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Isolation and Expression Analysis of Meristem Gene *CLV*2 and Carpel Generation Gene (*CRC*) from Multi-Carpel Structure of *Thermopsis turcica*

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Abstract

Thermopsis turcica is an endemic species of wild legume in Türkiye. The plant produces flower pistils with unusual morphological properties. The aim of this study was to determine the factors controlling the interesting feature of this plant. Particularly the controlling features of the flower meristem and the pistils that mutations in two genes and helps occurring the number of flower organs and can cause abnormalities, This study has attempted to explore the controlling feature of *Thermopsis turcica*, the young bud *Arabidopsis CLV*2, and *CRC* gene homologs were isolated and analyzed in different tissues. First, with total RNA isolated from the first thread degenerated primers synthesized cDNA expressions, second with a partial length cDNA cloning process made and the replicated set of analyses. The full-length cDNA of genes related to RACE analysis was carried out with the sequencing process. The real-time PCR method was applied for gene functional analyses with buds, sepals, petals and stamen and relative expression analysis of genes in the tissue of pistils. Full-length cDNA sequence for the gene *CLV*2 2557 bp, the encoded protein 716 amino acids, *CRC* 901 for full-length cDNA sequence of the gene—bp, the encoded protein 174 identified as amino acids. Alignment and phylogenetic analyses of *CLV*2 and *CRC* genes were found to be similar to other types of legumes in homologous sets. Real-time (RT-qPCR) PCR results relative to study courses with genes.

Keywords

Thermopsis turcica, *CLV*, *CRC*, cDNA, PCR, RACE, RT-qPCR, *In Situ*

1. Introduction

Meristem tissue on the shoots and apical ends contains meristematic or undifferentiated cells that are able to divide rapidly for developing new tissues and organs, which can go for further differentiation under optimum conditions, and provide the fundamental structure of the plant body [1]. In favorable environmental conditions, an inflorescence meristem can be transformed from the shoot apical meristem by modifying into sepals, petals, stamens, and carpels of the flower, which is called as flowering stage. In this developing stage, plants are able to distinguish and respond to various environmental factors, *i.e.* temperature, day length, etc. which activates developmental regulation by signalizing a complicated gene network [1]. These genes exhibit both combined and independent functions for turning the shoot meristem into an inflorescence meristem by integrating the response to characteristic photoperiod, vernalization and gibberellins (GA) level [2].

The *Thermopsis* genus includes in total of 25 species that are naturally distributed in mountainous moist regions of Central Asia and North America [3]. (*Thermopsis* is only represented in Türkiye by an endemic species known as *Thermopsis turcica* [4], which is most abundant in the south region of Lake Eber of Afyon province and the south-west coasts of Akşehir Lake in Afyonkarahisar and Konya provinces of Türkiye. *T. turcica* and other species of *Thermopsis* are rhizome-producing perennial herbs belonging to the family Fabaceae (Leguminosae). The majority of legume plants usually contain pentamerous flowers with five sepals, five petals, two rings of five stamens each and a single median carpel, in total 21 flower organs [5]. Nevertheless, polycarpellary condition or uniform occurrence of three free carpels containing flower has been reported only in *T. turcica* which is a very unique character for Leguminosae, and could be a potential source of the gene pool, which could be transferred into cultivars for creating multiple free fruits in more economic ways. Hence, analyzing the molecular structure of this flower is very significant to understand its genetical constitution which could be helpful for further advanced research.

Intended to accomplish this abovementioned reasons, two meristem genes of *T. turcica*, namely, CLAVATA2 (*CLV2*) [6] and CRABS CLAW (*CRC*) [7], capable of causing that were structural and functional characterization of *T. turcica.*

In this study, CLAVATA2 (*CLV2*) [6] and CRABS CLAW (*CRC*) [7], which are *T. turcica* meristem genes and can cause multiple carps in case of mutation, were performed.

The unique character of *T. turcica* is the uniform occurrence of three free carpels. The polycarpellary condition of *T. turcica* is clearly derived, rather than being a primitive character since the whole Papilionoid pollination syndrome has obviously evolved around the presence of a single median carpel [8] [9]. Although the presence of polycarpellary condition has been previously documented in normal members of the Mimosoideae and Caesalpinoideae, apart from teratological forms, this is

the first record in the Faboideae [8].

In this study, structural and functional characterization of CLAVATA2 (*CLV*2) [5] and CRABS CLAW (*CRC*) [7], which are *T. turcica* meristem genes and can cause multiple carps in case of mutation, were performed. For this purpose, RNA isolation was made from the relevant tissues of the plant. Degenerate primers and the first thread cDNAs of flower buds were used to partially sequence the isolation of genes. Rapid duplication (RACE) analysis of cDNA ends was used to determine full-length cDNA sequence. Isolation of target genes has been proven by testing nucleotide and amino acid sequences with a bioinformatics tool. Additionally, the probable numbers of copies of these genes in the *T. turcica* genome were analyzed by the Southern blotting method.

Finally, semi-quantitative expression analysis of 18 different reproductive, fruit and vegetative tissues of 2 genes, which have direct or indirect relation with flowering and whose full-length cDNAs were determined in this study, were performed successfully.

2. Materials and Methods

2.1. Plant Sampling

The samples of *T. turcica*, which spread in the conservation area on the southern shores of Eber Lake, Afyonkarahisar, were sampled between April 20 and May 20, 2015. It is wrapped in aluminum foil packs, to be 150 - 1000 mg depending on the presence of vegetative and reproductive tissues to be used in gene sequence and quantitative PCR studies. Plant sampling was done at four different times, before pollination, after pollination, young fruit period and mature fruit period. The samples of the study consisted of seven different buds from the top end of the *T.turcica* plant in the racemose flower state, according to their size, seven different buds, from the flowers before and after pollination, sepal, petal, stamen, carpel tissues, vegetative (shoot stem, young and mature leaves) and fruit upright (young fruit, ripe fruit, ripe fruit peel and ripe fruit seed) tissues.

The sampled plant tissues were packed in one gram, frozen in liquid nitrogen and stored in a −86˚C ultra freezer until working time. Sampling operations were carried out quickly in order not to damage DNA/RNA assets in tissues.

2.2. Total RNA Isolation from *Thermopsis turcica* Buds

Total RNA isolated from the young flower buds of *T. turcica* was used in gene isolation. Total RNA isolation was performed from all of the tissues mentioned (**Figure 1**) for use in qPCR analysis. The environment, bench, devices and consumables using RNA isolation have been tried to create RNase-free enzyme-free medium using RNaseZapTM (Ambion), 75% EtOH, dense bleach and UV lamp. Also, during studies, extreme care was taken to sterile study to prevent RNase contamination.

