (taken photo as black and white; ocular measurement is 50 micrometer (µm))

One day before anthesis, 300 flowers of *V. faba* and 300 flowers of each population of *T. turcica* were emasculated at balloon stage in the Nezahat Gokyigit Botanical Garden of Istanbul, hand pollinated (100 flowers per treatment) using a small paint brush and covered with a tracing paper (Figure 2.13). To prevent accidental pollination, separate brushes were used for each pollination.

After hand pollination, pistils were covered with a cotton bag until 10 days after pollination (DAP) and subsequently the cotton bag was removed to aerate the pistils. Hybridization studies were conducted at Nezahat Gokyigit Botanical Garden (NGBG) and in a greenhouse at Sabanci University (Figure 2.14).

Figure 2.13. (A) After hand-pollination, pistils were covered with a cotton bag; (B) After fertilization cotton bags were removed, and each experiment was labeled

Figure 2.14. Plant samples in greenhouse at Sabanci University

T. turcica is a self-pollinated species. It means that stigma has strong pollen receptivity before pollen is released from a male parent in the same inflorescence. Thus, emasculation is required in hybridization study to prevent self-fertilization by pollen. 100 flowers per treatment were either self-pollinated or cross-pollinated in all possible combinations as seen in Table 2.3.

2.3.2.2.1.1. Results and Discussion

After hybridization of *T. turcica* with *V. faba* and *P. vulgaris* at NGBG, pods at 10th day of pollination were collected and directly taken to the laboratory for in ovule-embryo culture. All pollination samples conducted with *P. vulgaris* were burnt (Figure 2.15). Therefore there is no valuable data for this species. Heat condition in the field could affect the development of pistil of *P. vulgaris*. However, at least three samples from each combination conducted between *T. turcica* and *V. faba* were collected and fixed in FPA-70 solution. Collected samples were stored at +4 °C until histological analysis.

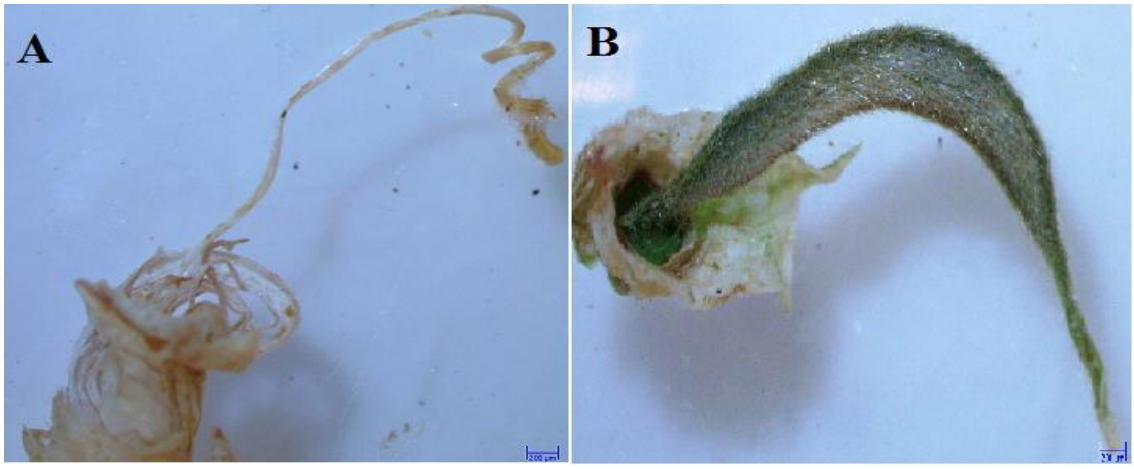

Figure 2.15. Burned (A) and undeveloped pistil (B) samples of *P. vulgaris* (arrow: 200 µm; magnification: x20)

In all cross-pollinated *T. turcica* (♀) X *V. faba* (♂) samples at 5th day of pollination, pistil development was observed, however, all pistils about 10 DAP started to die (Figure 2.16 and 2.18). Pistils became dry within ten days after pollination.

Based on this result, it is stated that *T. turcica* is self-pollinated. Moreover, in all self-pollinated *T. turcica* samples, pistils developed, and ovules formed. Self-pollinated *T. turcica* samples could be easily recognized from intergeneric crossed samples 10 DAP (Figure 2.17 and 2.19).

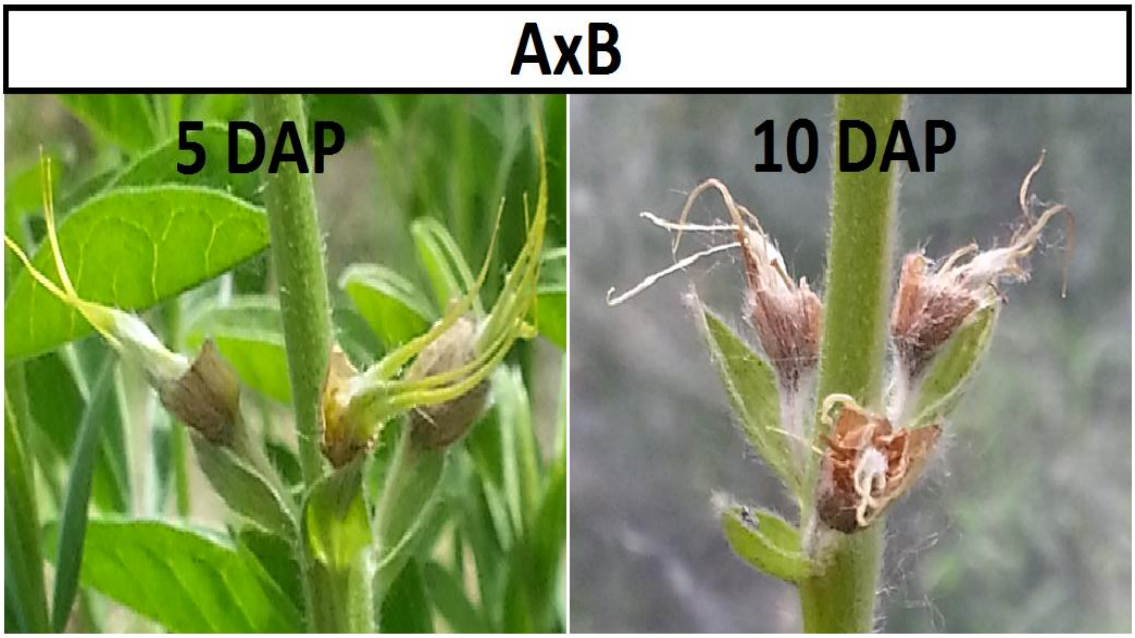

Figure 2.16. Cross-pollinated *T. turcica* (A: *T. turcica*-Aksehir population; B: *V. faba*; DAP: Day After Pollination)

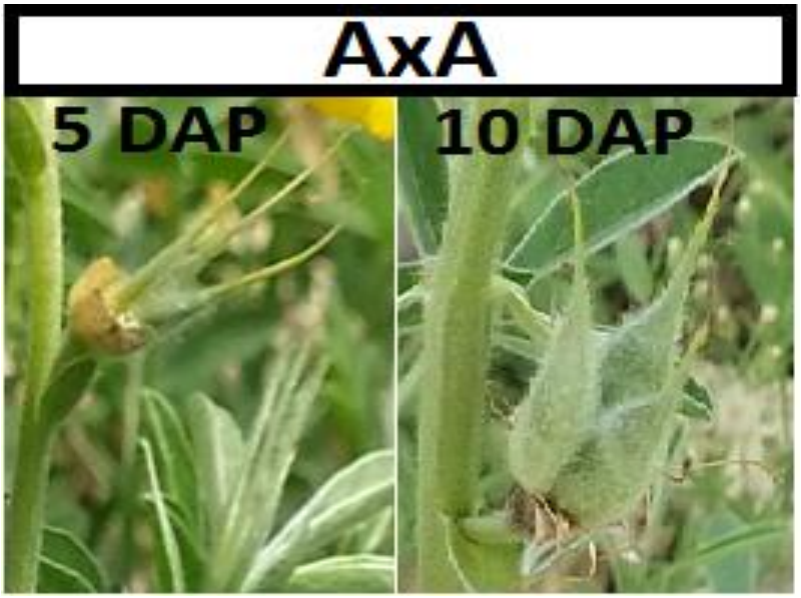

Figure 2.17. Self-pollinated *T. turcica* (A: Aksehir population; DAP: Day After Pollination)

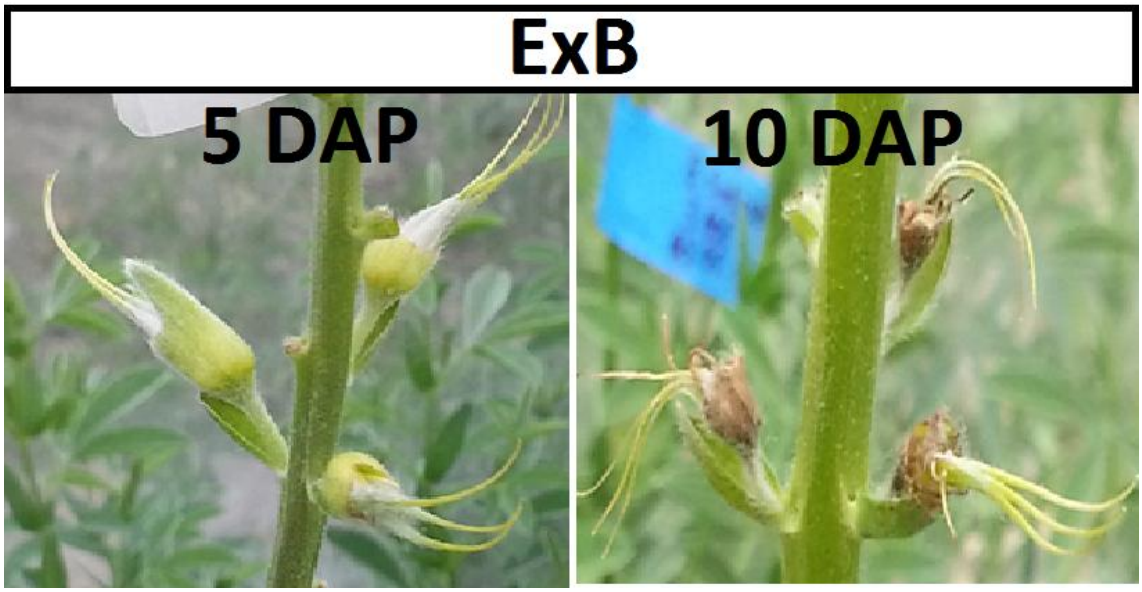

Figure 2.18. Cross-pollinated *T. turcica* (E: *T. turcica*-Eber population; B: *V. faba*; DAP: Day After Pollination)

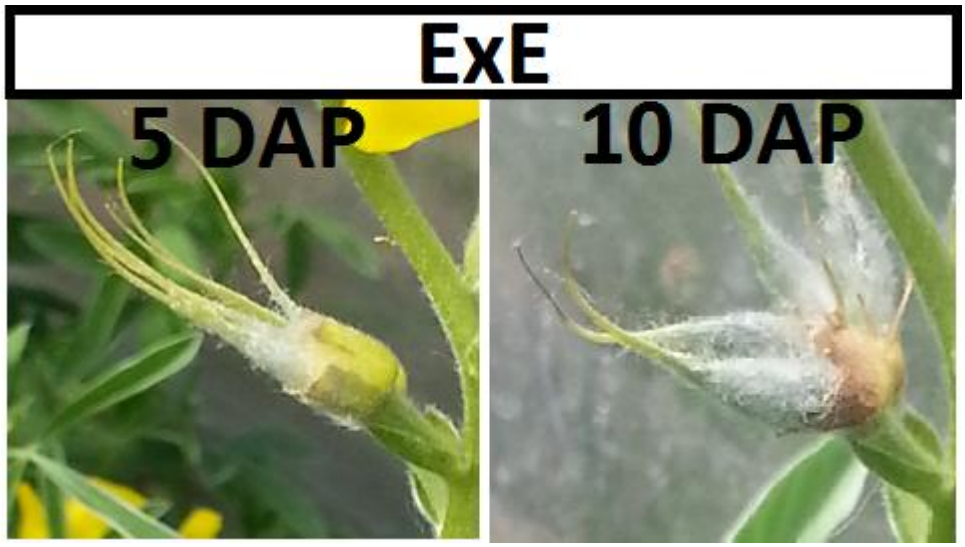

Figure 2.19. Self-pollinated *T. turcica* (E: Eber population; DAP: Day After Pollination)

Low pollen germination, low stigma receptivity and crossing direction may affect the fertilisation although a large quantity of pollen of *V. faba* was applied to the stigmas of *T. turcica* and for these reasons pistils may maintain their liveliness until 10 days after pollination. As shown in Figure 2.16 and 2.18, in the crosses that *T. turcica* used as a recipient, hybridization is not fruitful and incompatible. As a result, the effect of genotype on intergeneric crosses is important concerning hybridization achievement.

The effect of genotype on interspecific and intergeneric hybridization has been reported in a number of studies [49–51]; For instance, in the crosses between *Phaseolus vulgaris* and *Phaseolus polyanthus*, the use of *P. polyanthus* as the maternal parent has hardly given embryos, but in the reciprocal cross embryo formation was observed. In addition, the change of crossing direction may affect the development of hybrid embryo, since the interaction between embryo sac and maternal tissue and the cytoplasmic genes in the endosperm and embryo can change by the reversal of crossing direction. Furthermore, incompatibility between hybrid embryo and endosperm may be another reason. In self-pollinations, ovules formed mature seeds 10 days after pollination. It was observed that fertilization was successful, and the pod developed quickly (Figure 2.17 and 2.19).

To test if *T. turcica* can fertilize with any other pollen coming from the environment by air or by insects, pistils were left free of pollination. Free pollinated samples of *T. turcica* at the fifth day of pollination became wrinkled and started to die (Figure 2.20). Stigmas may lost their pollen receptivity and then their life. This result is also an evidence to explain experimentally that *T. turcica* is a self-pollinated species. Furthermore, if ovule fertilization occurred, the cause of not developing embryo in *T. turcica* X *V. faba* crosses may be due to the degeneration of endosperm.

Figure 2.20. Free pollinated samples at fifth day of pollination

2.3.2.2.2. Histological Analysis in Pollinated Samples

Six pistils from each of self- and cross-pollinated species were collected from first to the tenth day of pollination to establish pollination possibilities. All pistils were fixed in FPA-70 solution and stored at +4 °C until microscopic observations. The histological analysis was carried out according to previous studies [45,47].

The primary focus was to analyze pollen tube growth in all pollinated samples. Once this goal was achieved, ovule fertilization analyzes were conducted. Moreover, to determine embryo formation, paraffin block analysis was implemented. During the histological analysis of paraffin step, all samples were stained with hematoxylin and then observed under a fluorescent microscope.

2.3.2.2.3. Fluorescence Microscopy Analysis

For microscope observation of pollen tube growth, pistils 1, 2 and 3 DAP fixed in FPA-70 solution were washed with tap water for 24h and then taken into 8N NaOH solution for 3 hours. Pistils were washed under tap water to remove NaOH from the softened tissue for 24h and stained with aniline blue [45,47]. After staining, pistil samples were cut into two parts (stigma + style and ovary) and were further cut longitudinally, split into two parts. Pollen tube growth was monitored. Cutting samples were observed under a fluorescence microscope (Olympus BX51-DP72).

To determine embryo formation, the paraffin block analysis was implemented.

<u>Paraffin Block analysis;</u>

Fixed tissues into FPA-70 solution were processed as follows:

1. Samples were taken at 70%, 85%, 95% and 100% ethanol for 3 hours, respectively,

2. After ethanol bath, the surface air of the samples was removed in a desiccator,
3. After vacuum operation, samples were incubated in Tertiary Butyl Alcohol (TBA) solution at 25-30^{o}C for overnight. This step was consecutively repeated three times,
4. Samples were taken into molten paraffin wax and kept at 55°C for three days.
5. Samples were transferred to cold plate and kept for 30 minutes for solidification of the paraffin,
6. Samples and paraffin were attached to the wood cassettes to form a block for sectioning step,
7. Samples were sectioned using a microtome and cut 10 µM sections,
8. 10 µM sections were placed on a slide with a forcep and a paint brush,
9. Slides were put in a heat block at 37°C for 2 days,
10. Slides were washed twice with xylol and isopropyl solutions for 10 and 5 minutes, respectively,
11. Slides were taken into %70, %40, %20 alcohol dilutions and lastly ddH_2O for 3 minutes, respectively,
12. Slides were taken in a slide rack and stained with hematoxylin dye. Stained samples were washed with fresh ddH_2O for 15 min. Then, slide rack taken into 20%, 40%, 70% and 96% alcohol bath for 3 minutes, placed in isopropyl twice for 5 min, lastly kept in xylol for 5 min,
13. Slides were removed from rack and dried on a paper towel. After slides dried completely, 3 drops of entellan were put onto the slides and covered with a small glass slides. All slides were kept in an incubator at 30°C for 3 days and slides were then examined under the fluorescence microscope.

2.3.2.2.4. Results and Discussion

To identify pollen tube growth in reciprocal, self- and cross-pollination samples were analyzed in all combinations as shown in Table 2.3.

It is observed that in all pollinated samples 1 day after pollination, pollen tubes reached the ovary followed by pollen germination (Figure 2.21, 2.22, and 2.23). It is known that pollen grains can germinate on the style or ovary wall instead of stigma [52], but in the present study when the reciprocal crosses were implemented using two populations of *T. turcica*, pollen grains of *T. turcica* germinated on the stigma on the first day of pollination. In addition, pollen grains produced thick pollen tubes, and when the tubes penetrated into style, the intensity of fluorescence did not change and, for this reason, pollen tubes could be observed clearly until they reached the ovary. In some species pollen tubes are filled with callose, which is a plant polysaccharide, thus pollen tubes are visible [46,52]. As a result of pollen tube growth study in this dissertation, it was observed that some extended and/or twisted pollen tubes grew down to ovary, as well. Furthermore, as a consequence of the present hybridization study there was no problem concerning pollen-pistil interaction. Pollen tubes entered the ovary without any problem.

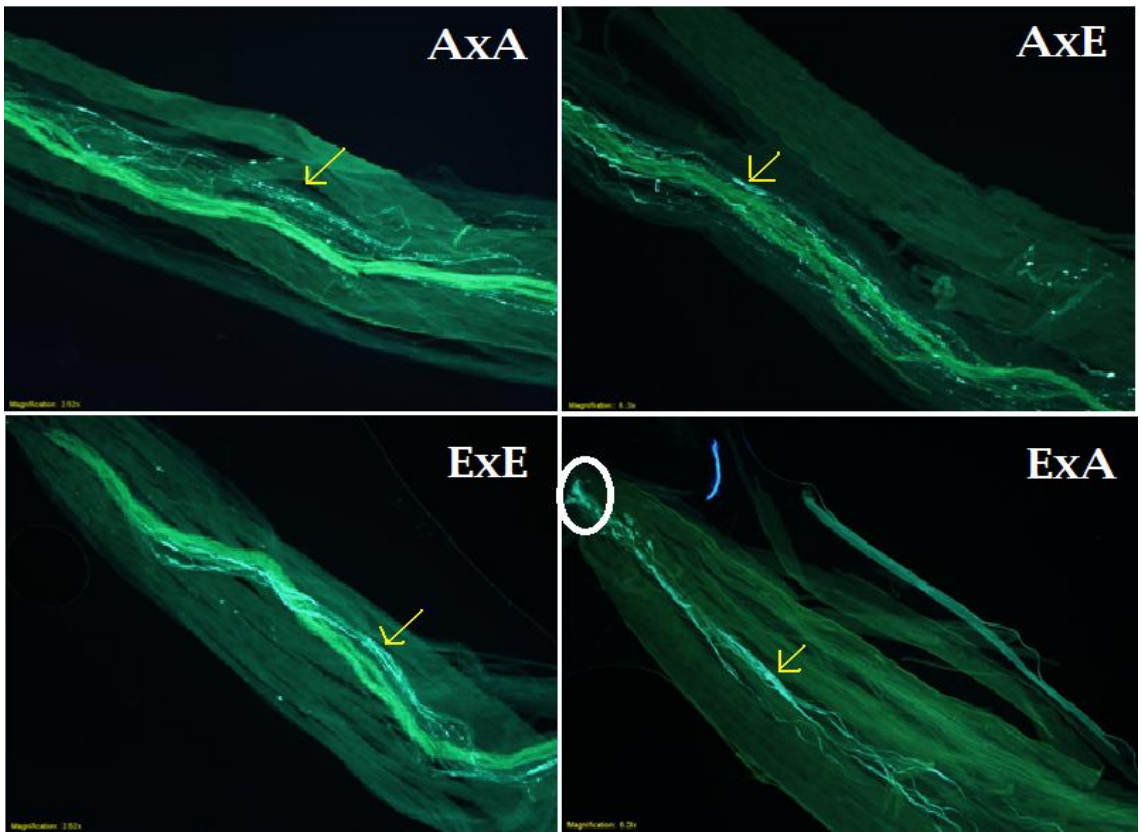

Figure 2.21. In all self- and reciprocal-pollinated *T. turcica* samples of 1 DAP, pollen tube growth was observed (A: *T. turcica*-Aksehir population; E: *T. turcica*-Eber population; arrows: pollen tube; circle: pollen germination) (Magnification: 6.3x); Filter: UMVIBA3; Dichronic: 505; Emitter: 510-550; Exciter: 460-495

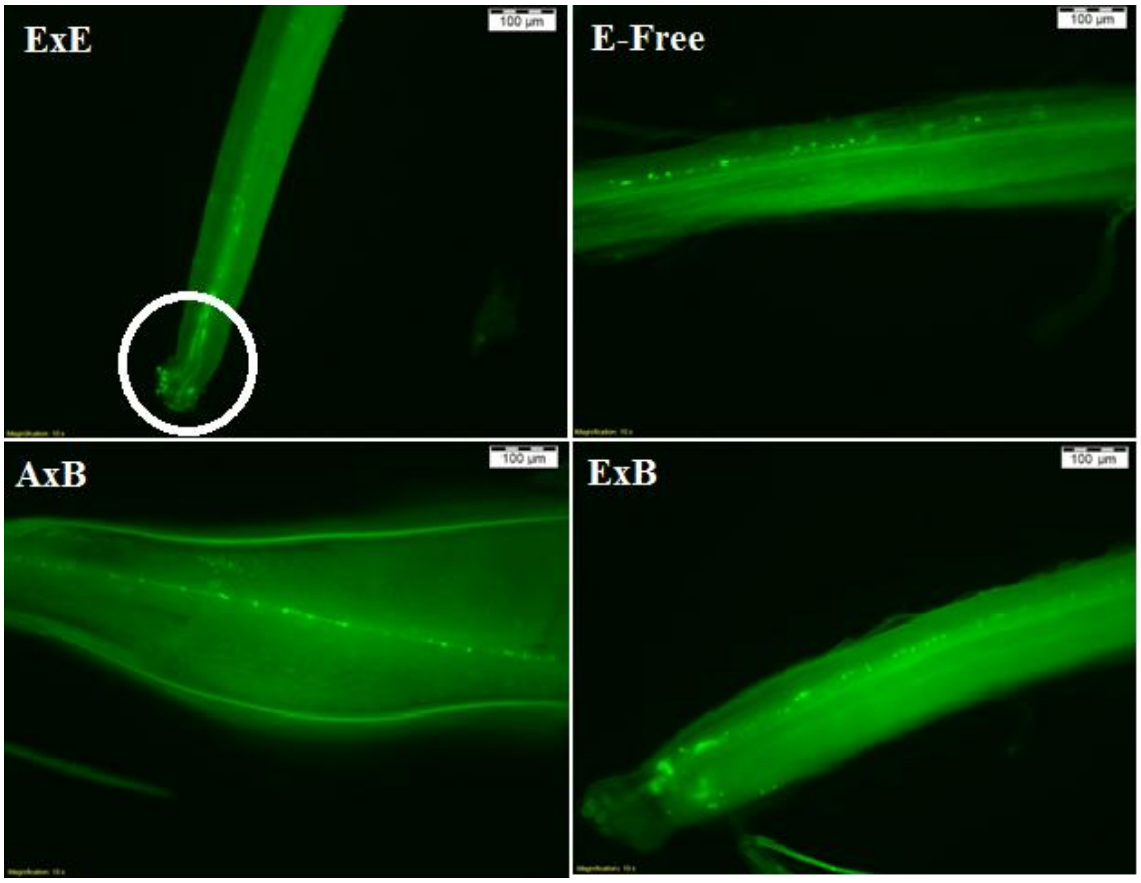

Figure 2.22. In all 1 DAP samples (self- cross- and free-pollinated) pollen tube growth was observed (A: *T. turcica*-Aksehir population; E: *T. turcica*-Eber population; B: *V. faba*) (Magnification: 10x; circle: pollen germination; measurement: 100 µm); Filter: UMVIBA3; Dichronic: 505; Emitter: 510-550; Exciter: 460-495

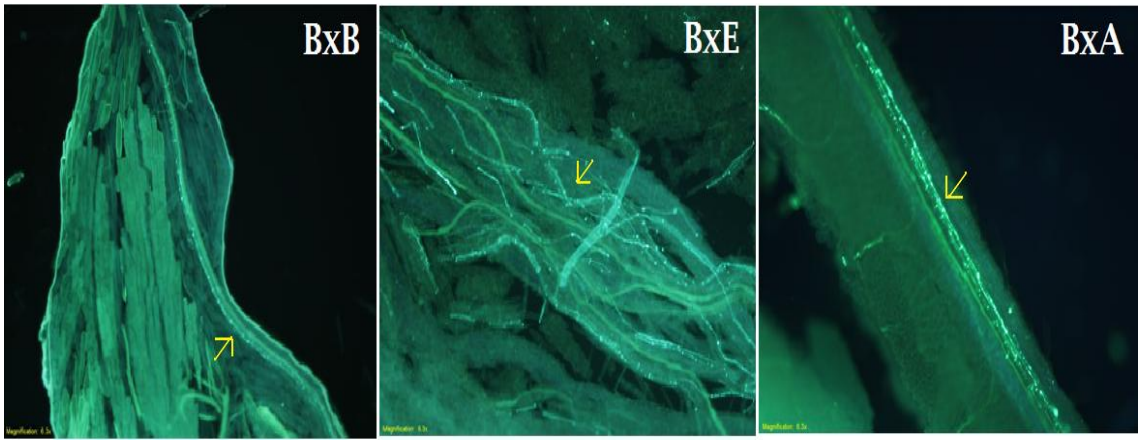

Figure 2.23. In all 1 DAP samples, pollen tube growth was observed (B: *V. faba*; A: *T. turcica*-Aksehir population; E: *T. turcica*-Eber population; arrows: Pollen tube) (Magnification: 6.3x); Filter: UMVIBA3; Dichronic: 505; Emitter: 510-550; Exciter: 460-495

In all samples 4 DAP of *T. turcica* (all self- and reciprocal pollinated samples), ovule fertilization was observed (Figure 2.24). By virtue of fertilization observations in the samples of *T. turcica* in which reciprocal crosses were made, the pollen tube penetrated into embryo sac from micropylar opening, hence the pollination type in *T. turcica* is considered as 'porogamy' [53,54].