RNA extraction was performed in accordance with the kit protocol using the PureLinkTM RNA Mini (Ambion, USA) RNA isolation kit. In summary, 250 mg

Figure 1. *Thermopsis turcica* bud sizes after pollination.

tissue sample was poured into liquid nitrogen in cooled sterile mortar and brought to henna consistency. 150 mg of forged tissue sample was transferred to a pre-cooled 1.5 mL Ependorf tube and 1 mL lysis buffer was added to the tube. After a short vortex, the tube was centrifuged at high speed for 5 minutes at room conditions. 1 mL supernatant was transferred to a clean tube. 0.5 mL of absolute ethanol was added and vortexed briefly, and the mixture was loaded into spin cartridges. After centrifugation at 12,000 g for 90 seconds, the RNA sample was cleaned with wash buffer I and II. RNA was eluted by centrifuging at room conditions by adding 100 µl of RNase-free water to the cartridge taken into a clean tube.

RNA extract was removed from possible DNA contamination using the DNAfreeTM DNase (Ambion, USA) kit. Briefly, 10 µl of DNase I Buffer and 1 µL of rDNase were added to the RNA extract and mixed gently. After 30 minutes of incubation in a 37˚C water bath, the reaction was terminated by adding 5 µL of DNase inactivation agent to the mixture. The transparent liquid phase was carefully transferred to a clean tube after centrifugation at 10,000 ×g for 2 minutes at room temperature.

Quantification of the RNAs obtained was performed by measuring the Qubit photometer 2 device using the QubitTM (Invitrogene) Assay kit. The measurement mixture was prepared according to the QubitTM protocol and measurement was made with RNA sample between 1 - 20 µL. For each sample, the mixture was prepared with 199 µL of Qubit buffer and 1 µL of Qubit agent. The sample and measurement mixture were transferred into the same tube, with a final volume of 200 µL, and briefly vortexed. The measurement was carried out by incubating for 2 minutes. The measured samples were stored in ultra-freezer at −86˚C.

Synthesis of cDNA from total RNA isolated from tissues RevertAid was performed using the first thread cDNA synthesis kit (Thermo Scientific, USA). Sterile conditions, free from RNase contamination, were achieved in the study with total RNA samples stored at −86˚C and studied in cDNA synthesis. In summary, 1 µL of Oligo (dT) 18 primers (100 mM), total RNA (0.1 - 5 µg) determined after the measurement for the tissue, up to 12 µL of water were added, and it was kept

at 65˚C for 5 minutes. 4 µL 5x reaction buffer, 2 µL 10 mM dNTP mixture, 1 µL 20 U/µL Rıbolock RNase Inhibitor, and 1 µL 200 U/µL RevertAid M-MuLV RT were added to the tube, and incubated at 42˚C for 60 minutes. The first strand cDNAs were either used immediately or stored at −86˚C until use.

2.3. Reproduction of the Target Gene Using the cDNA Pattern

In this study, *CLV*2 and *CRC* gene homologs were isolated from the *T. turcica* plant. In the degenerate primer design, especially the nucleotide and protein sequences previously delivered to the gene bank were used for leguminous plants. The common amino acid sequence and nucleotide sequence that can be used in degenerate primer design were determined for the study target genes and especially the sequence obtained from legume plant species was compared with the BLASTp/n and CLUSTALw programs. While designing degenerate primers, protein and nucleotide sequence determined from legume plants were obtained for each gene (Table 1). Protein sequences were aligned with the CLUSTALw program to identify common amino acid sequence regions. Then, nucleotide sequences encoding these common sequences were identified and eventually degenerated primers were designed.

The names and sequence of the degenerate primers for *CLV*2 and *CRC* genes isolated from *Thermopsis turcica* are given in Table 2.

2.4. PCR Setup Using cDNA

Partial region of the gene of interest using cDNA from total RNA action mix, 21.5 µl dH_2O, 10 µl 5× Fusion HF buffer, 5 µl 0.2 mM dNTP mix, 3 µl 50 mM

Table 1. RACE-PCR: primers used in quantitative RT-PCR analysis.

Gene name	Primer name	Primer sequence
β-ACTIN	TtACTINF*	AGCTCAGCTGTTGAGAAGAGC
	TtACTINR*	ACATCGCACTTCATGATCGAG
RACE universal pirmers	Long UPM	CTAATACGACTCACTATAGGGCAAGCAGT GGTATCAACGCAGAGT
	Short UMP	CTAATACGACTCACTATAGGGC

Table 2. *CLV*2 ve *CRC* degenerate primer sequences (Y = C and T, R = A and G, H = A, C and T).

Gene name	Primer name	Primer sequence	DNA length
*CLV*2	dCLV2F	CYGAGTTGAAAAGYTTGCAGGCYTTG	700 - 850 nt
	dCLV2R	GARATGTTTCCYGGGATATGTCCHGA	
CRC	dCRCF	TACGTTCGTTGCAACTTCTGYAACAC	405 - 417 nt
	dCRCR	GCRCTGAAAGCTTCTCGATGTGGGAT	

$MgCl_2$, 2.5 µl, specifically designed forward and reverse primers (dCLV2F & R, dCRCF & R), 5 µl 50 µl total reaction was prepared with cDNA and 0.5 µl 2 U/µl Fusion high-reliability DNA polymerase (Thermo Scientific).

Polymerase chain reaction was performed with Applied Biosystem ProFlexTM PCR thermal loop device. Reaction cycles: first, denaturation 98˚C 2 minutes, 5 cycles 98˚C 0.15 seconds, 59˚C 0.30 seconds and 72˚C 30 seconds, 35 cycles 98˚C 0.15 seconds, 66˚C 0.30 seconds and 72˚C 0.30 seconds and finally, 72˚C for 5 minutes.

Confirmation of the PCR products obtained as a result of the reaction was done by gel visualization. 1 × TBE (Trizma Base, Boric Acid, EDTA and d H_2O) mixture was used for gel buffer and solution preparation. By weighing 1.8 g of agarose, solid particles in 100 mL of TBE buffer were completely dissolved by temperature, thus a 1.8% agarose gel was prepared. RadSafeTM gel dye (Nucleic Acid Staining Solution) was added to 100 mL gel and 8 µl of images were obtained under UV light. The PCR product was mixed with the loading buffer and the products were loaded onto the gel and run at 120 volts for 1 hour 15 minutes.

The visualization of DNA fragments was performed with Gen Box SDR Bio-Imaging System. Image-Plaza program was used for determine of the DNA sizes.

PCR products were purified before DNA samples were sent for sequence. In summary, the PCR product was taken into a 1.5 mL ependorph tube. 1 in 10 of the product in volume of 3 M sodium acetate (pH: 5.2). Pure ethanol preserved at −20˚C in 2.5 times the volume of the mixture was added, the tubes were gently mixed and kept at −20˚C overnight. It was centrifuged at 4˚C for 20 minutes at maximum speed.