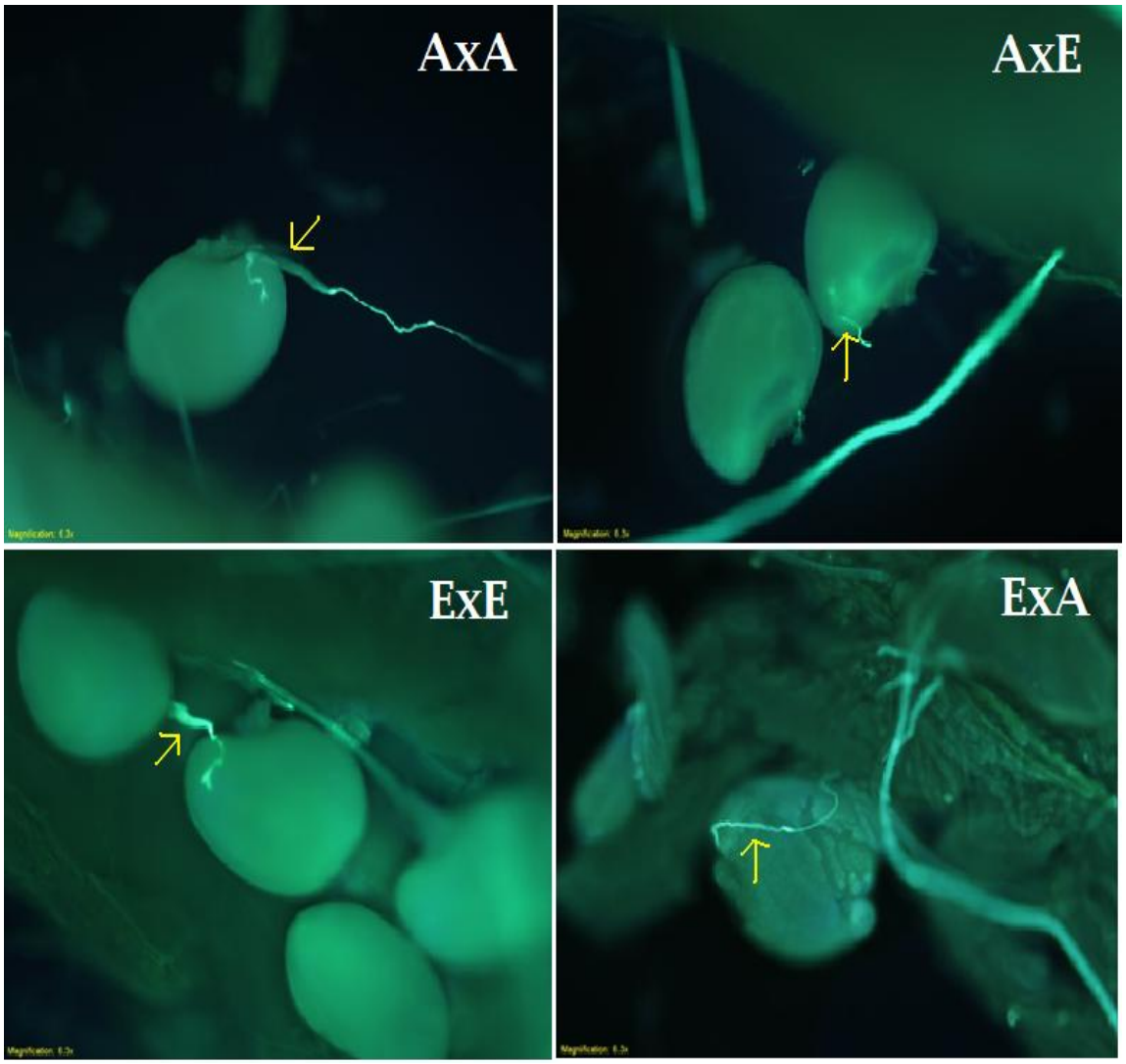

Figure 2.24. In all 4 DAP samples in *T. turcica* ovule fertilization was observed (A: Aksehir population of *T. turcica*; E: Eber population of *T. turcica*; arrows: pollen introduction to ovule) (Magnification: 6.3x); Filter: UMVIBA3; Dichronic: 505; Emitter: 510-550; Exciter: 460-495

On the other hand, although pollen germination and pollen tube growth were observed in all samples (hybrid candidates and free pollinated samples), ovule fertilization was not captured in these samples.

However, ovule development was maintained in hybrid samples of 5 DAP (Figure 2.25-2.28) whereas it stopped in free-pollinated samples. Due to observing ovule development in cross-pollinated samples in which *V. faba* used as female, further analysis was conducted to determine embryo formation. During histological analysis, 8 N NaOH was applied to the samples to test ovule fertilization and for this reason tissues of *V. faba* soften much more than those of *T. turcica*. It is possible that ovule fertilization in the pistil sample of *V. faba* was not seized because of tissue fragmentation.

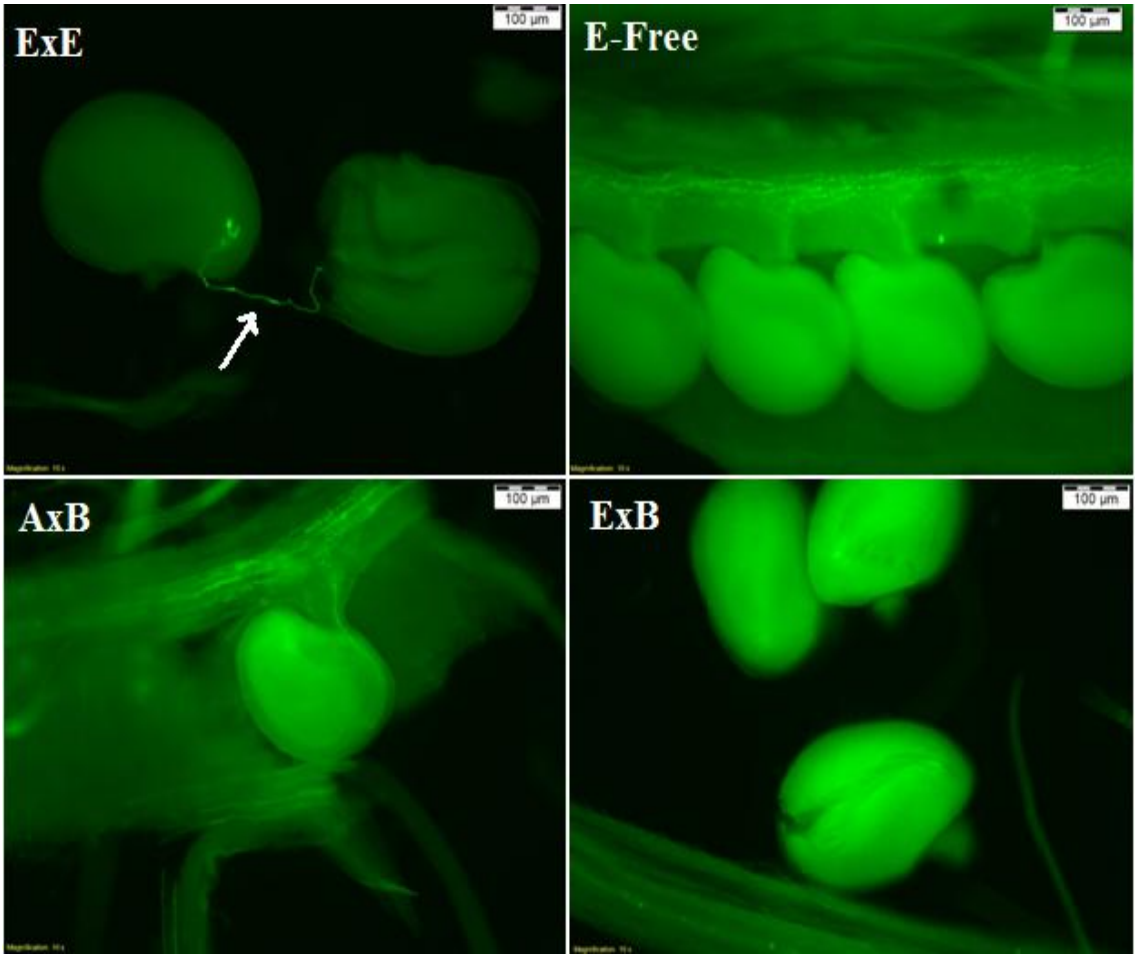

Figure 2.25. In self-pollinated samples in *T. turcica*, ovule fertilization was observed at the 4th day of pollination (A: Aksehir population of *T. turcica*; E: Eber population of *T. turcica*; B: *V. faba* arrows: pollen grain) (Magnification: 6.3x); Filter: UMVIBA3; Dichronic: 505; Emitter: 510-550; Exciter: 460-495

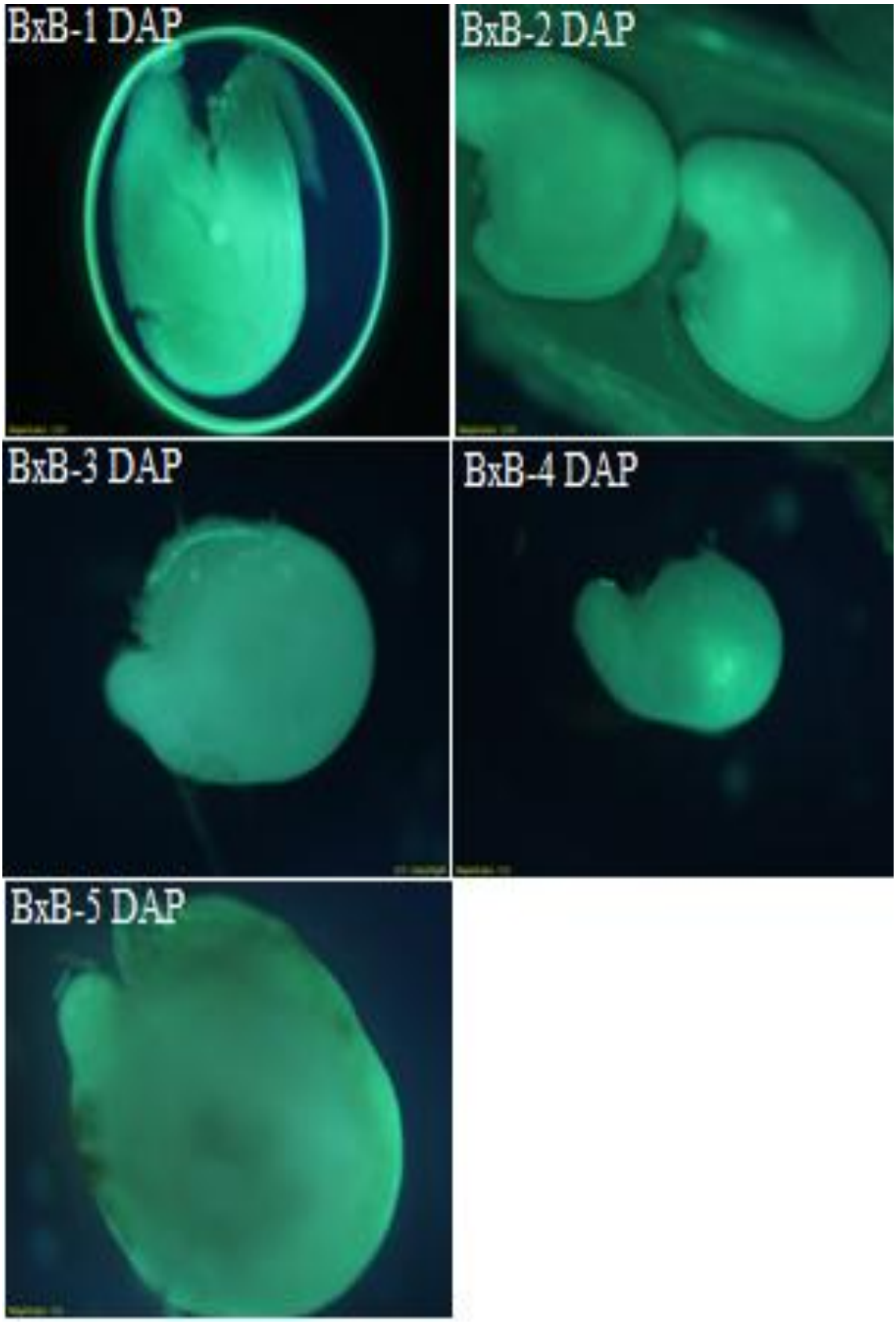

Figure 2.26. Self-pollinated *V. faba* (B: *Vicia faba;* DAP: Day After Pollination) (Magnification: 6.3x); Filter: UMVIBA3; Dichronic: 505; Emitter: 510-550; Exciter: 460-495

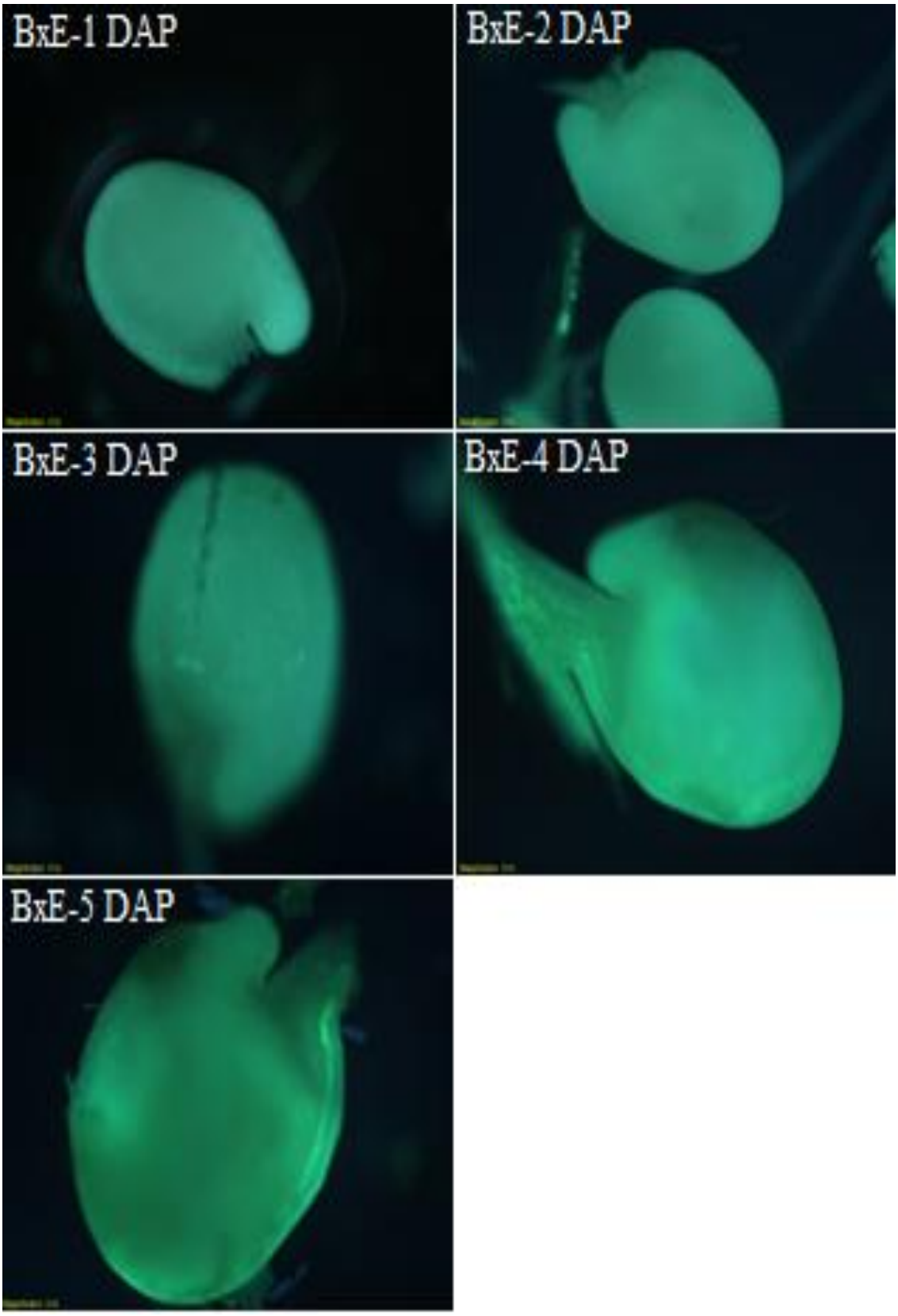

Figure 2.27. *V. faba* X *T. turcica* (B: *V. Faba*; E: Eber population of *T. turcica;* DAP: Day After Pollination) (Magnification: 6.3x); Filter: UMVIBA3; Dichronic: 505; Emitter: 510-550; Exciter: 460-495

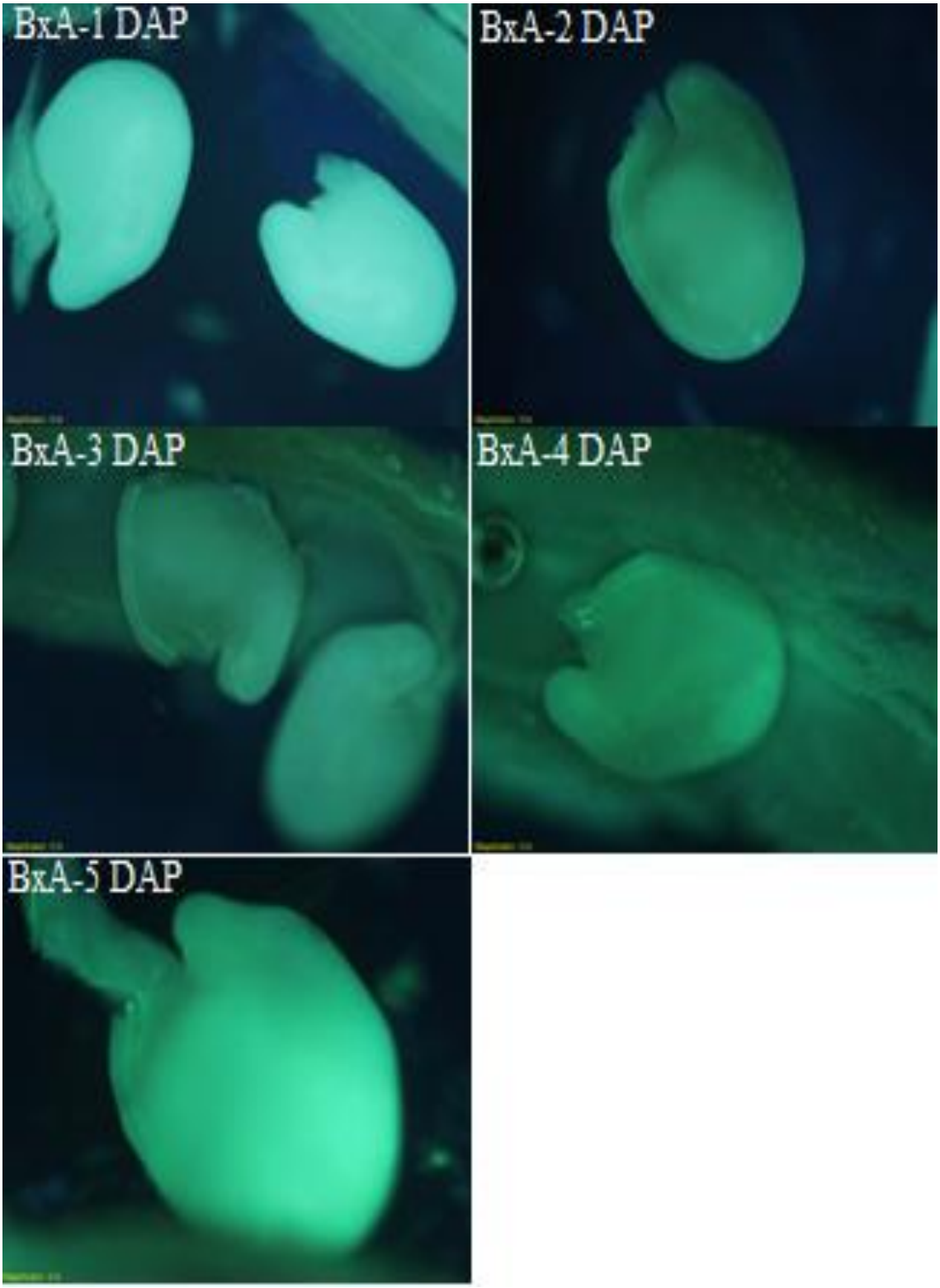

Figure 2.28. *V. faba* X *T. turcica* (B: *V. Faba*; A: Aksehir population of *T. turcica;* DAP: Day After Pollination) (Magnification: 6.3x); Filter: UMVIBA3; Dichronic: 505; Emitter: 510-550; Exciter: 460-495

As a result of the histological analysis, all samples of *T. turcica* have been clearly identified as self-compatible. In self-pollinations, ovules formed mature seeds after a month. In contrast, in intergeneric crosses, no seed formation was occured, but globular embryo formation was observed in these samples. Globular embryo formation was seen in all samples (self- and cross-pollinated samples of *T. turcica* (Figure 2.29) and *V. faba* (Figure 2.30)) at the eighth day of pollination. In addition, endosperm was seen in all ovules harvested from self-pollinated and crossed samples.

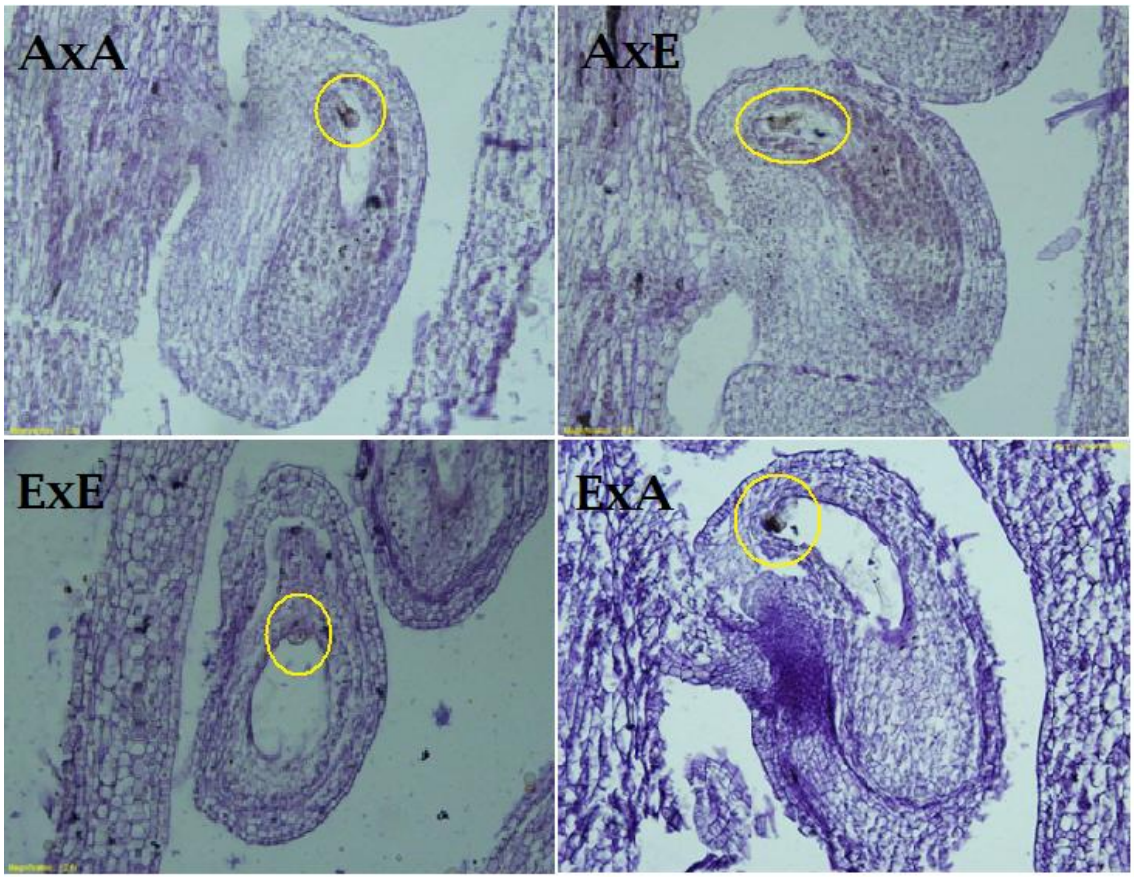

Figure 2.29. Globular embryo formation in all self- and cross-pollinated samples of *T. turcica* (A: Aksehir population of *T. turcica*; E: Eber population of *T. turcica*; circles: globular embryo) (Magnification: 12.6x); Filter: UMVIBA3; Dichronic: 505; Emitter: 510-550; Exciter: 460-495

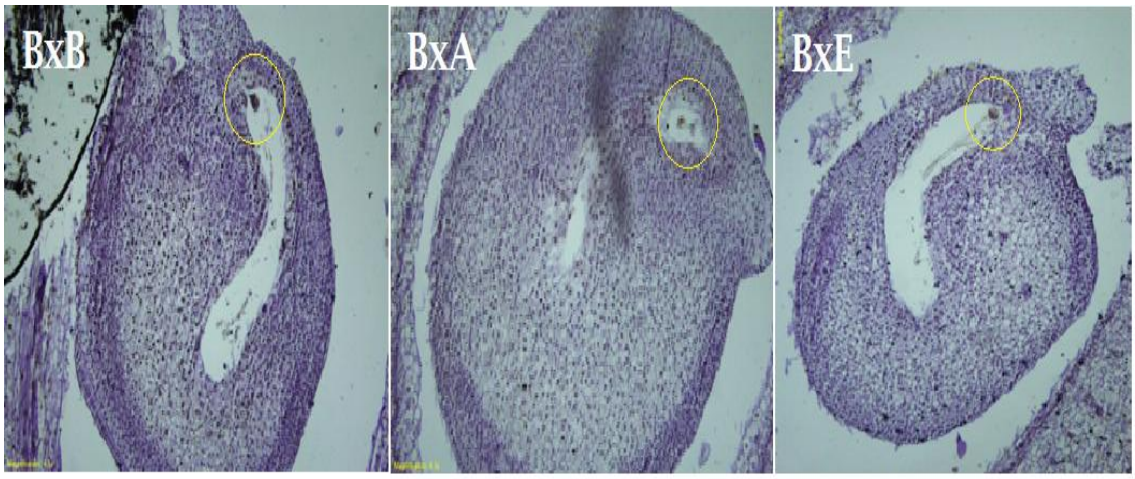

Figure 2.30. Globular embryo formation in self- and cross-pollinated samples of *V. faba* (B: *V. faba*; A: Aksehir population of *T. turcica*; E: Eber population of *T. turcica*; circles: globular embryo) (Magnification: 12.6x); Filter: UMVIBA3; Dichronic: 505; Emitter: 510-550; Exciter: 460-495

T. turcica and *V. faba* differ very distinctly both in morphological and genetic traits. *T. turcica* is yellow-flowered and endemic to Turkey; it is a perennial herbaceous species actively growing and flowering in spring. On the other hand, *V. faba* is the world's largest crop species; it is annual and has a white-pruple flower. Intergeneric crosses and self-pollinations were made using two populations of *T. turcica* and *V. faba*. From the data obtained from histological analysis, two major conclusions were drawn. First of all, Embryo formation was occured in the cross *V. faba* (♀) X *T. turcica* (♂) but not in *T. turcica* (♀) X *V. faba* (♂) crosses. In contrast, crossing in which *V. faba* used as donor and *T. turcica* used as the recipient, no embryo formation occured. Secondly, reciprocal crosses in two populations of *T. turcica*, Eber, and Aksehir, have led to viable progeny.

Observation of pollen germination, pollen tube growth, and ovule fertilization showed that pre-fertilization crossability barrier did not occur in intergeneric crosses of *V. faba* (♀) X *T. turcica* (♂). Based on these results, the barriers are of post-fertilization in nature. In addition, since successfull pollen germination and pollen tube growth was observed in the hybrids between *V. faba* and *T. turcica*, no surface specifity was seen in *V. faba* as declared in previous study [54,55]. Endosperm development was poor, and embryos could not develop beyond the globular stage. In addition, post-fertilization barriers can be brought down by applying plant growth regulators.

In spite of our efforts to synchronise the flowering times, the flowering seasons of *T. turcica* and *V. faba* were markedly different. Due to this reason, pollens of the samples have a very short lifetime. It was known from the previous studies that even when stored at 4°C *in vitro* pollen viability was reduced [50].

2.3.3. Conclusion

It seems likely that there will be an economic benefit in terms of increasing yield from producing hybrids of *V. faba* (♀) X *T. turcica* (♂). It was observed that the globular embryo was formed in hybrids obtained from crossing in which *T. turcica* was used as a donor. In contrast, in all crosses in which *T. turcica* was used as a maternal parent, the embryo was not obtained, and viability of the pistil did not exceed ten days after pollination. Also, in the samples left to free pollination, pistils died in a few days after anthers removed just before pre-anthesis. Overall, *T. turcica* can be accepted as 'cleistogamic'.

Since hybrids from the cross *V. faba* and *T. turcica* could be obtained from intergeneric crosses, crossing of *T. turcica* is feasible. Due to the fact that pollen tubes reached the ovary and fertilized it, pre-fertilization barrier characterized by incompatibility between pollen and pistil was not the major reason causing the failure of obtaining hybrid seed. Instead post-fertilization barriers such as endosperm failure, embryo abortion, and a rapid proliferation of endothelium are likely should be the main reasons for the failing of seed set of the cross *V. faba* and *T. turcica*. However, steps to enhance the obtaining hybrids and number of hybrids comprise embryo rescue, use of new recipients in hybridization program, and a large number of crossings. Embryo rescue will be a way to obtain optimal hybrid embryos from an intergeneric cross between *V. faba* and *T. turcica*.

2.4. Embryo Culture and Embryo Rescue

2.4.1. Introduction

Embryogenesis in higher plants can be divided into six phases which are preglobular, globular, transition, heart, torpedo and mature stages in dicots [4,51,55]. During this development, pattern formation which is a process including the formation of all organs and tissues in a seedling. The pattern comprises the shoot apical meristem, cotyledons (embryonic leaves), hypocotyl (embryonic stem) and radicle (embryonic root) [56].

At the preglobular stage, protodermal cells are established and anticlinal division. At the globular stage, the inner cells undergo anticlinal and periclinal, endowing the embryo a recognizable axis. At transition stage, there is more differentiation in the radial axis. At heart stage, the apical meristem is well-developed, and root apical meristem is developed in the basal region. At torpedo stage, vascular differentiation is visible and cotyledons and hypocotyl are enlarged. In a mature embryo, cell layers are clearly visible (Figure 2.31). Clearly embrionic excisions made in the late developmental stages such as heart stage and the torpedo have a high potential for survival due to the autotrophic growth of the embryo. During early embryogenesis which is referred to as morphogenesis stage, fundamental characteristics of a plant’s body take shape. During late embryogenesis that is also known as the maturation phase, storage macromolecules such as proteins, lipids, and starch are accumulated by the embryo [4,51,55,57,58].

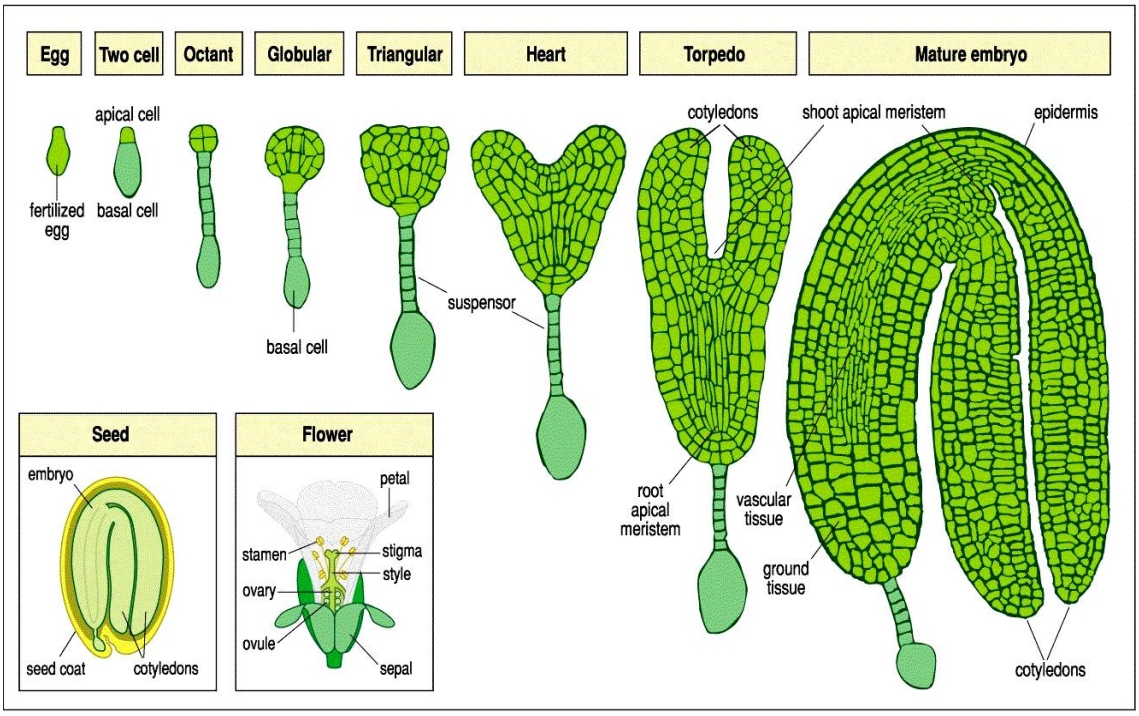

Figure 2.31. Developmental stages of an embryo, structure of a seed and a flower in an angiosperm (adapted from [56])

Embryo culture is one of the useful techniques to rescue hybrid embryos prior to abortion. An embryo culture technique consists of 6 main stages: dissecting embryos, culturing isolated embryos on a suitable medium, embryo development, embryo germination to grow into plantlet, rooting of developed plantlet, and subsequent hardening of micro propagated plants from the embryo. Medium complexity decreases as the developmental stage of the embryo advances. Embryos at a very early age often abort, thus to dissect embryos and to develop them *in vitro* are nearly impossible [49,51].

In the family of Fabaceae, embryo rescue technique has been widely conducted to recover the hybrid progenies from starvation between various species such as, *Cicer* [42,60], *Phaseolus* [49], *Lens* [50,61], *Lotus* [62], *Medicago* [63], *Trifolium* [64], and *Glycine* [65]. Many intergeneric studies have been conducted to date; *Festuca X Lolium* [66], *Menziesia* X *Rhododendron* [67], *Baptisia* X *Thermopsis* [68], *Chrysanthemum grandiflorum* X *Ajania przewalskii* [54] and *Dendranthema crissum* X *Crossostephium chinese* [69].

Vegetative propagation normally occurs by the partition of the rhizome in *T. turcica* and is often vigorous in the natural habitat of this species. For this reason, micropropagation by embryo culture is very important for maintaining of this species. Embryo culture is one of the *in vitro* culture techniques used for known the culturing of isolated embryo or/and ovule in a medium [70–73].

2.4.2. Embryo Culture

Embryos from developing pods of *T. turcica* were cultured to answer the below questions before the start of hybrid embryo rescue for intergeneric crossed samples:

1. Which development age of embryos can be suitable for *in vitro* culturing?
2. Which medium is beneficial for *in vitro* embryo development in *in vitro* condition?

Few embryos were available to find the answers to the above questions due to various causes such as lack of personnel and time limitation for the study due to pollination period of the plants, decreasing work productivity during the hybridization study due to summer time. Based on these, it was necessary to find the most comprehensive medium for embryo culture from a literature review.

2.4.2.1. Materials and Methods

2.4.2.1.1. Materials

2.4.2.1.1.1. Plant Material

T. turcica (Eber population) fruit seeds were collected from Istanbul's Nezahat Gokyigit Botanical Garden, in late June of 2011. *Thermopsis* fruit seeds were sterilized; embryos were then isolated and cultured.

2.4.2.1.1.2. Growth Media

A modified medium [74] which is a more complex one for embryo development (Table 2.6 and 2.7), was used for the ovule-embryo culture; for developing of the *T. turcica* embryos into plantlets, a modified medium [14] was employed.

Murashige and Skoog's Basal Medium [75]: For 1 L MS media, 4,3 g MS Basal was dissolved in 1 L ddH_2O and then autoclaved at 121°C for 15 minutes. Autoclaved medium was poured into sterile Petri dishes after cooling down to 50°C. Sterile solid agar plates were kept at 4°C.

Table 2.6. A modified culture medium composition for *T. turcica* ovules [74]

Culture medium compositions	***Per liter***
Murashige and Skoog salt mixture	4.3 g
Sugars	
Sucrose	100 g
Vitamins	
Myo-Inositol	100 mg
Nicotinic acid	0.5 mg
Pyridoxine-HCl	0.3 mg
Thiamine-HCl	0.1 mg
Gel and other	
Agar	7.5 g
Hormones	
IAA	0.2 mg
GA_3	0.5 mg
Zeatin	0.5 mg
pH: 5.7	

Table 2.7. *In vitro* embryo culture stages and media

Stages	***Media***
Ovule-Embryo Culture (2 weeks under dark conditions)	Cohen et al. (1984) modified medium [74]
Embryo Rescue in Ovule (2 weeks under a 12 h photoperiod)	Cohen et al. (1984) modified medium [74]
Regeneration of Plantles (around a month under a 16 h photoperiod)	Cenkci et al. (2008) modified medium [14]

2.4.2.1.2. Methods

2.4.2.1.2.1. Fruit Surface Sterilization

Fruit pods collected were dipped into 70% ethanol and kept in 20% sodium hypochlorite solution for 5 minutes. The fruits were then rinsed three consecutive times with sterile distilled water in a laminar flow cabinet. After straining the water, seeds were pitted open under sterile conditions. The ovule-embryos were isolated from the seeds and were placed directly on the culture medium.

2.4.2.1.2.2. Ovule-Embryo Culture

Only one ovul including embryo was taken on a modified medium [74] (see Table 2.6 for compositions), to avoid contamination by another samples. Only the micropylar end of the ovule was contacted directly with the medium. Control cultures were maintained on a hormone-free Murashige and Skoog's (MS) medium [75].

Ovule-embryos was grown for two weeks in a growth chamber at 25±1°C under complete darkness. Open ovules at the opposite end to that of the micropyle were cut, and embryos from the ovular integuments were carefully excised. All embryos that were longer than 10 mm were cultured in an upright position with the medium given in Table 2.6. The medium used for rescued embryos was free from gibberellic acid (GA_3) and contained 0.5 mg l^{-1} zeatin (ZEA) and 30 mg l^{-1} sucrose, reaching up to the hypocotyl for 2 weeks in a growth chamber at 25±1°C exposed to a 16 h photoperiod. GA_3 was not used for this step. After 2 weeks, the rescued embryos were transferred to test tubes containing a modified medium [14].

2.4.2.1.2.3. Regeneration of Plantlets from Rescued Embryos

The test tubes containing the embryos were cultured for approximately one month in a growth chamber so as to develop into plantlets; for this step, a modified medium [14] was used. The composition of this modified medium is 4.3 g MS basal salt, 1 mg^{-1} ml MS vitamin solution, 4.0 mg^{-1} L indole-3-butyric acid (IBA), 30 g^{-1} L sucrose, and 7,5 g agar. The medium's pH was adjusted to 5.7±0.2 before the addition of the gelling agent and autoclaved at 121°C for 15 min. As a control group, some samples were maintained on an MS medium without a plant growth regulator. All samples were cultured in a growth chamber at 25±1°C exposed to a 16 h photoperiod. Table 2.7 offers *in vitro* embryo culture steps.

2.4.2.2. Results and Discussion

Ovule-Embryo Culture*:*

With regard to the protocol [74], a modified medium was used due to the complex nutritional elements of the immature embryos in the present study.

After ovules had been collected, the embryos were cultured on MS medium supplemented with 100 g L^{-1} sucrose, 0.2 mg L^{-1} IAA, 0.5 mg L^{-1} GA_3, 0.5 mg L^{-1} ZEA, and 7.0 g L^{-1} agar. Contol groups were placed on MS medium free from plant growth regulator. After two weeks in the culture under complete darkness, all ovules planted in control groups turned brown, and embryos died. On the other hand, the embryos placed on the modified medium [74] remained viable. After the embryos had been released, they were placed on MS medium free from GA_3 and containing 0.5 mg l^{-1} ZEA, 0.2 mg L^{-1} IAA and 30 mg l^{-1} sucrose. It is known that zeatin should be added to the medium to grow rapidly embryos [74]. In this study, the developmental stage of extracted embryos from ovules was a good variable, from globular to cotyledonary.

By applying GA_3 into the ovule, medium sized viable embryos were obtained. Also, it is noticed that all the legumes obtained from the Eber population contained healthy fruit seeds.

The main differences between the medium used in the present study and recommended medium [74] is the amount of ZEA and agar added to the media. At the stage of ovule-embryo culture, the media were supplemented with 7.5 g L^{-1} agar in the present study, whereas the suggested media were supplemented with 9 g L^{-1} agar. In addition, the medium used for culturing excised embryos in this study was supplemented with 0.5 mg l^{-1} ZEA, whereas those in the proposed study were supplemented with 0.2 mg l^{-1} ZEA. After culture for two weeks on a modified suggested medium [74], embryos developed rapidly (Figure 2.32 and 2.33). In contrast, it is observed that the presence of plant hormones embryos developed abnormally with the induction of multiple buds without any rootlet. On the given medium, shoots were elongated. Possibly, root formation of regenerated shoots of *T. turcica* is affected by other unknown factors based on medium composition. In contrast, no callus induction was observed. According to previous study [76] if only *in vitro* plant tissue culture lacking callus proliferation, unaltered genetically stable plants can be regenerated. It is observed that the plantlets are morphologically similar to parents without callus formation.

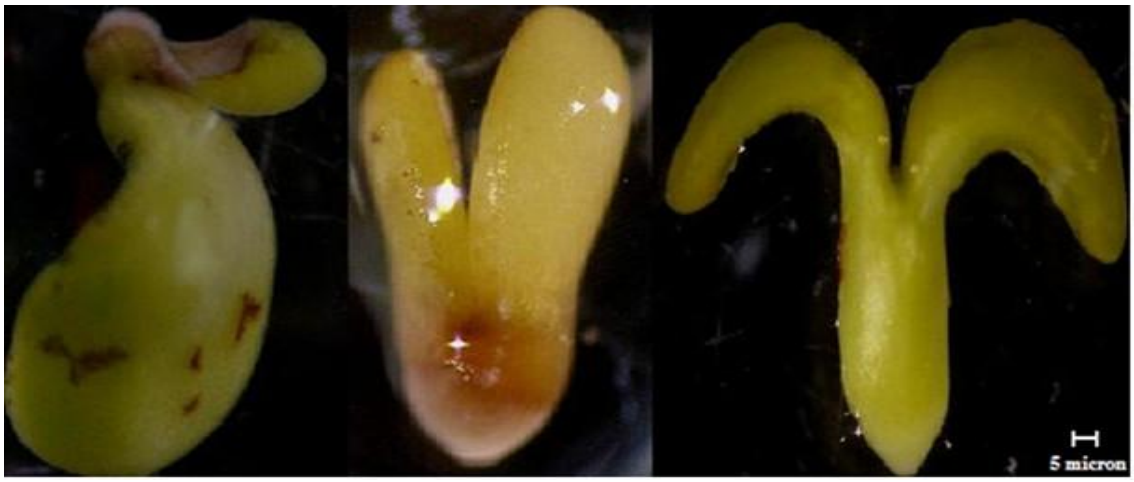

Figure 2.32. Different embryos at various ages embryos extracted from ovule (scale bar: 5 microns)

Figure 2.33. Embryos cultured *in vitro* during two weeks (scale bar: 5 microns)

On the basis of these results, the rescue of embryos extracted from the ovules of *T. turcica* could be possible by the improvement of an *in vitro* technique for culturing embryos. However, the research presented here shows earlier germination and plantlet development because of the absence of time-consuming actions in breaking seed dormancy and removing the seed's hard coat.

Regeneration of Plantlets from Cultured Embryos:

In this study, the development of plantlets from embryos was successful. Although shoot development was observed in plantlet development from embryos; on the other hand, during the present experiment, no root induction occured when the proposed medium [74] was used for subculturing (Figure 2.34).

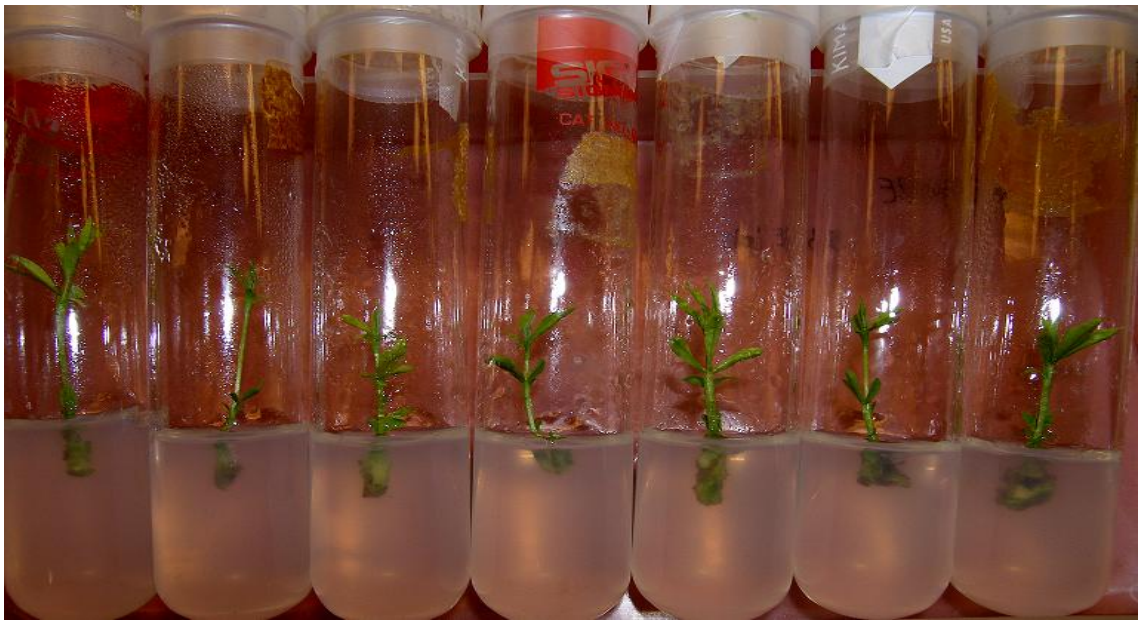

Figure 2.34. Plantlets which lack rootlet after 15th day of culture

Due to absence of root formation, a modified medium [14] was used to obtain plantlet with rootlet. Regenerated shoots were cut off from their forming tissue and transferred to suggested medium [14] for rooting. A month after placing the small plantlet lacking rootlet on the modified suggested medium [14], developed roots were formed (Figure 2.35). For control groups obtained from embryo culture, MS medium free from plant growth regulator was used. The difference between the modified medium [14] and used the medium in the present study was the concentration of IBA. Whereas researchers used 0.5 mg L^{-1} IBA, 2.0 mg L^{-1} IBA was used in present study. Upon the roots' emergence, the color of root explants darkened.

Figure 2.35. Root and shoot growth in 2.0 mg/L IBA rooting media

There was no observable root formation on control groups that were cultured on a hormone-free MS medium. The results of this study indicate that the given medium was suitable for developing of roots.

Based on a review of the literature**,** no fruit embryo rescue has to date been performed on *T. turcica*. In this study, an efficient rescue protocol has been obtained to recover *T. turcica* embryos. To increase the number of viable embryos via *in vitro* techniques, future work should entail various hormones and concentrations of different embryo ages. Further studies are needed to understand the germination, physiology, and sexual reproduction of this important Turkish endemic, rare plant. In addition, it is recommended that the findings of the present study be used as a source for hybridization and breeding purposes related to *T. turcica* for future experiments.

2.4.3. Embryo Rescue

2.4.3.1. Materials and Methods

2.4.3.1.1. Materials

2.4.3.1.1.1. Plant Material

After hybridization of *T. turcica* with *V. faba* at NGBG, pods at 10th, 15th and 20th day of pollination were collected and directly taken to the laboratory for ovule-embryo culture.

2.4.3.1.1.2. Chemicals

All the chemicals used in this thesis is listed in Appendix A.

2.4.3.1.1.3. Equipment

All the equipment used in this thesis is listed in Appendix B.

2.4.3.1.1.4. Growth Media

The media and culture conditions described in 2.4.2.2.1.2. were implemented.

2.4.3.1.2. Methods

2.4.3.1.2.1. Collecting the samples

After hybridization had been carried out, pods at 10th, 15th and 20th day of pollination were collected and taken to the laboratory.

2.4.3.1.2.2. Embryo Rescue Study

To eliminate the possible contamination factors, surface sterilization (3% sodium hypochlorite for 30 min. following immersion in 70% ethanol for 30 s.) was done. For the ovule-embryo culture of *T. turcica*, the medium mentioned in Table 2.6 was used. All laboratory studies conducted at Plant Biotechnology Laboratory, Sabanci University under strict sterile conditions in the laminar air flow cabinet.

Pistil samples 5 to 20 DAP were collected and moved to the laboratory as quickly as possible. After surface sterilization immediately both outer and inner integuments were immediately removed, and ovules were dissected. Excised ovules were cultured in 100x15-mm sterile petri dishes containing 20 ml of selected nutrient medium as given Table 2.6. Four ovules were cultured on each plate. Ovules were maintained in a plant culture room under controlled conditions. Responses of ovules were observed and recorded.

2.4.3.2. Results and Discussion

It was recognized that the development of testa maintained until 20 days after pollination in all samples (Figure 2.36). Although Testa grew, the seeds of hybrids developed until 15 days after pollination (Figure 2.37).

On the other hand, the seeds of self-pollinated samples maintained their development until the 20th day of pollination (Figure 2.36). The seeds of intergeneric crossed and self-pollinated samples were different in size; however, the seed coat (outer integument) of intergeneric crossed samples developed well, remained green and seemed healthy (Figure 2.36). In contrast, the inner integument of both intergeneric crossed and self-pollinated samples were yellow or green, thin and hairy (Figure 2.37).

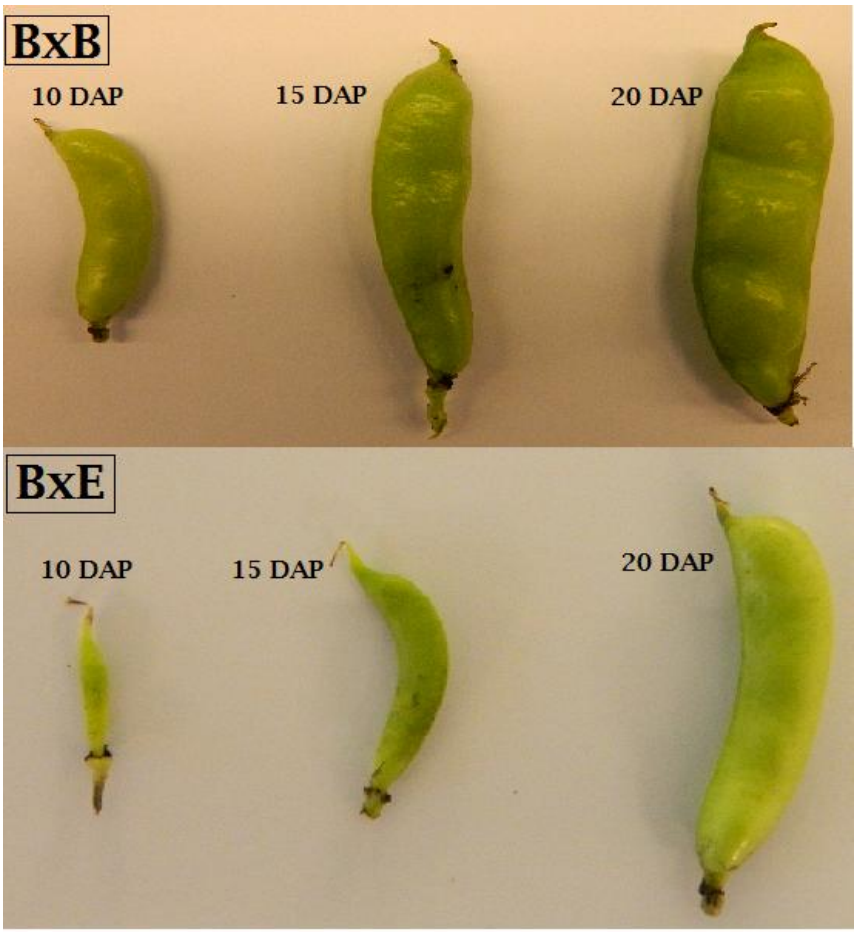

Figure 2.36. Testa development of the samples at different days of pollination (B: *V. faba*; E: Eber population of *T. turcica*; DAP: Day After Pollination)

After surface sterilization, the ovules were extracted and put on petri dishes including the medium mentioned in Table 2.6 Although the pods of hybrids from the cross *V. faba* and *T. turcica* appeared well-grown, the seeds inside it were smaller than those of self-pollinated samples. Since the ovules were too small to separate the embryos from the ovules, the ovules were cultured. All samples were cultured under controlled growth conditions. After 3 weeks of incubation, all of the ovules had died.

In addition, all ovules grown on the MS basal medium without a plant growth regulator had also died. As a result of this study, the modified medium [74] was demonstrated to be a sufficient for ovule-embryo culture of *T. turcica* but was not suitable for the ovules of *V. faba* (Figure 2.39).