The supernatant is removed and 70% ethanol is added to the pellet and centrifuged for 5 minutes under the same conditions. The supernatant was removed and the DNA pellet was dissolved in 30 µl of TE or dH_2O.

After adding 2 times the volume of PCR reaction volume of pure and cold ethanol, the sample tube was kept on ice for 5 minutes. After centrifugation at high speed and 4˚C for 10 minutes, the supernatant was removed from the tube. After the residue was dissolved in 32 µl of very pure water, it was mixed with 8 µl of 5M NaCl (final concentration 0.5 M). 40 µl of 22% PEG8000 was added to the tube and mixed (final concentration of 11% PEG will precipitate DNA fragments larger than 180 bp). Tubes were kept on ice for at least 20 minutes and then centrifuged at 4˚C for 10 minutes. After the supernatant was carefully removed, the pellet was dissolved in 20 µl of 0.3 M sodium acetate (NaOAc) and 2.5 volumes of 95% ethanol was added and mixed. Tubes left on ice for 15 minutes were spun in microfuge for 15 minutes. After removing the supernatant, the pellet was washed with 250 µl of 70% ethanol. After 5 minutes centrifugation, the supernatant was removed and the pellet was dried in dry air for 3 minutes and dried. The DNA pellet was dissolved by adding 20 µl of deionized water.

DNA fragments of *CLV*2 and *CRC* genes that were reproduced in RT-PCR were made by the company of Macrogen (Korea) through BM Laborsis Company (Ankara). The sequences obtained by the Sanger method were evaluated in the FinchTV program. Sequence information received was subjected to blast analysis in NCBI and it was proved that the sequences belong to our target genes.

2.5. RACE Analysis

Full-length cDNA sequence analysis of target genes was performed using the SMARTerTM RACE cDNA Amplification Kit (Clontech Laboratories, USA). In summary, for the cDNA synthesis reaction of 10 µl each, 2 µl of 5× first yarn buffer, 1 µl of DTT 20 mM (dithioethiol), 1 µl of dNTP mix 10 mM was gently pipetted and maintained for other stages in room conditions. 2.75 µl of RNA, 1 µl of 3' CDS primer A for 3' RACE Ready cDNA into 2 separate sterile microcentrifuge tubes. For 5' RACE Ready cDNA, 3.75 µl RNA, 1 µl 5' CDS primer A was added, spinning briefly in a microcentrifuge. The tubes were incubated for 3 minutes at 72˚C and after 2 minutes of cooling at 42˚C, the tubes were spinned at high speed for 10 seconds. Incubation and cooling steps were performed on the thermal cycler. 1 µl of SMARTer IIA oligo was added to the 5' RACE ready cDNA tube and the tube was vortexed briefly and centrifuged. Then, the reaction mixture was prepared by adding 4 µl master mix, 0.25 µl (40 U/Ml) RNase inhibitor, and 1 µl (100 U) SMARTScribeTM Revers Transcriptase, prepared in the first stage to both tubes. The mixture was first vortexed and briefly centrifuged. After the tubes were incubated in the thermal cycle device at 42˚C for 90 minutes, the reaction was terminated by keeping it at 70˚C for 10 minutes. The products were diluted by adding 100 µl of Tricine-EDTA buffer and maintained at −86˚C until cDNAs were used.

Two pairs of gene-specific primers (F: forward and R: reverse) were designed for *TtCLV*2 and *TtCRC* using partial cDNA sequence of genes obtained with degenerate primers to perform RACE and qPCR expression analysis (**Table 3**). Since the 5' cDNA end of *TtCLV*2 could not be reached in RACE analysis, 3 additional primers were synthesized.

PCR setup from cDNA synthesized for RACE was performed using the Advantage® 2 Polymerase Enzyme System (Clontech, USA). Reactions in accordance with the protocol prescribed by the kit were established using the components in the kit. In summary, each reaction tube contains 34.5 µl PCR-scale water, 5 µl 10× Advantage 2 PCR buffer, 1 µl dNTP, 5 µl 10× UPM (Universal Primer Mix), 1 µl (10 µM) gene-specific primer was briefly vortexed and then centrifuged. The reaction mixture was prepared by adding 2.5 µl of 5' RACE Ready cDNA to the reaction tube for the reproduction of 5' cDNA fragments, 3' RACE Ready cDNA, 1 µl 50× Advantage 2 Polymerase for the reproduction of 3' cDNA fragments. PCR cycles in BIO-RAD CFX96 thermal cycle device at 94˚C for 2 minutes, 5 cycles of 30 seconds at 94˚C, 30 seconds at 70˚C, 3 minutes at 72˚C,

Table 3. Primers used in RACE and RT-qPCR analysis of *T. turcica CLV*2 and *CRC* genes.

Primer name	Primer sequence	T_m Value (Füzyon Taq)	RT-PCR product Length (bp)
CLV2F1	ACCTAGTTGGTTGTTCGCGTTTGAAGC	72 °C	156
CLV2R1	ACCTTTCTTGCTGCAACCAATGGCTC		
CLV2F2	GCCATTGGTTGCAGCAAGAAAGGTTCAA		161
CLV2R2	CAGCTAGGCCAAATAAGCCCCTTGGA		
CLV2R5	GGCAGTGAACCTCCCAAATCAGGGTT		
CLV2R4	ATCCAACTAGGCAGTGAACCTCCCAA		
CLV2R3	GCTGCAAAGCAAGGCAAAGTACCCG		
CRCF1	ACTGCAGCAACCTCTCCTTTCTCAGC		172
CRCR1	TGGAGCTGCTTTAGGGGACACTGACT		
CRCF2	ACCATCGATCACACCCTCACTCTCCA		171
CRCR2	GTTGTAAGCAGATGGGAGACGGTGCT		

30 cycles of 30 seconds at 94˚C, 30 seconds at 68˚C, 3 minutes at 72˚C, it was carried out at 72˚C for 5 minutes.

2.6. Detection of *CLV*2 and *CRC* Gene Expressions

Total RNAs isolated from 22 different tissues were used for gene expression analysis. Synthesis of cDNA from total RNA isolated from tissues RevertAid was performed using the first thread cDNA synthesis kit (Thermo Scientific, USA). In summary, 1 µL Oligo (dT) 18 primers (100 mM), 1 µg total RNA, up to 12 µL water were added and it was kept at 65˚C for 5 minutes. 4 µL of 5× reaction buffer, 2 µL of 10 mM dNTP mixture, 1 µL of 20 U/µL of Ribolock RNase Inhibitor, 1 µL of 200 U/µL of RevertAid M-MuLV RT were added to the tube and spinned after vortexing the reaction mixture. CDNAs incubated at 42˚C for 60 minutes were stored at −86˚C until use.