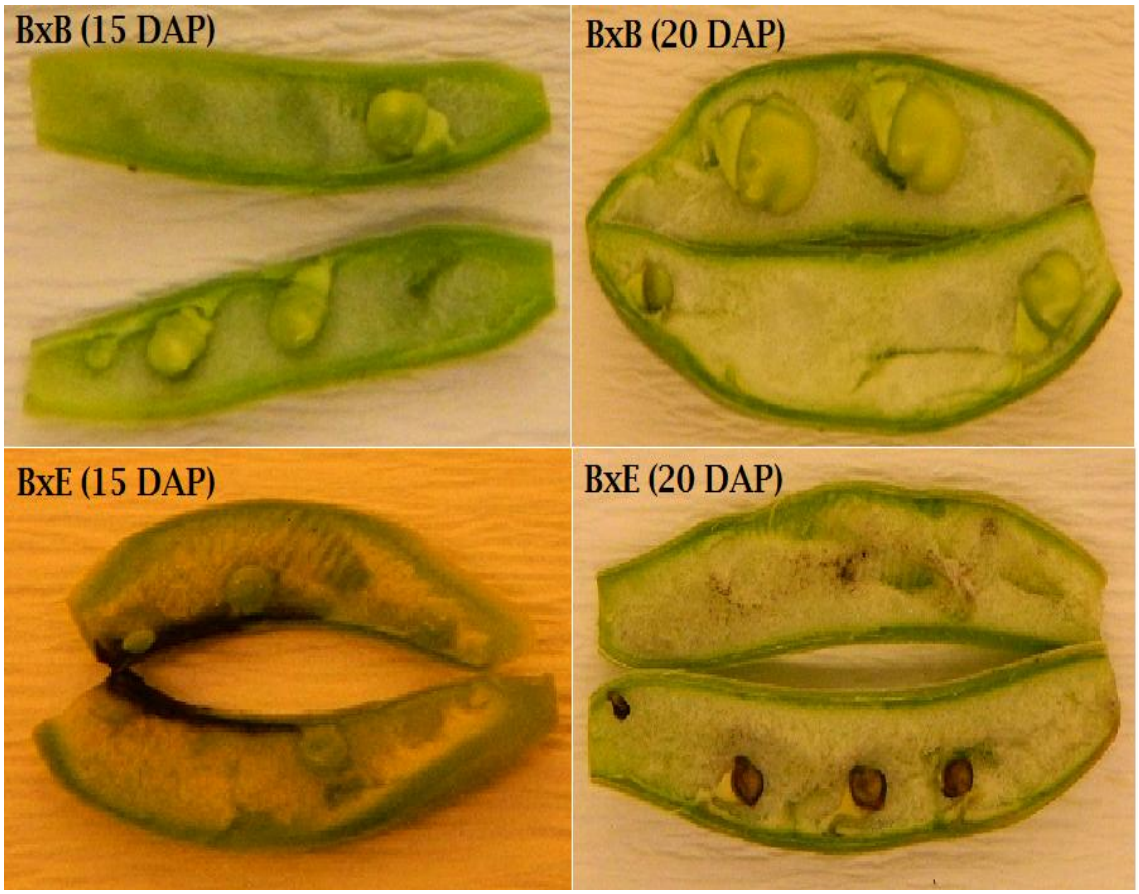

Figure 2.37. The situation of the seed development (B: *V. faba*; E: Eber population of *T. turcica*; DAP: Day After Pollination)

In embryo rescue, it is essential to establish when the embryos are thrown, so that the embryos can be dissected prior to abortion, and their development can be maintained under *in vitro* conditions. To determine the efficient culture medium and the proper age of the embryo to be cultured are critical points in obtaining hybrids by embryo rescue [51,52]. In the present study, embryo abortion was observed in hybrid seeds of 15 DAP; due to this event, embryo rescue should be carried out for the samples at or before the 15th day of pollination. Embryo abortion may be based upon the taxonomic distance between species used in hybridization and the endosperm defect.

Since immature embryo rescue is very difficult in many plant species, after fertilization at least a week is needed for ovule development. For this, pistil samples 10 and 15 DAP were collected for embryo rescue. Although ovule development in all self-pollinated samples was good enough for embryo culture, ovule growth in all cross-pollinated samples was not sufficient (Figure 2.38).

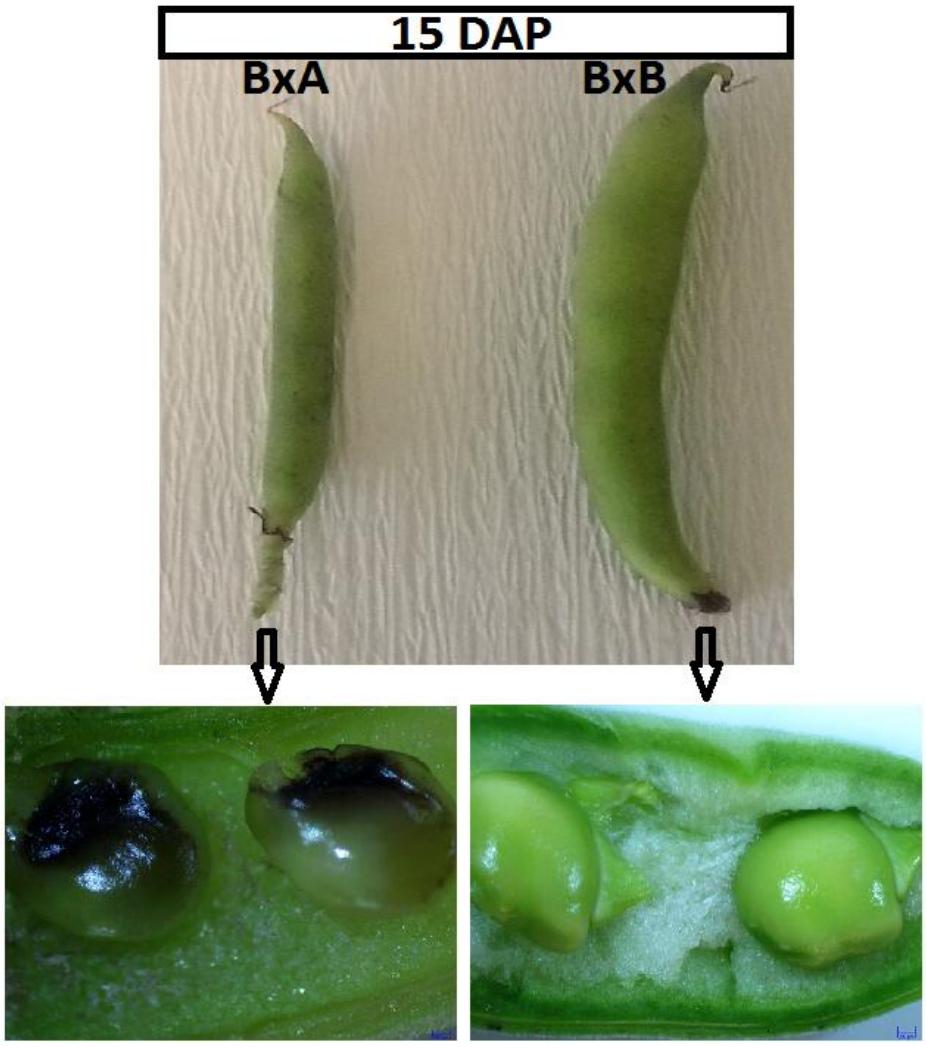

Figure 2.38. The situation of the seed development (A: Aksehir population of *T. turcica*; B: *V. faba)* (scale bar: 200 µm)

Plant regeneration or even induction of organ development have not been observed in the *V. faba* + *T. turcica* hybrids. This event may in part be caused by the unequal distribution of chromosome in hybrids. Since *V. faba* and *T. turcica* differ particularly in chromosome number, the hybrids were not able to grow for a long time on the culture medium.

With the change in the genotype direction in crossing, the osmotic adjustment of the medium may not be sufficient for embryo growth and can lead to differences in growth characteristics of excised embryos. The dissected embryos (0.1-0.2 mm in size) were not developed on the culture medium, remained at the globular stage for a while during the culture and then died. This event should possibly happen at the early ages of hybrids. The proportion of the regeneration from ovules or embryos can often increase as the age of (DAP) embryos or ovules increases. From the previous studies, it is known that the elongation of small embryos (below 0.7 mm in size) is very difficult and due to the natural requirements of immature embryos, the more complex medium is needed [77]. The key components of a medium and vital steps for the development of an embryo *in vitro* were explained in detail in a book [51]. Plant regeneration has been difficult in intergeneric hybridization studies through embryo culture; to overcome this problem in the present study ovule culture was attempted instead of embryo culture. By twenty days, no tissue regeneration was observed in crossed samples cultured on both the modified and MS medium, but only self-pollinated samples 20 DAP cultured on a modified medium generated root-like structure that was white and soft (Figure 2.39).

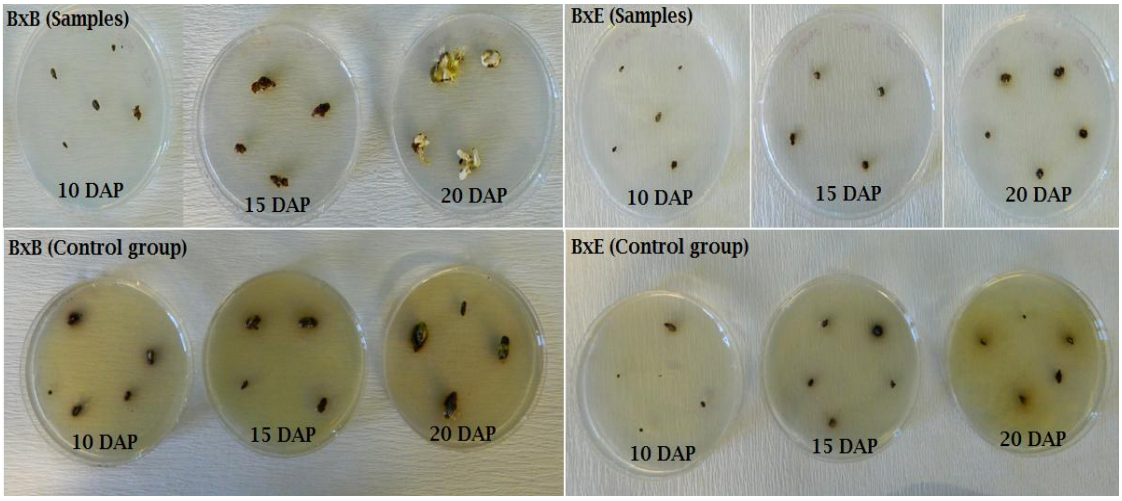

Figure 2.39. General view of the ovules extracted at 10th, 15th and 20th on the selected medium and MS medium free from hormone as a control group (B: *V. faba*; E: Eber population of *T. turcica*; DAP: Day After Pollination)

As a result, in intergeneric hybridization between *T. turcica* and *V. faba* it is very difficult to obtain a plantlet by classical crossing techniques without an embryo culture; due to post-zygotic barriers which are commonly related to endosperm failure and successive unusual differentiation and denutrition of immature hybrid embryos [51].

There is, however, a requirement for more in-depth analysis of embryo rescue for the hybrids obtained from crossing in which *T. turcica* is used as the paternal parent. In interspecific hybridization, the embryo normally grows for only a maximum of a week. Similar results were also reported in some studies [51,60]. In the present study, intergeneric crosses were carried out between *V. faba* and *T. turcica*, so it was very difficult to regenerate embryos into intact plantlets due to the distant relationship. The endosperm is vital for the nourishment of the embryo. Endosperm Balance Number (EBN) is crucial for normal endosperm development, and EBN consists of a 2 maternal: 1 paternal ratio [51]. Any alteration in EBN can affect normal endosperm development and due to this reason hybrid embryo abortion often takes place in intergeneric crosses.

Problems encountered during hybridization studies in the first year of the thesis studies:

In the first year of the hybridization study, various problems affected the success of the research, but in the later years, these problems were limited. These issues can be specified as follows:

1. In the crosses in which *T. turcica* was used as a maternal parent, internode elongation was a big problem for covering the pistil with a material. After pollinated pistils were bagged, due to increasing the distance between two nodal segment, covering failed and a large number of samples were excluded from the study. In addition, differences in flowering time between the buds in the apical inflorescence of *T. turcica* caused a problem for the hybridization study (Figure 2.40).

Figure 2.40. Flower bud structure and flowering situation of *T. turcica*

2. The presence of *Epicometis hirta* in the field at NGBG was a serious problem in achieving goals of the study. The workers of NGBG sowed the seeds of *V. faba* in the same place as *T. turcica*, so once the predator named *Epicometis hirta* arose, it badly damaged both *V. faba* and *T. turcica* (Figure 2.41). Because of this reason, a large number of flowers could not be used for crossing.

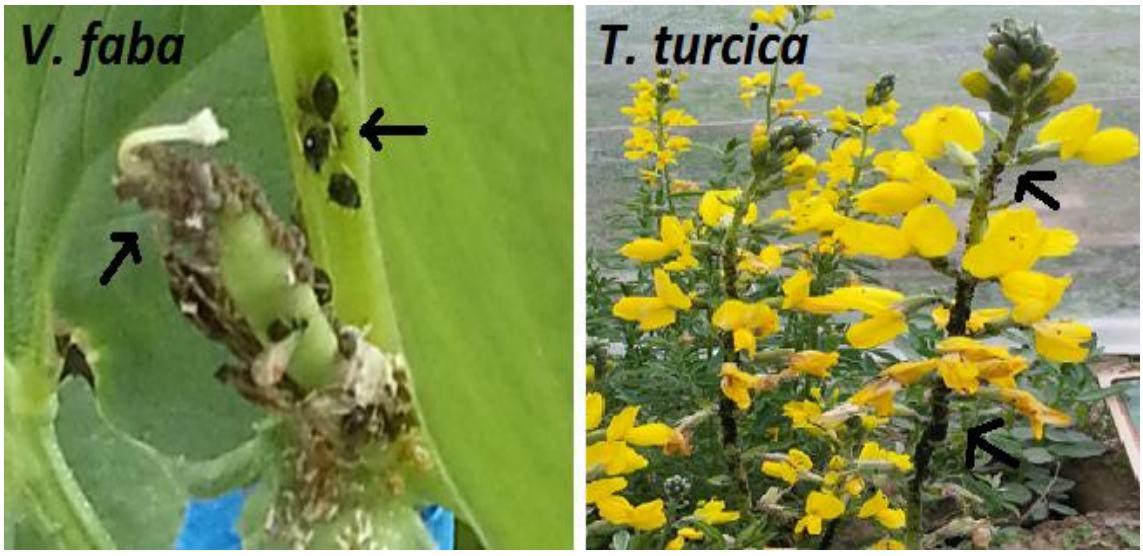

Figure 2.41. The loss of pistils of *V. faba* used as female in crossing and eliminated flowers from the research due to the presence of *Epicometis hirta*

2.4.3.3. Conclusion

This study demonstrates that incompatibility between *V. faba* (♀) and *T. turcica* (♂) has not occurred after fertilization. Embryos (dissected 15 days after pollination) were not elongated and remained globular on the MS medium and then died in few days of incubation. The selected medium for the embryo rescue study was not efficient for hybrid immature embryos extracted from *V. faba* X *T. turcica.* The small size of the embryos made it impossible to regenerate any tissue. On the other hand, self-pollinated samples of 20 DAP cultured on a proposed medium showed root-like structure compared with the control groups cultured on an MS medium free from any plant growth regulators. Plant regeneration achieved extracted ovules or embryos from *T. turcica*; however, no regeneration was observed in the ovules or embryos obtained from *V. faba*. The crossability between *V. faba* and *T. turcica* may be hampered by the post-fertilization barrier as endosperm failure was followed by embryo starvation. Since globular embryos were observed from the cross *V. faba* X T. *turcica*, it seems that the difficulty of obtaining hybrids via intergeneric crossing is the phylogenetic distance between *T. turcica* and *V. faba*; thus, the main attack in preventing the hybrids is embryo rescue. Based on this, further studies should be conducted to determine what medium is suitable for embryos obtained from crossing *V. faba* (♀) and *T. turcica* (♂) for embryo rescue. In addition, the content of the medium used to maintain embryos is a crucial point of prospering plant culture. These results are promising for further studies about obtaining new cultivars using *T. turcica*.

CHAPTER 3

ISOLATION OF THE PARTIAL HOMOLOGUES OF THE GENES RESPONSIBLE FOR MULTICARPELLARY FEATURE IN *Thermopsis turcica*

3.1. INTRODUCTION

In plant kingdom, two meristems: (1) shoot apical meristem (SAM) and (2) root apical meristem (RAM) are the groups of the cells which are in charge of the forming of the intact adult plant. The SAM consists of a group of undifferentiated cells and provides leaves (vegetative tissues) and flowers (reproductive tissues) [78]. A flower carries four types of laterally connected organs in consecutive whorls referred to as sepals, petals, stamens, and carpels. Some significant floral characteristics such as organization of organs in whorls, number of portions in whorl, carpel status, combination the features of organs within a whorl or different whorls, and if both reproductive organs (pistil and stamen) are located in the same flower (hermaphroditism) are imperative to determine floral ontogeny among plant families (Figure 3.1) [5]. Two main hypothesis named the ABC model (Figure 3.2), and the floral quartet model (Figure 3.3) explained how pattern formation were present [5,79].

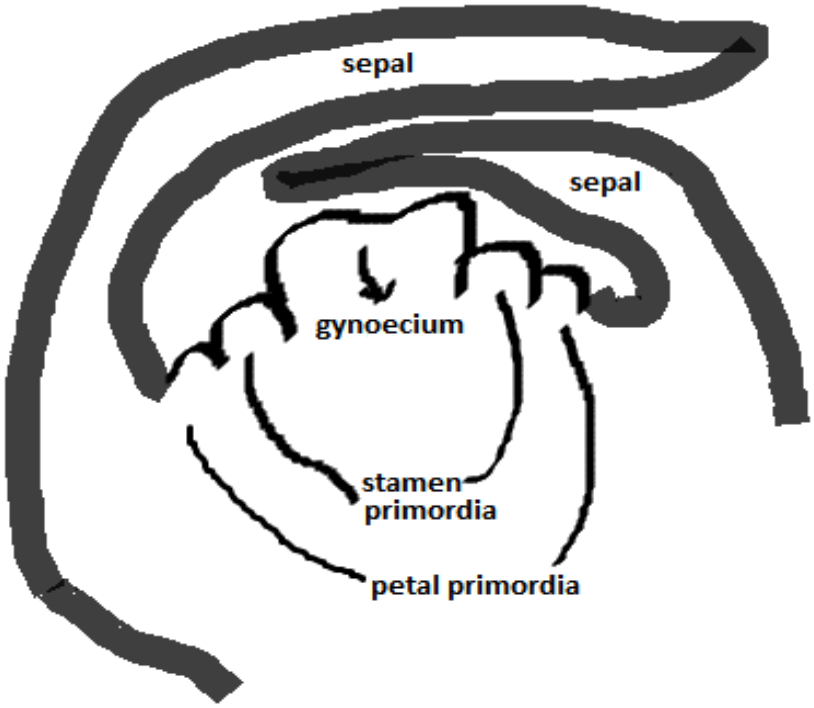

Figure 3.1. Schematic architecture of a young flower

ABC model is a hypothesis that claims all organs of a whorl occur concurrently and first sepals alike vegetative leaves, then petals (coloured), stamens (the male reproductive organ) and carpels (the female reproductive organ) initiate, respectively. A set of gene (floral organ identity genes or homeotic selector genes) or gene combinations influence which organ will be initiated. According to this hypothesis, class A genes alone specify sepal formation, class A + B genes generate petals, class B + C genes produce stamens, and class C genes alone initiate the development of carpel [5,79–81]. The class A and class C genes negatively regulate their expression. ABC model has focused on two species, *Arabidopsis thaliana* (Brassicaceae) and *Antirrhinum majus* (Scrophulariaceae). It was reported that there is no legume in which carpel is produced last, after male organ (stamens) is generated, based on this knowledge in the same report it was claimed that the ABC model hypothesis did not apply to legume flowers (Figure 3.2) [5].

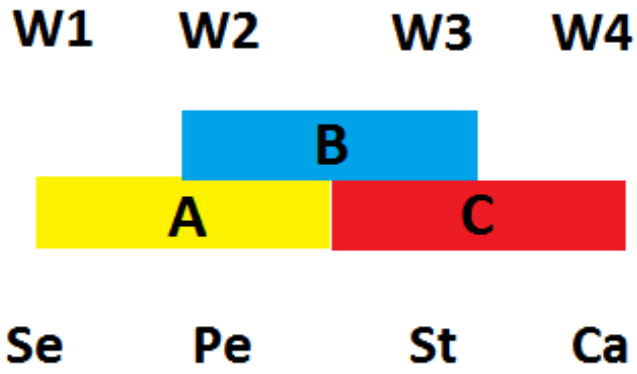

Figure 3.2. The ABC model: The A function specifies sepals (Se) induction in the outer whorl 1 (W1), The A + B genes induce the form of petals (Pe) in whorl 2 (W2), The B + C genes produce stamens (St) in whorl 3 (W3) and the class of C genes specifies carpels (Ca) in whorl 4 (W4)

The floral quartet model is also termed as ABCD model. Class D genes initiate ovule identity. In the ABCDE model by addition class E genes, *SEPALLATA* (*SEP*)-like genes are responsible for the development of all models of floral organs and A + E genes produce sepals, A + B + E genes specify petals, B + C + E produce stamens, C + E genes required to produce carpels and D + E genes produce ovules. Class C protein homodimers produce female reproductive organ when organ identity is characterized by dimer, whereas class E and C genes specify female organ according to the floral quartet (tetramer) model (Figure 3.3). The homeotic transition of floral organ identity genes causes the flowers with different architectures [79,82].

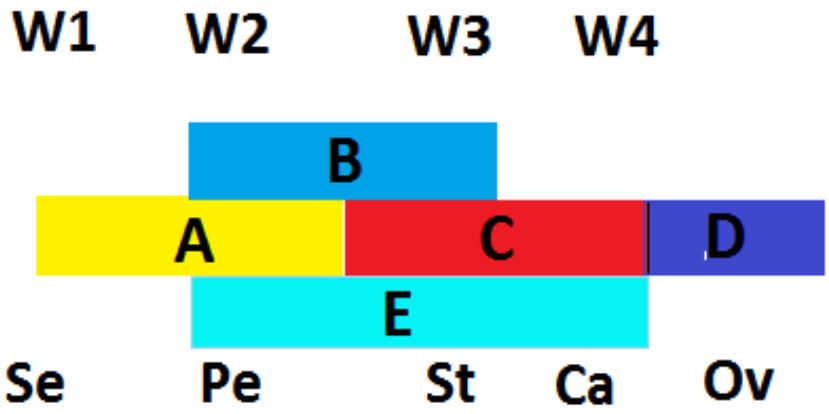

Figure 3.3. The floral quartet model which is a pattern obtained from the extension of ABC model by the addition of the D-function gene, which is responsible for ovule (Ov) development, and E-function gene, which is needed for the determination of floral organ (petal, stamen and carpel) identity.

Recent studies on floral ontogeny in various species led to a conclusion that the unusual multicarpellate state arose in different clades independently and via distinct mechanisms [2,9,83]. In addition, although in many species having multicarpellary gynoecium tends to form big flowers in size, increasing in the number of carpels may induce disruption of compitum function [41]. Normally organ number in a flower does not change from whorl to the whorl, and there may be three different mechanisms affecting gynoecium polymerization [41]:

1. a rise in organ number in the perianth (composition of calyx and corolla) is already available (for example, *Munroidendron* (Araliaceae), *Aeonium* (Crassulaceae)) [41]
2. while the perianth is present as oligomerous, rising in organ number occurs in androecium (stamens of a flower) (for example, *Limnocharis* (Limnocharitaceae), *Alisma* (Alismataceae)) [41,84]
3. perianth and androecium are present in small segments, but organ number increases in the gynoecium (for example, *Beiselia* (Burseraceae)) [41,85]

The overview of presence of multicarpellate gynoecia in plant kingdom was implemented using the classification of Angiosperm Phylogeny Group (APG III) in flowering plants [86] in 2014 and according to this classification, it is clarified that 138 families in angiosperms (flowering plants) have multicarpellate gynoecium. 28 of the 138 families have carpels in two whorls, whereas 110 have carpels positioned in one whorl and based on this information the carpel number emphasized determines that the total carpel number per flower, not the number of whorl [41]**.** This review article published in 2014 was used to create a table including plant species having multicarpellate gynoecium identified so far (Table 3.1).

Table 3.1. The occurence of multicarpellate gynoecia in Angiosperms (flowering species) [41,79,86]

CARPELS IN TWO WHORLS	
Groups	**Families**
***ANITA* or ANA Grade**	Austrobaileyaceae, Cabombaceae
Magnoliids	Winteraceae, Atherospermataceae, Calycanthaceae, Monimiaceae, Siparunaceae, Annonaceae, Eupomatiaceae, Himantandraceae
Monocots	Alismataceae, Butomaceae, Juncaginaceae, Potamogetonaceae, Ruppiacea, Triuridaceae
Early branching eudicots	Circaeasteraceae, Lardizabalaceae, Menispermaceae, Ranunculaceae, Nelumbonaceae
Core eudicots	Paeoniaceae
Rosids	Rosaceae, Lythraceae, Gyrostemonaceae, Sterculioideae, Malvoideae, Rutaceae
CARPELS IN ONE WHORL	
***ANITA* or ANA Grade**	Amborellaceae, Cabombaceae, Schisandraceae
Magnoliids	Canellaceae, Aristolochiaceae, Annonaceae
Monocots	Alismataceae, Aponogetonaceae, Araceae, Hyrocharitaceae, Limnocharitaceae, Scheuchzeriaceae, Cyclanthaceae, Pandanaceae, Triuridaceae, Melanthiaceae, Asparagaceae, Asteliaceae, Hypoxidaceae, Arecaceae, Centrolepidaceae, Cyperaceae, Poaceae, Sparganiaceae
Early diverging eudicots	Eupteleaceae, Lardizabalaceae, Papaveraceae, Ranunculaceae, Trochodendraceae,
Core eudicots	Dilleniaceae, Aizoaceae, Cactaceae, Caryophyllaceae,Montiaceae, Phytolaccaceae, Portulacaceae, Crassulaceae, Prnthoraceae,
Rosids	Zygophyllaceae, Celastraceae, Cephalotaceae, Connaraceae, Cunoniaceae, Elaeocarpaceae, Achariaceae, Calophyllaceae, Caryocaraceae, Clusiaceae, Euphorbiaceae, Acolyphoideae, Euphorbiadeae, Medusagynaceae, Achnaceae, Passifloraceae,

	Phylanthaceae, Putranjivaceae, Quiinaceae, Rafflesiaceae, Rhizophoraceae, Salicaceae, Scyphostegiaceae, Begoniaceae, Coriariaceae, Tetramelaceae, **Fabaceae**, Polygalaceae, Fagaceae, Dirachmaceae, Myrtaceae, Onagraceae, Lythraceae, Melastomaceae, Strasburgeriaceae, Capparaceae, Resedaceae, Tovariaceae, Cistaceae, Cytinaceae, Malvaceae, Muntingiaceae, Neuradaceae, Thymelaceae, Anacardiaceae, Burseraceae, Kirkiaceae, Meliaceae, Nitrariaceae, Ruteceae, Sapindaceae, Simaroubaceae
Asterids	Hyrdangeaceae, Cornaceae, Actinidiaceae, Ebenaceae, Ericaceae, Lecythidaceae, Marcgraviaceae, Sapotaceae, Scytopetalaceae, Ternstroemiaceae, Theaceae, Boraginaceae, Icacinaceae, Rubiaceae, Solanaceae, Aquifoliaceae, Campanulaceae, Rousseaceae, Paracryphiaceae, Araliaceae

According to a literature review, it is known that the multicarpellary can rarely be seen in legumes [5,87]. The first studies of multicarpellate gynoecia in legumes were published in 1839 [88] and 1872 [87]. Although the subfamily of Caesalpinioideae has a multicarpellary ovary, no multilocular ovaries existed in this subfamily were reported [2]. *T. turcica* is unique due to its carpel polymerization in the subfamily of Papilionoideae (Table 3.2).