Maxima SYBR Green qPCR Master (Thermo Fisher Scientific, USA) kit was used for qPCR setup. Kit components consist of hot start polymerase, dNTP, SYBR Green I dye, and reaction buffer. Expression analysis of the related genes in the tissues was performed on the BIO-RAD CFX96TM Real-time PCR device using the Bio-Rad CFX Manager 3.1 Software computer package program. As the control group gene, the *β*-Actin gene was previously cloned from the *T. turcica* and sequenced. The primer pair of the *β*-Actin gene is TtActinF and TtActinR primers, which yield 164 bp for qPCR analysis. The TtActinF primer sequence is designed as 5'-AGCTCAGCTGTTGAGAAGAGC-3' and TtActinR primer sequence 5'-ACATCGCACTTCATGATCGAG-3'. The *CLT*2F2 and *CLV*2R2, which gives 161 bp product and *CRVF*2 and *CRCR*2 primers, which are given in Table 3 of the *TtCLV*2 and *TtCRC* genes to be used in the reactions.

It was tested both in the design phase and the experimental phase that these primer pairs used did not form dimers. In all qPCR studies, no cDNA was added to the primer pairs (No Template Controls, NTC), to demonstrate that there was no contamination or primer dimers. The RT-qPCR mixture was prepared as 9 μL ddH_2O, 12.5 μL Maxima SYBR Green mixture, 0.75 μL forward and reverse primers, 2 μL cDNA components in total 25 μL per reaction. RT-qPCR cycles were performed as 10 min 1 cFycle at 95˚C, 30 sec 35 cycles at 95 sec at 30 sec 72˚C at 95˚C. Finally, melting cruve data was obtained by keeping 10 minutes at 95˚C for each qPCR.

The Southern blotting method was used to determine how many copies of the target genes were represented by *T. turcica*. Genomic DNAs from *T. turcica* leaf tissues were extracted using the CTAB method (Doyle and Doyle 1990). For this purpose, 300 mg leaf tissue was transferred to a clean tube after it was thoroughly grounded in liquid nitrogen. After adding 1 mL of CTAB buffer to the sample, the extract was left at 60˚C for 1 hour. After centrifugation at 12,000 rpm for 5 minutes, an equal volume of isoamilalkol: chloroform (1/24 v/v) was added to the supernatant transferred to the sterile tube. After the tubes were mixed by inverting for 5 minutes, they were centrifuged at 20,000 rpm and the supernatant transferred to the clean tube. This phase was repeated at least twice. The genomic DNA pellet to be precipitated with isopropanol after RNase application was washed with 70% ethanol, dried in room medium and dissolved in TE buffer. The amount and quality of genomic DNA was determined by spectrophotometry. Additionally, genomic DNA quality was confirmed by 1% agarose gel electrophoresis. 30 μg genomic DNA was cut with restriction enzymes and DNA fragments were separated by agarose gel electrophoresis following to the southern blotting analysis. It was then transferred to a nylon membrane. The target gene fragment was amplified from cDNA by RT-PCR and used as a probe. The DNA probe is marked with the "DIG DNA marking kit" (Roche protocol applied).

Tissues that were harvested in the season and grouped according to the working principle, were prepared in fresh and cold 4% paraformaldehyde (PFA) fixative. By applying vacuum to the samples on ice, the fixing process was performed in the best way and kept until it was used at 4˚C.

Tissue embedding is based on dewatering, washing and finally forming paraffin blocks. Samples are washed in two replicates of cold 1xPBS for 30 minutes. Then, dewatering was performed by passing through ethanol series at certain concentrations and for certain periods. It was kept in pure ethanol containing eosin for 1 night. Since eosin paints cell walls, it ensures that samples are visible during embedding and sectioning. The next day, he was passed through histoclear (HC) series to remove tissues from ethanol. At the end of the day, some paraffin was added into HC and paraffin saturation of tissues was initiated. HC paraffin replacement process continued for a few days. Liquefied paraffin was poured on the tissue placed according to the shape to be sectioned in the mold

and left to cool. The paraffin blocks obtained were stored until they were used at 4˚C.

DH5α and TOP 10 *E. coli* strains were used in cloning studies. TOP 10 and DH5α are very close genotypes. They can be prepared for transformation by chemical or electro methods. Although both *E. coli* cell lines were stored in glycerol stocks at −86˚C, DH5α *E. coli* strain was used in our cloning studies.

Luria-Bertani (LB) food is the most used nutrient solution in studies with *E. coli* cultures. Peptone, the chemical digestion product of the LB casein enzyme, contains yeast extract and sodium chloride salt. As a solidification agent, agar can be added optionally. It is sterilized by autoclaving for 15 minutes at 121˚C under 1 atm pressure. After cooling the LB medium to 50˚C, antibiotics can be added. LB agar is poured 20 - 25 mL LB per petri dish (90 mm) using sterile technique. LB food can be stored for 1 month at 4˚C, and LB foods with antibiotics can be stored for 15 days.

As an initial culture, an "isolated" colony on the LB agar was transferred into a 50 mL falconi tube containing 5 mL of LB food, with sterile toothpick. Only sterile toothpick was added to the tube for negative control. Tubes were kept in heated shaker (Zhicheng, China) at 37˚C, 260 rpm for 16 hours at overnight.

DH5α *E. coli* cells to be cloned were prepared at −86˚C by preparing the glycerol stock. The process steps are in order: the starting culture was prepared the night before. After preparing an equal volume of glycerol and distilled water (v/v) solution, it was sterilized by autoclaving. 300 µL of 50% sterile glycerol and 900 µL of starting culture were mixed in the tube. After the tubes were gently mixed, they were stored at −86˚C without pre-freezing in liquid nitrogen.

The plasmid was linearized by digesting approximately 5 µg of DNA with restriction enzymes. Gel electrophoresis was performed to confirm completion of the reaction. *In vitro* transcription mix, 1 µl purified DNA (1 µg/µl), 2 µl 10× transcription buffer, 2 µl 10× DIG RNA labeling mix, 1 µl RNAase out, up to 20 µl H_2O. It was incubated at 37˚C for 1 - 2 hours and visualized on the gel. It was purified by adding 1 µl of tRNA (100 mg/ml), 1/10 volume of 3 M NaAc pH 5.2, 2.5 volume of cold 100% ethanol, and incubated at 8˚C for 30 minutes. RNA was centrifuged at high speed for 20 minutes at 4˚C the supernatant was removed and the pellet was washed with ethanol. The centrifuged liquid portion was removed again and the RNA pellet was dissolved in µl of 50% deionized formamide.