Table 3.2. The distribution of polymerous gynoecia in legume family [2]

POLYMEROUS GYNOECIUM IN FABACEAE	
Subfamilies	**Species**
Caeselpinioideae	*Amherstia nobilis* *Cassia lutea*
Mimosoideae	*Archidendron lucyi* *Archidendron glabrum* *Inga congesta* *Albizia lophantha* *Acacia celastrifolia*
Papilionoideae	***Thermopsis turcica***
TRIBE **Swartziae**	*Swartzai dipetala* *Swartzia polycarpa* *Aldina diplogyne* *Cordyla pinnata*

Polymerous gynoecia can be found in the developmental mutants of the model species, such as *Pisum sativum* and *Medicago truncatula* [2]. Normally multicarpellate gynoecia are typical for all other families of the order Fabales (Polygalaceae, Surianaceae, and Quillajaceae). In Fabaceae, multicarpellate gynoecium has often been found in the subfamily Mimosoideae of Fabaceae [6,7] and in tribe Swartzieae of subfamily Papilionoideae [8,9]. Although a gynoecium consisting of a single carpel is dominant in legumes, gynoecium multicarpellate can rarely be formed by mutations, environmental shock or unknown reasons [8,89].

The studies on the floral ontogeny of known mutants of pea (*Pisum sativum*) with a multicarpellate gynoecium uncovered three possible mechanisms of gynoecium multiplication [2]. These are the homeotic replacement of stamens with carpels, enlargement of floral meristem providing enough space for additional carpels, and fusion of few separate floral meristems resulting in a solitary multicarpellate structure. The first mechanism is conditioned by the existence of genes which govern the identity of different floral primordial according to so-called ABC-model [90]. An ectopic expression of C-class genes in primordia predisposed for stamens (B + C expression) results in the production of supernumerary carpels. Such phenotype is known in pea as caused by the mutation *stamina pistilloida* [91].

Sizes of meristems are usually regulated by the interaction between gene *WUSCHEL* (*WUS*) and its negative regulators, such as genes *CLAVATA* (*CLV*) and *FASCIATA* (*FAS*) [92,93]. A number of positive regulators of *WUS* expression are lower. The distortions in negative regulation of *WUS* result in a stem fasciation which means the folding of an enlarged floral apex [41,94]. *WUS* is a gene which plays a vital role in establishing the shoot apical meristem (SAM), and ectopic expression of *WUS* is paramount in the formation of SAM [57,93,95]. *FASCIATA* gene family includes *FA*, *FA2*, and *FAS*. This family provides SAM stability, negatively regulates SAM size, inhibits generation of terminal flowers and restricts SAM extension [96]. The gene series of *CLV* such as *clavata1*, *clavata2*, and *clavata3* affect the number of the organ in a whorl and meristem size [97]. It is identified that *CLV*1 encodes a receptor kinase in kind of Leucine Rich Repeat (LRR) and a study conducted on a mutant of *CLV* reported that the number of cycling cell of SAM increased in the mutants.

Based on this result, a recessive mutation in the gene *CLV* may be a reason in the increasing embryogenic capacity in the mutants [98,99]. The mutant of *STP1* causes the transformation of stamens into carpels [2]. In the present study, it was thought that the study of orthologous homeotic genes in C-class might most plausibly explain the gynoecium polymerization in *T. turcica.* Table 3.3. gives the function of selected genes for this thesis.

Table 3.3. The function of targeted gene

Gene	**Class**	**Function**	**The function of mutated gene**	**Gene encodes**	**References**
CLAVATA	*CLV1*	Enlargement of SAM	Restriction of SAM size	Leucine-rich repeat receptor kinase	[98–102]
	CLV2			Leucine-rich repeat transmembrane protein	
	CLV3			Small protein	
WUSCHEL	*WUS*	Activate class C expression	Specification of cell identity in SAM Center	Homeodomain	[95,98,102–104]
STAMINA PISTILLOIDA	*STP*	Normal development of petals and stamens	Homeotic replacement of stamen with carpel	F-box	[2,91,104]
FASCIATA	*FAS*	Cellular and functional organization of SAM	Flower fasciation	chromatin assembly factor-1	[2,105]

Although no cases of flower anomalies was reported for fasciated mutants of legumes to date [1], one may expect that production of supernumerary organs (including carpels) may be due to an increase of floral meristem.

Although *T. turcica* is outstanding in terms of its carpel multiplication in the subfamily Papilionoideae, to date no study has been conducted on the mechanism of its multicarpellary feature according to a literature review. Floral ontogeny may point at the mechanism of carpel multiplication and to investigate flower development may give a clue to understanding better polymerous gynoecia in *T. turcica*.

Examination of expression pattern of key genes (orthologs of *CLV*, *WUS*, *STP1*, *FAS* etc.) in developing flowers of *T. turcica* in comparison with related species may provide information on the mechanism of gynoecium polymerization in this unusual species. Quantitative and qualitative analysis of expression together with sequencing of genes themselves is the central task of the present thesis.

3.2. Aims of the study

The research objectives are as follows:

- obtaining comprehensive information on vegetative and reproductive development with an emphasis on the molecular mechanisms of the multicarpellary gynoecium and flowering in *T. turcica*.

- analysis of regulatory pathways leading to multicarpellary in *T. turcica*.

3.3. Materials and Methods

3.3.1. Materials

3.3.1.1. Plant Materials

Eber population of *Thermopsis turcica*, of which are still present in Nezahat Gokyigit Botanical Garden (NGBG) used as a main plant material for the aim of research.

In addition, *Phaseolus vulgaris* which has an unicarpellary ovary provided by breeders around Adana and used for as a control group in the study. Flower buds at the different growth stage for each species (*T. turcica* and *P. vulgaris*) were collected and directly taken into liquid nitrogen for molecular analysis.

3.3.1.2. Buffers and Solutions

Standard buffers and solutions used in the project were prepared according to the protocols in a manual laboratory book [106].

Agarose Gel: For 100 ml 2% w/v gel, 2 g of agarose powder was dissolved in 100 ml 0.5X TBE buffer by heating. 0.01% (v/v) ethidium bromide was added to the solution.

Calcium Chloride ($CaCl_2$) Solution: 60mM CaCl2 (diluted from 1M stock), 15% Glycerol, and 10mM PIPES (pH 7.00) were mixed and the solution was filter-sterilized and stored at +4°C.

Tris-Boric Acid-EDTA (TBE) Buffer: For 1 L 10X stock solution, 104 g Tris base, 55 g boric acid, and 40 ml 0.5M EDTA (pH 8.0) were dissolved in 1 L of ddH2O. The solution is kept at room temperature.

3.3.1.3. Commercial Molecular Biology Kits

- Qiaquick Gel Extraction Kit, 28706, QIAGEN
- SensiFAST™ SYBR® No-ROX Kit, BIO-98005, BIOLINE
- Tetro cDNA Synthesis Kit, BIO-65042, BIOLINE

3.3.1.4. Enzymes

All polymerases and their corresponding buffers used in this study were from Fermentas.

3.3.1.5. Primers

The primers used in this thesis are listed in Table 3.4.

Table 3.4 The list of the designed degenerate primer, control and housekeeping gene primers employed in this research. Primer names, their sequences, and their product sizes are given

TARGET	**NAME**	**SEQUENCE (5'- 3')**	**DEFINITION**	**PRODUCT SIZE (bp)**
WUSCHEL A	*WUS1*	CAAAGTAGTACAMG GTGGAC	*WUS1*/sense primer	211±100
	WUS2	CAGAAGTGAACCTTT TCTTC	*WUS2*/antisense primer	
WUSCHEL B	*WUS3*	CTTCTGYTGARATGR TTACTG	*WUS3*/sense primer	294±100
	WUS4	GCATAGGGAATAAA GGGAG	*WUS4*/antisense primer	
FASCIATA A	*FAS1*	TGGAGGAGAGYGAV CTTCC	*FAS1*/sense primer	229±100
	FAS2	TCCCTMGTCTCCCAA CACC	*FAS2*/antisense primer	
FASCIATA B	*FAS1*	TGGAGGAGAGYGAV CTTCC	*FAS1*/sense primer	134±100
	FAS3	AAACAACACRCTACT YTTCAC	*FAS3*/antisense primer	

STAMINA PISTILLOIDA A	*STP1*	ATGGATGAACAGYA GRATATGGAG	*STP1*/sense primer	192±100
	STP2	TTGAAGAABAWGAA CCAGTG	*STP2*/antisense primer	
STAMINA PISTILLOIDA B	*STP3*	TCWCCWCVYYBYCA CTGGTTC	*STP3*/sense primer	217±100
	STP4	CAGAAACMMARCAD ASTAAACC	*STP4*/antisense primer	
CLAVATA A	*CLV1*	GCTSTYTGKTTTGAA GG	*CLV1*/sense primer	168±100
	CLV2	TAAGGRGTTTSAGGC T	*CLV2*/antisense primer	
CLAVATA B	*CLV3*	GGGASMCTTCTGRTT G	*CLV3*/sense primer	215±100
	CLV4	ATGATGYAGHGGRT CTGGACC	*CLV4*/antisense primer	
18S rRNA	18S-F	TACCGTCCTAGTCTC AACCATAA	18S/sense primer	Reference [107]
	18S-R	AGAACATCTAAGGG CATCACA	18S/antisense primer	
P. vulgaris-(internal control)	PV-F	GGAAAAGTGTTGGA GGTG	PV/sense primer	189
	PV-R	GTACGCTTGCTTCTC CTC	PV/antisense primer	

3.3.1.6. DNA and Protein Molecular Weight Markers

DNA ladders and protein molecular weight markers used in this thesis are listed in Appendix C.

3.3.1.7. DNA Sequencing

Sequencing service was commercially provided by MCLAB, CA, USA. (http://www.mclab.com/).

3.3.1.8. Software, Computer-Based Programs, and Websites

The software and computer based programs used in this project are listed in Table 3.5.

Table 3.5. The list of the software, programs, and websites used in this research. Name of the software, programs, and websites, their producers/websites and purpose of uses are given

SOFTWARE, PROGRAM, WEBSITE NAME	COMPANY/WEBSITE ADDRESS	PURPOSE OF USE
NCBI BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi	Basic local alignment search tool
Clustal W2	http://www.ebi.ac.uk/Tools/msa/clustalw2/	Degenerate primer design
Multiple sequence alignment	http://www.ebi.ac.uk/Tools/msa/clustalo/	Degenerate primer design
NCBI	http://www.ncbi.nlm.nih.gov/nucleotide/	Degenerate primer design
CLC Main Workbench	CLC bio	DNA sequencing analysis, DNA alignments, etc
EMBOSS Needle	http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html	DNA sequencing analysis

Expasy Translate Tool	http://web.expasy.org/translate/	Searching protein sequences
MUSCLE 3.8.31	http://www.phylogeny.fr/one_task.cgi?task_type=muscle	Comparison of amino acids
TreeDyn 198.3	http://www.phylogeny.fr/one_task.cgi?task_type=treedyn	Phylogenetic tree construction
LightCycler 480 II	ROCHE	Analysing qRT-PCR results

3.3.2. Methods

3.3.2.1. Total RNA Isolation

Total RNA was extracted using TRI Reagent (PeqLab). Total RNAs were isolated from the reproductive tissues (pistils at various ages) of *T. turcica* and *V. faba* by TRI Reagent method. ~500 mg sample and 3 mm tungsten carbide beads were added to 2 ml tubes, and then frozen in liquid nitrogen. The tubes were placed into the adapter sets, which were fixed by the clamps of the TissueLyser. For homogenization, frozen plant tissue samples were disrupted by TissueLyser. The disruption was performed in two minutes 30 Hz shaking steps. TRI reagent (1 ml) was added, vortexed for 1 min. followed by incubation for 10 min. at room temperature. 400µl chloroform was added, shaken vigorously for 15 s and incubated for 10 min at room temperature before being centrifuged at 12,000 x g for 15 min at 4ºC. The supernatants were transferred to a new tube. 600 µl Isopropanol was added, well mixed, incubated for 10 min., and then centrifuged at 12,000 x g for 10 min at 4ºC. The RNA pellets were washed with 1 ml 75% (DEPC-treated) ethanol and centrifuged at 9,000 x g for 5 min at 4ºC. RNA pellets were air-dried for 10 min. at room temperature. RNAs was dissolved in 40 µl RNase-free water and heated to 55°C for 15 min to activate its RNA protection ability.

The quantity and quality of total RNAs were measured using a NanoDrop spectrophotometer (ND-1000). Their integrity was checked by visualization of ethidium bromide-stained RNA separated on 2% (w/v) agarose gel following stored at -80°C.

3.3.2.2. DNase I Treatment

DNase I treatment of the isolated RNA samples was implemented in 50 µl reactions containing 4 µl RNA (30-40 ng), 5 µl reaction buffer, 2 µl DNase I, 0,5 µl RNase inhibitor, and 38,5 µl DEPC-H_2O. The reactions were kept at 37°C for 30 min. in a water bath. 50 µl DEPC-H_2O and 100 µl Phenol: Chloroform: Isoamyl alcohol (25:24:1) were added to the mixture. Reactions were centrifuged at 10.000 for 5 min. at room temperature. The upper phase (~100 µl) was transferred. 100 µl Chloroform was added into each tube and centrifuged at 10.000 for 5 min. at room temperature. The upper phases (~100 µl) were transferred again and µl NaOAc (3M, pH: 5.2) and 250 µl cold 100% EtOH were added. Reactions were kept -80°C for 20 min., and then centrifuged at 12.000 for 10 min. at 4°C. The upper phases were discarded, and the pellets were washed with cold 70% EtOH (with DEPC water). Reactions were centrifuged at 12.000 for 10 min. at 4°C and pellets were dried and dissolved in a suitable amount of DEPC-water (20 µl). Isolated RNAs were quantified by measuring the absorbance of samples at 260 nm using a NanoDrop spectrophotometer (ND-1000). RNA samples were kept at -80°C until the usage.

3.3.2.3. First-Strand cDNA synthesis

The first strand cDNA was synthesized using a Tetro cDNA Synthesis Kit (Bioline) according to manufacturer's protocol.

3.3.2.4. Degenerate primer design

Firstly, in order to find possible orthologs of genes regulating floral meristem fate the database on protein sequences of legumes (*Medicago truncatula, Lotus japonicus, Cicer arietinum,* and *Phaseolus vulgaris)* together with known fragments of other species (such as pea) was searched. Degenerate primers of 16-24 nucleotides were designed (Table 3.4) and used for isolation since there is no previous molecular and genetic information of *T. turcica*. In order to isolate coding sequences of each particular target gene for avoiding the false isolation of any co-existent paralogues or genes from other families, a series of orthologue amino acid sequences translated from protein for each gene of interest obtained from GenBank was aligned using the Clustal W2 program. Identified mRNA sequences conserved within the close species of *T. turcica* were aligned again, and the degenerate primers were designed. Two forward primers (F) and two reverse primers (R) were designed for each target gene. Sequences of these primers were as follows: (N: ATGC, M: AC, R: AG, Y: CT, W: AT, K: GT, S: GC, H: ACT, B: CGT, V: CG, D: AGT). Oligonucleotides were synthesized by Sentegen.

3.3.2.5. Reverse-Transcriptase (RT) PCR Analysis

Optimized PCR conditions are shown in Table 3.6. The thermal cycler conditions were as follows: initial denaturation at 95ºC for 10 min followed by 35 cycles (at 95ºC, for 30 seconds), annealing step (at a temperature specific for every primer pair, for 30 seconds) and an extension step (at 72ºC, for 1 min). These cycles were then followed by a final extension step at 72ºC for 7 min. Determined the best Tm_s for each primer set were as shown in Table 3.7.

Table 3.6. Optimized PCR conditions

PCR Reaction	Volume Used	Final Concentration
First strand cDNA	x µL	5 ng
10X Taq Polymerase Buffer (+KCL; -$MgCl_2$) Fermentas: Lot: 00061586	2.5 µL	1X
dNTP mix (10 mM each) Fermentas: #R0192	0.5 µL	0.2mM
25 mM $MgCl_2$ Fermentas: 00061590	2.5	2.5mM
Forward Primer (100 µM)	2 µL	0.8µM
Reverse Primer (100 µM)	2 µL	0.8µM
Taq DNA Polymerase (2.5U/µL) Fermentas: #EP0402	0.125 µL	0.125 U/µL
Betaine Sigma: 1 vial B-0300 Lot: 086K6045	6 µL	-
ddH_2O	Up to 25µL	-
Final volume	25µL	-

Table 3.7. Optimized Tm conditions

Primer Name	Tm (°C)
WUS1-WUS2	50
WUS3-WUS4	50
FAS1-FAS2	55
FAS1-FAS3	55
CLV1-CLV2	50
CLV3-CLV4	50
PV-F-PV-R	50

3.3.2.6. Agarose Gel Electrophoresis

PCR products were separated and visualized on agarose gels. Gels were prepared by dissolving the required amount of agarose (2 g depending on the sizes of the DNA fragments in the samples) in 100 mL 0.5X TBE. In order to fully dissolve the agarose, the mixture was heated in a microwave oven. The solution was then cooled down, and 2µl of ethidium bromide was added. After mixing properly, the gel was cast in a gel apparatus and cooled down and solidified. DNA samples were mixed with DNA loading dye were loaded on the gel, which was run at 100V for 45 min in 0.5X TBE and the bands were visualized using UV light on a Biorad Imager. DNA bands approximately the expected size were excised from the agarose gel and extracted using a Qiagen Gel Extraction Kit according to the manufacturer's protocol.

3.3.2.7. DNA Sequencing and Sequence Verification

After successful amplification, purified DNA bands from the agarose gel were sent for sequencing with the same degenerated primers as those used for PCR amplification. Both forward and reverse primers were used in separate sequencing reactions to obtain sequence information from both DNA strands. Sequencing service was commercially provided by MCLAB **(**Molecular Cloning Laboratories), CA, USA. (http://www.mclab.com/). Raw DNA sequence data were checked manually and then forward and reverse complement sequences were aligned using Pairwise Sequence Alignment (Nucleotide) Tool constructed by EMBL-EBI. A consensus sequence was searched via The GenBank database at the National Center for Biotechnology Information (NCBI) using BLAST (Basic Local Alignment Search Tool) search program [108]. To confirm whether the target gene homologs are coming from sequencing, both cDNA and amino acid sequence for each edited sequence were analyzed through the GeneBank database.

3.3.2.8. Quantitative Real-Time (qRT) PCR Analysis

Quantification of cDNA in RT-PCR in combination with SybrGreen I is based on the monitoring of the enhancing fluorescence intensity after each PCR cycle. [109]. Real-Time PCR conditions for SybrGreen quantification were determined based on Roche LightCycler 480 II SybrGreen I Master data sheet. Relative quantitative PCR results were carried out using SensiFAST™ SYBR® No-ROX Kit according to the manufacturer's instructions. mRNA levels were assessed by qRT-PCR using degenerate primers. Primer derived from the 18S rRNA of Fabaceae were used as an internal control, and the expression was normalized to 18S rRNA expression. The qRT-PCR analysis was performed for the partial homologs of *CLV*, *FAS* and *WUS* to analyze the developmental age of a pistil specific expression in *T. turcica.*

20%

3.4. Results and Discussion

On the basis of the results of the morphological and ontogenetic examination of flowers of *T. turcica* in comparison with the monocarpellate flowers of distant species, we have a primary conclusion on a mechanism of gynoecium polymerization in a studied species.

Total RNA Isolation:

Total RNAs, which were needed for cDNA synthesis, were isolated from the pistils of *T. turcica* (target) and *P.vulgaris* successfully (control). Total RNA isolation is one of the most determinants for cDNA synthesis and subsequently qRT-PCR. In the present study, total RNAs were isolated using TRI reagent method as described in method section. The quality of isolated RNAs was in good quality, with a 260/280 ratio of 1.8-2.0.

The integrity of isolated RNAs was determined using agarose gel electrophoresis and was found to be of good quality, with 18S and 28S ribosomal RNA bands (Figure 3.4 and 3.5).

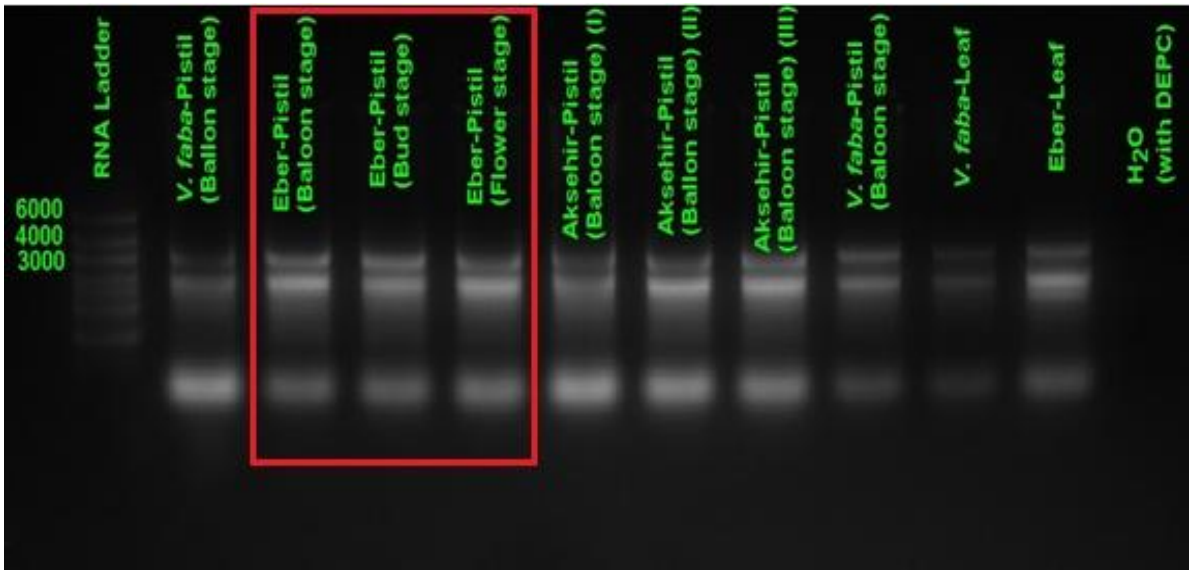

Figure 3.4 Agarose gel electrophoresis results of total RNAs isolated from the pistil samples of *T. turcica* and *V. faba* (without DNase treatment) (RNA Ladder: Thermo Scientific RNA Ladder SM1821) (red frame: pistil samples of *T. turcica* used in the present study)

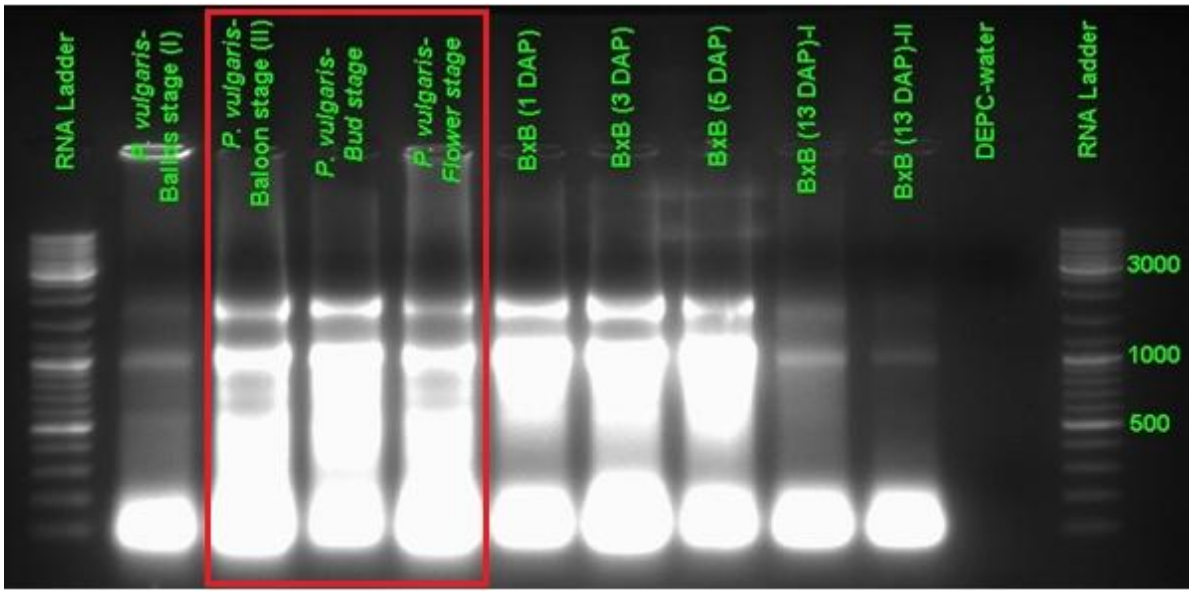

Figure 3.5. Agarose gel electrophoresis results of total RNAs isolated from the pistils of *P. vulgaris* and self-pollinated *Vicia faba* (without DNase treatment) (DNA Ladder: Thermo Scientific DNA Ladder SM0333) (red frame: pistil samples of *V. faba* used for cDNA synthesis)

Reverse Transcription Polymerase Chain Reaction (RT-PCR):

In the present study, cDNAs were used as the template for the PCR-based amplification using degenerate primers designed for target genes. The identification of unknown sequence regarding known sequences is a worthful research in plants in terms of searching biological function.

In the present study, using degenerate primers were important for the purpose of identifying the genes of interest. In the literature, previous studies reported that degenerate primers may not be useful for the identification of unknown sequences using degenerate primers due to designing of these primers on the conserved regions between the sequences of distant relatives [110,111]. However, in the present study, designed degenerated primers were sufficient to identify partial putative homologs of *CLV, WUS,* and *FAS* in targeted species, *T. turcica.*

CLV, *FAS,* and *WUS* genes were identified by the strategy of degenerate primer. Although degenerate primer was also designed to isolate *STP*-like genes in *T. turcica,* the amplification was not achieved for *STP* gene. There are few possible causes for this failure regarding the isolation of *STP*-like genes. First of all reasons are very likely led by degenerate primer design step. For the design of degenerate primer, *STP*-like genes from *Pisum sativum*, *Phaseolus vulgaris*, *Medicago truncatula*, *Cicer arietinum* were selected. Based on the inheritance distance within the species designed primers for *STP* gene might not be sufficient in *T. turcica*. Another reason may be related to amplification process (RT-PCR). PCR conditions such as Tm, the number of the cycle, time of the cycle, etc., might negatively affect the amplification of the desired genes. Degenerate primer design is based on the reverse translation of multiple aligned sequences; however, to amplify a proposed target gene is often a failure due to codon degeneracy and the requirement of the additional degeneracy. Both reasons cause failure in determining suitable annealing temperatures and primer lengths.

The *FAS*, *CLV,* and *WUS* cDNAs amplification was successful by RT-PCR and PCR products were first visualized on 2% agarose gel. The bands in 229 bp were produced by RT-PCR in which *FAS* (1-2) primers were used (Figure 3.6).

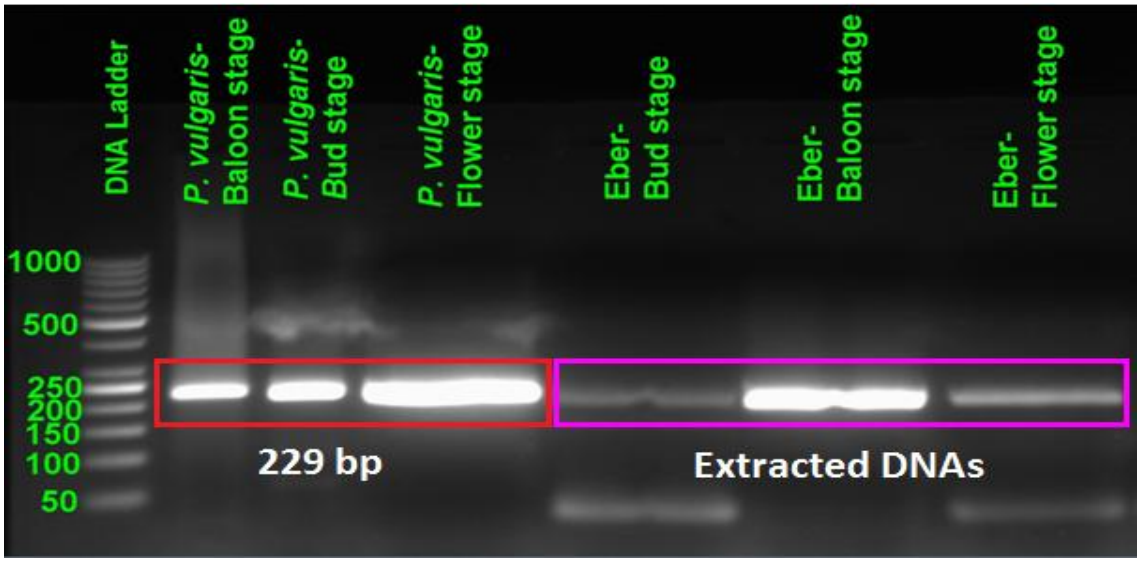

Figure 3.6. Agarose gel electrophoresis results of cDNA synthesis in which *FAS* (1-2) primers were used (DNA Ladder: Thermo Scientific DNA Ladder SM1133) (red frame indicates the region of the samples isolated from *V. faba* and magenta frame determines the regions of *T. turcica* that were excised and used for gel extraction)

To confirm the isolated total RNAs are free from genomic DNAs, RNAs with and without DNase I treatment were amplified using *FAS* (1-2) primers with the same PCR conditions mentioned in method section of this chapter. As a result, after RNA isolation, little genomic DNA contamination was observed, but when DNase I treatment were applied, no contamination was seen. This result was a major RT-PCR study in which used cDNA as a template and genomic DNA was not amplified during reverse transcriptase polymerase chain reaction and subsequent qRT-PCR, verifying the purity of isolated RNA samples (Figure 3.7).

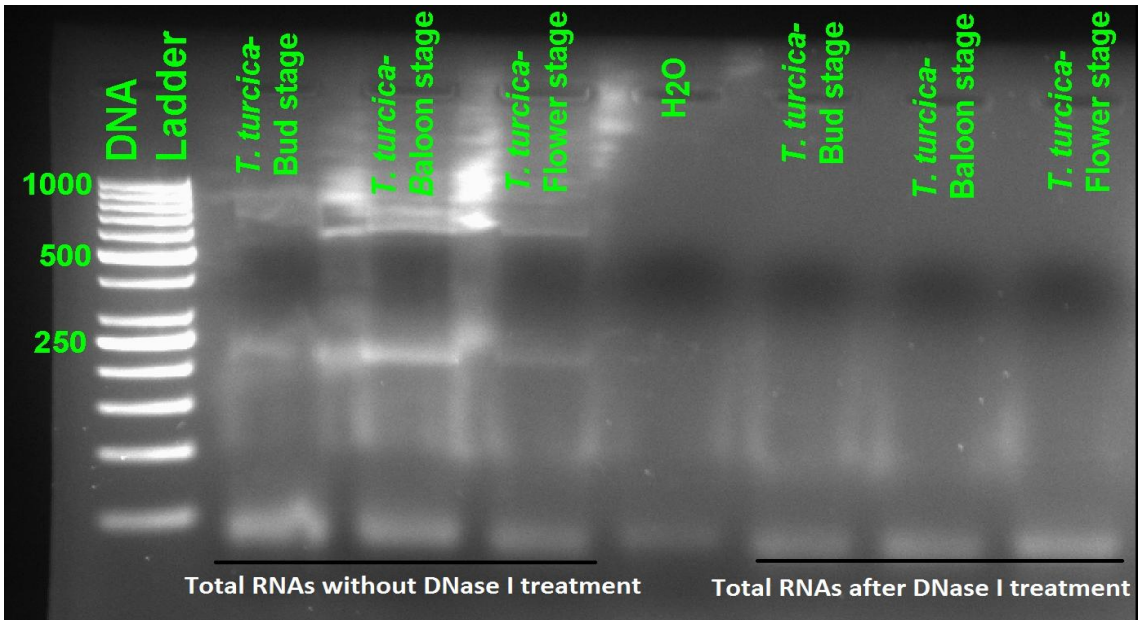

Figure 3.7. Agarose gel electrophoresis results of total RNAs that were amplified using the primer of *FAS* (1-2) (DNA Ladder: Thermo Scientific DNA Ladder SM1133) (the first three samples were the RNAs without DNase I treatment and the last three samples that were applied DNase I)

A nearly 134 bp putative *FAS* homolog for *T. turcica* was obtained when *FAS* (1-3) primers were used (Figure 3.8).

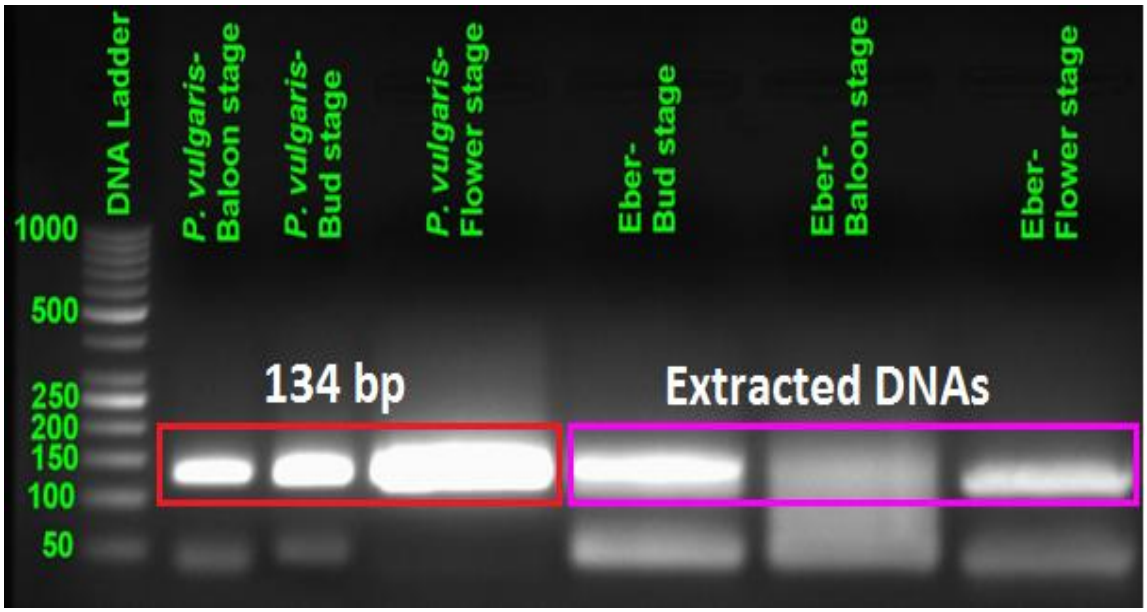

Figure 3.8. Agarose gel electrophoresis results of cDNA synthesis in which *FAS* (1-3) primers were used (DNA Ladder: Thermo Scientific DNA Ladder SM1133) (red frame indicates the region of the samples isolated from *V. faba* and magenta frame determines the regions of *T. turcica* that were excised and used for gel extraction)

As a consequence of RT-PCR in which *WUS* (1-2) primers were used, the bands in different brightness levels were produced. The difference between the more intensive band and the weaker band is possibly related to PCR conditions. Same amount of cDNAs were used, and no differences found between the sequences of the pistil samples at different developmental ages. Based on this, this result was not related to alternative splicing.

A nearly 250 bp putative *WUS* homolog in *T. turcica* was obtained when *WUS* (1-2) primers were used (Figure 3.9).

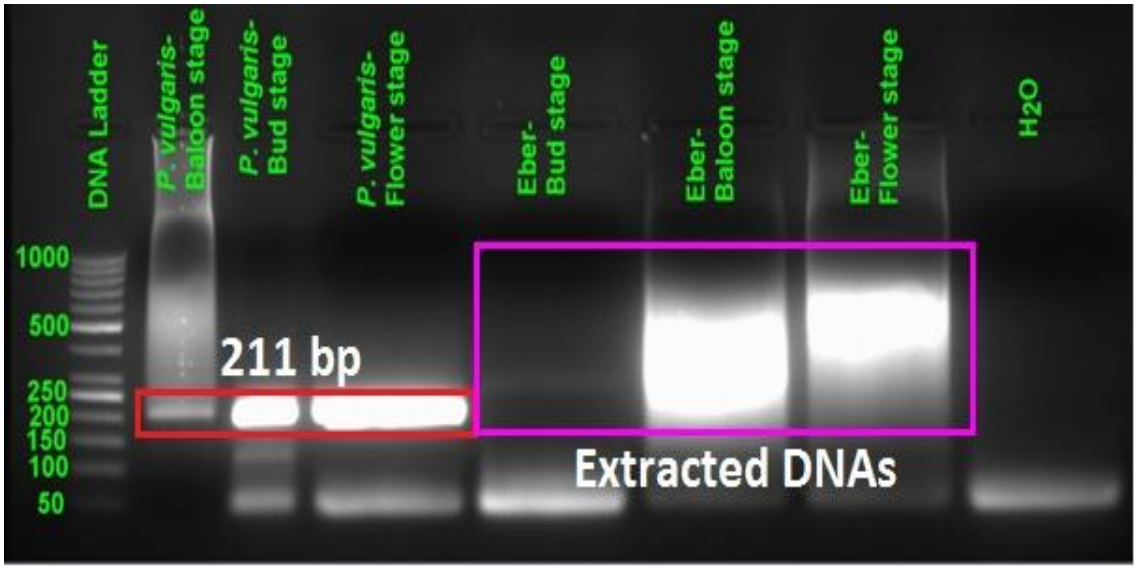

Figure 3.9. Agarose gel electrophoresis results of cDNA synthesis in which *WUS* (1-2) primers were used (DNA Ladder: Thermo Scientific DNA Ladder SM1133) (red frame indicates the region of the samples isolated from *V. faba* and magenta frame determines the regions of *T. turcica* that were excised and used for gel extraction)

A nearly 300 bp putative *WUS* homolog for *T. turcica* was obtained when *WUS* (3-4) primers were used (Figure 3.10). The bands in different lengths were produced by RT-PCR in which *WUS* (3-4) were used as the primers. In the present study, in both control species, *P. vulgaris*, and *T. turcica* the bands which were in unexpected size were seen on the agarose gel. Due to Tm condition non-specific synthesis may have occured. Moreover, since a large number of primers do not attend in amplification of the desired gene, artifactual amplification can be seen in PCR product [110].

In addition, in PCR products which were obtained by the amplification of cDNAs synthesized from the pistil samples at balloon and flower stages no band was observed.

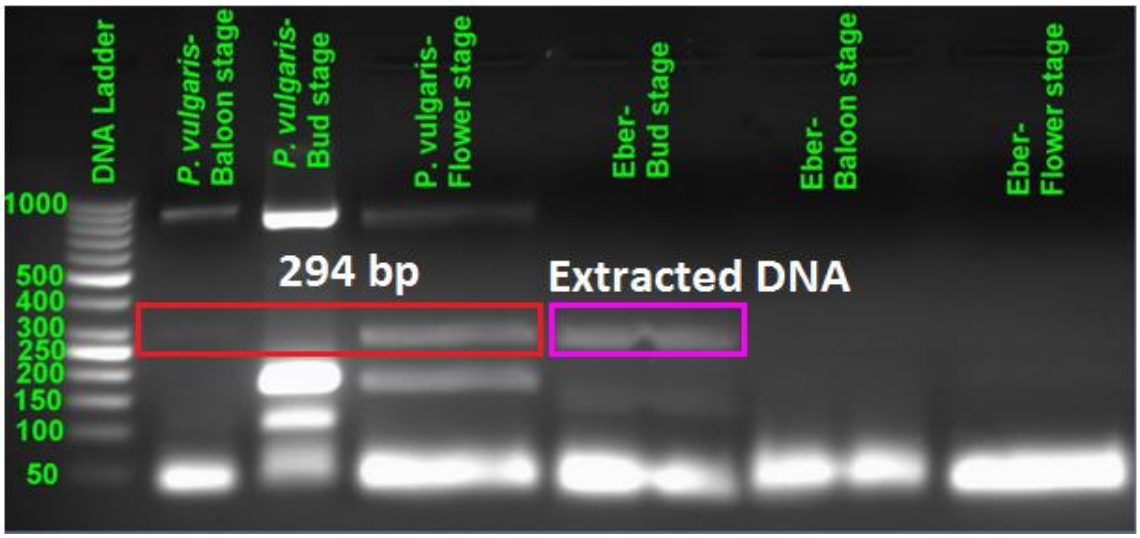

Figure 3.10. Agarose gel electrophoresis results of cDNA synthesis in which *WUS* (3-4) primers were used (DNA Ladder: Thermo Scientific DNA Ladder SM1133) (red frame indicates the region of the samples isolated from *V. faba* and magenta frame determines the regions of *T. turcica* that were excised and used for gel extraction)

A nearly 200 bp putative *CLV* homolog for *T. turcica* was obtained when *CLV* (1-2) primers were used (Figure 3.11). Unexpected band size might have occured due to mismatch between the targeted gene and the primer [110].

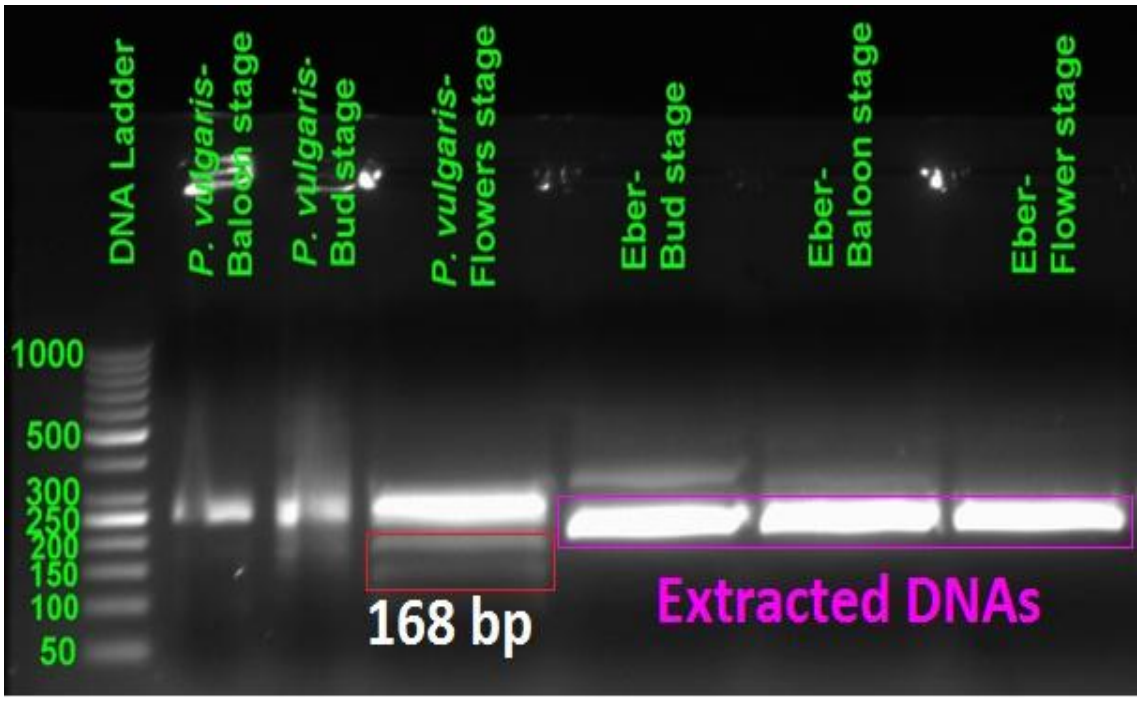

Figure 3.11. Agarose gel electrophoresis results of cDNA synthesis in which *CLV* (1-2) primers were used (DNA Ladder: Thermo Scientific DNA Ladder SM1133) (red frame indicates the region of the samples isolated from *V. faba* and magenta frame determines the regions of *T. turcica* that were excised and used for gel extraction)

A nearly 200 bp putative *CLV* homolog for *T. turcica* was obtained when *CLV* (3-4) primers were used (Figure 3.12).

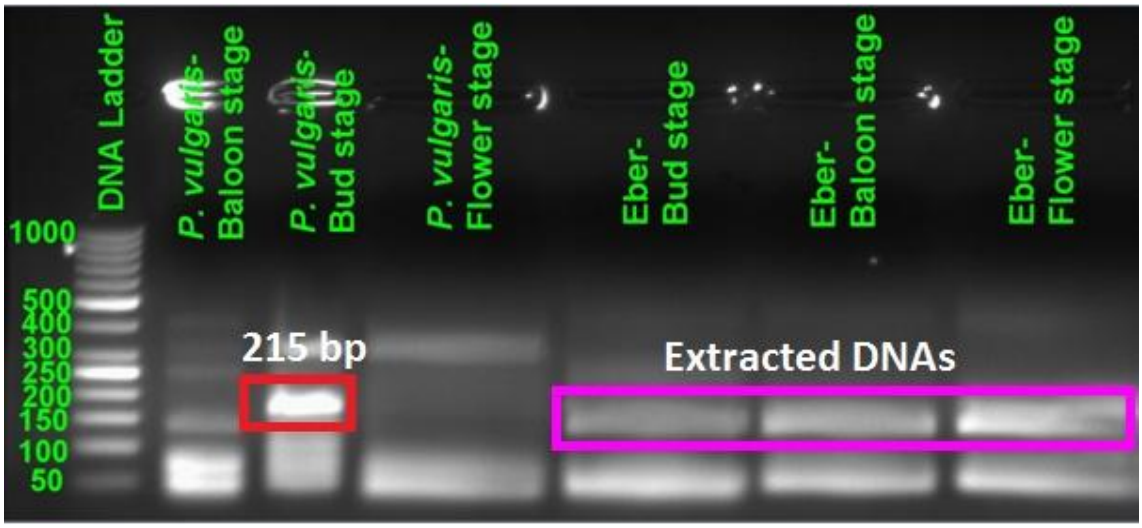

Figure 3.12. Agarose gel electrophoresis results of cDNA synthesis in which *CLV* (3-4) primers were used (DNA Ladder: Thermo Scientific DNA Ladder SM1133) (red frame indicates the region of the samples isolated from *V. faba* and magenta frame determines the regions of *T. turcica* that were excised and used for gel extraction)

Sequencing:

PCR products were obtained using cDNAs of *T. turcica*'s pistils at different developmental ages. To identify the putative orthologues of *FAS, WUS,* and *CLV* in *T. turcica*, purified PCR products of cDNAs were amplified by RT-PCR with degenerated primers. These primers were conceived from conserved regions of proteins available in the GenBank database. Isolated and purified PCR products were directly sequenced.

A 216 bp putative *FAS* homolog for *T. turcica* was obtained and named as TtFasc-1. These partial sequences were deposited the NCBI GenBank with accession number KT001128 (Appendix E). The confirmed cDNA sequence and the deduced amino acid sequences are as given in Table 3.8. Similarly, a verified 283 bp sequence was obtained from PCR product in which *WUS* (1-2) used as a primer pair and was considered to be a putative homolog of dihydropyridine dehydrogenase. It was named Tt-dihydropyridine dehydrogenase (NADP+) and was also deposited the NCBI GenBank with accession number KT182937 (Appendix F). Due to Tm, PCR cycle, primer length and the dominance of the primers unused in PCR, unexpected amplification may have occured [110,111]. The partial putative homolog of dihydropyridine dehydrogenase was obtained based on the reasons mentioned above.

The confirmed cDNA and deduced amino acid sequences of the fragment of each target gene are as shown in Table 3.8.

Table 3.8. Confirmed cDNAs sequences (Open reading frames (ORFs) are highlighted in red)

Gene	**Primer**	**Confirmed cDNAs**	**Translated AA seqs.**
FAS	*FAS* (1-2)	TTTCTGGAGGAGAGTGAACTTCCGCTGTCG CGGCTCGTGTATGATATTTATGAAAAGTTG AAGGTCGCGGCGTTGGTTGAGCCTGTCACG TGCGCGTCGGTGAAGAGTAGCGTGCTTTCT GTTGGGCAGAGAATGGCGTACGGCGTGCC CAATTCTGAAGCTGATGTATTGGAGGACCA TTCCGAATCTTGTTTTTGGTGTTGGGAGACT AGGGAA (216 bp)	FLEESELPLSRLVYDIY EKLKVAALVEPVTCAS VKSSVLSVGQR**M**AYG VPNSEADVLEDHSESC FWCWETRE

FAS	*FAS* (1-3)	TGGAGGAGAGTGAACTTCCCGTTTTCTTGC CCATTTCTCGAACTTTAAGATCCTTCTGGA ACCAATAAAAGAAAAACTCTGCCTTCAATG GGCTCATGGTTTCGTTGGTATTATTGGTTCC TTAATCTTATATGTGTGAAGAGTAGCGTGT TGTTT (156 bp)	WRRVNFPFSCPFLELS DPSGTNKRKTLPS**M**GS WFRWYYWFLNLICVK SSVLF
WUS	*WUS* (1-2)	TCAAAGTAGTACAAGGTGGACCCGACCCA ATAACAAATGGGTTAGGCATGTGCAACCC ATTCACAGTAACACTAAGAGCAGGTTCTGC AGAACCCTGAGTCTCAGAAGCAAAGACCT TAAACCCAATTCTGCTAGGACCAGCATGAA CCTTCTTAGAACAATTCAAAGCAAACCCAG ATGCAGAATTCCCAGTTCTGATCTGGGTCA TGCTCAAAGATGCCATTGTTGAAAGAAAA ATCTCAACTTTCTGCAAAGTGAAGAAGAAG AAAAGGTTCACTTCTGA (283 bp)	SEVNLFFFFTLQKVEIF LST**M**ASLS**M**TQIRTGN SASGFALNCSKKVHAG PSRIGFKVFASETQGSA EPALSVTVNGLH**M**PNP FVIGSGPPCTTL
WUS	*WUS* (3-4)	CATGGGGAATTATGGGTATGGATCTGTGCC CATGGAAAAGAGTTTTTTAGGGACTGCACA ATATCAGCTGGAGGTAGCAGTGGCCATGTT AGGTGGTGGTGCTATAAATCACAATTTGGG ATATTTTGGTGCGGATCCATATTCTTCAGC CTACACTTTCTTTGACAAAATAAGACCAAG TGGAGAAACCCTTGAAGAAGAACAAGTTG AGGATGGTTCCCCTGAAATTGAAACTCTCC CTTTATTCCCTATGC (254 bp)	**M**GNYGYGSVPMEKSF LGTAQYQLEVAVAML GGGAINHNLGYFGADP YSSAYTFFDKIRPSGET LEEEQVEDGSPEIETLP LFPM
CLV	*CLV* (3-4)	GGGACACTTCTGATTGAGCTTTTGTGAATT TGGGGAGTCTATCACCCACTCGATGCTCTC ATCAGTCATGGGCATAGTCACCATACTAGA TATAACAGGATGTGCATACCATCGCTCAAA AACCAAAATGGTCCAGATCCACTACATCAT (150 bp)	GHF**S**LSFCEFGESIHS**M** LSSV**M**GIVTILDITGCA YHRSKTK**M**VQIHYI

Analysis of *FASCIATA* homologues gene in *T. turcica*:

The partial homolog sequences of *FAS* gene identified using *FAS* (1-2) primer pair were aligned with the selected species with regard to *FAS* gene. The result of sequence alignment indicated that *T. turcica* and *M. truncatula* shared the max. identity (70.1%) at the nuclotide level (Figure 3.13) and the identity between *T. turcica* and *M. truncatula* was very high at the amino acid level (Figure 3.14).

Phylogenetic tree was constructed using the translated sequences (Figure 3.15) and it was confirmed that *M. truncatula* and *T. turcica* placed well into the same class. The result of BLAST searching using the amino acid sequences in the GenBank database indicated that the fragment was *FAS1*-like homologue protein. This information confirmed that the isolated partial gene in *T. turcica* was most probably homolugues of *FAS* gene for tested species.

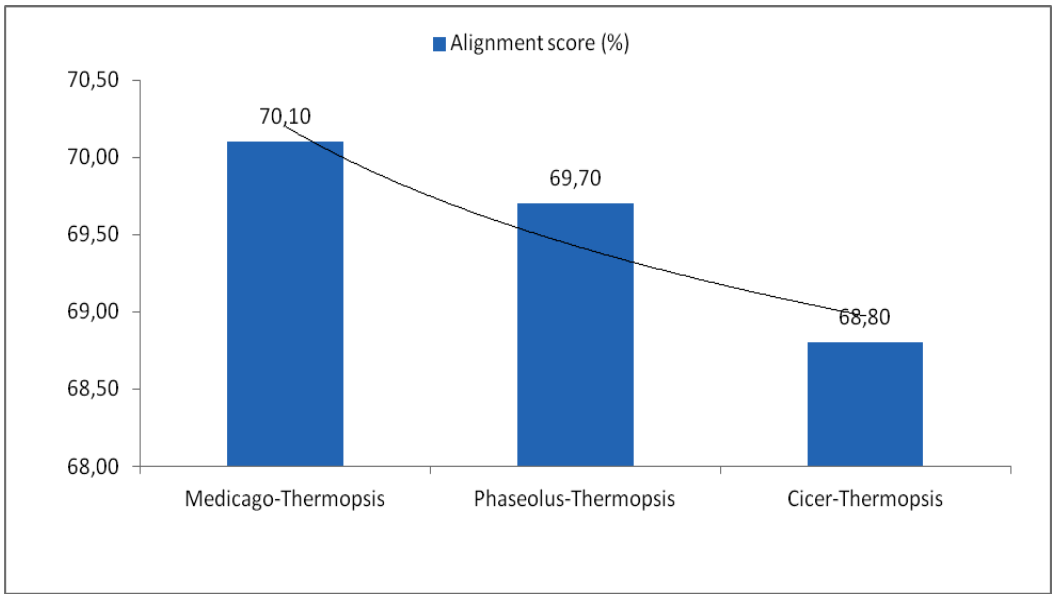

Figure 3.13. Pairwise sequence alignment score between *T. turcica* and other species (*Cicer arietinum*, *Medicago truncatula*, and *Phaseolus vulgaris*) in terms of *FAS* gene amplified by *FAS* (1-2). Species and their GenBank accession numbers are described in Appendix D.

```
Thermopsis      MaYGVPNSEADVLEDHSeSCfWCWET----------Re
Medicago_t      MMYGVPNADADILEDHSDSCLWCWET----------R-
Cicer_arie      MMYGVPNADADILEDHSDSCLWCWET----------R-
Phaseolus       ------metptlmEt-------CwrTtrsrasgvgrRg
```

Figure 3.14. Comparison of amino acid sequences of FAS translated from *FAS* gene amplified by *FAS* (1-2) primer pair in *T. turcica* with FAS proteins of *Cicer arietinum*, *Medicago truncatula*, and *Phaseolus vulgaris* in terms of *FAS* gene amplified by *FAS* (1-2). Species and their GenBank accession numbers are described in Appendix D. Average BLOSUM62 score: Max: 3.0 Mid: 1.5 Low: 0.5

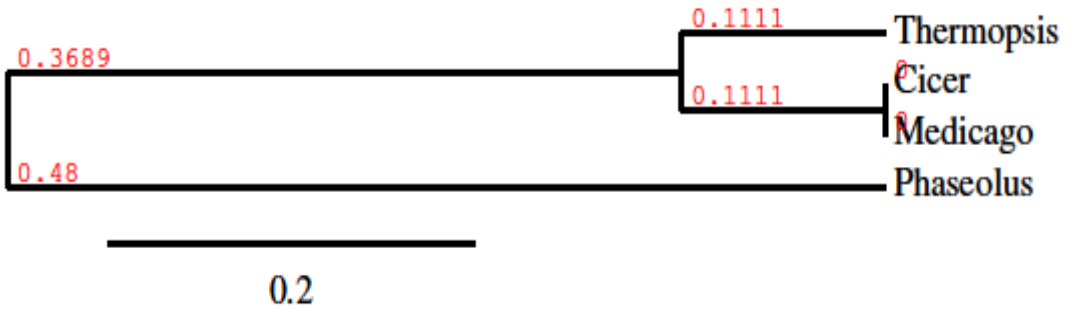

Figure 3.15. Phylogenetic relationship of the amino acid sequences of *T. turcica*, *Cicer arietinum*, *Medicago truncatula*, and *Phaseolus vulgaris* in terms of *FAS* gene amplified by *FAS* (1-2). Species and their GenBank accession numbers are described in Appendix D.