Probe evaluation and DIG-UTP junction were evaluated by dot blot analysis. Labeled DIG-UTP RNA probed with an *in vitro* transcription kit (Roche). 1xTBS buffer was prepared with 100 mM Tris-HCl pH 7.5, 150 Mm NaCl. RNA probes prepared by serial dilution are fixed on the membrane. The membrane is equilibrated with TBS on the shaking platform, treated with blocking buffer, washing is performed in TBS, 45 minutes incubation with 1 µl anti-dioxygen in 10 ml 1xTBS is required. 100 mM Tris-HCl pH 9.5 in TBS was kept in TN buffer prepared with 100 Mm NaCl. Due to the membrane staining of NBT/BCIP 1/50

(v/v) in 1xTN buffer, it is necessary to wash and visualize it under UV.

Paraffin blocks kept at 4˚C were kept to reach room temperature and the sections were optimally structured. Block microtome placement is for sections with a thickness of 8 - 10 µm. Sections were distributed on marked slides with 1 mL of H20 and tissues were fixed by heating. In this way, it can be stored for a further period of 4˚C.

3. Results

In this study, two different transcription gene isolations were carried out from *Thermopsis turcica*, the endemic of Afyonkarahisar province of Türkiye: the exile dission gene *CLAVATA*2 (*TtCLV*2) and the *CRABS CLAW* (*TtCRC*) gene involved in the formation of carpel. As part of the study, the full-length cDNA nucleotide sequences of both genes were reached by RACE analysis. The level of transcripts of both genes in 18 different *T. turcica* tissue is determined by RT-qPCR analysis. Additionally, expression analyses were performed in the tissues of different sizes of both genes using *in situ* technique.

Some used tissues sampled from Afyonkarahisar province of Türkiye, the natural spread area of *T. turcica* were shown in Figure 2. MRNAs isolated from young buds were used in partial and full-length cDNA sequence. Quantitative RT-PCR analyses were used 18 different tissues. These are, vegetarian (body, shoot, young leaf and ripe leaf), reproduction (flower bud, sepal before and after pollination, and fruit (young fruit, young fruit seed, young fruit peel, ripe fruit seed and ripe fruit peel) are tissues.

In determining partial and full-length cDNAs, total RNAs of the bud were used in quantitative RT-PCR analyses, and total RNA isolated from 18 different

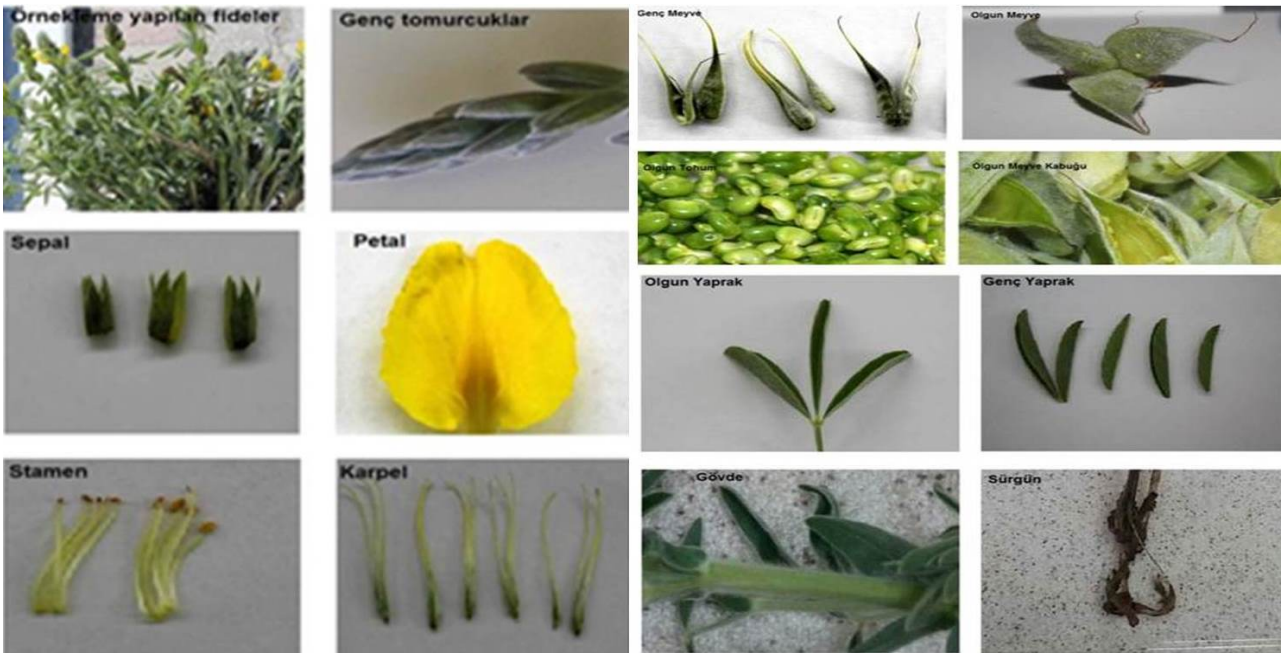

Figure 2. *T. turcica* tissues used in analysis (Before pollination sepal, petal stamen and carp tissues are not shown).

tissues were used. After the possible DNA contamination in isolated total RNA was cleared with DNase I application and RNA quantity determinations were made with Qubit assay. RNA quality is determined by RNA formaldehyde agaroz gel electrofore (Figure 3).

Total RNAs isolated from bud tissue were used in full-length cDNA determination studies. The first strand cDNAs obtained with normal revers transcription were used in partial cDNA determination. RACE-ready cDNAs obtained from bud total RNAs were used to determine both ends of cDNAs.

Degenerate primers prepared for *TtCLV2* and *TtCRC* from the bud cDNA mold and DNA fragments that have proliferated in PCR. PCR products of approximately 400 bp sizes for *TtCLV2* and 762 bp targeted for *TtCLV2* have been successfully obtained and single fragment. After the PCR products were purified, the service was sent to the service edifying process. Partial nucleotide and protein sequence from both genes are given below. The partial *TtCLV2* protein sequence was found to be a Leusin-rich rereceptor-like protein and a *CLAVATA2-like* gene that had previously been delivered to the gene bank for other legume plants. On the other hand, the partial *TtCRC* protein sequence has been identified as a protein belonging to the YABBY TF gene family (Figure 4). After these analyses, gene specific primers were designed. Primer adhesion zones for both genes are shown in nucleotide sequences.