The result of sequence alignment of identified partial sequence using *FAS* (1-3) primers showed that *T. turcica* and *M. truncatula* shared 58.9% identity at nucleotide level (Figure 3.16). 6 residues were highly conserved within the species and phylogenetic tree was constructed using the amino acid sequences across all the analyzed species (Figure 3.17 and 3.18).

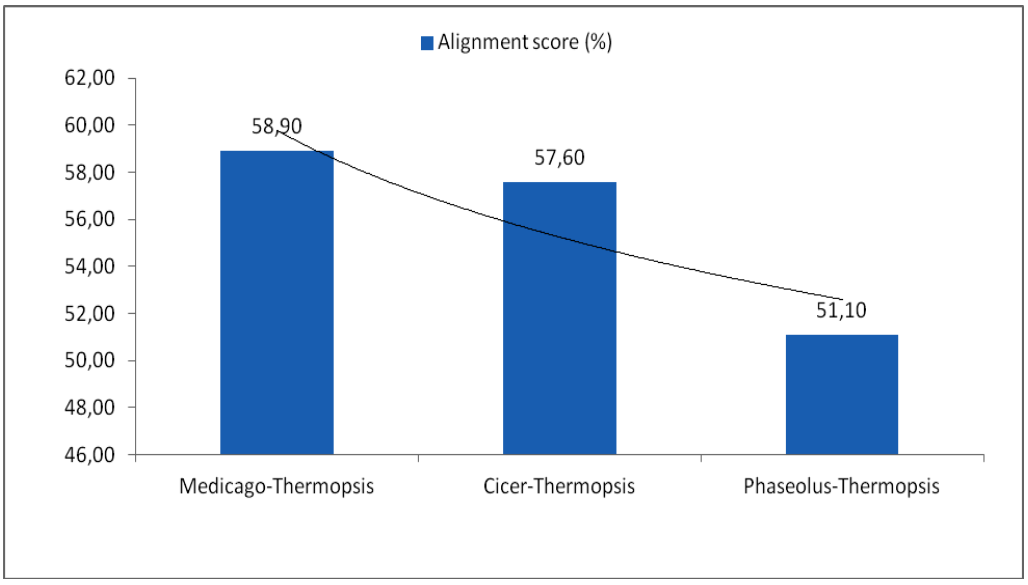

Figure 3.16. Pairwise sequence alignment score between *T. turcica* and other species (*Cicer arietinum*, *Medicago truncatula*, and *Phaseolus vulgaris*) in terms of *FAS* gene amplified by *FAS* (1-3). Species and their GenBank accession numbers are described in Appendix D.

```
Cicer_arie      -----MrFMgsMgeLCWRKvLtLLW
Medicago_t      -----MrFMrrMrvLCWRKgLiLLW
Thermopsis      mgswfrwyywflnliCvkssvlf—
```

Figure 3.17. Comparison of amino acid sequences of FAS translated from *FAS* gene amplified by *FAS* (1-3) primer pair in *T. turcica* with FAS proteins of *Cicer arietinum* and *Medicago truncatula* in terms of *FAS* gene amplified by *FAS* (1-3). Species and their GenBank accession numbers are described in Appendix D. Average BLOSUM62 score: Max: 3.0 Mid: 1.5 Low: 0.5

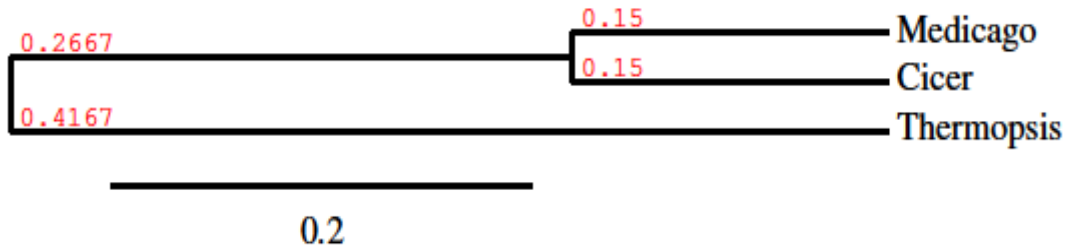

Figure 3.18. Phylogenetic relationship of *T. turcica*, *Cicer arietinum* and *Medicago truncatula* in terms of *FAS* gene amplified by *FAS*(1-3). Species and their GenBank accession numbers are described in Appendix D.

Analysis of *WUSCHEL* homologues gene in *T. turcica*:

Pairwise sequence alignment of the identified partial homolog sequence of *WUS* gene using *WUS* (1-2) in *T. turcica* indicated that the max. sequence alignment score (49,4%) was between *P. vulgaris* and *T. turcica* as shown in Figure 3.19. Amino acid sequences of tested species were somewhat similar; 9 residues were mostly conserved (Figure 3.20). The phylogenetic construction showed that *T. turcica* was different from tested species (Figure 3.21).

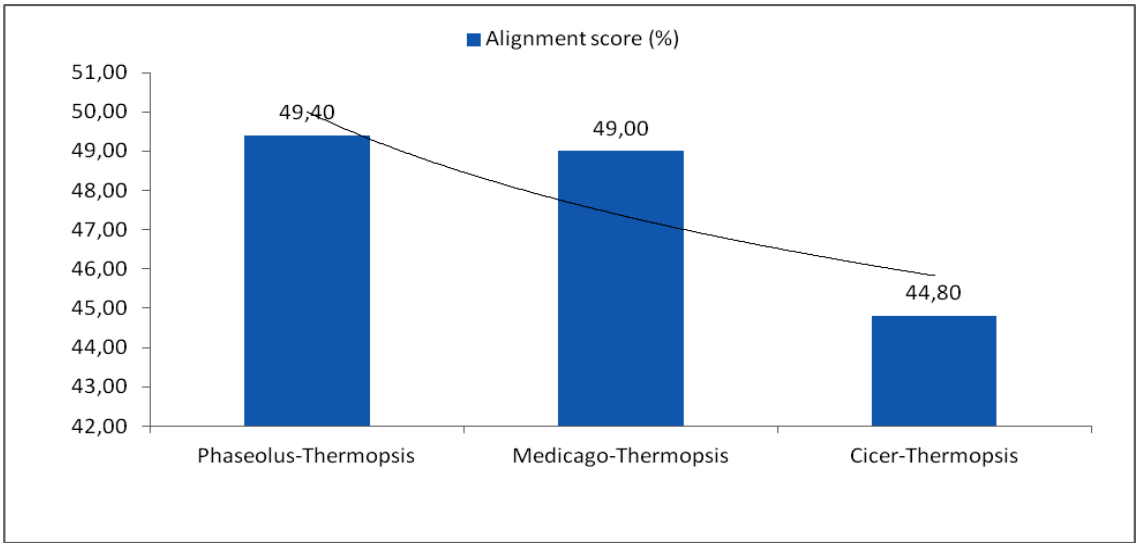

Figure 3.19. Pairwise sequence alignment score between *T. turcica* and other species (*Cicer arietinum*, *Medicago truncatula*, and *Phaseolus vulgaris*) in terms of *WUS* gene amplified by *WUS* (1-2). Species and their GenBank accession numbers are described in Appendix D.

```
Phaseolus       metELDpQVQsRFRGSLLGM----------etSFIGsrtTKLEKgRRKGSLL--------
Cicer_arie      --MELDpQVQnRFRGSLLGM------------SFIGFKITKLEKDRRKGSLL--------
Medicago_t      --MELDhQVQnRFReSLLGMv-----RLkAkmSFIGFKITKLEKDRRKGSLL--------
Thermopsis      --MaslsmtQiRtgnSasGfalncskKVhAgpSrIGFKVfasEtqgsaepaLsvtvnglh

Phaseolus       ----------------
Cicer_arie      ----------------
Medicago_t      ----------------
Thermopsis      mpnpfvigsgppcttl
```

Figure 3.20. Comparison of amino acid sequences of WUS translated from *WUS* gene amplified by *WUS*(1-2) primer pair in *T. turcica* with WUS proteins of *Cicer arietinum*, *Medicago truncatula* and *Phaseolus vulgaris* in terms of *WUS* gene amplified by *WUS*(1-2). Species and their GenBank accession numbers are described in Appendix D. Average BLOSUM62 score: Max: 3.0 Mid: 1.5 Low: 0.5

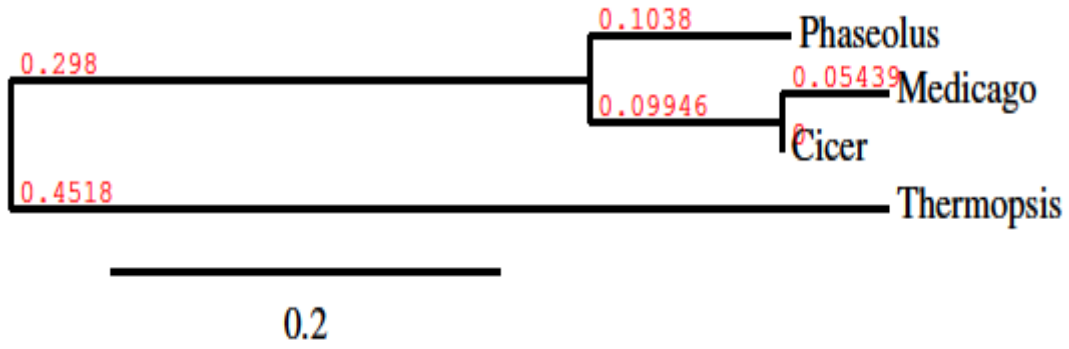

Figure 3.21. Phylogenetic relationship of *T. turcica*, *Cicer arietinum*, *Medicago truncatula*, and *Phaseolus vulgaris* in terms of *WUS* gene amplified by *WUS* (1-2). Species and their GenBank accession numbers are described in Appendix D.

The result of BLAST searching using the amino acid sequences of the identified *WUS* gene using *WUS* (1-2) primer pair in the GenBank database indicated that the fragment was dihydropyrimidine dehydrogenase (NADP+)-like homologue protein.

The isolated partial *WUS* gene using *WUS* (3-4) showed that the idenity of the sequences within the tested species were higher than that of *WUS* (1-2). The max. identity was found between *C. arietinum* and *T. turcica*, with 71,6% identity (Figure 3.22). The result of BLAST searching using the amino acid sequences in the GenBank database indicated that the fragment was *WUSCHEL* ortholog.

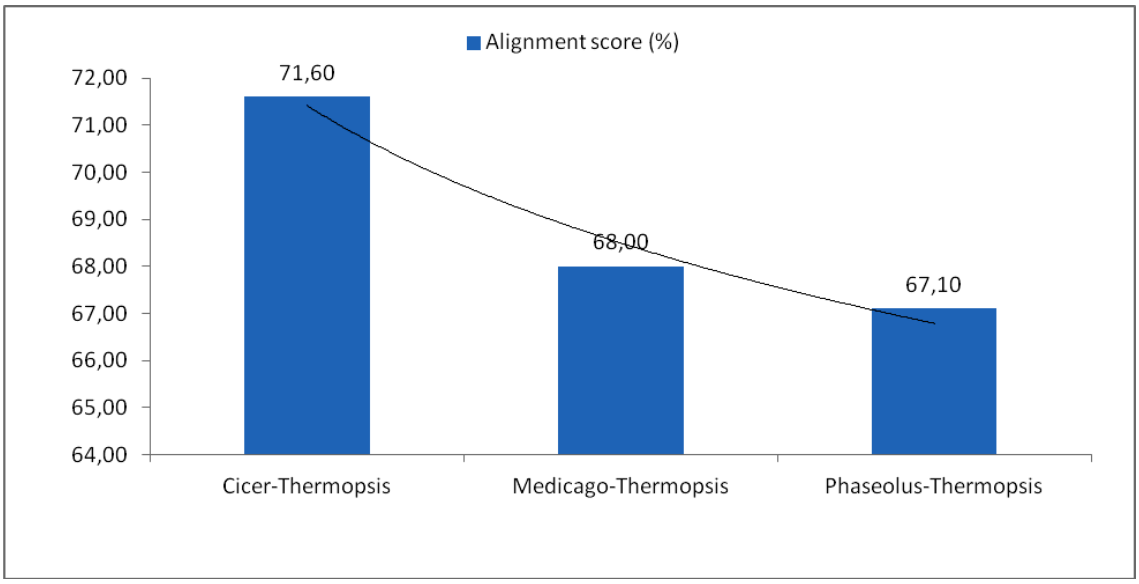

Figure 3.22. Pairwise sequence alignment score between *T. turcica* and other species (*Cicer arietinum*, *Medicago truncatula*, and *Phaseolus vulgaris*) in terms of *WUS* gene amplified by *WUS* (3-4). Species and their GenBank accession numbers are described in Appendix D.

The amino acid sequences clearly confirmed the relationship of these species in terms of *WUS* gene. Amino acid sequences of tested species were mostly conserved (Figure 3.23). The phylogenetic tree constructed using amino acid sequences and all species tested were grouped well into the *WUS* gene family (Figure 3.24).

```
Thermopsis      ------mGNYGYGSVPMEKSFlgtaqyqlEvavamlg-----GgaINHnLGyfG--aDPY
Phaseolus       MiTVGQmGNYGYGSVPMEKSF-------RdCsISAGgSSGHVG--INHnLGWVG--VD--
Cicer_arie      MVTVGQIGNYGYGSVPMEKSF-------RECTISAGCSSGHVGSTINHHLGWVG-HVgPY
Medicago_t      MVTVGQIGNYGYGSVPMEKSF-------RECTISAGCSSsqVGSTINpHiGWIGhHVDPY

Thermopsis      SSAYt-FFDKIRPsgEtL-------EEEQvEDGSPEIETLPLFPM
Phaseolus       SSAYANFFDKIRPNEEtLeeeeeEEEEEEEEDGgaEIETLPLFPM
Cicer_arie      SStYANlFeKIRPeEEIm------EEEqEEEnGSPEIETLPLFPM
Medicago_t      SSAYANlFeKIRPNEEIm-----EEydqgQEnGSPEIETLPLFPM
```

Figure 3.23. Comparison of amino acid sequences of WUS translated from *WUS* gene amplified by *WUS* (3-4) primer pair in *T. turcica* with WUS proteins of *Cicer arietinum*, *Medicago truncatula* and *Phaseolus vulgaris* in terms of *WUS* gene amplified by *WUS* (3-4). Species and their GenBank accession numbers are described in Appendix D. Average BLOSUM62 score: Max: 3.0 Mid: 1.5 Low: 0.5

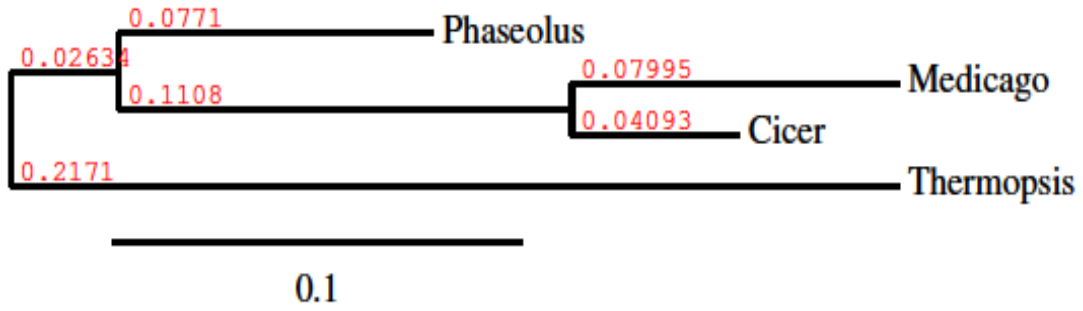

Figure 3.24. Phylogenetic relationship of *T. turcica*, *Cicer arietinum*, *Medicago truncatula*, and *Phaseolus vulgaris* in terms of *WUS* gene amplified by *WUS* (3-4). Species and their GenBank accession numbers are described in Appendix D.

Analysis of *CLAVATA* homologues gene in *T. turcica*:

The partial homolog sequences of *CLV* gene identified using *CLV* (3-4) primer pair were aligned with the selected species with regard to *CLV* gene. The result of sequence alignment indicated that *T. turcica* and *C. arietinum* shared the max. identity (47.4%) at the nuclotide level (Figure 3.25) and at the amino acid level, the identity between *T. turcica* and *C. arietinum* was higher than that of other species (Figure 3.26). Phylogenetic tree was constructed using the translated sequences (Figure 3.27) and was confirmed that *T. turcica* and *C. arietinum* fell well into the closely class.

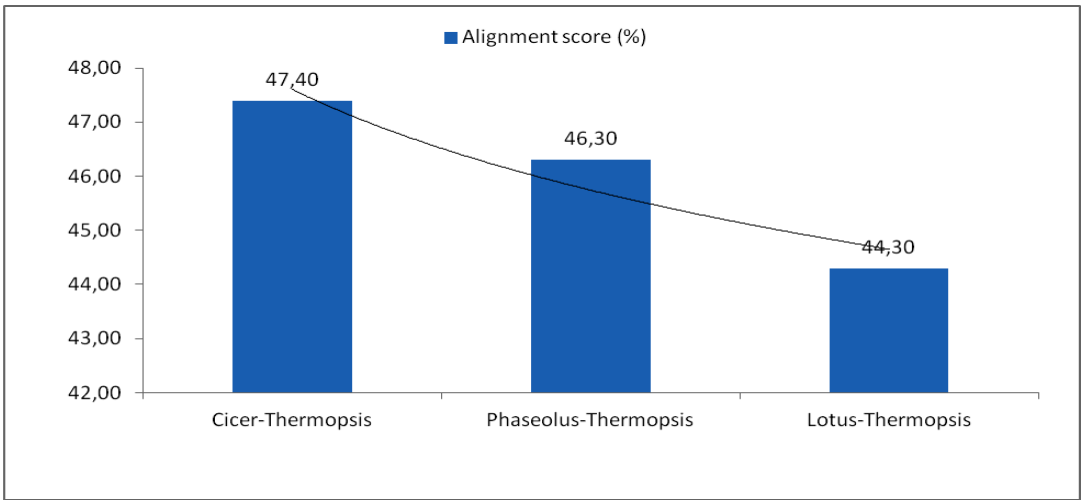

Figure 3.25. Pairwise sequence alignment score between *T. turcica* and other species (*Cicer arietinum*, *Phaseolus vulgaris, and Lotus japonicus*) in terms of *CLV* gene amplified by *CLV* (3-4). Species and their GenBank accession numbers are described in Appendix D.

```
Phaseolus       mlddsCklafREvf---LsfktdSAflfgmspRTaalapKhsyaePqPegs
Lotus_japo      -----mlpmVaqql--------------------------------vPrrf
Cicer_arie      -----mlfgLKDkkslMvnlkgSStrikydeKSligelRkvptGPdPlHH
Thermopsis      ----mCipsLKNqn-------------------------------GPdPlHH
```

Figure 3.26. Comparison of amino acid sequences of WUS translated from *WUS* gene amplified by *WUS* (1-2) primer pair in *T. turcica* with WUS proteins of *Cicer arietinum*, *Medicago truncatula* and *Phaseolus vulgaris* in terms of *WUS* gene amplified by *WUS* (1-2). Species and their GenBank accession numbers are described in Appendix D. Average BLOSUM62 score: Max: 3.0 Mid: 1.5 Low: 0.5

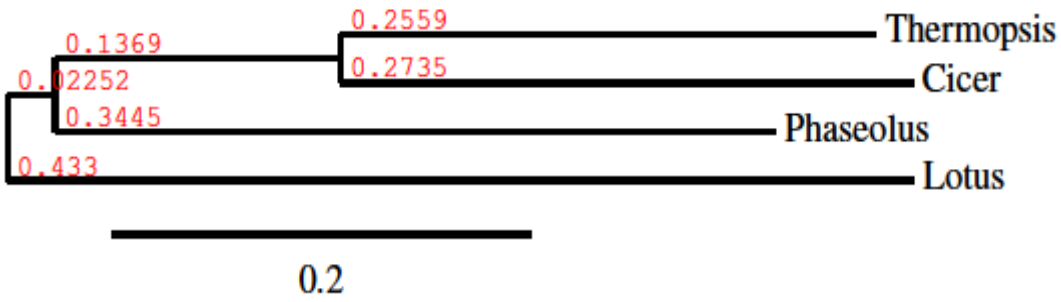

Figure 3.27. Phylogenetic relationship of *T. turcica*, *Cicer arietinum*, *Lotus japonicus*, and *Phaseolus vulgaris* in terms of *CLV* gene amplified by *CLV* (3-4). Species and their GenBank accession numbers are described in Appendix D.

According to previous studies, multiple homologue genes can be found multiple in the same species [107,112]. In the present study, only PCR products of proper size were sent to MCLAB for the sequencing and southern hybridization assay was not implemented.

Therefore, the copy number of the identified genes in *T. turcica* cannot be determined. In addition, degenerate primers were used during RT-PCR followed by sequencing due to the circumstance that no molecular data was available in *T. turcica*. Using degenerate primers may have caused some problems. These include the amplification of more than one fragment in proper size during PCR and sequencing all of them [107]. To cope with these eventualities, at least three separate PCR products for the same reaction were sequenced in this dissertation. However the identities of putative gene sequences at amino acid level were remarkably low. This could have occured due to wide taxonomic distance within the tested species and the variations between the homologues from each gene class. Since the identified sequences using two primer pairs designed for the same gene covered different regions of the gene of interest, the variation between the putative sequences might not be effective on the whole gene level, but could effect the positions of the identified sequences in the phylogenetic tree.

It was reported that ectopic expression of *AGAMOUS* (AG) in *Arabidopsis thaliana* influences carpel development alone [97]. Moreover, *LEAFY* (*LFY*) and *APETELA1* (*AP1*) are together sufficient to activate class C genes [90]. Besides *LFY* and *AP1* genes, there are negative regulators of floral organ identity genes termed as cadastral genes which are boundary lines. The *SUPERMAN* (*SUP*) gene of *A. thaliana* and *LEUNIG* (*LUG*) are in the cadastral genes [113]. Furthermore, the *COCHLEATA* (*COCH*) gene is another factor of the increasing carpel number in Fabaceae [2]. Based on this knowledge, while the genes *CLV*, *WUS*, *STP* and *FAS* may strongly influence the gynoecium polymerization in legumes, further investigation on the effects of the genes *AG*, *LFY*, *AP*, *SUP*, *LUG*, *COCH* in multicarpellary feature in *T. turcica* will give an idea on carpel polymerization.

From the point of view of the present study, RT-PCR was unable to determine the full-length of the sequences (*FAS, WUS,* and *CLV*); however partial sequences of desired genes were identified and these data obtained from the sequencing have moved the molecular study related to carpel polymerization in *T. turcica* a step forward.

However, further studies related to expression patterns and functions of identified sequences in different tissues of *T. turcica* should be performed to answer these questions: (1) Have these identified genes same roles in the different tissues of *T. turcica* and (2) in various plant species such as *C. arietinum*, *M. truncatula*, *P. vulgaris*, and *L. Japonicus*?

qRT-PCR Results:

The expressions of *WUS*, *FAS*, and *CLV* in the different developmental stage of the pistil isolated from *T. turcica* were investigated in a 96 well plate in a total volume of 20µl by qRT-PCR which is more useful to state small amounts of samples. This data helped us to focus our expression studies on a certain group of genes.

During qRT-PCR analysis, 18S rRNA identified in the family of Fabaceae [107](Table 3.8) was used as an internal reference to analyze the results of the PCR reactions and its amplification in the linear phase at low cycle number was important to quantitate the expression levels of the targeted genes.

The qRT-PCR analysis revealed that the partial ortholog gene isolated using *FAS* (1-2) primer expressed in the pistils at the different development stage in *T. turcica;* however, the max. expression was calculated in the bud stage of the pistil of *P. vulgaris*, with almost 8 fold compared to *T. turcica*. On the other hand, in *T. turcica*, the gene of interest expressed in all developmental stage of the pistil, with low expression level. In addition, the gene amplified using the primer *FAS* (1-3), the expression was seen only in the control group with high expression level at balloon stage. Thus, *FAS* gene was mostly active at the early age of pistil in *P. vulgaris* (Figure 3.28).

The gene amplified using *WUS* (1-2), the gene of interest expressed in all samples tested in *T. turcica*, with low expression level as seen in the usage of *FAS* (1-2), but the expression levels were opposite in *WUS* (1-2) to *FAS* (1-2). In control group, *P. vulgaris*, the max. expression was seen at flower stage.

It is, hence, likely that the *WUS* gene was mostly active at late age of pistil in *P. vulgaris*. In the expression analysis in which used *WUS* (3-4) primer pair, the signal in some samples was not detected, so the results obtained when the using *WUS* (3-4) was not efficient to give an idea on the expression pattern of *T. turcica*. Because of this reason, only the result coming from the usage of WUS (1-2) was considered (Figure 3.28).

CLV gene amplified using *CLV* (1-2) primers was detected in the pistils at balloon stage of control group whereas no signal was detected in those of *T. turcica*. On the other hand, *CLV* gene amplified using *CLV* (3-4) primers expression was detected in all samples isolated from *T. turcica*, but max. expression level was quantified when the development of pistil was at balloon stage in *T. turcica*. In the control group, the expression level of the target gene was also expressed in the pistil at all development stage, with low expression level when compared in *T. turcica*. Based on this result, the level of expression of *CLV* increases sharply at the early development stage of the pistil in *T. turcica* and for this reason, *FAS* and *WUS* gene expression may be inhibited.

The PCR efficiency may be affected by different primer pairs in the same gene due to the different size of primer, product length, and annealing temperature [107].