RACE analysis was used in determining full-length cDNA. It has been checked for both genes whether gene specific primers produce single fragment in cDNA

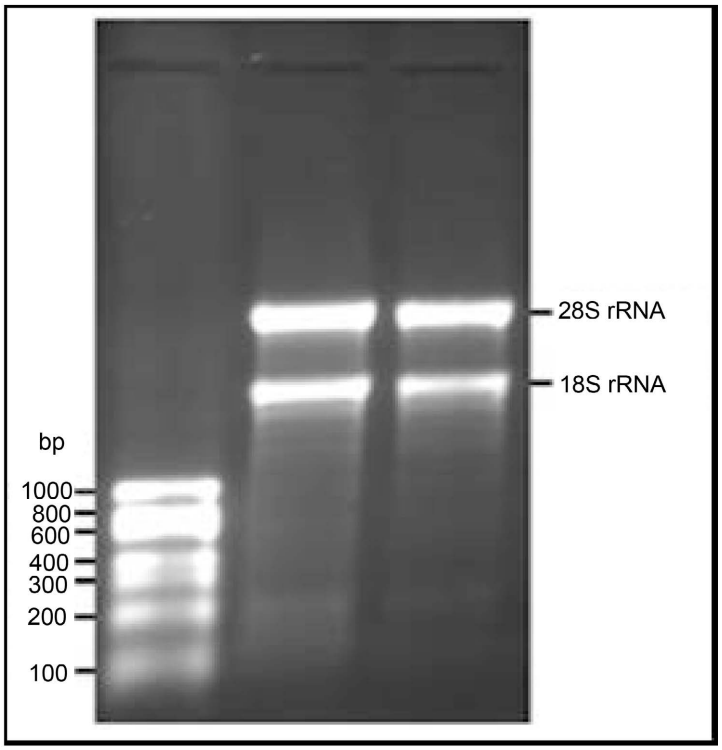

Figure 3. *T. turcica* flower bud total RNA. 2 µg (midline) and 1 µg (right line) total RNA. First line marker RNA.

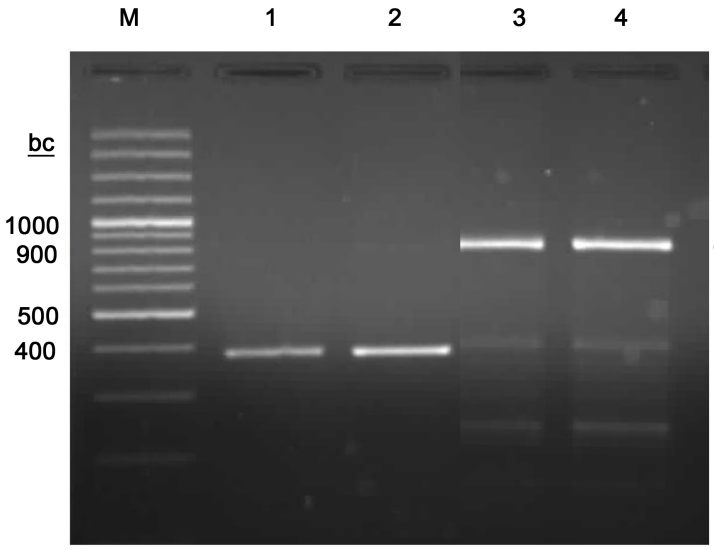

Figure 4. Fragments for 762 nt *TtCLV2* (3rd and 4th lines) and 366 nt *TtCRC* (1st and 2nd lines) produced with RT-PCR from bud cDNA extract (M: marker).

mold. These primers were used in race analysis with universal primer. The *TtCLV2* gene has a very long nucleotide sequence, so a full cDNA sequence has been reached in one stage. RACE-PCR products, which have proliferated in *TtCRC* 3'- and 5'-RACE analyses, are given in **Figure 5**.

T. turcica CLAVATA2 Protein [*Thermopsis turcica*], signal peptide, 716 aa. LRRNT_2-domain (N-tip Lösince rich region) and LRR-8-domain. There is also lrr-ri (leukaerich receptor RNaz inhibitor-like zone, but not all of them can be shown because it is intertwined).

Blastp analyses of genes with full-length cDNAs have been analyzed and shown to be target genes (**Table 4**). In BALSTp analyses, the genes obtained in the current study were determined for other legumes and were found to be highly similar to homologues delivered to the gene bank. As shown in **Table 4**, each target gene is compared to the sequences designated for *Cicer arietinum*, *Glycine max*, Medicago *truncatula* and *Pisum sativum* to make a meaningful comparison. As an external group, the target genes are compared to *A. thaliana* homologues.

Genes are a great resemblance to legume homogues (80% - 95%). *A. thaliana* homogues and lower (60% - 70%) similarities were determined. However, it has not been evaluated because there is no sequence detection for *CRC*-like protein for *P. sativum*.

Southern Blotting method was used to determine how many copies of genes targeted for isolated and quantitative PCR analyses are represented in the *T. turcica* genome. For this purpose, genomic DNA from *T. turcica* leaf tissue was extracted by the classic CTAB method. The quantity and quality of genomic DNA is determined by spectrophotometer. For southern Blotting analysis, 30 µg genomic DNA was cut in separate reactions with three different restriction enzymes,

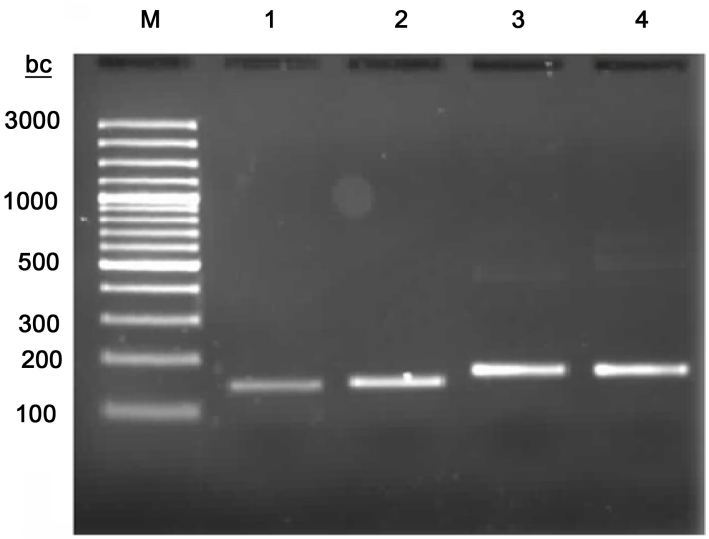

Figure 5. Testing of primers designed for *TtCLV2* (line 1 and line 2) and *TtCRC* (line 3 and line 4) genes used in RACE and qPCR analyses. Primers used in RT-PCR: Line 1: *TtCLV2F1/R1*, Line 2: *TtCLV2F2/R2*, Line 3: *TtCRCF1/R1* and Line 4: *TtCRCF2/R2*.

Table 4. BALSTp analysis *for TtCRC* and *TtCLV2* target proteins.

T. turcica CRC							
Species	**Protein**	**Maximum score**	**Total score**	**Coverage**	**E value**	**Similarity**	**Protein code**
Cicer arietinum	*CRC*	278	278	95%	3e-92	86%	XP_004509753.1
Glycine max	*CRC*	304	304	98%	2e-102	92%	XP_003520126.1
Medicago truncatula	*CRC*	265	265	98%	2e-86	79%	XP_003614201.1
Pisum sativum	-	-	-	-	-	-	-
Arabidopsis thaliana	*CRC*	199	199	90%	4e-64	68%	NP_177078.1
T. turcica CLV2							
Cicer arietinum	*CLV2/RLP10*	1155	1155	100%	0.0	82%	XP_004492826.1
Glycine max	*CLV2/RLP10*	1197	1197	100%	0.0	85%	XP_014626310.1
Medicago truncatula	*CLV2/RLP10*	1142	1142	99%	0.0	82%	XP_003624130.1
Pisum sativum	*CLV2/RLP10*	1132	1132	97%	0.0	83%	BAK39954.1
Arabidopsis thaliana	*CLV2/RLP10*	844	844	96%	0.0	64%	AAF02655.1

and DNA fragments were separated by agaroz gel electrophoresis.

The nylon membrane was transferred and the target gene was replicated with RT-PCR from cDNA and used as a probe (Table 5).

Table 5. CT values determined in qPCR analysis. Cycle (C_T, Threshold Cycle) values corresponding to the threshold point for 12 different genes determined individually in 18 different *T. turcica* vegetative and reproductive tissues.

Tissue	*TtCLV2*	*TtCRC*	Tissue	*TtCLV2*	*TtCRC*
Green leaf	20.5	19.2	Powdered Sepal	21.4	24.8
Mature leaf	ND	ND	Powdered Petal	19.5	19.9
Stem	ND	ND	Powdered Stamen	22.3	18.4
Shoot	21.7	18.6	Powdered Karpel	22.4	18.8
Bud	21.1	20.7	Green fruit seed	22.6	24.2
Nonpowdered Sepal	21.3	17.5	Green fruit	21.0	17.5
Nonpowdered Petal	23.0	17.2	Green fruit skin	21.3	17.8
Nonpowdered Stamen	23.4	18.7	Matured fruit seed	21.1	18.7
Nonpowdered Karpel	25.1	18.7	Matured fruit skin	22.4	19.4

4. Discussion

Developmental process from vegetarian stage to flowering, installation of flowering, formation of flower organs sepal, petal, stamen and carpel identities, developmental process of flower organs, pollination, embryo development and eventually fruit formation constitutes one of the most interesting research subjects in science. Flower mersion and flower organ identification genes often encode homeotic transcription factors. These factors regulate the expression of the relevant structural genes, enabling the transition of the plant to flowering and the formation of plant flowers. Flower structure and shape, such as the number and petal color of flower organs, are unique to plants. Plants have different flowering plans at the level of species, breed and family. However, despite these differences, genes that encode homeotic transcription factors working in the installation and formation and development of flower organ identity are preserved in flowering plants.

Within the scope of plant architecture, intensive research has been carried out since the early 1990s to illuminate the molecular mechanism of flowering and fruit formation. In plants, the transition from vegetative meristem tissue to flower meristem tissue and the complex molecular mechanisms in determining the time of flowering are taken and continue to be taken. Most of the information obtained from studies on flower organ identity genes was obtained from single-year herbaceous plant Arabidopsis and Antirrhinum model plants. Although much of the information about the molecular biology and physiology of flowering comes

from plants, similar research studies wild and cultural plants with intriguing flower morphology, or economically important plants [9] [10]. Information obtained from such plants contributes greatly to the universalization of acquired information about the molecular foundations of flowering or determining exceptions. In this context, the *T. turcica* wild plant with free much carpel flower morphology and legumes pouring fruit has attracted attention. Since *T. turcica* also enters the perennial herbaceous plant group, it is no doubt that the information obtained from this plant will provide valuable information about flowering in multi annual herbaceous plants.

The legumes plant family *Thermopsis R.* genus plant species usually spread in mountainous humid regions of Central Asia and North America [11]. However, in Anatolia, *T. turcica* of this genus is the only plant and has non-normal multi-free carpel flowers for legumes [3]. The "gynoecium", which means female + house in Latin, can be defined as the female organ that protects the seeds of the flower and in many cases is called carpel. The gynoecium component in plants can be in the form of single carpel (as in legumes), multiple free carpels (family of wedding flowergils or strawberry species) and multiple fused carpels (tulips and many flowers) [12]. This morphological feature of *T. turcica* has given the idea that it can lead to an increase in crops for legumes, whose characteristics such as chickpeas, peas, soy and beans are agriculturally important. However, multi-free carp or fruit production from single flowers is not a very common feature among plants. There is also no information on the molecular foundations of this uncommon morphological feature in the literature. In fact multiple (four or more) free carpel flower plan Rassulaceae, Paeoniaceae and Ranunculaceae plant is a prominent feature for some species within plant families [8]. It is given in **Figure 6** and **Figure 7** with the aim of comparing sample species belonging to the plant families and multi-free tiles or fruits belonging to the same flower for *T. turcica*.

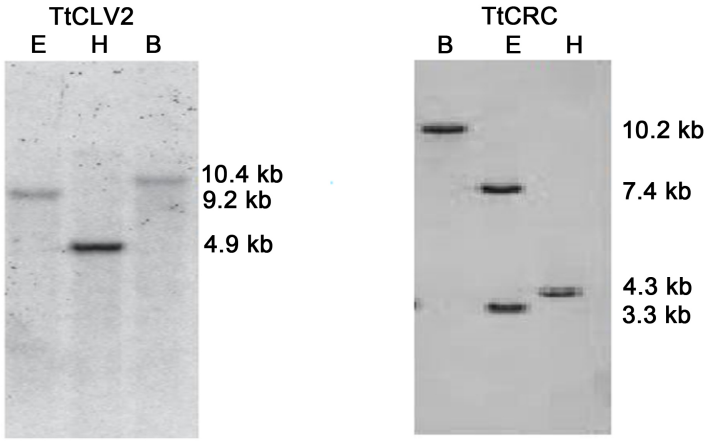

Figure 6. Southern Blotlama analysis of 2 genes in the *T. turcica* genome. Target genes (*TtCLV*2 and *TtCRC*), restriction enzymes (B = BamHI, E = EcoRI and H = HindIII) and bloted DNA fragment sizes are shown on the figure.