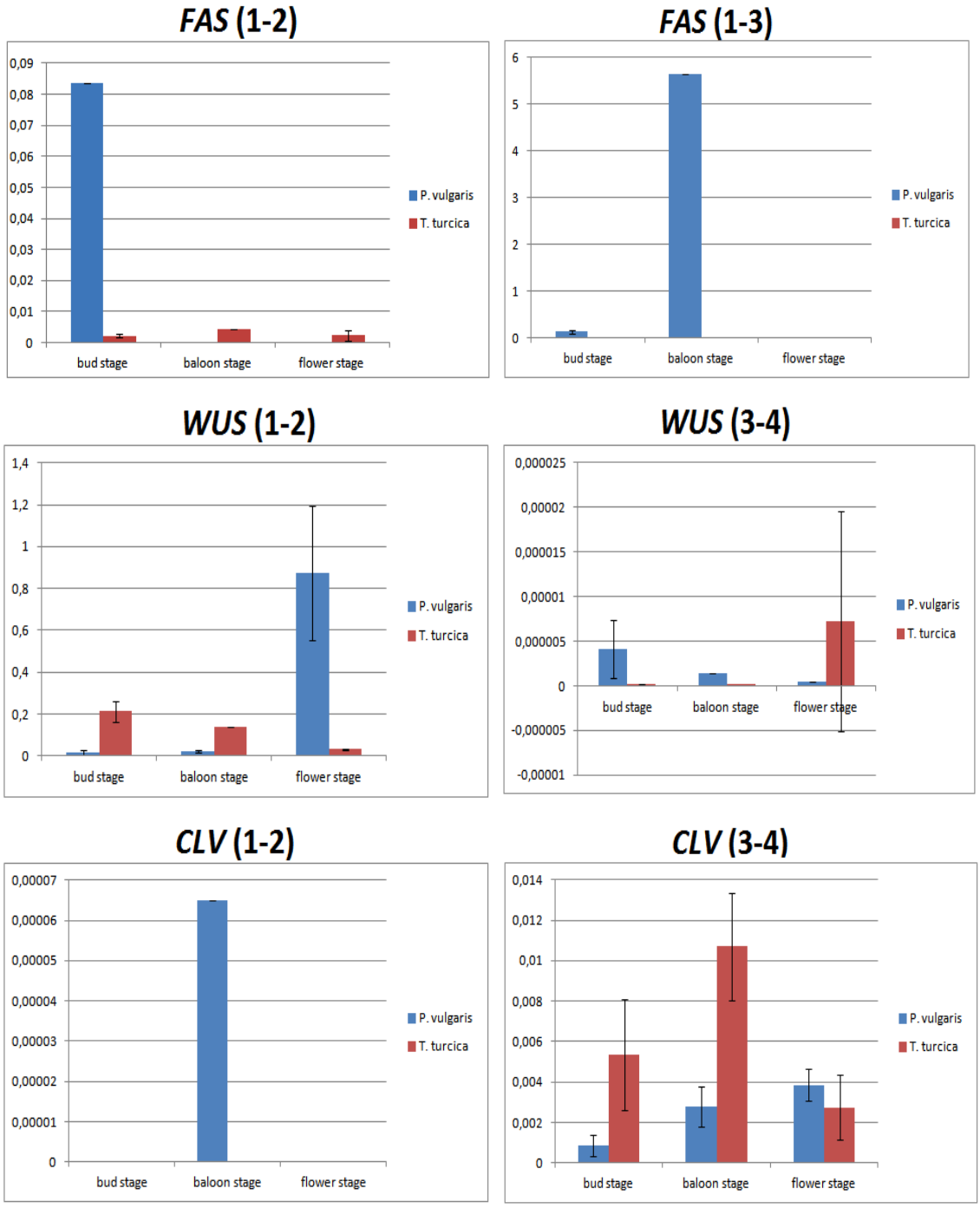

Figure 3.28. Comparison of the expression level of cDNA synthesized using designed degenerate primers. Values were the mean relative Ct(Cp) values of three replicates. 18S rRNA was used as an internal reference

As a result, qRT-PCR results were not in good agreement with RT-PCR data. It might have happened due to several reasons. Possibly, during qRT-PCR degenerate primers were used and based on the degeneracy of primers PCR reactions were affected.

Alternatively, it is more probable that the quantity of the transcript may be too low to detect the signal during qRT-PCR, to exemplify, if the gene of interest is expressed at a very low level or temporarily expressed. It seems that further analysis should be conducted using newly designed primers across the identified partial orthologs of the *CLV*, *WUS*, and *FAS* genes.

3.5. Conclusion

In the present study, degenerate primers were designed across the multiply aligned protein sequences of the selected species for each target gene.

As a result of molecular analysis of the genes related to carpel polymerization in *T. turcica*, little but highly important data was obtained. Partial homologs of *CLV*, *FAS*, and *WUS* in *T. turcica* were identified using designed degenerate primers. However, the full length of the target genes were not determined but data generated from the present study will lead to reaching this aim. Furthermore, from the study in which *WUS* (1-2) primers were used to amplify the target sequence dihydropyrimidine dehydrogenase (NADP+)-like partial sequence of cDNA synthesized from the pistil of *T. turcica* was identified.

In the future, research into the function of identified putative orthologs in *T.turcica* will serve as a clue to better understand the mechanism of polycarpellary gynoecium in legumes. These data may, on one hand, provide information on the evolution of studied genes within a family and, on the other hand, uncover regulatory mechanisms of the ontogeny of the multicarpellate leguminous flower.

4. GENERAL CONCLUSION AND FUTURE WORK

There are primarily two outcomes of the presented dissertation: the fertilization biology of *T. turcica* was identified at first, and the putative partial homologs of key genes in flower buds of *T. turcica* were isolated. These results might be used for other related crops of economic importance in order to obtain cultivars having polycarpellary feature and will represent the first scientific report about the molecular basis of the multicarpellary feature in *T. turcica*. The other outcomes obtained from this thesis and studies expected to be implemented in the future are as follows:

1. Hybridization was conducted for the first time between *V. faba* and *T. turcica* during this dissertation. However, as a consequence of histological analysis followed by crossing the prerequisites including compatibility between the parents embryo age to be cultured for embryo rescue were determined and no hindrance in pollen germination and pollen tube growth was observed. Furthermore, crossability of *T. turcica* and the influence of cross direction on the success of obtaining hybrids were revealed by histological analysis.

2. The transfer of useful traits of *T. turcica* (2n=2x=18) into another species by sexual crosses may be restricted with the same ploidy level and therefore somatic hybridization can be an alternative way to overcome the fertilization barriers for hybridization studies in *T. turcica.* Furthermore, *in vitro* fertilization can be utilized to identify the molecular mechanisms in the embryogenesis in *T. turcica* as an experimental system.

3. The knowledge obtained from Chapter 2 can be easily used in practical applications to improve new cultivars with desirable properties. In addition, in the present study, to avoid the damage of the embryo during the isolation of the embryos, ovule culture was carried out to prevent embryo injury and it is achieved when the hybrids between *V. faba* and *T. turcica* were used.

4. Perturbation auxin level in *T. turcica* may affect the gynoecium structure in early embryogenesis, for this reason, further study related to auxin influx, and efflux may be implemented. As a result of embryo rescue study, due to the change of cross direction hybrids from the cross *V. faba* X *T. turcica* were not achieved to grow *in vitro*. Based on this, the post-fertilization barrier may be broken by applying plant growth regulators, either alone or in combination in the medium. As can be seen from this knowledge, different media should be tested for understanding the artificial environment beneficial to grow hybrids.

5. Increasing the number of cross pollinations may provide an excellent chance to obtain hybrid plants by embryo culture. Furthermore, hybridization between *T. turcica* and other species such as *Lens*, *Phaseolus*, *Cicer*, etc. should be conducted for not only improving new cultivars but also revealing the possibilities of *T. turcica* in breeding programs to increase yield in legumes. Moreover, with regards to pollinator potential of *T. turcica* various species in Fabaceae for crossing combinations should be chosen, as well.

6. To obtain hybrids between *T. turcica* and any species in Fabaceae *in vitro* fertilization (IVP) following *in vitro* culturing may be an alternative way.

7. In the present study, in hybridization used *T. turcica* as a female parent, hand-pollination was implemented on the surface of the stigma, and there was no penetration of pollen into the ovule. In further studies, pollens may be directly introduced to the excised gynoecium of *T. turcica*, so that pre-fertilization barriers such as the incompatibility between parents may be eliminated.

8. In this dissertation, degenerate primers were used in expression analysis and the results were not completely clear. Therefore, it will be better to analyze the expression levels of identified partial sequences using the primers synthesized newly as sequence specific and are not degenerate.

9. To search the genes related to multicarpellary feature of *T. turcica* will be a source for the hypothesis named 'hopeful monster' [80,82,114] which is macromutation occured in controlling genes that regulate developmental process of a plant at early age. This mutation may occur in the timing, and the position of the developmental process and its effect may be seen in the phenotype of a plant. Based on this, *T. turcica* can give an idea of the evolution and the phylogeny of developmental process in plants due to its 2-4 free carpellary ovary.

10. Finally, *T. Turcica* can serve as a model in the search for the answer of the remaining question; how does multicarpellary gynoecium influence the fruit differentiation?

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LIST OF PUBLICATIONS

This publication states the term, September 2009 – August 2015 executively, since I have accepted as a Ph.D. student at Sabanci University.

A. PUBLICATIONS:

1. Tekdal D., Cetiner S. 2014. Efficient Embryogenic Callus From Filaments With Anther In *Thermopsis turcica. Turkish Journal of Agricultural and Natural Sciences,* 1: 1242-1246 (Research Article).
2. Tekdal D., Cetiner S. 2014. In-Ovule Embryo Culture of *Thermopsis turcica*. *Journal of Animal and Plant Sciences*, 26(6): 1673-1679 (Research Article).
3. Tekdal D., Cetiner S. 2014. *In vitro* plant regeneration derived from leaf and stem explants of endemic *Thermopsis turcica. Biologia*, 69(7): 863-869 (Research Article).
4. Tekdal D., Cetiner S. 2014. Determination of Mineral Element Content of *In vitro* propagated Turkish endemic *Thermopsis turcica* Kit Tan, Vural & Kucukoduk (*Fabaceae*). *Bagbahçe-Bilim,* 1(1): 35-43 (Research Article).
5. Tekdal D., Cetiner S. 2014. Determination of Self-Compatibility Status of *Thermopsis turcica* Through Histological Analysis. *Journal of Applied Biological Sciences,* 8(1): 64-67 (Research Article).
6. Tekdal D., Cetiner S. 2013. The Effects of Different Combinations and Varying Concentrations of Growth Regulators on The Regeneration of Selected Turkish Cultivars of Melon. *In: Current Progress in Biological Research, Agricultural and Biological Sciences (ed. Silva-Opps, Marina),* InTech, Croatia (Book Chapter).

B. PROCEEDINGS:

1. Tekdal D, Cetiner S. 2014. 'Efficient Embryogenic callus from filaments with anther in Thermopsis turcica,' Balkan Agriculture Congress, Trakya University Plant Breeding Research Center, Edirne, Turkey. Abstract Book.
2. Tekdal D, Cetiner S, Yalcin-Mendi Y, Eti, S. 2014. Histological analysis of hybrids from *Thermopsis turcica* and *Vicia faba*. New Phytologist Next Generation Scientists Meeting, 29-30 July, John Innes Center, Norwich, UK. Abstract book, pp. 46.
3. Tekdal D, Cetiner S, Eti, S. 2014. Determination of Self-Compatibility Status of *Thermopsis turcica* Through Histological Analysis. 3rd International Congress of Molecular Biology and Biotechnology, Sarajevo, Bosnia-Herzegovina. Abstract Book, pp. 2
4. Tekdal D, Cetiner S. 2013. Plant Regeneration via Organogenesis in Turkish Endemic, *Thermopsis turcica*. 1th Central Anatolia Region Agriculture and Food Congress (TARGID). Abstract Book 1, Plant Production, pp. 388
5. Karadag BN, Yildirim EC, Tekdal D. 2013. The Comparison of The Plant Regeneration Between *Jerusalem artichoke* and The Peruvian Potato Cultured on MS medium with different Concentrations and Combinations of Plant Growth Regulators. 3rd International Congress on Bioscience, Biochemistry and Bioinformatics, Rome, Italy. International Journal of Bioscience, Biochemistry and Bioinformatics, 3(2): 120-124.
6. Tekdal D, Cetiner S. 2013. Molecular and Genetic Characterization of ovarium development genes by cDNA-AFLP in *Thermopsis turcica*. Black Forest Summer School-Bioinformatics for Molecular Biologists. Abstract Book, pp. 13
7. Tekdal D, Cetiner S. 2013. Ovule-embryo Culture and Plant Regeneration of *Thermopsis turcica*, Critically Endangered Turkish Endemic. In Vitro Cell. Dev. Biol. 49 (Suppl.): 35-36
8. Tekdal D, Cetiner S. 2012. Isolation and Characterization of *Agrobacterium tumefaciens* strains from the artificial lake water. 15th European Congress on Biotechnology. 23-26 September, Istanbul, Turkey. New Biotechnology, 29 (Suppl.): 177

APPENDIX

APPENDIX A: Chemicals Used in the Study

Chemicals and Media Components	Supplier Company
Agarose	peQLab, Germany
Boric Acid	Molekula, UK
Bromophenol Blue	Sigma, Germany
Distilled water	Millipore, France
DNA Gel Loading Solution, 5X	Quality Biological, Inc, USA
EDTA	Applichem, Germany
Ethanol	Riedel-de Haen, Germany
Ethidium Bromide	Sigma, Germany
Hydrochloric Acid	Merck, Germany
Isopropanol	Riedel-de Haén, Germany
Liquid nitrogen	Karbogaz, Turkey
Methanol	Riedel-de Haen, Germany
Monoclonal Anti-HA Antibody	Sigma, Germany
Phenol-Chloroform-Isoamyl alcohol	Amersco, USA
RNase A	Roche, Germany
Tris Hydrochloride	Amresco, USA

APPENDIX B: Equipment Used in the Study

Equipment	Company
Autoclave	Hirayama, Hiclave HV-110, Japan
Balance	Sartorius, BP221S, Germany
	Schimadzu, Libror EB-3200 HU, Japan
Centrifuge	Eppendorf, 5415D, Germany
	Hitachi, Sorvall RC5C Plus, USA
Deepfreeze	-80°C, Forma, Thermo ElectronCorp., USA
	-20°C, Bosch, Turkey
Distilled Water	Millipore, Elix-S, France
Electrophoresis Apparatus	Biogen Inc., USA
	Biorad Inc., USA
Electroporator	Neon Transfection System - Life Technologies, USA
Gel Documentation	Biorad, UV-Transilluminator 2000, USA
Heater	ThermomixerComfort, Eppendorf, Germany
Ice Machine	Scotsman Inc., AF20, USA
Incubator	Memmert, Modell 300, Germany
	Memmert, Modell 600, Germany
Laminar Flow	Kendro Lab. Prod., Heraeus, HeraSafe HS12, Germany
Liquid Nitrogen Tank	Taylor-Wharton,3000RS, USA
Magnetic Stirrer	VELP Scientifica, ARE Heating Magnetic Stirrer, Italy
Microliter Pipettes	Gilson, Pipetman, France
	Eppendorf, Germany
Microscope	Olympus CK40, Japan
	Olympus CH20, Japan
	Olympus IX70, Japan
	Zeiss Confocal LSM710, German
Microwave Oven	Bosch, Turkey
pH meter	WTW, pH540 GLP MultiCal, Germany
Power Supply	Biorad, PowerPac 300, USA
Refrigerator	Bosch, Turkey
Shaker Incubator	New Brunswick Sci., Innova 4330, USA
Spectrophotometer	Schimadzu, UV-1208, Japan
	Schimadzu, UV-3150, Japan
Thermocycler	Eppendorf, Mastercycler Gradient, Germany
Vortex	Velp Scientifica, Italy

APPENDIX C: DNA Molecular Weight Markers

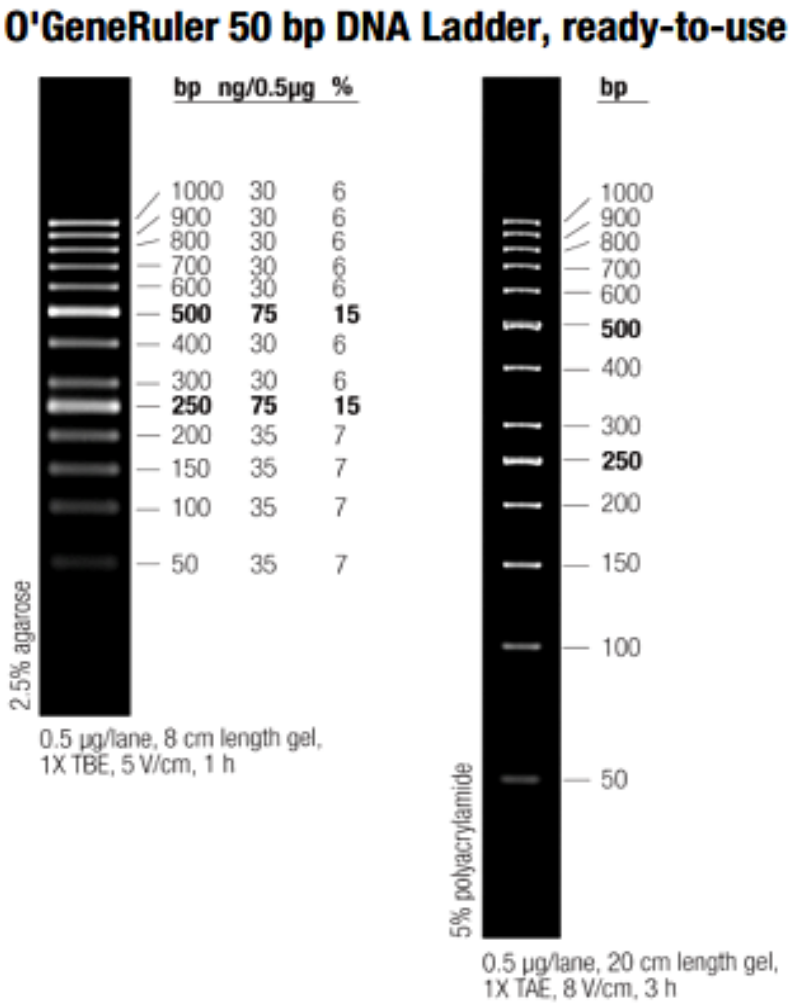

Figure C.(1). DNA Ladder (Fermentas SM1133)

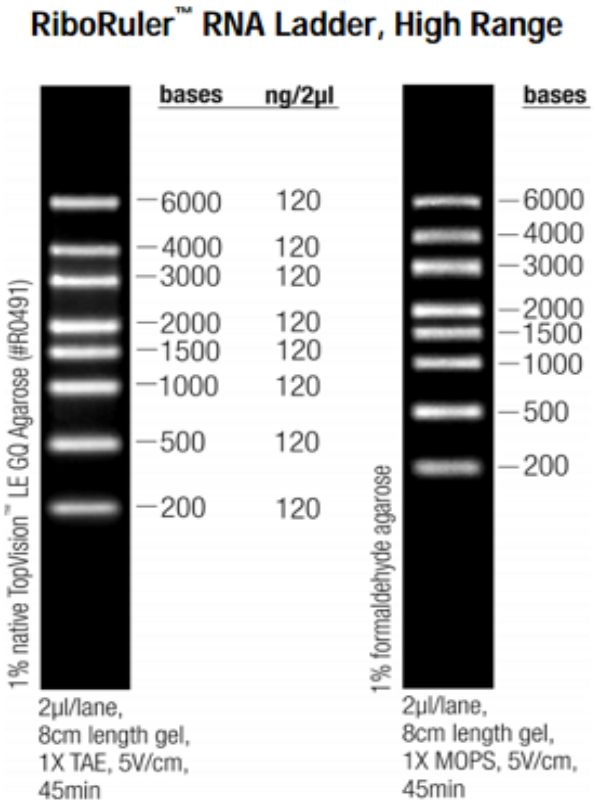

Figure C.(2). RNA Ladder (Fermentas SM1821)

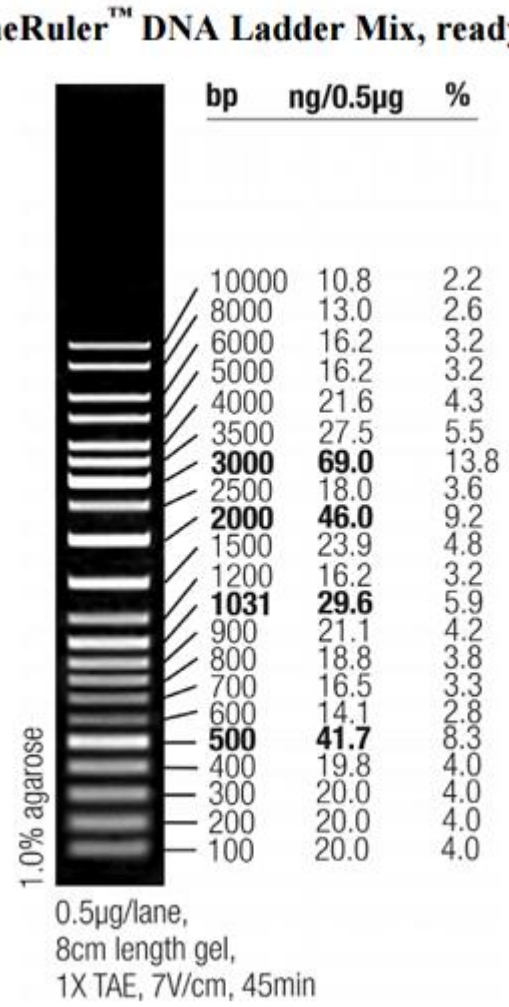

Figure C.(3). DNA Ladder (Fermentas SM0333)

APPENDIX D: *CLV, STP, FAS,* and *WUS* gene homologues used for mRNA sequences comparison and analysis of *T. turcica* putative genes

Family	mRNA	Species	Accession No.
CLAVATA (*CLV*)			
Fabaceae	*CLAVATA* 3-like	*Cicer arietinum*	XM_004487668.1
Fabaceae	*CLAVATA*3	*Lotus japonicus*	AB709900.1
Fabaceae	PHAVU_005G120600g	*Phaseolus vulgaris*	XM_007149973.1
FASCIATA (*FAS*)			
Fabaceae	*FAS*1-like	*Cicer arietinum*	XM_004492810.1
Fabaceae	MTR_7g080500	*Medicago truncatula*	XM_003624168.1
Fabaceae	PHAVU_008G051800g	*Phaseolus vulgaris*	XM_007139637.1
STAMINA PISTILLOIDA (*STP*)			
Fabaceae	LOC101505902	*Cicer arietinum*	XM_004504590.1
Fabaceae	MTR_126s0008	*Medicago truncatula*	XM_003638251.1
Fabaceae	PHAVU_002G188300g	*Phaseolus vulgaris*	XM_007158804.1
Fabaceae	*STP*	*Pisum sativum*	AF004843.1
WUSCHEL (*WUS*)			
Fabaceae	*WUSCHEL*-like	*Cicer arietinum*	XM_004512172.1
Fabaceae	*WUSCHEL* (WUS)	*Medicago truncatula*	FJ477681.1
Fabaceae	PHAVU_002G109400g	*Phaseolus vulgaris*	XM_007157862.1

APPENDIX E: Documents recieving from NCBI related to depositing *FASCIATA*-like partial gene sequences to GenBank

Sabancı Üniversitesi

Dilek Tekdal <dilektekdal@sabanciuniv.edu>

GenBank KT001128

7 ileti

gb-admin@ncbi.nlm.nih.gov <gb-admin@ncbi.nlm.nih.gov> 2 Haziran 2015 21:00
Alıcı: dilektekdal@sabanciuniv.edu

Dear GenBank Submitter:

Thank you for your direct submission of sequence data to GenBank. We have provided a GenBank accession number for your nucleotide sequence:

Updated_TtFasc.sqn TtFasc-1 KT001128

The GenBank accession number should appear in any publication that reports or discusses these data, as it gives the community a unique label with which they may retrieve your data from our on-line servers. You may prepare and submit your manuscript before your accession is released in GenBank.

Submissions are not automatically deposited into GenBank after being accessioned. Each sequence record is individually examined and processed by the GenBank annotation staff to ensure that it is free of errors or problems.

You have requested that your data are to be held confidential until:

May 31, 2017

They will not be released to the public database until this date, or until the data or accession numbers appear in print, whichever is first.

Since the flatfile record is a display format only and is not an editable format of the data, do not make changes directly to a flatfile. For complete information about different methods to update a sequence record, see: http://www.ncbi.nlm.nih.gov/Genbank/update.html

Any inquiries about your submission should be sent to gb-admin@ncbi.nlm.nih.gov

For more information about the submission process or the available submission tools, please contact GenBank User Support at info@ncbi.nlm.nih.gov.

Please reply using the original subject line.
This will allow for faster processing of your correspondence.

Sincerely,

Mark A. Landree, PhD
Contractor

The GenBank Direct Submission Staff
Bethesda, Maryland USA
****

gb-admin@ncbi.nlm.nih.gov (for updates/replies to GenBank entries)
info@ncbi.nlm.nih.gov (for general questions regarding GenBank)

APPENDIX F: Documents recieving from NCBI related to depositing dihydropyrimidine dehydrogenase (NADP+)-like partial gene sequences to GenBank

Sabancı Üniversitesi

Dilek Tekdal <dilektekdal@sabanciuniv.edu>

GenBank KT182937

gb-admin@ncbi.nlm.nih.gov <gb-admin@ncbi.nlm.nih.gov> 19 Haziran 2015 23:35
Alıcı: dilektekdal@sabanciuniv.edu

Dear GenBank Submitter:

Thank you for your direct submission of sequence data to GenBank. We have provided a GenBank accession number for your nucleotide sequence:

Tt.sqn Tt-dihydropyrimidine KT182937

The GenBank accession number should appear in any publication that reports or discusses these data, as it gives the community a unique label with which they may retrieve your data from our on-line servers. You may prepare and submit your manuscript before your accession is released in GenBank.

Submissions are not automatically deposited into GenBank after being accessioned. Each sequence record is individually examined and processed by the GenBank annotation staff to ensure that it is free of errors or problems.

You have requested that your data are to be held confidential until:

Dec 30, 2015

They will not be released to the public database until this date, or until the data or accession numbers appear in print, whichever is first.

Since the flatfile record is a display format only and is not an editable format of the data, do not make changes directly to a flatfile. For complete information about different methods to update a sequence record, see: http://www.ncbi.nlm.nih.gov/Genbank/update.html

Any inquiries about your submission should be sent to gb-admin@ncbi.nlm.nih.gov

For more information about the submission process or the available submission tools, please contact GenBank User Support at info@ncbi.nlm.nih.gov.

Please reply using the original subject line.
This will allow for faster processing of your correspondence.

Sincerely,

DeAnne Olsen Cravaritis, Ph.D.

The GenBank Direct Submission Staff
Bethesda, Maryland USA

gb-admin@ncbi.nlm.nih.gov (for updates/replies to GenBank entries)
info@ncbi.nlm.nih.gov (for general questions regarding GenBank)
www.ncbi.nlm.nih.gov/books/NBK51157/ GenBank Submissions Handbook