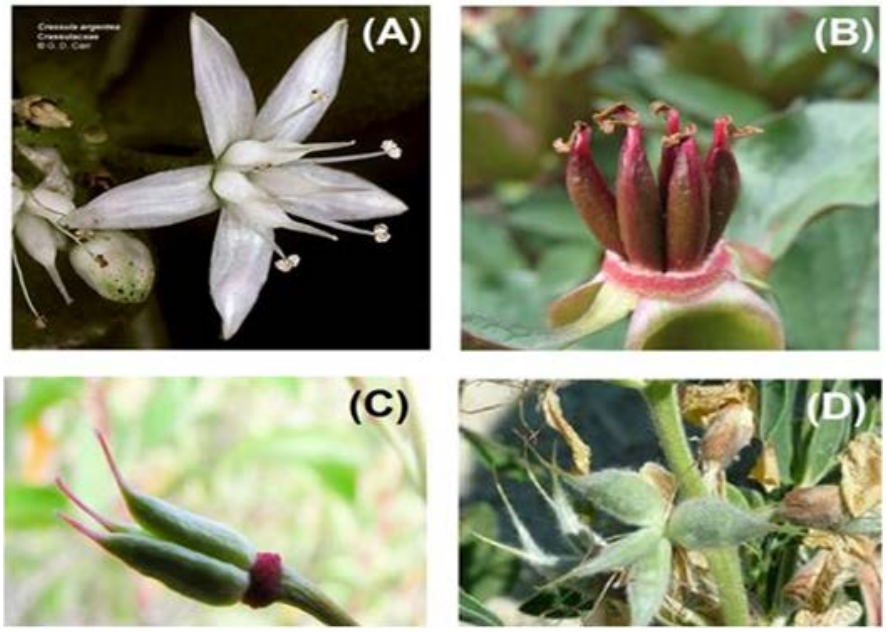

Figure 7. Examples of multiple free carpel plants. (A) *Crassula argentia*, (B) *Paeonia cambessedesii* from the Crassulaceae family, (C) *Delphinium cardinale* of the Ranunculaceae family and (D) *Thermopsis turcica* of the Leguminosae. (Photo Credit: (A) http://www.botany.hawaii.edu; (B) http://mundani-garden.blogspot.com.tr; (C) https://www.flickr.com; (D) Dr. Suleyman Cenkci).

Our ultimate goal is to investigate the effects of *CLV*2 (flower meristem gene) and *CRC* (carpel formation gene) genes in the production of multi-carpel fruit in Turcica, reach molecular information, compare them with the literature and to bring. However, this is to bring this feature to other economically important legumes plants.

Meristem gene, *TtCLV*2: the *TtCLV*2 protein, which is estimated to consist of 716 amino acids, is rich in leukemia in terms of amino acidand repeats the region 12 times the rich of leukaemia. *CLAVATA*1 (*CLV*1) and *CLV*2 receptor proteins activate cell proliferation in The Shoot end in Arabisopsis when they are connected to *CLV*3 ligands by activating the suppression mechanism of the expression of the transrips factor WUSCHEL [5]. The size of the exiled stem cell population is regulated by the CLV signalling system, and the elements of the system consist of *CLV*3, receptor-like kinase, CLV1 and CORNYE (CRYNE) and receptor-like protein *CLV*2, which is a small secretion peptide (Buy muller meat. Therefore, the *CLV*2 gene is not a transcription factor. The *TtCLV*2 full-length cDNA of *CLV*2, also known as Arabidopsis LRR10, consists of 2557 and ORF consists of 2155 nucleotides.

TtCRC gene, the full-length cDNA of the *CRC* homologue, which is not a MADS box and which describes increases in the number of carp in the event of Arabidopsis mutations, is isolated from *T. turcica*. Estimated to consist of 174 amino acids, the *TtCRC* protein has two preserved regions.

In harmony with the information obtained from functional analyses, tran-

script for the Highest *TtCRC* transcript in flower tissues is determined in carp tissue [7] [13]. According to our findings, high *TtCRC* expression was also determined in stamen tissue. On the other hand, young and mature seeds have a pronounced *TtCRC* expression in fruit shells, while the term *TtCRC* gene is almost nonexistent. The highest transcript of the *TtCRC* gene was found in the mature fruit shell.

CRC gene homologous, which is known to be related to carpel development, has also been found to have a similar function to *T. turcica*. It is determined by the expression *TtCRC*, where this gene can contribute to seed development.

*CLAVATA*2 and *CRABS* claw mutants or excessive expression of these genes were determined in mutant studies with Arabidopsis, where there may be increases in the number of flower organs, including the number of main carppels [5] [14] [15] [16] [17]. Functional analysis of the *TtCLV*2 and *TtCRC* gene can be done in a model plant like Arabidopsis or similar, determining the functional analysis of these genes.

In this study, *CLAVATA*2 (*CLV*2) and *CRABS CLAW* (*CRC*) Arabidopsis gene homologous were intended to be cloned and characterized in *T. turcica*. In achieving this goal 1) obtaining and sequencing short parts of the target genes by taking advantage of degenerate primaries, 2) designing gene-specific primaries for RACE analysis from these sequences, 3) 3 of the cDNAs of gene-specific primaries and target genes. and rapid reproduction, cloning and syntax of 5'-ends, 4) determination of full-length cDNA nucleotide sequences by combining cDNA sequence fragments, 5) analysis of nucleotide and amino acid sequences with software programs, 6) analyzing the number of representations in the genome of target genes by Southern Blotting method, 7) nucleotide and amino acid sequences of the target genes, present in gene banks it is aimed to analyze phytogenetic and molecular evolutionary relationships by comparing homologues, and 8) determination of developmental and organ-specific expressions of target genes by semi-quantitative RT-PCR method.

According to our results, high *TtCRC* expression was also determined in stamen tissue. On the other hand, young and mature seeds have a pronounced *TtCRC* expression in fruit shells, while the term *TtCRC* gene is almost nonexistent. The highest transcript of the *TtCRC* gene was found in the mature fruit shell.

*TtCLV*2 *T. turcica*'*s* young leaf has high transcript levels in the shoot tip, flower bud, sepal textures and mature fruit peel. From these tissues, the tissue with the highest transcript of *TtCLV*2 is the mature fruit shell. A very small *TtCLV*2 gene expression has been detected in other fruit tissues. In flower tissues other than sepal, the expression *CLV*2 is very small or almost nonexistent.

5. Conclusion

This study has attempted to explore the controlling feature of *Thermopsis turcica*, the young bud *Arabidopsis CLV*2, and *CRC* gene homologs were isolated and

analyzed in different tissues. First, with total RNA isolated from the first thread degenerated primers synthesized cDNA expressions, second, with a partial length cDNA cloning process made and the replicated set of analyses. The full-length cDNA of genes related to RACE analysis was carried out with the sequencing process. The real-time PCR method was applied for gene functional analyses with buds, sepals, petals and stamen and relative expression analysis of genes in the tissue of pistils. Full-length cDNA sequence for the gene *CLV*2 2557 bp, the encoded protein 716 amino acids, *CRC* 901 for full-length cDNA sequence of the gene—bp, the encoded protein 174 identified as amino acids. Alignment and phylogenetic analyses of *CLV*2 and *CRC* genes were found to be similar to other types of legumes in homologous sets. Real-time (RT-qPCR) PCR results relative to study courses with genes.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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